Lipid Polarity and Sorting in Epithelial Cells

1

SUBUNITS H1 AND H2 HAVE DIFFERENT ROLES FOR INTERNALIZATION OF THE OLIGOMERIC ASIALOGLYCOPROTEIN RECEPTOR

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The human asialoglycoprotein (ASGP) receptor is a hetero-oligomeric complex composed of two highly homologous subunits H1 and H2 which are both required for ligand binding and internalization. We have previously shown that the major subunit H1 alone, although not capable of binding ligand, undergoes constitutive endocytosis and recycling and that its efficient clustering into coated pits depends on a tyrosine residue in its cytoplasmic domain. In contrast, we show now that H2, when expressed alone in fibroblasts, is endocytosed only slowly and that mutation of a cytoplasmic Phe residue at the homologous position of the critical Tyr in H1 does not influence internalization. Furthermore we analyzed the relative importance of the H1 and H2 tails within the hetero-oligomeric complex by coexpressing Tyr-mutated H1 with wild-type H2, wild-type H1 with Phe-mutated H2 or both mutants together. Internalization and degradation of ligand proceeded at normal rates when H2-Phe was mutated, but was reduced to the same low level either when only H1-Tyr or both were mutated. Thus, H2 cannot compensate for mutant H1 in the oligomeric ASGP receptor and appears to lack an active internalization signal in its cytoplasmic domain.

2

Role of polymeric Ig receptor phosphorylation in IgA transcytosis using a co-culture system that allows efficient IgA transport.

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We have developed a co-culture system with poly-Ig receptor expressing MDCK cells and IgA producing hybridoma cells. In this system 5 ng of IgA/hour are transported by $4x10^6$ MDCK cells.
The wild type poly-Ig receptor and two phosphorylation-mutants were expressed in MDCK cells. The routing of the receptor and/or the capacity of IgA transcytosis was altered in the mutants.
Phosphorylation of Ser664 plays a role in vectorial release of SC while IgA transport is unaffected. In contrast, Ser726 affects rate of IgA transcytosis (4-5 fold reduction), while vectorial release of SC is unaltered.

3

Autophagic and endocytic pathways in Hela cells

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Autophagic and endocytic pathways were studied in Hela cells using immunoelectron microscopy and stereological techniques. Without autophagic stimulation, characteristic particulate endocytic structures could be identified that possessed multiple layers of membranes. These received fluid phase endocytic markers after 25 minutes uptake and contained the majority of the immunogold labelling obtained with a monoclonal antibody directed against a lysosomal glycoprotein. When autophagy was stimulated there was a quantitative depletion of these multilamellar endosomes and autophagosomes now contained multilamellar structures that possessed not only a similar structure to multilamellar endosomes but also showed similar volume and surface density of internal membranes. Autophagosomes now also contained the majority of the labelling obtained with the monoclonal antibody. These results are best explained by fusion of multilamellar endosomes with 'nascent' autophagosomes which could represent the convergence of the endocytic and autophagic pathways.

4

Polarized regulation of endocytosis by antidiuretic hormone in cultured kidney cells

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Hormonally regulated transepithelial Na^+ reabsorption is displayed by A6 *Xenopus laevis* kidney cells cultured on porous supports. It was investigated whether antidiuretic hormones (ADH: vasopressin and vasotocin), which produce a 3-fold increase of Na^+ reabsorption (measured as amiloride-sensitive transepithelial short circuit current), induce also membrane movements. Domain-specific endocytosis was measured during the response to ADH, using horseradish peroxidase (HRP) as fluid phase marker. Internalization of HRP was found in control conditions to be 15-30 times lower from the apical than from the basolateral surface. Upon stimulation with ADH, apical uptake of HRP increased by a factor of 2-3, when measured for short periods (10 min), and by a factor of 1.4 for a longer period (30 min), while basolateral uptake of HRP was not stimulated. Furthermore, the apical to basolateral transcytotic pathway was quantitatively of low importance, since basolateral efflux of HRP remained low in the course of apical marker uptake. These results suggest that ADH produces an increase of both fluid phase endocytosis and recycling to the plama membrane, which is restricted to the apical surface-domain and endosome. It is currently being investigated whether the induction of membrane movements, which could mediate the translocation of Na^+ channels to the apical surface, is a prerequisite for the ADH effect on Na^+ reabsorption.

5

DEXAMETHASONE REGULATES EXOPEPTIDASE ACTIVITIES IN BRAIN-DERIVED ENDOTHELIAL CELL LINES

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Blood-brain barrier function in the central nervous system is a property of endothelial cells. By retroviral transfection introducing the polyoma middle-T oncogene into fetal rat brain endothelial cells, two cell lines which express g-glutamyltranspeptidase (gGTP), angiotensin converting enzyme (ACE), aminopeptidases A (APA) and B (APB) have been obtained. Dexamethasone has the potential to reduce brain edema and angiotensin II (Ang II) regulates the hydric balance. Dexamethasone added to the culture medium increases ACE activity (3x), but decreases the expression of gGTP and APA (0.5x), thus possibly potentiating the effects of Ang II. Exogenous Ang II augments the incorporation of thymidine, and the effect of Ang II is potentiated by inhibition of APA. By regulating their exopeptidase activities, dexamethasone is thus able to regulate the proliferation of brain endothelial cells.

6

Structural Analysis of Glycosylphosphatidylinositol membrane anchors from *S. cerevisiae*.

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We purified milligram amounts of glycosylphosphatidylinositol membrane anchors from *S. cerevisiae* without previous purification of individual proteins. Many GPI anchored yeast proteins carry N-glycans close to the anchor attachment site and have in that region a discrete set of amino acids in common which include mainly Asn/Asp, Ser, Gly and Thr. Analysis of the complete structure of purified anchors revealed that yeast GPI anchors also contain the highly conserved $(Man)_3$-GlcN-myoinositol core structure with an ethanolamine on the third Man residue, linking the anchor to the protein. All of the analyzed anchors contained an additional fourth Man and 20% a fifth one linked to the core. The vast majority of yeast GPI anchors contain a ceramide as lipid moiety, while the GPI anchor of the purified 125 kD protein contains a diacylglycerol instead. Thus, the same cell synthesizes GPI anchors with substantially different lipid moieties.

7

CHARACTERIZATION OF GLYCOLIPID INTERMEDIATES IN THE BIOSYNTHESIS OF GLYCOPHOSPHATIDYLINOSITOL ANCHORS IN THY-1-NEGATIVE LYMPHOMA LINES.

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Several mammalian mutant cell lines do not express the glycophosphatidylinositol (GPI)-anchored Thy-1 protein on their surface. Some of these mutants, when labeled with [^{3}H]*myo*-inositol, [^{3}H]mannose or [^{3}H]ethanolamine, accumulate abnormal lipids which remain undetectable in the corresponding parental cell lines. Some of these lipids were isolated from the SIA^{-b} mutant line and characterized using biochemical methods. The partial structure elucidation of their carbohydrate moiety allowed us to determine that they represent intermediates of the GPI-biosynthesis and suggest that the SIA^{-b} mutant lacks the third mannosyltransferase of the GPI-anchor biosynthesis. Interestingly, the inositol of the main intermediate is acylated while this acyl is absent on a second, less abundant intermediate and also cannot be found on mature Thy-1 molecules of the SIA parental cell line. Lipid intermediates and mature Thy-1 are heterogenous since both diacylglycerols as well as acyl-alkylglycerols can be found.

8

GENERATION OF GLYCOSYL-PHOSPHATIDYLINOSITOL MUTANT IN P815 CELLS

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P815 mastocytoma cells , expressing the glycosyl-phosphatidylinositol-anchored (GPI-anchored) murine T cell marker Thy-1 were mutagenized by treatment with ethyl methanesulfonate. Thy-1 negative cells were selected by successive negative sorting of anti-Thy-1-stained cells. After limiting dilution culture, 13 clones were selected for further analysis. Northern blot analysis revealed that Thy-1 was still expressed at the mRNA level. The synthesis of Thy-1 protein was demonstrated by immunoprecipitation. However Thy-1 antigen was not present at the cell surface, suggesting a defect in a post-translational processing, such as GPI anchoring. Supporting this interpretation, the expression of another GPI-anchored polypeptide was shown to be affected. Finally, using metabolic labeling with [^{3}H]myo-inositol and analysis by TLC, the putative intermediates in GPI biosynthesis were studied.

9

PURIFICATION AND CHARACTERIZATION OF 2 ISOENZYMES OF RAT INTESTINAL ALKALINE PHOSPHATASE (IAP1 AND IAP2)

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IAP1 and IAP2 (EC 3.1.3.1) have been purified to over 97% homogeneity by the classic procedure [Potmann & al. (Helv. Chem. Acta 65 (1982) 2668] modified by an affinity chromatography (hystidylphosphonic Sepharose) and by a gel filtration chromatography (FPLC, Superose 12). For the first time it was possible to separate IAP1 and IAP2.
IAP1 and IAP2 have a specific activity of about 1800 IU/mg protein and both present a single band on SDS-PAGE. The apparent Mr of their monomer subunit are 63.6 KDa (IAP1) and 87.5 KDa (IAP2). The Mr of the dimers determined by gel filtration (Superose 12, FPLC) are 144 KDa (IAP1) and 176 KDa (IAP2). IAP1 and IAP2 have a different sugar molar content: IAP1 (13-14 mol fuc, 65 mol man, 9 mol gal, 40 mol glcNH$_2$, 12 galNH$_2$); IAP2 (6-7 mol fuc, 77 mol man, 4-5 mol gal, 27 mol glcNH$_2$, 25 mol galNH$_2$).
Despite a close similarity in aminoacid composition, significant differences in the sequences of the N-terminal regions prove not only a different glycosylation, but also a different protein backbone of these 2 isoenzymes.

IAP1: VIPVEEENPVFWNQKAKEALDVAKKLQPIQTSAKNLILFLGDGM
IAP2: VIPVEEENPAFWNQKAADALNVAKKLQPIQTSAKNLIIFL

10

INFLUENCE OF LIGHT AND TEMPERATURE ON THE FATTY ACID COMPOSITION OF PHOSPHATIDYLGLYCEROL IN SQUASH COTYLEDONS

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Phosphatidylglycerol (PG) appears to play several roles in the thylakoid membrane, among which its involvement in the mechanism of chilling sensitivity of plant is most important. Here, we study the influence of light and temperature on the fatty acid composition of PG in cotyledons of squash, a chilling sensitive plant. We show that during growth, the sums (mol %) [16:0 + t16:1] and [18:0 + 18:1 + 18:2 + 18:3] in PG remained constant. Although the proportion between the fatty acids of the C_{18} series did not change during growth, t16:1 increased at the expense of 16:0. The rate of this change depended on the plant growth rate, on the day length and on light intensity. However, lowering the temperature increased the unsaturation of the C_{18} series fatty acids. We conclude that in a given plant variety, the constant level of the C_{16} and C_{18} series fatty acids in PG is controlled genetically. In contrast, the changes occurring in the C_{18} fatty acids depend on the temperature and those in the C_{16} fatty acids on the light. These results are discussed in terms of the pathways of fatty acid synthesis.

11

THE ß SUBUNIT AFFECTS THE K^+ ACTIVATION OF THE Na,K-ATPase

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The TBM 18-23 cell line is a toad bladder clonal cell line which displays vectorial Na transport upregulated by aldosterone. Molecular cloning of α and ß subunit cDNAs of the Na,K-ATPase expressed in these clonal cell lines reveals expression of one α and two ß isoforms named $ß_1$ and $ß_3$. TBM $ß_3$ is 70% and 80% homologous with the TBM $ß_1$ or Xenopus laevis $ß_3$ subunits, respectively. To investigate possible functional differences between TBM $ß_1$ and $ß_3$ and to understand the physiological relevance of coexpression of two ß isoforms in clonal epithelial cell lines, we used the oocyte of X. laevis as an expression system, measuring Na,K-pump activity as ouabain sensitive-potassium-induced currents (in presence of 5 mM barium). TBM $ß_3$ subunits form functional heterodimer when associated with TBM $α_1$. TBM $α_1ß_1$ and TBM $α_1ß_3$ share similar ouabain sensitivity (Kd [in µM]:52.5±2.8 [$α_1ß_1$, n=7], and 56.9±3.0 [$α_1ß_3$, n=8] NS) but differ in their K^+ half activation constant ($k_{\frac{1}{2}}$) ([in mM]:1.0±0.1 [$α_1ß_1$, n=10] and 1.43±0.09 [$α_1ß_3$, n=12], p<0.005). Thus, the ß subunit could affect some functional properties of the Na,K-ATPase.

12

EXPRESSION OF RAT LIVER 11β-HYDROXYSTEROID-DEHYDROGENASE (11β-OHSD) IN TBM CELLS, AN ALDOSTERONE RESPONSIVE CELL LINE

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The mineralocorticoid receptor (MR) displays equal affinity for aldosterone and corticosterone. It has been proposed that mineralocorticoid specificity is achieved by enzyme protection of the MR through the conversion of corticosterone by 11β-OHSD into the inactive metabolite 11-dehydrocorticosterone (11-DHC). To test this hypothesis, we have transfected rat liver 11β-OHSD cDNA into TBM cells, a sodium transporting cell line which responds equally well to aldosterone and corticosterone, indicating that endogenous 11β-OHSD is expressed at a low level in these cells. Exogenous 11β-OHSD was expressed at high levels in transfected cells but mineralocorticoid specificity was not observed. By contrast, 11-DHC was readily converted into corticosterone, a potent agonist for sodium transport. Our results indicate that rat liver 11β-OHSD behaves predominantly as a reductase in TBM cells. Another kidney specific isoform is likely to be responsible for the dehydrogenase reaction in aldosterone responsive cells.

13

A NEW HPLC METHOD FOR THE SEPARATION OF MYCOBACTERIAL LIPID-ANTIGENS

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To characterize antigenetic properties of lipids from different mycobacteria, a one-step HPLC method for the separation of total lipids was developed.
After extraction and quantification, 200µg of mycobacterial lipids were adsorbed on a 5µm-sphere Cyano-Si-column (Beckman) in 20µl solvent A (hexane/dichlormethane/2-propanol/methanol/water; 30:56:3:10:1). Lipids were eluted within 24 min using a solvent gradient of exponentially increasing polarity. The gradient was formed by substituting solvent A with solvent B (hexane/dichlormethane/2-propanol/methanol/water; 25:24:4.5:42:4.5). Lipid fractions were monitored by high performance thin layer chromatography.
Obtained lipids were checked for their binding properties to serum antibodies of tuberculosis patients by a solid phase-immunoassay system.

14

MELANOCYTE DISTRIBUTION IN REISSNER'S MEMBRANE

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The melanocyte distribution of human Reissner's membrane (RM) were investigated in 12 cochleae from 4 patients with varying types of hearing loss (SNHL) and from 3 patients with age-related normal hearing (ARNH). RM is composed of an epithelium, a basal lamina with melanocytes and a mesothelium. The melanocytes were analyzed by light and electron microscopy on microdissected RMs. The percentage of melanocytes was in all patients 4 times less in the apical and lower basal turns than in the middle and upper basal turns. The number of melanocytes was increased in the apical and middle turns of RM from patients with SNHL in comparison to specimens from patients with ARNH. Melanogenesis was more active in cases with SNHL than in those with ARNH. Migration and activation of melanocytes along the cochlear turns are discussed in relation to possible pathogenesis of hearing loss.

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Quantitative electron microscopical analysis of Golgi apparatus disassembly induced by okadaic acid

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In interphase HeLa cells the phosphatase inhibitor okadaic acid induces Golgi apparatus disassembly to produce Golgi fragments similar to those in mitotic cells (Lucocq et al., 1991 J Cell Sci. In press). Stereological methods show that after 60 minutes of treatment with 1µM okadaic acid the amount of Golgi stack in suspension HeLa cells is reduced by >90%. This is explained mainly by a marked reduction in the extent of the stack and not the number of cisternae within it. After 40minutes tubular structures possessing coated buds appear but these are transient and by 60 minutes vesiculo/tubular clusters resembling mitotic Golgi clusters are observed in the cells. In ultrathin cryosections the gold labelling density for the Golgi marker protein galactosyltransferase, measured over Golgi stack profiles after 0,40,50 and 60 minutes treatment, does not vary by more than 20% . Within the clusters labelling is located mainly over indistinct structures that in optimum sections appear as a fine membranous reticulum. Qualitative observations indicate that coated or uncoated vesicular profiles within the clusters are not labelled. Taken together, these results indicate that during okadaic acid treatment disassembly of the Golgi cisternae is largely coordinated and proceeds toward Golgi clusters through a tubular intermediate. Galactosyl transferase appears to leave the stack but may not enter Golgi vesicle buds or vesicles, remaining in a fine reticulum within the Golgi clusters.

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A TEMPERATURE SENSITIVE MUTANT OF CHINESE HAMSTER OVARY CELLS EXPRESSES KEY PHENOTYPIC CHANGES ASSOCIATED WITH BREFELDIN A TREATMENT.

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The ts CHO cell mutant DS28-6 is pleiotropically defective in protein secretion. At nonpermissive temperature a time-dependent disassembly of the Golgi apparatus (GA) was found by electron microscopy. In parallel, a microtubule- and energy-dependent redistribution of GA mannosidase II (MII) and galactosyltransferase into an ER-like pattern was observed by immunofluoresence. Galactosylation of proteins in the ER occurred and was shown by lectin-gold staining. Further, NBD-ceramide exhibited an energy-dependent redistribution into the ER. In the mutant cells, the 110 kD GA-associated protein (ß COP) was co-redistributed with MII under nonpermissive temperature. All these effects were fully reversible upon shift to permissive temperature. Thus, DS28-6 cells exhibit key features of the Brefeldin A phenotype which suggests that Brefeldin A effects result from interference with a normally occurring cellular process.

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REDISTRIBUTION OF TWO TRANS-GOLGI MARKERS IN RESPONSE TC BREFELDIN A IN A RAT HEPATOCYTE CELL LINE.

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The behaviour of the *trans*-Golgi apparatus (GA) in response to Brefeldin A (BFA) has been studied in a rat epithelial hepatocyte cell line, RL-19, using immunofluorescence to follow changes in the distribution of two *trans*-GA markers, sialyltransferase (ST) and galactosyltransferase (GT). In untreated RL-19 cells, both GT and ST were localised as a perinuclear cluster of stain. GT labelling was restricted to this site whereas diffuse ST staining extended throughout most of the cytoplasm. 2-5 min after addition of BFA, tubules positive for GT were seen radiating from the GA. Similar structures were not seen by ST immunofluorescence. 5 min after the addition of BFA both Golgi enzymes showed a more extensive, punctate staining pattern. Further dispersal of GT and ST staining continued until after 30 min no cells showed a perinuclear spot of Golgi enzyme staining. Instead a weak fluorescence was seen in the region of the endoplasmic reticulum, identified by its glucosidase II immunoreactivity. When BFA was removed from cells previously exposed to the drug for 60 min, perinuclear GT and ST staining started to reappear after 5-10 min, typical GA staining being fully restored by 30 min.

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EFFECT OF BILE DUCT LIGATION (BDL) AND SUBSEQUENT RECANALIZATION (BDL-R) ON THE DISTRIBUTION OF DOMAIN-SPECIFIC ANTIGENS IN RAT HEPATOCYTES

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We characterized events resulting in BDL-induced impairment of cell polarity by combining immunofluorescence with the determination of protein secretion and receptor-mediated endocytosis. Our data demonstrate that BDL (1) leads to accumulation of pericanalicular vesicles containing canalicular (can=apical) antigens, (2) causes staining of can antigens in the basolateral (bl) domain associated with appearance of bl antigens in the can membrane, (3) does not impair secretion rate of albumin and transferrin, and (4) does not alter clearance of ASOR from circulation. BDL-R (5) results within minutes in a decrease of pericanalicular vesicles, (6) but does not restore the loss of domain specificity of can and bl antigens in 1 hr. We conclude (i) that altered domain-specific antigen distribution which may involve lateral diffusion through impaired tight junctions is restored slowly, (ii) that ER-to-bl and endocytic vesicular pathways are not affected significantly by BDL, and (iii) that inhibition of later steps in the bl-to-can pathway, possibly due to a fusion block, is relieved rapidly by BDL-R.

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PABA PEPTIDE HYDROLASE FROM HUMAN SMALL INTESTINE IS A MEMBER OF A NEW FAMILY OF METALLOPEPTIDASES

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PABA peptide hydrolase (PPH) is a metalloendopeptidase consisting of 2 subunits and is located in the microvillar membrane of human small intestine. Oligonucleotide-screening of a cDNA library from human small intestine yielded a near full-length clone for one of the subunits. This revealed the presence of a short pro-peptide on the N-terminus and a hydrophobic (membrane)-segment near the C-terminus. In addition, the putative metalloprotease domain with the pentapeptide motif HEXXH, known to represent a Zn^{2+}-binding site, was identified. Based on further similarities around this motif a new family of metalloendopeptidases can be defined. The simplest member of this family is Astacin, a crayfish metalloprotease (200 aa) which may be considered as the homologue of a protease domain in the other members which also include human bone morphogenic protein BMP-1, X. laevis UVS.2, and Tolloid from Drosophila.

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MATURATION OF HUMAN LACTASE-PHLORIZIN HYDROLASE

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Lactase-phlorizin hydrolase (LPH) is an enzyme of the microvillus membrane of small intestinal epithelial cells. LPH is synthesized as a precursor which is intracellularly cleaved to the mature enzyme. The questions regarding the site of cleavage and the protease(s) involved have been addressed using a colon carcinoma cell-line (Caco2) Incubation of Caco2 cells at 15°C or in the presence of Brefeldin A prevented proteolytic maturation of LPH, indicating that the cleavage of the precursor occurs after the Golgi.
Isolated LPH-precursor was hydrolyzed by an intracellular membrane fraction prepared from human small intestinal mucosa. One of the products showed electrophoretic properties analogues to mature LPH. The formation of this product was abolished by the serine protease inhibitors.Mature LPH on the other hand was resistant to hydrolysis by this fraction.

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PHOSPHATIDYLCHOLINE (PC) FLIPPASE IN CANALICULAR PLASMAMEMBRANES (cLPM) OF RAT LIVER

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Biliary secretion of phospholipids (PL) is selective for PCs, which do not spontaneously cross membrane bilayers. Two views are held: Secretion of PLs via exocytosis or release of PLs from the outer leaflet of the cLPM mediated by bile acids. This latter process could be mediated by a flippase in the cLPM. Using a filtration assay (Cell 52, 51 (1985) we determined the uptake of water-soluble L-α-dibutyroyl-(N-methyl-^{14}C)-PC (di-C_4-PC). Uptake of di-C_4-PC was linear in microsomal, basolateral (bLPM) and cLPM vesicles up to 1 min. and reached equilibrium at 5 min. In all three fractions the uptake occurred into an osmotic sensitive space, exhibited saturability and was not stimulated by ATP. At equilibrium, the uptake of di-C_4-PC into cLPM was only 285 % of that in bLPM and 185 % of that in microsomal vesicles. These data indicate the presence of a transport system for PC in the canalicular membrane.

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ESTABLISHING THE TWO-DIMENSIONAL MAP OF RED BLOOD CELL PROTEINS

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To detect posssible modifications of red blood cell (RBC) proteins under pathological conditions we are using two-dimensional gel electrophoresis. The aim is to identify RBC proteins by the position of their spots on the 2-dimensional map. Only few RBC proteins had been map earlier. Using commercially avaible RBC enzymes and other purified proteins, we extended our map. So far we identified: 6-phosphogluconic dehydrogenase, glyceraldehyde-3-phosphate dehydrogenase, glutathione peroxidase, superoxide dismutase, cholinesterase acetyl, and spectrin. In parallel we also establish a map of the RBC membrane proteins.

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PROCESSING OF PROINSULIN IN TRANSFECTED HEPATOMA CELLS

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We have transfected FAO (Hepatoma) cells with the rat insulin II gene driven by muMLV-LTR (DOL vector) in order to follow proinsulin II release and cleavage in cells expressing only the constitutive pathway. Immunoreactive products (IRI) released by the cells was greatly augmented (30 fold) by adding dexamethasone ($5x10^{-7}$M) to the medium; cellular IRI content was also increased, but, in keeping with constitutive release, little IRI was stored relative to that released. HPLC analysis of immunoreactive products released showed 3 major peaks, one coeluting with proinsulin II, the second with a conversion product (des 64-65 split proinsulin II) and the third with insulin II. Analysis of cellular extracts showed intact proinsulin II as the major product. Pulse-chase experiments confirmed these data, however the putative conversion product could be detected in cellular extracts. The generation of des 64-65 split proinsulin and insulin was not due to proteolysis of proinsulin after its release, but rather to an intracellular event. These data suggest that rat proinsulin II can be converted in the constitutive pathway albeit less efficiently than in secretory cells, the conversion compartment of the regulated pathway.

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ELECTROPHYSIOLOGICAL CHANGES INDUCED BY MERCURY AND SILVER AT THE BASOLATERAL SIDE OF RENAL EPITHELIAL CELLS (LLC-PK_1 AND MDCK-I)

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Two renal epithelial cell lines with proximal (LLC-PK_1) or distal (MDCK-I) characteristics were used to study the early electrophysiological changes induced by nephrotoxic heavy metals (Hg and Ag). Short-circuit current (Isc) and transepithelial resistance (R_T) were determined by using a modified Ussing chamber allowing separate perfusion of the apical and basolateral sides of cells grown on permeable filters. $HgCl_2$ or $AgNO_3$ (10 µM) applied to the basolateral side of the MDCK-I cells induced within 5 min an increase of 0.5 and 3.0 µA/cm^2, respectively over the basal Isc (0.4 µA/cm^2). In contrast for LLC-PK_1 cells in the same conditions, Hg and Ag induced a decrease of 5.5 and 8.2 µA/cm^2, respectively from the basal Isc (7.7 µA/cm^2). For both types of cells, Hg and Ag induced a decrease of the R_T. The presence of basolateral Ba (1 mM) markedly decreased the Hg- and Ag-induced Isc for MDCK-I cells. Hg and Ag at the basolateral side produce opposite effects depending on the basal characteristics of the cell line used. For MDCK-I cells, the Hg- and Ag-induced Isc is sensitive to Ba. Thus, Hg and Ag appear to open a Ba sensitive pathway to small cations in renal cell membrane.

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ASTED™ (Automated Sequential Trace Enrichment of Dialysates), A NEW METHOD FOR THE SCREENING OF BENZODIAZEPINES IN BLOOD SAMPLES.

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Up to now, most efforts have been devoted tc improve the analytic procedures whereas sample processing, particularly its automation, has been neglected. Here we report preliminary results on analysis of blood samples extracted with a Gilson ASTED™ automate. The procedure was as follows : blood samples (containing or spiked with benzodiazepines) were automatically 1) mixed with the internal standard and then 2) dialysed against t-butylmethylether in order to extract free drugs. After concentrating the ether down to 150 µl, extracted benzodiazepines were analysed by GC (HP-5 capillary columns) with dual injection, separation and detection (ECD and NPD). This procedure was compared with a conventional liquid-liquid extraction method, using n-butyl acetate as solvent. Our results show that both methods give clean separations and allow benzodiazepine detection. The strategy used for optimising the ASTED™ method and the results obtained will be discussed.

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ELECTRON MICROSCOPY OF SEMINAL VESICLES FROM MONKEYS EXPOSED TO LEAD: A 9-YEAR STUDY

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Effects of persistent exposure to lead (Pb) were studied on the seminal vesicles (SV) of cynomolgus monkeys. This is part of a project designed to examine the long-term reproductive effects of Pb-exposure to the animals. The neonates were divided into control (n=3), 400-day-to 9 year (n=2), and 9-year-exposed (n=3) groups. The exposed animals were given 1,500 µg/kg BW/day of Pb. SV specimens were obtained following euthanasia, fixed in phosphate-buffered 2% glutaraldehyde, and were processed by conventional methods. Images of SV from unexposed animals showed the gland acini lined by two cell types: secretory, that was predominant, and basal. In the former, apical secretory droplets consisted of pleomorphic matrices that were surrounded by halos; RER cisternae with narrow lumina were abundant, bulk of which were contained in the cell, proximally. In Pb-exposed animals, SV secretory epithelium had droplets with atypical morphology, and proximally, abnormal amounts of lipid droplets. The quantity of RER cisternae had apparently diminished. There was no evidence of a Pb-induced effect on circulating levels of gonadotropins or testosterone; the spermatogram was also unaffected. Conclusion: morphological alterations were induced by the Pb-exposure in the SV without evidence of functional changes.

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PARTITION COEFFICIENT: I. LIPOSOMAL VERSUS OCTANOL/ BUFFER SYSTEM FOR DETERMINATION

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The partition coefficient is an important parameter for drug design. It characterizes the lipophilicity of a molecule and thus its in vivo distribution, protein binding, metabolism and excretion. In vitro determinations traditionally have been performed in the n-octanol/buffer system, however, published values show large variations. As we could demonstrate, part of the problem stems from the fact that octanol itself shows pH-dependent partitioning in buffer, which in addition is also strongly dependent on the respective volumes of the lipophilic and hydrophilic phase. To generate a more reliable partition system, liposomes are being studied as an alternative lipophilic phase. As a model drug the β-adrenergic blocking agent dl-propranolol is used. Partitioning of this molecule between liposomes and buffers of various pH values is determined by a two chamber equilibrium dialysis. The use of ^{3}H-labeled drug and ^{14}C-labeled lipid bilayers provides a very sensitive analytical procedure for the measurement of the respective concentrations.

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PARTITION COEFFICIENT: II. CELL CULTURES AS A BIOLOGICAL PARTITION SYSTEM

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The pharmacokinetic behaviour of a drug is determined by its lipophilicity. The latter is expressed as the partition coefficient examined traditionally in the octanol/buffer system. In vivo, the nephron for instance acts as a "biological polarimeter": cell membranes represent the lipophilic, urine the hydrophilic phase. The amount of a drug excreted unchanged through the kidney directly depends on its partition behaviour. Therefore, we decided to design an in vitro partition system based on cell cultures. As lipophilic phase we chose kidney epithelial cells (MDCK) cultured on microcarriers, which can be exposed to various hydrophilic phases (buffers, pH). As a model substance ^{3}H-propranolol, a beta-adrenergic blocking agent, is used. For quantification of the lipophilic phase, cells are labeled with ^{14}C-amino acids. Partitioning of the drug under various conditions is studied by means of a two chamber equilibrium dialysis procedure.

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SPONTANEOUS LIPID TRANSFER BETWEEN LIPOSOMES DEPENDS ON THE NATURE OF THE TRANSFERRED LIPID AND ON THE LIPOSOME SIZE

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One possible interaction between lipid bilayers is spontaneous lipid transfer. We investigate this transfer by using three model markers: [^{14}C]dipalmitoylphosphatidylcholine (DPPC), [^{3}H]glycerol trioleate and/or the fluorescent lipid octadecyl-rhodamine B (R18). These markers are incorporated in small or large unilamellar liposomes, composed of phosphatidylcholine, phosphatidic acid, cholesterol and ganglioside G_{D1a}. After incubation, the liposome mixture is separated according to size by column chromatography and the amount of the markers recovered in donor and acceptor vesicles is determined. We find significant transfer for all three markers, the extent being largest for R18, intermediate for DPPC and smallest for glycerol trioleate. This correlates well with the number of lipophilic tails which serve as anchors in the membrane. In addition, transfer depends on membrane curvature, since much less transfer occurs, when large instead of small liposomes are used as donor vesicles.

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PARTICLE INHALATION UNDER ARTIFICIAL VENTILATION

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To investigate deposition, retention and clearance of inhaled particles, the breathing mode (tidal volume, respiratory frequency, end-expiratory lung volume or "level of respiration") during exposure has to be standardized. Therefore, we improved our exposure system for small rodents with a setup for controlled continuous-negative-pressure-ventilation. Anesthetized and paralyzed hamsters were differently ventilated and exposed to monodisperse 6 µm polystyrene particles via tracheal cannula. Particle concentration and flow rate were measured on-line by laser-light-scattering photometry and pneumotachography. Few minutes after exposure the lungs were fixed by intravascular perfusion and then processed for light and electron microscopy. The number of retained particles assessed stereologically was in good agreement with the photometrically obtained number of deposited particles, suggesting that no significant amount of particles was cleared until fixation of the lungs.

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COMPOUNDING AND STORAGE OF ALL-IN-ONE (AIO) ADMIXTURES OF TOTAL PARENTERAL NUTRITION (TPN): MICROBIAL SAFETY ASPECTS

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AIO TPN admixtures can not be sterilized after preparation. Thus, safe compounding has to respect strict aseptic technique. The compounding procedure was validated in a hospital pharmacy over a period of 14 months. To imitate standard TPN compounding procedure corresponding volumes of sterile peptone medium were filled from individual infusion glass bottles into 1008 two liter mixing bags. TPN and media bags were prepared in parallel under laminar air flow conditions. Incubation of the media-filled bags at 33°C (14 days) and subsequently at 20-25°C (2 days) revealed a contamination rate of 2.0% : 19 Bacillus sp., 1 Penicillium sp. These results documented a proper aseptic manufacture technique without contamination by human pathogens. Microbial growth characteristics of 9 different test strains in artificially contaminated TPN admixtures depended on species, osmolality, presence or absence of fat, and temperature. In conclusion, AIO TPN admixtures may be prepared safely by a validated aseptic preparation technique. To prevent microbial growth, storage of such admixtures at 2-8°C until administration is mandatory.

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ATYPICAL PROPOFOL BINDING TO HUMAN SERUM ALBUMIN FOUND BY A NEW, HIGHLY SENSITIVE ASSAY FOR THIS PHENOLIC ANESTHETIC

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In order to investigate propofol (P) binding to human serum albumin (HSA) equilibrium dialysis was used in 1/15 M phosphate buffer (pH 7.4). P concentration was determined by HPLC; the apparatus consisted of two pumps (A and B), an extraction- and analytical-column (both filled with RP-18), a loop-type injector, a 6-way valve and a fluorescence detector. An aliquot of the sample was injected without any sample preparation directly onto the extration column in a polar eluate (pump B). The polar sample constituents such as HSA passed through and were eluted to waste, whereas the highly lipophilic P was retained on the extraction column. After to 2 min the valve was switched, whereby the analytical eluate (pump A) backflushed the loaded P from the extraction to the analytical column. P was detected by fluorescence (excitation 276 nm, emission 310 nm). Using 1% HSA the percentage of P bound **decreased** with lower P concentrations from 75% at 100 µg/ml to 50% at 10 ng/ml. It is assumed that P causes conformational change of the HSA molecule with the effect of an enlarged binding capacity for itself at higher substrate concentrations.

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MOLECULAR MIMICRY OF TRIFLUOROACETYLATED PROTEIN ADDUCTS BY CONSTITUTIVE POLYPEPTIDES

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Trifluoroacetylated protein adducts (TFA-adducts) arise upon the oxidative, cytochrome P-450 dependent, metabolism of the anesthetic agent halothane. Sera of patients with halothane hepatitis (a fulminant massive liver necrosis associated with halothane) but not those of according control individuals contain antibodies directed against such TFA-adducts.
A monoform antibody (anti-TFA antibody), obtained by affinity purification of a polyclonal anti-TFA-RSA serum on a N-ε-TFA-L-lysine matrix, recognized TFA-adducts of distinct molecular masses on Western blots of liver homogenates of rats exposed to halothane. Two crossreactive constitutive polypeptides of apparent molecular masses of 52 kDa and 64 kDa were also recognized on Western blots of control homogenates of several rat tissues and of human liver. Experimental data indicated that epitopes on these constitutive polypeptides confer molecular mimicry of epitopes on rat and human liver TFA-adducts, of which the TFA-moiety is a dominant part. Additional data suggested that the absence or low levels of expression of these constitutive polypeptides may render human individuals susceptible for development of halothane-induced hepatitis. Currently,the constitutive 64 kDa crossreactive polypeptide of the rat heart is being identified as the E_2 subunit of pyruvate dehydrogenase.

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METABOLISM OF HYDROCHLOROFLUOROCARBONS (HCFCs): IDENTIFICATION OF TRIFLUOROACETYLATED POLYPEPTIDES IN THE LIVER AND KIDNEY OF RATS

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Due to the high ozone-depleting potential of chlorofluorocarbons (CFCs), which are widely used as refrigerants, solvents, foam blowing agents and aerosol propellants, hydrated CFCs (HCFCs) are being developed as non ozone-depleting substitutes. Cytochrome P-450 dependent metabolism of HCFCs such as FC-123 (2,2-dichloro-1,1,1-trifluoroethane) leads to the formation of reactive intermediates which may covalently bind liver cellular macromolecules.
A monoform antibody directed against trifluoroacetylated protein-adducts (TFA-adducts) recognized a variety of TFA-adducts of distinct molecular masses on Western blots of liver homogenates of rats exposed to FC-123. Surprisingly, TFA-adducts were also detected in the kidney. These results were corroborated by immunohistochemical analysis of tissue samples of rats exposed to FC-123 in which TFA-adducts were detected in the liver and also in the kidney. TFA-adducts could be generated in vitro by incubating FC-123 with liver microsomes but not with kidney microsomes. FC-123 could be substituted for by the anesthetic agent halothane (2-bromo-2-chloro-1,1,1-trifluoroethane), a structural analog of FC-123. Liver-derived GSH-conjugates of the reactive intermediates of metabolism of both FC-123 and halothane are discussed as mediators of TFA-adduct formation in the kidney.

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FREE FATTY ACIDS AND LOW CONCENTRATION OF FETAL CALF SERUM STABILIZE XENOBIOTIC METABOLISM OF PRIMARY CULTURES OF RAT HEPATOCYTES.

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The maintenance of an intact xenobiotic metabolism in cultured hepatocytes is an essential prerequisite for the performance of toxicity studies in vitro. During the first three hours of culture, low concentration of fetal calf serum (0.05%) and over the whole culture period of two days free fatty acids (palmitic acid 2.35µM, palmitoleic acid 0.21µM, stearic acid 0.88µM, oleic acid 1.18µM, linoleic acid 2.71µM and linolenic acid 0.43µM) were added. Attachment of cells, morphology, membrane integrity (LDH release), protein content, hepatocellular functions (albumin secretion) and cytochrome P-450 content as well as activities of enzymes involved in xenobiotic metabolism (aldrin epoxidase and ethoxyresorufin-O-deethylase) were determined. Free fatty acids and low concentration of fetal calf serum stabilized membranes of cultured hepatocytes. Furthermore cytochrome P-450 content and enzyme activities of xenobiotic metabolism were increased two fold compared to standard culture conditions.

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How does Vitamin E inhibit drug accumulation in cultured cells?

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The presence of vitamin E (10-100µM) decreased the accumulation of cationic amphiphilic drugs (CAD) in exposed cultured cells. This effect was accompanied by a decrease in drug induced lysosomal phospholipid (PL)-storage. Initial CAD uptake was hardly inhibited by vitamin E, whereas longterm and repetitive CAD exposures were increasingly affected in a dose and time dependent manner.
To test for the hypothesis that vitamin E interferes with the formation of lysosomal CAD-PL complexes an equilibrium dialysis technique was used. Desipramine as a model-CAD was dialysed against suspended liposomes of various PL-compositions at pH-values of 7.4 and 5.4 respectively. Liposomes showed high affinities and capacities for the binding of CAD. These binding parameters were, however, not altered by the presence of vitamin E under the chosen conditions. From this negative result of the equilibrium dialysis we conclude that the effect of vitamin E cannot be explained by direct interference with the CAD-PL complex formation.

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DETECTION OF COCAINE AND ITS METABOLITES (BENZOYL-(BE) AND METHYL-ECGONINE (EME)) IN BIOLOGICAL SAMPLES: COMPARISON OF DIFFERENT METHODS.

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Cocaine abuse is becoming in Switzerland an important problem for Public Health.
Laboratories are challenged to find reliable methods able to detect trace amounts of cocaine in biological matrixes of various complexity (e.g. urine, hair). Many extraction, derivatisation and analytical procedures for cocaine analyses have been published recently but their efficiencies have not been compared as yet. Results concerning the evaluation of two derivatisation methods are reported here (perfuoropropionylation and silylation). Cocaine and the derivatives of its two major metabolites (BE and EME) were analysed by GC/MS under electron impact and chemical ionization modes (positive and negative ions). The advantages and disadvantages of each procedure will be discussed.

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A CELL CULTURE MODEL FOR THE STUDY OF DRUG DISTRIBUTION

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Cell culture models are successfully used for the study of cellular drug kinetics. In order to investigate competitive uptake, distribution and redistribution of drugs between different cell types a culture model system was developed. Different cell lines were separately grown on glass slides. At confluency they were transferred into one culture plate and exposed to cationic amphiphilic drugs. Both, the kinetics of competitive drug uptake and of drug redistribution could be monitored in short and long term experiments. With this multi-culture model system and the choice of appropriate cell lines, in vivo conditions of drug distribution can be simulated.

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PIROXICAM 5'-HYDROXYLATION IS CATALYZED BY HUMAN CYTOCHROME P450TB (CYP2C)

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The NSAID piroxicam (PX) is extensively metaboiized by liver oxidation in humans. The role of a specific cytochrome P450 isozyme (P450TB, *CYP2C* subfamily) in its major biotransformation pathway, oxidation to 5'-hydroxy-piroxicam (OPX), was evaluated in human liver microsomes. PX and tolbutamide (TB), the model substrate of P450TB, were incubated and the production of both OPX and hydroxytolbutamide (OTB) was measured by HPLC with UV detection. OTB production was determined in microsomes from one poor and 4 extensive metabolizers of debrisoquine (*CYP2D6*). Reciprocal inhibition experiments were performed and the influence of sulfaphenazole, a selective and potent inhibitor of P450TB, on both oxidations was investigated. Piroxicam (Km = 54±13 μM, Vmax = 5.9±2.4 nmol/mgP/h, CLint = 107±.29 L/mgP/h) and tolbutamide (Km ≈ 150 μM, Vmax ≈ 23 nmol/mgP/h, CLint ≈ 150 L/mgP/h) exhibited Michaelis-Menten kinetics. The debrisoquine phenotype did not influence OPX production. PX competitively inhibited TB hydroxylation (Ki ≈ 40 μM) and, conversely, TB competitively inhibited PX 5'-hydroxylation (Ki ≈ 300 μM). Sulfaphenazole was an equally potent competitive inhibitor of both PX and TB oxidations (Ki < 0.2 μM). These results demonstrate that cytochrome P450TB plays a major role in piroxicam biotransformation in human liver.

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DICLOFENAC 4'-HYDROXYLATION IS CATALYZED BY HUMAN CYTOCHROME P450TB (CYP2C)

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The NSAID diclofenac is extensively metabolized by liver oxidation in humans. Our goal was to identify the isozyme responsible for the major biotransformation pathway, 4'-hydroxylation, of this high clearance drug. In vitro inhibition data for major human P450s indicated P450TB (*CYP2C* subfamily) as the most likely candidate. Diclofenac was incubated in human liver microsomes and the production of 4'-hydroxydiclofenac (HOD) was measured by HPLC with UV detection. The inhibition potency of tolbutamide, the model substrate of P450TB, and sulfaphenazole, a selective and potent inhibitor of this isozyme, were determined in microsomes from one poor and 4 extensive metabolizers of debrisoquine (*CYP2D6*). Diclofenac exhibited Michaelis-Menten kinetics (Km ≈ 5 μM, Vmax ≈ 350 nmol/mgP/h, CLint ≈ 70 L/gP/h). The debrisoquine phenotype did not influence HOD production (270 ± 217 nmol/mgP/h, diclofenac 10 μM). Sulfaphenazole competitively (Ki ≈ 0.2 μM) inhibited the reaction. Inhibition by both tolbutamide and sulfaphenazole was similar in all livers, with values (51 ± 5 and 48 ± 6 %, resp.) predicted from their previously determined affinities for P450TB and from diclofenac affinity determined in a separate experiment. These results demonstrate that cytochrome P450TB plays the major role in diclofenac biotransformation in human liver.

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EFFECTS OF MOCLOBEMIDE TREATMENT ON PLASMA L-DOPA AND CATECHOLAMINE METABOLITES IN HEALTHY VOLUNTEERS

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Plasma L-Dopa, catecholamines (CA) and their deaminated metabolites were measured (HPLC-EC) in 8 healthy subjects (aged 20-26) after treatment with placebo (PL, 4 days) and the monoamine-oxidase (MAO) inhibitor moclobemide (MO, 2x200 mg p.o., 7 days). Blood was sampled on the last day of each treatment before and for 4h after a dose of PL or MO (200mg). Two-way ANOVA showed a significant effect of treatment on plasma L-Dopa ($P<0.01$) and 3,4-dihydroxyphenylglycol (DHPG, $P<0.05$) concentrations. Both substances were lower (by 30-40%) after MO vs PL at corresponding times ($P<0.05$-0.01, by paired 't'). Plasma CA did not change after MO, while the other CA metabolites measured decreased not significantly (MHPG -29% P=0.06, vanilmandelic acid -26% P=0.09, DOPAC -31% P=0.09, homovanillic acid -20% P=0.08, vs PL at 4h). The decreases in DHPG, a product of intraneuronal deamination of noradrenaline, and in L-Dopa, a CA precursor whose synthesis by tyrosine-hydroxylase is inhibited by a rise in cytosolic CA, may be viewed as indices of MAO-A inhibition by MO treatment.

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SMALL SCALE FIELD TEST EXPERIMENT OF TRANSGENIC BINTJE POTATO RESISTANT TO PVY

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Transgenic Bintje potato plants (Bt6) that express the PVY^N 605 coat protein gene were obtained. A small scale field test experiment was performed to determine if the good PVY-resistance observed in the greenhouse was maintained in open air, and if the Bt6 plants are similar morphologically and physiologically to the ordinary Bintje potatoes. Having received the authorizations of the KOBAGO and SKBS commissions and of the federal government, the tubers were planted the 10th of May 1991. Tubers infected with PVY^N or PVY^O were disseminated in the plot to serve as virus source for the aphid-mediated transmission. The heavy aphid infestation of the spring led to a very rapid transmission of the viruses, as by mid June the first symptoms were seen on the ordinary Bintje. In the beginning of July, all the Bt6 were still symptomless whereas all the non-transformed Bintje were infected. Three ELISAs performed on the young leaves during the summer confirmed this result : 98% of the ordinary Bintje and only 22% of the Bt6 plants were PVY-infected. The tuber were harvested Sept. 4th.
An ELISA performed on three sprouting tuber per plant showed that all the ordinary Bintje were infected, and that all the Bt6 were virus-free. This good result is very promising. A general observation is that the Bt6 plants were greener and more juvenile than the ordinary Bintje, and we could see that 35% of the Bt6 tubers had a very elongated shape.

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ISOLATION AND CHARACTERIZATION OF ELICITORS AND SUPPRESSORS OF THE PLANT STRESS RESPONSE IN TOMATO

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We have used the induction of ethylene biosynthesis in suspension cultured tomato cells as a bioassay to characterize elicitors derived from yeast glycoproteins. Using a multi-step procedure involving ion-exchange chromatography, lectin affinity chromatography and high-performance liquid chromatography, we have purified several glycopeptide elicitors to apparent homogeneity. The most active of these elicitors induce ethylene biosynthesis half-maximally at concentrations of 5 nM. Chemical modification and enzymatic digestion show that both the glyco- and the -peptide parts are essential for activity. The peptide parts alone have no apparent biological activity. However, the oligosaccharides act as potent suppressors of elicitor activity. They inhibit elicitor-dependent induction competitively and specifically, suggesting that both, the glycopeptide elicitors and the oligosaccharide suppressors bind to the same recognition site.

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STIMULATION BY CALMODULIN OF Ca^{2+} TRANSPORT IN ENDOPLASMIC RETICULUM VESICLES FROM MAIZE ROOTS

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Maize (*Zea mays* L., cv LG 11) root membranes were fractionated by sucrose density gradient centrifugation (in the presence or absence of Mg^{2+}). Without Mg^{2+}, a single peak of calcium uptake was recovered at a density of 1,11 g/cm³ together with a protein peak corresponding to low density membranes (tonoplast, ER and dictyosomes). The Ca^{2+} transport activity was slightly inhibited in the presence of monensin (a proton ionophore), whereas vanadate was strongly inhibitory. When Mg^{2+} was added to the homogenization and gradient buffers, the apparent ER density increased to 1,16 g/cm³, with the ER being separated from most of the PPase activity (tonoplast marker). Under these conditions, two peaks of Ca^{2+} uptake were recovered in the gradient. The first peak, co-migrating with the PPase, was inhibited by monensin and corresponded to the tonoplast Ca^{2+}/H^+ antiport system. The second peak, co-migrating with the NADH-cytochrome c reductase (ER marker), was vanadate-sensitive and corresponded to the ER Ca^{2+}-ATPase. The Ca^{2+} transport activity into ER vesicles was strongly stimulated by calmodulin (2 µM). The plasmalemma vesicles were never sealed under our experimental conditions, and no Ca^{2+} transport can be associated with this membrane.

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PURIFICATION OF THE PYROPHOSPHATE-DEPENDENT PROTON PUMP FROM *RUBUS* CELL CULTURES

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Two proton-translocating activities are present on the tonoplast of higher plant cells: an anion-sensitive ATPase and a cation-sensitive pyrophosphatase (PPase). Both pumps generate an electrochemical potential difference of protons, serving as the driving force for several transport processes (uniport, antiport and symport) across the vacuolar membrane. The PPase from dicotyledonous plants is composed of one major polypeptide with a Mr of about 65'000 (red beet) or 73'000 (mung bean), whereas the PPase from maize roots (a monocotyledonous plant), is composed of two polypeptides with Mr values of around 40'000 and 41'000. A high activity of H^+-PPase is present on the tonoplast of *Rubus* cell cultures. The PPase from *Rubus* cell cultures was solubilized and purified, with the aim of understanding the differences in the polypeptide composition among monocotyledonous and dicotyledonous plants.The procedure was as follows: solubilization in presence of detergent (Triton X-100), gel filtration chromatography (Superose 6 Prep Grade), anion exchange chromatography (Mono Q HR) and SDS-PAGE. Data on the biochemical and immunological characterization of the PPase from *Rubus* will be presented and discussed.

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PATHOGEN-INDUCED GENES IN WHEAT

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The onset of induced resistance in wheat to the fungal pathogen *Erysiphe graminis* f.sp. *tritici* (powdery mildew) correlates with the activation of putative defense genes that were named Wheat Induced Resistance (WIR) genes (Schweizer et al., Plant Mol. Biol.12, 643-654, 1989). We have identified 4 classes of WIR genes. Two gene classes encode well known stress proteins, namely peroxidases (WIR 3) and thaumatin-like proteins (WIR 2). The WIR 5 genes encode glutathione-S-transferases (GST). WIR 1 encodes a novel class of small proteins with a hydrophobic N-terminus and a hydrophilic C-terminal domain rich in glycine and proline. The WIR 1 gene product is speculated to be an integral membrane protein with its C-terminal tail sticking into the cell wall.To learn more about their function, attempts are in progress to localize some of the WIR gene products in induced wheat and in transgenic tobacco plants overexpressing the WIR proteins.

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REGULATION OF THE ß-1,3-GLUCANASE B PROMOTER IN TRANSGENIC TOBACCO.

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The basic, vacuolar ß-1,3-glucanases are hormonally and developmentally regulated in tobacco. The enzymes are localized in the lower leaves and roots of the plant and are induced in leaves by treatment with the stress hormone ethylene or pathogens such as TMV. Although neither auxin nor cytokinin alone are sufficient, combinations of the two hormones block the ethylene mediated induction in cultured cells.These ß-1,3-glucanases are encoded by a small gene familiy of ≈ 4 members. We transformed tobacco cells with an expression vector containing 1.6 Kb of 5' flanking sequence of one member of this family, the ß-1,3-glucanase B gene, transcriptionally fused to the coding sequence of E. coli ß-glucuronidase (GUS). The GUS activity varied markedly in comparable leaves of plants regenerated from independent transformants (range 2.2-238 pkat/g FW, 21 transformants). Nevertheless, GUS activity and ß-1,3-glucanase contents exhibited similar patterns of regulation. Therefore the 1.6 Kb promoter region must contain the elements necessary for correct developmental, hormonal and pathogen regulation. The identification of cis-acting elements controlling the regulation of this promoter is in progress.

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STUDY OF THE PHYSIOLOGICAL FUNCTIONS OF ß-1,3-GLUCANASE IN ANTISENSE TOBACCO (NICOTIANA TABACUM) TRANSFORMED PLANTS

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The basic, vacuolar isoforms of ß-1,3-glucanase (class I) are induced in tobacco plants by ethylene treatment or infection with pathogens. Moreover, they exhibit a tissue-specific localization in roots and lower leaves. In cultured cells, ethylene-dependent expression of class I ß-1,3-glucanases is down-regulated by the hormones auxin and cytokinin. Here we used antisense transformation to identify possible physiological functions of these enzymes. Plants were transformed with a Ti-plasmid binary vector containing part of the class I ß-1,3-glucanase A coding sequence in reverse orientation regulated by Cauliflower Mosaic Virus 35S RNA expression signals and a chimeric gene encoding kanamycin resistance as a selectable marker. Tissues of plants homozygous for the transgene exhibited levels and activity of class I ß-1,3-glucanases ca. 10% or less that of comparable tissues of control plants. Induction of the enzymes by ethylene treatment was also markedly inhibited in antisense transformants. Virus infection (TMV: tobacco mosaic virus) also did not induce class I ß-1,3-glucanases in antisense plants. Nevertheless, these plants exhibited normal growth, development, and the susceptibility to the pathogen tested was apparently not changed. The possible role of tobacco class I ß-1,3-glucanases as "housekeeping" function and their role in plant defence mechanism will be discussed.

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ABCSISIC ACID REGULATION OF ß-1,3-GLUCANASE IN TOBACCO (*NICOTIANA TABACUM*) PLANTS.

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In tobacco cell culture the basic, vacuolar class I ß-1,3-glucanases are strictly regulated by plant hormones. They are induced by ethylene and down regulated by auxin and cytokinin in the early phase of the culture. We are interested in knowing if other phytohormones are involved in the regulation of class I ß-1,3-glucanases. Cultured tobacco cells were treated with abcsisic acid (10^{-7} to 10^{-5} M). Cells cultured in auxin and cytokinin medium show an initial lag phase of 6 days before class I ß-1,3-glucanases increase in the cultures. Addition of abcsisic acid resulted in a dose-dependent prolongation of this inhibitory phase up to 5 days. The physiological and developmental significance of this effect will be discussed.

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MOLECULAR CLONING OF ABSCISIC ACID-REGULATED GENES OF SPIRODELA POLYRRHIZA

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The aquatic angiosperm Spirodela polyrrhiza forms dormant buds (turions) in response to low temperature and exogenous abscisic acid (ABA). The role of ABA in the transduction of the low temperature signal is under investigation. Differential screening of a cDNA library from ABA-treated fronds has led to the isolation of three ABA-induced cDNAs. Analysis of the temporal and spatial regulation of these genes by Northern blot analysis and in situ hybridisation will be presented.

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INTRACELLULAR ROUTE AND MATURATION OF TOBACCO VACUOLAR β-1,3-GLUCANASE

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Class I β-1,3-glucanases are vacuolar enzymes implicated in the defense reaction of plants to pathogens. We used pulse-chase experiments and cell fractionation to trace the processing and intracellular transport of the tobacco enzyme to the vacuole. The results are consistent with the following model: The enzyme is produced as a preproprotein with an N-terminal signal peptide, which is removed co-translationally as the nascent protein enters the ER. The protein is also co-translationally N-glycosylated at a single site near its C-terminus. The resultant N-glycoprotein is transported through the endomembrane system to the vacuole where a C-terminal N-glycopeptide is removed by proteolytic cleavage to give the mature protein which is not N-glycosylated. Experiments with the inhibitor tunicamycin indicate that neither maturation of the protein nor its targeting to the vacuole require N-glycosylation.

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THE VACUOLAR TARGETING SIGNAL OF TOBACCO BASIC CHITINASES.

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Tobacco (*Nicotiana tabacum*) plants infected by pathogens produce several different chitinases. The basic isoforms are localized in the vacuole while the acidic isoforms are extracellular. The basic isoforms differ from the acidic isoforms not only by many single amino acid changes but also by additional sequences at the C-terminal and N-terminal ends. By oligonucleotide mutagenesis and subsequent in vitro rearrangements the N- and C-terminal sequences of the basic chitinase were individually removed and added to another unrelated protein, the extracellular chitinase of cucumber. The various constructions were placed under the control of the CaMV 35S promotor and introduced into *Nicotiana sylvestris* plants by *Agrobacterium* -mediated leaf disc transformation. The transgenic plants were analysed by the extraction of intercellular washing fluid and by the isolation of protoplasts and vacuoles. The hevein domain was found to be irrelevant for both localization and enzymatic activity of the basic chitinase. Deletion of the 7 C-terminal amino acids caused secretion of the tobacco chitinase. Addition of this C-terminal sequence to the cucumber chitinase caused the vacuolar accumulation of the modified chitinase. Experiments are in progress to determine the minimal sequence required for vacuolar targeting.

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PROTEINS AND CHITINASE IN *PARTHENOCISSUS QUINQUEFOLIA* CULTURED *IN VITRO*

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Protein content, protein synthesis and degradation were analysed in *Parthenocissus* callus, in relation with growth. Its latent phase is characterized by an increase - and the growth phase by a decrease - in protein level. This decrease results from a high protein synthesis and from an enhancement of aminopeptidase content.

Chitinase activity is also correlated to cell growth. Chitinases are shown to be extracellular proteins, but a small amount of their activity is found in vacuoles.

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INDUCTION OF ENZYME ACTIVITIES IN GROUNDNUT LEAVES INFECTED WITH *CERCOSPORA ARACHIDICOLA*

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Necrosis of groundnut (*Arachis hypogaea* L.) leaves, upon infection with the early leafspot pathogen (*Cercospora arachidicola* Hori), is characterised by the accumulation of phenolic compounds and the rapid disappearance of starch granules. β-Glucanase and peroxidase activities in the plant apoplast increase significantly during infection to reach a maximum 20 days after inoculation when the symptoms are fully developed. On the other hand, chitinase activity remains at a low and constant level. The β-glucanase activity, which is endogenous, increases about 20-fold and is represented by 1 basic and at least 2 acidic isoforms which all exhibit endo-(1→3)-β-glucanase activity. The hyphal cell wall of the pathogen is mainly composed of (1→3)-β-glucan (30%) and a lesser amount of chitin (8%). Incubation of the purified cell walls with leaf apoplastic fluid produces oligosaccharides, containing essentially glucose, which elicit phytoalexins in suspension cultures of groundnut cells. It seems reasonable to suppose that the role of the enhanced β-glucanase activity is to attack the fungal cell wall and thereby constitutes a defence mechanism.

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INHIBITION OF TOMATO PECTIN METHYLESTERASE BY KIWI PECTIN METHYLESTERASE INHIBITOR

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The inhibition of tomato pectin methylesterase (PME) by kiwi pectin methylesterase inhibitor (PMEI) is reported. Tomato PME was consecutively purified by CM Sephadex C-50, Concanavalin A Sepharose 4B and Mono S chromatography, while kiwi PMEI was purified by Q-Sepharose and Sephacryl S-200 chromatography. Inhibition of PME activity under optimal conditions (0.125 M NaCl, pH 7.5) was found to be uncompetitive with an apparent Ki of 0.18, 0.16 and 0.11 % apple pectin for PME/PMEI ratios of 0.2, 0.5 and 1.0, respectively. Inhibition of PME activity was independent of the PME/PMEI ratio at around 50 % of the maximal uninhibited activity, and it was most effective in the pH range of 4 to 7. Neither heating to 120°C nor trypsin digestion affected the inhibitory power of PMEI towards purified tomato PME.

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CHARACTERIZATION OF CHORISMATE SYNTHASE cDNA CLONES FROM TOMATO

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Chorismate synthase (CS; EC 4.6.1.4) catalyzes the last common step in the biosynthetic pathway of the aromatic amino acids. This shikimate pathway occurs exclusively in plants, fungi and microorganisms. We report on the first isolation of CS specific cDNA clones of tomato.
Two degenerate oligonucleotides, corresponding to highly conserved sequences of known CS have been used for a Polymerase Chain Reaction (PCR) with ss cDNA as template. Fragments of the expected size were subcloned and sequenced. One fragment which showed homology to the known CS sequences was used to screen a tomato cDNA library. Two different classes of cDNA clones were isolated, suggesting at least two CS genes per haploid tomato genome. Results of the analysis of these genes will be presented.

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New stilbenes in grape berries before and after flowering

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Before flowering, free phenolics are mainly represented by three unidentified stilbenes (about 60 % of total free phenolics) with high retention time (26-30 min) on HPLC (C_{18}, 250x4.5 mm, gradient MeOH-H_2O 5:95 to pure MeOH). Two weeks after flowering these stilbenes diseapear and three other stilbenes (about 50 % of total free phenolics) with lower retention times (10 to 14 min) appear. The other phenolics present at both developmental stages of berries are identified as caffeic, p-coumaric, ferulic and 3,4 dihydroxybenzoic acid as well as resveratrol (3,5,4'-trihydroxy stilbene). The stilbenic character of these six important phenolic compounds are shown by their characteristic UV spectra. The identification of their strucutres are in progress.

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Betalain synthesis in *A.muscaria*

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Betalains are yellow or violet pigments found in plants belonging to the *Caryophyllales* and in some fungi, e.g. *A. muscaria*. The biosynthetic pathway of betalains is controlled by three *loci* in higher plants. The key enzyme is DOPA-4,5-dioxygenase: cleavage of the aromatic ring of DOPA gives rise to seco-DOPA and then to the betalain chromophore betalamic acid (pale yellow).
The purification procedure for DOPA-4,5-dioxygenase was improved, so that several mg of the protein could be isolated and polyclonal antibodies were raised. It is shown that the accumulation of betalains and of DOPA-dioxygenase is developmentally regulated and that enzyme expression is tissue specific. The similarity of fungal DOPA-4,5-dioxygenase with the corresponding enzymes from higher plants was investigated by Western blotting and by immunoprecipitation of radiolabèled extracts.

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The Effect of Initial Carbon Concentration on Culture Growth and Betalain Biosynthesis in High Betacyanin-Yielding Cell Suspensions of Beta vulgaris L.

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The betalains, a class of pigments comprising both the yellow coloured betaxanthins and the violet betacyanins, are found naturally occurring in plants of the order *Centrospermae* and certain fungal species (mushrooms), such as *Amanita muscaria*. Violet coloured *B. vulgaris* cell suspension cultures were derived from a high betacyanin-yielding callus line. Cell suspensions were maintained in liquid media containing a range of initial sucrose concentrations. We report here the effect of initial carbon concentration on biomass and betacyanin accumulation in *B. vulgaris* cell suspensions and examine the effects of switching from carbon- to nitrogen-limited culture growth on cell metabolism.

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LIPOXYGENASE IN *PHASEOLUS VULGARIS*

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Lipoxygenase (linoleate:oxygen oxidoreductase EC 1.13.11.12) is a dioxygenase catalysing the hydroperoxidation of fatty acids containing a *cis,cis*-1,4-pentadiene structure, *e.g.* linoleic, linolenic and arachidonic acids. In animals the hydroperoxides produced from arachidonic acid by different lipoxygenases are precursors of important classes of chemical messengers such as leukotrienes and lipoxins. In plants no clearly defined physiological role has been demonstrated for lipoxygenase, but the hydroperoxides formed from linoleic and linolenic acids are further metabolised and produce several substances with pronounced physiological activity. Rôles for lipoxygenase in plant growth and development, senescence, wound responses and resistance against pathogens and pests have all been proposed. We have isolated bean cDNA and genomic LOX clones and present information on gene organisation and developmental and organ-specific expression in bean.

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CLONING AND EXPRESSION OF *ARABIDOPSIS THALIANA* GENES ENCODING HOMOLOGS OF MAMMALIAN P-GLYCOPROTEINS.

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Resistance of mammalian tumor cells to many lipophilic cytotoxic drugs can result from an increased drug efflux that is caused by the overexpression of the P-glycoproteins, membrane associated drug efflux pumps encoded by the multi-drug resistance (*mdr*) genes. We have become interested in possible plant homologs of P-glycoproteins and have cloned two *Arabidopsis* genes that both are homologous to the mammalian *mdr* genes. Sequence analysis of one of these genes, *atpgp1*, showed that the encoded protein is internally duplicated as are the mammalian P-glycoproteins, with which it shares about 55% sequence similarity. A comparison of intron positions suggests that P-glycoproteins evolved by duplication and subsequent fusion of an intron-containing primordial gene prior to the evolutionary separation of plants and animals. Analysis of transgenic plants harboring chimeric *atpgp1* promoter-reporter gene constructs reveal a highly specific expression pattern.

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A CYTOSOLIC Cu,Zn SUPEROXIDE DISMUTASE FROM *ARABIDOPSIS THALIANA*: ITS ROLE IN RESISTANCE TO INFECTION AND PARAQUAT

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We present the complete amino acid sequence of a putative cytosolic Cu,Zn superoxide dismutase (Cu,Zn SOD) from *Arabidopsis thaliana* ecotype Columbia, as derived from the sequence of a cDNA clone isolated using a homologous probe from tomato (*Lycopersicon esculentum*). The protein consists of 152 amino acids and has a calculated pI of 5.09. Cu,Zn superoxide dismutase metal binding signatures were present at amino acids 42 to 50 and 59 to 65. The protein was similar to published sequences for other Cu,Zn SODs and showed 92.1% identical and 3.3% conservative amino acid residues to a cytosolic Cu,Zn SOD from *Brassica oleracea*. This cDNA was used as a probe on northern blots of RNA from *Arabidopsis* treated with paraquat or infected with *Peronospora parasitica*. The enzyme activities of SOD, ascorbate peroxidase and glutathione reductase were also measured in corresponding tissues. We discuss possible roles in resistance to both stresses.

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MOLECULAR-GENETIC CHARACTERIZATION OF THE EXTREME ANOXIA-TOLERANCE OF THE MARSH PLANT ACORUS CALAMUS

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The marsh plant Acorus calamus survives at least two months of complete oxygen deprivation, whereas most plants die after a few days. mRNA steady state levels for fermentation enzymes accumulate to high levels during the first few days of anoxia, but decline dramatically afterwards.
In contrast the enzymatic activity of at least one of those enzymes, alcohol dehydrogenase (ADH), is high even in air and remains high during 2 months of anoxic incubation. Only two out of at least seven ADH isozymes appear de novo during anoxic treatment.
Light treatment under laboratory conditions down-regulates the mRNA levels drastically in Acorus calamus but not in maize.
Surprisingly in the natural habitat of Acorus calamus, mRNA levels for the selected enzymes remain extremely low throughout the year.

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EFFECT OF DROUGHT STRESS ON CARBOHYDRATE METABOLISM IN LEAVES OF PIGEONPEA (*Cajanus cajan*)

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Pigeonpea (PP) is a tropical legume which is known to be dehydration tolerant. One major feature of drought stressed PP is the pronounced accumulation of D-pinitol (methyl-*chiro*-inositol) of up to 60 mg/g dwt. The aim of this study was to investigate this channelling of carbon into pinitol by following the change of activities of six key enzymes of carbohydrate metabolism during a five week drying cycle. Sucrose phosphate synthase (SPS) showed a fivefold increase of activity and acid invertase (AI) and sucrose synthase (SS) increased by a factor of twenty and four, respectively. As the maximum activities of SPS and SS after stress were similar, no net gain of sucrose is expected. Furthermore, as AI was the most active of these three sucrose enzymes throughout the experiment it is speculated that PP's strategy is to provide as much hexoses as possible for a buildup of drought tolerance (via pinitol synthesis). The observed 2.5-fold increase of inositol methyl transferase activity after one week of drought would fit this hypothesis. Amylase, a further potential provider of hexose units, is also quite active in PP leaves and its activity increased about fourfold during stress. The activities of these enzymes will be compared with the levels of starch, glucose, fructose, sucrose, inositol, ononitol and pinitol.

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PROTEIN REMOBILIZATION AND LEAF SENESCENCE IN YOUNG WINTER WHEAT

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Field-grown wheat plants were collected in intervals of two weeks during winter 1990/91, extracted and analyzed for soluble proteins, chlorophylls and several peptide hydrolase activities. In spring, the different leaf laminas were extracted separately in order to compare older (present throughout the winter) and younger (newly formed) leaves. Chlorophylls, proteins and peptide hydrolase activities were quite stable during the winter. In spring, proteins and chlorophylls declined rapidly in the older leaves. Aminopeptidase activities were already high in expanding leaves, while carboxypeptidase activity increased later. Endopeptidases peaked in the senescing leaves. The rate of protein synthesis as judged by the incorporation of radiolabelled methionine into the proteins of leaf segments during a 1d-feeding period was high in the young leaves and very low in senescing leaves. A considerable net protein degradation during such a 1d-period was detected by SDS-PAGE and immunoblotting in segments of senescing and mature, but not of young leaves. The physiological age of the leaves was obviously important for this protein loss.

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INFLUENCE OF LIGHT AND TEMPERATURE ON THE FATTY ACID COMPOSITION OF PHOSPHATIDYLGLYCEROL IN SQUASH COTYLEDONS

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Phosphatidylglycerol (PG) appears to play several roles in the thylakoid membrane, among which its involvement in the mechanism of chilling sensitivity of plants is most important. Here, we study the influence of light and temperature on the fatty acid composition of PG in cotyledons of squash, a chilling sensitive plant. We show that during growth, the sums (mol %) [16:0 + t16:1] and [18:0 + 18:1 + 18:2 + 18:3] in PG remained constant. Although the proportion between the fatty acids of the C_{18} series did not change during growth, t16:1 increased at the expense of 16:0. The rate of this change depended on the plant growth rate, on the day length and on light intensity. However, lowering the temperature increased the unsaturation of the C_{18} series fatty acids. We conclude that in a given plant variety, the constant level of the C_{16} and C_{18} series fatty acids in PG is controlled genetically. In contrast, the changes occurring in the C_{18} series fatty acids depend on the temperature and those in the C_{16} series fatty acids on the light. These results are discussed in terms of the pathways of fatty acid synthesis.

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SPECTROSCOPIC CHARACTERIZATION OF THE Fe-S CENTER IN SPINACH FERREDOXIN-THIOREDOXIN REDUCTASE

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Ferredoxin-thioredoxin reductase catalyzes the light dependent reduction of thioredoxins in chloroplasts. The enzyme contains a redox active disulfide and an Fe-S center. The nature and properties of the Fe-S center in the native and N-ethylmaleimide (NEM) modified enzyme have been investigated by resonance Raman, variable temperature MCD and EPR spectroscopies. The results show the presence of a single $[4Fe\text{-}4S]^{2+}$ cluster with T_d symmetry in the enzyme as prepared. Structural changes in the vicinity of the Fe-S cluster accompany NEM-modification of the active site disulfide and can be interpreted in terms of lowering of the [4Fe-4S] core symmetry to D_{2d} and/or changes in one or more of the cysteinyl Fe-S-C-C dihedral angles. The redox properties of the Fe-S cluster are also altered in the NEM-modified enzyme. Only in the modified enzyme is ferricyanide able to effect complete oxidation to give an S = 1/2 $[4Fe\text{-}4S]^{3+}$ cluster (g = 2.11, 1.99, 1.98).

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CHARACTERIZATION OF A NEW LIGHT-REGULATED GENE IN RICE

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We have cloned and sequenced the cDNA of a light-induced gene in rice (lir1). The cDNA sequence corresponds to a putative protein of 128 amino-acids with a possible signal peptide. Comparison of the DNA and the protein sequence with the current data bases do not reveal any significant homology to other known genes or proteins. The mRNA level of lir1 is highest at the end of the photoperiod and lowest at the end of the dark phase. When rice plants were shifted to continuous dark conditions the oscillation of lir1 mRNA persists. We are currently testing whether the oscillation also continues in constant light which would indicate that gene expression follows a circadian rhythm. Experiments to determine the nature of the photoreceptor involved in lir1 induction are in progress.

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THE COMPLETE AMINO-ACID SEQUENCE AND THE CRYSTALLIZATION OF R-PHYCOCYANIN FROM THE RED ALGA *PORPHYRIDIUM CRUENTUM*.

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As with cyanobacteria, the red alga *Porphyridium cruentum* contains extramembraneous light-harvesting antennae known as phycobilisomes. Phycobilisomes of *Porphyridium cruentum* were prepared by a modified method and phycobiliprotein-complexes were isolated by ion-exchange chromatograhy on DEAE - Fractogel. R-phycocyanin is a member of the phycocyanin family and possesses a phycoeryhtrobilin as a second pigment on the β-subunit rather than the usual phycocyanobilin found in the other phycocyanins. The complete amino acid sequence of R-phycocyanin from *P. cruentum* is presented. R-Phycocyanin was crystallized and preliminary x-ray diffraction analysis data are shown.

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PHOTOREGULATION OF HIGHER PLANT GENES IN THE MOSS PHYSCOMITRELLA PATENS

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The moss *Physcomitrella patens* is a useful model organism in developmental biology. Many steps in it's life cycle are regulated by light. Light effects are controlled by receptors of which phytochrome is the best characterised.

The light-harvesting chlorophyll a/b-binding protein gene (cab) and ribulose-1,5-biphosphate carboxylase small subunit gene (rbcS) are light regulated. The promoters of cab (wheat) and rbcS (tobacco), fused to the ß-glucuronidase (GUS) reporter gene, were introduced into *Physcomitrella patens* protoplasts using polyethylene glycol mediated transfection. GUS activity was monitored during the first 48 hrs. In this transient expression system, the higher plant gene promoters were properly light and phytochrome regulated. Their expression showed a red/farred reversibility under low fluence rate.

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AUXIN-SENSITIVE MUTANTS OF NICOTIANA PLUMBAGINIFOLIA

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Two mutants with enhanced sensitivity to auxins at the seedling level were found by screening M2 seed families after treatment of seeds with ethylmethanesulphonate. The sensitivity is the result of two monogenic recessive mutations, which are non allelic. The death of the mutants is induced by active auxins and 1-aminocyclopropane-1-carboxylic acid. One of the mutants was chosen to be further characterized. Using hypocotyl elongation as a criterion, we have shown the mutant's enhanced sensitivity to active auxin analogues, L-tryptophan and ethylene. The response to 6-benzyladenine, abscisic acid and D-tryptophan is similar to wild type. The mutant displays a distinct root phenotype on control media: roots are smaller, more branched and devoid of root hairs. Low concentrations of auxin stimulates root hair formation and silver thiosulfate induces elongation of the main root. The mutant produces more ethylene than the wild type in response to auxin treatment.

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ENHANCED AUXIN SENSITIVITY OF GENETICALLY CYTOKININ HABITUATED TOBACCO PLANTS AND TISSUES

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Cytokinin and auxin are two plant hormones essential for growth and morphogenesis of plant tissues in culture. Leaf tissues derived from mutant tobacco carrying the dominant allele(s) *Hl-1* and *Hl-2* exhibit the habituated phenotype, i.e. they do not require cytokinin for sustained growth in culture. Our studies of the auxin response of these tissues lead to following conclusions: a) The habituated phenotype is auxin dependent b) Leaf discs of both mutants show comparable increases in sensitivity to auxin for root formation relative to the wild type. Evidence that cytokinin independence and auxin sensitivity of the two mutants is due to different mechanisms will be presented.

Signal Transduction

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EXTRACELLULAR SIGNAL TRANSDUCING ENZYMES IN ACUTE MYELOID HUMAN LEUKEMIAS

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The ectoenzyme y-glutamyltransferase (yGT) and the PIP_2 phospholipid phospholipase-C (PLC), a second messenger generating enzyme located on the inner membrane bilayer, were investigated in leukemic cells isolated from patients with acute leukemia. Six groups were distinguished on the basis of morphology, cytochemistry and immunophenotyping, according to the FAB classification: M0, M1-M2, M3, M4, M5, and CML blast crisis(BC). The activity of PLC ranged from 0.6 to 14.5 nmol/min/mg without a significant difference among groups, whereas the yGT activity varied significantly from 0 to 31.6 nmol/min/mg. The highest mean activity was measured in monoblastic leukemia (M5), followed by groups M4, CML-BC and M0 (undifferentiated) while the lowest activity was found in M1-M2 and M3 groups. Within each group, activity distribution profiles of both enzymes never correlated with each other, suggesting that in leukemic cells functional constituents of both membrane leaflets were indipendently affected by the neoplastic process.

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DIFFERENTIAL REGULATION OF PROTEIN KINASE C (PKC) ISOZYMES BY PHORBOL ESTER AND ANGIOTENSIN II IN RAT RENAL MESANGIAL CELLS.

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Rat renal mesangial cells express the PKC-α, -δ, -ε and -z isoforms as assessed by immunoanalysis with isozyme-specific antibodies. No PKC-β_1, -β_2, -γ and -η are detected. Exposure of cells to phorbol ester caused translocation and down-regulation of the isoforms α, δ and ε with different time-courses, whereas PKC-z was not changed by phorbol ester treatment. In contrast, incubation of mesangial cells with the peptide hormone angiotensin II for 24 h resulted in a selective downregulation of cytosolic PKC-z, that was evident already after 30 s of stimulation. All the other isoforms were unaffected by angiotensin II treatment. These data show that PKC-isozymes are regulated differentially in response to hormone or phorbol ester stimulation. The corresponding functional consequences of such a differential regulation of PKC isozymes in mesangial cells is under investigation.

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Myelin Basic Protein-Kinase Activity is Increased in Mitotic- and Okadaic Acid Treated HeLa Cells

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Okadaic acid treatment of HeLa cells results in Golgi apparatus disassembly to form tubulo-vesicular clusters and Golgi vesicles very similar to these found in mitotic HeLa cells. It also induces the arrest of intracellular transport (Lucocq et al., 1991; J. Cell Sci., in press), an additional feature of mitosis. In order to further investigate these striking similarities, we assayed myelin basic protein (MBP)-kinase activity in both, okadaic acid treated and mitotic HeLa cells. Interphase cells incubated with 1μM okadaic acid and cell preparations enriched with mitotics both displayed a more than 5 fold increase in MBP-kinase activity. This kinase activity was neither sensitive to cAMP dependent protein kinase-inhibitor (PKI) nor was it calcium dependent. MBP is a substrate for MAP-kinases, which are known to be activated during M-phase in oocytes (Gotoh et al., 1991, Nature 349: 251-254) and also upon okadaic acid stimulation in other cell types (e.g. Miyasaka et al., 1990, Biochem. Biophys. Res. Comm. 168: 1237-1243). Since H1 kinase activity does not increase upon okadaic acid treatment of HeLa cells, MAP-kinase activation could be involved in inducing the observed Golgi-specific effects.

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IDENTIFICATION AND CHARACTERIZATION OF *RAC* PROTEIN KINASE

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Recently, by homology cloning, we have identified a new member of the serine/threonine protein kinase family that is closely related to the A and C kinases (*rac* kinase). Subsequently the kinase was identified as an oncogene from the AKT 8 virus as a *gag*-fusion protein. The predicted open reading frame of the c-*rac* kinase encodes a protein of 56 kDa that contains a SH-2 domain N terminal region to the kinase domain. Antibodies raised to the carboxy terminal end of the predicted *rac* protein recognize a protein of 58 kDa in a variety of cell lines and mammalian tissues. These antibodies specifically precipitate a protein kinase activity that phosphorylates histone H1 and myelin basic protein. We have also found that the *rac* protein kinase is modified *in vivo* by phoshorylation on serine but also on threonine residues. Treatment of MCF-7 cells with okadaic acid increased the phosphorylation level of the kinase. This might suggest a role for protein phosphatase 2A in the control of the phosphorylation state of the *rac* protein kinase in the cell.

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MOLECULAR CHARACTERIZATION OF THE *RAC* PROTEIN KINASE FROM *DROSOPHILA*

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We have recently identified a new member of the serine/threonine protein kinase family that shows 68% and 73% similarity to PKA and PKC respectively, and has been termed *rac* (related to the A and C kinases). Mammalian cells appear to contain two isoforms of this kinase, α and β, encoded by mRNAs of 3.2 and 3.4 kb respectively. In order to gain insight into a possible function for this protein kinase we have cloned the *D. melanogaster* homolog which shows 75% similarity in the predicted amino acid sequence. Northern analysis has shown the expression of two mRNAs of 2.9 kb and 4.0 kb differentially expressed during embryogenesis, suggestive of both maternal- and zygotic-specific transcripts. The gene has been localised to 89B on the right arm of the third chromosome. Further experiments will establish whether any known P-element insertions or other mutation at this locus correlate with this gene.

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UNPHOSPHORYLATED α-PKC EXHIBITS PHORBOLESTER BINDING BUT LACKS PROTEIN KINASE ACTIVITY.

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α-isoform of protein kinase C (α-PKC) is synthesized in the cell as a precursor molecule which is then phosphorylated yielding the active enzyme. We could demonstrate that the expression of α-PKC in E.coli -in contrast to eukaryotic cells- led to the accumulation of the unphosphorylated form. This precursor was purified and found to exhibit phorbolester binding but no protein kinase activity. In addition, the precursor molecule was able to specifically interact with phosphatidyl serine, threonine and the ATP analogon Cibacron Blue F3GA, respectively, indicating that phosphorylation of α-PKC is neither required for binding of the substrate-ATP complex nor of the effector phosphatidyl serine or of phorbolester activators. Therefore, our results suggest that posttranslational phosphorylation of α-PKC is only required for the transfer of phosphate residues to protein substrates, but not for binding of effector or substrate molecules.

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POSSIBLE ROLE OF CA^{2+}/CALMODULIN PROTEIN KINASE II IN HIPPOCAMPAL LONG-TERM POTENTIATION

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High frequency stimulation in area CA1 of rat hippocampal slices results in a long-lasting enhancement in the efficacy of synaptic transmission referred to as long-term potentiation. The molecular events contributing to this form of plasticity are still unknown, but among the possibilities is the activation of Ca^{2+}/Calmodulin protein kinase II (CaM kinase), an enzyme composed of several catalytic subunits which, following autophophorylation, may remain active in a Ca^{2+}-independent way. We have measured the Ca^{2+}-independent activity of CaM kinase II in potentiated slices and slices which received low frequency stimulation. Our results indicate that LTP induction, but not low frequency stimulation resulted in a long-lasting (at least 1 hour) increase in the Ca^{2+}-independent activity of CaM kinase II (21.7±5.4% increase). The effect was observed in acutely dissected hippocampal slices and in organotypic hippocampal cultures and could be blocked by treatment of slices with D-AP5, an antagonist of the NMDA type of glutamate receptors which prevent LTP induction. These results are the first indication that long-lasting modifications of CaM kinase II activity and of its degree of autophosphorylation may occur in response to specific patterns of synaptic activation (Work supported by FNRS 3.173.0.88 and 31.30980.91).

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THE ENDOGENOUS PROTEIN KINASE C INHIBITOR IN HEART TISSUE IS A SERINE/THREONINE PHOSPHATASE

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A potent endogenous protein kinase C (PKC) inhibitor has been detected in rat ventricular heart tissue. Its inhibitory action on PKC activity is completely abolished by 1 μM okadaic acid (OA) which is known to have a highly specific inhibitory action on type 1- and 2A.-phosphatases. The addition of OA makes it possible to measure PKC activity in non-purified cell fractions . This suggests that the endogenous PKC "inhibitor" is a type 1 or 2A serine/threonine phosphatase. Confirming this, PKC inhibitory preparations were found to contain phosphatase activity which was suppressed by OA. Furthemore, OA stimulated prostacyclin production in rat cardiomyocytes and aortic smooth muscle cells, and like the PKC activator phorbol 12-myristate-13-acetate (PMA), it augmented the prostacyclin formation induced by the calcium ionophore A_{23187}. Our results strongly suggest that the endogenous PKC inhibitor in ventricular heart tissue is a cellular phosphatase which plays an important role in regulating the phosphorylation level of PKC target proteins.

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OVEREXPRESSION OF Dd PK2 PROTEIN KINASE CAUSES RAPID DEVELOPMENT AND ALTERATION OF cAMP PATHWAY

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The *Dd PK2* gene codes for a putative protein kinase with a C-terminal half sharing 52% sequence identity with catalytic subunits of cAMP-dependent protein kinase from other organisms. We overexpressed in *Dictyostelium* either the complete *Dd PK2* coding sequence (A7-Neo) or a mutated version with a frame shift shortening by half the catalytic region (K-Neo). Both A7-Neo and K-Neo cells develop more rapidly than wild type, and show lowered internal cAMP levels, thus resembling rdeC mutants. A7-Neo and K-Neo cells differ in the timing of expression of prestalk and prespore genes. In addition, A7-Neo cells, but not K-Neo cells, are sporogeneous and show about 4 times more protein kinase A (PKA) activity than wild type cells. The association between elevated Dd PK2, PKA and sporogeneous phenotype further indicates a role for these kinases in the formation of *Dictyostelium* spores.

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PROTEIN KINASE SIGNALLING PATHWAYS IN BRAIN FOLLOWING EXPERIMENTAL DEMYELINATION

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Remyelination is a critical step in the recovery of a number of diseases of the central nervous system and there is evidence, both *in vitro* and *in vivo*, that it is under the control of different growth factors than those involved in primary myelination. We have used a PCR cloning approach to identify growth factor protein kinase receptors as well as intracellular protein kinases expressed by regenerating cultures of fetal rat brain cell aggregates. Five transmembrane protein kinases were identified, i.e. IGF1-R, trk B, bFGF-R, Tyro1 and a related novel gene. The first three are receptors for ligands with known neurotrophic function. The latter two belong to a gene family with more than eight highly related putative receptors for as yet unknown ligands. Also, ten intracellular protein kinases were identified including four novel genes. Currently, we are establishing RNA expression data and characterizing full-length cDNAs.

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ANALYSIS OF MICROSOMAL PROTEIN KINASES SENSITIVE TO K-252A, A POTENT INHIBITOR OF TRANSDUCTION OF THE ELICITOR SIGNAL

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Plant cells recognize the presence of pathogens with a sensitive perception system for chemical signals, so-called elicitors. We are interested in the signal transduction pathway, following elicitor recognition using stimulation of ethylene biosynthesis as an early symptom of the elicitor-induced responses. We found that K-252a and staurosporine, two known inhibitors of animal protein kinases, block the induction of ethylene biosynthesis in plant cells at submicromolar concentrations. They also block changes in protein phosphorylation that occur within minutes after elicitor addition. To examine directly whether the compounds are inhibitors of protein kinase, we assayed the enzyme in tomato microsomes with histone III-S as a substrate. Both K-252a and staurosporine proved to be highly potent inhibitors. Work is in progress to examine the role of these plant kinases in transduction of the elicitor signal.

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PHOSPHORYLATION OF THR-124 BY $p34^{cdc2}$ INHIBITS NUCLEAR IMPORT OF SV40 LARGE T-ANTIGEN FUSION PROTEINS

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Nuclear import of regulators of transcription is a cellular mechanism to effect transition in cell cycle, differentiation and transformation. Nuclear transport of the SV40 large T-antigen (T-ag), which is dependent on the nuclear localization sequence (NLS; residues 126-132), is regulated by two discrete phosphorylation sites in the N-terminal flanking sequences. The role of the cdc2-kinase site (Thr-124) on NLS-dependent nuclear import of T-ag was assessed by specifically phosphorylating T-ag-β-galactosidase (β-gal) fusion proteins in vitro using $p34^{cdc2}$ kinase purified from HeLa cells and measuring nuclear import kinetics at the single cell level in HTC rat hepatoma cells using confocal laser scanning microscopy. Thr-124 phosphorylation, which could be functionally simulated by substitution of Thr-124 by Asp, reduced maximal nuclear accumulation by up to 70%. A stoichiometry of phosphorylation of 1 per T-ag-β-gal-tetramer was sufficient for maximal inhibition, implying that cdc2-mediated inactivation of the NLS was unlikely to be the mechanism of inhibition of nuclear transport. It is likely that Thr-124 phosphorylation in vivo may regulate the low but functionally significant level of cytoplasmic T-ag present in SV40-infected cells.

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PHOSPHORYLATION OF XENOPUS NA,K-ATPASE BY PROTEIN KINASES

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In Xenopus oocytes, stimulation of protein kinase C reduces the maximum Na,K-pump current and reinforces the voltage dependence of K^+activation while stimulation of protein kinase A has opposite effects (Vasilets and Schwarz, J.Membr.Biol, in press). To elucidate the mechanism underlying these physiological responses, we have looked whether endogenous or exogenous Na,K-ATPases (NKA) expressed in Xenopus oocytes are potential targets for protein kinase phosphorylation. Our results show that, in microsomal fractions of Xenopus oocytes, NKA α-subunits can efficiently be phosphorylated by the protein kinase A catalytic subunit in the presence and to a lesser extent in the absence of Triton X-100. In addition, in homogenates the NKA α-subunit is phosphorylated upon stimulation of endogenous protein kinase A and C with dbc AMP (plus Triton) and phorbol-12-myriastate-13-acetate or 1,2 dioctonyl-sn-glycerol respectively.These data indicate that protein kinases might directly affect NKA activity in vivo

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INTERACTIONS OF T CELL MEMBRANE PHOSPHOTYROSYL PROTEINS WITH TYROSINE KINASES AND PHOSPHATASES

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Supramolecular complexes formed of the tyrosine kinase p59 fyn and phosphotyrosylproteins have been identified by coprecipitation of detergent-extracted, isolated membranes and characterized by 2-D electrophoresis. Two phosphoproteins of 85 and 110 kDa preferentially associate with p59 fyn. They are immunologically distinct from the phosphatidylinositol 3′ kinase consisting of 2 subunits of similar M_r and appear also distinct from phospholipase C-γ on the basis of M_r. Two additional membrane phosphoproteins of 55 and 80 kDa also bind to p59 fyn. The CD45 tyrosine phosphatase forms complexes with two surface labelled proteins of 80 and 100 kDa, as well as with ^{32}P orthophosphate, metabolically labelled phosphoproteins of 20-25, 50-60 and 80 kDa. The CD45-containing complexes are stabilized by inhibitors of tyrosine phosphatases, suggesting that phosphotyrosyl groups are required for protein-protein interactions involving CD45.

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Ras GTPase ACTIVATING PROTEIN: A POTENTIAL LINK BETWEEN PROTEIN TYROSINE KINASES AND G-PROTEINS IN T LYMPHOCYTES

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Ras GTPase activating protein (GAP) is a cytoplasmic factor that regulates the GTPase activity of $p21^{ras}$. Phosphorylation of GAP on tyrosine has recently been reported by several groups. To investigate whether GAP is a substrate of $p56^{lck}$ protein tyrosine kinase (PTK) and whether in T lymphocytes a link exists between PTKs and G-proteins we performed phosphorylation studies using purified, recombinant human GAP and purified, recombinant human $p56^{lck}$. Our experiments demonstrated that $p56^{lck}$ phosphorylates GAP on one specific tyrosine residue. Further we showed in co-immunoprecipitation studies that $p56^{lck}$ is bound preferentially to GAP phosphorylated on a tyrosine residue. In summary the experiments described suggest that GAP is an *in vivo* substrate of $p56^{lck}$, and that in T lymphocytes the function of GAP might be regulated through its phosphorylation on tyrosine and binding to the PTK $p56^{lck}$.

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REGULATION OF PROTEIN TYROSINE KINASES BY THE CD45 PHOSPHOTYROSINE PHOSPHATASE IN T CELLS

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Triggering of the TcR-CD3 complex, CD4, or CD8, leads to rapid tyrosine phosphorylation of regulatory proteins that participate in initiating T cell activation and proliferation. These signal transduction events require the presence of the TcR-CD3 associated protein tyrosine kinase $p59^{fyn}$ and the CD4/CD8 associated $p56^{lck}$, respectively. There is also evidence that the receptor phosphotyrosine phosphatase CD45 is involved in these receptor-mediated signalling events. We show here by capping experiments using double immunofluorescence techniques that CD45 and intracellular $p59^{fyn}$ and $p56^{lck}$ co-distribute in functional T lymphocytes. Furthermore evidence is provided that isolated $p59^{fyn}$ is a substrate for CD45 and that dephosphorylation is accompanied by an increase in the catalytic activity of the protein tyrosine kinase. These results suggest that members of the *src* family of protein tyrosine kinases, at least in T cells, are physiologically relevant substrates of CD45 and may be regulated by a similar mechanism.

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E.coli EXPRESSION, PURIFICATION AND CHARACTERIZATION OF THE PROTEIN TYROSINE KINASE p56*lck*

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p56*lck* is a *src*-like protein tyrosine kinase which is physically associated with the CD4 and CD8 T cell surface antigens and has been implicated in the signal transduction cascade. In T cells the activity of p56*lck* is suppressed by phosphorylation at a regulatory site (Tyr 505). In contrast, activated forms of p56*lck* are characterized by increased phosphorylation at an autophosphorylation site, Tyr 394. We have expressed human p56*lck* in *E.coli* and purified the enzyme by affinity chromatography on a monoclonal antibody column. In contrast to p56*lck* from T cells the heterologously expressed p56*lck* is phosphorylated at Tyr 394 but not at Tyr 505. This is in agreement with the view that phosphorylation at Tyr 505 requires the activity of a second kinase, and that this Tyr 505-kinase is not present in *E.coli*. We have performed experiments to determine the effect of phosphorylation at Tyr 394 on the kinase activity of the enzyme.

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CD2 FORMS A MOLECULAR COMPLEX WITH THE PROTEIN TYROSINE KINASE p59*fyn* IN HUMAN T LYMPHOCYTES

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T cell activation via CD2, a 50kD transmembrane glycoprotein, or the T cell antigen receptor-CD3 (TcR-CD3) complex leads to the phosphorylation of several intracellular proteins on tyrosine residues within seconds of activation. This suggests an involvement of protein tyrosine kinases very early in these activation processes. Signalling via CD2 seems to act independently from signalling via the TcR-CD3 complex since in $CD3^-$ T cells CD2 can still transduce activation signals. Recently we reported that the TcR-CD3 complex is physically associated with the *src*-like, nonreceptor protein tyrosine kinase p59*fyn*. Using capping experiments and double immunofluorescence techniques we now demonstrate a specific co-distribution of p59*fyn* with antibody-induced CD2 caps in viable human T lymphocytes. These results suggest that p59*fyn* is involved in CD2 signal transduction in T lymphocytes.

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THE PROTEIN TYROSINE KINASES p56*lck* AND p59*fyn* ARE ASSOCIATED WITH THE T CELL SURFACE RECEPTORS CD4/CD8 AND THE TcR/CD3 COMPLEX

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The T lymphocyte cell surface receptors CD4 and CD8, and the T cell receptor-CD3 (TcR-CD3) complex, are involved in activating several signal transduction pathways via coupling mechanisms that are poorly understood. Tyrosine phosphorylation of several transmembrane and intracellular proteins is one of the earliest events following triggering of T cells via these receptors. Using double immunofluorescence microscopy we demonstrate a specific co-distribution of the *src*-like, nonreceptor protein tyrosine kinase p56*lck* with CD4 and CD8 receptor caps, and of p59*fyn* with TcR-CD3 caps. Furthermore, using mouse T cell hybridoma cell lines transfected with mutant human CD4 *c*DNAs, we provide evidence that two cysteine residues in the cytoplasmic tail of CD4 are crucial for its interaction with p56*lck*. These results suggest that in T lymphocytes multiple protein tyrosine kinases are involved in receptor-mediated signal transduction following antigen recognition.

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NUCLEOLAR LOCALIZATION OF A NON-RECEPTOR TYPE PROTEIN TYROSINE-KINASE.

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We have recently identified a novel non-receptor protein tyrosine kinase, JAK1, the prototype of a new family of tyrosine kinases characterized by having a second kinase related domain immediatly N-terminal to the tyrosine kinase domain and no SH2/SH3 domains. The sequence of JAK1 contains two putative nuclear localization signals. Immunoprecipitation of metabolically labelled, fractionated cells revealed substantial amounts of JAK1 protein in the nuclear fraction. Immunofluorescence using affinity purified antibodies raised against a bacterially produced fusion protein revealed in addition to filamentous cytoplasmic and perinuclear staining, distinct nucleolar staining which could be competed by JAK1 protein. The same staining pattern was observed with an antiserum raised against a C-terminal peptide of JAK1. These results represent the first example of localization of a non-receptor protein tyrosine kinase to nucleoli.

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ACTIVATION OF $pp60^{c\text{-}src}$ DURING MITOSIS REQUIRES PHOSPHORYLATION BY $p34^{cdc2}$

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The activity of the cellular tyrosine kinase $pp60^{c\text{-}src}$ is regulated by phosphorylation of tyrosine 527 by unknown kinases and/or phosphatases. Kinase activity is high in mitotic and downregulated in interphase cells. It has been shown earlier that $pp60^{c\text{-}src}$ is phosphorylated by $p34^{cdc2}$ at thr 34, thr 46 and ser 72 and dephosphorylated at tyr 527 during mitosis. It is not clear whether the two events depend on eachother. $pp60^{c\text{-}src(527F)}$, a mutant carrying phe instead of tyr in position 527, showed constitutive high kinase activity throughout the cell cycle suggesting that dephosphorylation of this site is essential for kinase activity. Aminoterminal mutations, converting the cdc2 phosphorylation sites of $pp60^{c\text{-}src}$ to ala were tested for their ability to become activated in mitotic cells. A triple mutant where all three and a double mutant where two cdc2 sites were mutated were not activated while a single mutant containing ala in position 72 was still activated. This suggests that multiple site phosphorylation by $p34^{cdc2}$ is required for stimulation of $pp60^{c\text{-}src}$ during mitosis. Additional preliminary data suggest that dephosphorylation of tyr 527 is necessary but not sufficient for activation of this kinase during mitosis.

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MITOSIS-SPECIFIC PHOSPHORYLATION OF POLYOMAVIRUS MIDDLE-T ANTIGEN BY $p34^{cdc2}$

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Transformation of cells by polyomavirus is mediated by middle-T which forms complexes with src family tyrosine kinases ($pp60^{c\text{-}src}$, $pp62^{c\text{-}yes}$, and $pp59^{fyn}$), phosphatidylinositol-3-kinase (PI 3-K) and phosphatase 2A (PP2A). Middle-T is transiently phosphorylated during mitosis, presumably by $p34^{cdc2}$ resulting in an increase in the apparent M_r on SDS acrylamide gels. There are two putative phosphorylation sites for cdc2 kinase in middle-T (thr 160 and thr 291). We tentatively identified one of the phosphorylation sites as thr 160. We are interested in the role that these sites play in cell transformation and complex formation with cellular proteins. To further investigate mitosis-specific phosphorylation of middle-T two mutant middle-T genes converting the putative cdc2 phosphorylation sites to alanine (thr160- ala and thr291- ala) and a double mutant (thr291- ala, thr160- ala) were transfected into 3T3 cells. These mutants will be used to investigate the phosphorylation state of middle-T in the various phases of the cell cycle as well as complex formation with $pp60^{c\text{-}src}$, PI 3-K, PP2A, and cell transformation.

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CAFFEINE SENSITIVE CELL CYCLE ARREST CAN BE INDUCED BY RADIATION OR STAUROSPORINE.

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Using flow cytometric methods we have investigated the impact of the protein kinase inhibitor staurosporine on G2 arrest and its release by caffeine. The inhibitor induces a pure G2 arrest in both V79 Chinese hamster lung fibroblasts and TK6 human lymphoblasts. The cells are equally sensitive to the inhibitor and the induced delay is dependent upon its continued presence. Furthermore, caffeine abrogates the induced delay, release of the arrest occurring at concentrations identical with those which abrogate radiation induced arrest. The kinetics of caffeine induced release and subsequent mitotic traversal are essentially identical for both staurosporine- and ionizing radiation- induced G2 arrests. The results provide strong support for the involvement of caffeine in a signal control pathway which governs the G2/M transition and controls the cell cycle. They predict the existence in mammalian cells of an active intracellular response mechanism to radiation induced damage.

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CYCLIN B2 UNDERGOES CELL CYCLE DEPENDENT NUCLEAR TRANSLOCATION AND, WHEN EXPRESSED AS A NON-DESTRUCTIBLE MUTANT, CAUSES MITOTIC ARREST IN HELA CELLS.

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Cyclin proteins form complexes with members of the p34cdc2 kinase family, and they are essential components of the cell cycle regulatory machinery. They are thought to determine the timing of activation, the subcellular distribution, and/or the substrate specificity of cdc2-related kinases, but their precise mode of action remains to be elucidated. Here we report the cloning and sequencing of avian cyclin B2. Based on the use of monospecific antibodies raised against bacterially expressed protein, we also describe the subcellular distribution of cyclin B2 in chick embryo fibroblasts and in DU249 hepatoma cells. By indirect immunofluorescence microscopy we show that cyclin B2 is cytoplasmic during interphase of the cell cycle, but undergoes an abrupt translocation to the cell nucleus at the onset of mitotic prophase. Finally, we have examined the phenotypic consequences of expressing wild-type and mutated versions of avian cyclin B2 in HeLa cells. We found that expression of cyclin B2 carrying a mutation at arginine 32 (to serine) caused HeLa cells to arrest in a pseudomitotic state. Many of the arrested cells displayed multiple mitotic spindles, suggesting that the centrosome duplication cycle had continued in spite of the cell cycle arrest.

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STAGE AND TISSUE SPECIFIC EXPRESSION OF PROTEIN PHOSPHATASE 2A SUBUNITS IN *DROSOPHILA*

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The core subunits of all protein phosphatase 2A holoenzymes are the 36 kDa catalytic subunit and a regulatory subunit of 65 kDa. The core dimer can further associate with an additional regulatory subunit of either 54, 55 or 72 kDa. By homology cloning we isolated cDNAs encoding the *Drosophila* catalytic, 55 kDa and 65 kDa subunits. Analysis of the developmental expression of the different subunits revealed that the transcripts encoding the catalytic and 65 kDa subunits were expressed throughout development with highest levels in early embryos. In the larvae elevated levels of the transcripts could be observed in the brain, the imaginal discs and the salivary glands, whereas in adult female only the ovaries showed high expression levels. Two different transcripts encoding the 55 kDa subunit are expressed as splice variants from the same gene, using two different 5' exons. Both transcripts were maternally expressed and present at extremely high levels in early embryos, suggesting that the trimeric protein phosphatase 2A holoenzyme is important in early *Drosophila* development. *In situ* hybridization revealed a low localized expression in later development, associated with the germ line and also in other structures such as the malpighian tubes, hind gut and anal pads.

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IN VIVO MODULATION OF PROTEIN PHOSPHATASES 1 AND 2A LEVELS BY OKADAIC ACID AND CALYCULIN A

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Okadaic acid (OA) and Calyculin A (CA) are specific inhibitors of protein phosphatase 1 (PP-1) and PP-2A but differ in their affinity for both enzymes, OA being 100x more inhibitory towards PP-2A (K_i 0.5 nM) than PP-1, and CA being similarly potent against both phosphatases (K_i 1.0 nM). Treatment of MCF7 cells with 100 nM OA for 24h induced a 2-fold increase in the level of PP-2A but did not affect the level of PP-1 (as measured by Western blot analyses). The mRNA level encoding PP-2A did not significantly change during this treatment whereas there was a 60% decrease in the mRNA level encoding PP-1. Protein synthesis inhibitors only partially prevented the effect of OA on PP-2A, suggesting that this action was mainly due to a stabilization of the enzyme. CA-treatment (10 nM) also led to an increase in the level of PP-2A but contrary to OA induced a rapid (within 2h) disappearance of immunoreactive PP-1. Assay of PP-2A activity in extracts from OA- or CA-treated cells indicated that CA penetrated the cells much faster than OA. Both compounds are potent tumor promoters but could act in vivo through different mechanisms.

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A novel growth factor receptor tyrosine kinase differentially expressed during mammary gland development

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Protein tyrosine kinases (PTKs) play an indispensable role in signal processing and are excellent candidates for regulators of complex, externaly triggered differentiation events such as seen during mammary gland (MG) development. Using primers corresponding to highly conserved regions within the catalytic domain of PTK-genes and cDNAs from MG-derived cell lines we have isolated several tyrosine kinase specific clones. One of these clones -A67- represents a novel growth factor receptor related to the eck/elk/eph subfamily of PTKs. A67 detects three transcripts (3.5, 4.5 and 8 kb) in a wide range of organs. Interestingly, expression could be shown in ovary but is totaly absent in testes. Moreover only the shortest mRNA is presnet in lung. In MG-development highest expression is detected in virgin stages, declines during the course of pregnancy and is absent in lactating glands. At least one of the three transcripts encodes a truncated receptor missing nearly the entire catalytic domain. The co-expression of both forms of this receptor may imply a novel model for the regulation of its biological activity.

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CALCIUM HANDLING AMONG FIBROBLASTS EXPRESSING DIFFERENT PATTERNS OF CA^{2+}-BINDING PROTEINS

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Upon chemical transformation calmodulin expression was found to be enhanced in T14, T10 and T43 rat fibroblasts and oncomodulin was expressed to a high level in T14 cells whereas T10 exhibited low and T43 no oncomodulin expression. Since nothing is known about calcium homeostasis of these cells and the consequences of Ca^{2+}-binding protein levels with respect to calcium handling we started to measure intracellular free calcium concentrations with fura-2. Changes in intracellular $[Ca^{2+}]$ of the cell lines was monitored after increasing and reducing extracellular $[Ca^{2+}]$. The results obtained so far indicate that intracellular $[Ca^{2+}]$ is most susceptible to changes in extracellular $[Ca^{2+}]$ in T14 cells as compared to all other cell lines investigated. The question whether this characteristic is in some way correlated to the high level of oncomodulin expression in these cells is currently under investigation in our laboratory.

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RAPID AND LONG-TERM EFFECTS OF 17β-ESTRADIOL ON PIP_2-PHOSPHOLIPASE C SPECIFIC ACTIVITY OF MCF-7 CELLS.

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Activity of the enzyme phosphatidylinositol 4,5-bisphosphate phospholipase C (PIP_2-PLC) was demonstrated in MCF-7 human breast cancer cells. The addition of 10^{-9}M 17β-estradiol to the culture medium provoked two types of responses depending on the period of exposure. Enzyme activity was enhanced after 5 sec of treatment and attained a maximal activation at 10-15 sec. On the other hand, after 5 min, PIP_2-PLC activity was inhibited, and this effect continued at least until 48 h of exposure to the hormone. When 17β-estradiol was added *in vitro* to the total homogenate of untreated cells, enzyme activity was stimulated in a dose-dependent manner. Furthermore, the effects of estradiol on plasma and nuclear membranes were studied at the ultrastructural level and quantified by morphometric analysis of electron micrographs.

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Submicromolar calcium stimulates pyruvate carboxylation in liver mitochondria

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Hormonally regulated sites for gluconeogenesis are postulated to exist in hepatic mitochondria. It was shown that modification of the Ca^{2+} content of mitochondria secondary to hormone action plays an important role in regulating mitochondrial dehydrogenases. However, it is unclear how Ca^{2+} could influence gluconeogenesis, since none of the Ca^{2+} sensitive dehydrogenases (pyruvate dehydrogenase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase) studied (1) is in the gluconeogenic pathway. Ca^{2+} dependent positive modification of gluconeogenic relevant pyruvate carboxylase has so far not been shown. Long chain fatty acid CoA's are in vitro inhibitors of mitochondrial adenine nucleotide translocase. As a consequence this enzyme would be inactive in the fasted state. With atractylate as a model substance for translocase inhibition we show that increasing extramitochondrial Ca^{2+} from 0.1-1 μM allows ATP transport into mitochondria, thus increasing mitochondrial ATP/ADP ratio and concomitant stimulation of pyruvate carboxylation.

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Inositol-phosphate production in cultured smooth muscle cells from human airways.

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Smooth muscle cells from human airways were isolated by treatment with elastase and collagenase and cultured in a mixture of Ham's F12 and DMEM (1:1) culture medium. The cells reached confluence in 2-3 weeks and were subpassaged 8-10 times by mild trypsin treatment. They were characterized by immunocytochemistry, using anti-alpha-smooth muscle actin as a specific marker: at least 95 % of the cells expressed alpha-smooth muscle actin. Cells at confluence were grown for 3 days in the presence of *myo*-[^{3}H]-inositol and various isomers of inositol-phosphates were separated by HPLC, using a SAX column: 3 peaks corresponding to [^{3}H]IP_1, 1 peak for [^{3}H]IP_2, 2 peaks for [^{3}H]IP_3 (Ins 1,4,5-P_3 and Ins 1,3,4-P_3), plus [^{3}H]IP_4, [^{3}H]IP_5 and [^{3}H]IP_6, were separated. The production of IP_3 and its various metabolites was stimulated by carbachol and histamine. The effect of these agonists was observed following incubation for 5 sec or for selected duration up to 30 min. Dose-response relationships were established. The stimulation was selectively blocked by atropine or by the H_1-antagonist diphenhydramine. The results imply that the receptor-effector systems are functional in these cultured cells. This preparation seems to be a suitable model for studying the post-receptor events involved in pathological situations, such as asthma.

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VOLTAGE-DEPENDENT DISTRIBUTION OF NA-CA EXCHANGE MOLECULAR CONFORMATIONAL STATES

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After Ca^{2+}-binding Na-Ca exchange molecules undergo an electrogenic conformational rearrangement which produces a gating current (I_{conf}). I_{conf} was recorded with the whole-cell patch-clamp technique in cardiac myocytes while Ca^{2+}_i concentration jumps were produced by flash-photolysis of 'caged' Ca^{2+}. The amount of charge moved during I_{conf} presumably reflects the number of exchanger molecules facing the intracellular side of the membrane. Applying different membrane potentials prior to the Ca^{2+}_i jump revealed a weak voltage-dependence of I_{conf}. The increase of I_{conf} at negative voltages suggests that the distribution of exchanger states is largely determined by a molecular transition involving movement of positive charge across the membrane. At a given voltage, intracellular dichlorobenzamil (DCB_i) shifted exchangers to the inside, thereby increasing I_{conf}. This effect may result from DCB_i interfering with a molecular transition moving exchanger states outwards. This notion is supported by the finding that nominally Na^+_o- and Ca^{2+}_o-free solutions reversed the inward shift induced by DCB, presumably by interfering with molecular transitions moving exchanger states inwards. The redistribution of charge induced by voltage, DCB_i and Ca^{2+}_o is consistent with I_{conf} arising from a charge translocation mediated by the Na-Ca exchange molecules after binding of Ca^{2+}_i. Supported by the Swiss National Science Foundation.

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EXPRESSION AND PURIFICATION OF HUMAN CALRETININ FROM THE COLONIC ADENOCARCINOMA CELL LINE WIDR

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The calcium-binding protein calretinin is a member of the troponin C-superfamily and contains 6 EF-hand motifs. Its presence in human tissue has been confirmed in human brain. Here we report the expression of human calretinin in the WiDr (colonic adenocarcinoma) cell line. The screening of a cDNA library from these cells resulted in the identification of the calretinin DNA of 1451 base pairs (clone 3.6). The clone consists of approx. 50 base pairs of 5' non-coding sequence, followed by an open reading frame of 271 codons and terminating with the poly A-tail. A northern blot analysis of WiDr cell total RNA revealed a band at 1.6 kb suggesting that the isolated clone is almost complete with respect to the 5' non-translating sequence. Expression of the gene in a E. coli system resulted in 2 protein bands of 25 and 29 kDa. The protein at 25 kDa is due to an internal start of translation which was suppressed by the introduction of 2 point mutations that do not alter the sequence of the encoded protein. In a 2-step purification procedure, large amounts of pure human calretinin can be produced.

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Induction of the respiratory burst in bovine eosinophils by GTP-γ-S

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The aim of the present investigation was to study the burst by direct activation of G-proteins on α-toxin-permeabilised bovine eosinophils and with the non hydrolysable GTP analogue GTP-γ-S. α-Toxin alone did not induce a respiratory burst. The addition of 100 μM GTP-γ-S generated a high respiratory burst which was further enhanced by the addition of NADPH. At a high ratio of GDP/GTP-γ-S there was a reduction of the effect. The phospholypase A2 inhibitor bromophenacylbromide reduced the burst almost completely. The addition of low concentrations (1-3 μM) of arachidonic acid increased the burst by about 20%. The addition of high concentrations induced a byphasic burst. When eosinophils were activated by the platelet activating factor PAF only 10±5 (P= 0.95, n = 5) % of the burst was obtained compaired to GTP-γ-S. The addition of PAF to GTP-γ-S-stimulated eosinophils increased the burst 7.3±0.2 (P = 0.95, n = 5) fold. The permeabilisation of eosinophils with α-toxin enhanced the PAF-induced respiratory burst 3.3±0.6 (P = 0.95, n = 5) fold. Our results suggest that G-proteins, arachidonic acid (AA) or a metabolic of AA as well PAF are involved in the respiratory burst activation.

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CONFOCAL IMAGING REVEALS SPATIALLY RESTRICTED CA^{2+}-RELEASE IN CARDIAC MYOCYTES

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In cardiac myocytes, spontaneous Ca^{2+}-release from the sarcoplasmic reticulum (SR) occurs under conditions of Ca^{2+}-overload. Spontaneous Ca^{2+}-release is a highly regenerative process (large positive feedback) that spreads over the entire cell as a slow wave driven by a Ca^{2+}-induced Ca^{2+}-release mechanism (CICR). However, under physiological conditions (i.e. non Ca^{2+}-overload) CICR is thought to exhibit only low positive feedback because spatially restricted Ca^{2+}-release can be observed. We have used the fluorescent probe Fluo-3 AM and laser-scanning confocal imaging of the intracellular Ca^{2+}-concentration (Ca_i) to investigate spatial aspects of Ca^{2+}-release from the SR in cultured neonatal rat myocytes. Subcellular patterns of Ca^{2+}-release were monitored either by imaging a rectangular field (153x180 pixels) or by line-scans at high temporal resolution. Under conditions of experimentally induced Ca^{2+} overload spontaneous Ca^{2+}-release from the SR had focal properties in these cells ("hot spots") and did not propagate. The observation of apparently low positive feedback despite the Ca^{2+} overload may result from spatial characteristics of the poorly developed neonatal rat SR preventing the spread of a regenerative Ca^{2+}-wave. Additional results will be presented suggesting spatially resolvable differences in the time-course of Ca^{2+}-transients monitored in the cytosol and in the nucleus.

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EXPRESSION OF A FULL-LENGTH PLASMA MEMBRANE CALCIUM PUMP IN THE BACULOVIRUS SYSTEM

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A full-length cDNA coding for the human plasma membrane Ca^{2+} pump isoform 4b (hPMCA4b) has been assembled from overlapping partial cDNA clones in the pVL1393 transfer vector and recombinant Baculovirus particles were generated upon cotransfection of this vector and wildtype viral DNA into insect Sf9 cells. Selection of recombinants was performed by plaque-assays and dot-blots with hPMCA4 cDNA fragments as probes. Cells infected with purified recombinant virus produced large amounts of hPMCA4 protein of the expected M_r as judged by metabolic labeling, SDS-PAGE, Western blots and ^{125}I-calmodulin overlays. The expressed PMCA protein was at least partially functional as demonstrated by the formation of a phosphorylated intermediate and by an increased Ca^{2+}-ATPase and Ca^{2+}-uptake activity of crude membranes from infected vs. non-infected cells. Immunofluorescence staining with anti-human PMCA antibodies in combination with confocal microscopy revealed an accumulation of the recombinant protein in the periphery of infected cells, suggesting that a significant part of the overexpressed hPMCA4b may be properly targeted to the plasma membrane. Supported by Swiss NSF grants no. 31-27103.89 and 31-28774.90.

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BRADYKININ AND ATP AFFECT INTRACELLULAR FREE CALCIUM IN DIFFERENTIATED PC12 CELLS BY DIFFERENT MECHANISMS

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We have studied the pathways by which extracellular bradykinin and ATP elicit changes in intracellular free calcium ($[Ca^{2+}]_i$) in NGF-treated rat pheochromocytoma (PC12) cells. Both substances caused a significant rise in $[Ca^{2+}]_i$ within a few seconds as assessed by fura-2 based microfluorimetry. The bradykinin-induced response consisted of an initial Ca^{2+} mobilization from an internal pool, located opposite to the cell nucleus, followed by a sustained increase in $[Ca^{2+}]_i$ due to activation of a small inward current. The inward current was partially carried by Ca^{2+}. It began with a time lag of about 4 sec after the initial bradykinin-induced change in $[Ca^{2+}]_i$. Hyperpolarization of the plasma membrane after activation of the inward current by bradykinin caused a simultaneous increase in current amplitude and in $[Ca^{2+}]_i$, due to an increase in the driving force for Ca^{2+} influx. With ATP as an agonist the onset of inward current coincided with an increase in $[Ca^{2+}]_i$. Inward current and $[Ca^{2+}]_i$ were enhanced during hyperpolarizing steps indicating a substantial Ca^{2+} influx through ATP-activated channels. No release of Ca^{2+} from internal stores, but a large Na^+ inward current was observed in Ca^{2+}-free external solution after addition of ATP. While the bradykinin-induced responses were much more pronounced in cell bodies than in growth cones, the ATP effects were somewhat variable in cell bodies and more homogeneous in growth cones.

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PARATHYROID HORMONE (PTH) EFFECTS IN CULTURED MURINE PROXIMAL TUBULE CELLS

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In the kidney proximal tubule,Na/H exchange and Na/Pi-cotransport is inhibited by PTH.To analyze further the cellular mechanisms involved in the regulation of these transports,we have used MCT cells (a culture of SV-40 immortalized mouse proximal tubule cells) grown on permeant filter supports.Na/H exchange was measured using fluorescence technique (BCECF) and Na/Pi-cotransport by tracer techniques.MCT cells express an apical amiloride resistant (Na/H-2) and a basolateral amiloride sensitive Na/H exchanger(Na/H-1).Na/H-2 was inhibited by PTH and agents that activate protein kinase A (forskolin,8-Br-cAMP) and protein kinase C (phorbol ester).Na/H-1 was stimulated by PTH and by activation of protein kinase C ;it was inhibited by activation of protein kinase A.MCT cells also express PTH sensitive Na/Pi-cotransport in the apical membrane and a PTH-insensitive Na-dependent and Na-independent phosphate transport mechanism in the basolateral membrane.PTH stimulates production of IP3 and transiently elevates cytosolic Ca2+.These data suggest that PTH utilizes the phospholipase C / protein kinase C pathway to differently control Na/H exchange and to inhibit apical Na/Pi-cotransport in MCT cells.

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A NOVEL FORM OF GLYCOSYL-PHOSPHATIDYLINOSITOL PLD IN LIVER MEMBRANES

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It has been reported that rat liver membranes contain a glycosyl-phosphatidylinositol-specific phospholipase C (GPI-PLC) which may be involved in generation of phosphoinositol-glycan, a putative insulin second messenger. Using GPI-anchored acetylcholinesterase (AChE) from bovine erythrocytes as substrate, we attempted to isolate GPI-PLC from bovine and rat liver membranes. The tissues were homogenized in Tris-buffer, pH 8, centrifuged, and the pellet was solubilized with 0.25% (v/v) Non-idet-P40. The $6*10^6$ g*min supernatant was chromatographed on Fracto-gel TSK-DEAE. Two peaks with GPI-anchor degrading activity were obtained eluting at 60 and 110 mM NaCl, respectively. Using (I-125)-TID labelled AChE as substrate, radiolabelled diradylglycerol was obtained with both peaks. However, when the phosphatase inhibitors, NaF (50 mM) and orthovanadate (3 mM), were included in the assay systems, phosphatidic acid was detected in addition to diradylglycerol. This result suggested the presence of a GPI-PLD and a phosphatase rather than a GPI-PLC activity. Indeed, aliquots from both peaks cross-reacted on western blotting with antibodies ellicited against serum GPI-PLD. It could be further shown that the second peak of activity was due to contamination of the liver extract with GPI-PLD abundantly present in serum while the first activity peak seems to be genuine for liver cells, and thus represents apparently a novel form of a GPI-PLD which is membrane associated.

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MONOCLONAL ANTIBODIES AGAINST GLYCOSYL-PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE D

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Glycosyl-phosphatidylinositol-specific phospholipase D (GPI-PLD) was purified from bovine serum, and monoclonal antibodies (mAbs) were raised against reduced, SDS-treated, and heat denatured enzyme. The clones were screened by ELISA, and those reacting with both GPI-PLD from bovine and human serum were selected and subcloned. Two non-inhibitory mAbs, 117-1 (IgG_1, with k chain) and 117-2 (IgA, with k chain), were obtained. Competitive inhibition experiments revealed that mAb 117-1 and 117-2 did not compete with each other indicating that the epitopes to which the mAbs bound, were different. Further experiments showed that these two antibodies recognized continuous epitopes as both mAbs reacted with native and SDS-treated, and heat denatured GPI-PLD. Deglycosylation of GPI-PLD did not abolish the binding of mAbs indicating that the mAbs did not target N-linked carbohydrates. The determinants recognized by mAbs 117-1 and 117-2 are highly conserved in mammalian GPI-PLD since both mAbs crossreacted with serum GPI-PLD from human, bovine, goat, rabbit, rat, and monkey. The immunoactive peptides of bovine serum GPI-PLD cleaved by CNBr were purified and sequenced.

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PROPERTIES OF BOVINE AND HUMAN GLYCOSYL-PHOSPHATIDYLINOSITOL-SPECIFIC PHOSPHOLIPASE D

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Glycosyl-phosphatidylinositol-specific phospholipase D (GPI-PLD) was purified from human and bovine sera approximately 1200 fold with a recovery of 3-5%. Bovine serum contained about 40 µg/ml of GPI-PLD, about 10 times more than the amount determined in human serum. GPI-PLD is also present in mammalian cerebrospinal fluid and milk but to a much lesser extent than in serum. Both enzymes are N-glycosylated with about 25% of the total molecular mass being carbohydrates. Pure GPI-PLD was treated with CNBr, and 7 fragments were sequenced N-terminally. Compared with the cDNA-sequence of the enzyme from bovine liver, the fragments of the bovine serum enzyme were 100% identical, and they were 70% identical and 80% similar to the fragments of the human enzyme. GPI-PLD from both sources displayed amphiphilic properties as revealed by sucrose density gradient centrifugation and gel filtration in absence and presence of detergent. On density gradient centrifugation, both enzymes sedimented with an apparent sedimentation constant of 6.0 S in presence of 0.1 % Triton X-100, and formed aggregates up to 14.5 S in absence of detergent. Upon gel filtration, the enzymes migrated with an apparent molecular weight of 300 kD in presence of Triton X-100 and about 600 kD in its absence.

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DOES cAMP PRODUCTION PLAY A ROLE IN HEAT SHOCK PROTEIN SYNTHESIS IN MONOCYTES-MACROPHAGES (MΦ) ?

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The heat shock (HS) response is a universal cellular physiological response to stresses such as elevated temperature or oxidative injury, leading to the synthesis of specific proteins, the heat shock proteins (HSPs). Among various functions, HSPs probably play a role in inflammation and are induced in leucocytes during phagocytosis. The second messengers involved in the activation of HS genes have been studied. Whereas intracellular Ca^{++} is not involved in this process in mφ, the activation of protein kinase C participates in the synthesis of some stress proteins, but not others. Evidence has been provided that a cAMP-mediated activation of protein kinase A (PKA) leads to the activation of a transfected HSP70 promoter gene in fibroblasts.

We have investigated whether cAMP participates in a net detectable increase in HSP synthesis in intact mφ submitted to heat shock or to phagocytic stimuli, by labelling the cells with ^{35}S-methionine and resolving the proteins on SDS-PAGE. Incubation of freshly isolated human monocytes or alveolar macrophages during 2 or 4h with cAMP analogues or cAMP-stimulating agents such as dibutiryl cAMP (1mM), cholera toxin (20 µg/ml), forskolin (100 µM), isobutylmethylxanthin (1 mM) or PGE2 (100 µM), did not affect HSP synthesis either in control or stressed cells. Moreover, the specific PKA inhibitor, R-cAMPS (1mM), did not prevent HSP synthesis either in cells exposed to heat shock or during phagocytosis of opsonised sheep red blood cells. These results suggest that, in mφ, the adenylate-cyclase signalling pathway is not involved in HSP synthesis.

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PGD_2 AND PGE_2 STIMULATE ADENYLATE CYCLASE IN CHICK SPINAL CORD

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Chick spinal cord homogenates synthesize two main prostaglandins (PG_S), PGD_2 and PGE_2. The possibility that PGD_2 and PGE_2 interact with the adenylase cyclase system was tested as follows: chick spinal cord fragments were incubated with PGD_2, PGE_2, $PGF_{2\alpha}$ or Thromboxane B_2. The results showed that solely PGD_2 and PGE_2 enhanced the intracellular cAMP level (three-fold). As compared to classical stimulators such as forskolin, isoproterenol or adenosine the effects of PGD_2 and PGE_2 were similar to those of adenosine, but lower than those of forskolin or isoproterenol.
It is postulated that PGD_2 and PGE_2 act as local enhancers of adenylate cyclase in chick spinal cord.
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PURIFICATION AND CHARACTERIZATION OF ROLIPRAM-SENSITIVE cAMP PHOSPHODIESTERASE IN HUMAN MONONUCLEARE LEUKOCYTES

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Cyclic nucleotide phosphodiesterases (PDE's) have been classified on the basis of substrate specificity and pharmacokinetic properties ,into five isoenzyme families (Beavo 1988). Human mononuclear cell cAMP PDE was prepared by sonication and two steps of anion-exchange-chromatography in 33% overall yield.

Cyclic nucleotide PDE activity was stabilised by including 20% (v/v) ethylene glycol and 0.1 mg/ml 3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propanesulphonate (CHAPS). Under these conditions the cAMP PDE activity loss was between 20% and 30% after 60 min at 37°C.

The preparation was inhibited by the type IV-selective inhibitor Ro 20-1724 (IC_{50} between 8 and 10 µM, 1 µM cAMP as a substrate), but not by Trequinsin (type III-selective). cAMP PDE was partially purified from oligoclonal skin T-Lymphocytes (kindly provided by U. Reinhold, Bonn) with an overall yield of 20%. This preparation was inhibited by Ro 20-1724 (IC_{50} 1 µM) and by Trequinsin (IC_{50} 0.02 µM), respectively. IC_{50} values were not different in preparations obtained from the skin of patients with atopic dermatitis.

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INVOLVEMENT OF A 55 KD PROTEIN IN SIGNAL TRANSDUCTION AND METASTASIS

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Metastasizing cells differ from non metastasizing cells among other properties also in altered behavior in cell-cell and cell-extracellular matrix interactions. In order to characterize molecules possibly mediating such altered behavior we have raised a monoclonal antibody (Mab) against a 55 kD MW membrane protein in B16 F1 mouse melanoma cells. 2h treatment of B16 F1 cells with this Mab prior to tail vein injection can reduce the number of lung metastasis by 80%. Long term culture in the presence of this Mab, however, leads to an increased efficiency in lung tumor formation in this model system. It also leads to reversible morphological changes which correlates with pp60c-src autophosphorylation. The immunoprecipitated antigen itself has a kinase activity towards the exogenous substrate enolase. We are currently investigating if the kinase activity resides in the antigen itself or in an antigen associated protein kinase and if the src kinase is responsible for the enhanced invasion and change in morphology.

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IONIC SELECTIVITY OF GAP JUNCTION CHANNELS DETERMINED IN CELL PAIRS

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We explored the ionic selectivity of gap junction channels by measuring single channel conductance (γ_j) in the presence of various charge carriers (CsCl, Cs-Asp, KCl, K-Asp, LiCl, Li-Asp, TMA-Cl, TMA-Asp). Cell pairs of mammals (primary cell culture: neonatal rat hearts) and insects (cell line: Aedes albopictus, clone C6/36) were used in conjunction with a dual voltage-clamp method and cell dialysis via pipette. To visualize single channels, cells were exposed to 3 mM heptanol in order to reduce intercellular coupling. Heart cells revealed smaller values of γ_j than insect cells (CsCl, voltage gradient across gap junction V_j=50 mV: 33.3 pS versus 210.9 pS). In insect cells, γ_j of the various charge carriers followed roughly the sequence of the ionic conductivity in aqueous solutions. Furthermore, an inverse relationship between γ_j and V_j was found (CsCl: V_j=75 mV, γ_j=177,8 pS; V_j=50 mV, γ_j=210.9 pS; V_j=25 mV, γ_j=285.2 pS). Such a behavior was not encountered in heart cells.

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SIGNAL TRANSDUCTION IN CULTURED CELLS WITH DEFINED MEMBRANE DEFECTS

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Fibroblasts from patients with Zellweger's syndrome are characterized by alterations in the cellular lipid metabolism. We became interested in the consequences of the resulting altered membrane properties for signal transduction across the cell membrane. We recently showed that the membrane fluidity of fibroblasts with Zellweger's syndrome was consistently increased. As an example of signal transduction we determined β-adrenergic transmission, i.e. β-adrenoceptor density and isoproterenol-stimulated cAMP response. There was a big variation in receptor density between cells from different patients ranging from a lack of measurable receptor sites to receptor numbers which were higher than in normal fibroblasts. The isoproterenol stimulated cAMP responses did not correlate with the number of receptor sites. In order to localize the affected step of the transmission cAMP responses upon stimulation at the level of the G-protein and of the adenylate cyclase will be compared between cells from patients and normal volunteers.

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ENHANCED ACTIVITY OF MPF CORRELATES WITH CAFFEINE RELEASE OF G2 ARREST IN IRRADIATED CELLS

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Irradiation of hamster V79 fibroblasts with 7 Gy X-rays results in blocking the cell cycle progression both at the G2/M transition and during the S-phase. By using a synthetic T-antigen peptide as substrate we have measured MPF (maturation promoting factor) activity specifically. The activity of this protein kinase is low in cell populations arrested in G2 as opposed to cells that have been released from the block by caffeine treatment. In irradiated V79 cells (1.5 Gy) there is no significant change of the mRNA or protein levels for histone H4 and other chromatin associated proteins. However, the phosphorylation of nuclear proteins is known to change. The altered MPF actvity indicates that this protein kinase is involved in cell cycle disturbance of irradiated cells.

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STEROID BINDING DOMAINS AS REGULATORY CASSETTES

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The hormone binding domain of steroid receptors, a hormone-reversible HSP90 binding site, is an autonomous regulatory cassette. It can subject heterologous proteins to hormonal control by dominant hormone-reversible inactivation. We have now shown this for 3 of the 5 vertebrate steroid receptors (ER, GR, and MR), in contrast to the insect ecdysone receptor. Even a tyrosine kinase (activated c-abl), unrelated to transcription factors, becomes hormone-dependent by fusion to this domain. In fact, wild-type c-abl fused to a hormone binding domain can be dominantly activated as an oncogene, possibly due to activation of the tyrosine kinase by hormonally induced dimerization. We are currently analysing the role of HSP90 and other components of the complex genetically in yeast. Moreover, we are exploring a genetic yeast system to investigate protein-protein interactions.

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UNCOUPLING OF GROWTH AND EARLY GENE EXPRESSION IN MCF-7 CELLS

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The binding of growth factors to their receptors initiate a sequence of molecular events leading to DNA synthesis including the increased expression of a set of immediate early genes like the proto-oncogenes *c-fos* and *c-myc*. Expression of the *c-fos* and *c-myc* genes are considered to be key events in the development of the mitogenic response. However, recent findings indicate that increased *c-fos* and *c-myc* mRNA levels are not sufficient to stimulate cell growth. Thus, we correlated the mRNA levels of *c-fos* and *c-myc* in IGF-1 and EGF treated human mammary tumor cells MCF-7 with the stimulation of proliferative activity. Cell cycle analysis and growth experiments demonstrated that IGF-1 and EGF stimulate MCF-7 proliferation, with IGF-1 being 4 times more potent than EGF. In contrast, EGF is about 5 times more potent in stimulating *c-fos* expression than IGF-1. EGF and IGF-1 are equally potent in *c-myc* mRNA induction but compared to other mitogens very poor inducers. After addition of the growth factors, the maximum of *c-fos* or *c-myc* induction was reached at 20 minutes and 45 minutes, respectively. There is no direct correlation between *c-fos* and *c-myc* mRNA levels and the subsequent proliferation of MCF-7 cells, suggesting that induction of this proto-oncogenes are no obligatory events required for cell proliferation.

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STUDY OF CYCLOPHILIN ISOFORMS IN HUMAN EPIDERMAL CELLS OF DISTINCT DIFFERENTIATION

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Cyclosporin A (CsA), a specific immunosuppressive agent, is effective in the treatment of psoriasis. It has been suggested that cyclophilin (Cyp), an intracellular receptor for CsA, is involved in CsA's mechanism of action. Expression of Cyp isoforms were analysed by PAGE technique in non denaturating conditions from proteins extracts of human normal skin (HNS), psoriatic plaques (PP), non-differentiated (NDCK) and differentiating (DCK) cultured keratinocytes. Two specific Cyp isoforms were detected in HNS, both having a Mr of 17kDa. The major isoform was expressed at similar levels in all the samples studied. In contrast, the minor isoform was variably expressed; *in vivo*, it was decreased by 6-fold in PP as compared to HNS, whereas *in vitro* a 5-fold increase was observed in DCK as compared to NDCK. Our results suggest that the expression of the minor Cyp might be linked to the differentiation process of the keratinocytes and might be involved in the distinct response of the psoriatic plaque to CsA.

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ULTRASTRUCTURAL AND BIOCHEMICAL LOCALIZATION OF PROTEINS IN THE NUCLEUS

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The cell nucleus is a highly organized organelle. It is the place of genome replication, transcription and processing of cell function dependent genes. Upon an external stimulus, eg. growth factor, a signal is transferred, eg. via the phosphoinositide pathway, to the nucleus to activate the genes responsible for cell proliferation. Many reports assign the nuclear skeleton (nuclear matrix) an important part in signal transduction and gene expression. Methods were developed to localize nuclear proteins using immunoelectron microscopy and/or enzymatic assays. We found that important enzymes of the phosphatidylinositol pathway are located in the nuclear matrix: the PdtIns 4-kinase in the peripheral matrix, and the PdtIns(4)P 5-kinase, the PLC and the DAG kinase in the internal matrix.

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ISOLATION BY SUBTRACTION CLONING OF SEVERAL GENES UPREGULATED IN REVERTANTS OF H-RAS TRANSFORMED RAT FIBROBLASTS

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We have isolated phenotypic revertants of H-ras transformed 208F rat fibroblasts (Schäfer, R. et al. (1988) PNAS 85, 1590-1594). These cells have lost the transformed morphology, required anchorage for growth and were weakly tumorigenic.
In order to characterize the process of reversion at the molecular level we constructed a subtracted cDNA library from F9 cells which is highly enriched for revertant specific transcripts. In addition, we prepared a "normal minus transformed" fibroblast library from 208F cells.
From both libraries we were able to isolate several cDNAs of genes which are downregulated in cells transformed by activated H-ras but expressed at similar levels in revertant and 208F cells.
Some of these genes might contribute to the revertant phenotype of the F9 cells.

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INVOLVEMENT OF MITOCHONDRIAL BENZODIAZEPINE RECEPTORS IN ANGIOTENSIN II-, K^+- AND ADRENOCORTICOTROPIN-INDUCED STIMULATION OF ALDOSTERONE SECRETION IN ADRENAL ZONA GLOMERULOSA CELLS

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Mitochondrial benzodiazepine receptors (MBR) are particularly abundant in steroidogenic tissues and are known to be involved in hormonal activation of steroidogenesis in transformed adrenal and Leydig cells, presumably at the level of cholesterol transfer to the inner mitochondrial membrane. In the present study we examine the effect of peripheral-type benzodiazepine analogs on freshly isolated bovine adrenal zona glomerulosa cells stimulated in static incubations with angiotensin II (ANG II), K^+ or adrenocorticotropin (ACTH).

Flunitrazepam (Flu) and 4'-chlor-diazepam (CDZ) inhibit in a concentration-dependent manner ANG II-, K^+- and ACTH-induced aldosterone output, without significantly affecting basal secretion. The IC_{50} for Flu and CDZ are 50 and 25 µM, respectively, for all the tested activators. While the inhibition induced by Flu is only partial (78 %), the inhibition by CDZ is complete at 100 µM. MBR analogs do not reduce the sensitivity towards the activators but lower their maximal potency, as shown by unaltered values EC_{50} for ANG II and K^+. This, together with the fact that MBR are involved in both cAMP- and Ca^{2+}-mediated processes, suggests that MBR modulate aldosterone synthesis at a step distal to second messenger formation.

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CONVERGENCE OF FOUR DISTINCT SIGNAL TRANSDUCTION PATHWAYS THROUGH AN AP-1 LIKE ELEMENT IN THE UROKINASE PLASMINOGEN ACTIVATOR GENE PROMOTER

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Urokinase plasminogen activator (uPA) is an extracellular serine protease which is believed to play a major role in the proteolytic events associated with cell migration, tissue remodelling and metastasis. uPA is expressed in many different cell types and regulated by a variety of extracellular reagents, however in many cases very little is known about the signalling pathways which induce uPA gene expression. In LLC-PK_1 cells, a non-transformed renal epithelial cell line, uPA gene expression can be induced by the PK-A and PK-C pathways as well as by two less well understood pathways, one involving reorganisation of the cytoskeleton and another the inhibition of phosphatases. We show here that a footprinted region at -2009 to -1984 mediates all or some of the response to each of the above mentioned signalling pathways. Analysis of the *trans*-factors associated with this footprinted region has revealed some insight into the mechanisms involved in these signalling pathways, this will be discussed.

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GLYOXYSOMAL MALATE DEHYDROGENASE FROM SOYBEAN: ENZYME CLUSTER AND SEQUENCE OF cDNA CLONES.

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Glyoxysomal malate dehydrogenase [gMDH; (L)-malate:NAD^+ oxidoreductase, EC 1.1.1.37] and malate synthase [MS; (L)-malate glyoxylate-lyase CoA acetylating, EC 4.1.3.2] copurify as a complex of Mr ca 630'000. This complex is disrupted in the presence of 100 mM KCl and 5 mM $MgCl_2$. Polyclonal antibodies raised against the cluster recognize both gMDH and MS and several yet unidentified proteins localized in the glyoxysomes.
Immunoscreening of a cDNA library (constructed in λ-ZAP II from poly(A)+ RNA) with the anti-cluster resulted in the identification of a cDNA clone encoding gMDH.
In a comparison of deduced amino acid sequences, soybean gMDH exibits 92 % similarity and 84 % identity with the cucumber gMDH.
The pre-gMDH contains an N-terminal transit peptide.

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Cloning of the *puf* Operon and its 5' Flanking Region from a photosynthetically Incompetent Tn5-Induced Mutant, Rhodospirillum rubrum ST3

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The purple non-sulphur bacterium, Rhodospirillum rubrum was subjected to random Tn5 mutagenesis, and screening performed for mutants which were unable to grow photoheterotrophically. A green mutant (ST3) was isolated which was thought to contain a lesion in two overlapping gene clusters coding for the genes for bacteriochlorophyll and carotenoid synthesis, respectively. By analogy to the gene organisation of the related bacterium Rhodobacter capsulatus, it was likely that the *puf* operon was situated 4-6kb downstream from the Tn5 lesion. Chromosomal DNA from ST3 was partially digested with Hind III (which cleaves symmetrically about the kanamycin resistance gene of the Tn5 element) and then cloned into the vector Bluescript. Two clones were obtained after selection with kanamycin which were shown to include up to 5kb of the uptream and downstream flanking regions of the Tn5 element.. One of these (pBsT3H3) could be shown by PCR and Southern hybridization to encode both light-harvesting genes and at least part of the genes for the L and M subunits of the reaction centre. Sequence data for the regulatory region upstream of the *pufB* gene which codes for the b-polypeptide of the light-harvesting complex will be reported.

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High-Level Expression of Phosphoporin from E. coli Facilitates the Growth of Large Crystals diffracting to High Resolution.

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Although the E. coli outer membrane protein, phosphoporin (*PhoE*), has been crystallized some time ago, the crystals obtained for a certain space group have generally been small and poorly ordered. Until recently we have been expressing the protein from a pACYC184-derived vector, pJP12, using the natural "pho" promotor in strain CE1248 which allows constitutive expression from the pho box. To increase the yield of phosphoporin the *pho* box, the *PhoE* gene, and its terminator were subcloned into the pUC derivative, Bluescript, to generate vectors, pBsPHO1 and pBsPHO2, containing the *PhoE* gene in the same and oppposite orientation to the *lac* promotor. The new vectors were quite stable and upon insertion into the CE1248 background, showed a 2-5-fold increase of expression of the *PhoE* gene over that observed from pJP12 and was unaffected by the addition of IPTG during growth suggesting that the *pho* box is sufficient for maximal expression. The phosphoporin purified from this construct yields larger (0.25mm x 0.25mm x 0.2mm) crystals diffracting to higher resolution (3.0Å) than had previously been obtained. The pBsPHO vectors can be used for expression or site-directed mutagenesis, and may thus be useful for structural and functional analysis of phosphoporin.

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Gene Expression of the B875 Light-Harvesting Pre-Polypeptides from Rhodospirillum rubrum in Escherichia coli.

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The genes coding for the two pre-polypeptides of the B875 light-harvesting complex from the purple non-sulphur bacterium Rhodospirillum rubrum were obtained in tandem as a single 430bp fragment by directed PCR amplification from chromosomal DNA. The identity of the PCR product was confirmed by DNA sequencing and Southern hybridisation. The primers used for PCR amplification were synthesized so as to introduce an NcoI site at the ATG start codon of the first gene (*puf*B coding for the pre-β-polypeptide) of the tandem operon, thus allowing the insertion of this fragment into an high-level expression vector (pOTSNco12) at an optimal distance from the Shine-Delgarno sequence and upstream promotor. The pOTSNco12 vector utilizes the strong P_L promotor under control of the temperature-inducible cI857 repressor to express the target gene.

The construct pTSCG1 was grown in the expression strain AR58 at 30oC and then induced by a temperature shift to 42oC in the late exponential phase.

Contrary to expectations, no inclusion bodies were produced after induction, but immunoblotting techniques showed both polypeptides to be incorporated in low amounts into the membrane fraction of the cells. The membrane-bound polypeptides were identical in size to the mature polypeptides and could not be removed by washing with salt. Experiments to determine the role of the C-terminal pre-sequence in this process will be reported.

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EXPRESSION OF CARBONYL REDUCTASE AND NAD(P)H:QUINONE REDUCTASE IN HACAT AND MCF-7 CELL CULTURES AFTER TREATMENT WITH INDUCING AGENTS

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Induction of xenobiotic detoxifying enzymes by a number of chemically unrelated compounds such as polycyclic aromatic hydrocarbons, azo dyes, flavones and phenolic antioxidants is a major protective mechanism of the cells against cytotoxic and carcinogenic effects. NAD(P)H:quinone reductase (NQR, EC 1.6.99.2) and carbonyl reductase (CR, EC 1.1.1.184) efficiently catalyze the two-electron reduction of potentially cytotoxic quinones to hydroquinones which are further conjugated and excreted. Here, we report the expression of the two enzymes in spontaneously immortalized nontumorigenic human skin keratinocytes (HaCat) and in MCF-7 breast cancer cells after exposure to 3-methylcholanthrene (3-MC), ß-naphthoflavone (ß-Nf) and tert-butylhydroxyanisol (BHT). After administration of 3-MC and ß-Nf Western blots of extracts from both cell lines showed an increase of the two reductases which corresponded to an approximately 2-fold elevation in enzyme activity suggesting the presence of a genetic element responsive to xenobiotic aromatic compounds.

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A COMPLEMENTATION SYSTEM FOR SEARCHING COMPONENTS OF THE INTERFERON-γ SIGNAL TRANSDUCTION PATHWAY

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Interferon-γ (IFN-γ) is an immunoregulatory cytokine produced by activated T lymphocytes. At present the molecular events of IFN-γ-mediated signal transduction are widely unknown. Recently, cDNAs encoding the human and murine IFN-γ receptor (IFN-γR) have been cloned. Expression of the human IFN-γR in murine cells and vice versa revealed binding characteristics indistinguishable from those of endogenous receptors. Expression of a high affinity IFN-γR, however, proved not sufficient to confer biological responsiveness to the heterologous IFN-γ, suggesting that at least one additional species-specific component is needed for signal transduction. To identify this putative species-specific cofactor we designed a complementation approach based on the well established cDNA expression cloning strategy in COS cells. COS 7 cells were stably transfected with the murine IFN-γR together with a reporter construct consisting of a IFN-γ-inducible promoter element linked to a cDNA encoding the surface antigen CD 25 (IL-2 receptor α-chain). Since these COS cells respond only to human but not murine IFN-γ, complementation of the murine IFN-γ signalling cascade will be attempted by transient expression of a murine cDNA library and monitoring responsiveness to murine IFN-γ in terms of CD 25 expression at the cell surface.

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TARGETING OF THE MURINE INTERFERON-γ RECEPTOR GENE *IN VIVO*

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Interferon-γ (IFN-γ) is produced by activated T-cells and exerts a variety of biological effects including antiviral activity, activation of macrophages, and modulation of MHC antigen expression. Still, its physiological role remains poorly understood. IFN-γ exerts its effects through binding to a specific cell surface receptor. Recently, the human and murine IFN-γ receptor (IFN-γR) have been cloned. To further elucidate the physiological functions of IFN-γ we have inactivated the murine IFN-γR gene *in vivo* using recently established strategies for gene targeting by homologous recombination. A replacement vector containing 11 kb of the murine IFN-γR gene has been used for transfecting mouse embryonic stem (ES) cells. Homologous integration occurred with a frequency of 1/400 transfected colonies. ES cells carrying one disrupted allele of the murine IFN-γR gene were injected into blastocysts and these reimplanted into pseudopregnant foster mice. Breeding of chimeric offspring revealed germline transmission of the disrupted IFN-gR gene, and, recently, viable mice with a homozygous inactivation of the IFN-γR gene and no immediately apparent phenotypic alterations were obtained. The immune response of these IFN-γR-deficient mice is currently being investigated.

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SHAPE CHANGE AND ACTIN POLYMERIZATION IN HUMAN NEUTROPHILS INDUCED BY INTERLEUKIN 8 DERIVATIVES INEFFECTIVE IN EVOKING OTHER CELLULAR RESPONSES

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Human neutrophils respond to the chemotactic receptor agonist interleukin 8 (IL-8) by the whole response pattern including the extension of pseudopods. Pertussis toxin-sensitive changes in shape (detected by turbidimetry) and F-actin content (detected by rhodamine-phalloidin staining) were induced by IL-8 derivatives which are ineffective in evoking chemotaxis, $[Ca^{2+}]_i$ elevation, O_2^- production and enzyme secretion. IL-8 desensitizised neutrophils for subsequent stimulation with IL-8 derivatives, but not vice versa. These IL-8 derivatives do not compete with ^{125}I-IL-8 for binding to neutrophils. The results show a clear dissociation in signal transduction between cytoskeletal reorganization and other cellular events, possibly pointing towards the existence of a new G protein-coupled receptor associated with F-actin polymerization and shape change.

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LIPOPOLYSACCHARIDE (LPS) SYNERGIZES WITH TUMOR NECROSIS FACTOR (TNF) IN CYTOTOXICITY ASSAYS.

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The standard bioassay for determining tumor necrosis factor (TNF) consists in the determination of murine L929 fibroblast lysis. We demonstrate here that lipopolysaccharide (LPS) of gram-negative bacteria and TNF act synergistically in the lysis of L929 cells Upon addition of LPS during the TNF-target cell interaction, a significantly enhanced cytotoxicity induced by low concentrations (0.3 U/ml) of human recombinant TNF was observed. The effect was LPS dose-dependent, reaching a plateau at 200 ng/ml LPS (E.coli O111:B4). LPS alone was without effect. The augmenting activity could be blocked with polymyxin B. LPS and TNF had to be added at the same time. The described synergism may be of pathophysiological significance in gram-negative sepsis. Moreover, inclusion of LPS may render the L929 cytotoxicity assay more sensitive without losing in reproducibility.

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EXPRESSION OF AN AVIAN GLUTAMATE RECEPTOR VARIANT REVEALED BY IN SITU HYBRIDIZATION IN THE PIGEON BRAIN

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An avian glutamate receptor variant has recently been cloned in our lab from a pigeon cerebellum library. To investigate its pattern of expression in the pigeon brain, in situ hybridization on cryosections was performed using a radioactively labeled cRNA probe. In the telencephalon, neuronal labeling was mostly uniform in intensity, with the exception of a weaker hybridization signal in the nucleus basalis. In the optic tectum, the labeling pattern showed a layering characteristic of the layers of neuronal perikarya in this brain region. Interestingly, only a subset of cells in the subtectal nucleus isthmi, pars parvocellularis, was covered with dense accumulations of silver grains. In the cerebellum, labeling was strongest over Purkinje cell perikarya. Granule cells and, more strongly, the areas between Purkinje cells, i.e. the regions occupied by cell bodies of Bergmann glia, were labeled too. In the molecular layer stellate cells and presumptive processes of Purkinje and Bergmann cells were labeled. Thus, the great majority of neurons in the pigeon brain as well as Bergmann glial cells express the glutamate receptor subunit investigated.

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CHARACTERIZATION OF A NOVEL cDNA ENCODING AN AVIAN GLUTAMATE RECEPTOR SUBUNIT

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We have cloned a glutamate receptor subunit in a low stringency screening approach of a pigeon cerebellum cDNA library using a rodent homologue cDNA probe. Sequencing of positive phage clones revealed that one of the obtained cDNAs, designated pigeon Glu62, encoded for an AMPA-sensitive glutamate receptor. The predicted protein of pigeon Glu62 has like all members of the family of ligand-gated ion channels a large hydrophilic extracellular domain, four hydrophobic putative membrane-spanning segments and an extended cytoplasmic domain between the third and fourth transmembrane region. Within the second transmembrane region pigeon Glu62 contains a codon for an arginine residue that has been demonstrated to be characteristic of GluR-B subunits and to convey particular current-voltage relationships and ion conductances. Northern blot analysis performed on total RNA from cerebellum and from optic lobe of the pigeon with a radioactively labeled cDNA probe encompassing the entire coding region revealed hybridization to a transcript of >5 kb.

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CHARACTERISATION OF MEMBRANE PROPERTIES AND SYNAPTIC RESPONSES IN BIOCYTINE-IDENTIFIED MOUSE CINGULATE CORTEX NEURONS IN VITRO.

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Intracellular recordings were made from neurons located in layers III to VI in a submerged brain slice preparation. The neurons were first electrophysiologically identified, then injected with biocytine for morphological studies. Two types of firing pattern were observed : first, regular spiking neurons (N=22) with high spike frequency (275 Hz/nA) and time-dependent adaptation. Their mean resting membrane potential (RMP) is 75mV, the input resistance (Ri) 51MΩ and the time constant 11ms. Their spike amplitude and spike duration are 84mV and 2ms, respectively. Second, bursting neurons (N=4) respond to depolarizing current pulses with a typical cluster of 3 to 5 action potentials and show similar values of RMP (74mV), Ri (60MΩ), spike amplitude (86mV) and spike duration (1.7ms). I/V curves display a considerable inward rectification in both types of neurons. Synaptic stimulation via bipolar electrodes positioned on the corpus callosum elicited in either cell type a small (4mV) short-latency EPSP, usually included in a depolarizing IPSP (10mV). Increased stimulus intensities evoke a late-EPSP of irregular shape wich is occasionally masked by a late-IPSP. The sensitivity of these synaptic potentials to specific antagonists and their typical voltage-dependence indicate the involvment of non-NMDA, NMDA and GABAa receptors.

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VIP AND NA PROMOTE GLYCOGENOLYSIS FOLLOWED BY OVERINDUCTION OF GLYCOGEN SYNTHESIS IN ASTROCYTES.

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We have previously shown that VIP, noradrenaline (NA) and adenosine (Ado) promote a time- and concentration-dependent hydrolysis of glycogen in primary astrocyte cultures. Dibutyryl cyclic AMP (dBcAMP), the phorbol ester PdBu, as well as the β- and α_1-adrenergic agonists isoproterenol (Iso) and methoxamine (Mx) respectively, are also glycogenolytic, indicating the involvement of both proteine kinase A and C in glycogenolysis.

Glycogen synthesis can also be induced by appropriate treatments. Thus, the exposure of the cultures to insulin 1 μM or methionine sulfoximine 1 mM for 9 hours increases glycogen levels by two- to four-fold. More interestingly, when VIP (1 μM) is applied for 2 minutes and then removed from the medium, two time-dependent phenomena are observed : during the first 30 minutes the well-documented glycogen hydrolysis occurs; however this phase is followed by a time-dependent induction of glycogen resynthesis which results, after 9 hours, in glycogen levels that are six to ten times higher than those observed before VIP application. This effect is also observed with NA and Iso, as well as a prolonged pretreatment with dBcAMP, while Mx, Ado and PdBu are ineffective.

These results show that glycogen levels in astrocytes are regulated by various agents. They also show that certain glycogenolytic agents may trigger "rebound" mechanisms that result in increased glycogen levels.

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COMPUTER SIMULATION OF THE ELECTRICAL ACTIVITY OF SCHWANN CELLS.

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The electrical properties of axono-degenerated rabbit vagus nerve, where Schwann cells represent the only remaining cells, were studied with the sucrose-gap apparatus. In this preparation, a brief depolarizing pulse (0.4 msec) elicited a slow depolarizing wave (SDW). The SDW rose by a S-shaped phase to a peak, passed in a quasi-plateau and then slowly repolarized. The full SDW lasted about 5min. Low $[K^+]_{out}$ amplified the SDW, and a high one diminished it. An increase in $[Ca^{2+}]_{out}$ increased the amplitude and shortened the duration of the SDW, whereas lowering $[Ca^{2+}]_{out}$ decreased the amplitude and prolonged the duration of the plateau of the SDW. When EGTA was added to the nominally Ca^{2+}-free solution, the SDW further decreased, to completely disappear after a prolonged superfusion. The results are in agreement with the hypothesis that the SDW is initiated by a Ca^{2+} current, flowing through voltage dependent Ca-channels. This Ca^{2+} influx increases the concentration of internal free Ca^{2+}, which in turn activates the release of Ca^{2+} from some intracellular stores. This subsequent and prolonged increase in intracellular Ca^{2+} activates a potassium permeability, thus initiating an outwardly oriented repolarizing potassium current. This hypothesis was tested by a computer simulation using the ACSL (Advanced Continuous Simulation Language). The computed theoretical results were in excellent correlations with the experimentally recorded SDWs.

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SIMULTANEOUS MEASUREMENTS OF MEMBRANE POTENTIAL AND ACTIVITY-RELATED CHANGES IN EXTRACELLULAR POTASSIUM IN NONMYELINATED NERVE FIBRES.

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The rabbit vagus nerve consists of very fine caliber nonmyelinated axons. In this preparation, the dense packing and high surface-to-volume ratio is favourable for large activity-dependent changes in extracellular potassium concentration ($[K]_e$). Using a modified sucrose-gap apparatus in combination with ion-sensitive microelectrodes, we have measured the changes in the membrane potential simultaneously with the variations of $[K]_e$ during and after electrical activity. During the stimulation (15Hz 30sec), there was a depolarization of 6.0±0.6mV, paralleled by an increase in $[K]_e$ from the resting level (5.6mM) to 8.5±0.3mM (n=11). The period of activity was followed by a post-tetanic hyperpolarization (PTH), during which $[K]_e$ decreased below the resting level. Low-potassium solution or ouabain abolished both the poststimulation $[K]_e$ depletion and the PTH. High Ca^{2+} solution enhanced the activity-induced increase in $[K]_e$, without significantly affecting the PTH or the $[K]_e$ undershoot. These results indicate an involvement of the electrogenic Na^+-K^+ pump in the generation of the PTH and in regulation of the $[K]_e$. Furthermore, the effect of Ca^{2+} suggests a contribution of a Ca^{2+}-activated K^+ current to the observed activity-related changes in $[K]_e$.

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INTERACTION BETWEEN CLONIDINE AND LIDOCAINE ON C-FIBERS ACTION POTENTIAL.

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Administration of clonidine (CLO) together with lidocaine (LIDO) for a peripheral nerve block significantly prolongs the local anesthetic effect of LIDO, possibly by a direct action on the nerve. The present study examined the effect of CLO and its interaction with LIDO on C-fibers action potential (AP) of the rabbit vagus nerve.

CLO exhibited local anesthetic effects which did not differ from those of LIDO. CLO diminished the AP amplitude and slowed fiber conduction (500μM AP amplitude: 58.5±1.1%, peak delay 2.6±0.2msec n=5), but less than LIDO (500μM AP amplitude: 41.3±1.1%, peak delay 8.4±0.3msec n=11).

CLO decreased the AP area (50μM 83.0±1.0% n=11; 500μM 72.0±1.3%, n=5). In presence of LIDO, CLO led to a more significant decrease of AP area (50μM 63.0±4.4% n=4; 500μM 17.5±2.5% n=4). Moreover, 500nM of CLO added to 500μM LIDO, significantly decreased AP area (86.0±2.2% n=5).

In the presence of LIDO, CLO had synergistic effects at 500nM and above 50μM. The synergistic effect of the low concentration of 500nM CLO with 500μM LIDO might explain the clinical observation that CLO prolongs the action of LIDO in peripheral nerve block, when administered at a ~1000-fold lower concentration than LIDO.

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SUBUNIT STRUCTURE OF $GABA_A$-RECEPTORS IN CNS NEURONS: IMMUNO-HISTOCHEMICAL CO-LOCALIZATION OF THE α_1-, $\beta_{2,3}$- AND γ_2-SUBUNITS.

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A family of at least 15 genes provides the constituent subunits of the $GABA_A$-receptor, a chemically gated ion channel. Since each subunit displays a unique distribution pattern, the structure of the $GABA_A$-receptor is yet unresolved. We investigated by triple immunofluorescence the distribution of neurons which co-express the α_1-, $\beta_{2,3}$ and γ_2-subunits of the $GABA_A$ receptor. Free-floating sections were incubated with specific polyclonal antisera directed against the α_1- and γ_2-subunits and with the monoclonal antibody bd17 (which selectively recognizes both β_2- and β_3-subunits) and processed for triple-immunofluorescence, using secondary antibodies conjugated with red, green and blue fluorophores. Co-localization of the α_1-, $\beta_{2,3}$ and γ_2-subunits was apparent in numerous areas (e.g. olfactory bulb mitral cells, pallidum, basal forebrain, substantia nigra, hypothalamus, reticular formation, and cerebellum). All three subunits were similarly distributed at the (sub-) cellular level, as shown by confocal laser microscopy. In certain neurons, only two subunits were co-localized, e.g $\beta_{2,3}$- and γ_2-subunits in olfactory bulb granule cells, α_1- and γ_2-subunits in brainstem motor nuclei. The extensive co-localization of the α_1-, β_{2-3}- and γ_2-subunits suggests that they are co-assembled in a substantial population of $GABA_A$-receptors. The presence of only two subunits in certain neurons demonstrates a structural heterogeneity of $GABA_A$-receptors. Identification of $GABA_A$-receptor subtypes provides the basis for a functional analysis on the level of identified neurons. Support: Swiss NSF.

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ACTION OF NMDA ON FACIAL MOTONEURONES IN BRAINSTEM SLICES OF NEWBORN RATS.

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In a previous study, facial motoneurones were shown to respond to arginine vasopressin (AVP) by generating a voltage-dependent inward current which was mainly carried by sodium and was resistant to TTX (Raggenbass et al., J. Neurosci. 11:1609, 1991). In the present work, we have investigated the action of N-methyl-D-aspartate (NMDA) on these same neurones by using single-electrode voltage-clamp recordings. In 30 AVP-sensitive motoneurones, NMDA at 50 μM induced an inward current whose average peak amplitude was 0.24 ± 0.12 nA. NMDA acted directly, its effect was concentration-related and could be suppressed by the specific competitive antagonist D(-)-2-amino-5-phosphonopentanoic acid (D-AP5). The NMDA-evoked current was voltage-dependent : it increased in amplitude as the neuronal membrane was depolarized from the resting level toward 0 mV. It was potentiated following removal of extracellular magnesium; it was attenuated, or suppressed, when the magnesium concentration was increased from 1 to 10 mM and could be blocked by the noncompetitive antagonist MK-801. By contrast, none of these treatments affected the AVP-induced current. These results show that facial motoneurones in the newborn rat possess functional NMDA receptors and indicate that in these neurones NMDA and AVP generate separate voltage-dependent inward currents.

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REALISTIC COMPUTER SIMULATIONS OF MEDIAL VESTIBULAR NUCLEI NEURONS

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A program package for realistic computer simulations of single nerve cells running on UNIX workstations was developed. A window-based editor, under X-windows, allows for comfortable construction of cells and variation of parameters. An integration routine based on an implicit second order algorithm allows for fast and stable integration of coupled differential equations describing the kinetics of ion-channels, concentrations of ions, and membrane potential of different compartments.
We have constructed models of Type A and B guinea pig medial vestibular nuclei neurons, consisting of 22 branched compartments, based on published electrophysiological data (Serafin et al. Exp. Brain Res. 84:417-433). Each compartment contains up to six active ionic conductances: g_{Na}, g_{Ca}, $g_{K(DR)}$, $g_{K(A)}$, $g_{K(AHP)}$ and $g_{K(C)}$. The kinetics of conductances were obtained from voltage-clamp studies in a variety of preparations. Some kinetic parameters, as well as the distribution and density of ion-channels, were adjusted to yield the reported behavior of vestibular neurons. The models are able to faithfully reproduce the responses of real vestibular neurons as revealed in current-clamp experiments.

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CAFFEINE ENHANCES AN OUTWARD CURRENT INDUCED BY ACTIVATION OF METABOTROPIC GLUTAMATE RECEPTORS IN CEREBELLAR PURKINJE CELLS

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Intracellular recording in combination with microfluorometric monitoring of cytosolic calcium ($[Ca^{2+}]_i$) was used to assess the effects induced by the specific metabotropic glutamate receptor agonist trans-D,L-1-amino-1,3-cyclopentane-dicarboxylic acid (t-ACPD) in Purkinje cells from organotypically cultured slices of rat cerebellum. The preparation was continuously perfused in saline containing TTX (0.5 uM), bicuculline (10 uM) and cesium chloride (2 mM). Bath application of t-ACPD (100 uM, 30 s) under voltage-clamp conditions (holding potential -70 to -60 mV) induced a transient inward current, shown to be associated with a rise in somatic $[Ca^{2+}]_i$[(1)]. This inward current was followed by a transient outward current with a significant increase in membrane resistance. We show here that the t-ACPD-mediated outward current was enhanced both in amplitude and duration by short co-application of either caffeine (5 mM, 30 s) or the phosphodiesterase inhibitor isobutyl-1-methylxanthine (IBMX) (5 mM, 30 s) in a reversible way. Co-application of caffeine did not affect the t-ACPD-induced rise in $[Ca^{2+}]_i$. Control applications of caffeine of the same duration and concentration did neither elicit a detectable rise in $[Ca^{2+}]_i$, nor cause an outward current.

(1) Vranesic et al., NeuroReport 1991, in press

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NEUROTRANSMITTER RELEASE IN NATURAL SYNAPSES AND IN RECONSTITUTED SYSTEMS

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Synaptic transmission at the *Torpedo* nerve-electroplaque junction is quantal with one quantum made of ca. 10000 acetylcholine (ACh) molecules. The quanta are released at 800 nm from each other, activating adjacent receptor fields in the postsynaptic membrane. Each quantum is in turn composed of a preferential number of 10 subunits that are released within a very restricted area (< 300 nm). This conclusion was reached by comparing the signals recorded in standard or modified conditions to a bidimentional model simulating synaptic transmission.
The process of Ca-dependent ACh release has been induced into *Xenopus* oocytes injected with mRNAs extracted from cholinergic neurons. The primed oocytes expressed choline acetyltransferase, accumulated ACh and released the neurotransmitter in a manner very similar to what is seen in natural synapses. These oocytes also expressed the "mediatophore", a membrane protein able to translocate ACh. The role of the mediatophore in the mechanism of ACh is being precised by using antisens RNA probes.

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RELATION BETWEEN THE INTENSITY TO EVOKE A VOLLEY IN GROUP IA MUSCLE AFFERENTS AND A MONOSYNAPTIC H REFLEX IN MAN

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It is generally known that a stimulus which is applied to the posterior tibial nerve and which is threshold for the H reflex in resting condition can evoke a large H reflex just before the onset of an voluntary movement. The threshold for a volley in Ia afferents is thus lower than the threshold for an H reflex. The aim of the present investigation was to study the difference between the threshold intensities with two techniques.

In the first set of experiments, conditioning stimuli were applied by the same electrode as test stimuli which were delayed by 5 ms. In control conditions, test stimuli evoked an H reflex of 30% of a maximum control reflex whereas the intensity of the conditioning stimuli was variable but always below H reflex threshold. The conditioning stimulus facilitated the following H reflex till intensities of about 30% of the H reflex threshold depending on the subject. We assumed that this intensity corresponds to the threshold for the afferent volley.

In the second set of experiments, subject performed plantar foot flexions in a visual reaction time task. Stimuli of variable intensity were applied to the tibial nerve during reaction time just before movement onset. The threshold to evoke an H reflex during this period, the second estimator, was about 70% of the control threshold because of reduced presynaptic inhibition at Ia terminals.

Reasons for the difference between the two estimators are discussed.

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IDENTIFICATION OF A NOVEL 140 KD NERVE GROWTH FACTOR BINDING PROTEIN IN RAT PC12 CELLS.

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Rat pheochromocytoma (PC12) cells express two distinct nerve growth factor receptors (NGFRs), $p75^{NGFR}$ and $p140^{trk}$. To determine whether one or both of these proteins participate in the formation of the high affinity NGFR, we have established a method to selectively crosslink ^{125}I-NGF bound to $p75^{NGFR}$ or $p140^{trk}$ in PC12 cells where they are co-expressed. We find that BS^3 crosslinks ^{125}I-NGF to $p140^{trk}$, and to two high molecular weight bands, all of which are immunoprecipitated by a polyclonal antiserum raised against the cytoplasmic domain of the trk oncogene. These bands are not immunoprecipitated by mAb192, a monoclonal antibody specific for the $p75^{NGFR}$ protein. $p75^{NGFR}$ protein can be crosslinked to ^{125}I-NGF using EDC, and is immunoprecipitated by mAb192. EDC also crosslinks NGF to a 140 kD protein which is not recognized by trk antiserum or by mAb192, suggesting that it may represent a novel NGF binding protein.

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OXIDATION-REDUCTION PROCESSES AT THE CELL PLASMA MEMBRANE LEVEL IN CALF BRAIN SYNAPTIC MEMBRANES.

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The relevance of oxidation-reduction processes at the cell plasma membrane level and for signal transduction has been investigated using crude extracts or partially purified plasma membrane preparations from calf brain cortex. We have tested the effects of plasma membrane oxidoreductases (PMO) in neural tissue on β-receptor activity and on adenylate cyclase activity under various experimental conditions in the presence of varous electron donors (e.g. pyridine nucleotides, PN) and acceptors (e.g. quinones Q, p-benzoquinone, dichloro-phenol-indophenol, etc). In competition studies with ^{3}H-dihydroxyalprenolol, the K_D of (-)-Alprenolol and the B_{MAX} for β-receptors are slightly affected by Q and DCIP or by substrates for PMO, whereas PN have no effects on the system when added alone. Similarly adenylate cyclase is markedly inhibited by DCIP or Q, This inhibition is partially released with PNs. A similar PMO has been explored in intact cells from a neuroblastoma cell line NB41A3. The significance of these results, with respect to physiological responses towards oxidative stress and related processes in neural tissues is being discussed.

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EFFECTS OF BRIEF CHANGES IN TEMPERATURE ON SYNAPTIC TRANSMISSION AND LONG-TERM POTENTIATION IN HIPPOCAMPUS

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We used a specially designed chamber consisting of a Peltier element to study the effects of fast and brief reductions in temperature on synaptic transmission and plasticity in area CA1 of rat hippocampal slices. Slices were prepared and maintained at a standard temperature of 33°C and brief shocks to 30°C, 27°C and 24°C for 2 or 5 min were applied. Equilibrium to the new temperature was reached in about 30 sec. These cooling episodes resulted in marked modifications of the size and time course of synaptic responses. Changing the temperature for 5 min from 33°C to 24°C abolished synaptic transmission. Returning to 33°C was followed by a recovery period of about 20 min at the end of which synaptic transmission had fully recovered. This recovery period probably reflected changes in ionic gradients and cell excitability. The effects of these cooling shock on mechanisms of induction and expression of long-term potentiation (LTP) were analyzed. The results obtained in 15 different slices indicate that LTP can be abolished if such cooling episodes are applied during the first 20-25 min after stimulation, but not at later times. These results indicate that LTP is reversible and that a metabolic-dependent process may be involved during an initial phase for the generation of this form of plasticity. (Work supported by FNRS 31730.88 and 31.30980.91).

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LOCALIZATION OF MSH RECEPTORS ON MOUSE MELANOMA TISSUE SECTIONS

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The binding properties of MSH receptors on various cultured melanoma cell lines have been well characterized using radioiodinated α-MSH. The aim of this study was to determine the binding ability of α-MSH to its receptors expressed in a B16-F1 mouse melanoma tumour inoculated in C57Bl/6J mice. For this purpose frozen tissue sections were incubated with ^{125}I-α-MSH and the binding sites were made visible by subsequent autoradiography. Incubations of ^{125}I-α-MSH in the presence of increasing concentrations of α-MSH gave a dose-dependent displacement. By quantitative analysis of the autoradiograms a K_D of 1.9 nM was determined, similar to that obtained in a binding assay using isolated B16-F1 cells (K_D= 1.3 nM). This method may be applied to the detection of MSH receptors on human melanoma tissue sections from tumour biopsies. Therefore it could serve as a tool for the analysis of MSH receptor status of different tumour variants and in melanoma tumour diagnosis.

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CALCITONIN RECEPTOR GENE STRUCTURE

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Thyroid hormone calcitonin lowers serum calcium levels. Calcitonin executes its action by binding to plasma membrane calcitonin receptors (CTRs) which are coupled to protein kinase A and/or protein kinase C signal transducing pathways. The porcine kidney epithelial cell line, LLC-PK_1, which expresses CTRs and receptor defective mutants derived from this cell line are a good model system to study receptor gene expression. Recently a CTR cDNA was isolated by expression cloning using COS cells and a LLC-PK_1 cDNA library. The predicted amino acid sequence of CTR shows seven putative membrane domains but has no homology to any described G-protein coupled receptor. In order to study the regulation of CTR expression we have isolated genomic clones encoding receptor from a LLC-PK_1 library. The structure of calcitonin receptor gene will be presented.

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COMPARAISON OF THE PUTATIVE TRIPEPTIDE SIGNALS OF THREE GLYOXYSOMAL ENZYMES FROM SOYBEAN.

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The transport of glyoxysomal malate synthase [MS; EC 4.1.3.2], isocitrate lyase [ICL; EC 4.1.3.1] and malate dehydrogenase [gMDH; EC 1.1.1.37] is most likely a post-translational process. A C-terminal tripeptide (SKL or conservative mutant) is known to act as a signal sequence for MS and ICL. This tripeptide can also be found in the gMDH presequence. A cDNA library was constructed in order to isolate clones for further studying the transport. Our cDNA library was screened for MS, ICL and gMDH clones, using antisera prepared against the following antigens: denatured MS, denatured ICL, and a native enzyme cluster including at least gMDH and MS. The deduced C-terminal MS tripeptide (SKL) is also found in cucumber and cotton MS, whereas the castor bean, rapeseed and pumkin MS tripeptide is SRL. The deduced C-terminal ICL tripeptide (ARM) is also found in cotton and castor bean ICL. The gMDH tripeptide signal is GHL for soybean and AHL for cucumber. It is interesting to note that the tripeptide is not conserved among various glyoxysomal enzymes of the same species, but much more so among homologous enzymes in different species.

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MODULATION OF RED BLOOD CELL (RBC) MEMBRANE PHOSPHATIDYLSERINE (PS) ASYMMETRY BY ATP.

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Under normal metabolic conditions, human RBC membrane phosphatidylserine (PS) is located in the cytoplasmic membrane layer. Based on studies with exogenously added labelled PS, a protein mediated ATP-dependent transport mechanism has been suggested to be responsible for maintaining this asymmetry. In the present study we have determined the distribution of endogenous PS at various intracellular ATP-levels using an assay based on PS-dependent thrombin formation (prothrombinase activity) to detect PS in the outer membrane leaflet. ATP-depletion of RBC to 20% of the original value resulted in a three-fold increase of prothrombinase activity, suggesting a partial loss of PS asymmetry. The return to original ATP levels restored asymmetry. These results show that also with endogenous PS, an ATP-dependent inward transport is observed, in agreement with the results obtained with exogenously added lipid.

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ATP DEPENDENT AMINOPHOSPHOLIPID TRANSLOCATION IN VESICLES RELEASED FROM RED BLOOD CELLS

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Vesicles released from human erythrocytes by incubation with dimyristoylphosphatidylcholine contain essentially the same phospholipids and intrinsic red cell membrane proteins as the native cell but no membrane skeletal components. In the present study, red cell ATP levels were modulated prior to vesiculation and spin labelled lipids were used to monitor phospholipid transbilayer mobility and distribution across the vesicle membrane. An ATP-dependent inward translocation was observed for phosphatidylserine and phosphatidylethanolamine. The kinetic parameters of the translocation and the plateau levels reached for the asymmetric distribution were comparable for vesicles and intact red blood cells. The results suggest that aminophospholipid translocation across the red cell membrane does not require the presence of the membrane skeleton.

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CHARGE TRANSLOCATION BY THE NA-K-PUMP EXPRESSED IN XENOPUS OOCTES.

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The Na-K-pump transports successively 3 Na^+ out and 2 K^+ into the cell during each cycle, and net charge translocation is known to occur during the Na^+ transporting steps. To determine the number of charges moved across the membrane during these steps we have estimated the number of Na-K-pumps by ouabain binding and measured the ouabain-sensitive voltage-dependent charge displacement under conditions where normal pump cycling is prevented by the absence of external K^+. To increase the number of active Na-K-pump in the oocyte membrane, oocytes were previously injected with cRNA coding for the $\alpha 1$ and $\beta 3$ subunits of the Xenopus Na-K-ATPase (see PNAS 88:8397, 1991). Charge translocation was measured as the time integral of transient current induced by rapid voltage steps (from -140 to + 40 mV) using the two-electrode voltage-clamp technique. The number of ouabain binding sites averaged $38.3 \pm 3.8 \cdot 10^9$, while the maximal ouabain-sensitive charge displacement was 10.4 ± 1.3 µA.ms ($64.9 \pm 8.1 \cdot 10^9$ charges) per oocyte. Thus, the transport of 3 Na^+ ions through the pump is accompanied by translocation of about 1.7 charges per cycle.

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PROTEASES INVOLVED IN THE ACTIVATION OF NEUTROPHILS DESCRIBED BY SPECIFIC INHIBITORS.

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The involvement of proteases in the activation of neutrophils has been well documented: The addition of neutral proteases to neutrophils enhances the stimulatory effect of agonists such as fMetLeuPhe (fMLP). However, agonist induced activation may be inhibited by protease inhibitors and therefore seems to be mediated itself by endogeneous proteases.
Inhibitors specific for chymotrypsin-like enzymes such as TPCK, zLYCK and zAAFCK inhibited production of superoxide induced by fMLP or NaF with a K_i of 2-5 uM (TPCK and zLYCK),or of 8 uM for zAAFCK, respectively. TLCK which is specific for trypsin-like enzymes was less potent (K_i: 50 uM), while PMSF, a general serine esterase inhibitor, showed a K_i of 200 uM. 10 uM TPCK or zLYCK inhibited activation by PMA by ca. 60 %, while activation by arachidonic acid was not inhibited. This points to an involvement of proteases in an early step of the activation process. Cathepsin G and elastase are not inhibited by zLYCK and TPCK at concentrations of up to 100 uM. This excludes a role for these enzymes in the activation process. We therefore postulate that a novel enzyme mediates the agonist induced activation of neutrophils.

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CHARACTERIZATION OF A LUNG CULTURE SYSTEM

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**The aim of our study was to establish and characterize a novel culture system for adult rat lung tissue. In order to maintain the alveolar structure, the lung was filled with an agar solution. After solidification and using a special device, 500 um thick lung slices were obtained, which were cultured at 37°C on an inclined rotating disc.
Cell viability and morphology of the tissue were satisfactory for at least 7 days. Nevertheless two observations indicated, that the tissue did not behave as it would in vivo. 1) During culture, the synthesis of certain proteins decreased, while others were induced. 2) More than 20% of the cells incorporated ^3H-thymidine during an 8 hour pulse, yet in vivo the cells would be expected to grow very slowly. We found that mainly cells at the surfaces of the slices incorporated thymidine. Interestingly, the incorporation occured also if the culture medium was not supplied with FCS. Our findings support the existence of a stimulus for growth, induced either by the slicing of the tissue or by the culture environement itself. Candidate growth factors are discussed.**

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A SIMPLE LOW COST AND HIGH RATE PELTIER THERMOREGULATION SET UP FOR PHYSIOLOGICAL EXPERIMENTATION.

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We used a square Peltier element as bottom of a recording chamber and built an electronic device to control the amount of current and its sign across the Peltier thermocouple. The temperature within the chamber was continuously measured using a thermic probe and regulated by a feed-back loop to a preset level. The chamber was a ring of plexiglass of 2.5 cm internal diameter and 0.4 cm high adjusted on the Peltier element. With this system, rapid changes in temperature could be obtained (about 10°C per mn). The temperature could be precisely controlled with an accuracy of 0.1°C within a range 0-50°C. The appliance was stable during long periods of use and did not generate any noise. This system may be of interest for a wide range of applications and was used to analyse the effects of rapid changes of temperature on evoked and spontaneous release of transmitter at the *Torpedo Marmorata* electric organ. (This work is supported by FNRS 31-28780.90)

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EFFECT OF DIVALENT CATIONS ON CYTOSOLIC PEPCK.

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A cytosolic cell free system prepared from the liver of 48 hours fasted rats was used to study the effect of divalent cations on the activity of the gluconeogenic enzyme phospho-enolpyruvate carboxykinase (PEPCK), at micromolar steady state concentrations of oxaloacetate. 2 mM ITP (the GTP analogue) and 3 mM Mg(II) were also added. Micromolar concentrations of Mn(II), Fe(II) and to a lesser extent Zn(II) and Co(II) were shown to stimulate PEPCK activity. An increased V_{max} was observed, whereas the K_m (oxaloacetate) did not change upon addition of the divalent cations. The apparent K_a for Mn(II) and Fe(II) were around 1 μM. The chelating agent EGTA was added to the system in order to decrease the concentration of the free cations that are thought to be the allosteric regulators of PEPCK *in vivo*. PEPCK activity was almost unaffected by a 10-fold decrease of the Me(II) free concentration (lower limit was 10 nM). Possible causes for the lack of reversal of Me(II) activation are discussed. A comparison with arginase - a hepatic Mn(II) dependent enzyme that was inactivated by concomitant addition of Mn(II) and EGTA - is presented.

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CHARACTERIZATION OF THE INWARD BASOLATERAL K^+ CONDUCTANCES OF THE RENAL EPITHELIAL A6 CELLS

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The basolateral membrane of A6 cells (a cell line derived from the distal tubule of *Xenopus laevis* kidney) contains a barium-sensitive, inward rectifying K^+ conductance (G_K). To test for the presence of ATP-sensitive K^+ channels in this membrane we have studied the effects of tolbutamide, a sulphonylurea drug well known to act on the ATP-sensitive K^+ channels present in the pancreatic β cells. After the selective permeabilization with amphotericin B of the apical membrane of A6 cells grown on filters, the basolateral K^+ currents were mesured under voltage-clamp conditions. In the presence of 3 mM K^+ both barium and tolbutamide sensitive G_K are inward rectifying with equilibrium potential of -78.4 ± 3.4 mV and -83.4 ± 1.1 mV respectively. Tolbutamide inhibits 72.4 ± 4.9 % of the barium sensitive G_K with a K_i of 25.68 ± 2.95 µM. In the presence of both 5 mM glucose and 500 mU insuline (to increase the cellular ATP production) the effect of tolbutamide was totally abolished. Such results suggest that a part of basolateral G_K of the A6 cells is made of K^+ channels sensitive to the intracellular ATP concentration similar to those present in the pancreatic β cells.

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THE EFFECTS OF BENZOCTAMINE ON PERIPHERAL HISTAMINE RECEPTORS

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Benzoctamine (Tacitin®) is an anxiolytic and centrally-acting muscle relaxant found in ligand-binding studies to interact equipotently with central histamine H1 and H2 receptors (Williams, unpublished results). We therefore examined its effects on functional H1 and H2 receptors of the guinea-pig ileum and atrium respectively. Its effects on the H3 receptors of the electrically-stimulated guinea-pig ileum were also examined.

Effects of benzoctamine on contractions of the isolated guinea-pig ileum induced by histamine (H1 receptors) were measured using standard techniques. Effects on H2 and H3 receptors were investigated using histamine-stimulated guinea-pig atria (chronotropic effects) and electrically-stimulated guinea-pig ileum (Eu. J. Pharmac. 186, p343), respectively. Benzoctamine was found to have a moderate antihistaminic (H1 receptor antagonist) activity, (pA2=8.1). No measurable stimulatory or antagonistic effect on H2 or H3 receptors was observed. Higher concentrations of benzoctamine (above 10^{-6}M) reduced the basic rate of contraction of the atrium, probably due to a "membrane stabilising" effect, which is not unusual in molecules of this type. In conclusion benzoctamine is a reasonably potent antihistamine, but, in contrast to its effects on brain membranes, has no H2- or H3-receptor blocking activity in our system.

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DIVERSITY OF CYTOSKELETON IN DIFFERENT PHENOTYPES OF CULTURED MICROVESSEL ENDOTHELIAL CELLS (MVE) AND THE INFLUENCE OF INTERFERON-γ (IF-γ).

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For the first time, 5 different phenotypes of cultured MVE derived from bovine corpus luteum have been convincingly characterized at the light and ultrastructural level (Spanel-Borowski, K. 1991; Spanel-Borowski, K. and van der Bosch, J. 1990). This time we investigated the cytoskeleton and the extracellular matrix. Confluent MVE cultures were treated with bovine recombinant IF-γ (200 U/0,5 ml) for 3 days. The actin cytoskeleton which was localized with phalloidin-FITC showed peripheral bundles in type 1, a delicate polygonal network in types 3 and 4, and a perinuclear belt with peripheral radiations in type 5. By using immunofluorescence localization vimentin filaments formed a distinct honeycomb-like meshwork in type 1 unlike the actin cytoskeleton. Microtubuli were abundant in types 2 and 5. A fibronectin network was found in monolayers of types 3 to 5. Treatment with IF-γ caused no change of the cell specific cytoskeleton. Additionally, the number of microtubuli decreased in type 3 which became fibroblastoid. In type 5, mitotic spindles were noted. Thus, MVE express inherent morphological diversity maintained in cell culture.

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Localisation of the complementary binding site of fibrinogen involved in fibrin polymerisation

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Upon selective cleavage of fibrinogen's N-termini fibrinopeptide A and B by thrombin, fibrin monomers start to react with themself. The reaction takes place between the newly exposed N-termini of alfa and beta chains, and the D1 domain of the molecule containing C-termini of beta and gamma chains. Isolated D1 domains added to a fibrinogen solution compete to the homologous binding site inhibiting the polymerisation. Plasmin degradation of D1 produces D2 and D3 domains which have lost the inhibitory activity on the polymerisation. Plasmin degradation of fibrinogen in the absence of calcium produces D3* domains which retains antipolymerisation activity. D3* was composed by a D3 remnant and a peptide most probably cleaved from beta-chain C-terminus. Reconstitution of D3 with the peptide was necessary to elicit antipolymerisation activity.

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ISOPROTEIN DIFFERENCES ARE IMPORTANT FOR BIOGENESIS OF MUSCLE CYTOARCHITECTURE.

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Myosin light chain (MLC) form an isoprotein family with members typical for muscle others for non-muscle tissue. By utilization of the newly developed epitope tagging method it had been shown that intracompartmental sorting of MLC to nascent myofibrils in adult rat cardiomyocytes is isopro-tein-specific (Soldati and Perriard, Cell 66, 277-289, 1991). Using this assay the various MLC isoproteins can be arranged into a sequence of in-creasing affinity for their interaction with myofibrils. Experiments with chimeric constructs showed that information dictating the preferential incorporation into the sarcomeres is in the central MLC 1f/3f segment.

The actin protein family is composed of six isoforms, which can be subclassed into cytoplasmic, smooth muscle, and striated muscle isoforms. Isoactin cDNAs containing the VSV-epitope and cloned into expression vectors were microinjected into adult rat cardiomyocytes (ARC). Immunological staining of the epitope showed that muscle isoforms integrate into the sarcomeric organization. However, exogenous γ-cytoplasmic actin did not participate in the myofibrillar structures. Most of the γ-cytoplasmic actin was localized near the cellular circumference and morphological changes occured. Filopodia were induced, the cell area was increased, and interference with the sarcomeric organization was observed. It can be concluded that the various actin isoproteins have different tasks in the biogenesis of cytoarchitecture.

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CYTOSKELETAL BREAKDOWN AND RE-ASSEMBLY IN CULTURED ADULT RAT CARDIOMYOCYTES (ARC): A CONFOCAL MICROSCOPICAL STUDY

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The morphology of ARCs has been investigated by staining the cells at several stages for total F-actin with rhodamine-phalloidin and reconstructing the 3D pictures from confocal data sets by artificial shadowing using the newly developed software package 'Imaris' (Messerli et al., in preparation). Immunofluorescence has been used thereafter to localize several myofibrilar as well as cytoskeletal antigens

After isolation there are at least two populations of cells as can be discriminated by their morphological appearance. On one hand, cells having a rod shaped structure full of myofibrils (type I), on the other hand, spherical cells almost devoid of any discernible myofibrils (type II, see also abstract by S. Koch-Schneidemann et al.). After a period of two days the cells start to form an actin containing sheet like structure on the culture substrate. In type II cells this structure surrounds the cell body in type I cells it forms at the distal ends. Myofibrils of type II cells get progressively degraded as can be seen by the blurring and final disappearance of the sarcomeric pattern. It is not clear whether type I also loose their striated myofibrils completely as do type II or whether sarcomeres are added to the preexisting myofibrils. At around day 12 most cells are flattened out and are filled with regenerated myofibrils.

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VIDEO TIME-LAPSE RECORDING OF THE BEHAVIOR OF ADULT RAT CARDIOMYOCYTES ON SUBSTRATES CONTAINING LAMININ AND LAMININ FRAGMENTS

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Adult rat cardiomyocytes (ARC) were cultured for more than two weeks in an environmental chamber, mounted on an inverted microscope. The temperature of the microscope table was kept at 37°C and the chamber was supplied with humid and 5% CO_2 containing air warmed up to 37°C. This set-up allowed the observation of distinct cells over an extended time-period. Using a time-lapse video-recording system images were then taken at five minute intervals and stored on a magnetic optical disc.

ARC were cultured on different substrates: Gelatine and fibronectin as well as laminin-nidogen complex and laminin-fragments E8 and E1 (Konrad Beck et al. FASEB, Vol. 4, 148-160) all being a gift from Dr. Jürgen Engel.

Isolated ARC during redifferentiation in vitro may attach mainly in two ways to the substrate. On gelatine some cells attach without major morphological changes as rodshaped cells and begin to spread out along their longitudinal axis, while other cells first round up in suspension and only later attach to the substrate assuming a polymorphic shape (see abstract by M. Messerli et al.).On a laminin coated dish, however, ARC tend to attach rather fast and preferentially as rodshaped cells, indicating a possible role of laminin in the mode of attachment.

While laminin-nidogen complex promoted a longitudinal spreading of the more or less rodshaped cell, E8-fragment induced attachment followed by a rapid spreading out of the cells into a disc shaped structure.Fragment E1, on the other hand, known as a mitosis promoting domain, almost prevented attachment and only allowed very little redifferentiation of the ARC.

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Ca^{2+} CHANNELS AND Ca^{2+} STORES: THEIR CONTRIBUTION TO $[Ca^{2+}]_i$ STUDIED IN THE A7r5 SMOOTH MUSCLE CELL LINE USING SINGLE CELL MICROFLUORIMETRY WITH FURA-2

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Cultured cells from smooth muscle are convenient for studying some of the mechanism regulating vascular tone and blood pressure. We have used the A7r5 cell line as a model system for the study of vasoconstrictor hormone action as well as compounds acting at potential operated ('L-type') Ca^{2+} channels (POCs). In these cells vasoconstrictor hormones (vasopressin, serotonin, endothelin and platelet activating factor) as well as the Ca^{2+}-ATPase inhibitors thapsigargin and *t*-Bu-BHQ mobilized stored Ca^{2+} from the endoplasmic reticulum and stimulated Ca^{2+} influx. While the hormones stimulated influx through receptor-operated Ca^{2+} channels, we show that the Ca^{2+}-ATPase inhibitors stimulate capacitative influx (proposed by Putney in 1986). Evidence is also provided for the existence of at least two different Ca^{2+} stores; an agonist ($InsP_3$) sensitive and a ryanodine/caffeine sensitive Ca^{2+} store. The spontaneous Ca^{2+} oscillations which accompanied electrical activity of these cells were found to be mainly carried by 'L-type' POCs and could be modulated by Ca^{2+} antagonists and agonists. Oscillations in $[Ca^{2+}]_i$ ceased after hormone stimulation with the exception of endothelin which stimulated this activity.

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IDENTIFICATION OF 8 RFLPS AT THE LDLR GENE LOCUS AND CONSTRUCTION OF 370 HAPLOTYPES IN SUBJECTS FROM 51 SWISS AND 25 GERMAN FAMILIES WITH FAMILIAL HYPERCHOLESTEROLEMIA (FH)

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Restriction fragment length polymorphisms (RFLPs) at the low density lipoprotein receptor (LDLR) gene locus in 553 individuals from unrelated Swiss and German families with hypercholesterolemia were studied. For the *Taq I, Hinc II, Ava II,* and *Nco I* RFLPs, new rapid techniques based on PCR were used. In 51 Swiss and in 25 German families, FH was diagnosed. Analysis of linkage disequilibrium revealed non random associations among the RFLPs. Heterozygosity and RFLP information content values were compared with values calculated from literature (Italian, Icelandic, American, South African, and Japanese subjects). Differences of haplotype frequencies between the 7 population samples were revealed and confirmed by pairwise comparison and calculation of genetic distance between populations. For diagnosing FH, the *ApaLI 5', Ava II* or *Hinc II,* the *Nco I, Pvu II,* and *Taq I* RFLP were particularly helpful and FH can be diagnosed at the gene level in about 85% of the patients.

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CARBOHYDRATE RECOVERY IN 36 H FASTED - 4 H REFED RATS: EFFECT OF CARBOHYDRATE AND INORGANIC SALTS

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Previous experiments (J.Nutr. 1991, 121, 302-310) have shown that recovery of absorbed carbohydrates after refeeding 36 h fasted rats for 4 h (measured as increase of total liver + muscle glycogen + increases of blood glucose and plasma lactate) is about 40% higher with malt extract as the carbohydrate source than with starch or glucose. Since malt extract contains maltose, maltotriose, maltodextrines ($Gluc_4$-$Gluc_{10}$) as well as B-vitamins and inorganic salts, any of these compounds could be responsible for the observed effect. The same recovery of absorbed carbohydrates as with malt extract was now obtained when malt carbohydrates + inorganic salts (both as present in malt extract) were used for refeeding. With total malt carbohydrates or with maltodextrines alone, recovery was intermediate between malt extract and glucose. Maltose and glucose yielded similar carbohydrate recoveries. B-vitamins (as present in malt extract) had no effect. Portal blood glucose was higher after refeeding with malt carbohydrates ± inorganic salts than after glucose. This suggests that the different carbohydrate recoveries observed were due to differences in intestinal carbohydrate absorption.

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EXPRESSION OF FOUR PKC ISOFORMS IN RAT FIBROBLASTS

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Protein kinase C (PKC), the major receptor for tumor promoting phorbol esters, consists of a family of at least nine distinct lipid-regulated enzymes. How the various PKC isozymes are regulated *in vivo* and how they couple to particular cellular responses is largely unknown. We examined the expression and regulation of PKC isoforms in R6 embryo fibroblasts, a cell system we have previously used to study growth control and neoplastic transformation. Northern and Western blot analyses indicate that these cells express four PKC isoforms, cPKCα, nPKCε, nPKCδ, and nPKCζ; of which nPKCε and nPKCδ are the most abundant. Interestingly, the isoforms differ in their subcellular localization, Ca^{2+}- and phorbol ester-induced intracellular redistribution as well as in their capacity to be down-regulated in response to phorbol esters. Furthermore, transformation of these cells by activated c-H-ras, v-src or v-fos oncogenes causes increased expression of both cPKCα and nPKCδ, decreased expression of nPKCε, but no change in the expression of nPKCζ. Such effects are not observed in R6 cells transformed by v-myc, neu/erb-B2 or v-mos oncogenes. These results demonstrate that multiple isoforms of PKC can co-exist within a single cell type. Their differential regulation by extra- and intracelluar stimuli indicates that each isoform may influence growth control and transformation via distinct mechanisms.

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PLASMA LEVELS OF VITAMIN E CORRELATE WITH THE MORTALITY OF ISCHEMIC HEART DISEASE

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Classical risk factors for ischemic heart disease (IHD) like cholesterol (CHO), blood pressure (BP), and smoking habits have been determined as well as antioxidant vitamins and carotenoids in 16 study populations, mostly in Europe, with a sixfold difference of the age specific mortality from IHD. Significant correlations with IHD were found with the medians of CHO ($r2 = 0.29$) and diastolic BP ($r2 = 0.25$); inverse correlations with the medians of vitamin A ($r2 = 0.24$) and vitamin E ($r2 = 0.62$), both lipid standardized. Smoking habits and plasma selenium levels didn't show any association with IHD. In the stepwise regression analysis entering vitamin E, CHO, vitamin A and diastolic BP the following r2 were obtained: 0.62, 0.79, 0.83 and 0.87 which means that the mortality rates in these 16 populations can be explained to 87 % by two risk factors and two protective factors.

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THE ACE-INHIBITOR, CILAZAPRIL, SUPPRESSES INJURY-INDUCED NEOINTIMA FORMATION IN RATS, INDEPENDENT OF AGE, SEX, VESSEL, STRAIN AND INITIAL BLOOD PRESSURE

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Neointima (NI) thickening is part of the pathophysiology of restenosis after coronary angioplasty (RaCA). In the rat model NI formation is induced by vascular injury with a balloon catheter. NI formation could be markedly reduced by the angiotensin converting enzyme (CE) inhibitor cilazapril (cil) [1]. In order to exclude an inhibitory effect on NI which is specific only for the carotid artery in 4 month old male RORO rats which were used in our experiments [1,2,3], we examined the effect of 10 mg/kg/d cil in rats of different age (2,4,12 months), sex, in different vessels (carotid and iliac artery), with different iBP, (115-240 mmHg) or of another strain (Lewis). Results: Inhibition of NI formation with cil was not dependent on age, sex, arterial vessel, iBP, and could be observed in different rat strains. Conclusions: The mechanism by which CE inhibition modulates the response to vascular injury is unknown. We conclude, however, that this effect is not mediated via blood pressure reduction or limited to particular set of experimental conditions.

[1] Science (1989) 245, 186-188, [2] J Cardiol Pharmacol (1990) 16, Suppl 4 42-49, [3] JACC (1991) 17, 137B-142B.

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INTUSSUSCEPTIVE GROWTH OF CAPILLARY NETWORKS: A PRINCIPLE EFFECTIVE IN MANY ORGAN SYSTEMS

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In postnatal rat lung evidence was provided for a new type of capillary growth, termed intussusceptive microvascular growth: The lung microvasculature does not expand by endothelial sprouting, but by formation of slender transcapillary tissue pillars which give rise to new capillary meshes (Caduff et al., 1986, Burri and Tarek, 1990). Recently, in vivo microscopy combined with a TEM investigation of the capillary system of the chicken chorio-allantoic membrane (CAM) confirmed the existence of tissue pillars corresponding in size to those of the rat lungs (Patan and Burri, in prep.). In this study we investigated whether capillary growth by intussusception would also occur in other organ systems. We prepared Mercox casts of the vasculature of various organs in rats aged 4-9 weeks. Tiny holes (diameter ≈ 1.5 µm) corresponding in size to lung and CAM tissue pillars could be detected in the capillary networks of the choroid of the eye, submandibular gland, stomach, intestine, liver, kidney and ovary. These findings back the assumption that intussusceptive growth of capillaries represents a wide-spread principle.

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EFFECT OF HIGH ALTITUDE ACCLIMATIZATION ON AUTONOMIC REGULATION OF HEART RATE.

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Components of heart rate variability can be separated according to their frequency bands into two domains: low frequency (LF, ca. 0.1 Hz), due to both parasympathetic and sympathetic drive, and high frequency (HF, at the respiratory frequency) mediated by the parasympathetic alone. Series of 256 beat-to-beat intervals were used for spectral analysis of heart rate variability of 6 healthy male subjects (age: 32 ± 4 yr) supine and seated, both at sea-level and after 1 month of acclimatization to high altitude (5050 m). Subjects breathed in synchrony with a metronome at a frequency they choose (0.17 ± 0.02 Hz). Maximal heart rate was measured during a cycle ergometer test. At sea-level, a significant increase in LF and no change in RF were induced by the postural change, whereas at high altitude no change was seen in LF and a reduction in RF was observed (ie. a significant decrease in 5 out of the 6 subjects). This indicates that the cardiovascular adjustements brought about by a change in position from supine to seated at sea-level are mostly due to an increase in sympathetic activity whereas at high altitude this seems due to a decrease in parasympathetic activity. These results are concomittant with the reported reduced sensitivity of the heart to sympathetic drive, as well as with the hypothesis of increased in parasympathetic activity, after acclimatization to high altitude. This phenomenon may, at least partially, explain the decreased maximal heart rates (from 185 ± 10 to 153 ± 8 b/min) in altitude acclimatized human subjects. [This research was partially supported by the Ev-K2-CNR.]

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RECENT PROGRESS ON THE ENDOTHELIUM-DERIVED HYPERPOLARIZING FACTOR (EDHF) OF PIG CORONARY ARTERIES

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The left descending branch of the pig coronary artery are hyperpolarized and relaxed by kinins in an endothelium-dependent manner. Nitro-L-arginine, which inhibits NO synthesis, does not inhibit this hyperpolarization and relaxation. Therefore, in addition to NO, there exists another endothelium-dependent phenomenon that causes hyperpolarization and relaxation of smooth muscle. The signal, which originates from the endothelium could be either transmitted by electrical coupling or transmitted by a diffusible factor. The lack of dye coupling between endothelial and smooth muscle cells, plus the absence of effect of a gap junction uncoupler (halothane), and the fact that cytosolic calcium changes in an opposite direction in smooth muscles and in endothelium during stimulation by kinins, speak against an electrical coupling and thus favor the idea of a diffusible factor: the EDHF.

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A FLAG-TAGGED RAT PDGF RECEPTOR β ECTODOMAIN EXPRESSED IN SF9 CELLS RETAINS ITS HIGH AFFINITY BINDING

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Platelet-derived growth factor (PDGF) is a mitogenic and chemotactic peptide important for embryogenesis, growth and wound healing. It has been postulated to play a role in the pathophysiology of several diseases such as atherosclerosis and tumorigenesis. Three isoforms of PDGF can activate two different receptor subclasses: PDGF receptor (PDGFR) α and β. In order to investigate the role of PDGF in the response to vascular injury in the rat, we cloned the extracellular domain of the rat PDGFR β and expressed it in insect cells with the help of the baculovirus system. A hydrophilic octapeptide(FLAG) was added onto the N-terminus to enable purification via anti-FLAG affinity chromatography. Comparison of the FLAG-tagged protein with purified untagged PDGFR β ectodomain demonstrated that N-terminal addition of FLAG did not alter the affinity for PDGF. This method might be of general use in purification of proteins expressed by the baculovirus system.

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EXPRESSION OF CLUSTERIN (NA1/2 OR APO J) IN HUMAN FIBROBLASTS AND RAT SMOOTH MUSCLE CELLS

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Recent studies from our laboratory identified and characterised a novel, human plasma high density lipoprotein associated protein, termed NA1/2 or clusterin. The protein was simultaneously identified by other groups and this has led to a plethora of proposals as to its eventual function. These include a role in lipid transport, apoptosis and modulation of complement activity. To further investigate an eventual role in cell lipid homeostasis, we have examined, as potential models, cultures of two cell types for presence of the protein: human fibroblasts, the archetypal cell for studies of cell cholesterol homeostasis and rat aortic smooth muscle cells (SMC). Immunoblotting of whole cell extracts identified a major band (doublet) of Mr 45kD in both cell types: lower Mr bands of 36 and 25 kD (SMC) and 25 kD (fibroblasts) were also present. Subfractionation indicated that the higher Mr band was predominently associated with the membrane fraction, and the remaining bands with the cytoplasmic compartment. This is the first demonstration of the presence of the protein in fibroblasts and SMC and suggests that they could be useful experimental models for studies of the influence of cell lipid homeostasis on expression of clusterin.

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OKADAIC ACID AND $p13^{suc1}$ MODULATE THE REINITIATION OF MEIOSIS IN THE MOUSE OOCYTE.

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Short term exposure to okadaic acid (OA), a specific inhibitor of protein phosphatases 1 and 2A, induced resumption of meiosis including metaphase spindle formation in mouse oocytes treated with a phosphodiesterase inhibitor, while long-lasting incubations with OA arrested oocyte maturation at a step prior to spindle formation. Short-term exposure to OA led to tPA production and did not greatly affect the maturation-associated changes of protein phosphorylation. By contrast, a long-lasting application of OA did not result in tPA production and induced several changes in protein phosphorylation. Thus, inhibition of phosphatase 1 or 2A during a precisely time-regulated window of the cell cycle triggers resumption of meiosis and allows maturation to progress through M phase up to metaphase spindle formation. Microinjection into prophase oocytes of the product of the fission yeast gene, $p13^{suc1}$, known to inhibit $p34^{cdc2}$ kinase activation, prevented meiotic reinitiation. This effect was overcome by microinjection of OA, at concentrations higher than those required for induction of maturation in the absence of $p13^{suc1}$.

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SEQUENCE-SPECIFIC POLYADENYLATION CONTROLS DORMANT mRNA TRANSLATION IN MOUSE OOCYTES

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We have previously shown that tissue-type plasminogen activator (tPA) mRNA is stored, stable and untranslated, in the cytoplasm of fully grown primary mouse oocytes; this dormant mRNA contains an unusually short poly(A) tail. Its translational activation occurs during meiotic maturation and is accompanied by elongation of its 3'-poly(A) tract. By the injection of chimeric mRNAs containing part of the 3'-untranslated region (3'UTR) of t-PA mRNA, we have shown that polyadenylation and translation require both specific sequences in the 3'UTR and the canonical 3'-processing signal AAUAAA. More importantly, we have shown that the presence of a long poly(A) tract is necessary and sufficient for translation.
These results establish a role for regulated polyadenylation in the translational control of gene expression. We are currently studying the mechanism that prevents translation of tPA mRNA in primary oocytes, i.e. how is established the dormant status of a maternal mRNA.

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PURIFICATION OF A PERITONEAL FLUID FACTOR ABLE TO IMMOBILIZE HUMAN SPERMATOZOA.

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Substances present in peritoneal fluids (PFs) were shown to affect human sperm motility in vitro and in vivo. The aim of this study was to isolate the factor(s) present in PFs that are responsible for sperm immobilization. Fractionation of PFs on MW-sieving (Protein Pak 300) and on ion-exchange (SP MA7S and DEAE 5PW) HPLC columns allowed to purify a factor that, when tested by a sperm motility assay as function of time, could immobilize spermatozoa in 5 hours. The same functional assay was used after every purification step. The immobilizing factor (IF) was found to be tightly bound to albumin and if albumin was removed from the sperm immobilizing fraction, the immobilizing activity was lost (Sepharose Blue CL 6B). Consecutive separations of the immobilizing fraction on MW-sieving, SP-, and DEAE-columns respectively, allowed a purification of 150x of the IF, which was shown to have an apparent MW of 51.5-73.1 kDa. Extraction of the IF-fraction under acidic conditions (C7M/HCl; 200:100:1) showed that the IF was soluble in the organic phase. Furthermore, charcoal extraction of the active fraction completely eliminated the immobilizing activity. Thin layer chromatography (TLC; C/M/H_2O; 8:6:1) revealed a single band with a R_f=0.45. The staining of the TLC-plate for choline, serine and ethanolamine was negative.Extracting the band from TLC-plates and testing it on spermatozoa resulted in sperm immobilization confirming that the band contained the IF. Experiments are now in progress to determine the molecular structure of the IF.

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Characterization of POU genes in zebrafish embryos

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Transcription factors encoded by POU genes have been proposed to play important roles in establishing and maintaining the identity of various cell types in organisms as diverse as nematodes and mammals. The zebrafish is a model system that is particularly well suited to study developmental processes in vertebrates. To analyze the role of POU genes in the ontogeny of the zebrafish we have so far detected about a dozen genes or pseudogenes using a PCR-based screening approach. At least half of these are differentially expressed during embryogenesis. Currently we are isolating full-length cDNA clones of these genes in order to analyze their temporal and spatial expression patterns. This should provide us with clues about the potential function of those genes. Injection of anti-sense sequences or of dominant negative mutants should allow us subsequently to explore the role of the POU genes further.

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CARBONIC ANHYDRASE IN THE EARLY CHICK EMBRYO

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The presence, activity and distribution of the carbonic anhydrase (CA) (E.C. 4.2.1.1.) were studied in the whole chick blastoderm from laying onwards. Blastula, gastrula, neurula and two-day embryo were investigated. Three experimental approaches were used: western blotting method for detecting semiquantitatively the presence of CA, colorimetric delta pH micromethod for the assay of CA activity and immunohistochemical localization of CA II in embryo in toto. Two forms of CA were observed: a membrane-associated CA which was present but inactive at the earliest stages and a cytosolic CA which was first detected in gastrula and predominated in 2-day embryo. CA activity was first measurable in homogenate of young neurula (0.40 ± 0.24 U/embryo) and reached 2.2 ± 1.2 U in 2-day embryo. However, these activities corrected for protein were comparable (1.0 ± 0.5 U/mg). At gastrula and neurula stages, the highest CA II immunoreactivity was found in the embryonic area pellucida and in the ectodermal cells at the periphery of the rapidly expanding extra-embryonic area opaca. These findings suggest that CA could play a significant role in pH regulation in embryonic regions where CO2 production is high or might have other developmental functions.
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ENDOMETRIAL EXPLANT MODEL FOR THE INVESTIGATION OF UTERINE READINESS FOR IMPLANTATION IN HUMANS.

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The sequence of events leading to successful implantation of the embryo in the receptive endometrium is still poorly understood. Signals contributing to the pre-implantation dialogue between mother and conceptus can be local and/or endocrine. Functional endometrium is a dynamic tissue which includes the glandular epithelium and the stromal cells. We have developed an explant model which leaves the three-dimensional interactions between the individual cell types intact. Endometrial markers (prolactin, α_2-PEG, CA-125) were secreted and labelled precursor was incorporated into protein; this de-novo synthesis was inhibited by cycloheximide. In spite of a high variation in marker production from one tissue (patient) to another we could make out distinct patterns of secretion: paracrine vs. endocrine; cycle stage dependent or not. Platelet aggregating factor (PAF, suggested to play a role in the peri-implantation period) increased the production of α_2-PEG by cultured secretory endometrium. This was not the case with proliferative tissue, or for prolactin at any cycle stage. Growth factors and trophoblast material were also studied (co-cultures in the latter case).

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MEASUREMENT OF THE ENDOGENOUS ELECTRIC FIELD AND TRANSECTODERMAL POTENTIAL IN LIVING CHICK EMRYO

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Avian neural crest cells exhibit directed motility (galvanotaxis) in response to *in vitro* applied electrical field as low as 7 mV/mm, migrating towards the cathode. In this study, we measured *in vivo* endogenous electrical field (EEF) and transectodermal potential (TEP) in the last somite region of 20 somites chick embryo. Using glass microelectrode pairs, offset along their long axis by 30-50 µm, we measured dorsoventral components of the EEF ranging from 25-180 mV/mm (n=13) on 9 embryos. The field vector representing the flow of positive charges points ventrally from the dorsal ectoderm. In control experiments, using single microelectrodes, we found a mean TEP of 8.3 ± 2.3 mV (n=7), the inside of the embryo being positive. This TEP was reversibly decreased by phloridzin 10^{-3} mol/l (26 %), removal of glucose (10 %) and amiloride 5 10^{-4} mol/l (16 %). This EEF may help guide the neural crest cells ventrally, as the measured field amplitude is large enough and the polarity is consistent with their migration direction.

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TRANSITORY APPEARANCE OF CELLS WITH NEURONAL TRAITS IN THE DERMIS OF CHICK EMBRYOS

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In chick embryos, calbindin D-28k-positive cells are observed throughout the dermis. During development, they first appear between E8 and E10, then increase in number up to hatching, but later disappear progressively .Most of these cells display a bipolar shape : their ovoid cell body (10 µm diameter) gives rise to axon-like processes which extend up to 250 µm and occasionally mingle with nerve bundles. All these cells are immunoreactive to neuronal specific enolase, some of them are also positive for somatostatin 14, but none of them express substance P, CGRP or tyrosine hydroxylase. In electron microscopy, they contain numerous dense-core vesicles and they are surrounded by satellite cells.
The question may be raised whether these transient cells, which display some neuronal traits, originate from the neural crest.

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ION CHANNELS IN THE CHICK EMBRYONIC ECTODERM

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The early chick ectoderm generates organized trans- and pericellular ionic currents (Biol. Bull. 176S: 118,1989). The patch-clamp method has been used to identify the ionic channels in the ectoderm of the neurulating chick embryo. Single channel measurements at the dorsal ectodermal cell membrane showed 2 potassium channels and 3 non-selective monovalent cation channels. The most abundant is the high conductance calcium-activated potassium channel as indicated by the conductance, sublevels, mean open and close times, voltage-/ion-dependent activation range, and voltage-dependent sodium block. This channel is not active at the cell resting potential. The other potassium channel has a conductance of 9 pS and mean open and close times of 13 and 0.2 msec respectively. The non-selective channels have conductances of 5, 35, and 55 pS. The two latter types are active at the resting potential. The neural plate shows a higher density of both potassium channels and fewer non-selective channels than the extraembryonic ectoderm. No voltage dependent channels were found in the early ectoderm.

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ANALYSIS OF THE REGULATORY ROLE OF TRANSCRIPTION FACTOR AP-2 DURING MOUSE EMBRYOGENESIS

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AP-2 is a sequence-specific DNA binding transcription factor that was first identified and purified from human HeLa fibroblasts[1]. AP-2 is induced during retinoic acid dependent differentiation of human NT-2 teratocarcinoma cells[2] and RNA in situ hybridization to mouse embryo sections indicates that AP-2 has a restricted expression pattern during embryogenesis that is consistent with a regulatory role for AP-2 during retinoic acid-sensitive morphogenesis of several structures (notably the nervous system, limbs, and parts of the face)[3]. To assess the importance of AP-2 during embryogenesis, we are using a transdominant negative mutant strategy to block the function of AP-2 in specific tissues of transgenic mouse embryos. Using information from structure/function analysis of the AP-2[4], we have constructed vectors that express truncated versions of murine AP-2 which contain the dimerization domain but lack protein segments necessary for DNA binding and/or lack segments necessary for transcriptional activation. These truncated proteins form stable heterodimers with full-length AP-2 by *in vitro* cotranslation; furthermore, the mutant lacking the DNA binding specific segment abolishes DNA binding activity of wildtype AP-2 in bandshift assays. Experiments using the mutant proteins to block wildtype AP-2 functions will be presented.
[1] Mitchell et al. 1987. Cell 50:847-861, [2] Lüscher et al. 1989. Genes & Dev. 3:1507-1517, [3] Mitchell et al. 1991. Genes & Dev. 5:105-119, [4] Williams and Tjian. 1991. Genes & Dev. 5:670-682.

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IN VITRO ASSESSMENT OF CHRONIC DRUG-INDUCED HEPATOTOXCITY

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Hepatocyte cultures have been widely used to evaluate drug cytotoxicity in vitro, usually assessing the acute effects of single drug doses over periods of a few hours or days. However, therapeutically, drugs are often taken for several days or weeks. Therefore, we have used HepG2 hepatoma cells induced to express high levels of mixed function oxidase enzymes (1), to assess the hepatotoxic effects of acute (24 hr) or chronic exposure to amitryptiline, paraquat and ondansetron. We show that amitryptiline and paraquat are approx. 10-fold more potent hepatototoxic agents when added every 2 days up to 10 days in chronic exposure experiments, as assessed by morphological examination, than in the acute experiments where hepatotoxicity was assessed by morphology and biochemical assays. In contrast, ondansetron showed only modest hepatotoxicity after chronic exposure. The combined use of acute and chronic cytotoxicity studies using cells of human origin could be invaluable for assessing new drugs intended for human use.

(1) Hall, T.J., Cambridge, G. and James, P.R. (1991) Res. Comm. Chem. Pathol. Pharmacol. 72: 161-168.

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THREE IN VITRO TOXICITY / TERATOGENICITY TEST SYSTEMS VALIDATED BY USING TWELVE IDENTICAL CODED COMPOUNDS

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Toxic and teratogenic effects of 6 pairs of coded chemicals were assessed in 1) cultures of whole chick embryos 2) cultures of whole rat embryos and 3) aggregating brain cell cultures. (For methods, Experientia 44: vol 10, 1988).

Dose-dependent general toxic effects were obtained in all 3 systems. Similar types of malformations were induced in both chick and rat embryos. In the brain cell cultures, neuron- and glia-specific effects were distinguished. The compounds could be classified with respect to their teratogenicity/toxicity as follows: 1) $<10^{-6}$ M: retinoids (Ro 13-6307, Ro 1-5488), 6-aminonicotinamide, ketoconazole; 2) 10^{-6} to 10^{-3} M: sulfadiazine, 4-hydroxypyridine, sulfanilamide, theophylline, caffeine, metronidazole, methoxyacetic acid; 3) $>10^{-3}$ M: methoxyethanol. The results are comparable to data available from *in vivo* experiments.

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DISRUPTION OF HOST DEVELOPMENT BY PARASITIC WASPS: INVOLVEMENT OF POLYDNAVIRUS ?

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Parasitoids of the wasp genus Chelonus inject their eggs into eggs of various lepidopterous species. The parasitoid larvae develop within the developing host larvae; parasitisation causes hosts to enter metamorphosis one instar earlier than non-parasitised larvae. We analysed developmental and hormonal changes in parasitoids and corresponding hosts and searched for the mechanism by which host development is disrupted. The wasp females inject, together with the parasitoid egg, venom and calyx fluid which contains polydnavirus. In the electron microscope large quantities of cylindrical nucleocapsids were seen in nuclei of calyx epithelial cells and in calyx fluid. Gel electrophoretic analyses of viral DNA revealed the presence of at least 8 bands; after digestion with various enzymes very complex patterns were seen. Injection of calyx fluid, purified polydnavirus or venom into Spodoptera eggs did not cause precocious onset of metamorphosis indicating that polydnavirus or venom alone are not sufficient to cause disruption of host development. It appears that additional factors are necessary and/or that they act in concert.

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mtDNA REARRANGEMENTS AS A POSSIBLE INTRACELLULAR BIOLOGICAL CLOCK

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mtDNA-like fragments of the region of the genes coding for cytochrome oxidase subunit 3 and ATPase subunit 6 were analysed with the PCR assay in samples from human and rat liver biopsies and necropsies, and from human fibroblast cell culture. Numerous fragments were obtained, some of which are present in varying amounts in an age dependent and cell division number-dependent manner. The majority of the fragments (about 80%) can be recognised as deletions, and the rest as fragments with inversions and insertions. Sequence analysis has shown the presence of rearranged mtDNA sequences from the vicinity of the region being amplified in the PCR assay. Minor sequence differences indicate that these fragments are not pseudogenes, but generated during the cell life cycle or preserved during evolution. These fragments represent only a small fraction (approximately 5%) of the whole PCR-amplifiable population of mtDNA- and mtDNA-like fragments. The results suggest that mtDNA rearrangements may play an important role in the control of cell division and aging.

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LOCALIZATION AND IDENTIFICATION, WITH A MONOCLONAL ANTIBODY, OF NOVEL ANTIGENS IN THE MITOTIC SPINDLE AND IN THE CYTOPLASM OF *DICTYOSTELIUM DISCOIDEUM* AND OF MAMMALIAN CELLS.

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Using isolated, partially synchronized mitotic nuclei of the lower eukaryote *D. discoideum* we obtained by in vitro immunization a monoclonal IgM-antibody (MAb) called SCD. In double immunofluorescence experiments with SCD and TAT-1, an MAb specific for α-tubulin (Sherwin T., Gull K., Cell 57: 211; 1989), both labelled the mitotic spindle of *D. discoideum* throughout mitosis. With SCD it appeared as a fluorescent rod, solid in prophase and metaphase, but interrupted in the midzone of anaphase and telophase spindles. Spindle poles and asters were also revealed from metaphase onward. The filaments detected by SCD were thinner than microtubules. In interphase amoebae SCD also labelled the cytocenter and thin filaments emanating from it. In addition it revealed a spot-like fluorescence pattern in the cytoplasm throughout the cell cycle. In mitotic marsupial (PtK_2) and hamster (M3-1) cells SCD labelled the spindle poles and a few thin spindle filaments emanating from them and in interphase cells a bright spot near the nucleus that colocalized with the centrosome. Throughout the cell cycle SCD detected an array of cytoplasmic fibrils, some of which colocalized with single microtubules. Preliminary immunoblotting experiments with SCD revealed three polypeptides of ca. 44 kDa, 48 kDa and 64 kDa in a nuclear fraction from *D. discoideum*, and a doublet of polypeptides of ca. 29 kDa in total cell extracts of marsupial and hamster cells.

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INTRODUCTION OF A NORMAL HUMAN CHROMOSOME 11 INTO HUMAN RHABDOMYOSARCOMA CELLS.

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The development of human rhabdomyosarcoma has been associated with abnormalities of chromosome 11 in the region 11p15. To directly test the importance of sequences on chromosome 11, we introduced a normal chromosome 11 into the human rhabdomyosarcoma cell line RD via microcell fusion. The RD cells and the microcell hybrids express several markers of myogenesis, including myoD and myogenin. These factors have been shown to induce myogenesis in a number of different cell lines, but despite their expression RD cells differentiate only very poorly. Interestingly all RD cells fail to express myf5, in contrast to human primary myoblasts. To assay for in vivo tumorigenicity we injected the microcell hybrids in nude mice. One of the microcell hybrids is up to now tumor suppressed. Interestingly this clone is the only one showing a differentiated phenotype, although the expression level of the known myogenic transcription factors are not different than in the other microcell hybrids. We deduce therefore that there must be other factors which lead this clone to higher degree of differentiation, associated with tumor suppression.

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Deoxyribonuclease I is responsible for DNA degradation during apoptosis.

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Apoptosis occurs in a large variety of physiological events including embryogenesis, metamorphosis, endocrine-dependent tissue atrophy, removal of essential growth factors, maturation of the immune system and during immune responses *in vivo*. A hallmark of apoptosis is the internucleosomal DNA degradation for which a Ca^{2+}, Mg^{2+}-dependent endonuclease has been postulated. This nuclease activity, extracted from both thymocyte and lymph node cell nuclei, induced the ladder pattern of DNA fragments characteristic for apoptosis when transfered onto nuclei harboring only limited amounts of endogeneous nuclease. This activity was immunoprecipitated with antibodies to rat DNase I. Recombinant DNase I secreted by transfected COS cells displayed this "ladder forming" activity. In contrast to normal thymocytes and lymph node cells where DNase I was localised in the endoplasmic reticulum, a diffuse cytoplasmic distribution was observed in apoptotic thymocytes. We therfore propose that the loss of compartimentalisation of DNase I is responsible for DNA degradation during apoptosis.

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NOVEL ACIDIC GLYCANS AS MEDIATORS OF CELL ADHESION AND MIGRATION

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Cell adhesion in sponges is mediated by the new class of acidic glycans. Similar structures were localized by monoclonal antibodies in blastula and gastrula *L. pictus* sea urchin embryos within cell-cell contacts, attachment sites of apical lamina to microvilli and cilia, and blastocoel extracellular matrix. The Fab fragments of these antibodies completely inhibited cell reaggregation and also caused dissociation of living embryos. Both blocking effects were abolished by ether total sea urchin polysaccharides or purified new class of sponge acidic glycans, but not with mixture of glycosaminoglycans. Sea urchin cell adhesion and migration was promoted as in sponges via the same type of highly polyvalent acidic glycans of proteoglycan molecules. These carbohydrate structures were also found in mice embryos and adult normal and malignant human cells. Thus, the novel class of acidic glycans represents phylogenetically common cell adhesion and migration molecules.

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CARTILAGE FORMATION *IN VITRO;* EFFECTS OF α2,8-LINKED POLYSIALIC ACID REMOVAL BY ENDONEURAMINIDASE TREATMENT

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In mouse embryos, mesenchymal cells and precartilagenous mesenchymal condensates showed immunoreactivity for N-CAM and α2,8-linked polysialic acid (PSA) while embryonic cartilage was negative. Limb bud cultures were investigated by immunocytochemistry using antibodies specific for N-CAM and PSA (mAb 735 which recognises only PSA of ≥8 residues). An endoneuraminidase (Endo-N) which reduces PSA chain length to 5 units (not recognised by mAb 735) was also used. *In vitro* studies were performed using (1) whole limb buds and (2) dissociated cells. Treatment with Endo-N effectively removed all PSA *in vitro* as assessed by immunostaining.

Whole mouse limb buds were removed at embryonic day 13 and cultured *in vitro* for 3-4 days. PSA was seen in precartilagenous mesenchymal condensates, but not in areas of cartilage, as assessed by Alcian blue staining. In the presence of Endo-N, the overall size of cultured limb buds was significantly reduced, compared to controls. Structural changes in limb bud development were also seen, including the retardation of cartilage formation. Further investigations of cartilage formation *in vitro* are being carried out using high density limb bud micromass cultures.

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MAMMARY INVOLUTION-SPECIFIC GENE EXPRESSION

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We have studied changes in gene expression characteristic of mammary involution, the phase in which the mammary gland restructures after lactation. This process involves the programmed cell death of more than 90% of the epithelium. Using two approaches, a differential screening protocol and subtraction hybridization, we have cloned and sequenced genes expressed between 1 and 4 days after the end of lactation. The involution-specific genes identified fall into three categories: 1) genes associated with programmed cell death, 2) genes associated with tissue remodelling and 3) genes associated with re-activation of the mammary adipocytes for lipid storage. Some of the mammary-involution specific genes are additionally expressed transiently in early mammary development, others in the mammary gland in pregnancy. Significantly, certain tissue remodelling genes are over-expressed in association with mammary carcinoma, an observation which promises insight into the nature of epithelial:mesenchymal interactions across the basement membrane.

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PROTEIN TYROSINE KINASES IN MAMMARY GLAND BIOLOGY

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Protein tyrosine kinases (PTKs) play a fundamental role in normal cell growth control and differentiation, and there is compelling evidence for their involvement in neoplastic disease. Since carcinoma of the breast is the most frequent malignancy in women, a better knowledge of the involvement of PTKs in the growth control of the mammary epithelium will contribute to our understanding of mammary carcinogenesis. We have initiated PCR-cloning experiments to identify the constellation of PTK family members expressed at defined stages of normal mammary development (puberty, estrus cycle, pregnancy and involution). The isolated PTKs comprised known and so far not described members of the receptor, non-receptor and JAK families of PTKs. Distinct expression patterns were found in various organs and during mammary differentiation. Downregulation in the end-differentiated state, the lactating mammary gland was found consistently. Expression analysis during mammary carcinogenesis using Wap-ras and Wap-myc transgenic mice as model system revealed perturbations of expression during malignant transformation of the mammary gland.

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BOUNDARIES INSULATE SEGMENT-SPECIFIC REGULATORY REGIONS IN THE *DROSOPHILA* BITHORAX COMPLEX.

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The *Drosophila* bithorax complex controls segmental identity by selectively deploying three proteins, *Ubx*, *abd-A*, and *Abd-B* within specific segments along the body axis. Expression of these products within any one segment is under the control of large segment-specific *cis*-regulatory regions. In a model we had proposed, the segment-specific regulatory elements are sequentially activated along the chromosome by stepwise and independent opening of DNA domains. The results presented here provide very strong evidences that the segment-specific regulatory elements are organized in DNA domains that are separated and insulated of each other by boundaries regions. Inactive and active state of the segment-specific regulatory domains differ by their chromatin structure.

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Empty spiracles, A GAP GENE CONTAINING A HOMEOBOX INVOLVED IN *DROSOPHILA* HEAD DEVELOPMENT

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Mutations in *empty spiracles (ems)* cause severe defects in the head and the Filzkörper at the posterior end are missing. The *empty spiracles* gene has been isolated by homeobox homology using a probe from the *msh (muscle segment homeobox)* gene. In addition to the homeodomain, the gene product has a proline-rich domain and an acidic domain consistent with the role of the *ems* gene as a transcription factor. A 2.4 kb RNA is expressed in two phases of embryonic development. First expression is seen at the blastoderm stage in a single anterior band, correlating with it's function as an anterior gap gene. The gene is expressed in the preantennal, antennal and intercalary segments and is required for the development of the antennal sense organ, the optic lobe and parts of the head skeleton. Later during embryogenesis *ems* is expressed in the posterior spiracles as well as lateral regions of each segment where the tracheal pits form and lateral neuroblasts originate. Using ß-gal fusions we could identify at least five different regulatory elements in the *empty spiracles* promotor region responsible for tissue specific expression of the gene. Most of the elements seemed to be conserved in another *Drosophila* species, *Drosophila virilis*. The element responsible for the early expression is dependent on the maternal gene *bicoid*, the key gene of the anterior system. This dependence will now be tested at the molecular level.

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THE *DROSOPHILA SLOPPY PAIRED* LOCUS ENCODES TWO PROTEINS INVOLVED IN PATTERN FORMATION

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The *sloppy paired (slp)* genes are involved in the establishment of the metameric body plan of the *Drosophila* embryo. We have cloned the *slp* locus by P-element-mediated enhancer detection. We detected two transcription units in the *slp* locus, *slp*1 and *slp*2. The two genes show considerable sequence homology and share a region of 107 amino acids (76% identical amino acids). This region, termed the *fork head* domain, is also present in the *Drosophila* homeotic gene *forkhead* and three liver transcription factors in rat. The spatial expression patterns of the two transcripts are virtually identical suggesting common regulation of the two genes. However, *slp*2 is expressed later than *slp*1. The *slp* transcripts accumulate during early embryogenesis in three steps, each showing characteristics of one of the segmentation gene classes (gap gene, pair-rule and segment polarity class). We recovered additional *slp* alleles by remobilization of an enhancer detector transposon. In homozygous mutant embryos we find different aspects of the phenotype that correlate with each step of *slp* expression. Genetic analysis suggests that both genes contribute to the segmentation phenotype and interact functionally.

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Cell Communication Between Two Germ Layers is Involved in the Region Specific Expression of a *Drosophila* POU Box Gene in the Endoderm

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In *Drosophila*, the midgut forms from two endodermal primordia located near the embryonic poles. Whereas ectoderm and mesoderm are subdivided in the antero-posterior direction into parasegments by the action of the segmentation genes, regional specification of the midgut epithelium is dependent on the expression of homeotic selector genes that trigger expression of growth factor homologues in the adhering visceral mesoderm (VM). The midgut therefore represents an ideal system to study inductive events between germ layers in the *Drosophila* embryo.

We have recently cloned a *Drosophila* POU box gene (POU33F1) and characterized its expression during embryonic development. Interestingly, although the transcript and protein are present in the growing endodermal primordia until they fuse, the expression is modulated thereafter and turned off in two regions that are adjacent to visceral mesoderm cells that express the growth factor homologue *dpp*. We have found that *dpp* expression is required for the repression of the POU33F1 gene since that repression no longer occurs in various mutant embryos that eliminate *dpp* expression. Although repression seems to require signals from the adjacent cell layer, high level expression of POU33F1 in the most posterior midgut is independent of homeotic gene expression in the VM.

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PROPOSED MECHANISM UNDERLYING THE POLAR COORDINATE MODEL FOR REGENERATION IN DEVELOPMENTAL FIELDS

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The regenerative behavior of newt limbs, cockraoch legs and *Drosophila* imaginal disks are predictable on the basis of the polar coordinate model formulated by French et al. (Science 193, 969-981, 1975). However, the underlying mechanisms for these processes are still unknown. On theoretical grounds, we propose such a mechanim which forms the basis for a computer program by which the regenerative behavior in developmental fields can be simulated. The proposed mechanism is basically a variation of Turing's reaction-diffusion model, but it also takes cell proliferation into account. Furthermore, it assumes that the molecules which maintain the positional information also control cell proliferation and that all the gradients arising in a developmental field are local ones. The computer simulation shows that both the initial formation of the developmental field and the regenerative processes (including mirror-image duplication and supernumerary limb formation) can be explained by the same proposed mechanism.

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FUNCTION OF THE SEGMENTATION GENES *SLOPPY PAIRED* 1 AND 2 IN *DROSOPHILA* DEVELOPMENT

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Our laboratory has recently cloned and characterized the *sloppy paired (slp)* locus, which contains two genes involved in establishing the proper segmentation of the *Drosophila* embryo (Grossniklaus et al., submitted). The two proteins, *slp*1 and *slp*2, contain a putative DNA-binding domain (the *fork head* domain), also found in other fly developmental genes and hepatic transcription factors. Analysis of several *slp* alleles that delete either one or both genes suggests that the slp proteins have two functions in segmentation; a requirement in every other segment where *slp*1 is the major contributor and a second requirement in every segment, where both proteins have a redundant function. Analysis of the distribution of other segmentation genes in *slp* mutants revealed that *slp*1 is a repressor of the pair-rule gene *fushi tarazu (ftz)* in the posterior part of even-numbered parasegments (where *slp*1 and *slp*2 are normally expressed) and both slp proteins activate the segment polarity gene *wingless*, first in the even-numbered parasegments and later in every segment, consistent with the proteins' proposed pair-rule and segment polarity functions. Experiments to localize the cis-acting elements of *ftz* that respond to *slp*1 will be described.

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CONTROL OF DROSOPHILA MELONGASTER EF-1a EXPRESSION DURING DEVELOPMENT AND AGING

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Drosophila melongaster contains two EF-1a genes, F1 and F2, which seem to be developmentally regulated. The F1 gene has been implied to play a role in aging control (Shepherd et al. 1985). We are analyzing the regulation of the F1 gene expression. Throughout the lifespan of the flies, EF-1a mRNA and protein are measured by quantization of Northern blots and Western blots. The results suggest that EF-1a activity is not regulated only at the transcriptional level, but that post-transcriptional and/or post-translational mechanisms are also involved.

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MOLECULAR GENETICS OF THE *DROSOPHILA* LOCUS *HAIRLESS*

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Hairless is a dominant loss of function mutation affecting the formation of adult sensory organs. Genetic interactions of *Hairless* with various neurogenic loci suggest that it might be involved in the transduction of developmental signals during embryonic neural differentiation. In order to gain insight into the function of the *Hairless* gene product we have initiated a molecular analysis of the gene. In a chromosomal walk we have cloned the *Hairless* region and characterized genomic breakpoints of available *Hairless* alleles. Transcriptional analysis revealed that the region is heavily transcribed during embryogenesis. We have isolated and sequenced *Hairless* cDNA's indicating that all isolated cDNA clones fall into a single class although there seems to be a larval specific *Hairless* transcript which differs in size from a ubiquitous RNA. *Hairless* encodes a rather basic, serine rich polypeptide which has no homologies to other known proteins.

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CALF HAS TWO VERY SIMILAR ISOENZYMES OF THE INTESTINAL ALKALINE PHOSPHATASE (IAP)

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By PCR cloning of cDNAs, we have identified and partially analysed two isoenzymes of calf IAP (C-1 and C-2). The yet characterized regions of the two polypeptides share 95% of identity. The 514 amino acids of C-1 display four N-glycosylation signals (positions 122, 222, 249 and 410) and the hydrophobic C-terminus suggests addition of a glycosyl-phosphatidylinositol (GPI). C-2 on the other hand remarkably lacks the first N-glycosylation signal, which was found in every mammalian AP so far. All oligosaccharides attached to Asn 249 and 410 of C-2 are of the complex type. One or even two O-linked chains are possibly fixed in the sequon ProThrThr (479-481) preceding the GPI anchor, which will be analysed soon.

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CMD1, MYOGENIN AND CREATINE KINASES (CK) IN EMBRYONIC CHICK SKELETAL MUSCLE

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The B creatine kinase (B-CK) is expressed in developing myogenic cells while M-CK expression is typical for differentiated skeletal muscle. Using ^{35}S-labeled anti-sense cRNA probes for in situ hybridization, we have detected B-CK mRNAs at Hamburger-Hamilton stage 13 at high level, but M-CK mRNAs were not found before stage 19. The B-CK transcripts are detectable as soon as myotomes, the first skeletal muscle masses to form, are morphologically distinguishable. The mitochondrial CKs (Mi-CKs), always coexpressed with a cytosolic CK in adult tissue, will be analyzed by in situ hybridization, yet S1-data indicate that they appear later.

This expression pattern was compared to myogenic differentiation factor genes, which are thought to regulate skeletal muscle-specific gene expression, therefore also M-CK. CMD1 is expressed at the same time as B-CK in the myotomes, and Myogenin is only detected at stage 15. Both factors are expressed before M-CK. It has been proposed, that the myogenic factors directly regulate M-CK gene expression in the mouse by binding to its enhancer. If the regulation of the M-CK gene is similar in the chick and in the mouse as it has been shown for of the actin gene, our results indicate that these factors by themselves are not sufficient to initiate M-CK gene expression. There is no indication of direct regulation by these factors of the muscle specific mitochondrial Mib-CK gene.

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CULTURED AORTIC SMOOTH MUSCLE CELLS FROM NEWBORN AND ADULT RATS SHOW DISTINCT CYTOSKELETAL FEATURES.

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It is well known that arterial smooth muscle cells (SMC) from adult rat cultured in the presence of fetal calf serum (FCS) lose the expression of differentiation markers (Skalli et al., J. Submicrosc. Cytol. 18:481, 1986). In order to establish a model of in vitro differentiation, SMC were isolated from newborn rat aortic media and cultured in the same conditions used for adult animals. Compared to freshly isolated cells, primary cultures of SMC from newborn showed no change in the number of α-SM actin containing cells and a less important decrease in the number of desmin and SM myosin containing cells than that seen in primary cultures of SMC from adult animals; moreover, they showed an increase of α-SM actin mRNA level, α-SM actin synthesis and expression per cell. These features were partially maintained at the 5th passage. Cloned newborn rat SMC continued to express α-SM actin, desmin and SM myosin at the fifth passage. Thus, newborn SMC maintain, at least in part, the potential to acquire differentiated features in culture. When cultured in the presence of heparin, newborn SMC showed an increase of α-SM actin synthesis and content but no modification of the proportion of α-SM actin total (measured by Northern blots) and functional (measured by in vitro translation in a reticulocyte lysate) mRNAs compared to FCS treated cells. These results suggest that heparin action is exerted at a translational or post-translational level. Cultured newborn rat aortic SMC furnish an in vitro model for the study of SMC differentiation. (Supported by the Swiss National Science Foundation, Grant Nr 31.30796.91).

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CLONING OF NOVEL POU-DOMAINS FROM HUMAN MUSCLE

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Many homeobox-containing genes of Drosophila regulate pathways of differentiation. In Drosophila at least two homeobox-containing genes have been identified, that seem to have a regulatory function in mesoderm formation and myogenesis (S59, msh-2). Investigation on the mechanisms of myogenesis in vertebrates has revealed four related genes (MyoD, Myogenin, Myf5, MRF4) that belong to the helix-loop-helix family of DNA-binding proteins. Since several experiments indicate, that there might be additional factors involved in vertebrate myogenesis, we decided to search for homeobox-containing DNA-binding proteins in human muscle tissues.

Using a pair of degenerated oligonucleotides to amplify cDNA's from skeletal and heart muscle by PCR three novel homeobox-containing sequences were found, which belong to the family of POU-domain proteins. Until now we have concentrated on the characterization of clone BDOM130, since its linker sequence between the POU-specific and the POU-homeo-domain shows no significant homologies to any of the known classes of POU-domain proteins. Using the RACE protocol we cloned the 3'end of BDOM130. Preliminary results from Northern blot analysis of several human cell lines indicate, that expression of BDOM130 is not restricted to muscle. We are now cloning a full-length cDNA of BDOM130 in order to study the DNA-binding properties of the encoded protein.

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Smooth muscle hyperplasia and ODC activity in hypertrophic intestinal wall in rat.

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This study had been performed on the smooth muscular wall of the small intestine after a partial stenosis. In these conditions the muscular wall became thicher both because of cell hypertrophy and cell hyperplasia. Maximum thickness was reached 10 days after the intervention, and was mantained over the following days; Autoradiography after administration of Tritied-Thymidine demonstrated the presence of labeled smooth muscle cell nuclei in hypertrophic intestinal loops. The activity of ornithine-decarboxylase (ODC) in the smooth muscle increased progressively during the hypertrophic process reaching its peak ten days after the stenosis. On the fifteenth day ODC activity returned to basal levels, while the muscular wall hypertrophy remained.

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PURIFICATION OF HUMAN MUSCLE SATELLITE CELLS BY CLONING AND CELL SORTING

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Human muscle satellite cells (HMSC) are myogenic cells that are normally mitotically quiescent, but are activated in muscle growth or damage. We are interested in studying in vitro the biochemical and electrophysiological properties of these cells in normal and diseased muscle. HMSC were obtained from dissociated human muscle biopsies. In order to circumvent the problem of the contamination of cultures by fibroblasts, we established clonal cultures from dissociated biopsies. This revealed the presence of 2 types of cells: small myogenic cells (10μ diameter; 70% cloning efficiency) and large non-myogenic cells (20μ diameter; 27% cloning efficiency). From this observation, we developed a purification procedure based on cell size using the cell sorter. This allowed us to obtain pure mass cultures of HMSC, as well as clonal cultures which we used for detecting individual differences between HMSC.

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GROWTH POTENTIAL OF HUMAN MUSCLE SATELLITE CELLS IN CLONAL CULTURES.

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Human muscle satellite cells (HMSC) are small fusiform cells lying between the sarcolemma and the basal lamina of striated muscles. These cells are involved in the growth and regeneration of striated muscle. We studied the proliferation characteristics (lag time and doubling time) of HMSC in normal muscle. HMSC were obtained from muscle biopsies taken during orthopedic surgery on children. The proliferation of HMSC was studied in vitro in 30 clones coming from 3 different biopsies. Examination of the growth curves showed that 1) the lag time before the first division of the cloned cells ranged from 2 to 10 days; 2) cells divide at a constant rate with individual doubling times between 20 and 50 hours; 3) one of the 3 biopsies gave rise to cells having much longer lag times; 4) there was no significant variation in the doubling times of the cells coming from the 3 different biopsies.

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ELECTROPHYSIOLOGICAL PROPERTIES OF HUMAN MUSCLE SATELLITE CELLS IN CLONAL CULTURES

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Human muscle satellite cells (HMSC) are implicated in the growth and regeneration of skeletal muscles. Whole-cell recordings were performed in three different proliferating clonal cultures of HMSC. Three currents have been studied so far : an inward sodium current resistant to tetrotodoxin and two non-inactivating outward potassium (K^+) currents, one being activated by high internal calcium concentration. The systematic presence in our proliferating satellite cells of outward K^+ currents and the absence of the inward rectifying K^+ current differ with classically described fiber attached satellite cells and differentiating satellite cells. Within each clone, a marked cell to cell variability of membrane current amplitudes is observed (between 10-200 $\mu A/cm^2$), suggesting a differential expression of current amplitudes within the same progeny.

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CLONING OF THREE HUMAN MEMBERS OF THE S100 PROTEIN FAMILY AND FUNCTIONAL ANALYSIS BY CELL TRANSFECTION

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Despite the fact that there are at least nine known members of the S100 family of calcium binding proteins, their functional role remains an open question. Cell transfection experiments are in our mind the best approach to get some informations on the function they have in the cell. As a first step to construct expression vectors we isolated the full length cDNA clones of three members of the S100 protein family, S100alpha, CAPL and CACY, from human brain and heart muscle libraries. We determined the so far unknown sequences of human S100alpha and CAPL and compared them to the established sequences of the S100 protein family. Northern blots with RNA from various human tissues hybridized with these cDNAs as probes showed that the expression of S100alpha is mainly restricted to muscle and brain, whereas CAPL and CACY show a more general distribution. Given our particular interest for neuronal and muscular systems, we cultured human, muscle and rat cell lines of these origins under variant conditions and screened them by Northern hybridization with our probes. For the transfection experiments we chose both a cell line from neuronal and from muscular origin and we will present first datas on the transfectants.

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MODULATION OF PROSTAGLANDIN E_2 BIOSYNTHESIS IN CHICK SPINAL CORD DURING DEVELOPMENT

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Prostaglandin E_2 (PGE_2) is one of the major prostanoids synthesized in chick spinal cord. During the development of chick embryos from day E6 to E12 or chicken 6 days after hatching (6AH), PGE_2 synthesized in spinal cord homogenates was measured by enzyme-immunoassay immediatly after homogenization, after a 5 min incubation in Tris either free of or added with 0.3 mM arachidonic acid (AA).

	E6-8	E10-12	6AH
	pmol PGE2/mg protein		
After homogenization	1.3 - 2.0	0.4 - 0.7	11.4
+ 5 min in Tris	1.8 - 1.7	1.7 - 6.4	47.1
+ 5 min in Tris + AA	63.8 - 78.3	234.5 - 303.8	412.7

These results indicate that 1) the addition of AA enhances dramatically the formation of PGE_2 at each stage of development; 2) the amount of PGE_2 formed without or after addition of AA increases with age.

In conclusion, the synthesis of PGE_2 could depend on the concentration of free AA and on the increasing activity of PGE_2 synthase with age (Vesin and Droz, J. Neurochem. 57, 161, 1991).

SNF N°. 3.397-0.86.

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MYELIN/OLIGODENDROCYTE GLYCOPROTEIN: cDNA SEQUENCE AND mRNA EXPRESSION

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Myelin/oligodendrocyte glycoprotein (MOG) is expressed only in CNS myelin and found on the external myelin sheath and oligodendrocyte processes. This localization may provide an ideal target antigen for involvement in demyelination. We have positively identified a number of MOG clones following comparison of rat cDNA sequences with N-terminal mouse MOG peptide sequence. Northern blot analyses of MOG mRNA expression showed a single band of 1.6 kb. This expression was limited to brain and completely negative in all other tissues examined, including PNS sciatic nerve. In addition, peak MOG mRNA was expressed coincident with the active phase of myelination in mice (15-25 days of age). The sequence of a full-length rat MOG cDNA has been determined. This analysis has revealed a putative signal peptide of 27 amino acids, followed by 218 amino acids for mature MOG (calculated MW: 24962). Also, a single N-glycosylation site at Asn-31 was identified. Additional studies are ongoing to determine MOG function and its possible role in demyelination.

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AXON GUIDANCE GENE *UNC-51* IN THE NEMATODE *C. ELEGANS* ENCODES A PUTATIVE SERINE/THREONINE KINASE

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The adult hermaphrodite of the nematode *C. elegans* posseses only 302 neurons whose cell lineages and synaptic connections are completely known. Several genes were shown to be essential for the correct outgrowth and guidance of the axons of various neuron classes during the development of this nematode, among them *unc (uncoordinated)-51*. In *unc-51* mutants, several neurons have abnormally guided axons. The HSNs (hermaphrodite-specific neurons), e.g., are defective in their anterior growth. Such animals show an egl (egg-laying negative) phenotype, suggesting that their HSNs are impaired in the ability to stimulate egg-laying. The *unc-51* gene, which is located on chromosome V, was cloned by transposon tagging. A genomic probe of the unc-51 gene hybridizes to a single, 3.1 kb long mRNA species on Northern blots. A corresponding full length cDNA clone was isolated and entirely sequenced. Computer analyses revealed that it encodes a putative protein bearing all structural features typical for the catalytical domains of protein-serine/threonine kinases. Our results, therefore, suggest that the axon guidance gene *unc-51* encodes a protein-serine/threonine kinase.

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ISOLATION OF cDNA CLONES FROM RAT OLIGODENDROCYTES CODING FOR ZINC FINGER PROTEINS

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The function of the vertebrate nervous system is dependent on the appropriate myelination of its fiber tracts. In the CNS, oligodendrocytes produce the myelin sheath around the axons. In order to understand oligodendrocyte differentiation in more detail, we aimed at the cloning of putative transcription factors expressed in this particular glial cell type.

A cDNA library was constructed from highly enriched differentiated rat oligodendrocytes and screened with an oligonucleotide deduced from a conserved region of the Krüppel family of zinc finger proteins (the H/C link). 11 different cDNAs were obtained; 4 revealed an expression pattern consistent with a possible role in the oligodendrocyte cell lineage: while 3 of these 4 genes are upregulated in the spinal cord between birth and P20 (a phase in which extensive oligodendrocyte differentiation and myelination occurs in the rat spinal cord), the expression of 1 gene is markedly reduced during that time span. The corresponding mRNAs range in size from 2 to 5 kb. At present, we are analysing the cell type specificity by in situ hybridization as well as Northern blots of RNA from different areas of the nervous system. In addition, sequence analysis will be continued. So far, complete sequencing of 1 clone showed that the cDNA comprises the coding capacity for a new member of the zinc finger gene family; the deduced protein contains 12 finger domains.

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A DIFFERENTIAL SCREENING APPROACH TO ISOLATE NEW OLIGODENDROCYTE-SPECIFIC cDNA CLONES

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Oligodendrocytes play an important role in CNS development and regeneration, as well as in certain demyelinating diseases.In addition to forming myelin they also express specific neurite growth inhibitory proteins. Our project aims at the cloning and identification of new oligodendrocyte specific gene products and the study of their expression during oligodendrocyte differentiation in vitro and in vivo. We used single-stranded cDNA of P16 spinal cord (plus probe) and sscDNA of X-irradiated, oligodendrocyte free spinal cord (minus probe) to differentially screen a P16 rat spinal cord cDNA library. Liver mRNA was used to remove "house keeping genes" from both sets of probes by subtractive hybridisation. Clones coding for the known myelin proteins MBP,PLP,MAG and CNPase were identified and discarded. We have found several novel cDNA clones. Northern analysis shows that some of them are restricted to the CNS while others are also expressed in sciatic nerve. For several of these clones the developmental time-course is consistent with a function associated with myelin formation.

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ISOLATION OF A NEW OLIGODENDROCYTE SPECIFIC C-DNA

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Based on a differentially screened c-DNA library of rat spinal cord we preselected for oligodendrocyte specific c-DNAs (Schaeren-Wiemers et al., this volume). Northern analysis on CNS, sciatic nerve and liver m-RNA at different time points during development revealed a small set of potential oligodendrocyte specific genes.
One of these cDNA clones ("16.111"/CNS-3) corresponds to an m-RNA of 4.4 kb; its expression is restricted to the postnatal rat CNS and is higher at P16-20 than in the adult spinal cord. *In situ* hybridisation shows a selective, strong expression in oligodendrocytes in the brainstem, cerebellar white matter and corpus callosum. Existing sequences (80% of the full lenght c-DNA) do not correspond to any known oligodendrocyte or CNS protein.

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SCHWANN CELLS EXPRESS A NUCLEAR 3,5,3' TRIIODOTHYRONINE RECEPTOR IN RESPONSE TO A LOSS AXONAL CONTACT IN RAT AND HUMAN SCIATIC NERVE.

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The expression of nuclear triiodothyronine receptors (NT_3R) by Schwann cells was tested in adult rat or human sciatic nerves in presence or absence of normal axonal contact. The expression of NT_3R was investigated by immunostaining with a specific 2B3-NT_3R monoclonal antibody. In the intact sciatic nerve of adult rats, the nuclei of the Schwann cells were free of any NT_3R expression; after sciatic nerve transection, NT_3R expression was induced in Schwann cells of proximal and distal stumps adjacent to the section. Changes in NT_3R expression occurring *in vivo* after nerve transection were confirmed in dissociated sciatic nerve culture in which Schwann cells exhibited NT_3R immunoreactivity. In contrast, Schwann cells were devoid of NT_3R expression in explants of dorsal root ganglia in which they ensheath the growing neurites. Furthermore, Schwann cells in normal human sciatic nerve were free of NT_3R immunoreactivity. While in human Schwannomas, in which no axons are present in the body of the tumor, all the nuclei of the Schwann cells were NT_3R positive. In conclusion, with respect to possible mechanisms controlling NT_3R expression, it is suggested that the synthesis of these receptors is regulated by contact of Schwann cells with axons. (SFN 31-26410.89).

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EFFECTS OF LEAD ACETATE AND TRIMETHYLTIN IN BRAIN CELL AGGREGATE CULTURES

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In order to characterize the neurotoxic effects of low doses of metals chronically administered, and to find early markers of toxicity, aggregate cultures of fetal rat telencephalon were treated during 10 days with lead acetate (Pb) (10^{-8} to 10^{-4} M) or trimethyltin (TMT) (10^{-9} to $3{\cdot}10^{-7}$M) at two developmental stages (either undifferentiated or differentiated cells). The induced modifications were monitored by 1) determination of the activity of neuron- and glia-specific enzymes 2) analysis of structural proteins by immunoblot and immunohistochemistry 3) detection of microglia, using a specific lectin marker. Glial cells were most affected, showing selective changes: some glia-specific markers increased with TMT and decreased with Pb, in a development-dependent manner. The number of microglial cells increased only in differentiated cultures and at the lowest doses. Furthermore, their localization within the aggregates was different in the two treatments. Thus, TMT and Pb offer two model substances to study different mechanisms of gliotoxicity.

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NEURON-SPECIFIC ENOLASE IS EXPRESSED IN UNDIFFERENTIATED MURINE EMBRYONAL STEM (ES) CELLS

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Embryonal stem (ES) cells are primary, pluripotent cells derived from the day 4 mouse embryo which, under appropriate culture conditions *in vitro*, can differentiate into cells expressing neuronal markers (Wobus et al., Biomed. Biochim. Acta 47, 965-973, 1988; our own observations). Neuron specific enolase (NSE) is a glycolytic enzyme which is considered to be present exclusively in differentiated neurons - consistent with the fact that a reporter construct composed of the rat NSE promoter fused to the gene for bacterial β-galactosidase had been found to be expressed specifically in terminally differentiated neurons of transgenic mice (Forss-Petter et al., Neuron 5, 187-197, 1990). In order to visualize the appearance of such neurons in ES cell *in vitro* differentiation cultures, ES cell line 12957 (Eistetter, Eur. J. Cell Biol. 45, 315-321, 1987) was transfected with the NSE/β-Gal construct. Those clones which were found to have the reporter construct stably integrated into their genome unexpectedly displayed β-galactosidase activity in the undifferentiated state, already. Within 7±1 (n=3) days of culture under differentiation conditions *in vitro*, however, this activity decreased to basal levels. To confirm these results we performed RT/PCR studies on mRNA isolated from undifferentiated and differentiated stem cells using oligonucleotide DNA primers specific for the murine NSE gene. Interestingly, the endogenous, murine NSE promoter displayed an identical pattern of activity. These results suggest that NSE is expressed at early developmental stages in the mouse.

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NEURITE FORMATION OF DORSAL ROOT GANGLION CELL IN VITRO

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Dorsal root ganglion (DRG) cells at early stages of development are bipolar and later become pseudounipolar cells. We observed the morphological changes of cultured neurons from bipolar to pseudounipolar cells using a video recording system. The original process of the DRG neurons first disappeared within 30 minutes in culture and then the neurons elongated forming one or two new neurites. The DRG cells were spindle-shaped at 21 h, eccentric bulged at 23 h, bell-shaped at 30 h, and finally became short-stem unipolar cell at 34 h. The elongation and arborization of processes by the unipolar DRG cell continued after 48 h. We propose that DRG cells in the early developmental stages of culture repeat the morphological changes from bipolar to pseudounipolar cells and DRG cells become mature pseudounipolar neurons after two weeks of culture.

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STABLE EXPRESSION OF THE HUMAN LOW-AFFINITY NGF RECEPTOR IN MURINE EMBRYONIC STEM (ES) CELLS

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Exogenous nerve growth factor (NGF) has been shown to accelerate the appearance of neuron-like cells in *in vitro* differentiation cultures of murine ES cells (Wobus, A.M. et al., 1988, Biomed. Biochim. Acta 47: 965-73). The presence of NGF receptors (NGFR) on ES cells is postulated but has not been examined directly and thus the mechanism of this neuronal induction is unclear. The mammalian expression plasmid pMVE-1 encoding the human low-affinity NGFR (**p75**; Johnson, D. et al., 1986, Cell 47: 545-54) has been electroporated into ES cell line 12957. Ten independent clones were analyzed for expression of recombinant p75 using a monoclonal antibody (ME20.4) specific for the human p75. Seven clones stained positively for NGFR expression and clone 1N6 was chosen for further characterization. Fluorescent activated cell sorting (FACS) analysis using ME20.4 as the primary antibody and an FITC-labeled second antibody has demonstrated that human p75 can be detected on 100% of 1N6 cells. Pretreatment of 1N6 cells with 1 μM NGF reproducibly displaced the binding of 0.8 μg/ml ME20.4 to recombinant human p75. No displacement of ME20.4 binding was observed when cells were pretreated with an unrelated growth factor (bFGF) known not to bind to NGFR. Functional coupling of the p75 to signal transduction systems in 1N6 cells is currently being investigated.

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ASTROCYTE-CONDITIONED MEDIUM IMPROVES THE CULTURE OF DORSAL ROOT GANGLION (DRG) CELLS FROM CHICKEN AFTER HATCHING

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Dissociated DRG cell cultures from 8-day old chick survived with difficulty as compared to those from E10 chicks embryos. To improve the culture, a 1.04 percoll gradient was used to remove myelin and cell debris. Dissociated DRG cells were plated on 0.3% collagene and fed with astrocyte-conditioned medium (supernatant from 15-day old chick cortex astrocytes cultured 24 hours in F14 + Insuline 5 μg/ml + Transferrine 5 μg/ml + NGF 10 ng/ml) for the first day of culture. Under these conditions DRG cells were maintained for more than 13 days in F14 + Horse Serum 10% + NGF 10 ng/ml, and neurites were growing. Such a culture system will allow us to determine the presence, or absence, of quiescent neuronal precursors at late stages of development.

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SYNAPTIC DEVELOPMENT IN HIPPOCAMPAL ORGANOTYPIC CULTURES

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We have recently developped a new technique for preparing organotypic cultures of nervous tissue which has the advantage of easily allowing to investigate the events occurring during the first days and weeks in culture. In this study we have used morphological and electrophysiological techniques to characterize those changes and test whether they are related to the development that would have normally occurred in situ. In cultures prepared from 5 days old neonates, the synaptic density increased by a factor of about 20x between the first and 22nd day in culture, the changes consisting essentially of synaptic contacts on dendritic spines. These changes were correlated by an increase in the size of field synaptic responses. The ratio of glial to neuronal cells was stable over the first three weeks in culture except for a transient increase in microglia during the first days. Maturation of synaptic contacts occurred in vitro as indicated by the onset in long-term potentiation after a few days in culture. When comparing cultures prepared from 2 days old neonates versus 8 days old animals, the same changes could be observed, but they occurred with some delay and a slower time course. These results suggest that development in vitro is in many aspects similar to that described in in situ situation (Work supported by De Reuter Foundation and FNRS 31730.88 and 31.30980.91).

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NEUROTRANSMITTER MODULATION BY FIBROBLAST GROWTH FACTOR.

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Fibroblast growth factors (FGFs) are multifunctional and ubiquitous growth factors which promote neuronal survival and neurite outgrowth in various regions of the brain. Yet the physiological role(s) they may play in nervous system function and/or development are unclear. We report here that acidic and basic FGF differentially increase choline acetyltransferase (ChAT) and decrease tyrosine hydroxylase (TH) activity in avian sympathetic neurons in vitro without affecting neuronal growth and survival. The time-course of FGF action on the neurotransmitter phenotype is slow (1-2 days). FGFs thus appear to modulate the activities of ChAT and TH by differentially regulating the expression of the genes encoding these enzymes. These multifunctional growth factors may thus play a role in regulating neurotransmitter expression in sympathetic neurons during developmemt independently of any effect on neuronal survival.

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TRANSPORT AND METABOLISM OF GLUTAMIC ACID STUDIED IN THE HUMAN PLACENTAL TISSUE IN VITRO.

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In contrast to the other amino acids, the level of glutamic acid (Glu) during pregnancy is higher in the maternal than in the fetal circuit. It has been previously shown that there is no net transfer of glutamic acid across the placenta in either direction. Glu of fetal origin is removed from the umbilical circuit by uptake into the placenta. Placental transport and metabolism of Glu were studied using an in vitro perfusion method of human placenta with closed circuits separate for maternal and fetal side. Glu was added at 250 μmol/l together with ^{14}C-Glu (5 μCi) as a tracer to the perfusate in both circuits. Cold and radioactive amino acids were analyzed by HPLC after o-phthalaldehyde modification and enzymatic determination of Glu and glutamine (Gln). In most experiments (n=13), there was uptake of ^{14}C-Glu by the placenta from both sides with a drop of the concentration in maternal and fetal circuit to a level of 24 and 33% of the initial concentration respectively. The enzymatic determination of Glu and Gln indicated that Glu concentration dropped in maternal and fetal circuits to 90 and 40% of the initial level, respectively, while Gln was released into both circuits with a final concentration of 104 μmol/l on the fetal and 72 μmol/l on the maternal side. In addition, there was net release of Glu which was only observed into the maternal circuit (n=5) where the Glu final concentration was 275-348 μmol/l. HPLC analysis of the ^{14}C-label has clearly shown a conversion of Glu into Gln with a higher fraction released into the fetal circuit. The Gln fraction of total label was 15 and 30% in maternal and fetal circuit respectively. (Supported by Swiss National Foundation grant # 32-9003.86)

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NEUROTROPHIC FACTOR-3 (NT-3) INCREASES SPROUTING AND REGENERATION OF THE LESIONED RAT CORTICOSPINAL TRACT

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Spontaneous sprouting in response to transection is relatively weak in the corticospinal tract (CST) of young and adult rats. Nevertheless, sprouting fibers regenerate over distances of several millimeters in spinal cords deprived of oligodendrocytes, or in normal spinal cords where myelin-associated neurite growth inhibitory proteins (NI-35/250) have been neutralized by an antibody (IN-1). Two new members of the NGF family, NT-3 and BDNF, were analysed for effects on the lesioned CST. 0.5 - 1 ug of NT-3 or BDNF were injected at sites of spinal cord transection in 6 - 7 week old rats at the time of lesion. 2 weeks later, the CST of NT-3 treated rats showed an increased WGA-HRP labeling by anterograde transport of WGA-HRP, and a 2 - 3 fold increase in the number of sprouts per labelled axon rostral to the lesion site as compared to BDNF rats or untreated controls. Additional IN-1 treatment lead to regenerations over the entire length of the spinal cord (up to 20 mm). We conclude that NT-3 specifically acts on CST fibers and can enhance their sprouting and regeneration.

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NEURONAL AND GLIAL ENERGY METABOLISM IN AGGREGATING FETAL BRAIN CELL CULTURES AT HIGH [K^+]

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Aggregating cell cultures prepared from 15-day fetal rat telencephalon were grown in a chemically defined medium containing 30 mM K^+. Control cultures (CTR) consisted of both neuronal and glial cells. Neuronal cultures (N) were obtained by a single treatment at day 7 with cytosine-arabinoside (0.4 µM). Glial cell cultures (G) were obtained by treating aggregates between day 2 and 4 with cholera toxin (0.1 µM). Oxygen consumption (QO2) and lactate production were determined at day 14, 21, 28 and 35. QO2 was measured using a computerized spectrophotometric micromethod and lactate production was determined over periods of 24 hours. QO2 of N ranged from 100 to 520 nmol/h.mg protein (670 to 4700 nmol/h.mg DNA) and increased markedly between day 28 and 35 while QO2 of G and CTR was stable within the range of 60 to 180 nmol/h.mg protein (320 to 1400 nmol/h.mg DNA). The contribution of the oxidative phosphorylation to the total ATP synthesis was greater in N (60 %) than in G (31%) and CTR (41%) whereas lactate production was significantly higher in G than in either N or CTR (i.e. 1.8 ± 0.8 versus 0.8 ± 0.3 µmol/h.mg protein). These findings suggest that glial cells modulate the oxidative metabolism of the differentiating neurons.

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PRENATAL STRESS AND THE GLUCOCORTICOID RECEPTORS IN THE RAT HIPPOCAMPUS

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Two types of glucocorticosteroid receptors, the corticosteroid (CR), and the glucocorticoid type (GR) differing in their affinity for corticosteron (CORT), are found in the rat hippocampus. The CR type displays a high affinity for CORT. The GR type can efficiently bind CORT only during the diurnal peak, or during stress induced CORT elevation. The CR mediates a tonic influence on hippocampal associated functions, e.g. cognition, mood and affect. The GR mediates the feedback action of CORT on stress induced brain processes. While the CR is already present at birth, the GR is developed during the first two weeks of life. GR development can therefore be influenced by neonatal processes. The present study attemted to examine the influence of prenatal stress on the capacity and affinity of the CR and GR. Pregnant rats were subjected to repeated immobilisation stress during the 15th to 19th day of gestation. The binding of CORT and of a GR specific synthetic steroid (RU-28'362) to CR and GR in the hippocampus of the adult offspring were measured. The prenatally stressed rats (PS) had a smaller hippocampus than the prenatally unstressed rats (CON) and the binding capacity of GR was higher in PS than in CON females. [Supported by grants No. 0-15-056-90 (ETH) and No. 31-27502.89 (SNRF)]

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THE ISOLATION OF DEVELOPMENTALLY REGULATED cDNA CLONES FROM THE EMBRYONIC WHISKERPAD REGION OF THE MOUSE

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In line with our argument that, in the somatosensory whisker-to-barrel pathway of rodents, the periphery plays a major role in the creation and in the transfer of the vibrissal pattern to the central nervous system, where this pattern is reflected in a homeomorphic arrangement of multineuronal units, the "barrels", we decided to isolate developmentally regulated cDNA clones that might be involved in the genetic regulation of the vibrissal pattern in the embryonic whiskerpad (WP). To do this we used the approach of "subtractive hybridization" to identify clones present in WPs of embryonic day 10 (E10) and absent in WPs of E14. The 24 resulting clones are now being characterized in Northern blots of RNAs from tissues varying in age and origin and, as many of the clones are of low abundance, in quantitative PCR. Sofar, one of the clones was shown to be developmentally regulated and is now being sequenced.

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ABLATION OF THE PrP GENE IN THE MOUSE

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PrP^C is a cellular protein encoded by a single copy gene and is expressed on the outer surface of neurons and lymphocytes in all mammals examined. However, its function is unknown. It has been proposed that the infectious agent of spongiform encephalopathies such as scrapie in the sheep and Creutzfeld-Jakob disease in man is PrP^{Sc}, a posttranslationally modified form of PrP^C, and that infectivity is amplified by a conversion of PrP^C into PrP^{Sc}, elicited by PrP^{Sc} (Science 252,1515, 1991). Scrapie has been adapted to mice and infectivity can be passaged between mice by intracerebral inoculation with brain extracts from scrapie-diseased animals. We deleted the PrP gene in mouse ES cells by homologous recombination and transferred the deletion to the germ line of chimeric mice. Heterozygous ($PrP^{0/+}$) offspring were mated and homozygous ($PrP^{0/0}$) mice were obtained. $PrP^{0/0}$ embryos developed normally and no abnormalities have been noted 14 weeks after birth. This enables us to test the proposal that PrP^C is essential not only for the pathology of scrapie-like diseases but also for the formation of infectious agent. If this is true, $PrP^{0/0}$ mice should be resistant to scrapie infection.

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EHLERS-DANLOS SYNDROME TYPE VI IN TWO SIBLINGS.

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Ehlers-Danlos syndrome type VI is a rare, autosomal recessively inherited disorder, in the great majority of cases of which (Type VIA) a clinical syndrome of skin hyperelasticity and fragility, ligamentous laxity and joint hyperextensibility, progressive kyphoscoliosis, muscular hypotonia, and ocular fragility, apparently results from deficient activity of lysyl hydroxylase, which normally hydroxylates post-translationally specific lysyl residues within collagen. We report two female siblings, the children of healthy, consanguineous parents, who display typical features of the syndrome. The younger of the two (aged 10 years) is less severely affected than the elder (15 years), who required rodding of the spine to combat kyphoscoliosis at 7 years of age. The content of hydroxylysine in the dermis of the younger was significantly reduced, and the activity of lysyl hydroxylase in extracts of her cultured fibroblasts, in relation to that of prolyl hydroxylase, an unaffected reference enzyme, was only ca. 40% of normal levels under standard assay conditions (1mM ascorbic acid). If the concentration of ascorbic acid, an enzyme cofactor, was reduced to 0.2mM in the assay reaction mixtures, the deficiency of lysyl hydroxylase was more marked, residual activity being only 25% of normal.

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Biological databases grow with a tremendous speed. Today, about 60000 sequences are known, with an increase of 5% per month. To keep the local databases up-to-date, a procedure has been developed which transfers data from EMBL (Heidelberg) to Basel on a daily basis. After the necessary transactions, a new database is created from all sequences which are not present in the last quarterly release. This database, then, is reformatted into a format used by the (GCG) software package and transferred each night to various swiss universities. Further, a (anonymous) ftp file server has been set up (bioftp.unibas.ch). Besides the daily updates stored as single files, and the compressed databases as a whole, smaller databases are offered in ascii text file format for use on either mainframes or personal computers. Currently, all activities are financed and maintained by Basel University. The SMBQS (Swiss Molecular Biology Query System) Project has been submitted to the Nationalfond in order to get national funding for a national node to permit login for all biologists interested in a complete setup to permit research on sequence data analysis.

Gene Regulation

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REGULATION OF GLYCOGEN SYNTHASE EXPRESSION IN CULTURED ASTROCYTES.
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We have recently observed that a 10 minute "pulse" of 1 μM VIP induces 9 hours later a massive glycogen resynthesis (six- to ten-fold over basal levels) in cultures of mouse cerebral cortical astrocytes. This effect is completely abolished by either 1 μM actinomycin D or 100 μM cycloheximide, thus suggesting that new protein synthesis is a prerequisite to VIP stimulation of glycogen resynthesis. Since glycogen synthase is a key enzyme in the regulation of glycogen synthesis, we are presently investigating the possible induction of glycogen synthase by VIP in Northern blot analysis using a conservative 449 bp fragment from a human muscle glycogen synthase cDNA clone. Two transcripts of respectively ≈4 and ≈2 -kb were observed. However, in order to improve the rather weak signal obtained in mouse cortical astrocytes with the human muscle glycogen synthase cDNA fragment, we have now engaged in the cloning of the cDNA encoding the mouse astrocytic glycogen synthase. To this end, we have constructed a mouse astrocytic λZAPII cDNA library, size selected for inserts larger than 0.5 -kb, which exhibits a primary plaque number of $2 \cdot 10^6$ with only 1% of nonrecombinants. 200'000 clones were screened, from which six positives have been isolated and are presently being characterized.

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ISOLATION AND CHARACTERIZATION OF A VARIANT OF THE HUMAN PLATELET BASIC PROTEIN GENE

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Platelet basic protein (PBP), an α-granule protein released during platelet activation, is the parent molecule for a series of peptides with identical C-terminal sequences. PBP and platelet factor 4 (PF4) are platelet specific members of the super-family of small inducible genes (SIG). The genomic structure of PBP is of interest in understanding megakaryocyte-specific gene expression. Screening of a human genomic library with PBP cDNA led to a positive clone. A total of 4049 base pairs were sequenced, consisting of the protein coding region and 5′ and 3′ untranslated flanking regions. The predicted amino acid sequence was, however, only 70% homologous to that of PBP indicating that this is a variant of PBP, as has been described for PF4. PCR amplification of total platelet RNA with primers specific for our clone demonstrated the presence of specific RNA. Expression and function of this new platelet protein are still unknown.

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TWO TYPES OF CYTOCHROME P450-11-beta: SEPARATE INDUCTION IN RAT ADRENAL GLOMERULOSA CELLS

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Two different types of cytochrome P450-11-beta exist in the rat adrenal cortex. Type (1) is present in all the zones, but only catalyzes the conversions of DOC to corticosterone and 18-OH-DOC. Type (2) is found only in the zona glomerulosa; it catalyzes the three steps involved in the conversion of DOC to aldosterone.

Only type (1) activity was detectable in primary cultures of zona glomerulosa cells kept at a potassium concentration of 6.4 mmol/l. Expression of the gene encoding (1) was enhanced by ACTH. Aldosterone biosynthesis as well as mRNA encoding (2) could be detected only after at least one day of exposure to a high potassium concentration (18 mmol/l); pretreatment with ACTH had a potentiating effect. According to these results, separate control systems independently regulate the expression of the two genes encoding the two types of the enzyme in rat adrenal glomerulosa cells.

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VARIANT RBPs IN THE IMMUNE SYSTEM: DO THEY MODULATE V(D)J RECOMBINATION?

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V(D)J recombination is the process by which immunoglobulin gene segments are assembled to form a functional gene. Several proteins have been implicated in this process, one of which is called RBP-2 (recombination signal sequence binding protein-2; Nature 342, p.934, 1989). It has two features that make it a candidate for a component of the V(D)J recombinase: first it binds to the heptamer region of the recombination signal sequences surrounding the V(D)J gene segments, and second, it contains a sequence motif that is conserved in many prokaryotic recombinases of the integrase type.
We have cloned RBP cDNA from the same murine pre-B cell line (38B9) that served as a source of RBP in the original investigation. Screening was performed by hybridization with a probe that recognizes the DNA sequence encoding the integrase motif. We obtained 4 almost full length clones, which among themselves were found to be identical with respect to their restriction fragment patterns. This pattern was also identical to that published for the RBP-2 cDNA except for a completely different 5' end (upstream from nucleotide 136). By sequencing mouse genomic DNA we could show that the 5' end we found in our cDNA clones represents a separate exon. This exon is followed by an intron that contains typical eukaryotic promoter sequence elements (CAAT and TATA boxes) and a LINE-1 sequence. LINE-1 elements have been implicated in DNA recombination. Therefore we speculate that there exist several alternative RBP forms that may modulate V(D)J recombination.

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TOWARDS SINGLE LOCUS PROBES (MICRO- AND MINISATELLITES) IN DOGS

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Genetic markers form the basis of every genetic analysis. In the last decade Restriction-Fragment-Length-Polymorphisms (RFLPs) and Variable Number of Tandem Repeats (VNTRs) have gained tremendous interest in human as well as in animal genetics. Since these DNA sequences often show substantial polymorphism they are invaluable markers for different approaches in genetic problems such as gene mapping and linkage analysis. Contrary to human, in animals (beside the mouse) there exists much less information for genes and markers. At the moment the need for genetic markers (microsatellites and minisatellites) to establish well saturated chromosomal maps for all animal species is well recognized. On the basis of such a marker map it would become possible to look for genes which encode important quantitative/ qualitative traits or which are involved in disease resistance. We have started first to isolate species specific microsatellites from genomic dog DNA by constructing a cosmid library. Second we started with size-specific DNA fragments to characterize minisatellites. The two approaches and first results will be discussed.

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ANALYSIS OF MUTATED RNA HELICASE eIF-4A

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During initiation of translation 40S ribosomal subunits bind at or near the m^7Gppp cap structure of the mRNA and scan the leader sequence. During scanning the ribosomes may encounter inhibitory RNA secondary structure which can be melted by translation initiation factor eIF-4A, an ATP-dependent RNA helicase, together with eIF-4B. The putative ATP-binding site of eIF-4A contains an alanine which is conserved in helicases. We mutated this alanine residue and studied the properties of mutant eIF-4A in yeast in vivo and in an eIF-4A-dependent translation system in vitro. The results show that even the translation of mRNAs with little secondary structure in their leader sequence or with the AUG codon very close to the cap structure are still dependent on eIF-4A which is able to hydrolyse ATP.

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STRUCTURE AND TRANSCRIPTION OF THE psbD GENE OF EUGLENA CHLOROPLAST DNA

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The psbD gene coding for the D2 protein of photosystem II was sequenced. It extends over a region of approx. 11 kb, and the coding part is split by nine introns the largest intron being 3.7 kb. All introns have some structural features of class two introns. The 3.7 kb intron contains two major orfs encoding putative proteins of 282 and 506 aminoacids, respectively. Using several selected DNA probes we test the transcription products of the entire region. Unlike in higher plants the psbC gene is not adjacent to psbD, but we notice that the 3'end of the psbD gene could potentially code for the N-terminal part of the 44kD core protein (psbC gene). A possibility could be that the 5' terminal part is trans-spliced to the psbC transcript. Transcription start sites of psbD and psbC are identified by primer extension experiments.

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CONTROL OF THE PEROXISOMAL FATTY ACID ß-OXIDATION PATHWAY BY A NOVEL FAMILY OF NUCLEAR HORMONE RECEPTORS

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Three novel members of the *Xenopus* nuclear hormone receptor superfamily have been cloned. They are related to each other and similar to the group of receptors including those for thyroid hormones, retinoids and vitamin D3. Their transcription induction activity is regulated by agents causing peroxisome proliferation and carcinogenesis in liver. Together with a recently cloned mouse receptor, they form the new class of peroxisome proliferator activated receptors (PPAR). All three *Xenopus* receptors stimulate the acyl-CoA oxidase gene, which encodes the key enzyme of the peroxisomal fatty acid ß-oxidation, via a PPAR response element that has been identified. Thus, peroxisome proliferators may exert their hypolipidemic effects through PPARs, which activate the peroxisomal degradation of fatty acids. Furthermore, a differential expression of these receptors has been found in *Xenopus* suggesting specific functions for each of these receptors. Transcripts of two of them (xPPARα and xPPARß) were detected during oogenesis, embryogenesis and in all adult organ tested, whereas the third receptor (xPPARγ) is prominent only in fat body, kidney and liver.

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CAFFEINE, ESTRADIOL AND PROGESTERONE INTERACT WITH HUMAN CYP1A1 AND CYP1A2: EVIDENCE FROM CDNA DIRECTED EXPRESSION IN *SACCHAROMYCES CEREVISIAE*

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Human cytochrome P450IA1 and P450IA2 were expressed in yeast *S.cerevisiae*. Enzyme kinetics with recombinant yeast microsomes and 7-ethoxyresorufin as substrate resulted in K_m values of 17 and 520 nM and V_{max} values of 840 and 330 pmol/mg/min for CYP1A1 and CYP1A2, respectively. Both enzymes showed a distinct but overlapping substrate specificity determined with phenoxazone ethers, acetanilide and caffeine. The antimycotic drug ketoconazole, the isozyme specific CYP1A inhibitor a-naphthoflavone, caffeine as well as estradiol inhibited the CYP1A1 and CYP1A2 catalyzed ethoxyresorufin-O-deethylation. These data suggest a possible interaction of estradiol and caffeine.

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STRUCTURAL AND FUNCTIONAL ANALYSIS OF $GABA_A$-RECEPTOR GENE EXPRESSION IN THE CENTRAL NERVOUS SYSTEM

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$GABA_A$-receptors are physiologically and pharmacologically the most important targets for inhibitory neurotransmission in the mammalian brain. They constitute a heterogeneous population of multisubunit channel proteins that are differentially expressed in various regions of the CNS. The pharmacological heterogeneity of $GABA_A$-receptors is based upon the differential expression of a large number (≥15) of subunits in specific neuronal subpopulations. This results in combinatorial variations in the subunit composition of different $GABA_A$-receptor complexes.
Two representative $GABA_A$-receptor subunit genes, α1 and δ, have been used as a model to study neuron-specific gene expression. The α1 subunit is widely expressed and present in many different neurons of the CNS. The α1 gene is transcribed from two different promoters, separated by more than 700 bp. In contrast, δ is one of the most restrictively expressed subunits and present predominantly in granular cell neurons of the cerebellum. Cloning of the δ gene and mapping of its promoter has revealed characteristics typical for housekeeping genes: GC-rich promoter sequences, absence of a TATA box, a "good" initiator consensus sequence with multiple clustered transcriptional initiation sites. To determine the essential structural requirements for neuron type-specific expression we have generated transgenic mouse lanes carrying a lacZ gene driven by δ subunit promoter sequences. Reporter gene constructs have also been tested by transfection into primary tissue culture neurons from cerebellum.

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DOES THE FORMATION OF SOMATIC TELOMERES IN *ASCARIS LUMBRICOIDES* HAVE AN INFLUENCE ON THE EXPRESSION OF ADJACENT GENES ?

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Telomeres, the ends of eucaryotic chromosomes, are essential for the stable maintenance and replication of linear chromosomes in eucaryotic cells. They are thought to occupy unique locations within the nucleus, typically being associated with the nuclear envelope. Frequently, they show heterochromatic structures. It has been demonstrated that Pol II genes, if placed near the ends of chromosomes in *Saccharomyces cerevisiae* , undergo a position effect. The telomeres of the somatic chromosomes in *Ascaris lumbricoides* are of particular interest since they are newly formed during chromatin diminution. In the course of this process, which takes place in all presomatic cells of the early embryo of this parasitic nematode, developmentally regulated chromosomal breakage occurs within a specific, 2-3 kb long region, and is followed by the addition of many repeats of the telomeric sequence TTAGGC. Recently, we found a gene to be located within only 10 kb of such a *de novo* formed somatic telomere. Preliminary sequence data indicate that this gene may encode a protein which contains a GTP-binding domain. Its expression pattern in cells before and after the elimination process is now analysed in order to see whether the newly formed *Ascaris* somatic telomeres exert an influence on the transcription of adjacent Pol II genes.

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FORSKOLIN AND PHORBOL ESTER DEPENDENT REGULATION OF CYTOCHROME P450 SIDE-CHAIN-CLEAVAGE GENE EXPRESSION IN PRIMARY CULTURES OF BOVINE GRANULOSA CELLS.

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Throughout the ovarian cycle the production and secretion of progesterone and estrogen is regulated in a cyclic and highly coordinated fashion. Previous studies have demonstrated that, at least, part of this regulation occurs at the level of expression of the gene encoding cytochrome P450 side chain cleavage (P450scc), the rate-limiting enzyme in steroidogenesis. Treatment of primary cultures of bovine granulosa cells with forskolin (25µM) resulted in increased P450scc mRNA levels and progesterone production. The phorbol ester TPA added alone (10nM) slightly decreased progesterone production and P450scc mRNA levels. Co-treatment with forskolin and TPA reduced both P450scc activity and mRNA levels as compared to forskolin treatment alone. Since bovine granulosa cells treated with forskolin and TPA exhibited higher cAMP levels as compared to forskolin treatment alone, the inhibitory effect of TPA on P450scc gene expression appears to be distal to cAMP formation. Transfection of primary cultures of bovine granulosa cells with reporter gene constructs containing increasing deletions of the 5' regulatory region of the P450scc gene demonstrated that the regulatory cues of the PKA and PKC systems are mediated by one and the same stretch of DNA located at -118 to -83 bp. These results demonstrate that P450scc gene expression is differentially regulated by PKA and PKC and represent a first step towards the elucidation of the molecular mechanisms underlying steroid hormone production throughout the ovarian cycle.

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The phosphorylation state of the retinoblastoma (RB) protein in Polyoma infected mouse cells

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Polyoma virus induces a mitotic host reaction in Go-arrested mouse kidney cells. Using monoclonal antibodies to the retinoblastoma gene product (Rb), we studied alterations in Rb during virus-induced S-phase. An unphosphorylated species is the only detectable form of the Rb in quiescent Go-arrested cells. Time course analysis showed that in polyoma-infected cells induced to reenter the S-phase of the cell cycle, appearance of the phosphorylated forms of Rb coincided in time with the accumulation of large T-antigen and preceded DNA synthesis. Ongoing DNA synthesis was not required for the cells to phosphorylate Rb, indicating that this post-translational modification takes place during the activation of the cellular DNA-synthesizing apparatus. The underphosphorylated form of Rb was observed to immunoprecipitate with polyoma large T-antigen. These data add more evidence to the proposal that interactions of viral early proteins coded by DNA tumor viruses with the product of RB may play a pivotal role in the induction of S-phase.

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EXPRESSION OF TRANSCRIPTION FACTOR AP-2 IN GLIAL AND NEURONAL CELLS

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The transcription factor AP-2 seems to play a role in gene regulation in the developing nervous system of vertebrates, but its mRNA is also expressed in adult mouse tissues (e.g. cerebellum) and brain tumor cell lines. It binds as a dimer to the recognition sequence GCCN$_3$GGC. This sequence has been found in the promoter region of several genes including the transforming growth factor (TGF)β2 gene which is expressed during development of the central nervous system (CNS). When the embryocarcinoma cell line P19 is differentiated into neuron-like cells by treatment with retinoic acid (RA) increased levels of both AP-2 and TGFβ2 mRNA could be observed with RNase protection experiments. In contrast, there was no influence of RA on the mRNA expression in three glioblastoma cell lines, in primary astrocytes nor in two neuroblastoma cell lines. All these cells express different basal amounts of AP-2 and TGFβ2 mRNA with the exception of neuroblastoma. Among the different inducers of the classical signal transduction pathways only dibutyryl cyclic AMP increased AP-2 mRNA levels in astrocytes. By using the electrophoretic mobility shift assay we could confirm the presence of AP-2 protein with intact binding function in all mRNA positive cells. The colocalization of TGFβ2 and AP-2 mRNA in some types of cells in the CNS is consistent with a role for AP-2 as a transcriptional regulator of this gene.

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MINERALOCORTICOID BINDING SITES IN CELL CULTURES OF FETAL RAT BRAIN.

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Adrenal corticosteroids act via specific intracellular binding sites, the glucocorticoid (GC) and the mineralocorticoid (MC) receptors. These receptors have been characterized in the brain demonstrating high concentrations of MC binding sites in the septo-hippocampal region and a more abundant distribution of GC receptors in other brain areas.
We examined MC binding sites in brain cultures, employing [3H]-aldosterone in the presence of the selective GC receptor ligand RU 28362 (11ß,17ß-dihydroxy-6-methyl-17α-(1-propynyl)-androsta-1,4,6-trien-3-one), to block GC binding sites. Specific binding was observed in cultures of different brain regions and Scatchard analysis revealed a relative binding affinity of less than 1 nM for aldosterone, emphasizing the high affinity of these binding sites. Further characterization by competition analysis resulted in a typical MC binding pattern. Examination of binding sites at different time points in cortical cultures revealed no significant changes of receptors over time. Therefore, our culture conditions are suitable for the further analysis of MC receptors under in vitro conditions.

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CHARACTERISATION OF A GENOMIC CLONE COVERING THE MOUSE MYOD1 GENE AND ITS PROMOTER REGION

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We have isolated the mouse MyoD1 gene flanked by its promoter region by screening a genomic library with synthetic oligonucleotides. The structural gene is interrupted by two G+C rich introns. Transfection of the cloned gene inserted into an expression vector converts fibroblasts to myoblasts. Sequence analysis of about 650 bp of the 5' upstream region revealed the presence of several potential regulatory elements such as a TATA-box, an AP2-box, two SP1-boxes and a CAAT-box. In addition, there are three half palindromic estrogen response elements, a potential cAMP response element and various muscle specific elements such as a muscle-specific CAAT-box (MCAT) and four potential binding sites for MyoD1. Using S1 protection analysis the major start site of transcription in muscle and myoblast cells was mapped 3 bp upstream of the published cDNA 5' end. Promoter activity of the 650 bp upstream fragment was tested by *in vitro* transcription and by transfection analysis of myoblasts and fibroblasts. In all promoter test systems used, MyoD1 promoter activity was detected in myoblasts as well as in fibroblasts. Furthermore, DNA methylation was found to turn off MyoD1 promoter activity both in myoblasts and in fibroblasts.

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TISSUE SPECIFICITY OF MOUSE MAMMARY TUMOR VIRUS

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To study the tissue specificity of mouse mammary tumor virus (MMTV) gene expression, two series of transgenic mice were developed, containing the MMTV proviral DNA of mammary (GR) and kidney (C3H-K) origin. The expression pattern in the MMTV(GR) transgenic mice is very similar to that observed in infected animals, e.g. a strong preference for viral expression in the lactating mammary glands, and lower levels of expression in salivary glands, lymphoid tissues and male reproductive organs. One line of transgenic mice carrying the (C3H-K) provirus has a similar expression pattern, together with an unexpected high level of expression in the brain in very restricted areas, as shown by in situ hybridization, indicating that MMTV(C3H-K), despite a striking alteration in the U3 region of its long terminal repeat, can be expressed in the same tissues as the wild-type MMTV.

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TISSUE-SPECIFIC AND UBIQUITOUS FACTORS BINDING TO THE PROMOTER REGION OF MOUSE MAMMARY TUMOR VIRUS DNA.

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Transcription from the MMTV promoter is regulated by steroid hormones, but additional factors contribute to tissue specificity. We studied the binding in vitro of nuclear protein extracts of different origins onto the MMTV regulatory DNA. In the distal region (DR), a distinct binding activity (DRa) was observed on the 5' DNA adjacent to the glucocorticoid receptor binding site (DRb, -170 to -185). By DNase I footprinting and gel retardation analyses, the binding patterns of extracts from L cells and from tissues permissive for MMTV expression (spleen, salivary gland) are remarkably similar; they differ from that of liver, where MMTV is not expressed despite a high content of receptor. Tissue specificity was demonstrated by methylation interference, UV crosslinking and competition with mutated oligonucleotides. A DNA mutated in the DRb region, with a reduced hormone response in transfected L cells showed no binding of L cell factors to the whole DR region. In the basal promoter, the NF-I and NF-III binding sites were investigated using mutant DNA probes also tested in transfected cells for their basal and hormone regulated transcription activities.

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ANALYSIS OF *ARISTALESS*, A HOMEOBOX GENE INVOLVED IN GROWTH AND MORPHOGENESIS

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We have cloned and characterized the *paired*-type homeobox-containing gene *aristaless* (*al*). In *al* mutant flies, characteristic reductions of distal parts of the antennae (aristae), legs (claws), and scutellum are observed. Based on clonal analysis, Stern and Tokunaga suggested that the scutellar phenotype results from differential growth of the distal relative to the proximal part of the scutellar hypoderm. We will present evidence that *al* is a member of a genetic circuit including, for example, *expanded* and *dachsous* which are involved in establishing the growth pattern. In addition, we have determined the *al* mRNA expression pattern in embryos and in imaginal discs of third instar larvae. These results suggested that *al* is a potential target of various segment-polarity and homeotic genes. Our anlysis of its expression pattern in mutant embryos and imaginal discs confirms this hypothesis.

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THE MECHANISM BY WHICH DNA METHYLATION AFFECTS GENE EXPRESSION STUDIED BY IN VITRO AND IN VIVO FOOTPRINT

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Methylation of cytosine residues of CpG dinucleotides is one of the key mechanisms for the regulation of eukaryotic gene expression. However, the question of how DNA methylation affects gene expression is still largely unsolved. The footprint experiments, either in vitro with supercoiled DNA or in vivo with DNA microinjected within the nucleus of frog oocytes (where no replication nor cell division occurs), reveal a high similarity of protected regions between the methylated (transcriptionally inactive) and the non-methylated (transcriptionally active) HSV-*tk* promoter. The inactive promoter does not show the periodic DNAse I hypersensitive sites spaced at 10-bp intervals typical of nucleosomal regions. This finding means that there is no nucleosome present on the methylated inactive promoter and thus the presence of a nucleosome is not necessary to inhibit transcription. Furtheremore all the transcriptional factors are present on the methylated promoters and thus their abscence, even partial, is not necessary to inhibit transcription.

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FAR UPSTREAM ELEMENTS PROMOTE REGULATION OF AN ONCOGENE-INDUCED, GROWTH-ASSOCIATED GENE

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The T1 gene is transcriptionally activated in response to oncoproteins (ras and mos have been tested) and serum growth factors in NIH 3T3 mouse fibroblasts. No T1 mRNA is detected in quiescent cells. The transition from the G0 to the G1 phase of the cell cycle is associated with a dramatic increase of the T1 mRNA level. Thereafter it drops to an intermediate level which is maintained in growing cells.
Analysis of the T1 gene promotor region lead to the identification of an enhancer element between position - 3400 and - 3500. The sequence elements mediating growth factor and oncogene responsivness are located upstream of position - 3100.

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uPA GENE REGULATION IN POLYOMA mT-TRANSFORMED CELLS

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Urokinase-type plasminogen activator (uPA) is an extracellular protease involved in many biological processes. uPA expression in polyoma middle T (mT)-transformed endothelial cells is about 40-fold higher compared to non-transformed endothelial cells and NIH 3T3 fibroblasts. mT is known to be responsible for the development of the transformed phenotype in polyoma tumorgenesis. The high uPA in mT-transformed endothelial cells is correlated with high uPA mRNA levels. Therefore we investigated the mechanism leading to the high accumulation of uPA mRNA: first, uPA mRNA showed a long half-life ($T_{½}$ = 24h) in mT-transformed endothelial cells (eEnd2), compared to mT-transformed NIH 3T3 (108.4.2) cells (13h) and non-transformed NIH 3T3 cells (5h). These results give evidence for a mT-specific regulation of mRNA stability. We then found that the uPA gene promoter is more active in mT-transformed cells than non-transformed cells. This high activity is due to the absence of a silencer function in the region between -2.3kb and -35bp of the uPA gene promoter. Our results suggest that both transcriptional and posttranscriptional control is responsible for the high uPA gene expression in mT-transformed endothelial cells.

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TOPOLOGY OF DNA IN POLYTENE CHROMOSOMES

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Two topological characteristics of DNA were measured in isolated polytene nuclei of Chironomus thummi: 1.the fraction of the topologically non-closed DNA (tncDNA) and 2.the supercoiling density of the topologically closed unconstrained DNA (tcDNA). The fraction of tncDNA was found to be about 0.2. Evidence in favour of the tncDNA localization in transcriptionally active loci (puffs) of the polytene chromosomes is presented. The supercoiling density of tcDNA localized presumably in inactive loci (bands) of the polytene chromosomes is about -0.001. The localization of tcDNA in puffs and tncDNA in bands is presently being investigated..

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PARTIAL STRUCTURE OF THE HUMAN CAM-II CALMODULIN GENE

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The intracellular calcium receptor protein calmodulin (CaM) is encoded by a multigene family in the human genome. Structural information has been obtained for the CaMIII gene (Koller, M., Schnyder, B. and Strehler, E.E. (1990) Biochim. Biophys. Acta 1087, 180-189) and clones carrying parts of the CaMI gene have also been isolated (accompanying abstract by J.A. Rhyner, M. Ottiger and E.E. Strehler). A cDNA corresponding to an incompletely spliced human CaMII mRNA was fortuitously isolated in our laboratory; this cDNA lacks exon 1 but contains a part of intron 1 and the correctly spliced remainder of the CaMII coding and 3′ UT sequence. An intron 1 probe was used to isolate bona-fide CaMII gene sequences from a human genomic DNA library. Several kb of 5′ flanking DNA and exons 1 and 2 of the CaMII gene were characterized. The major transcriptional start site was mapped and about 1 kb of upstream sequence was determined. Comparisons with the rat CaMII gene revealed a high degree of conservation of the putative promoter and even of some intronic sequences. Several conserved elements that may represent binding sites for regulatory proteins were identified. Vectors with the growth hormone gene as reporter are now being constructed to analyze the CaMII gene promoter in more detail, and genomic walking is expected to result in the isolation of the complete CaMII gene. Supported by Swiss NSF grants no. 31-27103-89 and 31-28772.90.

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ANALYSIS OF THE HUMAN CAM-III CALMODULIN GENE PROMOTER

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Human calmodulin (CaM) is encoded by a multigene family. CaM levels vary, e.g., during the cell cycle and these changes may be ascribed, at least in part, to altered levels of transcription. 1.3 kb of the 5′ flanking DNA of the CaMIII gene were sequenced and found to contain numerous features typically present in housekeeping promoters. 6 GC-boxes (putative SP1 factor binding sites) and two AGGGA boxes also found in the promoters of other Ca^{2+} binding protein genes were identified. The CaMIII promoter is embedded in a CpG island from position -1000 to the first intron; 59 CpG dinucleotides are clustered between -253 and +47. Constructs of different fragments of the CaMIII gene 5′ flanking region in front of the human growth hormone gene were assayed for promoter activity in transient expression studies in teratoma and COS cells. One kb of upstream sequence was found to be maximally active; however, 252 bp (containing all 6 GC-boxes) were sufficient to drive high-level hGH expression in the cell types tested. Promoter activity dropped to 30% of the control level if only 114 bp of the CaMIII upstream sequence (4 GC-boxes) were present. The data suggest that the human CaMIII gene contains a strong "housekeeping-type" promoter whose regulation may depend on the methylation pattern and/or the presence of "silencing" factors.
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PARTIAL STRUCTURE OF THE HUMAN CAM-I CALMODULIN GENE

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In humans and other mammals, calmodulin (CaM) is encoded by a multigene family consisting of at least three members (CaMI, CaMII and CaMIII). For evolutionary comparisons and to study their regulation of transcription, the structures of all human CaM genes need to be elucidated. Previously, we have reported the isolation and complete exon-intron structure of the CaMIII gene (Koller, M., Schnyder, B. and Strehler, E.E. (1990) Biochim. Biophys. Acta 1087, 180-189). Using a CaMI intronic probe generated by PCR amplification of total human DNA, a lambda clone carrying the 3′ end region of the CaMI gene was isolated from a human genomic DNA library. Restriction enzyme mapping, Southern blotting and nucleotide sequencing revealed that it contained the exons specifying the C-terminal protein coding sequence and the 3′ untranslated region of the CaMI gene. Screening of a different library resulted in the isolation of a partially overlapping clone extending by several kb in the 5′ direction. The entire CaMI gene region covered by these two clones was mapped and the exon-intron structure determined. The results show a high degree of structural conservation between the human CaMI and CaMIII genes (e.g., identical intron location) and an even higher extent of homology between the human and the rat CaMI genes.
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ANALYSIS OF THE ROLES OF TWO NULEAR ENCODED FUNCTIONS IN THE TRANSLATION OF THE CHLOROPLAST *psbC* MESSAGE IN *CHLAMYDOMONAS*

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In *Chlamydomonas reinhardtii* the synthesis of the 43 kd PSII reaction core polypeptide P6 encoded by the chloroplast *psbC* gene is blocked by two nuclear mutations, F34 and F64 (Rochaix et al., 1989, EMBO J., 8; 1013-1021). P6 synthesis was measured during short ^{14}C pulse labelings and, therefore, the *F34* and *F64* encoded functions were proposed to be specifically required for the translation of the *psbC* mRNA. In order to test this hypothesis we have transformed the chloroplast genome with a chimeric reporter gene having the 5′ non-translated leader of *psbC* fused to the coding sequence of *aadA*, an *E. coli* gene which confers resistance to spectinomycin and streptomycin when expressed in the chloroplast. Our results from such *in vivo* expression studies and from *in vitro* RNA-protein binding experiments will be presented.

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FACTORS INVOLVED IN THE HEPATIC EXPRESSION OF THE XENOPUS LAEVIS VITELLOGENIN GENE B1.

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Vitellogenin genes are expressed under strict estrogen control in the liver of female oviparous vertebrates. Therefore they provide an ideal system for the study of stage-, sex-, tissue- and hormone-specific gene expression. Xenopus laevis liver nuclear extracts have been used to study the expression of the vitellogenin gene B1 in vitro. In addition to the well-documented estrogen-responsive-unit (ERU), several cis-acting elements have been characterized within the 5'-upstream sequences. Four binding sites are recognized by a CTF/NF-I-like activity that is required, together with the estrogen receptor, to mediate hormonal induction. In the absence of hormone the gene is repressed through the binding of a factor(s) to a negative regulatory element (NRE). This factor(s) is heat-stable and belongs to the C/EBP family. The interplay of these DNA-binding proteins modulates the transcription of the vitellogenin gene B1 in vitro.

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A CYTOLOGICAL STUDY OF CHROMATIN STRUCTURE USING A MOLECULAR APPROACH

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Digestion of chromatin using nucleases in combination with Southern blotting is a well documented molecular approach to the study of chromatin structure function relationship. The chromatin of transcriptionally active genes is more accessible to nuclease digestion compared to that of inactive genes due to its relative open-ness. Here, a technique is being developed which uses enzyme digestion to detect polytene chromosome decondensation cytologically. The gene chosen for this study is the BR6 gene of the dipteran *Chironomus*, since it can be conveniently induced or repressed. Southern blotting experiments show that restriction endonuclease digestion can discriminate between induced and repressed BR6. In the new method, salivary gland polytene nuclei are digested with a restriction enzyme to allow subsequent digestion by exonuclease III of one strand of the double-stranded DNA. The remaining strand serves as a target for *in situ* hybridization with specific DNA probes which are detected using fluoresence microscopy. Thus, DNA which is accessible to digestion (and hence in a relatively open conformation) is detected in this way. A great advantage of this approach is that the signal can be quantitated using confocal scanning laser microscopy, thus allowing a relative quantitation of chromatin decondensation.

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EXPRESSION AND LOCALIZATION OF AN INSECT STEROID HORMONE RECEPTOR HOMOLOGUE IN MAMMALIAN CELLS

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We have previously described the cloning of a cDNA sequence coding for a Chironomus tentans steroid receptor homologue which exhibits a good amino acid sequence homology to the Drosophila ecdysone receptor (Koelle et al. [1991]. Cell 67, 1371-1389). The DNA-binding domain has 95% and the hormone-binding domain 75% identity with the Drosophila receptor.
Expression of the putative Chironomus ecdysone receptor in monkey CV-1 cells and staining with polyclonal antisera raised against different domains of the protein shows that its localization is predominantly nuclear even without hormone stimulation. In contrast, in unstimulated Drosophila cells the receptor appears to be in the cytoplasm.
Currently we are in the process of performing hormone binding assays and we are testing for the transactivation potential of this receptor on corresponding regulatory DNA-elements using mammalian cells as a host.

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MODIFICATION OF HUMAN PAPILLOMAVIRUS (HPV) GENE EXPRESSION BY HERPES SIMPLEX VIRUS.

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HPV is a major etiological agent for cervical cancer. HPV DNA, encoding two oncogenes, E6 and E7, is found in about 80% of cervical tumours. However, HPV is not thought to act alone but along with cofactors. Herpes simplex virus (HSV) frequently infects the cervix and has been postulated as a possible cofactor for HPV.To investigate the effect of HSV infection on HPV gene expression, we infected HeLa cells (which contain integrated HPV-18 DNA) with various strains of HSV. After several hours of infection the RNA was extracted and the amount of HPV-18 RNA measured by RNAse protection. Infection by wild type HSV resulted in a dramatic decrease in HPV-18 mRNA compared to a control cellular mRNA. This effect was not due to host shut-off. Experiments with mutant viruses and with cycloheximide showed that the decrease in HPV-18 mRNA was not due to de novo protein synthesis but was dependent on proteins associated with the incoming virion.

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Myeloblastin: cDNA characterization and regulation of gene expression

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Myeloblastin (MBN) is a serine protease expressed in promyelocytic HL-60 cells which is rapidly downregulated during induced cell differentiation. The full molecular nature of MBN cDNA is presently not resolved, only partial sequences are published. We have used oligonucleotide primers defining a 5'sequence of MBN in conjunction with cDNA against poly A+ RNA isolated from HL-60 cells to generate a 5'MBN fragment by the PCR. With this fragment as a probe, we have isolated cDNA clones from an HL-60 lamba gt10 library. These clones are being presently characterized. Steady-state levels of MBN transcripts were downregulated after 3 hours induction by 60ng/ml PMA in HL-60 cells. This downregulation could not be inhibited by 40 µg/ml cycloheximide which is able to block ≥95% of the total protein synthesis. This suggests MBN possesses similar regulation pattern to that of myeloperoxidase (MPO) and that, therefore, MBN belongs to the family of primary response genes.

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ISOLATION AND CHARACTERIZATION OF A NOVEL HUMAN CYTOCHROME P-450 GENE POSSIBLY INVOLVED IN OLFACTION

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The olfactory neuroepithelium is the principal site of recognition for airborne molecules, such as odorants. Cytochrome P-450 dependent monooxygenases are extremely active in the upper part of the nasal cavity. The physiological connection between olfaction and the olfactory P-450 system remains to be elucidated. Here we describe the isolation and partial characterization of a new form of human cytochrome P-450. The clone was isolated from a human lymphocyte genomic library using rat CYP3A9 (P-450 olf2) as a probe. CYP3A9 was isolated from a rat olfactory specific cDNA library and is preferentially expressed in the olfactory epithelium.
Comparison of the derived amino acid sequence with that of other members of the P-450 family 3 showed similarities between 63% (3A2, rat), 67% (3A9, rat olf2) and 80% (3A3, human), indicating that the new gene belongs to family 3A.

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ISOLATION AND CHARACTERIZATION OF A VARIANT OF THE HUMAN PLATELET BASIC PROTEIN GENE

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Platelet basic protein (PBP), an α-granule protein released during platelet activation, is the parent molecule for a series of peptides with identical C-terminal sequences produced by proteolysis by various enzymes. PBP and platelet factor 4 (PF4) are platelet specific members of the superfamily of small inducible genes (SIG). The structure of the PBP gene is of interest in understanding megakaryocyte-specific gene expression. Screening of a human genomic library with PBP cDNA led to a positive clone. A total of 4049 base pairs were sequenced, consisting of the protein coding region and 5′ and 3′ untranslated flanking regions. The predicted amino acid sequence was, however, only 70% homologous to that of PBP indicating that this is a variant of PBP, as has been described for PF4. PCR amplification of total platelet RNA with primers specific for our clone demonstrated the presence of specific RNA. Expression and function of this new platelet protein are still unknown.

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In Vitro Detection of V(D)J Recombination

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DNA sequences encoding immunoglobulin domains are assembled from variable(V), diversity (D) and joining (J) segments by site specific recombination. These rearrangements require specific recombination signal sequences. V(D)J recombination, which takes place only during the early developmental stages of pre-B cells, was studied in the pre-B cell line 33.1.1-, known to be recombination competent. Another murine bone marrow cell line, 33.1.1+, of a more differentiated developmental stage and little recombinational activity, served as a control. We have developed an *in vitro* recombination assay using nuclear extracts or matrices with an extrachromosomal DNA as a substrate (pBluerec, assembled by F. Rougeon; see S. Kallenbach, M. Goodhardt, F. Rougeon (1990), Nucl. Acids Res. 18, 6730). Experimental conditions affecting *in vitro* V(D)J recombination will be described.

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COMPARISON OF IMMEDIATE-EARLY GENE PRODUCTS OF BOVINE HERPESVIRUS 1 STRAINS DIFFERING IN NEUROPATHOGENIC POTENTIAL

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Bovine herpesvirus 1 (BHV-1) strains Jura or K22 encode three major immediate-early (IE) RNAs. IER4.2 and IER2.9 arise by alternative splicing and are transcribed in divergent orientation to IER1.7. Among some minor IE transcripts, two are transcribed over circularized genome ends.

Northern blot analysis performed in this study with IE RNA from strain N569 exhibiting a neuropathogenic potential revealed three major IE transcripts with similar size and transcription pattern, except for slight differences in the 5′ terminal sequence of IER4.2 and IER2.9. However, significant differences were discovered for the minor IE RNAs transcribed over circularized genome ends. Four long transcripts were found to be abundant for strain N569, two of them were encoded in addition by sequences more than 2.4 kb apart from the left genome end.

Western blot analysis and radioimmunoprecipitation revealed three major IE proteins with sizes of 180 kd (IEP180), 135 kd (IEP135) and 55 kd (IEP55) for strain Jura. All three major IE proteins were found to be phosphorylated with IEP55 as major phosphoprotein. IEP180 was detected as major IE protein after methionine labeling. Strain N569 expressed major IE proteins similar to those of strain Jura, but in addition some abundant IE proteins determined as minor IE proteins for strain Jura (one with about 145 kd and three between 90 and 120 kd) were detected.

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MOLECULAR BASIS FOR THE DIFFERENCE OF EXPRESSION OF HLA-DRB1 AND DRB3 LOCI

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HLA class II genes encode molecules that present antigens to T lymphocytes. The DRB1 and DRB3 class II genes are the two ß chain genes of the DR subregion of DRw52 haplotypes. DRB1 is expressed at a 3-5 fold greater level than DRB3. In transcient transfection assays, a 200 bp promoter region is sufficient to obtain a 2 fold difference in the level of DRB1 versus DRB3 transcription. Sequence analysis of DRB1 and DRB3 promoters in 8 different DRw52 haplotypes has revealed isotypic-specific nucleotide differences. The two most prominent changes are situated within two highly conserved cis-acting elements, the class II X box and the inverted CCAAT sequence present in the Y box. The effect of these differences on the binding of nuclear factors to the X and Y box has been examined. Results indicate that the change within the Y box affects the relative affinity of two proteins : NF-Y and NF-Y*, suggesting that the differential level of B1 and B3 gene expression can be at least partially attributed to the sequence difference within the Y box.

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STUDIES ON THE TISSUE SPECIFIC TRANSCRIPTION OF THE ONCOMODULIN GENE

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Oncomodulin is a low molecular weight Ca^{2+}-binding protein, expressed in various rodent and human tumors and in the cytotrophoblasts of the rat and human placenta. It has been shown that a retroviral long terminal repeat (LTR) related to those of intracisternal A-particles (IAP) is inserted upstream to the oncomodulin gene in the rat genome. Retroviral LTRs of the IAP type are active in many tumors and in embryonic or developmental tissues but generally not in normal tissues. In order to measure promoter activities of IAP LTRs, especially the oncomodulin LTR, they were cloned upstream of a reporter gene (human growth hormone). Transient transfections were performed in chemically transformed rat fibroblasts which derived from the same origin but exhibit high (T14c), low (T10) or no (T43) oncomodulin expression. A 5-fold lower LTR promoter activity was observed in T43 as compared to T14c cells when the values were standardized with a control promoter. However, the activities of the isolated IAP LTRs in transient transfections did not reflect cell type variations of endogenous oncomodulin mRNA levels in the investigated cell lines. Therefore, it seems unlikely that the oncomodulin LTR per se is sufficient for cell type specific expression. Several experiments aimed to unravel the impact of the CG-methylation status and chromatin condensation on the oncomodulin gene expression have been carried out and will be presented.

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CHROMATIN STRUCTURE AND DNaseI HYPERSENSITIVITY IN THE PORCINE TNF GENE LOCUS

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The porcine TNF genes are arranged in a tandem repeat with TNF-ß located 5' of TNF-α and are independent transcription units. We have analyzed the accessibility of porcine chromatin in order to study the regulation of these genes. Isolated nuclei from peripheral blood mononuclear cells were digested with different nucleases. Both genes displayed faster digestion kinetics than bulk DNA as assessed with micrococcal nuclease. However, studies with DNaseI revealed distinct DNaseI hypersensitive sites (DH-sites). Whereas four DH-sites could be observed in the promoter region of the TNF-ß gene, only two DH-sites could be observed in the TNF-α gene, one in the promoter region and the other in intron 3. Interestingly, we found that the TNF-α promoter DH-site was displaced into intron 1 in a porcine macrophage-like cell culture. However, this pattern was totally absent in fibroblasts (freshly isolated from testicles) and in a porcine kidney cell line (PK15).

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POSITIVE AND NEGATIVE GENE REGULATION BY THE GLUCOCORTICOID RECEPTOR

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We have studied the behavior of wild type or mutated glucocorticoid receptor (GR) by expressing GR-cDNAs along with appropriate reporter genes and other non-ubiquitous trans-activators in mammalian cells. (1) We have analyzed the effect of several specific mutants in the Zn-finger region. So far, and in conflict with reports by others, we have been unable to separate the weak transactivation function contained within the Zn-finger region from DNA binding. (2) We have observed that in presence of relatively small amounts of GR, the action of ectopically expressed lymphoid-specific Oct2A is significantly impaired. Control experiments demonstrate is probably due to titration of one or more rate-limiting co-activator(s). (3) We have observed that there is a correlation between the size/shape of the transactivator and its ability to cooperate in the activation of clustered binding sites, in particular we suggest that the hormone binding domain may influence cooperativity in promoter/enhancer activation. (4) We have initiated a study on the role of monotonous aminoacid repeats in the GR and in other factors involved in transcription regulation. We can show that in the rat GR there are different effects depending on the frame by which the repeated (CAG)n segment is translated. We have data suggesting that there are tissue-specific sets of factors belonging to the repeat-containing class and we have devised a cloning strategy to better characterize them.

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The MHC class II gene transactivator RFX belongs to a novel familly of transcription factors capable of heterodimerization

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MHC class II molecules play a critical role in the immune response because they present peptides to T lymphocytes. MHC class II genes exhibit a complex pattern of cell specific and inducible expression that is mediated to a large extent by transcription factors binding to conserved cis-acting sequences in the promoter. We have recently cloned human and mouse RFX, a highly conserved factor binding to the X box of class II promoters. Transient transfection experiments have now confirmed that RFX is a transcription factor capable of transactivating the class II DRA gene. Low stringency screening of mouse and human cDNA libraries has allowed us to identify a familly of proteins showing strong homology (90%) in the DNA binding domain and in the structurally and functionally independent dimerization domain, but weak homology (25%-50%) in other regions of the protein, including the activation domains. These RFX proteins represent a new familly of transcription factors which contain novel DNA binding and dimerization motifs. RFX proteins can bind to the X box as both homo- and heterodimers. This suggests that the complex pattern of class II gene regulation may be achieved by a set of RFX homo- and heterodimers differing in their binding specificity and function.

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Transcriptional regulation of c-myc gene constructs microinjected into Xenopus oocytes and fertilized eggs.

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Promoter sequences from *X. laevis* c-myc I gene have been attached to the CAT reporter gene and assayed for transcriptional and translational activity after microinjection into oocytes and fertilized eggs. Deletions eliminating sequences from -1310 to -158 relative to the P2 promoter directed high expression of CAT activity. Deletion to -58 reduced activity twofold. This region contains two GC-rich hexanucleotides homologous to Sp1 binding sites. Deletion to -28 reduced CAT activity approximately 20 fold relative to the full length template. The region between -58 and -28 contains a further GC-rich hexanucleotide embedded within the sequence ATTTGGCGG, corresponding to a binding site for the transcription factor E2F. We have also constructed vectors containing Xl myc I exon1 and intron 1 sequences to determine whether a block to transcription elongation may operate as a control mechanism and to define the sequences involved.

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THE GENETIC COMPLEXITY OF THE HSP 65 GENE

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HSP 65 is a highly conserved chaperonin protein that can elicit cross-reactive T lymphocyte responses and that has been directly implicated in the pathogenesis of autoimmune diseases. Cloning and sequencing of PCR derived HSP 65 clones obtained from genomic DNA showed multiple highly related sequences, all different from the expressed protein and all with interrupted reading frames. From mRNA however, only the sequence expected for the expressed HSP 65 protein was obtained. This paradox was resolved by cloning and sequencing of genomic clones screened with a cDNA segment as probe : The majority of HSP 65 genomic clones corresponded to intronless genes having the characteristics of pseudogenes and different flanking regions. With the use of an oligonucleotide probe specific for the expressed HSP 65 sequence, we isolated several genomic clones corresponding to a unique functional HSP 65 gene. This gene is composed of multiple exons, some very short. The transcription start site was identified by primer extension and 900 base pairs of 5'flanking sequence determined. This unique expressed human HSP 65 gene is not induced by heat. We conclude that HSP 65 is encoded by a single highly fragmented gene, that coexist with multiple processed HSP 65 pseudogenes.

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CHARACTERIZATION OF NP-TC$_{II}$, A NEW LYMPHOCYTE SPECIFIC TRANSCRIPTION FACTOR

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We have previously described a group of nuclear proteins, now called NP-TC$_{II}$, which bind to the TC-II motif of the SV40 enhancer with a similar specificity as NF-κB (E.Espel et al. (1990) EMBO J. 9:929).

More thorough characterization of NP-TC$_{II}$ indicates first, that it is constitutively expressed in nuclei of all pre- or mature T and B cell lines tested, in normal resting B and T lymphocytes and in some non-lymphoid hemopoietic cell lines. But it is undetectable in all non-hemopoietic cell lines screened, including fibroblasts, carcinomas and pancreatic tumor cells.

Secondly, we present evidence that NP-TC$_{II}$ is a transcriptional activator. Expression of plasmids containing single point mutations of the TC-II motif was measured after transient transfection of thymoma cells. We found a strong correlation between the level of expression of these plasmids and the capacity of their mutated TC-II motif to compete for NP-TC$_{II}$ binding. Moreover, plasmids carrying mutations that prevent binding of NF-κB showed significative enhancer activity. Finally, biochemical and functional properties of NP-TC$_{II}$ rule out a close relationship with NF-κB and other proteins of the rel family and show that NP-TC$_{II}$ is different from other lymphocyte specific DNA binding proteins.

These data suggest that NP-TC$_{II}$ plays a role in lymphocyte differentiation.

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PURIFIED CO-FACTORS MEDIATE IN VITRO TRANSCRIPTION ACTIVATION BY CTF/NF-I: INVOLVEMENT OF HISTONE H1.

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To dissect the mechanisms of gene transcription control by human CTF/NF-I DNA binding factors, purified full length or truncated proteins were covalently coupled to a chromatography resin, which was then used to fractionate HeLa cell nuclear extracts. Several polypeptides which specifically interact with CTF/NF-I species were identified. In vitro reconstitution experiments implied that these polypeptide co-factors are involved in CTF/NF-I mediated induction of transcription. Interestingly, these co-factors possess both inibitory and stimulatory transcriptional activities, the latter being dependent upon CTF/NF-I. In addition, we found that histone H1-mediated repression of transcription can be counteracted by CTF/NF-I and its co-factors together, but not by CTF/NF-I alone. We therefore propose that CTF/NF-I and its co-factors regulate gene expression by stabilizing the association of the basal transcription machinery with the promoter, thereby preventing gene extinction by nonspecific DNA binding proteins, such as histone H1.

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SV40 ACTIVATES TRANSCRIPTION FROM THE TRANSFERRIN RECEPTOR PROMOTER BY INDUCING A FACTOR WHICH BINDS TO THE CRE/AP-1 RECOGNITION SEQUENCE.

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During the course of lytic infection by SV40, expression of both the viral late genes and certain host cellular genes is induced. The promoter of the cellular transferrin recpetor (TR) gene contains a DNA sequence which is similar to the AP-1 and AP-4-binding region in SV40 which has been implicated in the control of the viral late promoter. Expression of TR is needed for cells to enter S-phase and is therefore expected to be important for the SV40 lytic cycle. The level of TR mRNA in vivo is increased by SV40 infection. A factor which activates transcription from the TR promoter in vitro is specifically induced in SV40 infected cells. Characterization of the factor showed that it is a member of the AP-1/Jun/Fos family, suggesting that the virus can use a common mechanism to induce both viral and host cellular transcription.

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POSITIVE AND NEGATIVE CIS-ACTING ELEMENTS REGULATE MyoD1 PROMOTER IN *IN VITRO* TRANSCRIPTION.

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In muscle differentiation, the activation of a family of transcription factors (MyoD1, MyF5, Myogenin, and MRF4) plays a key role in both cellular determination and induction of muscle specific gene expression. The best characterized member of the myogenic regulators is MyoD1. MyoD1 is a nuclear phosphoprotein that trans-activates various skeletal-muscle specific genes. Furthermore, MyoD1 expression is controlled by serum, growth factors, electrical activity and by oncogenes such as ski, ras, myc, fos and jun.

In order to study how MyoD1 expression is regulated the MyoD1 promoter was tested in an *in vitro* transcription system. By competition with oligonucleotides containing putative binding sites for transcription factors we show that TATA box, CAT box, and SP1 are required for basal activity as well as a putative AP2 site. On the other hand, a cAMP-like element down regulates transcription activity. Using band shift assay and antibodies we show that the binding activity is related to AP1. Further studies are now in progress to show whether fos and jun are involved in the down regulation of the MyoD1 promoter.

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PURIFICATION AND CLONING OF A TRANSCRIPTION FACTOR WHICH ENHANCES THE cAMP RESPONSE IN THE PORCINE UROKINASE GENE PROMOTER.

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One of the cAMP regulatory regions in the porcine uPA gene located 3.4 kb upstream of the transcription initiation site comprises three adjacent protein-binding domains designated A,B and C. Domains A and B contain a core sequence TGACG of the cAMP response element (CRE) but require an adjoining C domain to confer cAMP responsiveness on a heterologous promoter. The C domain does not have a CRE, nor can it mediate cAMP responsiveness when used in isolation. The C binding protein enhances the binding of the A+B domain indicating that cooperative protein binding may play a role in mediating the cAMP response of the uPA gene. The domain C binding protein has been purified to near homogeneity from LLC-PK$_1$ nuclear extract by ion exchange and affinity chromatography. This protein appears to be about 10 times more abundant in the nuclei than other transcription factors. It shows an apparent molecular weight of 66 kD as determined by SDS-PAGE and South-Western analysis. It binds specifically a region of the porcine uPA promoter as shown by DNase I footprinting experiment with both crude nuclear extract and purified protein. The purification procedure and cDNA cloning will be discussed.

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DIRECT HOMEODOMAIN-DNA INTERACTION IN THE AUTOREGULATION OF THE *DROSOPHILA* SEGMENTATION GENE *FUSHI TARAZU*

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A major problem in the elucidation of the molecular mechanisms governing development is the distinction between direct and indirect regulatory interactions among developmental control genes. We describe here an experimental approach we applied to establish a direct interaction of the homeodomain-protein fushi tarazu (ftz) with the *ftz* cis-control region *in vivo*. Previous genetic studies have indicated that the *Drosophila* segementation gene *ftz* autoregulates its expression. We now find that mutations of *in vitro* binding sites for ftz protein strongly reduce the *in vivo* activity of an autoregulatory enhancer element of the *ftz* gene. This down-regulatory effect is suppressed by a DNA-binding specificity mutant of ftz that recognizes the mutant binding sites. This demonstrates a direct positive autoregulatory feedback mechanism in the regulation of this homeobox-gene. The described experimental approach to establish direct protein-DNA interactions *in vivo* is applicable to any organism amenable to transgenic studies and might help to unravel the complex regulatory networks governing development.

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Compatibility between upstream control elements and basal promoters determines transcriptional specificity of the two juxtaposed *gooseberry* genes in *Drosophila*

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We have dissected the cis-regulatory regions of the two closely apposed, divergently transcribed *gooseberry* (*gsb*) genes, *gsb* and *gsb neuro* (*gsbn*). *gsb* is mainly expressed in the epidermis, *gsbn* in the central nervous system. We are interested in the problem of how their transcriptional specificity arises. We show that different cassettes of upstream control elements are engaged in driving specific *gsb* or *gsbn* expression. Specificity of these control elements for their genes is provided by their restriction to act on their own basal promoter. Therefore, we propose that the compatibility between the upstream control elements and their corresponding basal promoter represents a mechanism ensuring transcriptional specificity.

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TRANSCRIPTIONAL ACTIVATION BY B-CELL SPECIFIC FACTOR OCT 2

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Oct 2 is a tissue-specific transcription factor involved in regulation of immunoglobulin gene expression in B lymphocytes. Promoters and enhancers of such genes contain a conserved octamer sequence ATGCAAAT to which this factor binds by means of a DNA binding "POU" domain. We used in vitro transcription systems to study the basic mechanism by which Oct functions to develop a better understanding of transcriptional activation. Recombinant Oct 2 produced in bacteria specifically stimulated transcription of octamer-containing promoters in a HeLa nuclear extract. Single-round transcription assays demonstrated that this increase was due to Oct 2 stimulating the formation of preinitiation complexes. Oct 2 gave similar levels of in vitro activation when tested with purified basal transcription factors, indicating that the stimulation is due to "true activation" and not just interference with inhibitors in the nuclear extract. The transcription factor appears to interact with TFIID-bound "adaptors": transcriptional stimulation was dependent on the presence of TFIID from HeLa cells while recombinant TFIID, which supported basal level transcription, did not allow Oct 2 activated transcription. Direct interaction of Oct 2 with elements of the basal transcription apparatus is being studied.

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DIFFERENT ACTIVATION DOMAINS STIMULATE TRANSCRIPTION FROM REMOTE (ENHANCER) OR PROXIMAL (PROMOTER) POSITIONS

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The lymphocyte-enriched transcription factor Oct-2A is able to activate, after transfection into non-B (HeLa) cells, synthetic or natural immunoglobulin promoters which contain the octamer sequence ATGCAAAT, but unable to stimulate transcription from multiple binding sites far downstream of a β-globin test gene. The latter sites represent, however, a functional enhancer in B-cells. The most obvious explanation is the lack of a B-cell specific adaptor, or a specific modification, in HeLa cells. An alternative possibility is that Oct-2A is generally unable to stimulate transcription from a remote position. We have tested a number of chimeric proteins, all containing the DNA-binding domain of Gal-4 attached to various activating domains including the glutamine-rich domain of Oct-2A. Our results provide a basis for a classification of activating domains in terms of their ability to activate transcription from a remote (enhancer) or a proximal (promoter) position. Our findings also explain why, in our hands, multiple binding sites for typical "promoter" factors, such as octamer factors and apparently Sp1, do not function as remote enhancers.

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cDNA CLONING OF N-OCT 3, AN OCTAMER-DNA (ATGCAAAT) BINDING PROTEIN EXPRESSED IN THE EMBRYONAL AND ADULT NERVOUS SYSTEM

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The "octamer" sequence, ATGCAAAT (ATTTGCAT), is a key element for transcriptional regulation of a number of genes in various cell types. Using the bandshift assay with extracts of mouse astrocytes and neurons, at least four N-Oct protein complexes (designated N-Oct 2,-3, -4, and 5) were detected (Nucl.Acids Res. 1990, p. 5495 ff.). The expression of N-Oct proteins can be effectively induced in P19 embryocarcinoma cells by addition of retinoic acid to the culture medium. The timing of N-Oct protein expression in these cells correlates well with the phenotypical differentiation to neuron-like cells. We have cloned several cDNAs from a human fetal brain cDNA library that encode octamer-DNA binding proteins, and identified by bandshift assay a cDNA clone that encodes the N-Oct 3 protein. It contains a POU domain as DNA-binding domain that was previously identified in embryonal and adult brain and partially cloned by others (He et al., 1989 Nature,340, p. 35-42). We observe N-Oct 3 protein expression in cell derivatives of the neural crest and the neural plate. Thus, N-Oct 3 may have an important role during neurogenesis. A functional analysis of this protein in co-transfection assays is in progress.

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NUCLEOSOME POSITIONING AND TRANSCRIPTIONAL ENHANCEMENT ON A XENOPUS VITELLOGENIN GENE

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We define a conserved DNA sequence that directs nucleosome positioning between the proximal (-30/-130) and distal (-300/-330) promoter elements of all four Xenopus vitellogenin genes. A nucleosome in this position creates a static loop that may mediate transcriptional enhancement in vivo and in vitro. In this model the estrogen receptor complex is brought into close proximity to the basal transcription machinery. This chromatin structure may form an integral part of the transcription process for these genes.

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The transcription factor CF1 regulates the c-myc, the IgH and the beta casein promoters.

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We are studying the mechanisms of gene regulation in mammary epithelial cells. The regulation of milk protein genes in these cells is mediated by the synergistic action of glucocorticoid hormones and prolactin. Expression of milk protein genes is restricted to quiescent mammary epithelial cells. 200 nucleotides of the 5' flanking sequences of the ß-casein promoter are sufficient for induction of a linked indicator gene. Nuclear proteins, which specifically bind to these sequences show differences in the binding pattern in hormonally induced or uninduced cells. Proteins prominently binding in the region between -100 and -150 derived from uninduced cells are downregulated in induced cells. Mutational analysis of binding sites shows that interference with binding of these proteins results in high constitutive activity of the promoter. Thus, one mechanism of ß-casein gene regulation is the relief of silencing by hormone induction. We have also identified a nuclear factor which specifically binds to sequences between -130 and -100. Mutation of the binding site prevents protein binding and results in downregulation of transcription in the induced state. This factor is present in similar concentrations in induced and uninduced cells and also found in different cell types. It is apparently identical with myc-CF1, as shown by mutational analysis of binding sites and by comparison of electrophoretic mobility shift and proteolytic clipping bandshift assay patterns with corresponding binding sites found within the c-myc and IgH-uE1 promoters. As in the beta casein promoter, myc-CF 1 recognises a positive regulatory element in these promoters, which is flanked by a repressor binding site and probably interacts with specific repressors.

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Regulation of a mammary gland specific transcription factor (MGF) in normal and transformed cells

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The expression of milk protein genes is regulated in a tissue and stage specific manner by steroid and peptide hormones. We have investigated the activity of a mammary gland specific transcription factor (MGF) which mediates hormonal influences on the transcription of the ß-casein milk gene promoter. Mutation in the MGF binding site (-85 to -100) leads to a complete loss of ß-casein gene inducibility by lactogenic hormones in cultured mammary epithelial cells. MGF expression is tightly regulated developmentally in vivo, being high at the end of pregnancy and during lactation. Withdrawal of the pups from their mother resulted in a rapid decrease of MGF activity that could be restored within 4 hours after the pups had been added back to their mother. The phosphorylation state of MGF plays a role in the regulation of the DNA binding activity of MGF. Various oncogenes have been implicated in human and rodent tumor development. We have analysed the influence of different oncogenes on the activity of the ß-casein gene promoter and on MGF binding to DNA. Cells harbouring Ha-ras or v-raf oncogenes lost their ß-casein gene inducibility. MGF activity could not be detected. In mammary tumors of WAP-c-myc transgenic animals constitutive milk protein expression independent of the hormonal status of the animal was found. MGF activity could be detected in these tumor cells.

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INTERACTION OF TRANSCRIPTION FACTORS WITH THE α1 AND α2 TYPE VI COLLAGEN PROMOTERS

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Type VI collagen is a hybrid molecule consisting of a short triple helix flanked by two large globular domains. These contain repeats which are homologous to sequences found in the von Willebrand factor. Type VI collagen is composed of three different polypeptide chains α1(VI), α2(VI) and α3(VI), which are encoded by three distinct genes. Transformation of fibroblasts by oncogenic viruses causes nearly complete inhibition of type VI collagen synthesis. This inhibition appears to occur at the transcriptional level. We therefore isolated and characterized the promoters of the α1(VI) and α2(VI) genes. Both promoters exhibit features characteristic of housekeeping genes and proto-oncogenes: They have a high GC content and lack the typical TATAA box. Transcription is initiated at several start sites. Gel mobility shift and footprinting analyses revealed several DNA sequences that may interact with transcription factors. The sequences closest to the first transcription initiation sites were analyzed in greater detail using synthetic oligonucleotides in combination with cloned SP1 and AP1 proteins. These experiments demonstrate that SP1 interacts with non-consensus sequences on both the α1(VI) and α2(VI) collagen promoter.

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CORRELATION OF AP-1 LEVELS WITH TERMINAL DIFFERENTIATION AND ONCOGENIC TRANSFORMATION OF MAMMARY EPITHELIAL CELLS IN CULTURE

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Prolactin, glucocorticoids and insulin induce terminal differentiation and milk protein synthesis (e.g. β-casein) in HC11 mouse mammary epithelial cells in culture. Based on the previously described inhibition of glucocorticoid receptor function by the transcription factor AP-1 (Fos/Jun) we measured AP-1 activity in HC11 cells during hormone-induced differentiation and after transformation with oncogenes which increase AP-1 levels in the cells. The activity of the β-casein promoter was very low in growing and confluent HC11 cells and it was high after lactogenic hormone treatment. Measurements of the level of transcription factor AP-1 by gel retardation analysis revealed high levels in confluent cells, and significantly reduced levels in hormonally stimulated cells. Transformation of the HC11 cells with retorviral vectors encoding Mos, Ras or Src (but not Myc) led to high levels of AP-1 (and *fos* and *jun* mRNA) and oncogene expression was accompanied by a complete inhibiton of the β-casein promoter in the absence and presence of lactogenic hormones. Similar results were obtained when a second mammary epithelial cell clone, LU-1/11, was used. Overexpression of AP-1 similarly inhibited glucocorticoid receptor function.

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Carboxyl- terminal domain of RNA pol II (CTD) and transcription initiation

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The largest subunit of RNA pol II contains a curious structure at its carboxyl terminus: the so called CTD consists of a heptad repeat (52 copies in mouse, 44 in Drosophila, 26 in yeast) of consensus YSPTSPS. This repeat is not found in RNA pol I and III. The CTD structure can change its phosphorylation state and has been implicated in the response to certain transcriptional activator proteins, since different promoters show a greater or smaller dependence on the CTD. We are using a genomic mouse clone of the big subunit of RNA pol II (RPOII215) and two CTD deletion mutations Δ31 and Δ5 which contain 31 and 5 repeats, respectively. These constructs in addition have an α-amanitin resistance mutation which allows the holoenzyme to initiate transcription when endogenous pol II is inhibited by α-amanitin at 2 µg/ml. In our transfection assay we cotransfect COS-1 cells with full tail, Δ31 and Δ5 together with different templates and transcription factors. Mapping the RNA by RNase- protection assay revealed that "enhancer factors" like glucocorticoid receptor or herpesvirus- derived VP16 fused to the Gal4 DNA binding domain, can initiate transcription with full tail and Δ31 but not with the Δ5 construct. "Promoter factors" like Octamer factors only work with the full tail construct. These findings were also consistent when various activation domains were fused to the DNA binding domain of Gal4. If the different tail usage of transcription factors is due to the quality of certain tail domains or simply a matter of the tail length remains to be analyzed in further experiments.

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IN VIVO FOOTPRINTING OF TWO ENHANCERS IN THE MOUSE κ IMMUNOGLOBULIN LOCUS

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During B-lymphocyte differentiation, transcription and rearrangement of the immunoglobulin heavy chain (IgH) locus precedes rearrangement of the immunoglobulin light chain lambda and kappa (Igl, Igk) loci. Using the ligation mediated polymerase chain reaction (Müller & Wold 1989, Science 246, 780), protein binding *in vivo* at two enhancer sequences of the murine Igk locus was analyzed in cell lines representing different stages of B-lymphocyte development. In plasmacytoma cell lines expressing an Igk gene, protein binding was found in the intronic enhancer (Picard & Schaffner, 1984, Nature 307, 80) as well as in the recently characterized 3' enhancer (Meyer & Neuberger, 1989, EMBO J. 8, 1959). 3 different sequences were identified in the 3' enhancer which bind proteins *in vivo*. Of the many proteins binding to the intronic enhancer *in vitro*, several were found to bind also *in vivo*, especially the transcription factor NF-kB. No protein-binding to the respective binding sites was found in cell lines representing pre B-lymphocyte or pro B-lymphocyte developmental stages.

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TRANSCRIPTION OVER CIRCULARIZED GENOME ENDS OF BOVINE HERPESVIRUS 1: THE *CIRC* PROTEIN.

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The genomes of herpesviruses are packaged as linear molecules in virions but replicated as circular structures in the nuclei of infected cells. It is not known when and how circularization occurs. We studied lytic infection with bovine herpesvirus 1 (BHV-1) and discovered immediate-early (IE) transcription over joined ends of the BHV-1 genome, which suggested that circles may be present at the initial stage of infection. Northern blot, primer extension, and polymerase chain reaction revealed an unspliced precursor (6.3 kb) and a spliced 1.5-kb IE RNA (IER1.5) spanning the genome ends. Exon 1 of IER1.5 consisted of the same noncoding leader RNA as exon 1 of IER4.2 and IER2.9, previously described major IE transcripts. Exon 2 of IER1.5 was colinear with a 1.1-kb RNA (LR1.1) transcribed from an alternative late promoter near the left genome end. IER1.5 and LR1.1 contained the same open reading frame (247 aa) for a putative *circ* protein with homology to the gene 2 product of varicella zoster virus. The function of *circ* remains unknown but may be sought in the area of BHV-1 genome replication, circularization, or cleavage.

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CHROMOSOME INACTIVATION BY A ZINC-FINGER PROTEIN

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Position-effect variegation is the inactivation in some cells of a gene translocated next to heterochromatin, the region of the chromosome that is permanently condensed. The number of copies of the Drosophila gene *Suvar(3)7* is a dose-limiting factor in this phenomenon, and seems from its sequence that it encodes a protein with five widely spaced zinc-fingers. This novel arrangement of zinc-fingers could help in packaging the chromatin fibre into heterochromatin, and also reflects a novel method of controlling the expression from DNA domains. The transcript encoding *Suvar(3)7* accumulates first in nurse cells of the mother's ovaries and is then transferred in oocytes around stage 10. Ubiquitous in early stages of embryogenesis, the message is seen in tissues active in morphogenesis later in development. The protein is in all nuclei throughout embryogenesis, and is chromosomal as seen on mitotic chromosomes.

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EXPRESSION OF THE MINOR U7 snRNA GENE IN HeLa CELLS

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The U7 snRNP is responsible for the formation of mature 3' ends of histone mRNAs. Its RNA moiety is most probably encoded by a single copy gene in mouse. Like other *U* genes, the *U7* gene is flanked by three regulatory elements: A conserved *U* enhancer in an inverted orientation and slightly modified versions of a snRNA TATA - like element and of a 3' signal. The functional significance of these elements was tested by transfecting HeLa cells with the *U7* gene in combination with either one, two or all three elements. To compare the *U7* gene with an snRNA gene of a major snRNP, we also transfected HeLa cells with a mouse *U1b* gene and analysed the corresponding RNAs. The U1b snRNA from the transfected gene was consistently 3 - 4 times more abundant than U7 snRNA. To test if this difference is depending on the *U7* and *U1b* specific regulatory elements, we combined the *U7* gene with *U1b* flanking sequences and vice versa. However, these constructs produced identical RNA levels as the wild-type genes, independent of the origin of their promoter and 3' flanking sequences. This indicates that the genes themselves are responsible for the different RNA levels. Using genes with *in vitro* mutagenized Sm-binding sites, we are testing the hypothesis that a less efficient assembly of the U7 snRNA (whose Sm-binding site diverges from the consensus sequence AAUUUUUGG) into snRNP particles could lead to increased RNA turnover and thus to lower stady state levels than for an efficiently assembled snRNA with a "better" Sm-binding site.

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ALTERNATIVE SPLICING OF TYPE VI COLLAGEN TRANSCRIPTS.

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The α2 subunit of type VI collagen is composed of one short triple helix flanked by three homologous repeats of a globular motif similar to the A domains of von Willebrand factor. We have isolated and characterized the entire gene for the α2(VI) subunit. It comprises 28 exons distributed over 26'000 bp. In addition, the 3' end of the gene contains a recently identified exon termed 28A with an open reading frame of 293 bp and an untranslated region of ~1000 bp. Exon 28 and the additional exon might be utilized in a mutually exclusive manner to generate two different gene transcripts. Alternative splicing would replace the carboxy-terminal 202 amino acids of the α2(VI) polypeptide, which correspond to exactly one of the three type A-like repeats, with a shorter domain of 98 amino acids and thus generate a variant polypeptide with new properties. The alternatively spliced mRNA could be detected in chicken heart, muscle and cartilage by northern blotting. We expressed the normal domain and its variant counterpart in an E.coli expression system. The recombinant proteins were used to raise polyclonal antibodies in rabbits. Using these antibodies and our DNA clones we intend now to study the tissue distribution of the normal and the variant form of the α2(VI) subunit by indirect immunofluorescence and by insitu hybridization.

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PURIFICATION OF PROTEIN FACTORS THAT FUNCTION IN THE INITIAL STEP OF NUCLEAR PRE-mRNA SPLICING

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The removal of introns from nuclear pre-mRNAs occurs in large multicomponent complexes (spliceosomes) by two consecutive cleavage and ligation events. We are investigating the different stages of the splicing reaction in vitro with components fractionated from HeLa cell nuclear extracts.

The initial step of the reaction, the formation of a pre-splicing complex, requires three protein factors (SF1, SF3 and U2AF) and U1 and U2 snRNPs which interact with the 5' splice site and branch site, respectively. SF1 has been purified to homogeneity; it is a heat-stable protein of 75 kDa and apparently does not interact with the pre-mRNA substrate directly. Partial amino acid sequence has been obtained and the cloning of the cDNA is underway. SF3 has been purified extensively and can be separated into at least two components. We are in the process of analyzing the interplay of these components during the assembly of the pre-splicing complex.

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A SPLICING FACTOR WHICH IS INACTIVATED DURING IN VIVO HEAT SHOCK IS FUNCTIONALLY EQUIVALENT TO THE TRI-snRNP-SPECIFIC PROTEINS

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The inhibition of pre-mRNA splicing during the heat shock response can be reproduced in vitro in extracts from heat-shocked HeLa cells. The block in splicing occurs at the level of spliceosome assembly prior to the cleavage and ligation reactions that generate mature mRNA.

We have isolated a protein factor (SF5) that restores splicing activity in an extract from heat-shocked cells. SF5 is distinct from previously identified splicing proteins and functions in the assembly of a U4/U5/U6 tri-snRNP particle, thus allowing the spliceosome to be formed. When purified snRNPs are used in the complementation assay only the tri-snRNP or its isolated proteins, but not the individual U4/U6 or U5 snRNPs rescue splicing. Thus, SF5 activity is equivalent to the tri-snRNP-specific proteins that function in the assembly of the U4/U5/U6 particle. Our results provide direct evidence that the tri-snRNP as such is essential for the formation of a functional splicing complex and further demonstrate that splicing is inhibited during heat shock at a basic step of the reaction.

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CHLOROPLAST RIBOSOMAL INTRON OF CHLAMYDOMONAS REINHARDTII: IN VITRO SELF-SPLICING, DNA ENDONUCLEASE ACTIVITY AND IN VIVO MOBILITY

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The chloroplast 23S ribosomal RNA gene of the green unicellular alga *Chlamydomonas reinhardtii* harbours a group I intron which contains an internal open reading frame (ORF). A precursor RNA encompassing the intron with its 5' and 3' flanking sequences is shown to be self-splicing *in vitro*. The intron ORF is shown to encode a DNA endonuclease. This endonuclease (I-*Cre*I) recognizes a sequence with a maximal length of 19 bp which spans the exon junction site present only in an intronless version or a cDNA of this 23S rRNA gene. The I-*Cre*I endonuclease generates a staggered cleavage - 1 and 5 bp downstream of the intron insertion site, respectively - resulting in 4-base hydroxyl overhangs at the 3' ends of the cleavage product. In vivo mobility of the *C. reinhardtii* ribosomal intron at the DNA level is demonstrated using a reverse genetic approach. A partial cDNA of the 23S rRNA which contains the exon junction sequence (the target site for intron transposition) was introduced into the chloroplast DNA of *C.reinhardtii* by partical gun mediated gene transfer. All the transformants which had the cDNA integrated at the expected position in the chloroplast genome had also the intron precisely inserted into this cDNA.

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A SELECTABLE MARKER FOR CHLOROPLAST REVERSE GENETICS IN CHLAMYDOMONAS : TRANS-SPLICING (tscA) AND PHOTOSYSTEM I (psaC) MUTANTS.

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The E.coli *aadA* gene (amynoglycoside adenyl transferase) was inserted in a chloroplast expresssion cassette and introduced into *Chlamydomonas reinhartdii* by particle gun bombardment. The chimeric *aadA* gene is stably expressed in the transgenic chloroplast and confers spectinomycin resistance to the transformed cells. It can be used as a portable selectable marker for transformation and also as a reporter gene. Gene disruption mutants of chloroplast photosynthetic genes can thus be directly selected. Disruption mutants of the *psaC* gene, coding for the 9 kD subunit of PS I with the F_A and F_B iron sulfur centers, show a complete loss of photosynthetic activity and also fail to accumulate other PS I subunits. The *tscA* gene encodes a small chloroplast RNA required *in trans* for the *trans*-splicing of *psaA* exons 1 and 2. Disruptions of *tscA* can lead to a complete block of *psaA* mRNA maturation depending on the site of insertion and the orientation of the *aadA* cassette. Experiments are in progress to introduce site-specific mutations in the genes to explore structure-function relationships.

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STRUCTURE AND FUNCTION OF PLANT SMALL NUCLEOLAR RNAs AND THEIR GENES

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Plant small nuclear RNA (U-snRNA) gene promoters consist of two conserved sequence elements, the plant snRNA gene-specific Upstream Sequence Element (USE) and the TATA box. These elements, with appropriate positioning, are sufficient for transcription by RNA polymerase II and III (Waibel and Filipowicz, Nature 346, 199).

We have characterized plant genes encoding the nucleolar RNAs U3 and MRP. In contrast to the U3 genes from other organisms, which are pol II transcribed, plant U3 genes are transcribed by pol III (Kiss et al., Cell 65, 517), and contain different 5'-terminal cap than metazoan U3 RNA. This is the only example of equivalent genes in different organisms being transcribed by different RNA polymerases. By manipulating the spacing between the USE and TATA elements we converted the plant U3 gene into a functional pol II-transcribed gene. These results emphasize the similarities between the pol II and pol II transcription systems.

The nuclear encoded MRP (mitochondrial RNA processing) RNA in mammals was reported to function in the mitochondrion. MRP RNA is the only reported example of an RNA imported into the mitochondria in metazoa (Clayton, TIBS, 16, 107). MRP RNA is also located in the nucleolus. We have characterized the gene and cDNAs encoding MRP RNAs in Arabidopsis and tobacco. In plants this RNA is also enriched in nucleoli but, using the very sensitive assays, is undetectable in purified mitochondria.

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Accumulation of chloroplast *psbB* RNA requires a nuclear factor in *Chlamydomonas reinhardtii*

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We have isolated and characterized a nuclear mutant, 222E, from *Chlamydomonas reinhardtii* which is defective in photosystem II (PSII). Polypeptide P5, the product of *psbB*, is not produced in this mutant, leading to a destabilization of other PSII components. The mutant fails to accumulate specifically *psbB* transcripts and displays an altered transcription pattern downstream of *psbB*. Pulse-labeling experiments suggest that mRNA stability and/or processing are affected by the alteration of a nuclear gene product in this mutant. We have shown that the *C. reinhardtii psbB* gene is co-transcribed with a small open reading frame, highly conserved in its location and amino acid sequence with respect to land plants. The 5' and 3' termini of the *psbB* transcript have been mapped to 35 bases upstream of the initiation codon and approximately 600 bases downstream of the stop codon. The 3' flanking region contains two potential stem-loops, of which the larger one (with an estimated free energy of -46 kcal) is near the 3' terminus of the transcript. Experiments are currently underway to 1) test the stabilizing ability of the 3' flanking region in fusion constructs, 2) isolate the corresponding nuclear gene, and 3) elucidate the function of the conserved ORF.

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CLONING AND EXPRESSION OF THE HUMAN IRON REGULATORY FACTOR

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The stability of transferrin receptor mRNA and the translation of ferritin mRNA are regulated by an RNA-binding protein, the iron regulatory factor (IRF). Binding of IRF to highly conserved hairpin structures, the iron responsive elements (IRE), is controlled by intracellular iron levels. We isolated three overlapping IRF cDNA clones with a probe derived from the sequence published by Rouault et al. (1990) using the polymerase chain reaction. After sequencing, two of them could be assembled into a full-length cDNA with an open reading frame for a polypeptide of 98,400 dalton. Northern- and Southern blot analysis indicated a single copy gene of about 40 kb specifying a 4.0 kb mRNA. After *in vitro* transcription and translation in a wheat germ translation system, we obtained active IRF which bound to a human ferritin IRE. This interaction could be inhibited by sulfhydryl oxidation with diamide and restimulated by β-mercaptoethanol in the same way as purified IRF from human placenta. The protein was also expressed after micro-injection of the *in vitro* transcribed mRNA into Xenopus oocytes. Deletions in the COOH-terminal region of the protein indicated that this part is involved in IRE-binding. The amino acid sequence of IRF shows homology to aconitase, a Fe-S-cluster containing enzyme. It should now become possible to analyse by site directed mutagenesis whether IRF has also an Fe-S cluster and how such a cluster is important for the RNA-binding in response to iron deprivation.

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REGULATION OF TRANSFERRIN RECEPTOR mRNA STABILITY BY INTRACELLULAR IRON LEVELS

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Transferrin receptor (TfR) expression is strongly influenced by the intracellular iron level. The iron-dependent regulation is mediated by a cytoplasmic protein, the iron regulatory factor (IRF), which inhibits degradation by binding to hairpin structures in the 3' untranslated region of TfR mRNA.

In order to investigate the mechanism regulating the degradation of TfR mRNA, we developed a method to determine the amount of bound IRF in parallel with TfR mRNA levels. Administration of iron salts to cells leads to release of the IRF from the mRNA and degradation of TfR mRNA. The transcription inhibitor actinomycin D prevents the iron dependent mRNA decay without affecting the release of IRF from the mRNA. The translation inhibitor cycloheximide stabilizes the TfR mRNA and delays the inactivation of the RNA binding protein. The results suggest the involvement of a labile component in the regulation of TfR mRNA stability.

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TRANSPORT OF MATURE mRNA FROM THE NUCLEUS AFTER MICROINJECTION IN XENOPUS LAEVIS OOCYTES.

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Export of mRNA from the nucleus to the cytoplasm was studied in mature Xenopus laevis oocytes. Radiolabeled mature (capped, spliced and polyadenylated) mRNAs were injected into nuclei and their appearance monitored in the cytoplasm by counting radioactivity or by RNA extraction and gel electrophoresis. mRNA export was saturable with a maximal rate of about 10^8 molecules per minute per nucleus at 20°C. Competitive inhibition between labeled and non-labeled mRNA or between two different mRNA species (5kb transferrin receptor mRNA and 2kb 4F2 antigen mRNA) could be demonstrated. This suggests that mRNA export corresponds to a facilitated process rather than diffusion. The transport of mRNA displayed properties characteristic of an energy-dependent mechanism since it was blocked at 4°C or by depletion of ATP in nuclei. mRNA export was also inhibited by prior injection of wheat germ agglutinin (WGA), a lectin known to inhibit active nuclear transport by binding to N-acetyl glucosamine containing proteins present in the nuclear pore complex. Indeed the coinjection of WGA and N-acetyl glucosamine abolished the WGA-induced inhibition of export. Co-injection of the dinucleotide cap analog (m^7GpppG) at 1mM together with capped mRNA blocked mRNA export. This is in agreement with a central function for the cap structure in mRNA export and with the suggestion by Hamm and Mattaj (Cell, 1990, 63:109-118) of a role for a nuclear cap-binding protein.

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MUTATIONAL ANALYSIS OF THE JE/I-33 mRNA: A NUCLEOTIDE SEQUENCE OTHER THAN AUUUA IS RESPONSIBLE FOR THE INSTABILITY OF THIS mRNA

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The murine JE/I-33 gene is an immediate-early growth response gene. The corresponding mRNA is rapidly turned over with a half-life of 20 minutes. A shorter version of the JE/I-33 mRNA arising by polyadenylation at a more upstream (220 bases) location, is stable and is identical in its sequence with the longer JE/I-33 mRNA. Since both versions of the JE/I-33 mRNA contain in their 3'untranslated region (UTR) a single copy of the pentanucleotide AUUUA believed to be an instability signal, it is likely that the AUUUA motif must cooperate with an additional nucleotide sequence, if it plays a role in targeting this mRNA for rapid degradation. Alternatively, other sequence elements functioning as instability determinants are contained in the 3'UTR of the longer JE/I-33 mRNA. Preliminary results from transfection experiments, where the JE/I-33 mRNA with deletions in the 3'UTR are expressed, support this notion and will be discussed.

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TISSUE SPECIFIC EXPRESSION AND REGULATION OF INSULIN-LIKE GROWTH FACTOR I (IGF I) AND ITS BINDING PROTEINS (IGFBPs) IN THE RAT

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IGF I mediates most of the actions of growth hormone (GH). Although the liver is the major source of circulating IGF I, IGF I is synthesized in most tissues as an auto-/paracrine growth factor together with IGFBPs.
We studied the tissue distribution and regulation of IGF I and IGFBPs in vivo. Male hypophysectomized rats were infused with either IGF I, GH or solvent and compared to normal rats. mRNA expression of IGF I and IGFBPs was studied in nine different organs. The IGF I message is present in all these tissues and is stringently regulated by GH in liver, muscle and white adipose tissue (WAT). IGFBP-3, the main BP in adult rat serum, is regulated by GH in liver and WAT and not GH-dependent in kidney, spleen and testes. mRNA for IGFBP-2, the predominant BP in the fetus, is found in liver only after hypophysectomy, but it is constitutively expressed in kidney, brain, testes, and WAT. IGFBP-4 mRNA is only present in liver. IGFBP-5 is regulated by GH in heart, brain, muscle and WAT and constitutively expressed in kidney. IGFBP-6 expression is restricted to spleen and thymus.
The tissue specific expression and regulation of IGFBPs suggests that these proteins play a crucial role in modulating para-/autocrine IGF I actions.

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ADRENALECTOMY DECREASES LIPOCORTIN-I M-RNA AND TISSUE PROTEIN CONTENT IN RATS. B. Vishwanath, B.M. Frey, M. Bradbury, M.F. Dallman & F.J. Frey. Medical Center, Univ. of Berne, Switzerland; Dept. Physiology, UCSF, San Francisco.
Clinical evidence suggests that acute withdrawal of chronic glucocorticoid therapy with suppression of endogenous cortisol production in RTX patients is associated with an increased susceptibility to rejection. Furthermore experimental and clinical observations revealed an enhanced immunological and inflammatory response in glucocorticoid deficiency. The antiinflammatory response of pharmacological doses of glucocorticoids has been tentatively attributed to the induction of lipocortin-I. In order to elucidate whether glucocorticoid deficiency causes lipocortin-I down regulation, the expression of lipocortin-I mRNA and protein was quantified in rats with and without adrenalectomy (ADX). The mRNA of lipocortin-I cDNA was quantified by polymerase chain reaction using a constant amount of a modified lipocortin-I cDNA transcript as an internal standard. The lipocortin-I mRNA was decreased by 56 ± 14% in tissue of ADX rats. This down regulation of lipocortin-I mRNA was not due to a nonspecific effect of ADX since the mRNA levels of other proteins (c-fos, c-myc, c -erbAbeta, methallothionein-II) remained unchanged. The decrease in lipocortin-I mRNA in ADX rats was reflected by a corresponding decrease in tissue (kidney, lung, spleen, liver) lipocortin-I protein content as assessed by quantitative Western blot analysis. Thus, ADX causes a decline in lipocortin-I message and protein, an observation in accordance with the increase susceptivility to inflammatory reactions in glucocorticoid deficiency.

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DEVELOPMENTAL ANALYSIS OF TWO SOYBEAN ELONGATION FACTOR EF-1α PROMOTERS ACTIVITY IN TRANSGENIC TOBACCO PLANTS

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Genes coding for the translation elongation factors EF-1α are present as a small multigene family. Two of these genes, (tefS1 and tefS2), have been analyzed in more detail (Aguilar et al., Plant Mol. Biol., 17:351-360, 1991). Both genes contain an open reading frame of 1341 nucleotides (nt), interrupted by a short single intron of 86 nt(S1) and 116 nt(S2) nt. The two genes diverge in 80 wobble positions (coding region), thus yielding an identical protein of 447 amino acids. cDNA analysis shows that the untranslated leader of both genes is interrupted by a large intron. Regulatory regions of genes tefS1 and tefS2 were fused to the coding region of the GUS reporter gene. Transgenic tobacco plants were regenerated by using binary vectors . Individual transformed plants were analyzed for GUS activity during vegetative and reproductive growth. Both genes are very active in all tissues analysed, with a higher activity in rapidly growing tissues. We currently study the activity of these promoters under various physiological conditions.

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INSULIN-LIKE GROWTH FACTOR I (IGF I) AND IGF I BINDING PROTEINS (IGFBPs) IN RAT WHITE ADIPOSE TISSUE (WAT)

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Screening various rat tissues for the expression of IGF I and IGFBPs, we found that IGF I mRNA levels in rat epididymal WAT are in the same range as in liver, the major source of serum IGF I, and lie far above the levels of other tissues. The synthesis of IGF I mRNA and protein in WAT decreases drastically after hypophysectomy and is restored to near normal by growth hormone (GH) treatement in vivo. IGF I expression is also regulated by GH in vitro: maximal stimulation of IGF I mRNA is achieved after 2 h. Rat WAT also contains the messages for IGFBP-3 and IGFBP-5. IGF I mRNA and IGFBP-5 mRNA are produced in both adipocytes and stromal-vascular cells, whereas IGFBP-3 expression is restricted to the stromal- vascular tissue.
Our results show that WAT contains high amounts of IGF I mRNA which is regulated by GH. We suggest that locally produced IGF I and IGFBPs play a critical role in the differentiation of adipose cells.

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VERTEBRATE p34^{cdc} PHOSPHORYLATION SITE MUTANTS : EFFECTS UPON CELL CYCLE PROGRESSION IN THE FISSION YEAST SCHIZOSACCHAROMYCES POMBE

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We have used the fission yeast *Schizosaccharomyces pombe* to analyse the effects of *in vitro* mutagenesis of the four known phosphorylation sites in the chicken p34^{cdc2} protein, Thr14, Tyr15, Thr161 and Ser 277, upon cell cycle progression. We have studied both the effect of overexpression of mutant proteins in a *cdc2*$^{+}$ background and assayed their ability to rescue null allele and temperature sensitive alleles of *cdc2*. Mutations of Thr14 and Tyr15 within the ATP binding domain of p34^{cdc2} which mimic constitutive phosphorylation cause dominant negative cell cycle arrest when overexpressed. In contrast, some substitutions which simulate permanent dephosphorylation of the corresponding sites advance mitosis. These data confirm the model that p34^{cdc2} is negatively regulated by phosphorylation of residues in the ATP binding site. Mutagenesis of the conserved residue Thr161 functionally inactivates p34^{cdc2}, and our data suggest that both phosphorylation and dephosphorylation events at Thr 161 are required for progression through the cell cycle. Mutations at the fourth site of phosphorylation, Ser277, lead to cold-sensitive cell cycle arrest, in minimal but not rich growth medium, suggesting that this site is involved in monitoring the nutritional status of the cell.

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ROLE OF HUMAN CTF/NF-I INTERACTION WITH ADPOL IN ADENOVIRUS TYPE 2 (Ad) DNA REPLICATION IN VITRO.

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CTF/NF-I proteins have been shown to bind to and activate Ad DNA replication origin. Previous results have suggested a direct interaction between CTF-1, a member of the CTF/NF-I human DNA binding protein and Adpol, the viral encoded polymerase. Our studies have suggested that AdPol interacts with a CTF-1 domain comprised between amino acids 68-150, which overlaps the DNA binding and replication activation domain. We have generated deletion and site specific mutations within this region of CTF-1. 11 mutant proteins, expressed in rabbit reticulocyte lysate, have been tested for their ability to form dimers in solution and to bind the viral origin of replication, as well as for their ability to activate initiation of Ad DNA replication. We show that dimerization is required for the binding activity of CTF-1, which in turn is necessary for activation of initiation. Mutant proteins which show reduced dimerization ability also display reduced binding activity. The interaction of the various mutants with AdPol and the role of this interaction in Ad replication will be discussed.

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TRANSLATIONAL REPRESSION OF ENDOGENOUS THYMIDINE KINASE mRNA IN DIFFERENTIATING MOUSE EMBRYONAL CARCINOMA CELLS

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During differentiation of F9 mouse embryonal carcinoma cells by retinoic acid we observed a decline of thymidine kinase (TK) enzyme activity to less than 10 % by 96 hours while full length TK mRNA was still present to 50 % by this time and 27 % still persisted by 192 hours. In order to study the molecular mechanism of this disparate behaviour a polyclonal antiserum against mouse TK was raised in rabbit. High level expression of full length mouse TK polypeptide in Escherichia coli was achieved by using a T7 RNA polymerase directed expression system. Using the antiserum in immunoblotting no indication for a pool of inactive enzyme was found during differentiation. In cytoplasmic extracts from cells labelled *in vivo* with [^{35}S]methionine we observed a 6-fold decrease in the rate of TK-protein synthesis after 4 days of treatment with retinoic acid. This was not due to increased turnover of the protein as measured in pulse chase experiments. During the whole differentiation process full length TK mRNA was associated with polysomes although some relatively stable degradation intermediates appeared in the polysome-free fraction. In comparison, no such free RNA species were observed for histone H4 mRNA. Taken together these results suggest that TK mRNA is translationally repressed in differentiating F9 cells.

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CLONING AND EXPRESSION OF SEMLIKI FOREST VIRUS CAPSID PROTEIN GENE

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Semliki Forest virus (SFV) contains a core consisting of the 49S genomic RNA of positive polarity complexed with a single-type capsid (C) protein (Mr 33 kD) surrounded by a lipid bilayer. The C protein exhibits karyophilic properties and contains an autoproteolytic activity. In addition, data suggest that it is responsible for the shut-off of host protein synthesis.
We intend to establish a convenient source of C protein by recombinant DNA technology. To this end the insect baculovirus Autographa californica Nuclear Polyhedrosis Virus (AcNPV) was used. The complete C fragment was ligated downstream from the AcNPV polyhedrin (PolH) promoter in a convenient plasmid. To produce a recombinant virus, Spodoptera frugiperda insect cells were transfected with a mixture of infectious AcNPV DNA and the plasmid mentioned above. Recombinant viruses were produced by homologous recombination which generated occlusive negative virions resulting from the insertional inactivation of the PolH gene.

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REGULATION OF V(D)J RECOMBINATION BY PROTEIN KINASES AND TRANSCRIPTION FACTORS

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Rearrangement of immunoglobulin genes by V(D)J recombination normally occurs only in pre B-cells but experimentally also in cells which have been stably transfected with the recombination activating genes rag-1 and rag-2. As the mechanisms which regulate V(D)J recombination are unknown, we treated pre B-cells and rag-1 and -2 transfected fibroblasts with activatiors and inhibitors of the protein kinases A and C. We also transiently transfected several transcription factors and measured the rate of V(D)J recombination with a cotransfected V(D)J recombination substrate. Preliminary results show that the protein kinase A activator caffeine enhances the rate of recombination approx. 4 fold, whereas TPA, an activator of protein kinase C, reduces it approx. 5 fold. Cotransfection of oct-2 genes increases recombination 3-4 fold, but their effect is totally reversed by additional cotransfection of submolar amounts of the glucocorticoid receptor. In addition the oct-2 factors reduce the effect of Co^{2+}, an inhibitor of protein kinase A.

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3' END FORMATION IN SACCHAROMYCES CEREVISIAE

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We have analysed the function of various polyadenylation sites of RNA polymerase II transcribed genes in the yeast Saccharomyces cerevisiae. We identified two classes of polyadenylation sites: (1) efficient sites (e.g. derived from GCN4) that were functional in a strict orientation-dependent manner and were without sequence similarities and (2) bidirectional sites (e.g. derived from TRP4) that had a distinctly reduced efficiency and carried on both strands the bipartite sequence TAG...TATGTA. (Irniger et al., 1991).
Since little is known about the first class of polyadenylation sites we have performed a deletion analysis of the GCN4 polyadenylation site. These deletions were tested for their ability to turn off readthrough transcription from the actin promoter in a test gene which was constructed in such a way that it can be integrated into the yeast chromosome. We find that the GCN4 site contains several elements which can act independently as polyadenylation sites and which will be further characterized.

Irniger S, Egli CM, Braus GH (1991) Different classes of polyadenylation sites in the yeast Saccharomyces cerevisiae. Mol.Cell.Biol. 11,3060-3069.

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Biochemical and genetic analysis of histone pre-mRNA 3' processing in vitro.

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The 3' ends of poly A⁻ histone mRNAs are formed by a specific cleavage reaction which was previously shown to be controlled by two conserved sequence elements flanking the cleavage site. By native gel analysis and UV- crosslinking, we demonstrate specific interactions of the upstream hairpin element with proteins of approx. 50-60 kDal and of the downstream spacer element with the U7 small nuclear ribonucleoprotein particle (snRNP). Mutational and competition analyses show that a strong U7 interaction is absolutely necessary for processing and is dependent on the stability of pre-mRNA:U7 RNA base-pairing. In contrast, processing is highly resistant to mutation or biochemical competition of the hairpin element. Thus the contribution of the hairpin to ensure efficient processing, most clearly demonstrated in vivo, appears to be only indirect. Further mutations indicate that certain nucleotides at the processing site are also critical for processing but are not involved in binding of the U7 snRNP.

We have also begun to map precisely the 5' and 3' cleavage products and to determine the chemical nature of the respective end groups. Preliminary results indicate that a synthetic histone H4 pre-mRNA of 85 nt is cleaved into a 45-50 nt upstream fragment and four downstream pieces of 33, 26, 23 and 20nt respectively, which only differ at their 5' ends. Thus, even the longest products appear to be lacking a few nucleotides suggesting that the reaction involves more than just an endonucleolytic cleavage.

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AUTOREGULATION OF A YEAST tRNA-SYNTHETASE

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The yeast mutant gcd5-1 was isolated by screening for strains resistant to amino acid analogs. Strains which show a gcd phenotype translate the mRNA encoding the transcription factor GCN4 at a constitutively high level. GCN4 coregulates over 30 genes encoding amino acid biosynthetic enzymes in a pathway known as General Control of Amino Acid Biosynthesis.

We cloned the wild type GCD5 gene by complementation of the slow growth phenotype of gcd5-1. By sequencing part of GCD5 we found it to be identical to KRS1 encoding the yeast lysyl-tRNA synthetase. *In vitro* lysyl-tRNA charging assays showed that gcd5-1 has a defect in lysine binding. Sequencing the mutant gcd5-1 revealed a single point mutation changing an arginine in the putative lysine binding domain to leucine. We show that reduced charging activity of the gcd5-1 enzyme leads to increased levels of the GCN4 transcription factor. GCN4 enhances transcription of gcd5-1 by binding to the promotor region thereby allowing the mutant strain to survive.

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GCN4-INDEPENDENT TRANSCRIPTION OF THE YEAST BIOSYNTHETIC ISOGENES ARO3 AND ARO4

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The two isogenes ARO3 and ARO4 code for the isoenzymes catalyzing the first step of the aromatic amino acid biosynthetic pathway in the yeast Saccharomyces cerevisiae. They belong to a group of at least 30 different genes in unrelated amino acid biosynthetic pathways coordinatively derepressed by the yeast transcription factor GCN4 as a response to amino acid starvation. Nevertheless all these genes are transcribed at a considerably high level even in the presence of an amino acid excess. This so-called 'basal' transcription is generally GCN4-independent. Our aim is the isolation and characterization of transcription factors other than GCN4 responsible for the basal transcription of the two isogenes. Therefore we have constructed integrative translational lacZ-fusions of 5'-deleted and in consensus sequences pointmutated versions of the two isopromoters and analyzed gene expression.

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REGULATION OF THIAMINE BIOSYNTHESIS IN SCHIZOSACCHAROMYCES POMBE

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The thiamine molecule consists of a pyrimidine and thiazole moiety. The two halfs are synthezised in separate (and still unknown) pathways and condense to thiamine monophosphate. We defined structural genes responsible for thiazole (thi2) and pyrimidine (thi3) synthesis and the condensation reaction (thi4) and showed that their expression is strongly repressed by thiamine. Regulatory mutants affecting expression of thiamine repressible acid phosphatase were selected. The mutants map in four genes, tnr1, tnr2, tnr3 and thi1. Tnr3 mutants over express thiamine 10 to 20 fold and thi1 mutants are auxotrophic for thiamine. All tnr mutants are derepressed for genes thi3 and thi2. Thi1 mutants are repressed for thi2 and thi3. These results suggest that the thiamine biosynthetic pathway is under complex regulation.

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DNA BINDING ACTIVITY OF XENOPUS CTF/NFI HOMOLOGUES IS NEGATIVELY REGULATED BY A C-TERMINAL DOMAIN.

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Three distincts cDNAs, xNFX1, xNFX2, xNFX3, were isolated from a *Xenopus* kidney cell line library by screening with a human CTF/NFI cDNA probe. Sequence analyses revealed homology with the hamster NFI/X subtype and suggest that the 3 *Xenopus* cDNAs originate from alternatively spliced mRNAs derived from a common precursor.

In vitro expression and DNA binding experiments show that the activity of the common N-terminal binding domain is negatively regulated by a novel type of C-terminal protein domain. Interestingly this negatively active domain does not prevent the DNA binding of heterodimers made of *Xenopus* NFXs and human hCTF1. This suggests a complex regulatory mechanism which involves the interaction of different CTF/NFI protein subtypes.

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STRUCTURAL AND FUNCTIONAL ANALYSIS OF A XENOPUS PROMOTER IN YEAST.

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Vitellogenin gene expression is strictly dependent on the binding of activated estrogen receptor to its promoter. Furthermore, the transcriptional activation requires interactions between the estrogen response element bound receptor and other transcription factors. Thus far, little is known as to the mechanisms of this transcriptional activation. In this context, we analyse the induction of the Xenopus Vitellogenin B1 promoter by the estrogen receptor in the presence and absence of another transcription factor CTF1/NF1 in Yeast. The chromatin structure of the transcriptionally active and silent promoter is also studied with respect to nucleosome positioning. This yeast model system proves to be a powerful tool to probe into the delicate mechanism of hormone-regulated gene expression.

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CYTOSKELETAL AND DNA STRUCTURE ABNORMALITIES RESULT FROM BYPASS OF REQUIREMENT FOR THE *cdc10* START GENE IN THE FISSION YEAST

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The *cdc10* gene of the fission yeast *Schizosaccharomyces pombe* is required for traverse of the start control in late G1 and commitment to the mitotic cell cycle. To increase our understanding of the events which occur at start, a pseudoreversion analysis was undertaken to identify genes whose products may interact with *cdc10* or bypass the requirement for it. A single gene, *sct1* (suppressor of cdc ten) has been identified, mutation of which suppresses all conditional alleles and a null allele of *cdc10*. Bypass of the requirement for $cdc10^+$ function by *sct1-1* mutations leads to pleitropic defects, including microtubule, microfilament and nuclear structural abnormalities. Our data suggest that *sct1* encodes a protein that is dependent upon $cdc10^+$ either for its normal function or expression, or be a component of a checkpoint that monitors execution of $p85^{cdc10}$ function.

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CHARACTERIZATION OF THE *S.CEREVISIAE* MUTATION *TRP5A*

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Genetic events such as point mutations, mitotic crossing-overs, homologous recombinations and gene conversions induced by test chemicals play an important role in the initiation and promotion of tumors. The *S. cerevisiae* tester strain *YHE2*, a *D7* derivative, provides the possibility of visualizing various events of this kind simultaneously.

For the detection of gene conversions we work with two mutations in the *TRP5* locus. One of these mutations named *trp5a* has been cloned on a 2μ plasmid, and the site of the mutation has been narrowed down to a 179 bp ClaI/ EcoRV fragment. Comparison of the DNA sequence of this fragment with the wild-type *TRP5* gene has revealed two mutations in the *trp5a* sequence: a) a transition A to G at position -3 before ATG and b) a transition C to T at position 168 after ATG which results in a stop codon. The individual influence of these two mutations on gene expression will be discussed.

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The influence of DNA-bending on nucleosome positioning tested in yeast minichromosomes

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Due to the protection of DNA by histone proteins, the position of nucleosomes with respect to the underlaying DNA sequence might play a decisive role in regulation of DNA dependent processes. DNA sequences that support bending around the histone octamer ('rotational setting') are considered as a major determinant of nucleosome positions. TG5 is an artificial positioning sequence containing 100 bp of a $(A/T)_3NN(G/C)_3NN$ motif repeated with a 10 bp period. It provides a strong rotational setting and is superior to natural sequences in nucleosome formation in vitro (Shrader and Crothers, Proc. Natl. Acad. Sci. USA (1989) 86, 7418-7422). To investigate the contribution of the rotational setting to nucleosome positioning in vivo, TG sequences were inserted in a nucleosome, at the edge of a nucleosome and in a nuclease sensitive region of yeast minichromosomes, and the chromatin structures were analysed. In none of the constructs, TG sequences were folded in a positioned nucleosome demonstrating that the rotational setting played a subordinate role in the rough positioning in vivo. The role of the rotational setting might be the fine tuning of positions. Positioned nucleosomes were found overlapping the ends of TG, indicating that a discontinuity of the 10 bp periodicity of $(A/T)_3$ and $(G/C)_3$ near the center of a nucleosome might be favorable for positioning and serve as a translational signal.

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Expression of histone H1 in yeast *Saccharomyces cerevisiae*

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The chromosomal protein histone H1 is tought to have different roles: 1) It stabilizes the nucleosomal structure. 2) It organizes and maintains the 30 nm chromatin fiber. 3) It might be involved in the spacing of nucleosomes. 4) It might serve as a general repressor of transcription. To investigate the role of H1 *in vivo*, a sea urchin histone H1 was expressed from a galactose inducible promoter. When H1 is expressed from a centromer vector (strain YCL1), low levels of H1 were detected copurifying with nuclei, but no obvious effects were observed on growth and phenotype. When gene dosage was increased using a 2μ vector (strain YCL7), growth on galactose was severely inhibited and the amount of H1 was similar to that of the individual core histones. We conclude that low levels of H1 is tolerated by yeast *S. cerevisiae*. We currently investigate the effect of high levels of H1 on nucleosome spacing, nucleosome positioning and on gene expression.

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MECHANISM(S) OF HOMOLOGOUS RECOMBINATION IN YEASTS: HYBRID DNA FORMATION

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Hybrid DNA formation is a central step in homologous recombination. Using biochemical assays, activities that can form hDNA *in vitro* have been purified in fungi, flies,plants and man. The strand exchange protein 1 (SEP1) from *Saccharomyces cerevisiae* has been purified to homogeneity as a Mr 132,000 polypeptide. As SEP1 shares many biochemical properties with the central recombination protein in *E. coli*, RecA, a role for SEP1 in yeast recombination is inferred. The sequence of the SEP1 gene revealed that this gene has been identified in several groups with different mutational analyses. Mutations in SEP1 show a very pleiotropic phenotyp: slow growth, defect meiosis, modest quantitative and qualitative effects on recombination, increased chromosome loss a.s.o.

In order to put strand exchange proteins in a general perspective we decided to analyze the yeast *Schizosaccharomyces pombe*. To this end we have partially purified a strand exchange activity from this yeast that co-purifies with an antigen that cross-reacts with two different anti-SEP1 monoclonal antibodies. Using biochemical as well as genetical techniques we want to isolate the *SEP1* homolog of *S.pombe*. This comparative analysis between the two yeasts might allow extrapolation to higher eukaryotic organisms.

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PHAGE-SPECIFIC CONTROL OF A TRANSCRIPTIONAL ACTIVATOR IN BACTERIOPHAGE P1.

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Gene *10* of bacteriophage P1 codes for a function required in the activation of late promoter sequences. The expression of gene *10* can be monitored 10 to 15 minutes following the onset of lytic phage growth,while during lysogenic growth, transcription of gene *10* is completely blocked. The promoter sequence of gene *10* (Pr94) was mapped. It is composed of an *E. coli* consensus-like promoter overlapped by a binding site (Op94) for the major repressor protein C1. However, *in vivo* and *in vitro* studies indicated that C1 alone is not sufficient to completely repress transcription from Pr94. A further phage encoded function, the Bof protein, was identified to tighten the repression exerted by C1. The fact that its expression is regulated by components of the P1 immunity system identified gene *10* as a direct mediator between early and late transcription during the lytic growth cycle of bacteriophage P1.

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COPY NUMBER CONTROL OF THE BROAD HOST RANGE PLASMID RSF1010

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The copy number control of the broad host range plasmid RSF1010 is accurately controlled by the plasmid encoded Rep proteins encoded by RepB' RepA and RepC. Expression of the *rep* genes, in particular *repC*, have a positive regulation of replication at the origin of replication *oriV*. The genes for replication and conjugal mobilization are mainly transcribed from a cluster of promoters P1/3 and P2 that are overlapping with the origin of transfer *oriT*. Three loci of deletion mutations have been found that affect the mobilization of RSF1010 and strongly increase its copy number in *E.coli*. A deletion mutation in *mobC* creates a plasmid with a four times increased copy number. In this mutant, the copy number effect can be restored *in trans* with a cloned *mobC* gene. A second deletion locus that removed *oriT* and part of the promoter sequence of P2 which transcribes *mobC* resulted in a three fold increased copy number. The third type of deletion that removes most of the N-terminal of MobA and presumably inactivates its primase activity for initiating the origin of transfer also results in a three times increased copy number. A small in-frame deletion in *mobA* only affects the conjugal transfer but not the copy number. Repression of the P1/3 promoter for the transcription of the *rep* genes and of P2 for the transcription of *mobC* by the presence deletion derivatives of RSF1010 and by the cloned *mobC* gene shows that the promoters P1/3 and P2 are negatively regulated by MobC and MobA proteins presumably by attachment of the Mob proteins to the *oriT* region of RSF1010. Both MobC and MobA seem to be required for maximal repression of the operon containing the replication genes and hence for the optimal regulation of the copy number control.

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Differences in nuclear chromatin of Trypanosoma b. brucei procyclic culture forms and blood stream forms

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T. b. brucei, a flagellate protozoan parasite, is a causative agent of Nagana disease of domestic ungulates in Africa. It represents a major animal health and economic problem in the affected countries. Chromatin of T. b. brucei blood stream forms was analysed by electron microscopy and gel electrophoretic techniques. Results were compared with those obtained from T. b. brucei procyclic culture forms and from rat liver chromatin.
As compared to procyclic culture forms, chromatin of blood stream forms showed a salt dependent condensation, but formed no 30 nm fiber. No H1-like histone could be demonstrated for procyclic culture forms and bloodstream forms in different gel systems. Additional proteins - which could not be demonstrated in procyclic culture forms - migrated in the core histone-region of bloodstream forms. Significant structural and functional differences exist not only between the chromatin of T. b. brucei and higher eukaryotes, but also between various stages of the life cycle of the parasite.

Extracellular Matrix and its Receptors

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TENASCIN VARIANTS AND THEIR LIGANDS

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Tenascin (TN) is a large hexameric extracellular matrix protein. In the chicken, three TN variants have been characterized that are generated by alternative splicing of 3 of its 11 fibronectin (FN) type three repeats. Recently, we identified a 12th FN type three repeat in the chicken TN gene, located between the previously identified repeats 7 and 8. These TN variants show distinct distribution patterns in cell cultures and tissues and they bind differentially to FN. Using the purified proteins in various types of solid phase binding assays, only the smallest TN variant bound strongly to FN. These biochemical data were paralleled by the observation that in chick embryo fibroblast cultures only the smallest form of TN could be detected in the FN-rich extracellular matrix network laid down by the cells. All TN variants bind equally well to heparin, have identical lectin-activity and agglutinate erythrocytes. This hemagglutination can be inhibited most efficiently by gangliosides containing two consecutive sialic acids, such as i.e. GD2 and GD3. We thus postulate that gangliosides could be cellular ligands of TN. Since GD2 and GD3 have been shown to be necessary for FN receptor function, we propose that TN interferes with FN-mediated cell adhesion by binding to the receptor-associated gangliosides.

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TWO *DROSOPHILA* TRANSCRIPTS WITH SIMILARITIES TO THE EXTRACELLULAR MATRIX MOLECULE TENASCIN

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Using PCR and low stringency hybridization we have isolated and analyzed two *Drosophila* transcripts with similarities to tenascin. Both transcripts derive from different genes, are large (7.2 kb and 11.5 kb in size) and exhibit the characteristic tenascin-type EGF-like motif. Surprisingly, no fibronectin type III repeats nor a fibrinogen homology was found. Both proteins appear to be secreted. The bigger transcript could encode for more than 2600 amino acids and reveals several heptad repeats at its NH_2-terminus enabling a multimeric structure as does tenascin. We have generated antibodies to test this hypothesis.
Both transcripts are mainly associated with neurogenesis (as has been determined by *in situ* hybridization) and transcripts are also present at muscle attachment sites. During metamorphosis, both transcripts are located in the eye. These findings are in good correlation with the reported sites of action of tenascin.

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LOCALIZATION OF A MAJOR HEPARIN BINDING SITE TO THE C-TERMINAL FIBRINOGEN-LIKE DOMAIN OF TENASCIN

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The extracellular matrix protein, tenascin, has recently been reported to bind to the heparan sulfate chains of a cell surface proteoglycan, syndecan. Tenascin is retained by heparin-Sepharose at 0.15 M NaCl and elutes at 0.3 M NaCl. We have previously localized a major heparin-binding site to a 60 kDa C-terminal fragment of tenascin subunits. Here we demonstrate that this heparin-binding site must be located within the 25 kDa fibrinogen-like homology of tenascin subunits, presumably at their very C-terminus. From a hybridoma bank, anti-tenascin mAb Tn4 was selected because it bound to a heparin-binding 25 kDa polypeptide included in the C-terminal 60 kDa fragment. By comparison with mAb Tn68 whose epitope on the 60 kDa fragment is known precisely, we could conclude that mAb Tn4 must recognize the fibrinogen-like domain. Accordingly, mAb Tn4 was found to bind to the distal globular domain of tenascin arms as revealed by electron microscopy. Binding of mAb Tn4 to immobilized tenascin was partially inhibited by heparin. However, we have evidence that the heparin-binding site is distinct from the mAb Tn4 epitope. At their C-terminus, tenascin subunits end with the sequence GRRKRA. A similar sequence in fibronectin has been proven to be required for its binding to heparin. We are currently testing whether such peptides inhibit the interaction of tenascin with heparin.

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NEURITE GROWTH VERSUS GLIAL MIGRATION ON TENASCIN

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The extracellular matrix protein tenascin is expressed by nerve satellite (i e. glial precursor) cells during migration, and it promotes neurite growth. To characterize the function of tenascin during these processes, we cultured 6 day embryonic chick dorsal root ganglia on purified tenascin, laminin or fibronectin. Neurites extended immediately on fibronectin or laminin and were accompagnied by satellite cells migrating on the substrate. On tenascin, neurites grew after a lag phase, but then with a similar velocity as on laminin and fibronectin. In contrast, satellite cells were not able to migrate on tenascin but moved on top of neurites in this case. Neurites and satellite cells were also confronted with borders of laminin/tenascin and fibronectin/tenascin. Neurites could cross borders from laminin to tenascin but were diverted away when they arrived at a low angle. Neurites at borders of fibronectin to tenascin were able to cross as well, whereas satellite cells remained restricted to the fibronectin substrate. To characterize the neurite growth domain of tenascin, a series of monoclonal antibodies (mAbs) against tenascin were tested. A mAb (TN 68) against a distal tenascin domain could partially inhibit neurite growth on tenascin and block it completely in the presence of secondary antibody. Control mAbs to other domains were inactive. Neurite growth on tenascin was inhibited by either JG22 (a function blocking mAb against ß1 integrins) or by heparin, suggesting that integrins and/or cell surface proteoglycans are involved.

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LAMININ VARIANTS IN THE CHICK

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We are interested in the expression and function of laminin variants during chick embryonic development. For this reason we isolated laminin from adult chick heart and chick gizzard. By rotary shadowing we identified laminin prepared from heart cross-shaped and T-shaped molecules as well as laminin aggregates. In the gizzard preparation the same cross-shaped and T-shaped molecules are found, many of which have a dumbell structure linked to one short arm. After reduction on SDS-PAGE, heart laminin gives rise to polypetides of 180, 190 and 200kD, 350, 400, 500 and 600kD. In contrast, the 350kD polypeptide is missing in gizzard laminin. Both laminin preparations were biologically active in a neurite outgrowth assay. We generated monoclonal antibodies against chick heart laminin. Two selected ones were directed against different subunits and labelled different sutructures in the 6-day-old chick embryo by immunofluorescence. One mAb called 3/E-8 stains all basement membranes as well as smooth muscle and mesenchyme, whereas another one called 9/F-10 binds only to basement membranes. Therefore we believe to have mAb's against different chick laminin chains which will enable us to separate laminin isoforms.

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LAMININ CHAIN ASSEMBLY BY TRIPLE- AND DOUBLE-STRANDED α-HELICAL COILED-COIL STRUCTURES

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Laminins are multidomain glycoproteins with multiple functions in cellular processes and in the supramolecular organization of the extra-cellular matrix. We studied the assembly of laminin from its three constituent chains A, B1 and B2 by means of fragments comprising the -helical coiled-coil domain in the 76 nm long arm of laminin. Reassembly studies of different chains mixed at different ratios demonstrated a high specificity of chain recognition which resides in this domain and may be compared with other systems (jun/fos, fibrinogen, cyto-keratins). Proper chain selection is important for correct assembly after non-coordinated biosynthesis of the laminin chains and for the formation of tissue specific and transiently expressed isoforms with distinct functions. In collaboration with D.A.D. Parry, Massey Univ., New Zealand, and K. Beck, Univ. Linz, we were able to supplement the experimental studies by computer aided prediction of the stability and selectivity of coiled-coil formation from sequence data.

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LAMININ SELF-ASSEMBLY IS MEDIATED BY MULTIPLE LOW AFFINITY SHORT ARM INTERACTIONS.

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Laminin self-assembly was further characterized using affinity retardation chromatography (ARC). The migration of proteolytic laminin fragments was compared on a "binding" column (laminin, or its fragments) and on a "reference" column (non-reacting; BSA, or uncoupled gel). Retardation of elution was used as a measure of relative binding activity. Elastase fragment E4 (B1 chain NH_2-terminal domains VI and V) binds to laminin and to fragment E1' (short arm complex without B1 chain NH_2-terminal domains IV-VI) but not to itself. E1' binds to itself, and possesses the ability to crosslink soluble E4 to immobilized one. We conclude that domain E1' binds to laminin, using more than one binding site, including self-interactions and dual binding of domain E4.

A mathematical model describing ARC was developed. Half maximal binding of a single domain E4 - laminin interaction was estimated to occur between 1 and 15 μM under physiological conditions (TBS, containing 1 mM $CaCl_2$, 37°C).

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Binding and calcium induced aggregation of laminin onto lipid bilayers

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Direct binding of laminin in the form of its complex with nidogen to planar lipid bilayers was demonstrated with total internal reflection fluorescence microscopy. Binding occurred equally well to zwitterionic (phosphatidylcholine) and negatively charged (phosphatidylglycerol) lipids and was enhanced by sulfatides but only at non-physiological molar ratios higher than 30 mol%. Strong interactions with lipid bilayers were also observed for BSA. This explains a strong inhibition of laminin binding by this protein. However, binding of laminin to sulfatide rich bilayers was not completely inhibited. Observable by the microscopic technique was the formation of laminin clusters on the surface of the bilayer which occurred concomitantly with binding. Both processes were strongly enhanced by the presence of calcium. These results show that calcium induced laminin selfassembly is enhanced at lipid surfaces.

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SYNTHESIS OF LAMININS AND PROTEOGLYCANS BY MACRO- AND MICRO-VASCULAR ENDOTHELIAL CELLS FROM HUMAN PLACENTA

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We have designed a method for the isolation of viable microvessels from chorionic tissue of human term placenta. When the microvascular fragments are plated either onto substrates coated by charged polymers, e.g. poly-DL-ornithin or heparin, or into protein gels, a high proportion of the fragments yield growing cell-populations of both endothelial cells and smooth muscle cells. By subsequent separation of the celltypes using magnetic beads coated with a lectin recognizing endothelial surface-antigens, we obtain pure cultures of the two cell species.
Primarily the cells will be used for studies of the differential expression of laminin isoforms and proteoglycans between micro- and macro-vascular endothelial cells and smooth-muscle cells.

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Identification of Gln^{726} in Nidogen as the Amine Acceptor in Transglutaminase-Catalyzed Crosslinking of Laminin-Nidogen Complexes

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The laminin-nidogen complex, the most abundant noncollagenous component of basement membranes, was recently shown to be a specific substrate for tissue transglutaminase (Aeschlimann and Paulsson (1991) J. Biol. Chem. 266, 15308-15317). Saturation experiments to determine the number of amine acceptor site(s) indicated a single reactive Gln residue in nidogen and none in laminin. Nidogen was labeled with [^{3}H]putrescine in the tissue transglutaminase-catalyzed reaction, and two major radioactively labeled fragments, T70 and T40, were isolated after limited trypsin digestion. N-terminal sequencing showed that T40 is contained in T70 and corresponds to the rod-like structure of nidogen, made up of EGF-like repeats. Three radioactively labeled peptides, obtained by extensive trypsin digestion of reduced and alkylated T40, were sequenced. In all a single residue, Gln^{726}, was found to contain label. Gln^{726} is located in an exposed loop between the second and the third EGF-like repeat in nidogen.

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HIGH AND LOW AFFINITY CALCIUM BINDING AND STABILITY OF THE MULTIDOMAIN EXTRACELLULAR GLYCOPROTEIN BM-40/SPARC/OSTEONECTIN

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Two classes of calcium-binding sites of very different affinity (K_d = 0.6 μM and $K_d \geq 10$ mM) were identified in BM-40 with spectroscopic and proteolytic methods. The data indicate that domain IV containing an EF-hand motif contributes the single high affinity calcium-binding site which may be important for stabilization and folding. Although the in vivo function of BM-40/SPARC/osteonectin is still a matter of dispute most of the reported functions have been found to be calcium dependent and both calcium-binding domains were identified to be directly involved in cellular activities. Low affinity binding of Ca^{2+} in domain I of BM-40 was detected by the proteolytic susceptibility against leukocyte elastase. It occurs in the millimolar calcium range where functions of BM-40 may be modulated by changes of extracellular calcium levels.

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ELECTRON MICROSCOPY OF NATIVE PROTEOGLYCAN AGGREGATES PURIFIED FROM THE SWARM RAT CHONDROSARCOMA

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Proteoglycans are the major non-collagenous constituent of the cartilage extracellular matrix where they form primarily aggregates. Chaotropic solvents such as 4M guanidine hydrochloride reversibly dissociate these aggregates and allow them to be extracted in high yields. Taking advantage of this behaviour we recently investigated the assembly of proteoglycans with hyaluronate and link protein by electron microscopy. The components employed in these experiments, however, had probably been denatured during isolation and the significance of the results depended on their ability to refold into a native conformation. Therefore we isolated proteoglycan aggregates from the Swarm rat chondrosarcoma under native conditions. These aggregates were examined with different electron microscopic techniques and exhibited a structure similar to dissociatively isolated and reconstituted proteoglycans from hyaline cartilage.

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ELECTRON MICROSCOPY OF NATIVE CARTILAGE OLIGOMERIC MATRIX PROTEIN PURIFIED FROM THE SWARM RAT CHONDROSARCOMA

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Cartilage matrix is a composite of collagens and a variety of noncollagenous proteoglycans and glycoproteins. With a few exceptions the functional role of this latter group of matrix constituents is poorly understood. A high M_r (>400.000) glycoprotein was identified by Fife and Brandt (1984) in articular and tracheal cartilage from several species, and consists of disulfide-bonded subunits of apparent M_r 116.000. We were able to isolate this protein in the native state from the Swarm rat chondrosarcoma. A crucial step was its selective extraction with EDTA-containing buffer. The purified protein was subjected to electron microscopy using rotary shadowing and negative staining. The images allowed the construction of a structural model. The bouquet-like protein consists of five 28 nm-long arms containing a peripheral globular domain, a flexible strand, and a central assembly domain, where the five arms meet in a cylindrical structure.

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COMP MEDIATES CHONDROCYTE ATTACHMENT

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Cartilage Oligomeric Matrix Protein (COMP) has been recently isolated and characterized in a native form from the Swarm rat chondrosarcoma. COMP is highly enriched in cartilage and its' function is not known.

COMP, fibronectin, or laminin were coated onto tissue culture wells. Primary bovine chondrocytes were isolated by collagenase digestion and placed in spinner culture for 24 to 48 hours to reconstitute their cell surface receptors. Cell attachment was measured using a crystal violet binding assay.

Chondrocytes were noted to bind best to fibronectin, intermediate to COMP and did not bind to laminin. Binding was dose dependent with maximum binding achieved at 3 ug/ml. Time of binding was also important with maximum binding achieved by 8 hours after plating. Inhibition of binding with RGD containing peptides occured in wells coated with COMP, but to a lesser extent and at a much higher concentration than with fibronectin.

In conclusion, native COMP mediates primary chondrocyte attachment and this may occur by an integrin receptor.

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ELECTRON MICROSCOPY OF FILAMENTOUS MOLECULES

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The visualization of thin filamentous proteins by electron microscopy currently present many problems owing to the poor image resolution of such molecules produced by routine preparative methods. Our aim was to improve the potential of the rotary shadowing technique for visualizing thin filamentous molecules. Proteoglycan core proteins obtained from rat chondrosarcoma cells were used as representative test molecules. Their morphological appearance was examined as a function of various preparative procedures. The following parameters were systematically varied: buffer salt (type of buffer, ionic strength, pH), decorating agent, molecular adhesion technique, surface properties of the carbon film, drying procedure and metal shadowing conditions. Fineness of metal film granularity and resolution were improved by solving molecules in volatile types of buffers and were significantly enhanced by lowering buffer salt ionic strength; highest resolution was obtained at ion-free solution conditions. Decorating agents, such as glycerol or polyethylene glycol, proportionally reduced the contrast quality of the images as a function of their concentration (and thus decoration effect). The highest yield (i.e. number of molecules per unit area) of well preserved individual molecules on carbon films was obtained following spraying samples on to positively-charged, hydrophobic surfaces and subsequent vacuum-drying. Contrary to a wide current belief that the production of a high degree fineness of metal granularity is dependent on low temperature shadowing conditions, we found that under the preparation conditions adopted, it was possible to obtain a comparable granularity degree at ambient temperature drying and shadowing. The usefulness of the suggested protocol is exemplified by an application in combination with monoclonal antibodies for mapping of structural and functional epitopes along filamentous molecules.

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Fibrillar Organisation in Cartilage

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17 d-old chick embryo cartilage matrix contains collagen fibrils with a uniform diameter of ~20 nm embedded into electron-translucent material consisting of proteoglycans and glycoproteins. The collagenous components include collagens II, IX, and XI, co-polymerising into heterotypic fibrils. Epitopes of collagen XI, but not collagens II and IX, are masked within the fibrils by adjacent collagen molecules suggesting that collagen XI may reside in the interior. This notion is supported by reconstitution experiments in which collagen XI is incorporated prior to the other collagens into fine filaments that mature into fibrils by accretion of collagens II and IX. Collagens II, IX and XI, in their native proportions, generate 20 nm-wide fibrils closely resembling authentic cartilage fibrils. By contrast, collagen II and mixtures of collagens II and XI or collagens II and IX produce short tactoids with little similarity to cartilage fibrils. Therefore, the diameter of cartilage fibrils is controlled thermodynamically by specific interactions between collagens II, IX, and XI.

In cartilage of fetal or young animals, fibrils frequently are oriented randomly to form a network containing the swelling pressure generated by the proteoglycan/glycoprotein-matrix between the fibrils. The surface of the fibrils is coated by small proteoglycans. They may mediate contacts between fibrils to stabilize their network or between the fibrils and the extrafibrillar matrix to confer to the tissue its mechanical properties.

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TYPE XII COLLAGEN: CHARACTERIZATION OF THE LARGE FIBROBLAST VARIANT

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Type XII collagen is an extracellular matrix protein with very large non-collagenous domains. The recently published complete cDNA sequence predicts a subunit molecular mass of 350 kDa. However, the only type XII collagen characterized so far has been isolated from tendon and has three subunits of only 220 kDa. From medium conditioned by chick embryonic fibroblasts, we have now purified a major collagenase-sensitive molecule with subunits of about 350 kDa. Peptide sequences obtained from chymotryptic fragments of our protein were identical to stretches of the collagen type XII sequence predicted from the cDNA. The collagenous domains seem to be similar in size in the two isoforms. In contrast to the tendon isoprotein, however, the large fibroblast variant of type XII collagen is sensitive to chondroitinase and can be metabolically labeled by 35S-sulfate. By electron microscopy, fibroblast type XII collagen has three flexible arms 90 nm in length, in contrast to the tendon protein which has arms 60 nm long. The fibroblast protein has a double globe at the tip and a third globe in the middle of its three arms. The cDNA sequence predicts two neighbouring von Willebrand factor A homologies at the N-terminus and a third about in the middle of the noncollagenous domain; these homologies are spaced by fibronectin type III repeats. Thus, our structural data confirm the cDNA-derived model of the large form of type XII collagen.

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ISOLATION AND SEQUENCING OF THE cDNA ENCODING HUMAN PLATELET CD36: COMPARISON TO MONOCYTE, ENDOTHELIAL AND HEL CELLS cDNA.

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Glycoprotein CD36 is a major platelet glycoprotein that bears the newly identified Nak^a alloantigen. This glycoprotein has recently been shown to act as a receptor to thrombospondin, collagen, Plasmodium falciparum infected erythrocytes and is involved in host defence mechanisms. A CD36-like glycoprotein has been reported to be present on monocytes, endothelial cells, fetal erythrocytes as well as a number of tumor cell lines. In addition, a glycoprotein (PAS-IV) present in human or bovine mammary epithelial cells has been found to be immunologically and structurally related to CD36. Very little is known at this stage if platelet CD36, PAS-IV and other CD36-like bearing cells represent a new family of receptors. The aim of this study was to clone platelet CD36 and investigate other forms of CD36-cDNA present in monocytes, endothelial and HEL cells. RNA from human platelets, monocytes, endothelial or HEL cells were reversed transcribed (RT), using specific primers for CD36, and amplified by the polymerase chain reaction (PCR) technique. Sequencing the different amplified platelet derived cDNA fragments, spanning the whole coding region, showed the identity between platelets and placenta cDNA. Platelet cDNA cross-hybridized, in Southern blots, with RT-PCR amplified cDNA originating from monocytes, endothelial and HEL cells. However, monocytes showed an RT-PCR amplified cDNA fragment (561 bp) that was present in platelets and placenta but not in endothelial or HEL cells. These results suggests that the structure of CD36 expressed in platelets is similar to that of placenta. Differences in apparent molecular weight between CD36 and CD36-like glycoproteins may be due to posttranslational modifications and/or structural variations.

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EFFECTS OF SPECIFIC INHIBITION OF 4-PROLYL HYDROXYLASE (PH) IN HUMAN SKIN FIBROBLASTS BY 2,4-PYRIDINE DICARBOXYLIC ACID (2,4-PDCA)

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PH is a resident luminal protein of the rough endoplasmic reticulum (rER) and provides hydroxylation of procollagen ∝-chains which only then form stable triple helices. The effects of 10 mM 2,4-PDCA, by electron and triple immunofluorescence microscopy, presented as dilation of the rER, endoplasmic retention and accumulation of procollagens I/III. Metabolically labelled cells displayed a dose-dependent degree of retention, overmodification and reduced mobility in SDS-PAGE of pro ∝ I/III chains. Conformational instability of procollagens synthesized in the presence of 2,4-PDCA was evident by decreased denaturation temperatures: retained procollagens I/III melted at 39/34°C, the secreted material at 40/37°C. Fully hydroxylated procollagens, retained in the rER through Brefeldin A, also showed significant overmodification, but melted normally (42/39°C). Our data confirm the sorting for secretion of procollagens by conformation in the rER.

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TEMPORAL EXPRESSION OF OSTEOPONTIN, OSTEOCALCIN AND OF THE E11 ANTIGEN DURING BONE DEVELOPMENT

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The purpose of this work was to compare the expression of osteopontin (OP), osteocalcin (BGP) and of the E11 antigen, during embryonal and postnatal bone formation in the rat. Expression of the E11 antigen, a cell surface marker characterized by a monoclonal antibody specific for a subpopulation of osteoblasts and for young osteocytes, was detected by immunohistochemistry while OP and BGP were studied by in situ hybridization. Both in metatarsals and in caudal vertebrae, OP and the E11 antigen appear simultaneously at the onset on bone formation. OP is expressed by hypertrophic chondrocytes and by osteoblasts. E11 is expressed by osteoblasts and at a higher level, by osteocytes. In contrast, BGP appears only later during bone development and is expressed only by osteoblasts. In conclusion, although OP and the E11 antigen are expressed simultaneously during bone formation, their expression seems to be related to different processes, OP being linked to mineralization and E11 to the differentiation of osteoblasts into osteocytes. On the other hand, BGP seems to be a marker for mature bone.

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DISCREPANCY BETWEEN BONE RESORPTION ASSAYED BY URINARY EXCRETION OF PYRIDINOLINE CROSSLINKS AND OF ^{3}H-TETRACYCLINE IN THE RAT.

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To evaluate urinary pyridinoline cross-links (Pyr) to assess bone resorption (BR) in the rat, we compared in the same animals Pyr with urinary ^{3}[H]tetracycline (^{3}[H]TC) excretion of chronically prelabelled rats. When stimulated by dietary calcium restriction, Pyr and ^{3}[H]TC excretion increased by about 50 %. The maximum increase of Pyr occurred after 2 days and after one day for ^{3}[H]TC. Daily injections of the bisphosphonate (BP) clodronate caused a continuous decrease of Pyr excretion reaching 50 % after 14 days. In contrast, ^{3}[H]TC excretion decreased by 70 % within 3 days. After TPTX, both decreased by about one third, Pyr after one week, ^{3}[H]TC after 2 days. Finally, 14-day infusion of PTH to the TPTX animals increased ^{3}[H]TC sevenfold starting after 2 days, whereas Pyr started to increase after 7 days and only doubled. Thus, ^{3}[H]TC responded more rapidly to manipulations which alter BR. The cause of this discrepancy is unknown, but could be related to a different kinetic of matrix and mineral degradation.

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CELL SURFACE PROTEOGLYCAN RECEPTORS FOR TENASCIN

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Cell surface proteoglycans are an important group of receptors for the extracellular matrix. The matrix glycoprotein tenascin has been shown to modulate cellular morphology, although the mechanism is unknown. Here we use an affinity-chromatographic approach to show that a chondroitin sulfate proteoglycan and three heparan sulfate proteoglycans from fibroblasts, PG125, PG48 and glypican, all interact with tenascin. Whereas the chondroitin sulfate proteoglycan binds weakly, eluting at low salt concentrations, the heparan sulfate proteoglycans elute in peaks at 0.25M and 0.5M NaCl. The latter peak disappears following heparitinase digestion of the proteoglycans. The heparan sulphate proteoglycans also elute from tenascin with either heparan sulfate or heparin but are not dissociated by free chondroitin sulfate or dermatan sulfate chains. The heparin-binding site is localised at the distal (carboxyl) end of the tenascin arms, as shown by rotary shadowing of tenascin-heparin and tenascin-glypican complexes.

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RECONSTITUTION OF INTEGRINS, RECEPTORS OF EXTRACELLULAR MATRIX COMPONENTS, INTO LIPID VESICLES

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Integrins are a class of transmembrane receptors which bind to extracellular matrix proteins (laminin, fibrinogen, fibronectin, collagen) and mediate cell-cell contact. Integrin $\alpha_1\beta_1$ was isolated from chick gizzard and $\alpha IIb\beta_3$ was purified from human platelets. The reconstitution of integrin into lipid vesicles was studied in detail. Detergent dialysis and the Biobeads method were used for the reconstitution procedure. The influence of the lipid composition and detergent was investigated. We tried to separate nonincorporated protein by sucrose gradient centrifugation. Electron microscopy was used to discriminate between vesicles with adhered protein and vesicles with correctly incorporated receptor; both showed similar behaviour in sucrose gradient centrifugation. The results show that only a few of the studied conditions give good reconstitution. Reconstituted integrins will now be used for quantitative binding studies.

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BINDING OF RGD LIGANDS TO INTEGRIN

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The integrin are a family of cell surface receptors important in mediating cell-matrix and cell-cell adhesion. The main recognition site of many natural integrin liagands appears to comprise an arginine-glycine-aspartic acid (RGD) sequence. Very recently evidence has been found that the RGD sequence is not only necessary for binding to integrin, but can also trigger conformational changes in the integrin receptor, leading to the activation of additional sites of higher affinity states. We investigated these complex binding phenoma by first depositing a layer of integrin onto a planar optical waveguide. The presence of protein molecules at the waveguide surface influences the optical propagation constants of the guided modes, from which the quantity of bound protein may be determined. Then solutions of fibrinogen and various RGD-oligopeptides were brought into contact with the integrin layer, sequentially and consecutively, in order to study binding and activation, respectively.

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Reconstituted plasma membrane Ca^{2+} ATP-ase imaged with the atomic force microscope

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The Ca^{2+} ATP-ases of the sarcoplasmic reticulum and of the plasma membrane differ in their cytoplasmic domain. Shape and size have been defined for the former but are still unknown for the latter. Taking advantage of the atomic force microscope (AFM), which can image soft samples immersed in physiological buffers, we observed molecules of the plasma membrane Ca^{2+} ATP-ase reconstituted in liposomes. These were produced by the method of detergent dialysis, dissolving 100 µg of enzyme purified from human erythrocytes in 10 mg of crude soya-bean phospholipid extract and were then supported on mica. We were able to visualize particles of diameter ranging from 10 to 30 nm protruding from the flat surface of the liposomes. The larger particles suggest a partial dimerization of the enzyme. Improvement of the resolution should allow us to study the detailed shape of the reconstituted Ca^{2+} ATP-ase and the dynamic changes of this enzyme on activation.

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Observation of the plasma membrane of living cells with the atomic force microscope

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Size, shape and movement of peripheral or integral proteins in the plasma membrane of cells can only be studied by electronmicroscopy using tissue that had been fixed, or in living cells with indirect immunological methods. Now, as the atomic force microscope (AFM) offers us the possibility of studying molecules directly at high resolution and under physiological conditions we have used it to observe the plasma membrane of cultured cells. Cells from a human lung carcinoma were grown on glass slides and observed in the AFM immersed in tissue culture medium. These large cells survived the multiple scanning of their surface with the AFM. We regularly observed waves travelling over the surface of the plasma membrane of the living cells. However, the movements of the cell surface never allowed us to increase the resolution to the point of enabling us to image structures resembling membrane proteins. Nevertheless, continuous improvements in the methods of specimen preparation will certainly offer new insights into the dynamic of membrane proteins.

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CONSTITUTIVE EXPRESSION OF THE INTERLEUKIN-2 RECEPTOR ON HUMAN MELANOMA CELLS, IN VITRO AND IN VIVO

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Melanoma cells can secrete several cytokines and express various cell surface molecules, such as the intercellular cell adhesion molecule ICAM-1, class II histocompatibility antigens, and the CALLA antigen, typically found in cells of the immune system. We have investigated the possible expression of interleukin-2 (IL-2) receptors in melanomas using monoclonal antibodies specific for the p55/α chain (TAC antigen) and the p75/β subunit. Flow cytometric analysis of cultured melanoma cells showed the presence of the TAC antigen on the cell surface of several lines, while the β chain appeared to be expressed at a much lower extent. Similar results were obtained in vivo by immunohistochemistry on cryosections prepared from cutaneous and ocular melanoma explants. Positive staining of various intensity was readily observed for the α chain of the IL-2 receptor in a high percentage of tumor cells. The β chain could also be detected, although with a weak staining and in a limited number of specimens. These results suggest that melanoma cells in vivo express high-affinity receptors for IL-2. The production of biologically active IL-2 by melanoma cell lines is currently assessed by testing culture supernatants on the IL-2 dependent murine CTLL-2 line.

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PORIN D2 AFFINITY OF QUINOLONES IN *PSEUDOMONAS AERUGINOSA*: STRUCTURE-ACTIVITY-RELATIONSHIP AND CONTRIBUTION ON ANTIBACTERIAL ACTIVITY

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The outer membrane porin D2 (D2) of *P. aeruginosa* catalizes facilitated diffusion of zwitterionic substrates like L-lysine, imipenem (I), and the quinolone (Q) sparfloxacin. Here we investigated apparent D2 affinity of various Qs (n=12) by assessing their inhibitory activity on [^{14}C] I labeling with a D2-producing *P. aeruginosa* strain. The potency of Qs to inhibit [^{14}C] I uptake correlated negatively with molecular weights of N1 and piperazine substituents in the Q molecule (r=-0.94). MICs of Qs for both a Q-resistant, D2-deficient strain (R) and its sensible parent (S) were assessed. MIC ratios (R/S) of Qs correlated with apparent D2 affinity (r=0.77). Inhibition of DNA synthesis by Qs in intact cells (IC) and EDTA-permeabilized cells (PC) of S was determined. Ratios of inhibition (IC/PC) by Qs correlated with apparent D2 affinity (r=0.86). So, drug design aiming at quinolones with improved activity against *P. aeruginosa* should pay special attention to N1 and piperazine substituents.

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CHARACTERIZATION OF 110 KD LAMININ-BINDING PROTEIN-LIKE IMMUNOREACTIVITY IN BRAIN.

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A 110 kD laminin-binding protein (110 kD LBP) isolated from newborn mouse brain extract, recognizes a neurite-outgrowth promoting 19-amino acid synthetic peptide (PA22-2) derived from the laminin A chain (Kleinman et al., *Arch. Biochem. Biophys.* **290**: 320, 1991). Antibody to 110 kD LBP strongly immunostains fibers and distinct cell populations in adult rat forebrain, in particular cortical pyramidal neurons with apical dendrites and hippocampal mossy fibers (Jucker et al., *Brain Res.* **555**: 305, 1991). Similar immunoreactivity was found in brains of adult mice and adult nonhuman primates. Electron microscopic analysis localized 110 kD LBP-like immunostain to the neuronal cytoskeleton. Dual immunolabelling demonstrated colocalization of intraneuronal laminin-like and 110 kD LBP-like immunoreactivity in cortical pyramidal neurons. Western blot analysis under reducing and nonreducing conditions revealed a 100/110 kD protein doublet and 140 kD protein depending on the fraction analyzed. Antibody fractions specific to the 110 kD and 140 kD immunoreactive protein species were cross-reactive on Western blots and revealed similar immunostaining in brain. In response to injury (transient ischemia, fimbria-fornix transection, and stab wound) 110 kD LBP-like molecules were also expressed in reactive glial cells. Since a putative laminin receptor is expressed in normal brain and in response to CNS injury, interactions of neurons and glial cells with laminin-like molecules are likely involved in normal brain function and repair.

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VINCULIN AND α-ACTININ IN PLASMA MEMBRANES ISOLATED FROM HUMAN NEUTROPHILS

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Our immunofluorescence studies have shown, that the putative actin-membrane linker protein vinculin and the actin-cross-linking protein α-actinin are concentrated in peripheral areas of spread neutrophils. We could demonstrate a stimulus-dependent interaction of α-actinin with the Triton X-100-insoluble cytoskeleton, whereas vinculin was not detectable in the cytoskeleton. We have now investigated possible membrane interactions of these two proteins. Subcellular fractions were isolated from human neutrophils by N_2 cavitation and Percoll density gradient centrifugation. The fractions were analyzed for the presence of vinculin and α-actinin by immunoblotting. Both proteins were clearly detectable in the plasma membrane fraction, but were not present in granule fractions. Preliminary data show that α-actinin was almost completely retained in the 200'000xg pellet of cell lysates, whereas vinculin was detectable both in the pellet and in the supernatant. We are now studying the effect of neutrophil activation by chemotactic stimuli on membrane association of these proteins.

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Pattern of Gelsolin Expression in Rat Epithelia and Fibroblastic Stromal Cells

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The expression of gelsolin in various rat cells and tissues was studied by means of immunofluorescence using antibodies against rat gelsolin. Gelsolin was found to be expressed differentially in fibroblasts from different tissues, supporting the concept of fibroblast heterogeneity. Immunostaining was negative in fibroblasts of subcutaneous tissue, present in tendons, in organ capsules (adrenal, lymph node, spleen, thymus, testis) and in the stroma of the uterus, ovary, and bladder, intense in hair follicle dermal sheats, in perineuria and in myotendinous junctions. In the thymus, positive stromal cells were abundant in the medulla and scattered in the cortex. In the spleen, stromal cells were highly stained in the red pulp, but poorly in the white pulp. Myoid cells of the testis and thecal stromal cells of the ovary were highly stained. Differences in gelsolin expression were significant in epithelia. Gelsolin was not detectable in intestinal epithelial cells, in bladder epithelium, in hepatocytes. In the stomach, it was present in mucous secretory cells, but not in parietal cells. In stratified epithelium of the esophagus, tongue, lip and skin, gelsolin was clearly expressed. Our results indicate that gelsolin is present only in certain organs or cells and that its expression can be modulated according to the cell location and activity. (Supported by the Swiss National Science Foundation, Grants Nr 31-30796.91 and 31-26614.89)

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DIFFERENTIATION-RELATED EXPRESSION OF CELL ADHESION MOLECULES BY NEUROBLASTOMA CELLS AND TUMORS

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The expression of cellular adhesion molecules (CAM) involved in cell adhesion and immune recognition was measured on an N-myc non-amplified neuroblastoma (NB) cell line, SK-N-SH and 3 phenotypically different variants, SH-SY, SH-EP and SH-IN, representing respectively, neuronal, glial/schwann and intermediate NB cell types. Flow cytometric analysis revealed a highly variable expression of HLA class I, LFA-3, CD54, CD56, CD2, VLA-2 and VLA-4 on the different variants. While CD56 expression appeared linked to the neuronal phenotype, expression of HLA class I, CD54, LFA-3 and VLA-2 was associated to the glial/schwann phenotype. Analysis of the expression of the same molecules on fresh tumor samples likewise revealed a co-expression of HLA class I, LFA-3, VLA-2 by low stages NB tumors with evidence of maturation, or by peripheral neuroepitheliomas (PN). CD56, however was uniformely and highly expressed by NB tumors of all stages, but only on 30% of PN. The CD56 or N-CAM molecule is characterized by several isoforms generated by alternate splicing of a single gene. Northern blot analysis of the N-CAM mRNA splicing pattern of NB cells, revealed mainly quantitative and no qualitative differences between the variants. In conclusion, expression of a majority of CAM analysed except CD56, appeared to be linked to differentiated cells or NB cells belonging to the schwann type.

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Phenotypic Modulation of Chondrocytes in Serum-Free Culture in Agarose Gels by Transforming Growth Factor-β

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Sternal chondrocytes derived from 17d chick embryo express their phenotype stably when cultured in agarose suspension. Biochemical markers of these cells are the cartilage collagens II, IX and XI. Upon anchorage-dependant culture, chondrocytes assume a fibroblast-like phenotype in a process called dedifferentiation, and synthesize type I collagen.

In serum-free culture in agarose gels, TGFβ causes cells to aquire a phenotype resembling chondrocytes dedifferentiated in monolayer culture. The factor arrests cartilage collagen synthesis and promotes a switch to type I collagen production. In addition, TGFβ inhibited the induction of terminal differentiation by serum, suggesting that TGFβ finely tunes the effects of other growth factors onto the cells.

In normal articular cartilage, chondrocyte hypertrophy is rare and takes place only near the subchondral bone. In degenerative diseases, articular chondrocytes undergo proliferation and hypertrophy, a possible consequence of an imbalance between TGFβ and other growth factors. Therefore degeneration, marked by synthesis of collagen I, may occur under the growing influence of TGFβ over factors promoting hypertrophy, whereas hypertrophy may be caused by a decreased control of TGFβ.

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Induction and Prevention by Growth Factors and Hormones of Chondrocyte-Hypertrophy in Serum-Free Suspension Culture

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Terminal differentiation of chondrocytes (hypertrophy) takes place during bone development, growth, and repair, and is prevented in joint cartilage. This process involves proliferation and subsequent hypertrophy of resting cartilage cells, is environmentally controlled, and can be mimicked *in vitro*. Biomechanical markers are cartilage collagens II, IX, and XI, expressed by chondrocytes at all stages of differentiation. Collagen X and alkaline phos-phatase are produced only by hypertrophic cells.

Terminal chondrocyte-differentiation is regulated by diffusible factors acting as agonists and/or antagonists. In addition, cells respond distinctly to signals depending on the stage they have reached in vivo. Serum strongly induces the terminal differentiation underscoring the need of serum-free culture conditions. Exogenously added IGF-1 induces proliferation and increased matrix production in resting cartilage cells. By contrast, IGF-1 stimulates hypertrophy in primed cells from proliferative cartilage. Autocrine/paracrine factors of resting cells inhibit this process.

As another example, hypertrophy is initiated without preceeding proliferation in a population of cells from resting and proliferative cartilage by thyroid hormones. In this case, hypertrophy cannot be prevented by factors derived from resting cells.

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ROLE OF EXTRACELLULAR MATRIX MOLECULES IN REGENERATION OF THE LEECH CNS.

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The aim of our experiments is to analyze how protein molecules in extracellular matrix of leech CNS induce regeneration of identified nerve cells. On a laminin-like molecule processes of Retzius cells are slender, straight and unbranched; on the plant lectin Con A they are thick, curved and branched. A different molecule tenascin, has now been purified by gradient centrifugation of high pH extracts of ECM surrounding leech CNS. On enriched tenascin fractions the pattern of neurite outgrowth resembles that on ConA. The laminin-like molecule appears in regeneration in vivo at new sites close to growing fibres. Microglial cells also accumulate at the lesion site. We are currently investigating whether these cells produce the laminin.

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HISTOCHEMICAL LOCALIZATION OF HYALURONAN IN MYELIN SHEATHS OF CENTRAL AND PERIPHERAL NERVOUS SYSTEMS

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Neural tissue of central (rat spinal cord) and peripheral origin (rat sciatic nerve, nerve fascicles of rat skin and iris and of human conjunctiva) was processed by osmium tetroxide/ microwave fixation and embedded in epoxy resin. Hyaluronan-binding proteins and link proteins coupled to 15-20 nm gold particles were used as markers in a one-step post-embedding procedure for identifying hyaluronan (hyaluronic acid) at the ultrastructural level. All myelin sheaths in both rat and human material were found to be intensely labelled. The specificity of the hyaluronan-binding probes was demonstrated by the total loss of labelling following treatment of sections with hyaluronidase or by preincubating either the probes with hyaluronan oligosaccharides or the sections with unlabelled hyaluronan-binding protein.

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MONOCLONAL ANTOBODIES AGAINST EXTRACELLULAR MATRIX MOLECULES OF THE LEECH CENTRAL NERVOUS SYSTEM

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To identify proteins involved in neurite outgrowth, monoclonal antibodies have been generated against an extracellular matrix fraction of the leech central nervous system. The leech was chosen, since so many properties of its CNS have been elucidated, including identification of specific cell-types, their electrophysiological properties, and their synaptic connections. Furthermore, a great deal is known about certain molecules of the extracellular matrix, such as laminin and tenascin, that play a role in neurite outgrowth and regeneration. Antibodies were produced against an extracellular matrix fraction known not to contain laminin or tenascin. Hybridomana supernatants were screened on immunoblots and cryosections of leech CNS. Five interesting antibodies have been selected and purified for further characterization: one is specific for muscle cells, one stains predominantly in the neuropil, two show specific staining for basement membrane, and one colocalizes with neuronal processes throughout the connectives and neuropil.

This work was supported by Boehringer Ingelheim Fonds

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BINDING AND KILLING PROPERTIES OF HUMAN γ/δ-TCR POSITIVE LYMPHOKINE-ACTIVATED KILLER (LAK) CELLS

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LAK cells, generated by cultivation of mononuclear cells in the presence of rIL-2, represent a heterogeneous cell population concerning the expression of surface molecules and many attempts were made to define the subgroup responsible for the killing activity. In binding studies, using K562 and Daudi cells as targets, γ/δ-TCR/CD3 positive LAK cells exhibited preferred adhesion affinity and the CD3 as well as the γ/δ-TCR molecules were found to shift towards the joining site with the tumor cells. Killing experiments, however, revealed reduced cytolytic activity of LAK cell fractions enriched with γ/δ-TCR positive cells in comparison to LAK cell bulk cultures. The killing activity could vigorously be enhanced by addition of anti-γ/δ or anti-CD3 mAb. These findings indicate that the γ/δ-TCR/CD3 complex may be involved in target cell binding and lysis.

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CONTROL OF BUD FORMATION IN *PHYSCOMITRELLA PATENS*

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We have chosen cytokinin induced bud formation in the moss *Physcomitrella patens* as an experimental system to investigate the possible role of extracellular proteins (ECPs) in tissue differentiation.
After induction of bud formation by cytokinin the overall amount of ECP increases and synthesis of a 13 kd ECP is initiated. A non bud producing mutant is deficient in a prominent 21 kd ECP that is also not detectable after inhibition of bud formation in the wt by tunicamycin.
These data suggest that ECPs are necessary for bud development in *Physcomitrella patens*.

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DEFINED OLIGOMER FOR DRUG THERAPY USING AN ANTIBODY-MEDIATED DELIVERY SYSTEM

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A new defined synthetic oligomer is described for conjugation to antibodies or their fragments for use in targetted drug therapy: $NH_2OCH_2CO\text{-}Gly_3\text{-}[Lys(Ser)]_5\text{-}Gly\text{-}OH$. Linkage of the oligomer to antibody or antibody fragment may be made through the aminooxy group attached to the amine terminus of the oligomer: this functional group reacts specifically with an aldehyde group placed on the antibody (or fragment) by chemical means in a prior step. Alternatively, use of an oligomer possessing a terminal aldehyde group permits site-specific attachment to the carboxyl termini of polypeptide chains which have been modified enzymically (see Bioconj. Chem. 2 1991 154-159). Once the oligomer is linked to protein, drugs may be attached through hydrazone bonds after mild oxidation of the Ser residues of the oligomer. Use of a defined oligomer leads to a relatively homogeneous product. Drug release is designed to be achieved through spontaneous decomposition of the hydrazone bonds after internalization in the target cell or external hydrolysis within its microenvironment. The preparation, properties and use of the defined oligomer will be described.

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PRENATALLY DIAZEPAM INDUCED IMMUNOSUPPRESSION: INVOLVEMENT OF CYTOKINES

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In this study we investigated the cytokine liberation in lipopolysaccharide (LPS) or Concanavalin A (ConA) stimulated rat spleen cells of prenatally diazepam treated and control male rats at the age of two, four and eight weeks. The cytokine tumour necrosis factor (TNF) -α has been shown to play an important role in immune responses. Its primary cell source are activated macrophages, LPS being one of the most potent activators. The TNF-α liberation was significantly lower in splenocytes of two and four week old treated than control animals, after 12 hours incubation. But at eight week of age prenatally diazepam treated animals showed a significantly higher LPS induced TNF-α production than control animals. We are currently investigating the liberation of IL-6 and IL-2. It appears that the prolonged suppressive effect of cellular immune responses seen in offspring of prenatally diazepam treated rats is possibly due to alterations in the cytokine network.

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CANCER PATIENT TREATMENT OPTIMIZED BY RAPID DETERMINATION OF TUMOR DOUBLING TIME USING Tpot.

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During radiotherapy, doses are given in small fractions to selectively reduce normal tissue damage. If the period between fractions is extensive, growth of cells can replace loss from the fractions and cause therapy failure. Here severe, accelerated therapy must be conducted, where the period between fractions is reduced to prevent such repopulation. The clinician must be aware of the tumor doubling time when diagnosing the therapy. Doubling times of tumors can be determined by analysis of their DNA synthesis. This is achieved by a single injection of the thymidine analogue: BUdR, four hours prior to taking a tumor sample. The sample is then stained with fluorescently labelled antibodies against the DNA-incorporated BUdR, stained with propidium iodide to measure total DNA content, and analyzed by flow cytometry. The method called T_{pot} produces doubling time estimates within hours of biopsy.

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TUMOR NECROSIS FACTOR RECEPTOR DISTRIBUTION IN HUMAN LYMPHOID TISSUE

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The nature and location of cells responding to TNFα were investigated in situ by immunohistochemistry using monoclonal antibodies (mAbs) directed against the p75 and p55 proteins of the TNF receptor. Receptor expression was found in the thymus and secondary lymphoid tissues. In the thymus the p75 receptor was confined to medullary lymphoblasts and dendritic cells which costain with the Tac protein of the IL-2 receptor. In lymph nodes and other secondary lymphoid tissues, the p75 receptor was expressed on activated lymphocytes and interdigitating reticulum cells of the T-cell area, whereas the p55 receptor was confined to the germinal center dendritic reticulum cells (DRC), which are the main site of TNFα production. TNF receptor proteins were upregulated in reactive hyperplasia together with increased TNFα expression. Surprisingly, no TNFR was detectable on non-lymphoid tissues. The data presented suggest that TNFα, which is produced by germinal center DRCs, might regulate an in vivo immune response through autocrine and paracrine pathways e.g. through the p55 and p75 receptor proteins, which are expressed at different sites of the lymphoid tissue.

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Interleukin-4 can substitute for prolactin and induce beta-casein transcription in mammary epithelial cells.

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Transcription of the beta-casein gene is induced by the lactogenic hormone prolactin, whose receptor belongs to the family of growth factor/cytokine receptors for growth hormone, erythropoietin, interleukins 2,3,4,5,6,7, granulocyte and granulocyte-macrophage colony stimulating factors. Of these, IL-4 was also able to induce beta-casein transcription in the mouse mammary epithelial cell line, HC11. The receptors for IL-4 and prolactin may use the same signal transduction mechanism but they have no sequence homology in their cytoplasmic domains. Both prolactin and IL-4 act synergistically with glucocorticoids and insulin, but not with other interleukins. They are inhibited by gamma-interferon or epidermal growth factor and IL-4 is also inhibited by the monoclonal anti-IL4 antibody, 11-B-11. Nucleotides -184 to -80 was the minimal region of the beta-casein promoter conferring prolactin responsiveness. Mutation of the binding site (-100 to -80) of the mammary gland specific transcription factor (MGF) abolished the response to both hormones but a 5' deletion to -170 did not affect the transcriptional induction by IL-4. IL-4 may act through different or a reduced set of transcription factors.

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INACTIVATION OF THE MURINE CYTOKINE GENE *IL4*

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The cytokine IL4 is produced by T helper cells and exerts a variety of functions including the induction of growth and differentiation of B and T cells. We used a genetic approach to analyze the function of this cytokine in more detail and introduced a null mutation in the *IL4* gene by homologous recombination in the embryonic stem cell line D3M. Homozygous mutant mice were obtained by intercrossing heterozygous offspring and analyzed for the effect of IL4 deficiency on the immune system. B and T cell development seems to be normal in IL4 deficient mice with respect to the total number of lymphocytes and the expression of several surface molecules including CD4, CD8, MHC class II, IL2R, IL4R, Pgp-1 and HSA. Isotype switch during B cell differentiation, however, was severely impaired in IL4 deficient mice. After challenging the mice with the parasite Nippostrongylus brasiliensis as well as with anti-δ antibodies, the serum level for IgG1 remained 50 times lower in the mutants as compared to control littermates and no IgE could be detected. These results indicate that IL-4 not only strictly controls the isotype switch to IgE, but also strongly influences the class switch to IgG1.

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COMPETITION BETWEEN LPS-BINDING PROTEIN (LBP) AND ANTI-LPS ANTIBODY IN LPS-INDUCED TNF SECRETION OF HUMAN MONOCYTES (Mo). P. Gallay[1], D. Heumann[1], C. Barras[1], R.J. Ulevitch[2], P.S. Tobias[2], M.P. Glauser[1] and J.D. Baumgartner[1]. [1]Div. Infectious Diseases, CHUV, Lausanne; [2]Dpt. Immunology, Scripps Clinics, San Diego.

It has been shown that anti-LPS antibodies (Ab) increase LPS clearance by the reticulo-endothelial system, including Mo, and suppress TNF secretion in animals challenged with LPS. In vitro, LPS reacts with LBP contained in serum, inducing a CD14-mediated TNF secretion by Mo. To determine the mechanism by which anti-LPS Ab suppresses TNF production in vivo, we studied in vitro the respective effects of anti-LPS Ab and of LBP 1) on the binding of fluorescent (FITC) O111 LPS on Mo using FACS analysis and 2) on TNF production. We found that the addition of LBP-containing serum to cultures of Mo induced a strong binding of LPS to Mo and induced TNF secretion, both of which could be inhibited by an anti-CD14 mAb (MY4). In contrast, when an anti-O111 LPS mAb (D6B3) was added to serum, most of the LPS binding could no longer be inhibited by MY4, suggesting that binding occured to receptors other than CD14. The binding pattern was different in the presence or in the absence of complement, suggesting a binding of LPS-anti-LPS immune complexes through Fc or complement receptors. When the effect of anti-LPS Ab on TNF secretion by Mo in the presence of LBP-containing serum was measured, we found that D6B3 totally prevented TNF secretion induced by 0.1 ng/ml of LPS, but only partially by higher concentrations. The TNF secretion occuring despite D6B3 was due to a triggering of CD14 since it could be suppressed by MY4. In conclusion, anti-LPS Ab mediate the binding of LPS to Fc or complement receptors, thus competing with the binding of LPS to CD14, and therefore decreasing TNF production by Mo. LPS-mediated TNF production was suppressed at LPS concentrations that are likely to occur in vivo.

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EXPRESSION OF TUMOR NECROSIS FACTOR-ALPHA (TNF-α) AND TNF-α RECEPTOR mRNAs IN HUMAN MONOCYTES AND MACROPHAGES

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Tumor necrosis factor α (TNF-α) is a potent inflammatory mediator. Two distinct TNF-α receptors of 55 kDa and 75 kDa have been cloned. We have used a well-defined culture system which allows terminal in vitro maturation of human blood monocytes into macrophages. The expression of TNF-α and its receptor mRNAs has been investigated by Northern blotting. A gradual decrease of both TNF-α and TNF-α receptor mRNA levels was observed during in vitro maturation of monocytes into macrophages. Treatment of monocytes and macrophages with dibutyryl cyclic AMP (1 mM) induced a marked increase in TNF-α receptor mRNA in a time-dependent manner. By contrast, TNF-α mRNA gradually decreased under the same conditions. In conclusion, our results provide a better understanding of the regulation of the TNF-α-system in human monocytes/macrophages.

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CHANGED OF HEPATIC HANDLING OF EGF IN AGED RATS

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Epidermal growth factor (EGF).is taken up by the liver through receptor-mediated endocytosis; most is degraded, but a small percentage is secreted intact into bile. In the aged liver induction of DNA synthesis by EGF is impaired. Changes in the hepatic handling of EGF may be important for these effects. We compared EGF handling in livers from young (5-10 week) and old (19-21 month) male SD rats. Plasma membranes from old animals showed a reduced EGF binding capacity (0.5±0.1 vs 1.3±0.31 pmol/mg). We studied intracellular handling by portal injection of ^{125}I-EGF and collection of 10 min bile samples for 60 min. In old animals, the peak for secretion of total radioactivity and intact EGF was shifted. The total secreted radioactivity in percentage of the injected dose over 60 min was reduced in the old animals (10.0±2.3% vs 16.5±5.7%). Expressed per gram of liver and µl of bile was the reduction even more pronounced (0.0002% vs 0.00094%) The percentage of immunological intact EGF over 60 min was also reduced in (0.76±0.2% vs. 1.49±0.51%) and again more pronounced per gram of liver and µl of bile (0.000016% vs. 0.000093%). This reduced ability for EGF handling linkt to the impaired EGF stimulation of DNA synthesis reported for aged hepatocytes in culture supports the idea of intracellular processing of EGF for signal transduction.

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EFFECTS OF FLUOROPYRIMIDINES AND INTERFERON-αA/D, IN COMBINATION OR NOT, ON HUMAN TUMOR XENOGRAFTS IN NUDE MICE

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Interferons exhibit inhibitory effects on a variety of tumor cells both *in vitro* and *in vivo*. A synergistic effect between IFN-α2a and 5'-deoxy-5-fluorouridine (5'-dFUrd) has been previously observed *in vitro*, using both liquid proliferation and clonogenic assays (Experientia 47: A61, 1991). A murine *in vivo* model has now been developed to determine the antitumor activity of IFN-αA/D, alone or in combination with fluoropyrimidines. The synergestic effect observed *in vitro* between IFN-αA/D and 5'-dFUrd has been confirmed with this *in vivo* model. IFN-αA/D used at doses ranging from 75,000 to 150,000 IU/mouse/day exerts a cancerostatic dose-dependent effect on WiDr colorectal xenografts in nude mice. Further results gave orignal evidence in favor of an enhanced activity of 5'-dFUrd by IFN-αA/D on the same cell line.

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THE HUMAN INTERFERON-INDUCED MxA PROTEIN: PROTEIN-PROTEIN INTERACTIONS AND INTRINSIC GTPase ACTIVITY.

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MxA is an abundant and ubiquitous protein induced by interferon-α in human cells. Upon full induction it can constitute 0.5-1% of cytosolic proteins. MxA can bind elements of the cytoskeleton such as actin and tubulins, and several larger cellular proteins. However these protein-protein interactions seem to be transitory.
A GTPase activity (GTP→GDP) is associated with native MxA protein purified by immunoprecipitation using affinity purified polyclonal antibodies directed against the C-terminal domain of MxA. The reaction is specific for GTP, and the approximate K_m is 0.2mM. The reaction has an absolute requirement for Mg^{2+}. The activity is abolished by polyclonal antibodies directed against the N-terminal domain of MxA (the domain which contains the GTP binding consensus elements). The turnover number is approximately 70 molecules of GTP hydrolyzed per min per Mx molecule. It will be shown that the human MxA protein has certain characteristics of the "stress proteins" (chaperones).

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EFFECT OF IMMUNOSUPRESSIVE DRUGS ON IL-2-SPECIFIC TRANSCRIPTION FACTORS

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We have functionally tested factors regulating IL-2 expression in the *Xenopus* oocyte system. A silencer present in primary resting T-cells represses ongoing basal transcription. Addition of proteins from induced cells fully reactivate the IL-2 promoter. CsA and FK506 have no effect if directly added to the injected genes or proteins. By contrast, when the donor T-cells are stimulated in the presence of drug, their extracts fail to derepress previously silenced genes and act as irreversible inhibitors. By injecting proteins from either resting, activated or drug-treated cells into the cytoplasm or nucleus of the host oocyte we found that drug treatment not only interferes with nuclear targeting of positive TF but, in addition, renders the silencer resistant to displacement by normal positive TF, even if the latter is added directly to the nucleus.

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A SILENCER OF INTERLEUKIN-2 GENE TRANSCRIPTION IN DIFFERENT HUMAN T-LYMPHOCYTE POPULATIONS

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Recent studies on the mouse IL-2 gene regulation have revealed a repressor (silencer) in resting T-lymphocytes (Mouzaki et al.,1991, EMBO J., 10:1399).
The current studies on the transcriptional regulation of the human IL-2 gene concern T-cell subsets, T-cell ontogeny, kinetics of cell activation and autoimmune diease. They revealed 1) that the repressor is mostly confined in CD4+ T cells and 2) strongly expressed in cord blood (naive) T-cells; 3) the repressor is replaced from its DNA-binding element within 20 min. of cell activation by a positive transcription factor which persists for at least 11 days. Finally, 5) in severe (untreated, 5 cases) systemic lupus erythematosous a repressor-like protein is present in peripheral blood T-cells but (in an Xenopus oocyte functional assay) exerts no detectable IL-2 repressor activity.

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GLIOBLASTOMA SECRETE MCP-1, A MONOCYTE CHEMOTACTIC FACTOR.

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Immunohistological studies on glioblastoma tumor sections have demonstrated the presence of variable amounts of infiltrating macrophages. To understand how these macrophages are recruited from the circulation through the blood brain barrier, we have tested for the presence of macrophage chemotactic factors such as MCP-1. MCP-1 is a human monocyte chemoattractant protein produced by several cell types such as fibroblasts, PMA stimulated PBMC and human glioma cell-line U-105MG. We found various constitutive levels of different specific MCP-1 mRNAs in 10/13 cell lines.The inflammatory cytokines IL-1b and TNFa could induce or increase the MCP-1 mRNA in all cell lines analyzed.To demonstrate in vivo MCP-1 production we showed that ex vivo extracted tumors contained MCP-1 mRNA, both in low and high grade astrocytomas. We are currently analyzing the cell line supernatants, CSF and cyst fluids of patients to check for the presence of soluble MCP-1 protein. These data will further be correlated with immunostaining on frozen sections to identify the MCP-1 producing cells.

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Purification and characterisation of FDGF, a potentially novel growth factor

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A fibroblast-derived growth factor (FDGF) was isolated from serum-free conditioned medium of growth-arrested primary cultures of chicken embryo fibroblasts. The mitogenic activity which was not suppressible by an antibody neutralizing the activity of TGF-β 1, 2 and 3 was purified to homogeneity more than 10,000-fold by isoelectric focusing, gel filtration, reversed-phase HPLC and nonreducing SDS-PAGE. FDGF is a 32-kDa disulfide-linked heterodimer of a 15- and a 17-kDa subunit with a pI of ~ 7. It is active only in its dimeric form and half-maximum stimulation was attained at ~ 5 pM. FDGF is trypsin-sensitive and exhibits partial stability towards heat-treatment. The biological, chemical and physical characteristics of FDGF suggest that it is not identical with any of the well known growth factors like IGF, FGF, PDGF, EGF or TGF-β but rather represents a novel type of growth factor.

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INTERLEUKIN-1α ENHANCES GSH TURN-OVER AND SUPEROXID DISMUTASE ACTIVITY IN CULTURED HUMAN DERMAL FIBROBLASTS: POSSIBLE PROTECTION AGAINST OXYGEN DERIVED FREE RADICALS ?

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Free oxygen radicals and their toxic intermediates may react with cellular components and subsequently damage essential molecules. This mechanism is involved in the pathophysiology of various diseases. Cytokines modulate certain immunological and inflammatory actions and may enhance the generation of free radicals in macrophages, leukocytes and other cells being in direct contact with oxygen. Human cells are protected to a certain extent by antioxidant defense systems against oxidative stress. (Glutathion (GSH), GSH-metabolizing enzymes and superoxid dismutases (SOD)). Effects of 300 U/ml Interleukin-1α (IL-1α) on cultured human dermal fibroblasts were studied in presence of 20% and 95% oxygen, respectively during 48 hours. Reduced GSH, lipid peroxides, lactat-dehydrogenase (LDH) for cell damage, Cu, Zn-SOD, GSH peroxidase, GSH reductase and GSH-S-transferase were measured. Our results showed an increase in cellular activity of Cu, Zn-SOD of about 18 to 35%, and an increase in the total activity of NADH-dependent LDH in the range of 26 and 66%. A 20% decrease in the activity of GSH peroxidase but no effects of IL-1α on GSH reductase and GSH-S-transferase activities were found. GSH levels were reduced by 10% and 10 to 20% lower levels of lipid peroxides were noted. At 95% oxygen an amplification of some of these effects could be demonstrated. IL-1α may temporarly protect fibroblasts from oxidative stress by increasing SOD activity and enhancing GSH turn-over. After 72h at 95% O2 the presence of IL-1α results in severe cell damage. The production of superoxide radicals by activating a NADPH-oxidase may lead to a loss of NADPH nessesary for GSH reduction and to an inhibition of GSH peroxidase.

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α-MSH RECEPTORS AND THE RESPONSE OF HUMAN MELANOMA CELLS TO INTERLEUKIN-1

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The peptide α-MSH has been shown to antagonize some of the effects of IL-1. Human melanoma cells of the A375 cell line carry receptors both for cytokines and α-MSH and represent a model for studying the interaction of these agents. Two strains of A375 were selected which differed in their sensitivity to the cytolytic effects of IL-1. Both strains carried one class of high affinity IL-1 receptors with a K_D of 0.2 nM. The receptor number was low on the IL-1 sensitive strain (200/cell) but increased 10-fold on loss of sensitivity. In resistant cells, these receptors could be further increased 2-4 fold by incubation with glucocorticoids and down-regulated by TNF-α. In contrast, α-MSH receptors were associated with loss of sensitivity. The α-MSH receptors, found only in the resistant strain, were not affected by either IL-1, TNF-α or dexamethasone. These results suggest that sensitivity to the cytolytic effects of IL-1 in A375 is associated with the absence of α-MSH receptors and could be an indication for an altered response to cytokines.

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Sequence Analysis of N-ras and p53 Genes in two Groups of AML Patients Characterized by Presence or Absence of Activating N-ras Mutations

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N-ras oncogenes are activated by point mutations of codons 12, 13 and 61 in 20-25% of acute myeloid leukemias and myelodysplastic syndromes. Studies were performed to address the question whether the N-ras oncogene could serve as a tumor marker during the course of disease. However, in a number of cases point mutations present at initial diagnosis were not detectable in later relapses. This leads to the prediction that N-ras mutations are not likely to play a central role in leukemia. Consequently, the N-ras mutation could just be indicative for a generally decreased genetic stability in the leukemia progenitor cell(s) and additional "atypical" mutations would be expected to occur as recently reported by Rovera et al. (Oncogene 4:867-72, 1989). A study was undertaken to determine the overall mutation frequency of N-ras and p53 genes in AML and MDS patients by direct PCR sequencing of the coding exons. Using gene amplification and oligotyping techniques 10 patients exhibiting an activated N-ras oncogene were found by screening 51 leukemia patients. Among them, 6 codon 12, 2 codon 13 and 2 codon 61 N-ras mutations were identified. Two groups, consisting of 10 individuals each, either with or without activating N-ras mutations are under investigation for atypical N-ras mutations and p53 gene alterations. Whereas the N-ras genes seem to display no further detectable mutations, genetic alterations are identified in the p53 tumor suppressor genes.

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SEQUENCE OF TUMOR SUPPRESSOR GENES IN THE NORMAL AND NEOPLASTIC TISSUES OF A PATIENT WITH TURCOT'S SYNDROME.

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Turcot's syndrome is characterized by the association of polyposis coli and primary neuroepithelial tumors of the central nervous system, particularly glioblastoma. We analyzed tumor suppressor genes, especially p53 and APC, in the normal and neoplastid tissues of a patient with Turcot's syndrome. This patient underwent three operations (glioblastoma in 1976, colon adenocarcinomas in 1981 and 1990). Using the PCR, we have amplified and sequenced the highly conserved regions of the p53 gene in these neoplastic tissues and patient's lymphocytes.We detected three different alleles in both colon cancer specimens;one wild type, one mutated at codon 235, and one with 3 mutations(codon 173, 228, and in the splice acceptor site of intron 6). These mutations could not be found in the DNA of lymphocytes and glioblastoma specimen. We are currently studying whether the patient carries germ line APC gene mutations.

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MOLECULAR CLONING AND FUNCTIONAL CHARACTERIZATION OF A PUTATIVE RAT TUMOR SUPPRESSOR

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We have obtained revertants from H-ras transformed rat FE-8 cells by transfection of normal human placental DNA. The revertants were anchorage-dependent. Their tumorigenicity in nude mice was reduced. Human DNA sequences (designated NTS-1) capable of conferring the reverted phenotype on FE-8 cells were moleculary cloned. (R. Schäfer et al,(1988) PNAS 85, 1590-1594)
We have now isolated homologous rat DNA sequences. Sequence comparison of human and rat NTS-1 DNA revealed a highly conserved 96 bp element. Transfection of genomic rat DNA harboring this element suppressed the anchorage-independent phenotype of FE-8 cells. Mobility shift assays have shown the specific binding of nuclear protein to the NTS-1 element suggesting that it may act as a regulatory element.

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T(14;18) TRANSLOCATION IN SWISS LYMPHOMA PATIENTS

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Bone marrow and peripheral blood were collected from 98 patients with non-Hodgkin's lymphoma (NHL) in order to study the frequency of the t(14;18) translocation in Swiss NHL patients. We developed a sensitive PCR assay for testing patient DNA which used a liquid hybridization step to enhance its sensitivity. The target translocation sequence coded for a 233 BP fragment of the major breakpoint region. We found the assay highly specific and 42% of the DNA samples from our patients series tested positive for the t(14;18) translocation. We also found the translocation within the DNA from 2 of 3 (66%) patients with a rare subtype of lymphoma called mucosa-associated lymphoid tissue (MALT) syndrome. Follow up studies in 20 NHL patients have been initiated and in some patients a close correlation between positivity and clinical course was found. In other patients the correlation was less clear.

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DEFICIENCY IN DEXAMETHASONE-MEDIATED ACCUMULATION OF αB-CRYSTALLIN AND CONCOMITANT ACQUISITION OF THERMOTOLERANCE IS A ras-TRANSFORMATION-SPECIFIC PHENOTYPE IN MOUSE NIH 3T3 FIBROBLASTS.

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αB crystallin is a small heat shock protein. It also accumulates in dexamethasone-treated NIH 3T3 fibroblasts. The forced accumulation of αB crystallin, mediated either by a transfected expression vector or in response to prolonged dexamethasone treatment led to the acquisition of thermotolerance. Sustained expression of a Ha-ras oncogene suppresses dexamethasone-mediated accumulation of αB crystallin and concomitantly the establishment of thermotolerance. Stable transfection assays with αB crystallin promoter-CAT reporter gene constructs indicate that the observed alterations in the αB crystallin expression pattern are regulated at the transcriptional level.

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MACROPHAGE COLONY-STIMULATING FACTOR (M-CSF) RESTORES BONE RESORPTION (BR) IN METATARSALS OF OSTEOPETROTIC (OP/OP) MICE IN VITRO

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Metatarsal from newborn op/op mice was cultured to investigate whether M-CSF restored osteoclastogenesis in op/op bone which is characterized by a lack of osteoclasts. BR was assessed by measuring the release of ^{45}Ca from prelabeled bone and by counting the number of tartrate-resistant acid phosphatase positive multinuclear cells. M-CSF, interleukin-6, 1,25(OH)2D3, parathyroid hormone (PTH) and tumor necrosis factor ∝ given alone did not stimulate BR. However, M-CSF in combination with 1,25(OH)2D3 or PTH enhanced BR, but had no effect with the other factors. Granulocyte-macrophage colony-stimulating factor (GM-CSF) instead of M-CSF was inactive. These results indicate that M-CSF plays an important role in osteoclastogenesis rather than GM-CSF and that, at least in vitro, the combination of M-CSF with PTH or 1,25(OH)2D3 is necessary.

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A NOVEL AU-BINDING PROTEIN POSSIBLY INVOLVED IN LYMPHOKINE mRNA TURNOVER

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IL-3 mRNA in PB-3c mast cells is extremely unstable, whereas expression of IL-3 in autocrine tumor counterparts is constitutive through an unknown mechanism stabilizing the mRNA (See poster, Hirsch *et al.*) The conserved AU-rich sequence in the 3' untranslated region of various lymphokine mRNA is known to regulate mRNA turnover. In fact,removal of the AU-boxes from the IL-3 gene leads to high constitutive levels of its mRNA in PB-3c cells (See poster, Hahn and Moroni). We have purified AU binding proteins from human brain using a synthetic AU rich RNA affinity column. The RNA binding activity was demonstrated by mobility shift and North-Western blot assays. Furthermore we have obtained partial amino acid sequence of two proteins, one found to be La, a known RNA binding protein, and the other, an unknown protein. A preliminary characterization and how proteins regulating mRNA decay may be involved in leukemogenesis will be discussed.

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POSTTRANSCRIPTIONAL STABILIZATION OF INTERLEUKIN-3 mRNA AS ONCOGENIC PRINCIPLE IN v-H-*ras* INDUCED AUTOCRINE MAST CELL TUMORS

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PB-3c is a non-tumorigenic, IL-3 dependent mast cell line from murine bone marrow. Expression of v-H-*ras* oncogene allows the generation of two types of IL-3 autocrine mast cell tumors *in vivo*. Class-1 type tumors show no polymorphisms at the IL-3 gene locus. Class-2 type tumors contain an insertion upstream to the IL-3 gene reminiscent of endogeneous retroviruses (IAP). Upon somatic cell fusion with the parental PB-3c, IL-3 expression was downregulated only in class-1 hybrids, resulting in IL-3 dependence *in vitro*, and suppression of tumor formation *in vivo*. IL-3 mRNA shows an extended stability in class-1 tumors, not seen in the class-2 tumors or in premalignant cells. Downregulation of the oncogenically expressed IL-3 mRNA in class-1 hybrids correlated with reacquired instability of the lymphokine mRNA. Thus, class-1 mast cell tumor formation may result from the loss of a tumor suppressor present in premalignant cells acting posttranscriptionally by destabilizing IL-3 mRNA.

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Molecular dissection of the pathways and mechanisms regulating IL-3 expression in PB-3c mastocytes.

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IL-3 gene expression is regulated by a calcium dependent post-transcriptional mechanism in mast cells. Treatment with calcium ionophores such as A23187 or ionomycin, leads to an accumulation of IL-3 mRNA by stabilizing the normally labile lymphokine mRNA. This calcium-mediated mRNA accumulation can be enhanced by the expression of activated *ras* oncogenes.
In our current examination we have focussed on other pathways influencing IL-3 gene expression in PB-3c mastocytes. We report that both cAMP and PKC responsive pathways modulate the extent of lymphokine mRNA induction by ionomycin.
We have furthermore undertaken a study to determine the calcium- and *ras*-responsive elements of the IL-3 gene using a series of deletion mutants and hybrid constructs. Our initial findings indicate that the AUUUA boxes located in the 3' UTR of the IL-3 gene are important destabilatory motifs; it is however not clear whether they are the targets for mediating calcium dependent mRNA stabilization.

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EVIDENCE FOR ENHANCED TRANSFORMATION RATE IN AN INHOMOGENEOUS RADIATION FIELD

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An extremely inhomogeneous radiation field was applied to a cell monolayer of C3H 10T1/2 fibroblasts, which are susceptible to transformation, as a model of hot particle irradiation. Thus, cells receiving a supralethal dose lay proximal to cells receiving only a sublethal dose and communication between these cells was possible. Cells, in the dose region with about 90 % survival, were isolated and grown in transformation assays. Derived transformation rates were compared to those of cells growing at the same dose levels, but prevented from communication with cells of other regions, i.e. cells receiving lethal doses, by isolation in an agarose trench. First results indicate that cell-cell communication in an inhomogeneous radiation field may enhance the transformation rate by a factor of about ten. Possible causes being investigated are growth stimulation by cell loss or the release of carcinogenic substances.

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POLYSIALIC ACID (PSA) ON N-CAM MAY MODULATE CELL-CELL INTERACTIONS OF HUMAN SMALL CELL LUNG CARCINOMA CELLS

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We have investigated the adhesive properties of small cell lung carcinoma cells using 2 cell lines with or without PSA cell surface expression. Both cell lines grow as clumps in suspension. PSA (a homopolymer of 8 or more α 2,8 linked sialic acids) was detected by immunohistochemistry using the monoclonal antibody Mab 735 which specifically recognises PSA. Endoneuramidase N (endo N) specifically cleaves only PSA leaving 5 or less α 2,8 sialic acid residues. Two assays were used. First, aggregation of a single cell suspension was assessed by quantifying the percentage of cells in clumps after 20 minutes under standard conditions using a microscope cell chamber. Cells with PSA showed reduced clumping compared to PSA negative cells. Secondly, a standard dissaggregation procedure was used which entailed 2 passages through a 21 gauge needle. The percentage of cells in clumps was assessed by counting the number of dissociated cells in relation to the total. Removal of PSA using endo N from PSA positive cells increases the resistance to dissaggregtion. The sensitiviy of these effects to $[Ca^{2+}]$ is being assessed using EDTA treatment.

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EXPRESSION OF M-CSF RECEPTOR (*c-fms*) AND F4/80 ANTIGEN IN +/? AND op/op MICE. W.Hofstetter, A.Wetterwald, R.Felix, H.Fleisch, M.G.Cecchini. Dept.of Pathophysiology, Univ. of Berne, Berne

The mononuclear phagocyte system (MPS) comprises a heterogeneous cell population, which depends on the CSF's produced at distinct tissue sites. The *op/op* murine osteopetrosis is defficient in M-CSF and thus is a model to investigate how the absence of M-CSF affects the MPS. Based on this, we studied the expression of the MΦ marker F4/80 and the localization of *c-fms* mRNA in normal *+/?* and osteopetrotic *op/op* 2d old littermates. In bones from *+/?* animals, *c-fms* mRNA was detected in cells corresponding, according to their size and location, to osteoclasts. F4/80 Ag is absent in OC, but highly expressed in marrow stromal and periosteal MΦ. In skin, a scattered cell population in the dermis expresses *c-fms* mRNA, as do cells in the epidermis. This is also the case for F4/80 Ag. In contrast, bones of *op/op* animals are negative for *c-fms* message. F4/80 Ag$^+$ stromal and periosteal MΦ are absent. In dermis, both *c-fms* mRNA and F4/80 Ag are not detectable, while in the epidermis both markers are expressed. In conclusion, (i) it was shown that in bones from *+/?* animals, *c-fms* is expressed at high levels only by osteoclasts. (ii) *c-fms* mRNA as well as F4/80 Ag are expressed in the dermis of *+/?* animals, but not of *op/op* littermates. (iii) In the epidermis both markers are also expressed in the op/op mutant. These findings support the hypothesis that MΦ at different anatomical sites are differently dependent on M-CSF.

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IN VIVO PHOSPHORYLATION OF B-TYPE LAMINS IN NORMAL AND NEOPLASTIC HUMAN LYMPHOID CELLS

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The nuclear lamina underlies the inner nuclear membrane and is important for the structural and functional organisation of the nucleus. In mammals it consists of two lamin-types: A-type lamins may be involved in the interaction between the lamina and chromatin and B-type lamins probably in linking the lamina to the inner nuclear membrane. Its dynamic structure is controlled by phosphorylation: Several kinases ($p34^{cdc2}$, PKC, PKA) and phosphatases (1, 2A) are believed to maintain M-phase and I-phase states. We have found, that in contrast to normal lymphocytes, neoplastic lymphoblasts show an altered morphology (increased nuclear/cytoplamic ratio), and differential expression of Lamin B_1 mRNA. We therefore suggest that B-type lamin phosphorylation of neoplastic cells could be altered compared to normal lymphocytes. By *in vivo* ^{32}P-labelling, immunoprecipitation and two dimensional tryptic peptide mapping, we have determined the phosphorylation pattern of B-type lamins in different human B-cell lines and and peripheral blood lymphocytes. Using cell cycle synchronizing agents and PMA treatment, we have identified phosphorylated tryptic peptides of M-phase, I-phase and PKC-dependent events. Initial experiments with bone marrow cells suggest differences in their phosphorylation pattern not previously observed.

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EXPRESSION OF RECEPTORS FOR NEUROTROPHINS IN NERVOUS AND IMMUNE SYSTEMS

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Nerve growth factor (NGF) is the best characterized protein in a family of related neurotrophic factors (neurotrophins) including brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3). The actions of these neurotrophins are mediated by the interactions with specific membrane receptors on target cells. Recent findings indicate that the high affinity NGF receptor is associated with the tyrosine kinase encoded by the trk proto-oncogene, whereas the trk B protein kinase is a component of the BDNF receptor. We investigated the developmental expression of trk and trk B in rat peripheral sympathetic and sensory ganglia as well as in thymus and spleen by reverse transcription combined with polymerase chain reaction. Our results show that NGF- and BDNF-receptors are developmentally regulated in a tissue-specific manner. In addition, trk expression was found to be induced on immune cells such as cyclic AMP-stimulated human monocytes. Analysis of the expression of trk or related receptors in nerve and immune cells should help define the biological role of neurotrophins in neuronal development and regeneration and in immunomodulation.

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Auxiliary proteins of DNA polymerases δ and ε

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Replication factor C (RF-C) originally was identified as a protein essential for *in vitro* replication of SV40 DNA. We have isolated RF-C from calf thymus nuclei by several chromatographic steps. Its activity was monitored by stimulation of DNA polymerase δ (pol δ). RF-C and the two other auxiliary proteins proliferating cell nuclear antigen and replication factor A stimulated pol δ on DNA-primed single-stranded DNA in the presence of ATP. Omission of any factor led to near inability of pol δ to utilize such a template. The activity of DNA polymerase ε (pol ε) on this single stranded DNA was not affected by those factors. However, in the presence of all three factors and ATP pol ε became much less sensitive to inhibition by salt. Detailed analysis of the auxiliary proteins of these two replicative DNA polymerases may clear the actual role of different DNA polymerases at eukaryotic replication fork.

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ORGAN-SPECIFIC METASTASIS: THE ROLE OF PARACRINE GROWTH STIMULATION.

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The B16-LS9 murine melanoma cell subline was selected for liver specificity by repeated in vivo passages through the liver. These cells colonize the liver more efficiently than the parental B16-F1 or the lung-specific B16-F10. We found that the adhesion of LS9 cells to hepatocytes or to liver-derived extracellular matrix was not different from that of F10 cells. Accordingly, the organ distribution pattern in vivo after tail vein inoculation was not different for the two sublines. However, LS9 cells grow slower than F10 cells either in vitro or in vivo after subcutaneous or intramuscular injection. Hepatocytes, or their membranes, can restore an efficient growth of LS9 cells, at least in vitro. Here we present data suggesting that LS9 cells have a decreased growth autonomy with respect to F10 cells, and therefore they may only succeed in colonizing those organs that provide them with an efficient growth stimulation, such as the liver.

Protein-Protein and Protein-Nucleic Acid Recognition

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STRUCTURE AND NOMENCLATURE OF ISOMETALLOTHIONEINS

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Metallothioneins (MTs) are small molecular-weight cysteine-rich proteins which form strong metal-thiolate clusters with transition metals such as zinc, copper and cadmium. In mammals, MT is encoded by a gene family of up to ten functional genes. In a particular organ or cell-line at least two isoMTs differing in their primary structure are expressed. By anion exchange chromatography they are generally separated into two main protein fractions, designated as MT-1 and MT-2, respectively. The difference in charge is the basis for the nomenclature of isoMTs and it is usually attributable to the replacement of a glycine residue in position 10 or 11 of the MT-1 sequence by an aspartic acid residue in the MT-2 sequence (Kägi J. H. R. and Kojima Y. (1987) in: "Metallothionein II", Kägi J. H. R. and Kojima Y., eds., Birkhäuser Verlag, Basel, p. 37).
In this report we document by sequence analysis that in rabbit kidney cells (RK-13) and in human liver the charge-separable MT-1 and MT-2 fractions contain isoforms which structurally must be classified as belonging to the opposite structural form. We propose that the nomenclature of isoMTs irrespective of the net charge be based on the above mentioned primary structure criterion.

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STRUCTURAL STUDIES OF HUMAN INTERLEUKIN-5

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We have produced large quantities of biologically active hIL-5 in *E. Coli* enabling initiation of structural studies. The protein is expressed in-inclusion bodies, and on refolding, adopts the disulfide-linked homodimeric topology found for the native murine protein. Although pure as estimated from SDS/PAGE, heterogeneity is observed with respect to pI. Mass-spectroscopic analysis by FAB-MS and electrospray show this heterogeneity to reside in N-terminal modifications. Crystals have been produced by the hanging-drop method, which diffract to a resolution of 2.0 A. We are currently searching for heavy metal derivatives, and exploring the possibilities of using selenium in multiwavelength anomalous dispersion experiments

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STRUCTURE OF THROMBIN WITH HIRUDIN DERIVATIVES

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Hirudin, a 65 amino acid protein isolated from the leech Hirudo medicinalis, is a specific inhibitor of the blood coagulation enzyme thrombin. It consists of a compact N-terminal core (residues 4 to 47) and a long, disordered C-terminal tail (residues 48 to 65). Upon binding, the core sits over the active site of thrombin while the C-terminal tail wraps around the enzyme along the fibrinogen-binding groove. We have crystallized and solved the structure of the complex of thrombin plus an active site inhibitor, d-Phe-Pro-Arg-chloromethyl ketone (PPACK) with the C-terminal tail (residues 55 to 65) of hirudin and the complex of thrombin with hirulog, a molecule in which PPACK has been joined to the C-terminal tail (residues 53 to 65) of hirudin by a linker consisting of a proline and four glycine residues. Details of the complex structures and interactions between thrombin and the hirudin-derived inhibitors will be presented.

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X-RAY STUDY OF THE SPINACH-CHLOROPLAST THIOREDOXIN F

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Thioredoxin f from spinach chloroplast is a monomeric protein of 113 amino acid residues. It has recently been crystallized in the monoclinic space group $P2_1$. The enzyme that plays an important role in a number of redox reactions exhibits 24% sequence identity with *Escherichia coli* thioredoxin of which the three-dimensional structure is known. Complete data sets to 1.9 Å (native) and 2.4 Å resolution (EMTS and $HgCl_2$ derivatives) have so far been collected. The two heavy atom derivatives have the same major binding site. The resulting M.I.R. map calculated after solvent flattening clearly shows the overall position of the molecule, but is of too poor quality to allow further interpretation at the atomic level. On the other hand, the molecular replacement method, using the refined structure of *E. coli* thioredoxin as a trial model, provides the rotation angles, but fails in finding the correct translation vectors. We are currently trying to correlate both these informations in order to finally solve the structure which we expect to be significantly different from that of *E. coli* thioredoxin.

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The Crystal Structure of OmpF Porin from *E.coli.*

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Porins are integral membrane proteins forming channels across outer membranes of Gram-negative bacteria, thus facilitating the diffusion of low-molecular-weight hydrophilic solutes. They exhibit an exclusion limit, with some of the porin proteins having a degree of specificity for transported substances. One of the porins found in *Escherichia coli* (OmpF porin) forms a non-specific channel which apparently excludes solutes only on the basis of molecular weight. In order to help establish the mechanism of diffusion, and also to learn more about membrane protein structure in general, crystallographic studies have been underway for some time. Here we present the structure determination of OmpF porin using multiple isomorphous replacement methods at 3Å resolution and describe features of the structure relevant to its observed properties.

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Crystal Structure of Dialkylamino Acid Decarboxylase, A Decarboxylating Transaminase

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Dialkylamino acid decarboxylase is an unusual pyridoxal phosphate-dependent enzyme that catalyzes both decarboxylation and transamination in its normal catalytic cycle. α-Methylalanine is oxidatively decarboxylated in the first half-reaction, while pyruvate is transaminated to alanine in the second. The structure of this α_4 tetrameric enzyme (46,500 Da/monomer) as determined by crystallographic studies at 2.8 Å resolution will be presented. The fold of the protein is similar to that of aspartate aminotransferase, a dimer, despite their lack of sequence homology. The monomers are comprised of two domains, a large PLP-binding and a small one. The largest differences between these two enzymes are found in the small domains. Comparison of their active site structures provides clues to the source of the unusual bifunctionality of the decarboxylase, which accomplishes carbon-carbon bond scission and proton transfer via a common binding site.

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PROBING CONFORMATIONAL EQUILIBRIA OF ASPARTATE AMINOTRANSFERASE WITH A SPIN LABEL

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Aspartate aminotransferase was selectively labelled at the conformationally sensitive Cys166 with a nitroxide spin label linked to maleimide. The modification decreased the enzymic activity to 30% of its initial value and lowered the K_m and K_d values for the various substrates and inhibitors 5 to 10-fold. The ESR spectra of both the pyridoxal and the pyridoxamine form showed a temperature-dependent partition of a strongly and a weakly immobilized component suggesting the existence of two different enzyme conformations. On formation of the adsorption complexes and the covalent intermediates during the transamination reaction a change in the ESR spectra was observed; i.e. the immobile portion increased whereas the mobile decreased. The ESR results are consistent with a shift of an equilibrium between two enzyme conformations, probably the "open" and "closed" conformations as defined by X-ray analysis, rather than with different conformations in the different steps of the reaction. The lifetime for the conformational transition was estimated to be $> 10^{-7}$ s.

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THE CRYSTAL STRUCTURE OF HUMAN RECOMBINANT TGF-β2

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Human recombinant Transforming Growth Factor Beta 2 (TGF-β2), a homodimer of 112 amino acids, is a member of a structurally highly conserved growth regulatory polypeptide family involved in cell proliferation and differentiation. TGF-β2 was crystallized ($P3_221$, a=b=60.6 Å, c=75.2 Å) and data to 1.8 Å were collected. The structure was solved at 2.8 Å by the MIR method, making use of anomalous dispersion and solvent flattening. The TGF-β2 monomer is a flat and elongated molecule consisting of 4 antiparallel β-strands, an α-helix connecting β-strand 2 and 3 and an N-terminal α-helix. There are 4 intramolecular disulfide bridges clustered together and forming a compact core. One cystein residue is involved in the only intermolecular disulfide bridge linking two monomers. The monomers are related by a crystallographic 2-fold axis. The structure and dimer interactions will be described.

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A RAPID PROTOCOL FOR AMINO-ACID ANALYSIS USING THE PHENYLISOTHIOCYANAT (PITC) PRE-COLUMN DERIVATIZATION METHOD

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The amino-acid analysis using precolumn derivatization and separation of the derivatives by reversed phase chromatography has gained in popularity during the past decade. It has been shown to be very sensitive (picomole - femtomole range) and represents a less time consuming and more economical methodology than the classical amino-acid analysis based upon ion-exchange chromatography. An improved protocol for the derivatization of free amino acids with PITC and the conditions for the separation of these derivatives by high performance liquid chromatography are described. The use of a new buffer-system (150 mM KH_2PO_4, 5 mM tetraethylammoniumacetate pH 6.4) ensures a rapid separation (15 min.) of the amino acids commonly found in a protein and guarantees a good reproducibility. The influence of salts, pH and temperature upon the separation of the PTC-amino acids is discussed.

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Purification of the B880 antenna complex and its possible subunit, the B820 complex, isolated from the nonsulfur purple bacterium *Rhodopseudomonas marina.*

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The photosynthetic nonsulfur purple bacterium *Rp. marina* has a photoreceptor complex consisting of the reaction center (RC) and the B880 core complex (B880) surrounding the RC. We purified the B880 to homogeniety by fractionated ammoniumsulfate precipitation and subsequent ionexchange chromatography. Furthermore we extracted the carotenoids from the lyophylised B880 by benzene treatment and titrated it with the detergent n-octyl-glucoside (OG) (Miller et al. 1987). This dissociated the B880 complex into a smaller subcomplex, which showed a maximal absorbance around 823 nm. After purifying the B820 by gelfiltration we could reassociate the B820 to its B870 form.

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THE RuvA AND RuvB PROTEINS OF *ESCHERICHIA COLI* MEDIATE ATP-DEPENDENT BRANCH MIGRATION OF HOLLIDAY JUNCTIONS

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The *ruv* locus of *E. coli* is required for the recombinational repair of ultraviolet light- or chemical-induced DNA damage. To investigate the role of the *ruv* gene products in repair, the RuvA and RuvB proteins have been purified and characterised. We found that the RuvA protein (22 kd) binds specifically to model Holliday junctions and that the RuvB protein (37 kd) has an ATPase activity. Together, the RuvA and RuvB proteins mediate branch migration of Holliday junctions *in vitro*. This reaction is dependent on ATP and Mg^{2+} and occurs in both possible directions.

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PURIFICATION AND IDENTIFICATION OF CHROMAFFIN GRANULE BINDING PROTEINS FROM BOVINE ADRENAL MEDULLA

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We are currently purifying chromaffin granule binding proteins by extraction from chromaffin cell membranes of bovine adrenal medulla. After sodium carbonate extraction at pH 11.5 the proteins are stabilized upon neutralization by the detergent Empigen BB. Further purification is achieved by cation exchange FPLC.
One major protein of MW 51kD purified by this procedure turned out to be cytochrome P450(SCC), an adrenal cortical enzyme involved in steroid hormone biosythesis. The presence of numerous islets of cortical cells within the medullary tissue readily explains the co-purification of this contaminant. Other chromaffin cell membrane proteins are further characterized by tryptic digestion and sequence analysis. Reconstitution into phospholipid vesicles and binding assays will be necessary to confirm their affinity for chromaffin granules.

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SYNTHESIS OF A POLY(ADP-RIBOSE) AFFINITY RESIN USING POLY(ADP-RIBOSE) POLYMERASE

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Posttranslational modification of nuclear proteins by poly(ADP-ribose)polymerase involves the covalent attachment and subsequent elongation of ADP-ribose (ADPR) units. We have observed that these covalently attached polymers provide very strong, non-covalent binding sites for histones. To identify other nuclear proteins which may interact non-covalently with poly(ADPR), we enzymatically synthesized a poly(ADPR) affinity resin. NAD^+- and ADPR-agaroses were recognized as acceptors by poly(ADPR)polymerase, which elongated the existing ligands to form polymers covalently linked to the agarose. Addition of ADPR residues depended upon a) poly(ADPR) polymerase activity, b) time of incubation, c) the concentration of free NAD^+ available as substrate, d) the amount of derivatized agarose, and e) the chemical moiety through which the ligand was linked to the agarose. When rat liver nuclear lysates were passed over the poly(ADPR) affinity resin, only H1 and small quantities of the other histones bound. These observations support a role for poly(ADPR) in chromatin structure.

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CHARACTERIZATION OF A REPETITIVE DNA SEQUENCE IN *BORRELIA BURGDORFERI*

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In the course of our studies on the genetic structure of the spirochete *Borrelia burgdorferi*, we cloned genomic DNA fragments and used them as probes in Southern blot experiments. One of the probes, a 3.8 kb *Eco*RI fragment originating from the supercoiled plasmid fraction of the type strain B31, hybridized to several additional *Eco*RI fragments of supercoiled plasmid DNA (1.4, 6.0 and 6.4 kb) as well as to one of the undigested linear plasmids (50 kb) and supercoiled plasmids (29 kb) of this strain, respectively. Experiments with other American and Swiss *B. burgdorferi* isolates gave similar results. In order to determine the extent and nature of the homologous DNA sequences, we cloned the DNA fragments of interest and characterized their cross-hybridizing segments. We also checked for homologies in other *Borrelia* species to evaluate the suitability of the repetitive sequence for use in DNA diagnostics of Lyme borreliosis.

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DIFFERENT RECOMBINANT REVERSE TRANSCRIPTASE FORMS OF HIV1 DERIVED FROM THE CARBOXYTERMINAL END OF p66

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The p15 carboxyterminal proteolysis product of the human immunodeficiency virus type 1 reverse transcriptase (HIV1 RT) p66 possesses DNA polymerase activity (Hafkemeyer, P. , Ferrari, E. , Brecher, J. and Hübscher, U. Proc. Natl. Acad. Sci. USA 88, 5262-5266, 1991). The p15 protein was encoded by pJS 3.7 harbouring the pol-open reading frame. In order to determine the minimal structure of this carboxyterminal protein to display RT activity we constructed two vector DNAs with newly formed coding regions for the p15 protein. Taking into account the unclarified region (tether region) of the protease cleavage site between p51 and p15 one strategy was to ligate the Nsi I/Sal I fragment of p6HRT15 (Schatz, O. Cromme, F. , Grüninger-Leitch, F. and LeGrice, S. FEBS-Letters 257, 311-314, 1989) ,the Bbv I/Nsi I fragment of pJS 3.7, a synthesized oligonucleotide with the start codon and Tyr 441 as the first amino acid into pGEMEX DNA. The second strategy was to cut the Kpn I (nucleotide 3863)/Bbv I fragment, the tether region and ligating it with the above mentioned fragments into pGEMEX DNA.

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OCTAMERIZING DOMAINS OF MITOCHONDRIAL CREATINE KINASE (Mi-CK): A PEPTIDE COMPETITION APPROACH

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Mi-CK exists as dimers and octamers. The latter consist of four dimers as the stable building blocks, which are arranged around a central cavity to form the symmetrical cube-like octamer. Therefore, at least two faces of each dimer have to interact. In order to get information about these octamerizing domains and to possibly locate them on the molecule, we try to identify proteolytic fragments of Mi-CK which inhibit octamer formation. Mi-CK octamers overexpressed in E.coli were purified to homogeneity, dimerized by inducing a transition state analogue complex (TSAC), incubated with ArgC and LysC-digested Mi-CK and allowed to reoctamerize by removing the TSAC-reagent. While a peptide mixture of ArgC-digested Mi-CK had no effect, the mixture of LysC peptides showed a clear concentration dependend inhibition of the reoctamerization. We are currently trying to identify the peptide(s) involved in this effect.

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MACROMOLECULAR RECOGNITION IN AN ELECTRON-TRANSFER COMPLEX: THE BINDING SITE FOR FERREDOXIN (Fd) ON FERREDOXIN-$NADP^+$-REDUCTASE (FNR)

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FNR catalyzes the reduction of $NADP^+$ by reduced Fd, which is generated by photosystem I in chloroplasts. FNR and Fd (both from spinach) form a 1:1 complex stabilized by electrostatic interactions. To map the intermolecular interface of the complex, free FNR and the FNR:Fd complex were treated with biotin-N-hydroxysuccinimide ester. Biotinylated FNR from the two experiments was isolated and digested. Biotinylated and non-biotinylated peptides were separated on avidin-Sepharose. When FNR was biotinylated in the FNR:Fd complex, one peptide was no longer biotinylated and one was much less biotinylated. The two peptides were sequenced and the non-biotinylated lysine residues identified: K18 and K153. In the crystal structure of FNR, residue 153 is located between the two domains of the molecule, close to the flavin prosthetic group. K18 is in a mobile area invisible in the crystal structure.

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PREDICTION OF CARBENE- AND NITRENE BINDING SITES FOR PROTEINS

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The photocrosslinker 3-trifluoromethyl-3-(m-isothiocyanophenyl) diazirine, a carbeneprecursor or p-azidophenylisothiocyanate, a nitreneprecursor were thermochemically coupled to aminopropylated glassfibers and polyvinylidene difluoride. Light induced coupling of amino acids revealed that the philicity of amino acid side chains for both, photogenerated carbenes and nitrenes is different for each individual amino acid. Therefore, proteins with clusters of carbene- or nitrenephilic amino acids at their surfaces provide the requirements for oriented immobilization. A computer program is presented that progressively evaluates the carbene- or nitrenephilicity of a given amino acid sequence and identifies protein domains which predictedly interact with a 'carbene' or 'nitrene' support.

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THE YEAST *WBP1* PROTEIN IS ESSENTIAL FOR N-LINKED CORE GLYCOSYLATION *IN VIVO*

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We have recently isolated a yeast membrane associated protein which binds the lectin wheat germ agglutinine. The corresponding gene was isolated and termed *WBP1* (wheat germ agglutinine binding protein 1). The essential *WBP1* protein is a 47kD type I transmembrane protein located in the endoplasmic reticulum; the large N-terminal domain is exposed to the lumen, the short C-terminus is located in the cytoplasm. Temperature-sensitive *wbp1*-alleles as well as depletion of the *WBP1* protein affect core-glycosylation of vacuolar proteases. The phenotype of *wbp1* mutants and the localization of the protein suggest an essential role of the *WBP1* protein in N-linked core-glycosylation.

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PRODUCTION AND SPECIFICITY CHARACTERIZATION OF POLYCLONAL ANTIBODIES DIRECTED AGAINST ISOCITRATE LYASE; IMMUNOAFFINITY PURIFICATION OF ISOCITRATE LYASE FROM SOYBEAN COTYLEDONS

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Isocitrate lyase (ICL; EC 4.1.3.1) is a specific enzyme of the glyoxylate cycle, which plays a key role in the initiation of gluconeogenesis in germinating oilseeds.
Polyclonal antibodies have been produced in rabbit from the SDS-PAGE ICL band of a partially purified extract. These antibodies specifically recognize the native as well as the denatured enzyme in a crude extract, as shown by ELISA and immunoblot tests. ICL activity was unexpectedly enhanced by the immunoprecipitation procedure, but the specificity of the antibodies was nevertheless demonstrated by analysis of the immunoprecipitate on SDS-PAGE.
The first steps of the ICL purification consits in the isolation of glyoxysomes (sucrose gradient) and the recovery of the organellar matrix (osmotic shock). ICL specifically adsorbs on an affinity column (Affi-prep Hz®) prepared with the antibodies. The enzyme is then readily eluted in the presence of 4 M $MgCl_2$.

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SHORT CHAIN PHOSPHOLIPID ANALOGUES: SYNTHESIS AND APPLICATION IN MEMBRANE PROTEIN CRYSTALLIZATION

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Solubilization and crystallization of membrane proteins require detergents to substitute for the lipid environment. Useful detergents should stabilize the membrane protein in its active conformation and have a critical micellar concentration (CMC) in the millimolar range. We have used short chain phosphatidylcholine (dipentanoyl-, dihexanoyl- and diheptanoyl-PC) as the starting material for the synthesis of the phosphatidyl-ethanolamine (PE) and -glycerol (PG) analogues. The headgroup exchange was achieved by a transphosphatidylation reaction using the enzyme phospholipase D from cabbage either in a biphasic ether/water system (PE), or immobilized on an octyl-sepharose resin (PG). CMC values have been determined by surface tension measurements and range from 1 to about 40 mM. The synthesized lipid analogues have been successfully used in the crystallization of the two integral outer membrane proteins OmpF and maltoporin (LamB) of E.coli.

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Mimick of a HLA-class I molecule using the TASP approach

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X-ray crystallographic analysis of three MHC class-I molecules, HLA-A2, Aw68 and B27, revealed that MHC molecules have a groove formed by two α-helices overlying an eight stranded β-pleated sheet. This groove was previously thought to be the binding site for antigenic peptides and this was recently confirmed in the case of B27. We decided to mimick the A2 peptide binding groove and raise specific antibodies to it. We used the TASP strategy (Template-assembled synthetic proteins), where secondary structure forming peptide blocks are assembled by covalent attachment to a carrier molecule (template). Part of the α_1-helix of the A2 molecule was synthesized including residues 59-75 and four such modeled α_1-helices were attached on the template. Circular dichroism studies showed that the TASP molecule adopts a helical conformation at a neutral pH and with an increase in helix content upon addition of trifluoroethanol. Antibodies against the MHC construct were raised in BALB/c mice and were detected by ELISA using the synthetic MHC-TASP as solid phase. Flow cytometry studies showed that these antibodies specifically recognize the A2 MHC class-I molecule on the surface of HLA-A2 positive cells.

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COOH-TERMINUS AMINO ACIDS REQUIREMENTS FOR GPI-ANCHOR ADDITION TO CELL SURFACE PROTEINS

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All surface proteins anchored in the plasma membrane via a glycosylated form of phosphatidylinositol (GPI) are originally synthesized with a C terminal stretch of hydrophobic amino acids which is cleaved soon after translation and replaced, in the endoplasmic reticulum, by a preformed anchor containing ethanolamine, carbohydrate and phosphatidylinositol. Three features have been shown to be essential for correct processing of the precursor protein: a suitable cleavage/attachment site, i.e a specific amino acid, a hydrophobic domain downstream of the attachment site and a spacing region separating these two elements. To test the importance of the primary amino acid sequence in this spacer, we used the GPI-anchored Thy-1 glycoprotein and introduced a number of specific amino acid deletion and/or substitution in the region comprised between the attachment site and the hydrophobic domain. These DNA constructs were expressed in Hela cells and stable clones were tested for the presence of a GPI-anchored Thy-1 molecule at the cell surface. Furthermore, we are currently replacing the Thy-1 spacer region by a random amino acid sequence of various length capable of giving rise to a correctly processed Thy-1 molecule. This approach should provide us with a large number of information on the amino acid sequence and the length of this spacer region. Finally, we also succeded in transforming a conventional transmembranous protein, the gD1 of HSV I, into a GPI-anchored molecule by deleting its C-terminal hydrophilic tail and thus providing all the requirements necessary for addition of a GPI anchor.

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Construction of prokaryote expression vectors containing B and T epitopes and use of the fusion proteins as immunogens in mucosal immunity.

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An immune response with the production of antibodies requires that the antigen contains appropriate antibody recognition sites (B-cell epitopes) as well as sites capable of eliciting T-cell help (T_H-cell epitopes). Different expression vectors that contain sequences for single or multimeric T_H epitopes (tetanos toxin) and a cloning site for B epitope sequence insertion have been constructed. The chimeric proteins have been used to immunize Balb/c mice. The immune response has been monitored by measuring the serum antibody concentration (ELISA) and by scoring the number of positive hybridomas following fusion of spleen lymphoblasts and myeloma cells. To facilitate purification of the recombinant immunogen, a C-terminal tag (His hexamer) has been inserted in the vector and the recombinant proteins purified using nickel nitrilo-acetic acid agarose.

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Mechanism of membrane insertion of human complement component C9

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After binding to the C5b-8 complex, C9 undergoes a drastic rearrangement from a water-soluble globular protein into an elongated molecule with the properties of an integral membrane protein. Based on membrane-restricted photoaffinity labeling, secondary structure predictions and sequence comparisons with perforin, the pore-forming protein found in cytotoxic T lymphocytes, a molecular model of the membrane-inserting region was predicted. The domain is formed by two amphipathic alpha-helices (292-308 and 313-333) separated by a short turn (309-312).
In order to evaluate this model, wild-type C9 and C9 point mutants were expressed in COS cells. The changes were designed to gradually affect the amphipathic character of either side of the putative helices. Recombinant wild-type C9 and mutations in which the amphipathic character at the membrane helices was not affected (i.e. Tyr 324 replacement by Phe) lysed C5b-8 coated erythrocytes as efficiently as native, serum-derived C9. In contrast, non conservative changes (i.e. Tyr 324 replacement by Glu) resulted in C9 molecules which were not only completely inactive but were even not secreted. The results obtained are in agreement with a crucial role for this amphipathic sequence motif in C9 structure and function.

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SOLUBLE T CELL RECEPTOR (TcR) SPECIFICALLY RECOGNIZES THE MHC PLUS PEPTIDE

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TcR is a key element in antigen specific, selfrestricted interactions between T cells and their targets. To study molecular details of TcR binding events, the soluble form of the cell bound TcR would be optimal. TcR from a T_H cell clone recognizing a hemagglutinin peptide of influenza virus on MHC class II was solubilized by genetic engineering. Biological activity of the purified soluble TcR was studied in cellular assays where the inhibition of antigen specific activation of different T cell hybridomas was measured. These assays with T cell hybridomas having either the same or a different specificity than the soluble TcR showed clearly that the soluble TcR inhibits specifically and completely those T cell hybridomas that recognize the same antigen and MHC class II molecule. T cells recognizing a different peptide on the same restriction element were only poorly inhibited. Furthermore, these experiments enable us to estimate the affinity of TcR to the MHC class II plus antigen.

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EPITOPE MAPPING WITH SYNTHETIC PEPTIDES SELECTS FOR A SMALL FRACTION OF ANTI-PROTEIN ANTIBODIES

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Synthetic peptides are widely used to map the antigenic structure of proteins. We have tested 103 synthetic hexapeptides covering the entire cytochrome *c* sequence with several rabbit antisera against native yeast cytochrome *c*. Peptides were bound to plastic pins (Geysen method). Affinity-fractionation of the antisera revealed the following: 1. Only antibodies that crossreact with denatured cytochrome *c* recognize synthetic hexapeptides. 2. These antibodies account for only 2-3% of all cytochrome *c*-specific antibodies in any of the antisera tested. 3. Most pin-bound peptides are recognized only by intact IgG but not by the corresponding Fab's, even if Fab's are highly concentrated. Hence, crossreactivity of anti-protein antibodies with synthetic hexapeptides is weak, as crossreactivity is limited to bivalent IgG's reacting with a surface that is densely covered with peptides.

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UNFOLDING OF PLASTIC-ADSORBED PROTEINS: CAVEATS FOR THE USE OF ELISA TO TEST ANTIBODY SPECIFICITY

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Polyclonal antibodies against native yeast cytochrome *c* strongly crossreact with denatured cytochrome *c* in an ELISA in which the protein antigen is adsorbed to plastic. Similarly, antibodies raised against denatured cytochrome *c* crossreact with native cytochrome *c* in the ELISA. In contrast, the same antibodies are highly specific for their cognate antigen when tested in a solution-RIA: anti-native cytochrome *c* antibodies discriminate against denatured cytochrome *c* by more than a factor of 1000. This discrepancy between results from different immuno assay procedures clearly indicates that adsorption to plastic alters the antigenic structure of even a stable protein such as cytochrome *c*. The results warrant serious thoughts about the crossreactivity of anti-protein antibodies with peptides, and vice versa, as observed by conventional ELISA.

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LARGE-SCALE PURIFICATION OF TWO MINOR LINKER POLYPRPTIDES FROM PHYCOBILISOMES OF THE CYANOBACTERIUM *MASTIGOCLADUS LAMINOSUS*

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Phycobilisomes serve as the primary light-harvesting antennae for photosystem II in cyanobacteria and red algae. These supramolecular complexes are composed of two main structural elements: a tricylindrical core and peripheral rods, arranged in a hemidiscoidal way around the core.
The core and the rods are composed of hexameric disks of phycobiliprotein complexes, which contain also chromophore-free linker polypeptides. These linker polypeptides are necessary for the assembly of the phycobiliproteins in the phycobilisomes.
The terminal linkers ($L_R^{8.9}$; $L_C^{8.9}$) of the rod and of the core respectively have been isolated in a large scale preparation through ion-exchange and reversed phase chromatography for the three-dimensional structure analysis of these linker polypeptides.

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PROTEOLYTIC TREATMENT OF CHLOROSOMES FROM CHLOROFLEXUS AURANTIACUS ANALYSED BY REVERSED-PHASE CHROMATOGRAPHY.

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The chlorosome is the main light-harvesting antenna of the green photosynthetic non-sulfur bacterium Chloroflexus aurantiacus (J-10-fl). It is an ellipsoid body that comprises several thousands of bacteriochlorophyll c molecules and four polypeptides (M_r 18, 11, 5.8, 5.7 kDa) according to Feick and Fuller (1984). Purified chlorosomes were analysed by reversed-phase chromatography and SDS-PAGE and the 5.7 kDa protein was found to be the predominant polypeptide. Two other proteins with M_r 11.8 and 15.8 kDa (Mr 11 and 18 kDa, Feick and Fuller, 1984) were found in smaller amounts. Purified chlorosomes were subjected to proteolytic treatment using trypsin. Both the 11.8 and the 15.8 kDa protein could be degraded, whereas the 5.7 kDa protein apparently resisted to the degradation. The UV/Vis absorption spectrum of the chlorosomes remained unchanged.

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PROTEIN-A CAPTURE ELISA (PACE): A NEW ELISA FORMAT TO OVERCOME UNFOLDING OF PLASTIC-BOUND PROTEIN ANTIGENS

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Adsorption to plastic may severely alter the antigenic structure of proteins through unfolding (C. Schwab and H.R. Bosshard, J. Immunol. Methods, in press). This problem is overcome in a new ELISA procedure in which the antibody (monoclonal or polyclonal) is first incubated with biotinylated antigen to attain binding equilibrium in solution. Second, the ag:ab complex is adsorbed to the microtiter plate pre-coated with protein-A. Third, the ag:ab complex is detected with phosphatase-coupled streptavidin. A competition ELISA is accomplished by co-incubation of biotinylated and non-biotinylated antigen in step 1, allowing for the determination of association constants. The procedure combines the assets of both the ELISA and the RIA, evading denaturation of plastic-bound antigen and the use of radioisotopes.

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MITOCHONDRIAL CREATINE KINASE MEDIATES CONTACT FORMATION BETWEEN MITOCHONDRIAL MEMBRANES

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Mitochondrial creatine kinase (Mi-CK) is involved in the energy transfer between mitochondria and cytoplasma. Mi-CK occurs in the mitochondrial intermembrane space, where it is preferentially located at contact sites between inner and outer membrane. We have investigated Mi-CK/membrane interaction. Mi-CK was shown to interact simultaneously with inner and outer mitochondrial membranes, thereby creating an intermembrane contact. Intermembrane contact formation was demonstrated by measuring the binding of inner membrane vesicles to outer membranes spread at the air-water interface. The two oligomeric forms of Mi-CK, octamer and dimer, differed in their ability to mediate intermembrane adhesion, the octamer being more potent. Beside influencing Mi-CK function, this property enables Mi-CK to participate in the stabilization and/or formation of contact sites *in vivo*.

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LIMITED PROTEOLYSIS TO INTRAMEMBRANE-BOUND LIGHT-HARVESTING PIGMENT-PROTEIN COMPLEXES: STRUCTURAL AND SPECTRAL EFFECTS

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The light-harvesting- and energy transfer systems of purple bacteria represent ideal model components for studying (protein)structural-(bacteriochlorophyll)spectral relationships. In order to obtain information regarding the structural requirements for the generation of distinct pigment populations (e.g. B880, B850 and B820) we have carried out a number of experiments in applying limited proteolysis to the diverse antenna pigment-protein complexes of *Rp. acidophila* (strains 10050 and 7050), *Rc. gelatinosus* and *Rc. tenuis*. Detergent-solubilized core- (B880) and peripheral antenna complexes (e.g. B800-850) were exposed to elastase and proteinase K and to the specific proteases chymotrypsin and trypsin. The time course of modification of the antenna complexes were monitored by absorption- and circular dichroism spectroscopy in the ultraviolet and in the near-infrared region. Analyses of the partially degraded antenna polypeptide components were performed by reversed phase HPLC using a C-4 matrix (7 µ, 1000 Å) and subsequent protein chemical analysis like Edman degradation and amino acid analyses.

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ISOLATION OF A PROTEIN FACTOR THAT ENHANCES BINDING OF PURIFIED XENOPUS ESTROGEN RECEPTOR TO ITS RESPONSIVE ELEMENT

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Estrogen receptor is a nuclear protein which regulates transcription in a hormone dependent manner. The vaccinia virus expression system was used for the production of the Xenopus estrogen receptor (xER) in eukaryotic cells. For further in vitro transcription and DNA binding studies, we purified the full length receptor to homogeneity using a DNA-affinity column. We found that purified xER does not form a detectable complex with an estrogen responsive element (ERE) under conditions where xER-ERE complexes are readily formed with crude cells extracts. We present evidence, that high affinity binding of the xER to its cognate response element is dependent on interactions with additional protein factor(s).

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PROTEIN BINDING TO CHLOROPLASTS AND ITS ENVELOPES

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Most chloroplast proteins are nuclear encoded, translated in the cytoplasm as precursors and imported into the chloroplasts. The first step of chloroplast protein import is the binding of a precursor protein to the organelle. We measured the binding of the precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase (pSS) to intact chloroplasts of Chlamydomonas reinhardii and to their isolated envelopes under conditions where no import did occur. Binding affinities of pSS to both the chloroplasts and the isolated envelopes were similar and rather high, as indicated by the low dissociation constants in the range of 10^{-9} M. The number of binding sites was found to be 4 to 12 x 10^4 per chloroplast in this organism. Most of the pSS once bound to intact chloroplasts could subsequently be imported into the organelle, demonstrating a physiologically significant binding in our assay. Binding of pSS to chloroplasts and to envelopes was specific also with respect to the presence of the transit sequence and to the type of the membrane.

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MEMBRANE TOPOLOGY OF THE ESCHERICHIA COLI GLUCOSE PERMEASE

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The glucose permease of *Escherichia coli* catalyzes the concomitant transport and phosphorylation of D-glucose. It is a complex consisting of a hydrophilic subunit (III^{Glc}) and a transmembrane subunit (II^{Glc}). II^{Glc} consists of two domains. The N-terminal hydrophobic domain contains the glucose binding site, the C-terminal hydrophilic domain includes the phosphorylation site. Secondary structure prediction methods suggest that II^{Glc} spans the membrane 6 to 8 times. To test this prediction we constructed a set of nested gene fusions between *ptsG* (encoding II^{Glc}) and '*phoA* (encoding alkaline phosphatase lacking its signal sequence) as well as between *ptsG* and *lacZ* (encoding the beta-galactosidase). About 90 fusions with reporter protein activity have been analysed. The membrane topology of the II^{Glc} subunit will be discussed.

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PHOTOIMMOBILIZATION OF MACROMOLECULES ON INERT SURFACES

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A new and unique immobilization procedure has been elaborated, by which covalent light-induced immobilization of ligands differing in chemical nature and complexity is attained under mild and non-destructive conditions. Reactive functional groups are not required, neither on the ligand nor on the surface. Photocoupling is effected by topical interaction of ligands (amino acids, peptides, proteins including enzymes, nucleic acids, carbohydrates) with organic or inorganic surfaces through the mediating action of linkermolecules. Polymers equipped with carbene generating photoactivable diazirines serve as linkermolecules. The exceptional reaction characteristics of carbenes, including fast reaction and diversity in substrate binding, render photogenerated carbenes powerful tools for universal ligand coupling.

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FUNCTIONAL ANION EXCHANGERS ARE EXPRESSED IN THE ENDOPLASMATIC RETICULUM (ER) OF TRANSFECTED HUMAN KIDNEY CELLS.

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AE1 and AE2 are members of a family of transport proteins which mediate Na-independent anion exchange. We have transiently expressed AE1 and AE2 cDNA in the human embryonic kidney cell line 293 to study their transport activities. Expression of AE2 leads to an increase in whole cell $^{35}SO_4^{2-}$efflux, consistent with its function as a plasma membrane (PM) anion exchanger. No such increase was observed in AE1 transfectants. In contrast, both AE1 and AE2 expression resulted in a significant increase in SO_4^{2-}/SO_4^{2-} and Cl^-/Cl^- exchange activity in crude microsomes (CM) prepared from transfected cells, showing that both AE1 and AE2 encode functional transporters. Pulse-chase experiments revealed that while ~60% of AE2 is processed to the cell surface of transfected cells AE1 is restricted to the ER.CM from transfected cells were separated into PM and ER fractions by concanavalin A affinity chromatography. Transport assays on these fractions confirmed that nearly 100% of the transport activity of AE1 CM was recovered in the ER fraction, while AE2 activity was divided roughly equally between PM and ER fractions. We conclude that AE1 and AE2 are processed to different intracellular destinations in transfected 293 cells and that posttranslational processing in a post-ER compartment is not required for the function of either protein.

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ALTERATIONS IN MEMBRANE PERMEABILITY IN SEMLIKI FOREST VIRUS-INFECTED AEDES CELLS UNDER FUSOGENIC CONDITIONS

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Semliki Forest Virus (SFV) infected aedes cells undergo cell-cell fusion upon exposure to mildly acidic pH. This model is used to study membrane fusion. As a highly controlled process, biological membrane fusion is generally considered to be non-leaky. We show here that fusion of SFV-infected aedes cells is accompanied by leakage of low molecular weight compounds (Mr < 1000) out of the cells. The leakage is pH dependent and cannot be seen in mock infected cells under the same conditions. It might therefore be mediated by viral spike proteins that are expressed in the plasmamembrane of the host cell. The leakage can be inhibited by Zn^{++} and Ca^{++} in the micromolar range. Fusion is still taking place in presence of divalent cations, indicating that the permeability change is not mandatory for cell fusion. The effect of anionic antiviral substances on permeability change and cell fusion was also investigated. Some of those compounds inhibit both fusion and leakiness, and there is evidence that they might block the irreversible conformational change of the viral spike proteins.

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RECONSTITUTION OF HUMAN ERYTHROCYTE BAND 3 INTO REGULAR ARRAYS

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Two-dimensional crystallization of membrane proteins is a valuable tool for determining their structural properties. Although a wealth of biochemical and functional data is available on the anion transporter band 3 from human erythrocyte membranes, no information exists on its structure. Purified band 3 dissolved in detergents tends to aggregate to oligomers that may exhibit intrinsic symmetries. Whereas in detergents of high cmc (e.g. octyl-POE) tetramers and hexamers have been found, the predominant form in detergents of low cmc (e.g. $C_{12}E_8$) is the dimer. Dialysis of band 3-lipid-detergent mixtures against detergent free buffer induced assembly of two-dimensional crystalline protein arrays. Averaged projections obtained by digital image processing of electron micrographs show the arrangement of band 3 protomers in a trigonal lattice (lattice constant: a=b=100 Å). Estimates of the area occupied by the protomers and the known cross-sectional area of transmembrane helices indicate that the protomers represent dimers of band 3.

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ORGANISATION OF THE ER-TO-GOLGI PATHWAY

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Due to the lack of appropriate markers the structural organisation of the ER-to-Golgi pathway has been elusive. We have taken a mAb approach to define this organisation in detail. A novel 63 kD protein (p63) was found that identifies a large tubular network in primate cells. The network overlaps and is in continuity with the ER-Golgi intermediate compartment (ERGIC) defined by the marker p53 as visualized by CLS-IF microscopy and immuno-EM. That the 63 kD compartment mediates protein transport from ER to Golgi was indicated by partial colocalization of p63 and VSV G protein in Vero cells cultured at 15°C. Low temperatures and BFA had little effect on the distribution of p63 suggesting that this novel marker is a stably anchored resident of the ERGIC. P63 and p53 were enriched to a similar degree by the same subcellular fractionation method. These findings establish the ERGIC as a permanent organelle of previously unanticipated dimensions and further clarify its relationship to ER and cis-Golgi.

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EXPRESSION OF HUMAN CYTOCHROME-P450IA1 AND HUMAN P450-REDUCTASE AS FUSION PROTEINS IN *S.CEREVISIAE*

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We intend to express particular important human cytochrome P450 genes in *S.cerevisiae* in order to use this organism as an in vitro genotoxicity test system capable of intracellular promutagen activation. P450IA1 and P450-reductase were expressed as fusion proteins in order to achieve optimal conditions for the interaction of these two enzymes. By expressing a hybrid gene consisting of P450IA1 sequences, human P450-reductase sequences and HMG1 sequences we expect three consequences: (1) synthesis of additional internal membranes (as a result of overexpression of HMG1), (2) integration of the chimeric protein into these membranes and (3) exhibition of cytochrome P450IA1 specific activity due to homologous interaction with the P450-reductase moiety. Expression of three alternative fusion proteins, P450IA1 in combination with (a) the human P450-reductase, (b) HMG1 and (c) the human P450-reductase and HMG1, will be shown.

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BIOSYNTHESIS AND INTRACELLULAR TRANSPORT OF α2,6-SIALYLTRANSFERASE IN RAT HEPATOMA CELLS

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We studied biosynthesis, intracellular transport and release of ß-galactoside α2,6-sialyltransferase (ST) in a dexamethasone-inducible rat hepatoma cell line. Following induction by dexamethasone, a pulse/chase protocol involving metabolic labeling with [^{35}S]met/cys followed by immunoprecipitation of ST and electrophoretic/fluorographic analysis was applied. The [^{35}S]-labeled enzyme was synthezised as a 46kD precursor, converted to an intermediate 47kD form within 1h chase and gradually to a mature form of 47.5kD within 4h. By means of either tunicamycin inhibition of N-glycosylation or cleavage of N-glycans from isolated ST using N-glycosidase F the sizes of the precursor and the mature form were reduced to 43kD and 44.5kD, respectively. After a 4h chase period, treatment with endo H revealed two distinct molecular forms of ST bearing either two complex or one oligomannosidic and one complex type N-linked sugar chain. In addition, ST was found to be sensitive to neuraminidase digestion as demonstrated by isoelectric focusing. On SDS/PAGE sensitivity to neuraminidase was detected only after 4h chase. The half-life of cellular [^{35}S]-ST was estimated to 6h. After 2h, 4% were detected as a soluble enzyme in the supernatant; after 8h, a peak value of 12% was reached; after 72h, 2% remained immunoprecipitable. Our results suggest that ST follows the secretory pathway as a membrane protein and is retarded at a late Golgi stage. A possible post-Golgi degradation of ST resulting in loss of immunoreactivity has to be further investigated.

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CHARACTERIZATION OF POLYCLONAL ANTIBODIES TO HUMAN RECOMBINANT α-2,6 SIALYLTRANSFERASE AND USE FOR DOUBLE IMMUNOFLUORESCENCE WITH GALACTOSYLTRANSFERASE IN HEPG2 CELLS: EVIDENCE FOR DIFFERENT COMPARTMENTS

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α-2,6 sialyltransferase {ST} is a Golgi enzyme which transfers sialic acid to penultimate galactose in glycoprotein sugar chains. Based on the sequence of human ST (Grundmann et al., Nucl. Acids Res. 18, 667, 1990) the cDNA was synthesized by PCR using mRNA of HepG2 cells (human hepatoma cells) as template. The cDNA was cloned and expressed as hybrid ß-galactosidase-ST fusion-protein {FP} in E. coli. The ST-FP was purified by SDS-PAGE and used to elicit a polyclonal antiserum, which crossreacted with purified rat liver ST as shown by ELISA. Affinity purified antibodies were characterized by immunoblotting and immunofluorescence in HepG2 cells which revealed a typical Golgi staining. This antiserum was then used for double immunofluorescence using monoclonal antibodies to 4-ß-galactosyltransferase {GT} in HepG2 cells. Co-localization of both ST and GT was demonstrated. However, treatment of HepG2 cells with monensin, a cationophor causing reversible dilatation of Golgi cisternae, led to scattering of GT-positive Golgi elements leaving ST-positive Golgi structures almost unchanged. We conclude that ST and GT reside in different Golgi subcompartments which differ by their sensitivity to monensin. Supported by grant 31-30757.91 of the SNSF to EGB.

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SUBSTRATE SPECIFICITY OF HYBRID PROTEINS BETWEEN THE GLUCOSE AND N-ACETYLGLUCOSAMINE SPECIFIC TRANSPORTERS OF ESCHERICHIA COLI

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The glucose and N-acetyl glucosamine specific transporters (II^{Glc} and II^{GlcNAc}) of the bacterial phosphotransferase system mediate carbohydrate uptake across the cytoplasmic membrane concomitant with substrate phosphorylation. The two proteins have 40% amino acid sequence identity. In order to map the substrate binding site, hybrid proteins were constructed (i) by homologous recombination of the respective genes *in vivo*, and (ii) by gene reconstruction *in vitro*. The sugar specificities of the hybrid proteins indicate that the substrate binding site is located in the N-terminal half of the polypeptide chain. The sugar specificity can be relaxed in hybrid proteins.

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CHARACTERIZATION OF TWO REPETITIVE NON-VARIABLE ANTIGENS FROM AFRICAN TRYPANOSOMES

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We have characterized two repetitive proteins representing common antigens of African trypanosomes which are conserved throughout the life cycle of the parasites. These antigens were identified by their ability to immunoreact with a serum from a cow taken during the early phase of a cyclic trypanosomal infection. One of the two antigens is MARP1 (microtubule associated repetitive protein 1) of *Trypanosoma brucei brucei* previously characterized as a high molecular weight protein of the membrane cytoskeleton containing more than 50 tandemly repeated, near identical 38 aa units. Starting from an immunological screening of a *Trypanosoma brucei gambiense* bloodstream form cDNA expression library we have identified a second repetitive motif consisting of 68 aa repeat units. These repeats have shown to be part of a protein (named GM6) which, like MARP1, is a high molecular weight component of the trypanosomal cytoskeleton. Immunofluorescence and immunogold electron microscopy revealed that GM6 is located on fibers which connect the microtubules of the membrane skeleton with the flagellum. Recombinant repetitive peptide fragments of the two proteins which were fused to the ß-galactosidase of *Escherichia coli*, demonstrated in a Western blot a strong immunoreactivity with many sera from *T. b. brucei* and *Trypanosoma congolense* infected cattle. The result from this preliminary immunological evaluation indicates a high immunodiagnostic sensitivity (90 %) of the two recombinant antigens which makes them interesting candidates for a immunodiagnosis of trypanosomiasis in cattle.

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MOLECULAR BIOLOGICAL CHARACTERIZATION OF A REPETITIVE PROTEIN OF *TRYPANOSOMA BRUCEI*

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We have identified a repetitive high molecular weight protein in *Trypanosoma brucei*. This protein, termed GM6, consists of a large number of repeat units of 68 amino acids and is a component of the trypanosomal cytoskeleton. Immunofluorescence and immunogold electron microscopy revealed that GM6 is located on fibers which connect the microtubules of the membrane skeleton with the flagellum. The aim of this project is to investigate the genomic organisation of the GM6 gene and to determine the nucleotide sequence of the non-repetitive portions of this gene. Besides the molecular biological investigations we intend to perform further biochemical and immunocytochemical studies in order to get more information about the cytological function of this protein.

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INVESTIGATION OF THE PARAFLAGELLAR ROD PROTEINS IN TRYPANOSOMA BRUCEI: GENORGANISATION, TRANSCRIPTION AND TRANSLATION

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The flagellum of the parasitic hemoflagellate *Trypanosoma brucei* contains, beside the microtubular axoneme, another cytoskeletal element, called the paraflagellar rod (PFR). This is a highly ordered, three-dimensional fibrous network of yet unknown function, which runs along, and is tightly linked, to the axoneme. The major structural components of the PFR are two immunologically related proteins of 69 and 73kD. They are coded for by two tandemly linked genes, whose genomic nucleotide sequences are, surprisingly, identical. Both genes are transscribed as two stable mRNAs of very similar length. These two mRNAs, however, are translated, in vivo and invitro, as two proteins of different molecular weights and of slightly different charge. This observation indicates,that no posttranslational modification occurs.

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Microtubule-associated repetitive proteins (MARPs) in *Trypanosoma brucei*.

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The membrane skeleton of the parasitic homoflagellate *T. brucei* consists of a single layer of subpellicular microtubules which are tightly crosslinked with each other, as well as with the overlying cell membrane, by a host of microtubule associated proteins (MAPs). We have previously identified two highly repetitive MAPs termed MARP1 and MARP2. Both proteins consist of over 50 tandemly linked, highly repetitive units of 38 amino acids . Within the same protein these repeats are virtually identical, however, between MARP1 and MARP2 they share only 50% homology. By expressing different fragments of the MARP-genes in heterologous cell lines (CV1. 3T6, Hela) we provide evidence that MARP1 interacts with microtubules via its repetitive units, while the non-repetitive C-terminus constitutes the microtubule binding site for MARP2

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IN VIVO AND IN VITRO EXPRESSION OF MOUSE MAMMARY TUMOR VIRUS ORF PROTEIN

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The Mouse Mammary Tumor Virus (MMTV) long terminal repeat contains an open reading frame (ORF) of 960 base pairs encoding a 36 kD polypeptide with a putative trans-membrane sequence and five N-glycosylation sites.This protein has never been detected in MMTV infected cells. Because of its high degree of conservation between different virus strains we speculated that this protein must exert a function. Transgenic mice bearing complete and truncated orf sequences from MMTV-GR regulated by a heterologous promoter were raised and three lines of each were isolated by Southern blot analysis. Mice harboring the complete ORF protein showed at 6 weeks of age a clonal deletion of Vbeta^{14} bearing T cells. The tissular expression pattern of messenger RNA was studied by polymerase chain reaction. To express ORF protein in vitro, different recombinants of vaccinia virus containing entire or truncated ORF sequences of MMTV-GR and MMTV-C3H-K were produced.

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IDENTIFICATION OF THE MOUSE MAMMARY TUMOR VIRUS CELL SURFACE RECEPTOR

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In an attempt to understand the cell tropism of Mouse Mammary Tumor Virus we want to characterize the cell surface receptor for MMTV gp52. Two MMTV strains that differ by 3% in their env sequences can be produced in tissue culture. Recombinant vaccinia viruses containing the MMTV env gene have been made, in order to produce gp52 in eucaryotic cells and to be able to produce mutant proteins for later characterization of the virus-receptor interaction. Binding assays will be done on cell types known to be infected in vivo, e.g. lymphocytes and epithelial cells. We are in the process of developing an infectivity assay for MMTV in these cells. Binding studies and crosslinking experiments will be performed to identify the receptor. Finally attempts will be made to clone the gene coding for the receptor.

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DIFFERENTIAL CYTOTOXIC ACTIVITY OF TWO δ-ENDOTOXINS OF BACILLUS THURINGIENSIS IN MAMMALIAN CELLS.

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δ-endotoxins are insecticidal proteins produced by Bacillus thuringiensis during sporulation; they are deposited as parasporal bodies in the sporangia. The toxicity of established endotoxin from B. thuringiensis subsp. israelensis (ISR) was tested in V79 Chinese hamster cells and compared with the endotoxin from a new isolate (K24) from sawfly larvae toxic for both diptera and saflies.Growing hamster fibroblasts were exposed for 15 min, 1, 4, and 8 hours at doseses between 0.01 -10µg. Toxicity was characterized by the cellular uptake of neutral red (NR test), the mitochondrial succinate dehydrogenase activity (MTT test) and extracellular lactate dehydrogenase activity (LDH relase). The time response and the relationship obtained sugests that the toxin interacts primarily with the cell membrane. In contrast, the new isolate K24 showed only a slight toxic activity at the highest doses and longest exposure periods (8h). The lower toxicity of the K24 endotoxin in mammalian cells combined with the broader insecticidal activity speak in favor of its use as a new insecticide.

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DNA BINDING SPECIFICITY OF THE OCTAMER FACTORS

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The Octamer factors Oct-1, Oct-2, Oct-3 and Oct-6 belong to the newly described POU proteins family and can bind in vitro or in vivo to an octamer site -ATTTGCAT-. Oct-1 is a nearly ubiquitous protein, Oct-2 is restricted mostly to lymphoid cells, Oct-3 is present in early embryonic cells and Oct-6 is mostly found in the brain.
These transcription factors are very dissimilar but share a high degree of relatedness in their DNA binding domain, the POU domain. This DNA binding domain is bipartite and consists of a C-terminal Homeodomain-related region and of a N-terminal "POU-specific" subdomain.
Because very little is known about the in vivo targets of octamer factors we set out to further define their DNA binding specificity. To this end we expressed the POU domain of each octamer factor either in bacteria as a GST fusion protein or in reticulocyte lysates. We then used a PCR assay to accurately identify possible binding sites.

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3-DIMENSIONAL SHAPE OF DNA IN SOLUTION AND ITS CONSEQUENCE FOR TRANSCRIPTION.

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Native DNA can be observed in a thin vitrified layer of solution by cryo-electron microscopy. The 3-dimensional path of the molecules can be visualized from stereo-pairs or can be reconstructed with the help of a computer. We are measuring properties of DNA fragments such as curvature, persistence and flexual length as well as writhe and twist in supercoiled molecules.
The observation of E. coli RNA polymerase actively transcribing on pUC 9 supercoiled DNA shows that the enzyme does not rotate around the DNA and that the RNA transcript does not become entangled during transcription while it is the DNA that rotates around its axis. An explanation for this fact is found in the interplay between the enzyme induced bent and the intrinsic rigidity of the DNA.

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THE PHOTODYNAMIC ACTION OF METHYLENE BLUE AND LIGHT ON VIRUSES PRODUCES CROSS-LINKING OF THE VIRAL GENOMES WITH THEIR CAPSID PROTEINS RESULTING IN VIRUS INACTIVATION

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Viral contamination, especially HIV-1 and Hepatitis B viruses, in human plasma used for infusion in humans poses a very serious problem to public health. In this study we have used methylene blue and light to inactivate viruses in human plasma. We show that $2x10^{13}$ viruses/ml (Semliki Forest, Hepatitis B, Herpes Simplex) are inactivated with an irradiation dose of 85 $mW.min/cm^2$. The virus particles remained intact after the photodynamic treatment. The spike proteins were not affected 2D-SDS-PAGE, retaining their antigenicity as well as their ability to adsorb to the cells. However, the main effect of the photodynamic process is the cross-linking of the viral genomes with their capsid proteins. This cross-linking is responsible for virus inactivation preventing the viral genomes from being transcribed or translated (DNA and RNA viruses).

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NUCLEAR LOCALIZATION OF MVM NS1 AND COTRANSPORT OF AN NS1 RELATED CYTOPLASMIC POLYPEPTIDE.

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By analogy to non-structural papovaviral T-antigens, the structurally similar protein (NS1) encoded by the murine parvovirus, is predicted to form oligomers for certain regulatory events in virus replication. To investigate this hypothesis we expressed wildtype and mutant NS1 protein with a transient expression system, using the recombinant vaccinia virus vTF7-3 and a plasmid containing the NS1 genes under control of the phage T7 promoter and an EMC leader sequence. We demonstrated that wildtype NS1 as well as two NS1 proteins containing different mutations in the putative purine triphosphate binding site (K405/R;K405/M) are able to translocate into the nucleus, whereas an NS1 related polypeptide lacking aminoacids 96 to 253 remained in the cytoplasm. We coexpressed the mutant NS1 peptide lacking the signals for nuclear localization with either wildtype NS1 or the nucleotide binding site mutants. In immunofluorescence and cell fractionation experiments we could demonstrate that wt NS1 as well as the arginine substitution (K405/R) are able to translocate the deleted polypeptide to the nucleus, implying that NS1 is able to form oligomers. Crosslinking studies as well as co-immunoprecipitations are in progress to investigate the interaction of different NS1 related proteins by a direct approach.

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PRODUCTION OF MONOCLONAL ANTIBODIES AGAINST ISOLATED CENTROSOMES BY IN VITRO IMMUNIZATION OF MURINE SPLEEN CELLS.

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Centrosomes contain the centrioles, consisting mainly of tubulin, and the pericentriolar material. Centrosomes can be isolated from cultured cells, blood lymphocytes or calf thymus tissue. 5 liters of cell suspension could be prepared per working cycle. By including an ultrafiltration step the yield could be enhanced to $3*10^9$ centrosomes or 70 µg protein. The in vitro immunization conditions and supernatants were established in our lab. Different immunization strategies were assayed with isolated centrosomes, (1) by absorbing solubilized centrosomes to the cell culture flask, (2) by directly adding solubilized centrosomes to the immunization mixture, (3) by absorbing solubilized centrosomes to silica beads and exposing the coupled antigen in the presence of macrophages to spleen cells and (4) by exposing intact centrosomes in the presence of macrophages to spleen cells. The last two stragtegies seem to be the most promising and the obtained antibodies are identified.

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SPECIFIC PROTEIN BINDING TO THE 3'-HAIRPIN OF PARVOVIRUS DNA AND ITS ROLE IN VIRUS REPLICATION

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The 3'-terminus of parvovirus replicative form DNA exists in two conformations, the hairpin and the extended forms. Using DNA of the autonomous parvoviruses Aleutian disease virus or the minute virus of mice, we showed that two factors from virus-infected cells, and one from uninfected cells, bind specifically to the hairpin form. One of the infected cell proteins is the viral capsid protein VP1. By hydroxyl radical footprinting we mapped the sites of interaction of the two infected-cell proteins within the 3'-hairpin. We are testing the effects of mutation of VP1 on viral DNA replication and using in vitro transcription and replication assays to determine the biological role of protein binding to the hairpin DNA.

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***IN VIVO* GENOMIC FOOTPRINTING OF THE TRANSCRIPTIONAL ACTIVATOR MCM1**

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The MCM1 protein activates transcription from both **a**- and α-specific UAS elements in the yeast *Saccharomyces cerevisiae*. Although MCM1 has similar affinity for both cell-type UAS elements (*in vitro* using purified proteins), it activates only **a**-specific genes in **a**-cells and α-specific genes in α-cells. The presence of MATα1 and MATα2 accounts for the different gene activities in α-cells. In **a**-cells, which lack these factors, our previous *in vitro* studies suggested that an active conformation is induced in MCM1 on binding to **a**-specific, but not α-specific UAS elements.

We have footprinted the **a**-specific STE2 UAS and the α-specific STE3 UAS in both cell types using DMS and DNaseI in conjunction with ligation-mediated PCR. Preliminary results show that MCM1 is bound to both elements in α-cells, but only to the STE2 element in **a**-cells. The lack of binding *in vivo* to the STE3 element in **a**-cells indicates a role for other factors, such as chromatin structure, not present in the *in vitro* studies.

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CHROMATIN REPLICATION *IN VITRO*

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We have used the SV40 *in vitro* replication system together with an *in vitro* chromatin assembly system to study the process of chromatin replication.
Using Xenopus egg or oocyte extracts plasmids containing the entire SV40 genome (pJym) were reconstituted into chromatin in the presence or absence of SV40 T-Antigen (T-Ag). Our data indicate that the binding of T-Ag inhibits the formation of nucleosomes around the SV40 origin and thereby increases replication efficiency. Analysis of replicating minichromosomes in the electron microscope shows that the segregation of the parental nucleosomes to the daughter strands is random. Furthermore the data indicate that the histone octamer assembly on the daughter strands occurs in two steps, starting with the deposition of H3/H4 tetramers on the DNA, followed by the binding of the H2A, H2B dimers.

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STRUCTURE AND CHARACTERIZATION OF THE COMPLETE CORE GENE SET FROM THE THERMOPHILIC CYANOBACTERIUM *MASTIGOCLADUS LAMINOSUS*

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Cyanobacteria harvest light energy through multimolecular antennae structures, the phycobilisomes. They are composed of a central three cylinder core from which six rods radiate. Incident light is mainly absorbed by the rod components and funneled through the core to the chlorophyll in the PSII. In the cyanobacterium *Mastigocladus laminosus*, genes for all core phycobiliproteins and their linker polypeptides have been cloned, sequenced and characterized. Of the six core proteins, allophycocyanin α^{AP}, β^{AP} and the so called core membrane linker L_{CM}^{120} seem to play major roles in terminal energy transmission. The genes for these polypeptides are located on the same operon together with the minor core linker. The apcEABC gene cluster, as well as the apcD and apcF genes, are compared to their counterparts in four other organisms.

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THE SYNTHESIS OF THE VIRAL PROTEINS IN GRANULOSIS VIRUS-INFECTED LARVAL TISSUES

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The replication of *Adoxophyes orana* granulosis virus (AoGV) was investigated in vivo. Three larval tissues, the fat body, the hemolymph and the midgut were chosen for detecting viral protein synthesis. In the fat body, SDS-PAGE revealed changes in the host proteins after AoGV infection. One host protein band (L26) disappeared gradually, while two other bands (V28, V34) increased in quantity. Immunoblot with three specific rabbit antisera against different viral structure demonstrated that the two last mentioned bands were different viral structural proteins: V28 was the granulin that forms the viral inclusion bodies (capsules), whereas V34 was a constituent of the enveloped nucleocapsids. The viral proteins were detected early on day 4 after the infection in the fat body and on day 7 in the hemolymph, but not in the midgut. Immunoblot also revealed that the two viral proteins were synthesized synchronously in the larval tissues. Another noticeable point is that one of our virus-specific antisera recognizes a larval protein. Whether this host protein is passively included into virus structures during viral replication or whether some viral component has a similar antigenic structure is unknown.

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STRUCTURAL STUDIES ON THE COMPLEX OF RECA PROTEIN WITH SINGLE-STRANDED DNA.

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The complex of recA protein with ssDNA is the active species for the *in vitro* reactions of strand-exchange (the event leading to homologous recombination) and cleavage of lexA repressor (the mechanism of control of the SOS system of repair). RecA forms a ~100 Å thick, helical coat on DNA with ~6 monomers of protein (38 kD) per turn.
Here we analyse the stroichiometry of binding with ssDNA as determined by various approaches; as a function of the input stoichiometry (nucleotides per recA), we measure the enzymatic activity (ATPase and lexA cleavage), the binding of DNA (DNAse protection and etheno-fluorescence) and two structural parameters by electron microscopy (the contour length and helical pitch).
The binding is found to be less "stoichiometric" than predicted in molecular models. We speculate that this "non-static" binding reflects the higher dynamism (enzymatic activity) of this complex as compared to the well ordered complex with dsDNA.

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GENETIC ANALYSIS OF ACBP: A SINGLE-STRAND ARS CONSENSUS BINDING PROTEIN FROM YEAST

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A protein of 67kDa has been purified from yeast nuclear extracts that shows a high affinity for the T-rich single strand of the ARS consensus (Hofmann and Gasser, 1991). The ARS consensus is essential for initiation of DNA replication in yeast. Point mutations in the 11bp ARS consensus that eliminate the ability of the ARS to promote initiation, also have significantly reduced affinity for ACBP. An antibody raised against the purified factor recognizes a single 67kDa protein in whole cell extracts from *S. cerevisiae* and also recognizes a protein of similar size from *S. pombe*, human and mouse cells.
Using an antibody to screen an expression library, the gene for ACBP has been cloned. The sequence reveals a leucine zipper motif and has homology to myosin and other proteins containing a coiled coil domain involved in protein-protein interaction. Studies have been pursued to ascertain whether or not ACBP is functional as a dimer. Gene disruption has been performed and the resulting phenotype will be discussed.

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Phosphorylation of Topoisomerase II: Effects on Protein:DNA and Protein:Protein Interaction

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In vitro phosphorylation experiments have shown that topoisomerase II is a substrate for casein kinase II, protein kinase C, Ca^{2+}/calmodulin-dependent protein kinase and $p34^{cdc2}$ kinase (Cardenas et al, 1992). In all cases phosphorylation *in vitro* enhances topoisomerase II activity, as measured by the kinetoplast decatenation assay. Studies with cell cycle-arrested yeast cells show that in intact cells topoisomerase II is 6-10 times more highly phosphorylated in mitosis than in G1. Among major phosphate accepting peptides, all but two comigrate with those phosphorylated by purified casein kinase II. The location of putative CKII phosphorylation sites in yeast topoisomerase II was determined by computer analysis and confirmed by 2D phosphoaminoacid analysis. The four modified peptides are in the C-terminal third of topoII and each contains multiple casein kinase II sites.
The most clear cut effect of topoII modification by CKII is a dramatic stimulation of its decatenation activity. Dephosphorylation of topoisomerase II renders the enzyme unable to decatenate kinetoplast DNA and its affinity for DNA is greatly diminished, as measured by a gel retardation assay. Consistent with a role in chromosome condensation is the enhancement of protein-protein interactions and aggregation upon hyperphosphorylation *in vitro*. To determine which sites play a role in the observed stimulation of activity requires *in vitro* mutagenesis of the acceptor sites, work that is currently in progress.

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CLONING OF MOUSE DNA POLYMERASE δ (pol δ)

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Cloning and sequencing of the yeast *Saccharomyces cerevisiae* pol δ (A. Boulet et al. EMBO J. 8:1849 (1989)) allowed the definition of homology boxes that are present only in herpesviral DNA polymerases and *S.cerevisiae* pol δ (δ-like boxes). In order to clone a mammalian pol δ from a mouse cDNA library we performed PCR using primers corresponding to conserved boxes and screened the cDNA library with one subclone of the PCR products. The sequence deduced so far spans 2135 bp which equals about two thirds of the open reading frame of *S.cerevisiae* pol δ. The amino acid sequence identity is about 55%, the similarity (taking conservative amino acid substitutions also into account) about 72%. Homologies to the meanwhile known sequences of *S.pombe* pol δ (Pignède et al. J.Mol.Biol. in press) and *Plasmodium falciparum* pol δ (Robert Ridley, personal communication) are in the same range.

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ANALYSIS OF POLY(ADP-RIBOSE) MOLECULES DURING DNA REPAIR *IN LIVING CELLS*

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We have recently shown that the poly ADP-ribosylation system may serve as a histone shuttle mechanism on DNA. The histone binding capacity is determined by the numbers, sizes and branching frequencies of ADP-ribose polymers (Panzeter et al., Biochemistry, *in press*). Using *in situ* radiolabeling of ADP-ribose polymers in cultured human keratinocytes, we have been able to study specific changes in their molecular properties (numbers, sizes, branching) during DNA excision repair in living cells. Results: The polymer pattern formed in response to DNA damage undergoes specific changes which include the numbers, sizes and branching frequencies. Interestingly, the fraction of branched polymers with very high binding affinity for histone H1, accumulated during a 45 min repair period, while the overall amount of poly(ADP-ribose) exhibited a transient increase during this time. These data suggest that different ADP-ribose molecules are processed in a differential manner during DNA excision repair *in vivo*.

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NUCLEOTIDE SEQUENCE IMAGES FOR RECOGNITION AND COMPARISON OF NUCLEIC ACID SEGMENTS.

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By assigning different vectors to each of the four nucleotides A, C, G, and T, any nucleic acid sequence (NAS) can easily be transformed into a 2-dimensional graph - the "nucleotidogram".

Thus, in contrast to conventional methods of NAS-analysis, a pictorial display of NAS is constructed. Complementary sequences are represented by vectors with inverted direction. Reverse sequences appear as a mirror image of the original sequence. When comparing different NAS, quick information is visually obtained, since related NAS show similar nucleotidograms. This allows easy localisation of different types of mutations. PCR primers or hybridization probes are matched to their target sequences by superimposing their graphs. Restriction enzyme recognition sequences that are palindromic appear as symmetrical patterns in the "nucleotidogram".

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Eukaryotic DNA helicases

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DNA helicases are enzymes that transiently melt double-stranded DNA in order to provide DNA and RNA polymerases with single-stranded DNA substrates. Four different DNA helicases (A, B, C and D) were isolated from calf thymus and could be distinguished by: (i) different molecular weights, (ii) nucleotide requirements, (iii) direction of movement, (iv) DNA binding properties, (v) ability to displace long DNA fragments and (vi) stimulation by replication factor A. Histones were shown to inhibit unwinding activity of DNA helicase A *in vitro*. The inhibition mediated by histone H1 could be overcome by an excess of DNA helicase A. Enzyme activity could also be restored by addition of ADP-ribose polymers to the *in vitro* DNA helicase assay. ADP-ribose polymers specifically interact with histones (Panzeter, P.L., Realini, C. & Althaus, F.R., Biochemistry, 1991, *in press*).

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C-terminal deletions of interferon-γ alter its ability to bind RNA and modulate the activity of ribonucleases

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Human and bovine recombinant interferon-γ (IFN-γ) inhibit the degradation of ss- and ds-RNA by bovine pancreatic RNase while they activate the activity of bovine seminal RNase on the same substrates (FEBS Letters, **270**:229-332, 1990). We prepared hu-IFN-γ with deletions of 5-6 or 17-18 amino acids from the C-terminus with the proteases Factor Xa and "Arg-C" respectively. Deleting 5-6 amino acids did not diminish the activity of IFN-γ in any of the assays. However, the Δ18 protein, which retained about 30% of the specific antiviral activity of the parent molecule, was no longer capable of binding RNA, nor did it inhibit RNase A. The Δ18 IFN-γ was about 1/3 as active as the wt molecule in activating the degradation of ssRNA by bovine seminal RNase, but its activation effect on the hydrolysis of ds-RNA was unaffected.

We conclude that the RNA binding site of IFN-γ lies in its positively charged C-terminus; its ability to bind RNA is related to its inhibitory effect on RNase A. These results, together with the effect of specific monoclonal antibodies on the assays, allow us to conclude that another area of the protein is responsible for the activation of cleavage of ds-RNA by selected nucleases.

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SITE-SPECIFIC INCORPORATION OF CHEMICAL PROBES INTO PROTEINS MEDIATED BY A CEMICALLY CHARGED SUPPRESSOR tRNA

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To probe the molecular environment of a selected site within a protein or polypeptide, a novel crosslinking approach is being developed. An amber suppressor tRNA was constructed and chemically aminoacylated (charged) by a derivatized cysteine. The high reactivity of the sulfhydryl group allows selective modification of the cysteinyl side chain either prior or after chemical acylation of the suppressor tRNA by a reagent of choice. Upon alkylation with a radioiodinated (125-iodine) thiol specific reagent the modified cysteine is shown to be incorporated into proteins in a cell free translation system (wheat germ) in response to an amber (UAG) codon. Alkylation with a radiolabeled, photoactivatable and cleavable reagent will lead to modified proteins which make unique probes for studying interactions with other macromolecules as well as for identifying nearest neighbors.

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THE SPECIFICITY OF THE RECOGNITION OF RETROVIRAL GENOMIC RNA BY THE VIRAL NUCLEOCAPSID PROTEIN

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An important aspect of the retrovirus life cycle is the packaging and dimerisation of its genomic RNA during virus assembly. We have shown that the NC protein of the *gag* gene is involved in this process and particularly the 2 Cys-His motifs of this protein. To further study the specificity of retroviral RNA packaging we have constructed chimeric Rous sarcoma virus (RSV) particles expressing either the NC protein of Moloney murine leukemia virus (Mo-MLV) or an NC protein derived from RSV in which either of the Cys-His motifs has been replaced by the equivalent sequence of Mo-MLV. These mutants viruses have been transfected into fibroblasts and tested for their ability to package either RSV or Mo-MLV RNA. In order to further delineate the recognition of the packaging sequence by the NC protein, the Psi packaging signal of Mo-MLV has been cloned into these chimeric mutants. The results obtained with each of these virus mutants will be presented.

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RNA-PROTEIN AND PROTEIN-PROTEIN INTERACTIONS IN THE SIGNAL RECOGNITION PARTICLE.

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SRP, a cytoplasmic ribonucleoprotein, plays an essential role in sorting proteins to the ER. During targeting, SRP effects a specific but transient inhibition of the synthesis of presecretory proteins Two proteins, SRP9 and SRP14, and the Alu-sequences of SRP RNA are essential for this function. SRP9 and SRP14 specifically bind SRP RNA only as a heterodimer. We are currently identifying structural elements in SRP9 and SRP14 which are essential for dimerisation, for SRP RNA binding and for conferring elongation arrest activity to SRP. Preliminary results indicate that the middle portion but not the C-terminal part of SRP9 are required for RNA binding. Interestingly, a fusion protein containing SRP14 and SRP9 sequences binds SRP RNA as well as the heterodimer SRP9/14. In addition, we found that c-myc epitopes fused to the C-terminal part of SRP9 and SRP14 do not interfere with SRP9 and SRP14 functions. We are now using these fusion proteins as well as tagged SRP19 protein to study SRP assembly and the subcellular distribution of SRP in vivo.

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INTERACTIONS OF BACTERIOPHAGE Qβ REPLICASE WITH Qβ PLUS AND MINUS STRAND RNA

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Replication of bacteriophage Qβ RNA by Qβ replicase proceeds via synthesis of complementary minus strands which serve as templates for Qβ plus strand synthesis. The reaction can be carried out *in vitro* with pure enzyme and is highly template specific. Earlier it was found that recognition of the plus strand template by replicase involved strong binding interactions at one or two internal sites of the RNA (depending on conditions), while binding at the 3'-end (where synthesis starts) was detectable only after initiation. We now prepared radioactive plus and minus strands by *in vitro* transcription from suitable plasmids containing the full-length phage sequence and studied their binding complexes with replicase by partial degradation with ribonucleases. Southern analysis of radioactive RNA fragments remaining bound to the enzyme confirmed the interaction at the expected internal site of the plus strand. Minus strand binding was generally less stable; the RNA fragments recovered from the complexes were derived from the 3'-end, the 5'-end, and from an internal site overlapping with the sequence complementary to the plus strand binding site. We conclude that replicase interaction with the minus strand, which is a better template than the plus strand, shows less sequence specificity, but that affinity to the 3'-end is relatively higher.

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BINDING OF CAMV TRANSACTIVATOR TO PLANT RIBOSOMES

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The genome of cauliflower mosaic virus (CaMV) is organized into seven ORFs, but only two mRNAs are available. The monocistronic 19S RNA covers ORF VI and the polycistronic 35S RNA spans the total viral genome. Polycistronic translation is unusual for eucaryotes and the 35S RNA is only translated in the presence of the ORF VI geneproduct. This so-called *trans*-activator (TAV) acts in *trans* at a posttranscriptional level and enhances the translation of downstream ORFs on polycistronic mRNAs. In order to study the *trans*-activation mechanism, I wanted to learn if TAV binds to the plant translation machinery. Polysomes from CaMV infected plants have been size fractionated in sucrose gradients and tested by Western analysis for the presence of TAV. A reconstitution assay has been developed to study binding of *E. coli* expressed TAV to plant ribosomes under various conditions. The experiments described above showed that TAV forms a 100-150S complex with polysomes. High salt concentration inhibited complex formation suggesting a non covalent binding of TAV to ribosomal 1- or 2-mers.

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Structural alterations of U7 snRNPs induced by block of cells in G1 phase or by heat treatment of extracts

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The U7 small nuclear ribonucleoprotein (snRNP) is involved in 3' end processing of histone pre-mRNA. Processing additionally requires a heat-labile factor (HLF), i.e. snRNP-depleted and heat-inactivated extracts complement each other. U7 snRNPs can be visualised on native gels by decoration with an oligonucleotide complementary to the 5' end of U7 RNA. In heat-inactivated extracts, this complex migrates slightly faster than normal. A similar mobility change can also be observed in extracts from cells arrested in the G1 phase of the cell cycle which are known to be deficient in HLF. A possible reason for this is that HLF may be a subunit of the U7 snRNP which is subject to cell cycle-dependent inactivation or dissociation. We have found that U7 complexes from heat-treated or G1-arrested cells still react with anti-Sm antibodies directed against the common snRNP proteins, but little is known, so far, about two recently described U7-specific snRNP proteins. Molecular size estimations using native polyacrylamide gradient gels and gel filtration chromatography are being performed to analyse the molecular basis for the observed mobility difference of U7 complexes. Additional faster migrating complexes observed in extracts treated with detergents may thereby serve as molecular markers for U7 complexes lacking individual protein subunits.

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RESPONSE OF HUMAN KERATINOCYTES TO LOW DOSES OF CARCINOGENS

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The most sensitive method to study DNA strand breaks is the nucleoid sedimentation technique. Nucleoids consist of RNA, supercoiled DNA and nucleoid cage proteins. DNA integrity is studied by measuring the distance sedimented by nucleoids through neutral sucrose gradients - nucleoids with damaged DNA sediment slower than those with undamaged, supercoiled DNA. Using this technique, nucleoids derived from human keratinocytes were studied after treatment with carcinogens. DNA damage was induced by H_2O_2 or N-methyl-N'-nitro-nitrosoguanidine (MNNG). When the cells were treated with very low, non-toxic concentrations of these agents (10-100 nM H_2O_2 or 1-50 nM MNNG), resulting nucleoids sedimented faster than those derived from untreated cells. Carcinogen treatment of cells on ice or in the presence of 2mM benzamide did not completely abolish the low-dose carcinogen response. Cellular response to low doses of carcinogens may involve an induction of DNA repair mechanisms and/or structural changes in the nucleoid cage influencing nucleoid sedimentation rates.

Synaptogenesis

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LESION-INDUCED SYNAPTIC REGENERATION IN ORGANOTYPIC HIPPOCAMPAL CULTURES

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We recently described a technique for preparing organotypic cultures of nervous tissue in which tissue slices are maintained on a porous and transparent membrane at the interface between culture medium and atmosphere. An interesting advantage of this technique is that the cultures are easily accessible for analyses or manipulations at any time in the culture. We have studied the effects of producing sections of fibers between groups of pyramidal neurones in hippocampal slice cultures. The sections were produced after different times in culture by cutting transversely the entire hippocampal slice between CA4 and CA1 areas with a razor blade. Electrophysiological recordings were carried out by stimulating a group of neurones on one side of the section and recording the synaptic responses elicited on the other side. The results show that synaptic regeneration occurs extremely quickly within 1-4 days in culture. Labelings of neurones using extracellular applications of Horse Radish Peroxidase and immunohistochemistry directed against axonal and synaptic components also showed clear evidence of regeneration. The speed with which regeneration occurred varied as a function of the age in culture, being slower in older cultures. This approach may represent an interesting model for studies of reactive synaptogenesis (Work supported by FNRS 31730.88 and 31.30980.91 and De Reuter Foundation).

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REPAIR OF INJURED NEONATAL MAMMALIAN SPINAL CORD IN CULTURE

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Little is known about how central nervous systems of embryonic and neonatal mammals regenerate after injury. The isolated CNS of the new-born opossum has been shown to survive in culture and provides a favourable system for studying repair. The entire CNS was isolated and the spinal cord crushed, abolishing all through conduction. Preparations were then maintained in tissue culture for up to 6 days, during which the ability of the cord to conduct action potentials was monitored. Three to 4 days after the lesion had been made, transmission through the spinal cord was re-established. By this time, axons stained with DiI or horseradish peroxidase had grown profusely through the lesion and several millimetres beyond it.

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SYNAPSE-SPECIFIC EXPRESSION OF AChR mRNAs IN MUSCLE FIBRES REGENERATED IN THE ABSENCE OF THE NERVE.

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Innervation of skeletal muscle fibres induces a local change in the pattern of expression of genes for the acetylcholine receptor (AChR) in the myonuclei at the synapse. This results in a switch in the subunit composition of the AChRs in the muscle membrane from $\alpha 2\beta\gamma\delta$ to $\alpha 2\beta\varepsilon\delta$ and in the functional properties of their ion channels. In addition, the expression of α-, β- and δ-subunits at the synapse, but not elsewhere, becomes resistant to the down-regulating effects of muscle activity. The nature of the neurotrophic signals that control synapse specific expression of AChR genes is not known. We now show that when adult rat soleus muscle fibres are destroyed and new ones are allowed to regenerate in the absence of innervation but within pre-existing basal lamina sheaths, ε- and α-subunit mRNAs are again expressed in an activity-resistant manner in the region of the original synaptic sites. Furthermore, AChR channels with functional properties characteristic of those containing the ε-subunit accumulate at these same sites. Thus, in spite of degeneration of the muscle fibres, factors survive at original synaptic sites which induce the synapse-specific expression of AChR genes in nuclei of regenerated muscle fibres in the absence of the nerve.

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CALCIUM INFLUX AND PROTEIN PHOSPHORYLATION MEDIATE METABOLIC STABILIZATION OF SYNAPTIC AChR.s IN MUSCLE

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When a neuromuscular junction is formed, the synaptic AChR.s become metabolically stabilized, their half-lives increasing from about 1 day to 10-15 days. This process is mediated by electrical activity induced in the muscle by the nerve. We have investigated the pathways linking muscle activity and AChR stabilization in chronically denervated rat soleus muscles in organ culture. AChR half-lives were estimated from the decay of endplate-bound ^{125}I-α-Butx-AChR complexes with time. Six hours of high frequency electrical stimulation were sufficient to stabilize the AChR.s. Stabilization was prevented in muscles stimulated in the presence of the Ca^{++} channel blocker (+)PN 200-110 and could be induced in nonstimulated muscles by the Ca^{++} channel activator (+)SDZ 202-791 in elevated K^+. The stabilization process was not prevented by cycloheximide, required shorter stimulation periods (4.5hrs) in the presence of the phosphatase inhibitor okadaic acid and could be brought about in inactive muscles by treatment with DBcAMP. AChR stabilization was not induced by the phorbol ester TPA nor could it be prevented in stimulated muscles by staurosporine, and thus is independent of kinase C activation.We observed activity-dependent DHP-sensitive phosphorylation of myosine light chain which was dependent on Ca^{++} influx through DHP-sensitive Ca^{++} channels. These findings are consistent with the notion, that muscle activity causes AChR stabilization via the activation of Ca^{++}-dependent protein phosphorylation reactions.

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BURSTING SPONTANEOUS SYNAPTIC ACTIVITY IN SPINAL CORD CULTURES: POSSIBLE INVOLVEMENT OF REENTRY.

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The formation of functional synaptic networks during development is believed to be influenced by the electrical activity within such networks. Thus spontaneous activity arising in isolated circuits may be of great importance during development. In tissue cultures of embryonic rat spinal cord, dorsal root ganglia and skeletal muscle, spontaneous postsynaptic potentials as well as synaptically evoked action potentials were routinely seen in the motoneurons. In 84% of the cells these potentials appeared randomly at frequencies of 4 to 200 per min. In 16% of the cells the synaptic activity was clustered forming bursts of postsynaptic potentials or action potentials which were then followed by silent periods. When the inhibitory potentials in the cultures were blocked by bicuculline, strychnine or both, the pattern of spontaneous activity changed from random to bursting. Strychnine was the most potent in inducing bursting activity. With 10 μM strychnine bursts were induced in 100% of the cells. The EPSPs within the strychnine induced bursts declined in amplitude. A similar depression of EPSPs was seen when they were evoked by focal electrical stimulation of the dorsal horns at frequencies similar to those during the bursts. When single stimuli were applied at the end of a spontaneous burst, depressed EPSPs were evoked. On the other hand, the spontaneous bursts were supressed during regular stimulation. These results suggest that the same synaptic pathways were repetitively activated during spontaneous bursts as during stimulation. Such a behaviour could be explained by reentering activity within positive feedback circuits. *Supported by SNF No. 31-27553.89*

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ONTOGENY OF DOPAMINE D_1 RECEPTORS IN RAT BRAIN : AN AUTORADIOGRAPHIC STUDY

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The development of D_1 dopamine receptors in the pre- and postnatal rat forebrain was studied using quantitative autoradiography. Long Evans rat pups and foetuses of different ages were frozen and sectioned coronally and sagittally at 10 μm. For the characterization of the binding to D_1 receptors the slides were incubated with the selective D_1 antagonist [^{125}I]SCH 23982. Labelled sections were apposed to ^{125}I sensitive film along with ^{125}I plastic standards for different exposure periods. In the postnatal period D_1 receptors increase in number from birth to about one month of age at which time receptor density is similar to that found in adults. The localization of dopamine D_1 receptors in the forebrain of the rat at PN1 is quite similar to the distribution of receptors found in the adult rat. The highest levels of receptors are found in regions like caudate-putamen, nucleus accumbens and the olfactory tubercle. The regional development of D_1 receptors in the foetus is under investigation.

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POSTNATAL MATURATION OF $GABA_A$-RECEPTOR SUBUNITS EXPRESSION IN RAT BRAIN: IMMUNOHISTOCHEMICAL ANALYSIS

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Maturation of the $GABA_A$-receptor α_1- and $\beta_{2,3}$-subunits was studied immunohistochemically in Long Evans rats between birth (pn1) and pn28. The distribution of these subunits was mapped in olfactory bulb, striatum, cerebral cortex, hippocampus and cerebellum. Rats were perfused with paraformaldehyde and free-floating sections were stained with the ABC method using anti-α_1-specific polyclonal antisera and the monoclonal antibody bd17 which selectively recognizes the β_2- and β_3-subunits. α_1-Immunoreactivity was faint at birth and increased to adult levels by pn28. In cerebral cortex, intense α_1-staining was observed in layers III-IV of visual and somatosensory areas between pn6 and pn14. In cerebellum, Purkinje cell dendrites were intensely stained in young animals, but not at pn28. In contrast to α_1-immunoreactivity, bd17-staining was intense at birth and increased further only in mitral cells, hippocampus and cerebellar granule cells. The results demonstrate a differential developmental expression pattern of the α_1- and $\beta_{2,3}$-subunits in immature rats suggesting that subunit composition of $GABA_A$-receptors changes during maturation.

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TERM1: A SURFACE LABEL FOR IDENTIFIED GROWTH CONES AND DEVELOPING SYNAPSES IN THE GRASSHOPPER.

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The chemoaffinity hypothesis postulates that neuron-specific molecular labels are involved in generating synaptic specificity. We are using monoclonal antibodies to isolate molecular labels of this type in the grasshopper embryonic nervous system The molecular label with the highest specificity is expressed on a pair of identified descending interneurons, which project from the brain to the segmental ganglia. During axonogenesis, this cell surface label is expressed on the axonal growth cones and on the ganglionic collaterals of these neurons. During synaptogenesis, the label becomes concentrated at the presynaptic terminals of these neurons. This neuron-specific label, which we call TERM1 for terminal recognition molecule 1, is not expressed on any other of the millions of neurons in the embryonic nervous system, nor on any other cell in the developing embryo. These findings suggest the existence of a highly specific molcular recognition system for developing neurons. The biochemical characterization of TERM1 and related molecules is now in progress. (Supported by the Swiss NSF).

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FUNCTIONAL MAPPING OF THE AMYLOID β-PROTEIN PRECURSOR.

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The secreted form of the amyloid β-protein precursor (APP) is involved in the growth regulation of fibroblasts (Saitoh et al., Cell 58:615-622, 1989). To determine which domain of the molecule is responsible for this activity, we used APP fragments made in *E. coli*. Plasmids which drive the synthesis of various fragments of the extracellular domain of APP-695 were constructed using a prokaryotic expression vector. These fragments were purified from bacterial lysates and tested for the ability to support the growth of A-1 fibroblasts, a cell line which produces very low levels of APP and depends on the presence of exogenous APP in the medium for normal growth. Lysates of bacteria carrying the expression vector were used as negative controls. Whereas some fragments showed no growth stimulating activity, others were active. The results suggest that this biological activity of the secreted form of APP-695 is contained within a region 40 amino acids in length. Indeed, a 40-mer peptide corresponding to this region stimulated A-1 cell growth. Experiments using small peptides covering this region are under way to further define the site of activity.

Structural Aspects of CNS Function

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How specific are synaptic connections in the hippocampus?

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The various afferent fiber systems in the hippocampus terminate in a laminated fashion. Previous studies have suggested that this lamination is largely due to spatio-temporal constraints during ontogenetic development. In these hippocmpal layers some afferent fibers establish synapses with all available target cells, whereas other afferents are restricted to distinct types of neurons. A particularly high degree of neuronal specificity is found in dentate axo-axonic cells which are restricted not only to specific types of target cells but also to distinct portions of the target cells' membrane. These data indicate that there are different levels of target cell specificity of the various hippocampal afferents. Different levels of neuronal specificity suggest that different factors are involved in the formation of the various hippocampal connections during ontogenetic development. Possible factors that determine the different levels of neuronal specificity in the hippocampus could be 1) soluble molecules from the target region, 2) positional cues for the ingrowing growth cone, 3) specific molecular interactions between pre- and postsynaptic membranes, 4) the naturally occurring cell death in some projections, and 5) the selective stabilization of synaptic contacts.

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CAUSES AND CONSEQUENCES OF EPILEPSY IN THE HIPPOCAMPUS. **Scott M. Thompson**

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The hippocampus is especially prone to epilepsy as a result of the organization of its synaptic circuits. When inhibitory synaptic potentials are reduced by convulsant drugs, powerful excitatory synaptic connections between CA3 pyramidal cells cause them to discharge synchronously. Synaptic inhibition can also become transiently decreased after repetitive activation, and this is sufficient to produce epileptic discharge in otherwise normal tissue. Several endogenous mechanisms that decrease inhibitory synaptic potentials will be described, as well as potential compensatory mechanisms.

Human epilepsy is accompanied by a characteristic pattern of cell damage in the hippocampus. We have developed an *in vitro* model of chronic epilepsy in hippocampal slice cultures with which to examine whether this injury is a **cause** or **consequence** of epilepsy. After 3 days in the presence of convulsants, cultured hippocampal neurons had swollen, vaculated somata and a drastically reduced number of dendritic spines, the site of excitatory synaptic contacts. These morphological changes were accompanied by a decrease in the size of excitatory synaptic potentials. Neither the number of inhibitory interneurons nor inhibitory synaptic potentials were affected, however. We can therefore conclude that such changes result from the epileptic activity *per se*, and this model will hopefully provide an *in vitro* alternative for development of anticonvulsant and neuroprotective drugs.

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THE GERBIL PERFORANT PATH CONTAINS PARVALBUMIN (PV): MORPHOLOGY AND FUNCTIONAL IMPLICATIONS

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PV is a high affinity Ca^{2+} binding protein found in fast spiking neurons in CNS. PV may act as early buffer of Ca^{2+} transients influencing ion conductances and Ca^{2+} triggered effector mechanisms. Immunocytochemistry revealed species specific, selective presence of PV in the terminal field of the perforant path projection in the hippocampus of the gerbil (Meriones unguiculatus), an animal species genetically predisposed to seizures. Ultrastructurally, PV was contained in axospinous boutons with asymmetric synaptic contacts in the molecular layer. PV staining disappears from this zone upon ablation of the entorhinal cortex. As a test for the functional characteristics of excitatory perforant path influence of elevated extracellular Ca^{2+} concentration on the activity of the dentate area was studied in gerbil hippocampal slices. A long-term potentiation (LTP) response did not develop in the gerbil in contrast to the effects known in the rat. LTP following exposure to high Ca^{2+} solutions depends on prolonged intraterminal elevation of the ion and consequent increase of neurotransmitter release per impulse. PV in synaptic terminals may prevent it by buffering residual Ca^{2+} lingering at release sites.

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LIFETIME CHANGES IN THE HIPPOCAMPAL MOSSY FIBER SYSTEM OF THE GUINEA PIG

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Behavior of mammals undergoes periods of change throughout life: pre- and postpubertal playfulness gives way to increasing predictability of behavior and habits form which resist extinction. This suggests protracted and slow structural modifications of limbic circuitry. We have investigated the proliferation of recurrent mossy fiber collaterals in the fascia dentata of guinea pigs aged 5, 10, 20, 40, 80, 160, 320, 640 and 1100 days (8 animals per group). Prior to morphometry of Timm stained hippocampi animals were tested for labyrhinth activity and shock escape behavior.
Density of recurrent mossy fiber terminals increased steadily until a first growth spurt around puberty. After a phase of stability a second massive spurt peaking at two years of age was observed. Thereafter we found a massive decline to postpubertal levels. Shock escape behavior showed age dependent changes that were however not in phase with mossy fiber growth. Labyrinth activity showed high variability across all ages. The observed structural changes may reflect the operation of an ultra-slow system stabilizing aquired patterns of neuronal activity by means of adaptive axonal growth.

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MODELLING FUNCTIONAL STATES OF THE BASAL GANGLIA WITH NEURONAL NETWORKS

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We have investigated the role of a sequential network with a structural analogy with the motor control system (Jordan) in assessing the functional properties of the basal ganglia. Trajectories of hand movements in Parkinsonian patients without medication -"off-states"- and with optimal dopaminergic substitution -"on-states"- were used as learning curves for a neuronal network with 4 neuronal layers. Movements in the working space of the arm could be identified using 54 interconnected neurons. Since mayor differences between "on" and "off" states were seen in the neuronal connections between state neurons and hidden neurons , these neuronal layers of the Jordan network can be interpreted as representing functional states of the striato-pallido-thalamic pathways of the motor circuit, which are mainly influenced by the dopaminergic therapy in Parkinson's disease.

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MAPPING OF c-FOS EXPRESSION AS A RESULT OF ELECTRICAL STIMULATION OF THE RAT SENSORIMOTOR CORTEX

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In order to distinguish, at cellular level, the neuronal populations activated directly (monosynaptically) from those activated indirectly (polysynaptically) by electrical stimulation of the principal forelimb area of the sensorimotor cortex, c-fos functional tracing with immunocytochemistry was combined with axonal tracing techniques using biocytin or phaseolus vulgaris-leucoagglutinin (PHA-L). c-fos-like immunoreactive neurons were seen in the rostral cortical forelimb area, caudate-putamen, globus pallidus, reticular, posterior and ventromedial thalamic nuclei, subthalamic nucleus, substantia nigra, tectum, red nucleus, pontine nuclei, inferior olive, external cuneate nucleus and cerebellum. As a result of injection of biocytin or PHA-L at the site of stimulation, dense anterogradely labeled terminal fields were seen to overlapp the c-fos-like positive neurons in each of these regions, except the globus pallidus and the cerebellum. It can be concluded that these two latter structures were activated indirectly, while all other structures were activated most likely directly. The combination of the c-fos functional and PHA-L or biocytin anterograde axonal tracing techniques represents a promising tool to establish the fine connectional properties of the various populations of neurons involved in a defined neural process and to determine the successive steps of the cascade of activations taking place in the corresponding chains of neurons.

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TOPOGRAPHY OF RETINOGENICULATE FIBERS IN TREE SHREWS

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Five tree shrews (Tupaia belangeri) were used to investigate the fiber pathway of a selected retinal ganglion cell (GC) population in the optic nerve (ON), the chiasm and the optic tract (OT). Nerve fibers and neurons were retrogradely labeled by pressure injections of wheat germ agglutinin conjugated to horseradish peroxidase (WGA-HRP) into electrophysiologically defined positions of the dorsal lateral geniculate nucleus. In the experiments performed so far, labeled GC populations were found in the superior, inferior and temporal parts of the retina. The axons of those neurons were located in the ON on the same side as in the retina and occupied wedge-shaped areas in cross sections. No transformations of the GC fiber trajectories along the ON were observed. The superior-temporal-inferior distribution of labeled axons in the ON was followed by a medial-lateral distribution pattern in the OT. Thus, the findings of this preliminary study indicate that in a highly developed mammalian visual system the GC fibers run without any major transformations along the retinogeniculate pathway.
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BILATERAL AND UNILATERAL PROJECTIONS FROM THE LATERAL LEMNISCUS NUCLEI IN THE RAT

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The projection from the dorsal (DLL), intermediate (ILL) and ventral (VLL) nuclei of the lateral lemniscus were studied using iontophoretical injections of the anterograde tracer, Phaseolus vulgaris-leucoagglutinin in the rat. The projection from DLL to the inferior colliculus (IC) was bilateral while the projections from ILL and VLL were only ipsilateral. The projection from DLL reached the central nucleus (CIC) and the external cortex of the IC. In the CIC, labeled fibers from the DLL ran parallel to the fibro-dendritic laminae of the CIC and were apparently tonotopically organized. The projection to the external cortex (ECIC) was restricted to its deep layers and its rostral part. The projection from ILL and VLL reached the three subdivisions of the IC. In the CIC, labeled fibers were obliquely oriented with respect to the orientation of the laminae. The strong projection to the dorsal cortex (DCIC) occupied most of its layers.
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INTRINSIC CONNECTIONS AT THE BOUNDARIES OF THE PRIMARY AUDITORY CORTEX (AI)

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Cat AI is surrounded by auditory fields AII, AAF, PAF, and the suprasylvian fringe (SF). We have studied the intrinsic connections in AI and at its boundaries in 4 adult cats of which each received an injection of PHA-L at an electrophysiologically defined location of AI. Patches of labelled neurons and axon arborizations were found around the injection site, both in AI and in the adjacent auditory fields. The labelled neurons were in layers II-VI (less dense in IV) and 80 % were pyramidal; no abrupt changes occurred at AI boundaries. Axons crossed the AI boundary within layers I-VI and in the white matter. Five types of intrinsic axons were found in AI and crossing its boundaries with AII, AAF, PAF and SF. In conclusion, the intrinsic connections do not change abruptly at AI boundaries and may link locations with similar characteristics on either side. There is tonotopic continuity characterized by a reversal of the tonotopic gradient at the AI/AAF and AI/PAF boundaries; little is known about the AI/AII and AI/SF boundaries.

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SELECTIVE SEROTONERGIC INNERVATION OF CALBINDIN-CONTAINING INTERNEURONS IN THE HUMAN CEREBRAL CORTEX.

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The serotonergic innervation of the mammalian cerebral cortex originates in the dorsal or median raphe nucleus. Serotonergic axon terminals of median raphe neurons are characterized by large varicosities surrounding selectively calbindin-containing interneurons in the rat and marmoset cerebral cortex. Calbindin and parvalbumin are reported to be found in two distinct subpopulations of inhibitory cortical interneurons. In freshly-fixed biopsies of frontal and temporal association human cerebral cortex, three morphologically distinct types (thick and smooth, or thin with small or large varicosities) of serotonergic fibers were visualized immunocytochemically. Double-labelling for the detection of 5-HT and calbindin (or parvalbumin), demonstrated a preferential association of 5-HT-containing large varicose baskets surrounding calbindin-containing interneurons. No such associations were found between 5-HT- and parvalbumim-containing structures. Thus, in the human cortex, an association between midbrain serotonergic afferent and GABAergic cortical interneurons is also present, providing a substrate for a fine tuning of intracortical processing by a brainstem nucleus.

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INNERVATION OF RAT CORTEX BY BRAINSTEM SEROTONERGIC FIBERS IN ORGANOTYPIC SLICE CO-CULTURES

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The serotonergic innervation of the mammalian cerebral cortex arises from the median and dorsal raphe nuclei of the brainstem. Originating from the dorsal raphe nucleus, fine axons arborize throughout the cortex whereas, from the median raphe nucleus, large varicose 5-HT axons have a restricted areal and laminar distribution. In order to study the interactions of the serotonergic system with the cerebral cortex in isolation, coronal slices of brainstem and cortex were obtained from 0- to 2-day-old rats and cultured side by side on a porous and transparent membrane, without coating. Using 5-HT immunocytochemistry, we demonstrate that the midbrain neurons survive, maintain their cytoarchitectonic organization and that 5-HT afferents provide a dense innervation to cortical explant with a pattern of laminar distribution comparable to that present in vivo. Differentiation of cerebral cortex is illustrated by the presence of GFAP-positive astrocytes, as well as calbindin-, parvalbumin-, tyrosine hydroxylase-immunoreactive neurons and SMI-32-immunoreactive large pyramidal cells.

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BRADYKININ IMMUNOREACTIVITY IN THE MOUSE BRAIN

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Twenty mice were anesthetized and perfused with Bouin or Bouin-Hollande fixative. After paraffin embedding, 7 µm-thick sections were incubated with rabbit anti-bradykinin (BK) serum and treated by the peroxidase-anti-peroxidase (PAP) method. Controls with normal sera, rabbit anti-myelin basic protein (MBP) and anti-glial-fibrillary acid protein (GFAP) sera were performed in parallel. The results showed BK-positive cells in discrete areas of the brain stem. The cells observed were large and had a typical neuronal morphology in the trigeminal mesencephalic and ventral cochlear nuclei. Similar but medium-sized cells were found in the principal trigeminal nucleus and around the infundibulum. BK(+) cells were also numerous in the pial and ependymal structures. The tanicytes of the infundibular recess were also strongly BK(+).

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EXPRESSION OF VASOPRESSIN RECEPTORS IN HAMSTER HYPOTHALAMUS IS SEXUALLY DIMORPHIC AND DEPENDENT UPON PHOTOPERIOD.

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The distribution of vasopressin receptors was studied in the brain of a photoperiodic animal, the siberian hamster. Attention was focused on [^{3}H]vasopressin binding sites located in the hypothalamic ventromedial nucleus, medial tuberal nucleus and ventral premammillary nucleus in males or females kept in long or short photoperiod conditions. Quantitative data obtained with a gaseous detector of β-particles, indicated that in the ventromedial nucleus and in the ventral premammillary nucleus of animals in long photoperiod, the number of β-particles emitted was significantly greater in males than in females. In the ventromedial hypothalamic nucleus, in both males and females, the number of β-particles emitted was significantly lower in short than in long photoperiod conditions. In the ventral premammillary nucleus, shortening of the photoperiod had a significant effect in reducing the amount of [^{3}H]vasopressin bound in females.
These data suggest that, the control of the expression of vasopressin receptors differ amongst various hypothalamic nuclei and may depend on the sex and/or on the level of circulating gonadal steroids.

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THE POTENTIATION OF THE MONOSYNAPTIC TRANSMISSION BY SUBSTANCE P IN THE HEMISECTED RAT SPINAL CORD PREPARATION

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Substance P (SP) is thought to be involved in the transmission of nociceptive information from the periphery to the central nervous system. After nociceptive stimulation of peripheral nerves SP is released from C-fibers in the dorsal horn of the spinal cord and exerts a powerful excitatory effect on dorsal horn interneurons. It has recently been demonstrated that in rats i.t. application of SP facilitates nociceptive flexor reflex[1],an effect which mimicks the effect of a cutaneous C-fiber stimulation. We wanted to see whether SP has also an effect on the monosynaptic reflex in the hemisected rat spinal cord preparation in vitro. Recording extracellularely from a ventral root we found that SP dose dependently (0.01-30 µM) increased the amplitude of the monosynaptic reflex whereas the amplitude of the polysynaptic response was reduced. This effect could be blocked by the novel SP receptor antagonist CP-96,345[2] (3-30 µM; Pfizer). In intracellular recordings from motoneurons SP increased the number of action potentials induced by dorsal root stimulation.Our results demonstrate that SP impaired the polysynaptic components but in addition facilitated the monosynaptic reflex components.

1: Woolf,C.J. and Wiesenfeld-Hallin,Z.,Neurosci.Lett.,66(1986)226-230
2: Snider,R.M. et al., Science, 251(1991)435-437

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Agonist-induced desensitization of the dopamine D-1 receptor in the bovine retina *in vitro*.

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Dopamine (DA)-receptor down-regulation has been reported in cultured cell lines, mammalian brain and in the retina *in vitro*. Since we have developed an assay to investigate the actions of various dopaminergic agents at the retinal D-1 or D-2 receptor (positively and negatively linked to adenylate cyclase) respectively, we have attempted to elucidate the mechanism(s) of D-1 receptor desensitization in bovine retinas *in vitro*. Isolated retinas were incubated for 60 min at 37°C in either Earle's physiological saline alone (controls) or with different concentrations of DA. The retinas were then extensively washed, homogenized, and then re-exposed to different concentrations of DA, in the presence of 0.5mM ATP and 0.01mM GppNHp. Accumulated cAMP was used as an index of D-1 receptor stimulation. Initial results suggest that a 60 min incubation with 10mM- and 50mM-DA did not lead to an attenuation of D-1 response to re-stimulation by DA. However, pretreatment with 100mM DA did lead to a significant down-regulation of the D-1 receptor, indicating a homologous desensitization of the receptor under these conditions *in vitro*.

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THE EFFECTS OF CAFFEINE AND D-AMPHETAMINE ON PERFORMANCE IN A DRL 18-S SCHEDULE

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Various psychoactive drugs have been found to disrupt DRL performance, a paradigm used to measure time estimation ability and/or response suppression. Aim of the present study was to compare the effects of the two psychostimulant drugs caffeine and D-amphetamine on performance in a DRL 18-s schedule. Presession systemic injection of caffeine (3, 6, 12, 24, and 48 mg/kg) did not alter the number of responses (lever presses) during the following session of 45 minutes. Efficiency (reinforcements/total lever presses) was dose-dependently decreased. Further, a clear shift of the inter-response time (IRT) distribution to the left was seen with higher doses of caffeine (24 and 48 mg/kg), but no increase in response bursts (responses separated by 0-3 seconds) occurred. In contrast to caffeine, D-amphetamine (1 and 2 mg/kg) injected immediately before a test session increased the number of responses and markedly increased response bursts. Similar to caffeine, D-amphetamine decreased efficiency and shifted the IRT distribution to the left.

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CARBOHYDRATE METABOLISM IN RETINAL GLIAL CELLS

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Glucose uptake and phosphorylation has been demonstrated by 3H-2-deoxyglucose autoradiography and biochemistry to occur in insect and mammalian glial cells. Here, we explore the fate of 14C(U)-glucose in slices of honeybee retina and suspensions of freshly isolated Müller glial cells from the retina of the juvenile guinea pig. In the honeybee glial cells about 2% of the radioactivity was incorporated into glycogen and about 60% in the dissacharide trehalose. The major labelled product of the metabolism of 14C-glucose was alanine (about 30% of total radioactivity). Glutamate and aspartate were also labelled but to a much lesser degree. Proline was not labelled. In the mammalian glial cells the major labelled product was phosphoenol-pyruvate and the major labelled amino acid was glutamate. The Müller glial cells produced also important amounts of labelled lactate whose major part was found outside the cells.

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POINT MUTATIONS AFFECTING AGONIST GATING OF $GABA_A$ RECEPTOR CHANNELS

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The location of the agonist site on the $GABA_A$ receptor channel has not yet been described. We have investigated this problem using the recombinant subunit combination $\alpha1\beta2\gamma2$ expressed in Xenopus oocytes. A point mutation in the N-terminal portion of the extracellular domain of a1 led to a 200-fold decrease in the apparent affinitiy of GABA dependent channel gating (increase in EC_{50}) and in the affinity of the competitive GABA antagonists bicuculline methiodide (BM). Homologous mutations in the $\beta2$- and $\gamma2$-subunits did not change the affinity for BM. The mutations in $\gamma2$ and $\beta2$ led to an only 5-fold and 30-fold change in the EC_{50}, respectively. Our results suggest a) localization of the agonist binding site on the α-subunit, b) involvement of relatively N-terminal portions of all subunits in gating of the channel, and c) differential inter-subunit transfer of conformational changes during the gating process.

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ETHOLOGICAL ASSESSMENT OF THE ANTIDEPRESSIVE EFFECT OF SLEEP DEPRIVATION (SD)

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One of the methodological problems in biological psychiatry is the lack of observer-independent criteria to asses the severity of a disease. Since disturbed interpersonal communication is a major component of psychiatric disease, an ethological approach may be justified. The behavior of 13 depressed (DSM IIIR) patients and 13 volunteers was videotaped during a standardized interview before and after SD. Before SD patients smiled less, looked more down and less up, displayed more mouth corners down, and had longer speech pauses (Mann-Whitney, all $p<0.02$) than volunteers. After sleep deprivation, no differences between patients and volunteers were found, indicating an increase in sociability by the patients.

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MAP2 can induce process information in non-neuronal cells

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The neuron-specific microtubule-associated protein MAP2 is believed to influence neuronal morphogenesis by promoting the formation, stabilization and cross-linking of microtubules in neuronal processes. To investigate the molecular mechanism rat cDNAs for both high-molecular weight MAP2 (the adult isoform), and low-molecular weight MAP2c (the embryonic isoform) were transfected in non-neuronal and neuronal cell-lines. In both the human hepatoma cell-line PLC, which does not express endogenous MAP2, and in the rat neuroblastoma cell-line Bl04, which expresses low levels of endogenous MAP2, the exogenous protein binds to microtubules and bundles them in a concentration dependent manner. In Bl04 neuroblastoma cells transfected MAP2 also induced the formation of processes, which never happended in cells transfected with β-galactosidase. In PLC cells no processes were induced by MAP2. If however, after transfection PLC cells were treated with the actin-depolymerizing drug Cytochalasin B for 30 min processes were formed in cells which showed bundles microtubles. This was fully reversible. Process formation by MAP2 without Cytochalasin in neuronal cells indicates that these cells differ in their cortical cyto-skeleton or have factors that locally depolymerize actin.

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INFLUENCE OF SUBSTRATE AND CALCIUM ON THE REGULATION OF GROWTH OF LEECH NEURONS IN CULTURE

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Leech neurons plated on different substrates grow with distinctly different morphologies (Chiquet and Acklin, 1986, PNAS 83:6188-6192).To understand the influence of the substrate on the regulation of neurite outgrowth, the effects of membrane depolarization (by raised extracellular K^+) were analyzed in cells grown on the plant lectin Concavalin A (Con A) and on leech extracellular matrix (ECM).In cells on ECM depolarization led to neurite retraction. This retraction was inhibited by Mg^{2+} and was likely to be a Ca^{2+} mediated effect. Cells grown on ConA did not show an equivalent retraction of their neurites after depolarization.This substrate dependent behavior was further analyzed by comparing morphologies of growth cones and cytoskeletal organization of cells grown on ECM and ConA under normal culture conditions and after depolarization.

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HYDROPHILIC AND AMPHIPHILIC CHOLINE ACETYLTRANSFERASE OF DROSOPHILA MELANOGASTER ARE ENCODED BY A SINGLE cDNA

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We have previously shown that an hydrophilic and an amphiphilic form of choline acetyltransferase (ChAT) exist in *Drosophila melanogaster*.
Aiming to understand how the amphiphilic form is attached to membrane, we screened a head *Drosophila* cDNA library with oligonucleotides synthesized on the basis of the sequence published by Itoh *et al.* (1986). A full length ChAT cDNA was isolated. The insert was subcloned in an expression vector under the control of SV40 promotor. ChAT activity was expressed in both rat fibroblasts and in *Xenopus* oocytes. Triton X114 fractionation of transfected and non transfected oocytes showed that in the transfected cells hydrophilic and amphiphilic ChAT activities were encoded by a single cDNA species. Similar ratios of amphiphilic to hydrophilic enzyme were found in transfected oocytes and in *Drosophila* head extracts.

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EXPRESSION OF MYELIN PROTEIN GENES IN *pt* MUTANT RABBITS

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Paralytic tremor *(pt)* is an X-linked recessive mutation characterized by coarse body tremor and limb paresis. CNS myelination is delayed, prolonged and deficient. The axons are wrapped with irregular, thin and often uncompacted myelin sheaths. The amount of myelin in the 4-week-old mutants was reduced to about 30% of that in controls. Myelin proteins were analyzed by radioimmunoassay, PAGE and immunoblotting. In total homogenates, myelin proteins in mutants were reduced to a degree corresponding to hypomyelination with the exception of proteolipid protein (PLP) which showed a more drastic reduction. In purified myelin, PLP was reduced to about 50% of the normal amount, whereas myelin-associated glycoprotein and myelin basic protein were decreased to a lesser extent. Proteins present in oligodendrocyte cytoplasm, such as 2',3'-cyclic nucleotide 3'-phosphohydrolase, or at the external surface of oligodendrocytes and myelin sheaths, such as myelin/oligodendrocyte glycoprotein were relatively spared. Northern blots showed that the PLP RNA was of normal size but its level was reduced to a greater extent than messages for the other myelin proteins. Our results indicate that the *pt* mutation in rabbits affects primarily PLP gene expression.

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SEARCH FOR THE ANTI-PRION PROTEIN

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The prion gene complex (*Prn*) has been shown to be involved in spongiform encephalopathies in various ways: First, it comprises the *Prn-p* gene which encodes both the cellular prion protein (PrP^C) and its abnormal isoform PrP^{Sc}. PrP^{Sc} is thought to be part or all of the infectious agent. Second, *Prn* contains a gene controlling the scrapie incubation time in mice, *Prn-i*, which is genetically linked to *Prn-p* and might be identical to it. Third, the locus responsible for the species barrier has been mapped to the *Prn-p* ORF region. Fourth, there is genetic linkage between *Prn-p* mutations and occurrance of Gerstmann-Sträussler disease in humans.

The only *Prn* products identified so far are the two PrP isoforms encoded by *Prn-p* in a single ORF. On the minus strand of the PrP transcriptional unit there is an ORF spanning the whole region of the PrP ORF. The biological significance of this "anti-PrP" ORF has not yet been elucidated. We have used various strand-specific riboprobes to analyze RNA transcripts within *Prn* on Northern blots and have identified a candidate for a potential anti-PrP messenger RNA.

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CELLULAR PROTEINS BINDING TO THE PRION PROTEIN

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The molecular mechanisms causing scrapie and other prion diseases are still unknown. Interactions of the PrP isoforms with cellular proteins of the infected animal have therefore been analyzed using either ligand blots or binding of PrP to native proteins in frozen sections. Two proteins of 110 and 45 kDa (Pli 110 and Pli 45) were found to bind to PrP on ligand blots. Pli 45 was shown to be glial fibrillary acidic protein (GFAP) which is produced by astrocytes. The identity of Pli 110 is still unknown. Partial purification suggests that it might be membrane-associated. *In situ* binding of radiolabeled PrP to normal hamster brain sections showed a distinct pattern: pyramidal cells of the hippocampal formation and granule cells of the dentate gyrus strongly bound radiolabeled PrP 27-30. Binding sites were also detected in the cerebellum which appears to be the primary lesion site in the Gerstmann-Straussler syndrome. Further analysis of cellular proteins interacting with PrP may give us insight into the normal function of PrP as well as the cellular processes underlaying the degenerative changes observed in prion diseases.

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BRAIN MACROPHAGE INDUCED NEUROTOXICITY INVOLVES REACTIVE OXYGEN INTERMEDIATES AND GLUTAMATE ACTING VIA NMDA RECEPTORS

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Recently we observed that microglial cells induce death of neuronal cells in cultures. The cytotoxicity detected follows two pathways: one being due to secretion of hydrogen peroxide, the effect of which can be inhibited by the oxygen radical scavenger catalase; the other pathway involves microglia-derived neurotoxin(s), which interact with the neuronal NMDA receptor complex. In the latter pathway the neurotoxic effects can be neutralized by NMDA receptor antagonists but not by drugs which inhibit non-NMDA receptor mediated pathways or calcium influx. The neurotoxic molecule in supernatants of microglial cell was identified as glutamate. Reducing the glutamate level of microglial cell supernatant, either by exposure to astrocytes or by enzymatic degradation abolished the toxic effect. In contrast to reactive oxygen intermediates and L-glutamate, microglia-derived cytokines, namely TNF-α, IL-1β, IL-6 and IFN-α/-β caused no significant neuronal damage when added to murine cerebellar granule cell cultures. These data indicate that in addition to the classical view that microglia phagocytose neuronal debris, they may also directly damage neurons and may therefore play a role in vivo in traumatic and cerebrovascular brain lesions.

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NEUROTOXICITY OF ALUMINIUM ON EMBRYONIC CHICK BRAIN CULTURES

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Toxic damage of brain cells by aluminium (Al) is discussed as a possible factor in the development of the senile and presenile dementia of the Alzheimer type in humans. In order to investigate the sensitivity of various brain tissues to Al, serum-free cultures of mechanically dissociated embryonic chick (stage 28-29) brainstem, forebrain and optic tectum and for comparison meninges cells were prepared. Cultures were treated with Al (0-1000 µM $AlCl_3$) from day 1 to 8 in vitro. On day 8 viability (MTT- and NR-assay) and differentiation (expression of microtubule-associated protein type 2, glial fibrillary acidic protein and neurofilament 68kD antigens) were measured. Results suggest that differentiation and viability expressed as IC50 were influenced at about 10 times lower concentrations in cultures of optic tectum (IC50: around 100 µM Al) than necessary to affect the other cultures tested.

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NEUROTOXIC EFFECTS OF CISPLATIN AND ORG.2766

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Effects of cisplatin and the ACTH(4-9) analog ORG.2766 were investigated using serum-free chick embryonic brain cell cultures. After a treatment period of 7 days the cisplatin concentration added to the medium affecting brain cell cultures and cisplatin concentration in blood resulting in peripheral neuropathy in humans are comparable. Differentiation of the astroglia cells was influenced at lower concentrations (EC50: 2 µM) than necessary to affect nerve cells (EC50: 10 µM for neurofilament 68kD). The binding of cisplatin to proteins is quick. Dilution studies suggest that only free cisplatin is toxic for glia and nerve cells. ORG.2766 is reported to enhance recovery from and to protect animals for cisplatin neuropathy. In our cultures ORG.2766 did not influence sensitivity to or affect recovery from a cisplatin treatment. ORG.2766 itself increased at very high concentrations lysosomal activity and astroglia cell differentiation.

Other Aspects of Neurosciences

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ELECTROPHYSIOLOGICAL PROPERTIES OF BASAL FOREBRAIN CHOLINERGIC NEURONS IN GUINEA PIG SLICES.
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Although electrophysiological studies of basal forebrain (BF) neurons have been performed in both in vivo and in vitro preparations, the intrinsic properties of identified cholinergic neurons have not yet been described. In this study, neurons were recorded in guinea pig brain slices through the substantia innominata, where choline acetyl transferase (ChAT)-immunoreactive neurons were localized. The recorded cells were filled with biocytin for subsequent morphological and histochemical study. A majority of the recorded BF neurons were characterized by the presence of a large afterhyperpolarization and a transient rectification due to the presence of an A-current. In addition, in the hyperpolarized condition, low threshold spikes (LTS) were demonstrated and found to be associated with firing in bursts. The LTS, which was determined to be calcium-dependent, appeared moreover to play an important role in generating the slow rhythmic firing of these cells. Combined staining for ChAT and biocytin revealed that the recorded cells with these properties were cholinergic. (Supported by the Swiss NSF and the Canadian MRC).

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NEUROMODULATION OF BASAL FOREBRAIN NEURONS IN GUINEA PIG SLICES.
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Providing a major innervation to the cerebral cortex, cholinergic neurons within the basal forebrain (BF) sit in the path of ascending fiber systems including noradrenergic, serotoninergic, cholinergic and also glutamatergic fibers. Extending from preliminary results that revealed a class of BF neurons exhibiting oscillatory properties to be cholinergic, the potential influence of the chemically specific afferent input upon this class of cells was further investigated in guinea pig slices. Cells were recorded within the region of the substantia innominata, and filled with biocytin in order to study their morphological and histochemical identity. The cells were strongly depolarized and excited by noradrenaline (beta receptor-mediated effect). In contrast, they were consistently hyperpolarized and inhibited by serotonin (5HT1a receptor). The responses of these neurons to cholinergic agonists demonstrated that they are depolarized and excited by nicotine but hyperpolarized by muscarine. Excitatory aminoacids (AMPA, NMDA or trans-ACPD) were all excitatory as was substance P. One of the most striking features of the excitatory effect of several transmitters (in particular NMDA and noradrenaline) was that in certain cells they could induce a long-lasting and sustained repetitive burst firing. Such responses could be important in processes of cortical activation. (Supported by the Swiss NSF and the Canadian MRC).

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PHARMACOLOGICAL STUDY OF NUCLEUS GIGANTOCELLULARIS NEURONS IN GUINEA PIG BRAINSTEM SLICES.
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Nucleus Gigantocellularis neurons (NGCn) belong to networks involved in a variety of functions. In a first step, two neuronal cell types, A and B NGCn, differing by their electrophysiological and pharmacological properties, were identified in the guinea pig using either brainstem slices or an isolated whole brain preparation (Serafin et al., Experientia 44, A70). In a second step, intracellular injections of Lucifer Yellow have confirmed that both A and B NGCn were gigantocellular neurons. Thus far, no attempts have been made to study their neuromodulation in vitro. We therefore undertook to study the response of these cells to the neurotransmitters that were shown to be present in this nucleus. Type A and type B NGCn were reversibly depolarized and excited by excitatory amino acid agonists such as NMDA, AMPA or trans-ACPD. In contrast, they were both hyperpolarized by $GABA_A$ and $GABA_B$ agonists (muscimol and baclofen respectively). Monoamines also appear to play a crucial role in controlling the activity of these cells. Indeed, bath-application of serotonine, noradrenaline as well as histamine resulted in a reversible membrane depolarization of A and B NGCn with a concomitant increase in their firing rate. (Supported by Swiss NSF grants no 31-26495.89, 3.288.0.85 and 3.560.0.86).

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PHARMACOLOGICAL STUDIES OF MEDIAL VESTIBULAR NEURONES IN GUINEA PIG BRAINSTEM SLICES.
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We have identified in the guinea pig vestibular nucleus (MVN) two main neuronal cell types, A and B MVNn, differing by their intrinsic membrane properties (Serafin et al. (I, II), Exp. Brain Res. 84, 1991). These cells were selected for further pharmacological studies. We first tested their sensitivity to excitatory and inhibitory amino acids. All MVNn were strongly depolarized and excited by NMDA, AMPA and trans-ACPD. They were hyperpolarized and inhibited by both muscimol and baclofen, which are specific agonists for GABA A and B receptors, respectively. These results are in accordance with the hypothesis that glutamate is the major transmitter released by primary vestibular afferents, whereas GABA would be one of the major mediator of both commissural and cerebellar afferences to the vestibular nuclei. A and B MVNn were also generally slightly depolarized by application of various monoamines such as serotonin, noradrenaline and dopamine; these effects were accompanied by a decrease in membrane resistance. Whereas for the above mentioned transmitters, the responses of the two types of MVNn were similar, some other transmitters revealed major differencies between A and B MVNn. Indeed, in contrast with B MVNn which were always depolarized and excited by either nicotine or muscarine, responses of A MVNn were more heterogeneous. This discrepancy between A and B MVNn could also be observed with substance P, whose depolarizing effect was restricted to B MVNn. Generally speaking, type A MVNn appear quite more heterogeneous than type B MVNn in their responses, and this probably reflects a functional diversity which remains to be investigated. (Supported by a Swiss NSF grant no 31-26495.89 and the French Ministère des Affaires Etrangères)

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DIAMETRICALLY OPPOSITE CNS-EFFECTS BETWEEN THE ENANTIOMERS OF 2 OPTICALLY ACTIVE 1,5-BENZODIAZEPINES IN THE RAT
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7- as well as 8-Chloro-3-ethyl-1,3-dimethyl-5-phenyl-1H-1,5-benzodiazepine-2,4 (3H,5H)-dione (rac. 1 and 2, respectively) were synthesized. S(+)- and R(-)-enantiomer of 1 and 2 were obtained using optically active precursors. The water insoluble substances were injected i.v. in Wistar rats (weight about 200 g) dissolved in 30% Cremophor EL^R /0.9 % NaCl. S(+)-(5 mg/kg) and rac. 1 (10 mg/kg) caused immediately after administration pentetrazol-like seizures (loss of consciousness) whereby a strong tonic phase was followed by clonic convulsions (n=8-15); most animals died during seizures if higher doses of both substances were given. S(+)- and rac. 2 caused also CNS-excitation (seizures), however, slightly higher doses were necessary. In contrast, rats which received R(-)-1 and -2 (15 mg/kg) did not show any signs of CNS-excitation (n>13); hexobarbital sleeping time (after i.v. 25 mg/kg, given 5 min after pretreatment with R(-)-1 or -2, n= 5-7) was slightly prolonged vs. control.

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ELECTROCHEMICAL DYNAMICS OF EXCITABLE MEMBRANES
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It has recently been shown that chemical kinetics can be cast into a formulism common to most other sorts of dynamic processes (Sieniutycz, Chem.Eng. Sci.42:2697,1987; Shiner, Adv. Thermo. 5,1990). With chemical and nonchemical processes given by the same formalism it is possible to study systems with chemical-nonchemical coupling in terms of the one formalism. This is much simpler than treating the chemical aspects with traditional chemical kinetics, treating nonchemical aspects with their traditional formalisms and then taking the coupling into account. The new formalism will be applied to the dynamics of excitable membranes, in particular the Hodgkin-Huxley model (AL Hodgkin & AF Huxley, J.Physiol 117:500, 1952) extended to take ion chemistry into account explicitly. Variational, minimal and network formulations (Sieniutycz & Shiner, Information Phys. Life Sci,submitted,1991) will be presented.

Supported by the Sandoz Foundation and the Swiss National Science Foundation (grant no. 3.033-0.87).

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INSECT GAP JUNCTIONS: MODULATION BY THE INTRACELLULAR ELECTRICAL FIELD

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Cell pairs of an insect cell line (clone C6/36; Aedes albopictus) were used to study the electrical properties of gap junctions. A dual voltage-clamp approach was used. The conductance of the gap junction membrane (g_j) was found to be sensitive to voltage across the junctional (V_j) and non-junctional membrane (V_m). Depolarization of V_m gave rise to a time- and voltage-dependent decrease in g_j. This was caused by a reduction in single channel conductance and the number of channels open. The experimental data are compared with predictions derived from a mathematical model describing a cell pair as electrical circuit. The model assumes two separate gates, a V_m-gate sensitive to voltage across the gap junction channel wall, and a V_j-gate sensitive to voltage across the channel pore.

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INFLUENCE OF CATIONS ON GAP JUNCTION CONDUCTANCE.

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Cell pairs of neonatal rat hearts were used to study the influence of cations on the conductance of gap junction membrane (g_j). Experiments were carried out using a dual voltage-clamp method in conjunction with dialysis of the cytosol via patch pipette. A dose-dependent decrease in g_j was found when the intracellular concentration of Ca^{2+} or H^+ was raised. Pipette solutions containing 5 mM Ca^{2+} or 10 µM H^+ (pH = 5.0) produce complete uncoupling within 3 - 4 min. The effects of Ca^{2+} and H^+ were additive. No change in g_j was observed when pipette solutions contained Mg^{2+} or Ba^{2+} ions (up to 5 mM). Sr^{2+} (5 mM) resulted in a partial uncoupling which developed slowly. This results suggest that gap junctions of neonatal and adult hearts exhibit different ionic sensitivities.

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ETHANOL, ROTAROD PERFORMANCE AND PSYCHOGENETICALLY SELECTED LINES OF RATS

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Four groups each of 12 adult, male RHA/Verh(H) or RLA/Verh(L) rats were tested on a rotarod: controls (C), pre-/postnatally exposed to ethanol through mothers drinking a 10% solution(PN) and chronic consumption of ethanol following either history(C-E and PN-E). The number of replacements on the rod necessary during each 200 sec session was counted. LCs performed better than HCs during 4 days of initial training at 10rpm, as did LPNs vs HPNs and LPN-Es vs HPN-Es. After 4 days at 20rpm, most rats performed more poorly during 4 days at 30rpm, particularly HPNs vs HCs and HPN-Es, and LCs vs LC-Es. Finally, 4 night sessions (when the rats actually drink) were conducted at 20rpm. LCs once again performed better than HCs, as did both L-E groups vs both H-E groups. Summary: Ls generally outperformed Hs. No harmful effects of moderate ethanol consumption were observed, but PN was detrimental to Hs at the highest speed. Supported by an SSA/FSRA grant.

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IS THERE A SEASONAL RELATIONSHIP BETWEEN URINARY MELATONIN AND CORTISOL?

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Low melatonin (MEL) and elevated cortisol (CORT) levels have typically been reported during depression. Furthermore, based on the observation of an inverse relationship between MEL and CORT in some severely depressed patients, Wetterberg et al. (Lancet,1979) suggested a direct link between these hormones. We here report findings from a seasonal study of MEL and CORT that may help to address this issue.
Analysis of nighttime urinary MEL and CORT, investigated monthly over 14 months in healthy volunteers (11m,9f; age range: 23-52 years) did not reveal a temporal association : MEL exhibited a marked seasonal variation ($F=17.63$, $p<.0001$) with high levels during the dark months, while CORT showed multiple peaks around the year without a seasonal trend ($F=0.87$, n.s.). Nevertheless in several subgroups a seasonal relationship between MEL and CORT became apparent, being positive in morning- ($r=.71$, $p<.01$), inverse in evening type individuals ($r=-.41$, $p=.10$).
These results exclude a simple link between MEL and CORT, suggesting more complex mechanisms (including homeostatic and chronobiological ones) controlling their balance as well as their disturbance in depression.

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INFLUENCE OF THE MENSTRUAL CYCLE ON THE SWEATING RESPONSE MEASURED BY DIRECT CALORIMETRY IN WOMEN.

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The whole body sweating response was measured at rest in eight lean women during the follicular (F) and the luteal (L) phases of the menstrual cycle. Subjects were exposed for 30-min to neutral (N) environmental conditions (T_a 28°C) and then for 90-min to warm (W) environmental conditions (T_a 35°C) in a direct calorimeter. At the end of the N exposure, tympanic temperature (T_{ty}) was 0.18±0.06°C higher in the L than in the F phase ($P<0.05$), whereas mean skin temperature (T_{sk}) was unchanged. At the onset of sweating, the tympanic set point temperature (T_{tyset}) was higher in the L phase (37.18±0.08°C) than in the F phase (36.95±0.07°C; $P<0.01$). The magnitude of the shift in T_{tyset} (0.23±0.07°C) was similar to the L-F difference in T_{ty} observed at the end of the N exposure. It is concluded 1) that the internal set point temperature of resting women exposed to warm environmental conditions is shifted to a higher value during the L phase as compared to the F phase of the menstrual cycle; 2) that the magnitude of the shift corresponds to the difference in internal temperature observed in neutral environmental conditions between the two phases.

Proteinases and their Inhibitors

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EXPRESSION OF MAMMALIAN PROLYLENDOPEPTIDASE IN E. COLI

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Prolylendopeptidase (PE) is an 80 kDa serinprotease which hydrolyses polypeptides carboxyterminally of proline residues. The aim of our studies is to investigate the reaction mechanism and the possible use of PE for enzymatic peptide synthesis. However, the relatively small amount of protein available has limited such investigations so far. - Therefore, the cDNA coding for PE from porcine brain (Rennex et al. 1991 Biochemistry 30,2195) was cloned into the polylinker region of the expression plasmid pDS56-2 (Bujard et al. 1987 Meth. Enz. 155, 416) in three different ways: in the inverse orientation (No.2); in the correct orientation (No.3); in frame with the E. Coli heat labile toxin subunit B-signal sequence (No.4).
On Coomassie stained SDS-PAGE, a prominent protein band (M_r 75 kDa) was present in aliquots of the sonicated cellular extracts of clones No. 3 and 4. However, when incubated with ^{3}H-DFP, a labelled band could only be detected in aliquots of clones No. 3. Also when we used Z-Gly-Pro-p Nitroanilide as a synthetic substrate for PE, only in the extracts of No. 3-clones, PE activity was detected. We concluded from this, that the construction No. 4 was properly, but inactively expressed. A 2 liter culture of clone 3B5 was thus used to produce recombinant PE. Approximately 5-10 μg/ml active PE were then purified from the cellular extract in 5 steps ($(NH_4)_2SO_4$ precipitation, DEAE-, Hydroxylapatite- and Phenylsepharose column) to apparent homogeneity on SDS-PAGE.

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TRANSIENT EXPRESSION and PROTEOLYTIC PROCESSING of HEPATITIS A VIRUS POLYPROTEINS.

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Hepatitis A virus (HAV) RNA encodes a single giant polyprotein that is thought to be processed in a proteolytic cascade to mature proteins by viral proteases. Most cleavage sites on the polyprotein have yet to be demonstrated experimentally. To study proteolytic processing in detail defined regions of the HAV genome were expressed in a vaccinia virus/T7 hybrid system. Hence, we identified with the aid of specific antibodies predicted protein precursors containing structural as well as nonstructural proteins, and observed their processing to mature proteins by the major viral protease 3Cpro. Furthermore, we demonstrated that the second putative protease 2Apro does not release structural precursors from the polyprotein as its poliovirus analogue does.

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PROCESSING AND PRESENTATION OF TETANUS TOXIN BY HUMAN B CELL TO HUMAN T CELL.

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T lymphocytes recognize antigens as proteolytic fragments in association with MHC class II molecules. The accessory cell internalize the antigen into an acidic compartment where a proteolytic degradation takes place. These fragments are then associated with MHC molecules, and the complex MHC-peptide is expressed at the cell surface for presentation to T cells. Several specific T cell epitopes for *tetanus toxin* have been determined in our laboratory. It was also reported that several human EBV transformed B cell lines and PBL, displaying the proper MHC molecules differentially processed *tetanus toxin* as assessed by their ability to present *tetanus toxin* antigenic determinants to different T cell clones. We have now established that the tridimentionnal structure of the *tetanus toxin* plays an important role in this differential processing. We have extended these studies to other DP restricted epitopes and determined that the APC incapable to present 947-67 tt epitope from native tt to the T cell clone DR5 restricted, is able to present 947-67 tt epitope to the T cell clone DP restricted. These data are consistent with hypothesis that different indivivuals possess different enzymes involved during the antigen processing.

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MODULATION OF SPECIFICITY AND INHIBITORY FUNCTION OF EGLIN c BY SITE-DIRECTED MUTAGENESIS

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Eglin c, naturally occuring in the leech Hirudo medicinalis, is a strong 70 amino acids inhibitor of the serine proteinases elastase, cathepsin G, α-chymotrypsin, chymase and subtilisin. Contrary to many other proteinase inhibitors eglin c lacks disulfide bridges for structure stabilization. As is known from crystallographic and NMR data, eglin c has a basket-like shape with a hydrophobic core and a solvent exposed binding loop, which is held in its conformation by a hydrogen bond network.
Mutants have been prepared in order to modify specificity (P1- and P1'- position) and in order to investigate the contribution of specific amino acids involved in the hydrogen bond network to the stability of the binding loop.
Some of the mutants could be co-crystallized in complex with subtilisin, which allows an interesting comparison of kinetic and crystallographic data.

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u-PA/u-PAR expression and the invasive phenotype: In vitro behavior of u-PA transfected human colon carcinoma HT29 cells.

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Urokinase (u-PA) and the urokinase receptor u-PAR are thought to play an important role in invasive behavior and metastatic properties of cancer cells. We surface u-PA binding sites, but no PA activity and therefore is unable to generate plasmin in presence of plasminogen. In contrast, significant plasmin generation is observed, when cells are preincubated with high molecular weight u-PA, but not with low molecular weight u-PA. HT29 cells have been transfected with a human u-PA cDNA plasmid, pcUKLTR6, and pSVneo as a resistance marker to investigate whether secreted u-PA could enhance *in vitro* extracellular matrix degradation and whether inhibition of u-PA binding to the cell surface is modulatory. Fifty clones were isolated and some selected for stable expression of high PA activity. These clones were, in presence of plasminogen, capable of marked degradation of R22 smooth muscle-derived extracellular matrix, whereas the parental cell line contributed to an insignificant breakdown only. Aprotinin, recombinant PAI-2, and coculture with PAI-1 producing mouse L cells significantly inhibited this degradation. In addition, a peptide displacing u-PA from its receptor could decrease the breakdown by as much as 80%. These results show that both secreted and receptor bound u-PA contributes to matrix breakdown by HT29 u-PA transfectants.
Supported by the Swiss Science foundation.

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MODULATED EXPRESSION OF THE CATHEPSINS B, C, H, AND L, IN TEN HUMAN COLON ADENOCARCINOMA CELL LINES:

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In previous studies we have shown that homogenates from human malignant colon tissues have increased Cathepsin B (Cath.B) activities compared to adjacent colon mucosa. Cath.H and L activities were barely detectable.

Since tumor tissues can be variably infiltrated by inflammatory and stromal cells we checked whether established human colon carcinoma cell lines could synthesize Cath.B. To assess the tissue-specificity of this expression, we assayed three other lysosomal cathepsins, Cath.C, H, and L. Among ten colon cell lines tested (HT29, SW480, SW620, SW1116, CaCo2, LoVo, Colo205, Co112, Co115, and CoSut) seven expressed predominantly Cath.B activities. Cath.L activities were too low to be clearly identified, and Cath.H- and C-like activities were present at low levels.

All cell lines studied secreted Procath.B and C into their culture medium. This secretion could be enhanced several fold by treatment of cells with the lysosomotropic agent, ammonium chloride (10mM). Both proenzymes could be activated by pepsin in a dose and time dependent way.

Intraperitoneal injection into nude mice of 3x10*6 Co115 cells resulted in the production of an ascitic fluid with high levels of Procath.B, similar to those obtained from ascites of cancer patients.

These *in vitro* studies are in good agreement with our results obtained on colon carcinoma tissues, and point out that Cath.B may have a specialized function within colonic mucosa.

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DO CATHEPSIN B AND THE NATURAL INHIBITOR CYSTATIN C CONTRIBUTE TO EXTRACELLULAR MATRIX DEGRADATION BY COLON CARCINOMA CELLS IN VITRO?

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Human colon carcinoma cells in culture were previously found to secrete both the cysteine proteinase cathepsin B and its inhibitor cystatin C. Cathepsin B is mainly secreted as a pro-enzyme, activable in vitro by a pepsin treatment.

A reconstituted extracellular matrix (ECM) produced by smooth muscle rat cells was used to define the role of cysteine proteinases in ECM breakdown induced by colon carcinoma cells. Iodinated Laminin was also used in a monosubstrate degradation assay.

Conditioned media as well as cells cultured on the matrices, were unable to produce a degradation sensitive to cysteine proteinases inhibitors (rCyst C, E64 and specific diazomethane inhibitors), in the conditions of the assays. Inhibitors of serine proteinases were found to inhibit partially the ECM breakdown.

These results suggest that, in the conditions used, the level of active secreted cathepsin B may be too low to induce a specific measurable degradation, and that neither cell-matrix contacts nor interactions with other secreted proteinases, can activate the secreted pro-form into a functional enzyme. Inactivation of the enzyme by the co-secreted inhibitor cystatin C could also be part of these regulatory events.

Studies are currently in progress to define whether exogenous activators(s) released by non-malignant cells may contribute to activation of the pro-enzyme or whether autocrine interactions with cystatin C may modulate the activity of secreted cysteine proteinases in ECM lysis.

Supported by the Swiss Science Foundation and the Ligue Neuchâteloise contre le Cancer (O.C.).

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The PA/plasmin system during tissue invasion processes

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Proteolytic cascades are thought to be involved in the modulation of the cell-extracellular matrix interactions that accompany tissue remodeling processes. To explore the contribution of plasmin-mediated proteolysis in tissue invasion, we have analyzed the expression of plasminogen activators during murine embryogenesis and in human neoplasia. By using in situ hybridization and histological zymography, we have demonstrated the transient production of urokinase (uPA) by murine trophoblasts invading the uterine endometrium. Similarly, we have shown that malignant cells of certain human cutaneous carcinomas endowed with metastatic properties consistently produce uPA, although in variable amounts, while non-metastasizing tumors do not express the enzyme. Further analysis has revealed that, during embryo implantation, the expression of plasminogen activator activity is tightly controlled in time and space by the local production of plasminogen activator inhibitors (PAIs); in contrast, only restricted subsets of neoplastic cells produce PAIs. Our observations suggest that the production of PAIs might contribute to limit the extent of plasmin-catalyzed proteolysis and that imbalanced plasminogen activation may account for some of the observed differences between cell invasion during physiological versus pathological processes.

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OVARIAN GRANULOSA CELLS SYNTHESIZE ANTICOAGULANT HEPARAN SULFATE PROTEOGLYCANS.

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Maturation of the ovarian follicule and its rupture at ovulation is triggered by gonadotrophins and involves controlled proteolysis. We have detected on rat follicular granulosa cells (GC) heparan sulfate proteoglycans that bind and activate antithrombin (aHSPG). aHSPG have been described on the extracellular matrix of endothelial cells. GC kept in vitro in serum-free medium can be stimulated by FSH to synthesize estradiol but do not spread on the culture dish. In contrast GC grown in presence of serum form monolayers but do not respond to FSH. To determine wether the expression of aHSPG by GC is modulated by FSH we have found conditions in which GC proliferate but also retain their sensitivity to FSH. A brief exposure to chicken serum allowed the cells to spread and divide and after 4 days of subsequent culture in serum-free media GC responded to FSH stimulations by a 15-fold increase in estradiol production. We measured aHSPG on the cells and in the medium using specific ^{125}I-antithrombin binding assays performed on the cell layers and on nitrocellulose immobilized soluble aHSPG. The amounts of aHSPG detected on GC surfaces were comparable to those found on endothelial cells. aHSPG were also released by GC in the media but the signal obtained was about 3-fold lower than on the cells. After 48h of stimulation by FSH GC cell surface aHSPG were decreased by approximatly 40% whereas aHSPG in the media remained unchanged. These data suggest that FSH modulates the expression of aHSPG by GC, which could favour proteolytic activities in the inner follicle at the time of ovulation.

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Purification of a metalloprotease expressed by glioblastoma cells to overcome the inhibitory substrate effect of CNS myelin.

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Rat C6 glioblastoma cells are highly invasive tumor cells in the CNS. Due to a specific membrane-bound metalloprotease C6 cells can overcome the inhibitory substrate effect of oligodendrocytes and CNS myelin (Paganetti et al.; J.Cell Biol. 107; 1988). The blocker profile of this enzyme is different from that of the known metalloproteases. By using a specific peptide degradation assay we characterized the protease as a tightly bound plasma membrane protein. The enzyme activity can be solubilized with an 1% CHAPS buffer. The protease was further purified with a FPLC anion exchange column, a size exclusion column and an affinity column in series. The enrichment of the protease was assessed by the peptide degradation assay, SDS PAGE and a biological assay testing for inactivation of myelin-associated neurite growth inhibitors.

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PRESENCE OF GLIA-DERIVED NEXIN IN GERBIL HIPPOCAMPUS AFTER ISCHEMIA-INDUCED DELAYED NEURONAL CELL DEATH

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Transient bilateral occlusion of common carotid arteries for 5-7 min in Mongolian gerbil results in selective delayed death of pyramidal cells in hippocampal CA1 sector. GABAergic interneurons, however, as well as most of afferents persist in CA1. We were interested if the serine protease inhibitor glia derived nexin (GDN), which is expressed after lesions in peripheral nervous system, plays a role in the mainteance of these neuronal elements. GDN-immunoreactivity (GDN-IR) was found in CA1 at the peak of neuronal degeneration and persisted there for up to 90 days. Immunostaining for glial fibrillary acidic protein and ultrastructural analysis verified that GDN is located in reactive astrocytes. It was never observed in endothelial cells. In case of thrombotic infarction, occasionally occurring in hippocampus or striatum, the infarction center is free of GDN-IR. GDN staining is present, however, in the surrounding penumbra. These observations indicate a role for GDN in limiting proteolytic activity and migration of phagocytes, in addition to its suggested permissive function in neuronal rearrangement

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DIFFERENTIAL EXPRESSION OF GLIA-DERIVED NEXIN AFTER 6-HYDROXYDOPAMINE OR IBOTENIC ACID INDUCED LESIONS OF RAT SUBSTANTIA NIGRA

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Glia derived nexin (GDN), a serine protease inhibitor originally isolated for its neurite promoting activity in vitro, is present in adult CNS in continously de- and regenerating olfactory system. In order to study its possible role in lesion induced neuronal plasticity, substantia nigra (SN) was unilaterally lesioned in rats by local application of either 6-hydroxydopamine (6-OHDA) or ibotenic acid (IBO). In SN, from the 2nd day on after injection of excitotoxin IBO, astroglia developed intense GDN-immunoreactivity (GDN-IR). In contrast no GDN response was detected around the 6-OHDA injection. This suggests that the nature of degeneration influences expression of this protease inhibitor and thus local regenerative potentials. In nigral target areas, however, particularly in striatum GDN-IR astrocytes were found 1 to 4 weeks after deafferentation, irrespective of type of SN lesion. IBO also induced GDN-IR in nigrotectal fields. In view of demonstrated synaptic rearrangements following SN lesion, presence of GDN is a further indication for its active permissive role in neuronal plasticity.

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REGULATION OF GLIA-DERIVED NEXIN IN PRIMARY SCHWANN CELLS

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Glia-derived Nexin (GDN) is a 43 kDA neurite promoting glycoprotein with serine protease inhibitory activity. In vivo experiments have shown that the expression of GDN is dramatically induced after injury of the rat sciatic nerve (Meier et al., Nature 343, 548-550, 1989).

In explant cultures of dorsal root ganglia, the GDN level is down regulated and, as in vivo, can be induced following lesion of the processes emerging of the explants. The GDN increase detected by immunocytochemistry is restricted to the Schwann cells distal to the site of injury, where cell proliferation is induced following the loss of axonal contact. Neurotrophic factors, also known to be induced after injury, have been tested for their effect on GDN expression in pure Schwann cell cultures. A significant increase of GDN mRNA and protein is seen after NGF treatment. NT-3 and BDNF remained inactive. These results suggest that neuron-glia interactions, both by cell-cell contact and secretion of macromolecules, play an important role in the regulation of GDN.

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TISSUE-SPECIFIC AND HIGH-LEVEL EXPRESSION OF THE GLIA DERIVED-NEXIN GENE IN TRANSGENIC MICE

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Glia derived-nexin (GDN), also known as protease nexin I, is a serine protease inhibitor, that in vitro promotes neurite outgrowth from neuroblastoma cells, sympathetic and hippocampal neurons. In vivo, GDN is constitutively expressed in the olfactory system, where axonal degeneration and neurogenesis occur continuously throughout life, and it is also upregulated following lesion of the rat sciatic nerve. Therefore GDN could influence both degenerative events and/or axonal regeneration in vivo. To investigate the biological role of GDN, we generated several strains of transgenic mice carrying a chimeric Thy-1-GDN gene, which directs the expression of high levels of rat GDN in the brain. Transgenic mice of three strains went through a whole series of behavior tests. In any of the observation tests used (global behavioral assessment, rotarod, motility) no differences were noted between transgenic mice and non-transgenic littermates. However, some transgenic mice of one strain (6/40) did not learn at all in the Morris water maze test. Immunohistochemistry and in situ hybridisation analyses are in progress to determine possible consequences of the overexpression of GDN on brain organisation.

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MOLECULAR ORGANIZATION OF THE RAT GLIA-DERIVED NEXIN PROMOTER.

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Analysis of rat genomic clones has identified the three first exons and the promoter of rat glia derived nexin (GDN). A 1.6 kb fragment containing the first exon and the 5'-flanking sequences was sequenced. The promoter and the first exon were located within a CpG-island. A TATA box but no CAAT box was found. The rat GDN promoter contains 5 SP1 sites, 4 sites for MyoD and 1 binding site for the early NGF response genes NGFI-A and NGFI-C. The rat GDN gene had one transcriptional start site. A 1600 bp promoter fragment was shown to induce luciferase expression after transfection in different cell lines. An enhancer was localized in region -1575 to -377. A silencer is found between -377 and -238. GDN was transcriptionally silenced by CpG methylation of the promoter in rat hepatoma cells. These results provide the first insights in the gene organization and regulation of the rat GDN gene.

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THROMBIN-INDUCED NEURITE RETRACTION IN NEURONAL CELLS

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The mechanism through which thrombin causes neurite retraction was studied in the mouse neuronal cell line NB2a. Thrombin-induced retraction in these cells was rapid starting within seconds and was complete after few minutes. The effect of thrombin involved an interaction between its anion-binding exosite and the cellular target. The neurite retraction in response to thrombin was mimicked by a 14-amino acid peptide starting at Ser^{42} of the deduced amino acid sequence of the recently cloned human thrombin receptor (Vu et al., 1991; *Cell*, 64: 1057). It has been proposed that the thrombin receptor was cleaved before Ser^{42} and that interactions with the newly created N-terminal region of the receptor led to activation. The retraction could be blocked by protein kinase inhibitors staurosporine and H7. It is concluded that thrombin-mediated neurite retraction is caused by cleavage of a cell-surface receptor that bears homology to the human receptor. The activation of this receptor leads intracellularly to stimulation of protein kinases.

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TOWARDS THE STUDY OF INSULIN DEGRADATION IN MAN USING MASS SPECTROMETRY

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In order to work in Man without using radioactivity, we have prepared insulins labelled with stable isotopes (deuterium). Such insulin derivatives of different molecular weights are authentic (the native structure is rigorously maintained), non-radioactive and can easily be distinguished from endogenous insulin by mass spectrometric analysis.

Labelled insulins have been extracted and purified from plasma samples of a few ml volume with more than 50 % recovery. Results of analysis of such samples at very high sensitivity by mass spectrometry will be presented.