STEROID/THYROID/RETINOIC ACID SUPER GENE FAMILY

Organizers: John Cidlowski, Malcolm Parker and Jan Ake Gustaffson

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Keynote Address

K 001 MOLECULAR AND GENETIC DISSECTION OF THE RETINOID SIGNALLING PATHWAY, Pierre Chambon, Andrée Dierich, Phillipe Kastner, Marianne LeMeur, David Lohnes, Thomas Lufkin, Manuel Mark, Cathy Mendelsohn. Laboratoire de Génétique Moléculaire des Eucaryotes (LGME/CNRS and U.184/INSERM), Institut de Chimie Biologique, Faculté de Médecine, 67085 STRASBOURG CEDEX, France.

The teratogenic effects of both excess or deficiency of retinoic acid (RA) during early embryogenesis suggests that this retinoid plays a critical role during development in addition to being essential for post-natal life. Two families of RA receptors, the RARs and the RXRs, transduce the RA signal. These receptors are thought to control the transcription of target genes by binding as RAR:RXR heterodimers to RA response elements. Each RAR and RXR type gene (α, β or γ) is expressed as a series of N-terminal variant isoforms. The interspecies conservation of RARs and RXRs, both in sequence and pattern of expression, suggests that each receptor type and isoform may play a unique role during development.

In order to address this question, we have created mouse lines in which a specific RAR or RXR type isoform has been disrupted by gene targeting. Knockouts of the isoforms RARα1, RARβ2 or RARγ2 have no apparent effect on development or viability. In contrast, disruption of all isoforms of RARα, RARγ or RXRα results in a number of developmental and post-natal defects, some of which have been previously observed in vitamin A deprivation studies. These defects include post-partum growth deficiency with a high degree of mortality (RARα or RARγ disruption), testicular germinal epithelium degeneration (RARα disruption), squamous metaplasia of the seminal vesicles and prostate, Harderian gland agenesis, skeletal malformations (RARγ disruption), and embryonic lethality due to heart defects (RXRα).

The lack of an effect for the RAR isoform knockouts and the limited phenotype for the RARα, RARγ and RXRα knockouts, compared to the known patterns of expression of these genes, suggest a high degree of functional redundancy amongst RARs and RXRs. To address this possibility, we have created a number of different combinations of RAR and RXR double mutant lines. In all cases null double mutants die before or shortly after birth. A detailed analysis of the defects in these mutants will be presented.

Steroid/Thyroid Receptor Structure

K 002 FUNCTIONAL AND STRUCTURAL BASIS OF DNA TARGET SELECTIVITY BY THE VITAMIN D_3 RECEPTOR, Leonard Freedman, Boris Cheskis, and Terri Towers, Memorial Sloan-Kettering Cancer Center, New York, NY.

Dimerization facilitates cooperative, high affinity interactions between protein subunits and DNA. Steroid receptors form symmetrical homodimers on palindromic half-sites that are mediated by residues within the DNA-binding domain; dimerization is enhanced by residues in the ligand-binding domain. Other nuclear receptors bind with high affinity to variously spaced direct-repeat elements, either as homodimers or as heterodimers with RXR. We and others have found that this class of receptors bind DNA in such a way that the interactions favorably align monomeric subunits to bind cooperatively to optimally spaced half-site targets. Analogous to the mechanism of steroid receptor-DNA interactions, discrimination of differentially spaced direct-repeat elements appears to be mediated principally by a putative asymmetric, head-to-tail dimer interface formed between DNA-binding domain subunits, and is enhanced by a dimerization region co-localized to the ligand-binding domain. We have mapped this dimer interface in the DNA-binding domain of the vitamin D3 receptor. Reflecting the organization of the DNA target, the interface maps to the tip of the first zinc finger module, and to the N and C termini of the second zinc module. A stereochemical model for these interactions will be presented.

The fact that receptor dimerization strongly influences DNA-binding affinity and selectivity, and given that one of two dimerization regions co-localizes to the C-terminal ligand-binding domain, suggest that a key control point of steroid/nuclear receptor function by ligand may be in regulating the association or dissociation of receptor dimers and/or receptor-nonreceptor complexes. Ligand appears to act on steroid receptors principally by stimulating the dissociation of heat shock proteins from the receptors, resulting in the formation of receptor homodimers, as well as a derepression of nuclear localization, DNA binding, and transcriptional regulatory activities. The precise role of ligand in the regulation of many nuclear receptors, however, is much less clear, since these receptors appear to be localized to the nucleus and bound to DNA constitutively, and do not associate with heat shock proteins. Conceivably, ligand could effect receptor homo- or hetero-dimerization, kinetics of DNA binding, or interactions with other components of the transcriptional machinery. Using overexpressed and purified human vitamin D_3 receptor and RXR, we have begun to examine the role 1, 25 dihydroxyvitamin D3 has on receptor dimerization and DNA binding. We find that VDR homodimerization is DNA-dependent but ligand-independent, and that vitamin D3 actually acts to destabilize a DNA-bound VDR homodimer while at the same time actively favoring the formation of a VDR-RXR heterodimer. These results may rationalize how a nuclear receptor can bind DNA constitutively but still act to regulate transcription in a fully hormone-dependent manner.

K 003 ROLE OF THE GME ELEMENT IN MODULATION OF GLUCOCORTICOID INDUCTION OF TYROSINE AMINOTRANSFERASE GENE TRANSCRIPTION. S. Stoney Simons, Jr., Hisaji Oshima, Daniele Szapary, Clayton D.Collier and David A. Jackson LMCB/NIDDK/NIH, Bethesda, MD 20892.

The current model for steroid hormone action predicts that the only components required for induction of a basally expressed gene are the receptor-steroid complex and its cognate HRE. However, closer analysis of the induction properties of agonists and antagonists for the liver-specific, tyrosine aminotransferase (TAT) gene in transformed rat liver cells (Fu5-5) indicated that other DNA elements are operative. Transfection assays in Fu5-5 cells were used to locate a 21 bp sequence of the rat TAT gene that was able to convey the unique properties of the endogenous TAT gene to homologous or heterologous promoters and genes. Furthermore, this 21 bp sequence, which has been called a glucocorticoid modulatory element (GME), modulates the response to sub-saturating concentrations of agonists, and saturating concentrations of antagonists, at the level of correctly initiated transcripts. Gel shift assays were used to show that the biological activity of the GME is mediated by the binding of a yet unidentified *trans*-acting factor. The mechanism of action of the GME initially appeared similar to synergism. However, further studies revealed numerous differences. Most significant is that GME activity was seen at subsaturating concentrations of glucocorticoids but not at the saturating steroid concentrations that are characteristic of synergism. This property has major implications regarding steroid control of gene expression during development, differentiation, and homeostasis. Further studies have identified two other elements of the TAT gene that regulate the activity of the GME. Thus, glucocorticoid induction of the TAT gene involves a multicomponent system that may have parallels in other steroid induced genes.

Steroid/Thyroid Activation

K 004 **REGULATION OF RETINOID AND THYROID HORMONE ACTION THROUGH HOMO AND HETERODIMERIC RECEPTORS**, *Magnus Pfahl, Cancer Center, La Jolla Cancer Research Foundation, La Jolla, CA 92037.*

In contrast to the steroid hormone receptors that function predominantly as homodimers, thyroid hormone receptors (TRs) and retinoic acid receptors (RARs) require interaction with the retinoid X receptors (RXRs) for efficient DNA binding and transactivation. RXRs heterodimerize in addition with several other receptors including a novel orphan receptor. RXRs thus have a central role in controlling the activity of a number of hormone receptors that bind structurally unrelated ligands. In the presence of 9-*cis* retinoic acid (9-*cis* RA) or selective synthetic retinoids, RXRs can also form homodimers that bind only to a subset of RAREs and thus allow a different retinoid response. Interestingly the heterodimer and homodimer activities of RXRs can be separated by mutations. More recently we have observed that formation of RXR homodimers can lead to repression of the thyroid hormone response, an example of how RXRs allow crosstalk between different hormonal signals. The orphan receptors COUP-TF and ARP-1 (or COUPα and β) can also participate in the regulation of the retinoid signals. COUP receptors bind to some (but not all) of the RAREs with high affinity and can thereby restrict the action of the retinoid receptors on those response elements. One such RARE is located in the HIV-1 promoter.

Like RXRs and COUPs, TRs can also bind to a certain TREs highly efficient as homodimers. These TREs are inverted palindromes (IPs) with 4 to 6 bp spacers. However binding to these sequences is sensitive to T3. When placed adjacent to the TATA box, IP-TREs can function like bacterial operator sequences to which TR homodimers bind as ligand sensitive repressors. Thus, a complex network of receptor interaction has been unraveled that promises a better understanding of thyroid and retinoid hormone regulation of fundamental biologic processes and in diseases.

K 005 HEAT SHOCK PROTEINS AND THE ASSEMBLY OF THE UNACTIVATED PROGESTERONE RECEPTOR COMPLEX, David Toft, Jill Johnson, Robert Schumacher, Edward Diehl, Magdy Milad, William Sullivan, and Ronald Corbisier, Mayo Medical School, Rochester, MN 55905.

When analyzed in cytosol extracts, the avian progesterone receptor (PR) is complexed with five cellular proteins; the heat shock proteins hsp90 and hsp70 plus three proteins defined by size as p54, p50, and p23. David Smith and co-workers have recently shown p54 and p50 to be immunophilins that bind the immunosuppressant drug, FK506. They are both closely related to the mammalian receptor-associated protein, p59 (also called hsp56 or FKBP52). DNA cloning and sequencing show p23 to be a protein of unique sequence. P23 has been detected in several tissue types and animal species, and it exists mainly in complex with hsp90. When isolated PR is stripped of its associated proteins, these can all be reconstituted into a complex by incubation of the PR in rabbit reticulocyte lysate. This is an ATP-dependent assembly process that can be blocked or reversed by the binding of progesterone. When assembled in this way, two additional PR-associated proteins are observed. The first, p60, is related to the yeast hsp, STI1, and the second is a 40 kDa protein called cyp40 which belongs to a family of proteins (cyclophylins) that bind the immunosuppressant drug, cyclosporin A. These PR-associated proteins can be placed into three categories. The first includes hsp90, hsp70 and probably p60. These are hsp's that are likely to function as chaperones in protein folding. For example, when firefly luciferase is heat-denatured, its activity can be restored by incubation in reticulocyte lysate. This refolding activity requires ATP and it can be partially replaced by a mixture of purified hsp90 and hsp70. The second group of proteins includes p54, p50 (p59) and cyp40. These are all immunophilins that bind the immunosuppressant drug FK506 or cyclosporin A. They may also assist in protein folding through their proline isomerase activity, and they have been implicated as modulators of protein phosphorylation pathways in other systems. Finally, p23 is structurally unrelated to the above proteins. It is ubiquitous and exists in complex with hsp90 indicating that it may act to regulate or assist in a function of hsp90.

Steroid/Thyroid Modification I

K 006 STRUCTURAL ELEMENTS OF THE GLUCOCORTICOID RECEPTOR PROTEIN INVOLVED IN THE BINDING OF STEROID AND DNA, Jan Carlstedt-Duke[1], Per-Erik Strömstedt[1], Ulrika Lind[1], Johanna Zilliacus[2], Anthony Wright[2], Ulf Norinder[3], and Jan-Åke Gustafsson[1,2], [1]Department of Medical Nutrition and [2]Center for Biotechnology, Karolinska Institutet, Huddinge Hospital F60 Novum, S-141 86 Huddinge, Sweden, [3]Karo Bio AB, Box 4032, S-141 04 Huddinge, Sweden.

The steroid receptor proteins are modular proteins in which each of the primary binding functions related to the mechanism of action of the receptor is located in a separate autonomous domain. Interaction between receptor and steroid and between receptor and DNA each occur in separate domains of the protein. The function of ligand interaction within these two domains is entirely self-contained within the boundaries of the individual domains and therefore can be studied in either the intact protein or in isolated domains overexpressed in a number of different systems.

Three segments occuring at the steroid binding surface of the steroid binding domain (SBD) of the human (h) and rat (r) glucocorticoid receptor (GR) as well as the human progestin receptor (PR) have been identified by affinity labelling. Two of the segments are in proximity of the A-ring of the bound steroid (Met-604 and Cys-736 in hGR). Affinity labelling within the C-terminal of these two segments differs between GR and PR. The third segment (Cys-638 in hGR) is found in close proximity to the side chain of the bound steroid. Mutation of Met-601, Met-604 or Cys-736 resulted in receptor proteins with reduced affinity for glucocorticoids. Random mutation of hGR at positions 601 and 736 resulted in a limited number of functional mutants at each site. Mutation of Cys-638 to Ser had no effect on affinity for glucocorticoids.

The primary recognition site for DNA sequence specificity has been localised to the P-box which consists of Gly-439, Ser-440 and Val-443 in hGR. Initial studies comparing the binding specificity of these three residues with the corresponding residues in the estrogen receptor (ER) showed a complex correlation between individual amino acid residues and base pairs, both positive and negative effects, as well as synergistic effects between amino acid residues at these three positions. Further analysis of the components involved in base pair recognition was carried out by mutation at positions 439 and 440 to all twenty amino acids. The ER alternative, Glu, at position 439 as well as the GR alternative, Gly, were each shown to play a unique role in specificity of base pair recognition in comparison with the other 18 alternatives. Ser-440 appears to play a critical role in GR sequence selectivity. However, this effect is entirely dependent on its negative role, inhibiting binding to DNA sequences specific for ER and related receptor proteins. The function of Ser-440 appears to involve hydrogen bonding to other residues in GR and Ser can be functionally substituted by Cys or Thr.

K 007 AGONIST EFFECTS OF ANTAGONIST-OCCUPIED PROGESTERONE B-RECEPTORS: NEW MODEL SYSTEMS, Kathryn B. Horwitz, Departments of Medicine and Pathology, and the Molecular Biology Program, University of Colorado School of Medicine, Denver, CO 80262

Human progesterone receptors (hPR) occur naturally as two isoforms -- 94kDa A-receptors (hPR_A) and 120 kDa B-receptors (hPR_B) -- which form two homodimers (A:A and B:B) and one heterodimer (A:B). The dimers bind to DNA at progesterone response elements (PRE). B-receptors have a 164 aa N-terminal extension that we call the B-Upstream Segment (BUS). In transient expression systems, antagonist-occupied B-receptor homodimers stimulate transcription from a *tk* -CAT reporter in a PRE-independent manner, and from an MMTV-CAT reporter when cAMP levels are increased. By contrast, antagonist-occupied A-receptor homodimers are transcriptionally silent. In A:B heterodimers, A-receptors dominantly suppress the agonist-like effects of antagonist-occupied B-receptors. To show that these anomalous responses to antagonists persist when receptors are expressed at physiological levels, we developed new breast cancer cell lines that have only one receptor isoform. First, a monoclonal PR-negative T47D cell line (T47D-Y) was selected by flow cytometry. This cell line was then stably transfected with plasmids containing either hPR_A or hPR_B cDNAs, to produce cells expressing only A-receptors (YA) or B-receptors (YB). As in the transient systems, in these stable models, antagonists have inappropriate agonist-like transcriptional effects in YB cells but not YA cells. These cells also allow the study of long-term hormone effects on the mitotic cell cycle using flow cytometry. In YA and YB cells the agonist R5020 doubles the percent of cells in S-phase after 16 hours. Progesterone antagonists do not increase S-phase, and inhibit the increase produced by R5020. However, when cAMP levels are raised, the antagonist RU486 becomes an agonist -- it doubles the percent of cells in S-phase in YB cells but not in YA cells. To explain these unique properties of B-receptors we constructed expression vectors linking BUS to the DNA binding domain and nuclear localization signal of hPR (BUS-DBD-NLS). Transient transfection studies using a PRE_2-TATA-CAT promoter-reporter, show that BUS-DBD-NLS is a strong constitutive transactivator, stimulating CAT to levels comparable to those of full-length hPR_B. BUS-DBD-NLS stimulated transcription only when BUS was bound to DNA since no transactivation was obtained with BUS-NLS lacking the DBD, or with the DNA binding mutant, $BUS\text{-}DBD_{cys}\text{-}NLS$. The DBD alone had no intrinsic activation function, since a DBD-NLS fragment was transcriptionally inactive. The transcriptional activation function of BUS appears to be regulated by phosphorylation of *ser-pro* motifs located on this domain of B-receptors.
CONCLUSIONS: The agonist-like effects of progesterone antagonists, as assessed by transcription or by growth analyses in new cell models, are restricted to the B-receptors. These inappropriate effects of B-receptors may be mediated by a third transactivation domain, TAF-3, that is activated by antagonists, and is missing in A-receptors.

K 008 GLUCOCORTICOID RECEPTORS: ATP-DEPENDENCE, HORMONE-DEPENDENT HYPERPHOSPHORYLATION AND THE CELL CYCLE. **Jack Bodwell, Jiong-Ming Hu, Li-Ming Hu, Eduardo Ortí and Allan Munck.** Department of Physiology, Dartmouth Medical School, Lebanon, NH 03756-0001.

Early studies showing that hormone binding to glucocorticoid receptors (GRs) falls and rises with cellular ATP levels led to the hypothesis that GRs normally traverse an ATP-dependent cycle, possibly involving phosphorylation. Independent kinetic evidence also pointed to a GR cycle. Recently we have found that (i) after undergoing hormone-dependent activation and hyperphosphorylation, GRs recycle to unliganded form in hyperphosphorylated state; (ii) ATP depletion of cells causes accumulation of GRs unassociated with Hsp90. From these observations we conclude that the postulated ATP-dependent cycle can be accounted for by hormone-induced or spontaneous dissociation of GR•Hsp90 complexes, followed by re-association of unliganded GRs with Hsp90, Hsp70 and related components via ATP-dependent reactions like those other workers have demonstrated *in vitro*.

Mutation to alanine of several of the 7 phosphorylated sites we identified in the N-terminal domain of mouse receptors leads to significant loss of GR transactivating activity with reporter genes. Four of the 7 sites lie in consensus sequences for $p34^{cdc2}$ kinases important in cell cycle regulation. This and observations by others point to a potential role for receptor phosphorylation in causing the well-documented resistance of proliferating cells to glucocorticoids during G2-M phases of the cell cycle. We find that glucocorticoid treatment hyperphosphorylates glucocorticoid receptors in S, but fails to hyperphosphorylate in G2-M. Supporting a previous suggestion, basal receptor phosphorylation is higher in G2-M than in S. Resistance to glucocorticoid activity in G2-M is thus associated with raised basal phosphorylation levels of receptors, and absence of hormone-induced hyperphosphorylation.

Steroid/Thyroid Receptor Cell Biology

K 009 REGULATION OF STEROID RECEPTOR NUCLEAR TRANSPORT, Donald B. DeFranco, Anuradha P. Madan, Uma R. Chandran, and Jun Yang, Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260.

We have used transient heterokaryon analysis to monitor the nuclear export and nucleocytoplasmic shuttling of glucocorticoid (GR) and progesterone receptors (PR) *in vivo*. Both GR and PR were capable of exporting from transfected Cos-1 cell nuclei and importing into NIH3T3 cell nuclei in the presence of hormone agonist or antagonist. In contrast, in a cell line exhibiting constitutive nuclear localization of both PR and GR (i.e. CHO cells), PR, but not GR was efficiently exported from nuclei in the absence of hormone. Thus, although GR and PR exhibit differential nuclear export capabilities when unliganded, both receptors are capable of shuttling between the nuclear and cytoplasmic compartments when ligand bound. A GR chimera that possesses a segment of SV40 TAg encompassing its nuclear localization signal (NLS) fused to its amino terminus efficiently imports into nuclei when unliganded, but does not efficiently export from nuclei. Interestingly, hormone treatment relieves the repressive effect of the TAg NLS and permits nucleocytoplasmic shuttling of this TAg/GR chimera. In addition to *in vivo* studies of steroid receptor nuclear export, we have also initiated *in vitro* analyses of steroid receptor nuclear transport using a digitonin permeabilized cell assay. *In vitro* transport of GR derived from rat hepatoma cell cytosol into HeLa cell nuclei was observed to be ATP, temperature and hormone dependent and required receptor activation. Interestingly, while the *in vitro* nuclear import of SV40 TAg was efficiently inhibited by either adding an antibody against the 70 kilodalton family of heat shock proteins (hsp70) to cytosol, or depletion of hsp70 from cytosol, GR nuclear import was unaffected. Thus, unlike the NLSs present in TAg and other karyophilic proteins, hsp70 does not appear to required for GR NLS function *in vitro*.

Transcriptional Activation/Repression

K 010 MECHANISMS OF RESPONSE ELEMENT RECOGNITION AND TRANSACTIVATION OF RETINOIC ACID AND THYROID HORMONE RECEPTOR HOMO- AND HETERODIMERS, Hinrich Gronemeyer, Jia-yang Chen, Zhiping Chen, Sylvie Mader, Xiquang Shen, Christina Zechel, and Pierre Chambon, INSERM/U184 - CNRS/LGME - Institut de Chimie Biologique, Faculté de Médecine 11 rue Humann, 67085 Strasbourg, France.

Selective recognition of cognate DNA elements by a trans-acting regulatory protein is a key step in the cascade of events that ultimately lead to the regulation of specific programmes, which in the case of the nuclear receptor superfamily, control diverse aspects of development, cell growth and differentiation, and homeostasis. Thus, the mechanisms that define the DNA response element repertoires of nuclear receptors are of major importance in the signal transduction pathways initiated by steroid and thyroid hormones, retinoids and vitamin D3. We have determined the binding site repertoire of RAR, RXR and TR homo- and heterodimers to direct repeats of the basic PuG(G/T)TCA motif and found that the DNA binding domains (DBDs) dictate the response element recognition of the corresponding full length receptors by (i) steric hindrance phenomena, (ii) homocooperative and (iii) heterocooperative interactions. The regions within the DBDs that are responsible for these interactions have been defined. Two types of dimerization interfaces give rise to cooperative DR binding, both of which involve the second zinc finger of one RXR monomer. Type I interfaces, which are formed between RXR and TR or RAR on DR4 and DR5, respectively, involve overlapping, though distinct, regions in the first zinc finger of TR and RAR as the complementary heterodimerization surface. Type II interfaces are formed with the T-boxes of either RXR (DR1 homodimers) or RAR (DR2 heterodimers). Modelling of these various interactions, based on the 3D structure of the GR DBD, suggested that heterocooperativity requires anisotropic binding of the corresponding DBDs, with the RXR DBD occupying the 5' position of the corresponding DR element. Crosslinking demonstrated that this anisotropy is maintained in the full-length receptors and contributes to the efficiency of transactivation.

K 011 POSITIVE AND NEGATIVE REGULATION BY THYROID HORMONE: INSIGHTS FROM NATURAL RECEPTOR VARIANTS, T. Nagaya, Laird D. Madison, and J. Larry Jameson, Division of Endocrinology, Northwestern University Medical School, Chicago, IL 60611.

Although most studies have focused on transcriptional activation of positively regulated genes, it is apparent that transcriptional repression is also an important mechanism for physiologic control by thyroid hormone receptors. Transcriptional repression occurs in three different manners: 1) Silencing of basal transcription by unliganded receptor where repression is mediated through TREs and is relieved upon the addition of T3. 2) T3-activated repression which involves negatively regulated promoters such as the TSH α and ß genes. 3) Repression by functionally defective receptor isoforms such as $\alpha 2$ or naturally occurring receptor mutants which occurs by competition with the normal receptor for binding to TREs. In this report, we summarize data concerning the role of receptor dimerization in the processes of silencing, T3-mediated repression, and competition for target TREs. Our approach involves creation of mutations in the amino acid sequences surrounding the ninth heptad repeat at the carboxyterminal end of the thyroid hormone receptor. Various mutants distinguish receptor domains involved in the formation of receptor homo- and heterodimers. Mutants defective in homodimerization are incapable of silencing, suggesting an important role for the homodimer in this process. Mutants retaining homodimerization but incapable of T3 binding and heterodimerization with RXR lose the ability to block T3 dependent stimulation of positively regulated genes. These results suggest that in the presence of T3, TREs are preferentially occupied by heterodimers. Furthermore, dimerization mutants exhibit remarkable selectivity for different arrangements of TRE half-sites. For example, the homodimerization defective $\alpha 2$ isoform binds as a heterodimer to TREs arranged as direct repeats, but not to TREs arranged in a palindromic or inverted palindromic orientation. Consequently, $\alpha 2$ acts a specific antagonist of a subset of TREs. These results emphasize an important role for receptor dimerization as a mechanism for modulating different aspects of transcription repression.

K 012 DIFFERENTIAL EXPRESSION OF COUP-TF I AND II IN DEVELOPING MOUSE CENTRAL NERVOUS SYSTEM, Yuhong Qiu, Austin J. Cooney, Shigeru C. Kuratani[1], Sophia Y. Tsai, Ming-Jer Tsai, Dept. of Cell Biology, [1]Dept. of Biochemistry, Baylor College of Medicine, Houston, TX 77030.

COUP-TFs are members of the orphan subfamily of the steroid/thyroid hormone receptor superfamily. They bind to AGGTCA direct repeats and palindromes with various spacings. Some of these binding sequence are also the response elements for VDR, TR, RXR, and RAR. We have shown that COUP-TFs repress the transactivation function of these receptors in cell culture. Studies on COUP-TF expression in zebrafish and chick embryos suggest that COUP-TFs might be involved in the development of the neural tube. To study the physiological functions of COUP-TFs, we cloned COUP-TF counterparts from mouse (mCOUP-TF I and II). mCOUP-TF I and II showed 99% and 100% identity to human COUP-TF I and II, respectively. The spatial and temporal expression patterns of the two genes were examined by *in situ* hybridization on frozen sections of 7.5 to 18.5 days post coitum (p.c.) embryos using antisense RNA as probe. The results showed that the expression of mCOUP-TF I and II began around 8.5 days p.c., peaked at 14-15 days p.c., and declined by 18.5 days p.c. The expression of the two genes was very broad in the central nervous system. The distribution of mCOUP-TF I and II transcripts were overlapping, yet clearly showed distinct patterns. Differential expression was observed in the telencephalon, diencephalon, midbrain, and the spinal cord. Since RA and its derivatives have profund effects in organogenesis, COUP-TFs may exert their function by regulating or modulating the RA signal pathway or they might play very important roles in embryogenesis independently.

Tissue Specific Gene Expression

K 013 DISRUPTION OF HNF-4 BY HOMOLOGOUS RECOMBINATION. W. S. Chen, D. C. Weinstein, A. S. Plump, J. L. Breslow, and J. E. Darnell, Jr., The Rockefeller University, New York, N.Y. 10021

Hepatocyte Nuclear Factor 4 (HNF-4), a liver-enriched transcription factor also present in intestine and kidney, binds to the upstream elements of a very large number of liver-expressed genes. For example, HNF-4 is a potential activator of the apolipoprotein genes as well as the HNF-2 transcription factor. HNF-4 is the earliest known marker of hepatocytes, being present in the liver primordium. The Drosophila counterpart of HNF-4, also cloned in this lab, appears to play a critical role in gut development. We therefore felt that a disruption of the mouse HNF-4 would prove illuminating.

An HNF-4 fragment was constructed in which the DNA-binding domain was replaced by the neomycin gene. This construct was transfected into embryonic stem cells which were then selected for homologous recombination of the replacement gene. HNF-4-1-mice have been found to fail in an early step in gastrulation. We will discuss this defect and its implications.

K 014 MOLECULAR GENETIC ANALYSIS OF cAMP AND GLUCOCORTICOID SIGNALING IN DEVELOPMENT, Günther Schütz[1], Edith Hummler[1], Timothy J. Cole[1], Julie A. Blendy[1], Wolfgang Schmid[1], Ruth Ganss[1], Adriano Aguzzi[2], [1]Division Molecular Biology of the Cell I, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany, [2]Institute of Neuropathology, University of Zürich, 8091 Zürich, Switzerland.

To understand the role of cAMP and glucocorticoid signaling during development, we have disrupted the CREB and the glucocorticoid receptor gene by homologous recombination in mouse embryonic stem cells. Mice deficient in the CREB protein were generated with a distribution significantly deviating from the expected Mendelian frequency, however, surviving mutants appeared healthy and exhibited no impairment of growth and development. We therefore investigated the potential of other members of the CREB/ATF family to substitute for CREB in mutant mice. Analysis with an antibody selectively recognizing CREB after phosphorylation by protein kinase A demonstrated that two other members of the CREB/ATF family, CREM and ATF-1, are also targets for PKA phosphorylation and appear to be the only dimerization partners for CREB. Examination of CREM mRNA and protein levels in CREB -/- mice demonstrated overexpression of CREM in all tissues examined, but no change in ATF-1 levels. We conclude that a subset of CRE binding-proteins is able to transduce the cAMP signal to the nucleus, which in the absence of CREB can compensate for CREB function *in vivo*. In the case of the targeted mutation of the glucocorticoid receptor, most of the mice die in the perinatal period. The basis of lethality is presently being investigated.

Keynote Address

K 015 CHROMATIN STRUCTURE AND REGULATION OF GENE EXPRESSION BY STEROID HORMONES, Miguel Beato, Mathias Truss, Jörg Bartsch, Reyes Candau and Sebastián Chávez, I.M.T, E.-Mannkopf-Str. 2, 35037 Marburg, F.R.Germany.

Induction of gene expression is often accompanied by alterations in chromatin structure reflected as changes in accessibility of the corresponding DNA sequences to various nucleases. Whether these changes are a consequence or rather a prerequisite for transcriptional activation is still unclear. Transcription of the MMTV proviral genome is induced by steroid hormones, in particular glucocorticoids and progestins. The MMTV promoter contains a complex hormone responsive region (HRR) upstream of a binding site for the transcription factor nuclear factor I (NFI) and two octamer motifs between the NFI site and the TATA box. Optimal hormonal induction of MMTV expression requires the integrity of the HRR, the NFI binding site, and the octamer motifs. While the octamer transcription factor OTF1 cooperates with hormone receptors in transcription from the MMTV promoter *in vitro*, the role of NFI can not be reproduced in cell-free systems using free DNA as tempalte. In transiently transfected cells the HRR precludes access of NFI-VP16 to the MMTV promoter, probably due to its organization in chromatin. This repression is relieved upon hormone induction. In cells carrying MMTV sequences either as minichromosomes or integrated in the chromosomal context, the HRR, the NFI site and at least the octamer distal motif are organized into a phased nucleosome. High resolution analysis of the nucleosomal DNA by genomic footprinting shows that its rotational setting in the living cell is identical to that reported for reconstituted nucleosomes. The orientation of the double helix on the surface of the histone octamer is compatible with binding of the hormone receptors to two of the four sites on the HRR, but would precludes binding of NFI and OTF1. Hormone treatment leads to a rapid alteration in chromatin structure that makes the dyad axis of the regulatory nucleosome more accessible to digestion by DNaseI and restriction enzymes. As demonstrated by genomic footprinting, this structural alteration of the nucleosome is accompanied by partial occupancy of the four receptors binding sites of the HRR, the NFI, and the octamer motifs. Neither receptor nor NFI and/or OTF-1 binding appear to be sufficient for complete removal of the histone octamer from the MMTV promoter. The exact nature of the structural alteration in the regulatory nucleosome is presently unknown, but it is only induced by agonistic receptor ligands and is independent of transcription elongation. The potential role played by modifications of the core histones is discussed in connection with studies on MMTV promoter expression in yeast.

Mutant Receptors

K 016 ANDROGEN RESISTANCE DUE TO MUTATIONS IN THE ANDROGEN RECEPTOR GENE, Michael J. McPhaul, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX.

Mutations in the androgen receptor gene cause phenotypic abnormalities of male sexual development in affected individuals that range from a female phenotype (complete testicular feminization) to that of undervirilized or infertile men. Due to the location of the androgen receptor on the X-chromosome and the normal lifespan of subjects with androgen resistance, a large number of individuals with androgen resistance caused by mutations in the androgen receptor gene are available for ascertainment. These subjects have been studied and classified according to the abnormality identified in ligand-binding studies performed in their genital skin fibroblasts: absent androgen-binding, reduced levels of androgen binding, and qualitative androgen binding abnormalities. In approximately 10% of subjects, no abnormality of androgen binding is demonstrable.

Analysis of the androgen receptor coding sequence, immunoblot assays, and assays of androgen receptor function have permitted the definition of mutations in the androgen receptor gene and their effects on receptor function and abundance in a number of pedigrees. The defects are most often due to nucleotide changes within the androgen receptor gene that result either in the introduction of premature termination codons or single amino acid substitutions within the androgen receptor open-reading frame. Premature termination codons have been identified in several coding exons and, with one exception, such mutations lead to the synthesis of an androgen receptor truncated at its carboxy terminus that is inactive in ligand-binding and transactivation assays. In a single pedigree, a premature codon in place of amino acid residue 60 (Q60X) results in the synthesis of a shortened form of the androgen receptor truncated at its amino terminus. This smaller form of the androgen receptor (approximately 87 kDa) is derived from translation initiation at an internal methionine, binds hormone normally, is analogous to the A-form of the human progesterone receptor, and is synthesized in fibroblast strains established from normal individuals.

Amino acid substitutions causing androgen resistance cluster in the DNA-binding domain and two short segments of the hormone-binding domain. Analyses in transfected cells and in broken cell assays indicate that distinctive amino acid substitutions within the androgen receptor have different effects on the capacity of mutant androgen receptors to bind different androgens or to bind to target DNA sequences. Functional studies and immunoblot assays indicate that in most cases the phenotypic abnormalities observed in subjects with androgen resistance are paralleled by decreases in the function and/or level of the mutant androgen receptor expressed in patient fibroblasts.

Cross-Talk Between Signalling Systems

K 017 CROSS-TALK BETWEEN PEPTIDE GROWTH FACTOR AND ESTROGEN RECEPTOR SIGNALING SYSTEMS, Diane M. Ignar-Trowbridge[1], Maria Pimentel, Christina T. Teng, Kenneth S. Korach and John A. McLachlan, National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709 and [1]Glaxo Research Institute, Glaxo Inc., Research Triangle Park, NC 27709.

Estrogen-inducible polypeptide growth factors may act as mediators of estrogen action in normal and neoplastic tissues. Our laboratory has previously demonstrated that many of the physiological effects of estrogen in the murine female reproductive tract such as induction of DNA synthesis, lactoferrin gene expression, and phosphatidylinositol lipid metabolism, are reproduced by epidermal growth factor (EGF) administered in a cholesterol-based pellet implanted under the kidney capsule. However, the mechanisms of convergence between the estrogen and EGF signaling pathways are presently unknown. A specific estrogen receptor antagonist, ICI 164,384, inhibited the estrogen-like effects of EGF in the uterus. In addition, EGF elicited enhanced nuclear localization of uterine estrogen receptors and the formation of a unique nuclear ER form that is present after estrogen treatment. These in vivo observations suggest that some biological effects of EGF in the reproductive tract may be mediated by estrogen receptors. The possibility that peptide growth factors could activate transcription from a consensus estrogen-responsive element (ERE) in an estrogen receptor-dependent manner was assessed in Ishikawa human endometrial adenocarcinoma cells, which contain negligible levels of estrogen receptors, and BG-1 human ovarian adenocarcinoma cells, which contain a high level of estrogen receptors. EGF and transforming growth factor α (TGFα) induced transcriptional activation of a consensus ERE in an estrogen receptor-dependent manner. Transcriptional activation was inhibited by both ICI 164,384 and neutralizing antibodies to the EGF receptor. Interestingly, synergism between a physiological dose of estrogen and peptide growth factors was observed. The presence of cross-talk between EGF receptor and estrogen receptor signaling pathways suggests that interactions between growth factors and steroid receptors may regulate normal and aberrant function in mammalian cells.

K 018 ESTROGEN RECEPTOR INTERACTIONS WITH CELLULAR SIGNALING PATHWAYS, Benita Katzenellenbogen, Monica M. Montano, David J. Schodin, Pascale Le Goff, Susan M. Aronica, and Hyeseong Cho, Department of Physiology and Biophysics, University of Illinois, Urbana, IL 61801.

We find that protein kinase A and protein kinase C activators, and the growth factor IGF-1, increase the transcriptional activity of the estrogen receptor in a promoter-dependent and cell-specific manner. Transcriptional enhancement is also obtained by transfection of expression vectors for the protein kinase A catalytic subunits. Suppression of this activity by protein kinase inhibitors and by antiestrogen suggests the involvement of the estrogen receptor and a role for phosphorylation (1,2). In addition to altering transactivation of estrogen-responsive reporter gene constructs, cAMP, IGF-1 and estrogen regulate expression of the progesterone receptor in rat uterine and human breast cancer cells, and the use of inhibitors and other findings imply regulation of progesterone receptor expression at different levels by these agents. Our studies show that cAMP and estradiol stimulate phosphorylation of the estrogen receptor and that specific phosphorylation sites are associated with enhanced bioactivity of the estrogen receptor. Receptor mutants are being utilized to map regions of the estrogen receptor important in this transcriptional activation/synergism. The promoter-dependence of the phenomenon highlights the fact that factors in addition to the estrogen receptor are also important.

1. Cho, H. and Katzenellenbogen, B.S. (1993) *Molec. Endocrinol.* 7:441-452.
2. Aronica, S.M. and Katzenellenbogen, B.S. (1993) *Molec. Endocrinol.* 7:743-752.

K 019 ANDROGEN-SPECIFIC RESPONSE FROM NONSPECIFIC DNA ELEMENTS, Diane M. Robins, Adam J. Adler, Arno Scheller, Cameron O. Scarlett, and Stefanie Nelson, University of Michigan, Ann Arbor, 48109-0618.

Transcriptional specificity is a fundamental problem within several transcription factor families whose members recognize similar sequences. This is especially evident for the steroid receptors, since the consensus glucocorticoid (or hormone) response element (GRE, HRE) can be activated by androgen, progesterone and mineralocorticoid receptors as well. We have characterized a novel enhancer of the mouse sex-limited protein (*Slp*) gene that is activated by androgen but not by glucocorticoid in transfection, similar to expression of the gene *in vivo*. Induction requires both a consensus HRE and auxiliary elements also present within a 120 bp DNA fragment. Cotransfection with wild type and mutant receptors reveals that GR can bind but cannot transactivate from the HRE within the enhancer. Multiple nonreceptor factors are involved in specificity, with respect to both the elevation of AR activity and the inactivity of GR. Placing the HRE a short distance from the rest of the enhancer allows GR to function, suggesting that juxtaposition to particular factors represses GR within the androgen-specific complex. Therefore transcriptional response is determined by the context of the receptor binding site. Surprisingly, sequence variation of the HRE, within the context of the enhancer, alters stringency of hormonal response. GR shows distinct affinity differences for a set of HREs among which AR does not distinguish. Differences in sequence recognition of AR and GR are also evinced in *in vitro* footprinting assays on wild type and mutant enhancers. However, various HREs only confer hormonal responses corresponding to their differential receptor binding within the context of the enhancer. This suggests that these sequences act to alter interactions with other factors, perhaps via precise positioning of the receptor. Thus protein interactions that elicit specific regulation are established by the array of DNA elements and may be modulated by sequence variations within these elements that influence protein contacts. We are using a variety of approaches to identify the accessory factors involved in specificity, including *in vivo* footprinting assays to reveal which DNA elements are crucial for androgen regulation. A major site adjacent to the HRE is protected from DNaseI in male liver and kidney nuclei; this site acquires protection in females treated with testosterone. Other sites within the enhancer differ in tissue-specific as well as sex-specific manners. To confirm the relevance to *in vivo* expression of particular sequences identified in transfection, we are testing their specificity in transgenic mice. Ultimately, this may allow insight into promoter and cell-type specific steroid response.

Development

K 020 RETINOIC ACID RECEPTORS AND AMPHIBIAN LIMB REGENERATION, Jeremy P. Brockes, Ludwig Institute for Cancer Research, 91 Riding House Street, London W1P 8BT.

Limb regeneration in urodele amphibians such as the newt and axolotl is a key system for evaluating the morphogenetic effects of retinoic acid (RA). Amputation is followed by local formation of a blastema, a growth zone of mesenchymal progenitor cells surrounded by a wound epidermis. Three effects of RA on the regenerating limb are a) respecification of the positional identity of the blastema to a more proximal value, b) transient arrest of cell division, and c) induction of secretory differentiation in the wound epidermis (mucous metaplasia). At least six different RARs are expressed in the newt limb and limb blastema(1). Their function has been analysed by replacing the ligand binding domain with that of the Xenopus thyroid hormone receptor-α, transfecting the chimeras into cultured blastemal cells or into the wound epidermis in situ by use of a particle delivery system, and activating with T3 (2). Arrest of cell division is mediated by α1, whereas induction of secretory differentiation is mediated by δ1.
Cultured newt blastemal cells hve been transfected with plasmids that provide a normalised reporter system for RA. After implanting such cells into a host blastema, there is differential activation of the reporter consistent with activation of the retinoid response pathway during normal regeneration (3). The wound epidermis is a potential source of retinoid and this possibility has been evaluated by coculture of this tissue with reporter blastema cells.

(1) Mech. Dev. 40, 99-112; (2) EMBO J. 12, 3459-3466; (3) PNAS 89,11386-11390

K 021 THE REGULATION OF EMBRYONIC CELL DIFFERENTIATION BY RETINOIC ACID, Lorraine J. Gudas[1], Alexander Langston[1], John Boylan[1], Betsy Hosler[1], Pierre Chambon[2], and Melissa Rogers[1], Department of Pharmacology, Cornell University Medical College, New York, NY 10021, [2]Laboratoire de Genetique Moleculaire des Eucaryotes du CNRS, Institut de Chimie Biologique, INSERM-U 184, Strasbourg 67085, France

Retinoic acid (RA), a derivative of retinol, influences the proliferation and cellular differentiation of a wide variety of cell types. RA also exhibits striking effects on vertebrate development, lending support to the idea that RA plays a pivotal role in pattern formation during development. F9 murine teratocarcinoma stem cells closely resemble the pluripotent embryonic stem cells of mouse blastocysts and thus provide an important model system in which to study critical early events in mouse development during the time when initial cell lineage commitments are made. F9 teratocarcinoma stem cells differentiate into primitive endoderm in response to RA and into parietal endoderm, an epithelial extraembryonic cell type in the mouse blastocyst, in response to RA and dibutyryl cyclic AMP. When F9 cells differentiate, genes such as those encoding laminin B1 and collagen type IV(α1) are transcriptionally activated between 24 and 48 hours after exposure to RA. In contrast, within 2 hours after RA addition the ERA-1/Hox 1.6 (a1) gene is transcriptionally activated. We have shown that the RA activation of the Hox 1.6 gene is controlled through the activity of an enhancer that contains an RARE identical to the RARE of the RAR β (retinoic acid receptor β) gene; however, other sequences also influence the activity of this enhancer, suggesting the presence of binding sites for novel proteins which regulate Hox 1.6 expression. Experiments in F9 cells with Hox 1.6 minigenes in which lacZ expression is controlled by the Hox 1.6 promoter and enhancer demonstrate that it is the 3' enhancer which confers RA responsiveness on the endogenous Hox 1.6 promoter, as constructs which *lack* the enhancer do not respond to RA. Analyses of transgenic animals also point to the importance of this enhancer for Hox 1.6 expression. This identification of the RA responsive enhancer downstream of the Hox 1.6 gene demonstrates that RA directly controls the transcription of at least one member of a gene family that determines tissue identity in the vertebrate embryo.

Recent analyses of two F9 cell lines, one RAR γ -/- and one RAR α -/- (lines in which both chromosomal copies of the genes have been disrupted by homologous recombination), have shown that the *RAR γ* gene is required for the activation of *both* the Hox 1.6 (a1) and laminin B1 genes, but *not* for activation of the Hox 2.9 (b1) or the CRABP II gene. In contrast, RAR α is required for the activation of the CRABP II gene in response to RA, but not for the activation of the Hox 1.6 gene. Our results strongly suggest that different homeobox gene clusters are regulated by different RARs.

Preliminary analyses of the mechanism by which the expression of the REX-1 gene is negatively regulated at the transcriptional level by RA have also been carried out in both F9 cells and in mouse embryos. A region required for REX-1 promoter activity in F9 stem cells contains an octamer motif (ATTTGCAT), a binding site for "octamer" transcription factor members of the POU domain family of DNA binding proteins. REX-1 promoter/CAT reporter plasmids including this octamer site exhibit reduced expression in F9 cells treated with RA. A larger REX-1 promoter fragment, also containing the octamer site, was able to promote expression of the bacterial lacZ gene in mouse embryos at the morula stage. Such molecular studies provide a basis for understanding the actions of vitamin A and RA in embryogenesis.

Orphan Receptors

K 022 **THE DIOXIN RECEPTOR - ANOTHER LIGAND ACTIVATED TRANSCRIPTION FACTOR**, Jan-Åke Gustafsson, Murray Whitelaw, Anna Wilhelmsson, Lorenz Poellinger, Dept. of Medical Nutrition, Novum, Karolinska Institute, Huddinge University Hospital, Stockholm, Sweden

The intracellular dioxin receptor is a basic helix-loop-helix (bHLH) gene regulatory protein the activity of which is dependent on binding of the appropriate ligand. There is no known endogenous ligand for this receptor. However, its activity can be induced by binding of a variety of environmental contaminants including dioxin (2,3,7,8-tetrachlorodibenzo-*p*-dioxin). The conversion of the latent dioxin receptor to a transcriptionally active form is termed activation. The details of this process are poorly understood, but it is clear that several critical steps are involved in this pathway. In nonstimulated cells the receptor can be detected as an ~300 kDa heterooligomer containing the molecular chaperon hsp90 (Mr 90 kDa heat shock protein). Following binding of ligand hsp90 is released from the complex, and the ligand binding receptor subunit dimerizes with its bHLH partner protein arnt. These proteins dimerize with one another via their bHLH domains. The resulting heterodimer binds to the target DNA sequence of the receptor, termed xenobiotic response element, that mediates dioxin regulation of target promoter activity. Dioxin dependent activation of the DNA binding activity of the receptor has been reconstituted *in vitro* using crude or fractionated cytoplasmic extracts from non-treated wild-type or mutant hepatoma cells. These studies show that ligand dependent recruitment of arnt confers DNA binding specificity to the dioxin receptor. The construction of glucocorticoid/dioxin receptor fusion proteins allows the regulation of glucocorticoid receptor activity by dioxin in transient transfections of CHO and hepatoma cells. Thus, in the absence of dioxin, chimeric receptor constructs which contain large 500-720 amino acid C-terminal dioxin receptor fragments, but lack the N-terminal bHLH motif, confer repression upon the transcriptional activity of a glucocorticoid receptor derivative τDBD containing its N-terminal strong transactivating signal (τ) and its DNA binding domain (DBD). In the presence of dioxin, this repression is reversed. Importantly, these chimeric receptors did not require the bHLH arnt co-factor for function. A considerably smaller region of the dioxin receptor located between amino acids 230-421 showed specific dioxin binding activity *in vitro*. Moreover, dioxin binding *in vitro* correlated with the ability of receptor fragments to form stable complexes *in vitro* with the molecular chaperon hsp90. In summary, the dioxin receptor system provides a novel and complex model of regulation of bHLH factors that may also give important insights into the mechanisms of action of ligand-activated nuclear receptors.

K 023 RESPONSE ELEMENTS AND TRANSACTIVATION MECHANISMS OF PPARS, Walter Wahli, Hansjörg Keller, Grogorios Krey and Abderrahim Mahfoudi, Institut de Biologie animale, Université de Lausanne, CH - 1015 Lausanne, Switzerland.

Peroxisome Proliferator Activated Receptors (PPARs) are transcription factors belonging to the nuclear hormone receptor superfamily. At least three such receptors are expressed in *Xenopus laevis*, xPPARα, β and γ. The PPAR response elements of several target genes including those of acyl-CoA oxidase, enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydrogenase and cytochrome P450/4A6 fatty acid ω-hydroxylase, have been identified. This analysis reveals that the minimal PPRE is a DR-1 element (AGGTCANAGGTCA). All three xPPARs are able to bind to this element, but only as heterodimers with the receptor for 9-cis retinoic acid (RXR). Analysis of palindromic and inverted palindromic elements with different spacings between the half-site motifs demonstrated that the estrogen response element (ERE, pal3) is also a strong PPAR response element to which PPAR binds as heterodimer with RXR, too. Together, these results suggest that PPARs interact with different hormonal signaling systems. The PPRE of the acyl-CoA promoter is located 560 bp upstream from the transcription initiation site. Strong activation of this promoter depends on a functional interaction of PPAR with Sp1 for which several binding sites are present close to the transcription initiation site. Physiological concentrations of fatty acids, especially polyunsaturated fatty acids, activate xPPARα to the same extent as the known xenobiotic peroxisome proliferators. Cyclooxygenase, lipoxygenase and epoxigenase inhibitors did not affect activation of PPARs indicating that β-oxidation is not required for the generation of activating molecules. The potency of different activators was tested on PPARα, β and γ and functional diversity was revealed.

Poster Session I

K 100 TRANSACTIVATION PROPERTIES OF RETINOIC ACID AND RETINOID X RECEPTORS IN MAMMALIAN CELLS AND YEAST: CORRELATION WITH HORMONE BINDING AND EFFECTS OF METABOLISM, Elizabeth A. Allegretto, Michael R. McClurg, Steven B. Lazarchik, Marc G. Elgort, J. Wesley Pike, and Richard A. Heyman. Ligand Pharmaceuticals, Inc. 9393 Towne Centre Drive, San Diego, CA 92121.

To study the structural and functional properties of the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), they were recombinantly expressed in Sf21 cells and yeast, and specific antibodies were produced against each receptor subtype. The binding affinities of 9-*cis* retinoic acid (9-*cis* RA) and all-*trans* retinoic acid (t-RA) for RARα, β, γ and for RXRα, β, γ were determined using the recombinant receptor proteins, and were compared with each hormone's ability to activate transcription through the retinoid receptors in mammalian and yeast cell assay systems. The K_d values obtained for the binding of 9-*cis* RA to the RXR subtypes from Sf21 cells or yeast were in the range of 1.4-2.4 nM, 8-fold lower than previously reported. 9-*cis* RA also bound to the RARs, with K_ds ranging from 0.2 nM to 0.8 nM. The ability of 9-*cis* RA to bind to the RARs and RXRs correlated with its ability to produce similar transactivation profiles with these receptors in mammalian and yeast cell assays. t-RA bound to the RARs with affinities (K_ds = 0.2-0.4 nM) similar to that of 9-*cis* RA and activated transcription through the RARs in mammalian and yeast cells. In contrast, while t-RA did not bind to the RXRs, it did activate the RXRs, albeit less potently than 9-*cis* RA, in mammalian cells. In yeast, however, the RXRs activated transcription only in the presence of 9-*cis* RA, not with t-RA. While RARγ was activated in yeast by either t-RA or 9-*cis* RA to stimulate transcription through an RARE, the overall level of transcription was increased upon the addition of hormone-occupied RXR. Metabolism studies suggest that while there was no cell-dependent interconversion between t-RA and 9-*cis* RA in yeast, there was evidence of cell-dependent conversion of 9-*cis* RA to t-RA in mammalian cells.

K 101 HORMONE AND DNA BINDING FUNCTIONS OF THE ECDYSONE RECEPTOR REQUIRE EcR/ULTRASPIRACLE COMPLEXES, M. Arbeitman, M. Koelle, M. Bender and D. Hogness, Department of Developmental Biology, Stanford School of Medicine, Stanford, CA 94305-5427

Ecdysone is a steroid hormone that stimulates molting and metamorphosis in Drosophila. The active ecdysone receptor is a heteromeric complex between two members of the steroid receptor superfamily, the EcR protein and the Ultraspiracle protein (Yao and Evans; Koelle, Arbeitman and Hogness). EcR was originally identified as a component of the ecdysone receptor, as it partakes in binding ecdysone response elements (EcRE) and is required for ecdysone responsiveness in cultured Drosophila cells. However EcR expressed in yeast or mammalian cos-1 cells does not bind EcRE DNA or ecdysone, but can be activated for these activities with protein fractions that lack EcR.

Using proteins that are expressed in a yeast expression system, we found that EcR/USP complexes bind EcRE DNA, but neither protein alone is sufficient for binding. This is reminiscent of the requirement of vertebrate receptors for dimerization with RXR for binding to their DNA response elements. *usp* is the Drosophila homologue of the mammalian RXR gene. In addition, we observe hormone binding by USP and EcR together, but by neither protein alone. This result has no precedent in the studies of vertebrate steroid receptors. The EcR gene encodes three isoforms that are identical in the carboxy terminal domain and therefore share the same DNA and hormone binding domains. All three isoforms require USP for DNA and hormone binding.

A large number of EcR mutants have been identifed which map to the DNA and hormone binding domains of EcR (see poster by Bender et al.). These mutations provide a basis for the biochemical dissection of EcR/USP interactions. A biochemical analysis of these mutants will be presented.

K 102 IDENTIFICATION OF TWO DIFFERENT DOMAINS OF HSP90 INVOLVED IN THE MINERALO-CORTICOSTEROID RECEPTOR FUNCTION, Nadine Binart, Marc Lombès and Etienne Emile Baulieu, INSERM U 33, Lab-Hormones, 80, rue du Général Leclerc, 94276 Bicêtre Cedex, FRANCE

The 90kDa heat shock protein (hsp90) is associated with several proteins including the steroid receptors in their untransformed state. When coexpressed in the baculovirus system, the chick hsp90 (chsp90) and the human mineralocorticosteroid receptor (hMR) form a 9S heterooligomeric complex which was displaced in density gradients by BF4, a monoclonal anti-chsp90 antibody. To define the domains involved in the interaction between hsp90 and receptor, three mutants of chsp90 (ΔA, ΔB and ΔZ) have been created by deletion of the A (221-290) and B (530-581) regions rich in charged aminoacids, and the Z (392-419) region, a putative leucine zipper. Each of these mutants was coexpressed in combination with the hMR. After cell metabolic labeling, we analyzed the 35S-methionine labeled hsp90 complexed with hMR using a monoclonal anti-hMR antibody. The receptor was immunoprecipitated with either the wild type chsp90, the ΔB or the ΔZ mutant, but not with the ΔA mutant, indicating that the A region of the hsp90 is involved in the interaction with the hMR. Surprisingly, biochemical and immunohistochemical studies showed that when the receptor is coexpressed with the ΔB mutant, in contrast with the wild type chsp90, the ΔZ mutant or the receptor expressed alone, hMR lost *in vitro* its capacity to bind aldosterone and did not undergo *in vivo* hormone induced-nuclear translocation. This suggests that the B region is important for correct folding of the ligand binding domain of the hMR. In summary, we have demonstrated that the A and the B domains are involved in the role played by hsp90 in receptor function.

K 103 **POINT MUTATIONS IN THE HSP90-BINDING REGION OF THE RAT GLUCOCORTICOID RECEPTOR AFFECT THE STEROID-BINDING CHARACTERISTICS OF THE RECEPTOR.** C.A. Caamaño, M.I. Morano, S.J. Watson, F. Dalman*, W.. Pratt* and H. Akil. Mental Health Res. Inst. and Dept. of Pharmacology*, University of Michigan, Ann Arbor, MI 48109.

We have recently reported for the first time the use of recombinant corticosteroid receptors for the assembly *in vitro* of functional complexes containing the 90 kDa heat shock protein (hsp90) (Caamaño *et al.*, 1993, Biochemistry **32**, 8589). Purified bacterial fusion constructs bearing the DNA- and the steroid-binding domains of the rat mineralocorticoid and glucocorticoid receptors (MR and GR) treated with rabbit reticulocyte lysate resulted in receptor complexes that exhibited steroid-binding affinity/specificity, hydrodynamic behavior and DNA-binding characteristics identical to the native receptors. In order to further understand the structural requirements of GR, we produced mutant GRs as fusion proteins in bacteria or as full-length receptors in mammalian Cos-1 cells.. The selected mutations in the hsp90 binding region, according to the rGR amino acid seq., were: Pro-643 to Ala (**GRMO**) and Lys-597 to Ile and Pro-600 to Leu (**GRM8**). In Cos-1 cells, the affinity for corticosterone of both mutants was reduced (K_d values [nM]: **GR**, 5.3 ± 0.5; **GRMO**, 11.1 ± 0.6; and **GRM8**, 18.6 ± 0.3). A consistent reduction of the B_{max} was also observed (**GRMO**, -55 ± 10%; **GRM8**, -80 ± 5%). In the bacterial system, **GR** and **GRMO** showed the same correct steroid specificity (**RU**28362 > dexamethasone > corticosterone > cortisol > progesterone > aldosterone >> spironolactone ≥ estradiol) but the steroid-binding affinity and capacity of **GRMO** showed a 3-fold reduction. Upon activation, steroid-bound complexes of both **GR** and **GRMO** showed a similar increase in DNA-cellulose binding. A similar analysis of the bacterially expressed **GRM8** was complicated by its 1000-fold reduction in steroid-binding affinity. We conclude that the assayed point mutations affect the steroid-binding affinity and capacity but not the specificity of the receptor. A relationship between this effect and receptor-hsp90 stabilization is suggested. Supported by NIMH PO1-MH42251-06 and NIADDKD P30-AM34933.

K 104 COOPERATIVITY AND DIMERIZATION OF THE A AND B FORMS OF THE HUMAN PROGESTERONE RECEPTOR.
P. Carbajo, K.Christensen*,D.P. Edwards*, D.F. Skafar, Dept. of Physiology, Wayne State University School of Medicine, Detroit, MI 48201 and *Dept. of Pathology, Univ. of Colorado Health Sciences Center, Denver, CO 80262.
We are investigating the ligand binding mechanism and dimerization properties of the A and B forms of the human progesterone receptor (hPR). The hPR was overexpressed in *Spodoptera frugiperda* (Sf9) insect cells using a recombinant baculovirus system. Pellets from approximately 30 x 10^6 cells containing hPR A and B were lysed in TDEG buffer (40 mM Tris, 5 mM DTT, 0.1 mM EDTA, 10% glycerol) containing 20 mg/ml ovalbumin and clarified at 35,000 RPM. Aliquots of lysate were incubated with [^{3}H]progesterone at concentrations between 0.5 and 40 nM for two hours at 0 ^{0}C. Nonspecific binding was measured using a parallel set of incubations containing a 200-fold molar excess of unlabeled progesterone. Free and bound [^{3}H]progesterone were separated by a dextran-coated charcoal assay and quantified via liquid scintillation. Saturation binding experiments were performed on hPR A and B individually and when mixed together. The A and B forms had identical maximal Hill coefficients (n_H) at similar receptor concentrations. At receptor concentrations > 2.2 nM, hPR-A displayed a mean n_H of 1.6 $\pm$ .07 (n=5). Similarly, at receptor concentrations> 2.7 nM, hPR-B displayed a mean n_H of 1.60 $\pm$ .07 (n=5). Receptor concentration also influenced cooperativity: at receptor concentrations less than 2.2 nM, the mean n_H for hPR-A decreased to 1.33$\pm$ .11 (n=4). At receptor concentrations less than 2.7 nM, hPR-B also displayed a lower mean n_H , 1.1 $\pm$ .03 (n=5). Differences in cooperative binding were detected when A and B forms were mixed vs. separate. At a receptor concentration less than 4.2 nM there was little or no cooperative binding; the mean n_H was 1.14 $\pm$ .03 (n=8). This is in sharp contrast to the 1.6 value obtained for the individual forms at similar concentrations. However, at receptor concentrations > 5.8 nM the mean n_H was 1.53 $\pm$ .09 (n=3). These results suggest that a biological system having both forms of receptor will be less responsive to hormone than a system having only one form.

K 105 VITAMIN D RECEPTORS FORM HETERODIMERS WITH RETINOIC ACID RECEPTORS AND RETINOID X RECEPTORS ON DIRECT REPEATS, PALINDROMES AND INVERTED PALINDROMES. Carsten Carlberg, Clinique de Dermatologie, Hôpital Cantonal Universitaire, CH-1211 Genève 14, Switzerland
The transcription of vitamin D (VD) responsive genes is regulated by three different nuclear signalling pathways mediated by homodimers of VD receptor (VDR), heterodimers of VDR and retinoid X receptors (RXRs) and heterodimers of VDR with retinoic acid receptors (RARs). Here, the *in vitro* DNA binding affinity of all three receptor complexes was shown to be enhanced by the presence of VD. However, the specificity of the three pathways is dictated through differential affinities of the receptor complexes for VD response elements. Potential response elements are discriminated by the sequence, the distance and the relative orientation of the hexameric core binding motifs. It was found that both VDR-RAR and VDR-RXR heterodimers are functionally on all three response element configurations: direct repeats, palindromes and inverted palindromes. On direct repeats both heterodimer types showed no preference for one of the three principal core motifs PuGGTGA, PuGGTCA and PuGTTCA; moreover, they exhibit preferences for core motifs on palindromes but have identical spacing requirements. Inverted palindromes, however, formed the most specific response elements. A simple model explains a sterical link between the optimal spacings of direct repeats and inverted palindromes. Taken together, the experimental data and the model provide further indications for the screening of VD responsive genes.

K 106 CHARACTERIZATION OF A POTENTIAL MEDIATOR OF ESTROGEN RECEPTOR HORMONE-DEPENDENT TRANSCRIPTIONAL ACTIVITY, Vincent Cavaillès, Paul S. Danielian, Roger White and Malcolm G. Parker, Molecular Endocrinology Laboratory, I.C.R.F., London, WC2A 3PX
The estrogen receptor (ER) is a ligand-dependent transcription factor which contains two transactivation functions. The NH2-terminal function AF1 is constitutive, whereas AF2 in the ligand binding domain requires the presence of hormone. In order to study molecular mechanisms involved in ligand-dependent transactivation, we have identified AF2-associated proteins that might act as coactivators and mediate its transcriptional activity. We have constructed a vector (GST-K-AF2) allowing expression of the mouse estrogen receptor AF2 domain (residues 313 to 599) as a fusion protein with the glutathione-S-transferase. After *in vitro* ^{32}P labelling, the GST-K-AF2 probe was used in Far-Western blot experiments. Using nuclear extracts from different cell lines, we detected several bands, the most intense corresponding to a protein of 140 kDa. This interaction, observed in the presence of estradiol, was blocked by antiestrogens like tamoxifen or ICI182780 that prevent AF2 activity. Moreover, a probe containing a transcriptionally inactive AF2 function (mutations M547A/L548A) also failed to interact with this nuclear protein suggesting it may be involved in the mediation of ER hormone-dependent transactivation. By screening a ZR75-1 breast cancer cell cDNA expression library with the probe, we have isolated several overlapping clones containing inserts from 2 to 6 kb. Sequencing of these clones is in progress as well as transfection experiments in an attempt to demonstrate whether the encoded protein interacts also with AF2 *in vivo*.

K 107 TRINUCLEOTIDE REPEATS ENCODING POLYGLUTAMINE INHIBIT ANDROGEN RECEPTOR TRANSACTIVATION FUNCTION, Nancy L. Chamberlain*, Sharon D. Pascoe†, Erika D. Driver‡, and Roger L. Miesfeld*,†,§, Departments of Biochemistry* and Molecular and Cellular Biology§, Undergraduate Biology Research Program‡ and The Arizona Cancer Center†, University of Arizona, Tucson, AZ 85724
Expansion of trinucleotide repeats in the human genome has recently been associated with several human diseases. One of these is X-linked spinal and bulbar muscular atrophy (Kennedy's disease). Patients affected by this disorder are males who experience late-onset, progressive neuromuscular degeneration, accompanied by signs of androgen insensitivity. Expansion of the CAG trinucleotide repeat (from ~20 to >40) in the androgen receptor (AR) gene has been correlated with the incidence and severity of this syndrome. In order to study the effect of this repeat on AR transactivation function, we constructed receptors which varied either in the size of the trinucleotide repeat or in its position within the modulatory domain, and assayed for the abilities of the mutant receptors to bind the synthetic androgen R1881 and to activate transcription. We observed that elimination of the trinucleotide repeat in both human and rat AR resulted in elevated activity, indicating that the presence of a repeat is inhibitory to transactivation. We also found that the addition of 10, 24, or 52 CAGs to the trinucleotide repeat in human AR caused a progressive loss in transcriptional activation, while not affecting hormone binding. Importantly, a mutant receptor which contained a repeat three times the normal size retained >60% transactivation function. We postulate that the residual level of AR activity in patients with Kennedy's disease is sufficient to ensure normal development of male primary and secondary sexual characteristics, but may fall below a threshold level of activity necessary for maintenance of function of certain tissues, such as spinal and bulbar motorneurons.

K 108 ANALYSIS OF THE BINDING OF 3,3',5-TRIIODO-L-THYRONINE AND ITS ANALOGUES TO MUTANT HUMAN β1 THYROID HORMONE RECEPTORS: A MODEL OF THE HORMONE BINDING SITE, Sheue-yann Cheng, Steve Ransom+, Manoj K. Bhat, Peter McPhie+ and Bruce Weintraub+, NCI and +NIDDK, NIH, Bethesda, MD 20892

Thyroid hormone nuclear receptors (TRs) are members of steroid hormone/retinoic acid receptor superfamily. They are transcriptional factors which regulate growth, differentiation and development. The transcriptional activity of TRs is 3,3',5-triiodo-L-thyronine (T_3)-dependent, but the molecular basis for the T_3-dependence is not clearly understood. To understand the nature of interaction of T_3 with TR we characterized the binding of T_3 and its analogues to the wild type and eight naturally occurring mutated human β1 TRs (h-TRβ1s). The mutant receptors were derived from patients with thyroid hormone resistant syndromes and each has a point mutation in the hormone binding domain (ED:A312T; IR:D317H; KT:R333W; NN:G342E, OK:M437V; TP:L445H; AH:P448H and RL:F454C). Compared to the wild type h-TRβ1, binding of T_3 was lowered by 51, 92, 94, 95, 95, 96, 95 and 97% for the mutant KT, TP, IR, NN, AH, OK, RL and ED, respectively. Binding of D-T_3, L-thyroxine, 3,5-diiodo-L-thyronine and 3,3',5-triiodo-L-thyronine was respectively lower than those to the wild type h-TRβ1. However, the changes in average free energies in the binding of the analogues to the wild type and mutant receptors relative to those of T_3 binding to the wild type h-TRβ1 deviated from the sum of free energy changes resulting from the separate alterations in the structure of ligand and protein. These results suggest specific local interaction of analogues with the mutated residues in the receptors. Based on these data and the structure of the hormone binding domain as an eight stranded α/β barrel, we proposed the location of the hormone in the binding site of h-TRβ1. Ionic bonds anchor the hormone's alanine side chain to Loop 4 of the eight-fold α/β barrel. The phenyl ring lies across the amino terminal face of the domain with the phenoxy ring pointing downward into the barrel interacting with β strand 8 in the opposite side. Loops 1 and 7 which locate on the same side as the DNA binding domain fold over the top of the bound hormone. The T_3-induced conformational changes observed by us and others may be partly mediated by Loops 1 and 7. The present model should provide a basis for further studies to understand the T_3-dependent transcriptional activity of h-TRβ1.

K 109 ANALYSIS OF INTERACTIONS OF THE VITAMIN D_3 RECEPTOR WITH LIGAND, DNA AND RXR, **Boris Cheskis and Leonard Freedman, Cell Biology & Genetics Program, Memorial Sloan-Kettering Cancer Center, NewYork, NY 10021**

Nuclear receptors directly transduce signals presented by levels of hormones and other small molecules into effects on gene transcription. *In vivo*, the role of ligand-binding in the steroid receptors appears to be to unmask the nuclear localization and DNA-binding domains from a complex with auxilliary proteins such as the heat shock proteins. Among the nuclear receptors, however, the role of ligand is less clear, since these receptors are constitutively nuclear-localized, and are presumeably associated with DNA in an unliganded state. Conceivably, ligand could effect receptor homo- or hetero-dimerization, kinetics of DNA binding, or interactions with other components of the transcriptional machinery.

We have overexpressed human vitamin D_3 receptor (VDR) and purified it to homogeneity. The receptor binds specifically and with high affinity to both a target DNA (a direct repeat element spaced by three base pairs [DR+3]) and 1, 25 $(OH)_2D_3$. In order to begin to address the mechanism of VDR action, we have examined, by gel filtration chromatography, receptor complexes formed after pre-incubation with ligand, DNA, and/or its heterodimeric partner, RXR. VDR's apparent molecular weight, as assessed by gel filtration, is 60.8 kDa, in the presence or absence of 1, 25 $(OH)_2D_3$. VDR pre-incubated with DR+3-containing DNA gives monomeric and dimeric complexes with apparent molecular weights of 49.4 kDa and 92.2 kDa, respectively. These results indicate that the receptor can form homodimers, but that specific DNA is necessary for this dimerization to occur. The fact that the monomeric VDR-DNA complex has a lower MW than the receptor alone (49.4 vs 60.8 kDa) suggests that DNA binding induces a more compact VDR conformation. We find that ligand actually acts to destabilize a DNA-bound VDR homodimer, while at the same time strongly enhancing the formation of a VDR-RXR heterodimer. Further examination of the role of ligand on hVDR-DNA binding kinetics indicates that 1, 25 $(OH)_2D_3$ lowers hVDR's on-rate to a DR+3, while increasing the on-rate of the heterodimer to the same target. The data presented here suggest that a principal role of 1, 25 $(OH)_2D_3$ may be to induce the dissociation of the receptor homodimer, resulting in a monomer available to complex with RXR, which we show to be enhanced by the ligand. Thus, these results may rationalize how a nuclear receptor can bind DNA constitutively but still act to regulate transcription in a fully hormone-dependent manner.

K 110 CLONING THE MINERALOCORTICOID RECEPTOR FROM XENOPUS LAEVIS, Mark Danielsen, Tamas Csikos and Joyce Tay, Department of Biochemistry and Molecular Biology, Georgetown University Medical School, 3900 Reservoir Rd., N.W., Washington, DC 20007

We have isolated cDNA clones that encode the DNA and hormone binding domains of the Xenopus laevis mineralocorticoid receptor (MR). The protein sequence is highly homologous to the human and rat MR sequences. Of particular note is the presence of 4 extra amino acids located between the zinc fingers of the DNA binding domain. These 4 amino acids were found in 3 independent clones from larval and adult Xenopus cDNA libraries. Using PCR analysis we have been unable to detect alternate forms of xMR that do not contain these extra amino acids. Similarly, we have been unable to detect rat MR that contains these additional amino acids. Fusion of a glucocorticoid receptor transactivation domain to the xMR cDNA yields a receptor that activates MMTVCAT in response to mineralocorticoids and glucocorticoids in transfected COS-7 cells. The potency of these steroids for transcriptional activation follows the expected binding affinity to a MR rather than a GR. We are presently examining the expression of the MR during metamorphosis in Xenopus laevis.

K 111 THE HUMAN PROGESTERONE B RECEPTOR: INHIBITION OF TRANSCRIPTION ACTIVATION BY ANTAGONISTS IN CHO CELLS STABLY TRANSFECTED WITH RECEPTOR AND REPORTER, Rein Dijkema[1], Renée Teuwen[1], Joop C. Swinkels[1], and Willem G.E.J. Schoonen[2], Department of Biotechnology and Biochemistry[1] and Department of Endocrinology[2], Organon Scientific Development Group, Organon International bv, P.O.Box 20, 5340 BH Oss, The Netherlands

The human progesterone receptor (PR) is unique in the superfamily of nuclear receptors in that two isoforms with different molecular weight have been observed. Their difference comprise the NH-terminal 165 amino acids present only in PR isoform B (PR B). Since two in-frame ATG codons are present in the cDNA-derived amino acid sequence the two isoforms presumably arise from either alternative initiation of translation from a single mRNA or alternative transcription from two isoform-specific promoters.

In order to investigate the transcription activation function of individual PR isoforms a hormone-responsive transcriptional system has been made. The system is based on a primary stable transfection of receptor-negative CHO cells with complete PR cDNA (PR B: residues 1-933). Clones obtained this way were evaluated for stable expression of PR B by Western Blot and hormone binding assays. In addition, functional expression of PR B was analyzed by the effect of agonist/antagonist on transiently transfected pMMTV-reporters. Subsequently, single clones were subjected to a secondary stable transfection with pMMTV-LUC as a reporter. Clones obtained this way were evaluated for their stimulation of transcription activation by Org2058. As a result a permanent cell line has been selected that functionally expresses both PR B and PR B-mediated transcription activation. Therefore, this cell line represents a new tool to assay progestagenes and/or anti-progestagenes as illustrated by reporter gene transcription modulation.

As an example, different progestagen agonists were able to activate reporter gene transcription with a ranking that one would expect on the basis of their RBA as determined by hormone binding assays. In addition, different progestagen (type I and II) antagonists were all able to inhibit agonist-promoted gene transcription with a similar ranking as indicated by their RBAs.

K 112 THE MOLECULAR BASIS OF RU486 RESISTANCE IN THE TAMMAR WALLABY, MACROPUS EUGENII.

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RU486 binds to the uterine progesterone receptor (PR) of rats, rabbits, dogs, monkeys and humans with an affinity equal to or greater than that of progesterone but does not bind to the chicken and hamster PR. Recently, Benhamou et al [Science 255: 206, 1992] demonstrated that whereas the human and rabbit PR sequences have a glycine at position 722, the chicken and hamster PR sequences have a cysteine at the equivalent position. Substitution of a cysteine for a glycine in the human PR abrogated binding of RU486 but not the agonist. The tammar wallaby, Macropus eugenii is resistant to the antiprogestigenic effects of RU486. Binding studies reveal that RU486 is unable to displace ^{3}H-ORG2058 from PR in tammar uterine cytosol. To establish whether a cysteine for glycine substitution is responsible for this lack of RU486 binding, total RNA was extracted from tammar uterus for reverse transcriptase-polymerase chain reaction (RT-PCR) amplification of a PR cDNA using specific primers corresponding to conserved regions of the human and rabbit PR sequences. The primers were designed to amplify the region around position 722. Sequence analysis of the ~460 nucleotide PCR product confirmed that the cDNA encoded the tammar PR. This sequence shows >90% homology with that of the human PR. The amino acid sequences differ at only 10 residues. As the result of a single nucleotide change, rather than having a cysteine at the position corresponding to glycine-722 in the human PR, the tammar PR has an alanine residue. The closely related androgen and glucocorticoid receptors which bind RU486, have a glycine at this position; the mineralocorticoid receptor, which does not bind RU486, has an alanine at this position [3]. The observed resistance of the tammar both *in vivo* and *in vitro* to RU486 results from the presence of an alanine rather than the glycine found in the PR of those species in which RU486 is active. Glycine, the smallest of the amino acids, may thus be the only residue tolerated at this position in the PR if RU486 is to bind. The methyl side group of alanine, though small in comparison to the thiol group of cysteine, may provide steric hindrance for the phenyl group of the 11β-substituted synthetic steroids. In conclusion, the presence or absence of a glycine at this position does not affect binding of the physiological ligand, however the use of a synthetic steroid can compromise the otherwise relaxed structural constraints of this position within the 11β-pocket of the PR ligand-binding domain.

K 113 HETEROTETRAMERIC STRUCTURE OF UNACTIVATED STEROID HORMONE RECEPTORS, Ulrich Gehring, Petra Rehberger, Martin Rexin, and Bernd Segnitz, Institut für Biologische Chemie, Universität Heidelberg, D-69120 Heidelberg, Germany.

Our studies concentrate on the subunit structure of unactivated steroid hormone receptors, in particular the murine glucocorticoid receptor and the B form of the human progesterone receptor. Receptors contained in cell extracts were affinity labeled with appropriate [^{3}H]steroid ligands and submitted to progressive chemical cross-linking with dimethyl suberimidate. SDS gel electrophoresis revealed subunit patterns characteristic of hetero-tetrameric structures. Both receptors consist of **one** hormone binding polypeptide in association with **two** molecules of the heat shock protein **hsp90** and **one** p59 subunit, a protein that had previously been described. The heterotetrameric polypeptide stoichiometry was established by a combination of chemical cross-linking with cleavable bifunctional agents, extensive purification on immunoaffinity columns and analysis by gel electrophoresis and immunoblotting. It was demonstrated unequivocally that the heat shock protein **hsp70** is not a *bona fide* receptor component but rather copurifies with receptor complexes by unspecific binding to the immunomatrix; it can be released by treating the antibody columns with ATP.

Cross-linking studies using whole cells revealed the presence of the above described heterotetrameric glucocorticoid receptor structure in intact cells under physiological growth conditions and in the absence of steroid. We thus conclude that this heteromeric structure is the original form of the receptor in target cells which is ready for accepting the hormonal signal.

These results indicate that all steroid hormone receptors in their unactivated states may have a similar architecture. Recent experiments with the human estrogen receptor indeed support this view.

K 114 IDENTIFICATION OF A VITAMIN D_3 RESPONSIVE REGION IN THE PROMOTER OF THE CYTOCHROME $P450_{24}$ GENE, Christopher N. Hahn, David M. Kerry, Jack L. Omdahl[1] and Brian K. May, Department of Biochemistry, The University of Adelaide, Adelaide, Australia 5005, [1]School of Medicine, University of New Mexico, Albuquerque, NM 87131

The hormonally active form of vitamin D is 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), a secosteroid hormone that regulates many cellular processes including differentiation and calcium homeostasis. The circulating level of 1,25-$(OH)_2D_3$ is determined by the hormone's rate of synthesis and turnover which is mediated by specific renal mitochondrial cytochromes P450. Activation is regulated by the 1-hydroxylase enzyme ($P450_1$) whereas entry into the vitamin D degradation-pathway is initiated by the 24-hydroxylation of 1,25-$(OH)_2D_3$ and other 25-hydroxylated metabolites. This C24-oxidation pathway is controlled by the 24-hydroxylase enzyme ($P450_{24}$). The activity of this enzyme is modulated by various agents, but most notable is the feedback action of the 1,25-$(OH)_2D_3$ hormone to transcriptionally up-regulate expression of the cytochrome $P450_{24}$ gene. This gene-regulatory action of 1,25-$(OH)_2D_3$ is mediated by its binding to the vitamin D_3 receptor (VDR). In the current study, we report the hormone response element (HRE) that binds the 1,25-$(OH)_2D_3$:VDR complex during transcriptional activation of the rat cytochrome $P450_{24}$ gene.

A genomic clone for the rat 24-hydroxylase gene was isolated and approximately 1.5 kb of the promoter sequenced. Various lengths of the promoter were positioned upstream of the luciferase reporter gene and the resulting constructs co-transfected into COS-1 cells along with a VDR expression plasmid. Use of the promoter-reporter gene constructs resulted in the identification of a 1,25-$(OH)_2D_3$ responsive region in the first 298 bp which displayed a 20-fold induction upon treatment with hormone. Sequence analysis showed a cluster of five putative hormone responsive element (HRE) half-sites (i.e. AGGTC/GA-like sequences) between -124 and -171 bp. A synthetic double stranded oligomer encompassing this region was placed upstream of the Herpes Simplex thymidine kinase promoter attached to luciferase. A 4-fold response was observed in COS-1 cells treated with 1,25-$(OH)_2D_3$ ligand. Shorter oligomers and mutagenesis studies have been used to define the precise HREs involved in the response to 1,25$(OH)_2D_3$. HREs for other vitamin D_3 dependent genes are located 400-800 bp upstream of the transcription initiation site. However, the identified 1,25-$(OH)_2D_3$ responsive region in the cytochrome $P450_{24}$ gene is more proximally located.

K 115 THE GLUCOCORTICOID RECEPTOR SPECIFICALLY BINDS DNA AT SITES OF CONSERVED GENETIC INFORMATION INDUCING DNA BENDING AND UNWINDING,

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Binding at specific sites on DNA by regulatory proteins is essential for genetic regulation. This suggests that a code for recognition between DNA regulatory proteins and cognate DNA binding sites exists. This laboratory has long been interested in the origin of the genetic code and site specific DNA binding by DNA regulatory proteins and has made several key observations. We compared nucleotide sequences containing hormone response elements (HREs) and operator sites with cDNA coding for eukaryotic and prokaryotic regulatory proteins' DNA binding domains, respectively. Our findings indicate that there is a high degree of nucleotide similarity between the c-DNA encoding regulatory proteins' DNA recognition helices and the DNA sequences to which they specifically bind (HREs or operators). By model building, we observed that codon/anticodon nucleotides are spaced within the HREs and operator major groove half-sites compatibly positioned to align with cognate amino acid sidechains within the regulatory protein's DNA recognition helix. Based on these observations, we proposed a hypothesis for a common site-specific DNA recognition code which has an underlying mechanism of stereochemical complementarity between amino acid sidechains and their cognate codon/anticodon nucleotides within their specific DNA binding sites. Using molecular dynamics simulations of protein/DNA interactions, we observed that amino acids within eukaryotic or prokaryotic DNA regulatory proteins' DNA recognition helices interact with cognate codon/anticodon nucleotide base sites within the major groove halfsites of their specific HREs or operator sites. Our findings therefore indicate that conservation of genetic information is a determinate of site specific DNA recognition. Furthermore, our results indicate that our hypothesis, applied to genetic sequence analysis, secondary structural prediction and molecular model building, can be used as a predictive tool for determining specific sites on DNA regulatory proteins which recognize cognate DNA binding sites and vice-versa. We will show a videotape of molecular dynamics simulations of DNA regulatory protein/DNA interactions consisting of the glucocorticoid receptor/glucocorticoid response element computed in vacuo and in solvent on CRAY-2 and CRAY-C90 supercomputers at the Minnesota Supercomputer Institute. The explicitly solvated model consists of approximately 25,000 atoms.

K 116 A PHARMACOPHORE FOR ESTROGENIC ACTIVITY SUGGESTS DNA MAY BE A SECONDARY RECEPTOR, Lawrence B. Hendry and Virendra B. Mahesh, Department of Physiology and Endocrinology, Medical College of Georgia, Augusta, GA 30912

The primacy of classical receptor proteins has been unequivocally established as an integral part of the genomic mechanism of action of steroid/thyroid hormones. Receptors provide a critical role by recognizing a given ligand out of a host of cellular chemicals eventually leading to regulation of specific hormonally responsive genes. Several laboratories have shown that the extent of binding of various ligands to cytosolic receptors frequently does not correlate with degree of hormonal response. In the case of estrogens, receptor binding also does not always correlate with uterine uptake or binding in whole cell assays which has led to general speculation that an as yet undiscovered factor(s) must be involved. Here, we report new evidence that DNA may function as a secondary receptor for estrogens. In studies aimed at understanding why certain structures and not others exist in nature, we discovered that small molecular weight hormones exhibited stereochemical complementarity with cavities formed between base pairs in double stranded DNA. These findings provided a plausible explanation for how hormone structures evolved. However, it was also found that degree of complementarity or fit into DNA of natural and synthetic compounds as measured by standard energy calculations correlated with degree of estrogenic activity. By combining the molecular surfaces of active estrogens based upon their orientation of fit into DNA, a three dimensional pharmacophore was developed. The degree of fit of active and inactive ligands to the pharmacophore can now be used to predict estrogenic activity and thus to design new drugs. While the goal of our studies as explicitly stated in previous reports has not been to study mechanism, the discovery of an estrogen pharmacophore based upon DNA structure is intriguing. In light of the lack of correlation between receptor binding and estrogenic activity, these results raise the possibility that the mode of action of estrogens may involve direct insertion of the ligands into DNA. This insertion would not have biological significance without the receptor molecule which may direct the steroid to the right DNA site. The steroid binding to DNA may represent one of the transcription factors required for gene activation and could be enhanced by other transcription factors. In this manner, DNA would function as a secondary receptor governing the magnitude of the hormonal response with protein receptors determining the specificity of the response. Given that steroids by themselves bind only weakly with DNA, experiments to test this hypothesis will require careful study of steroid/DNA interactions in the presence of receptor proteins as well as other transcription factors.

K 117 ESTROGEN RECEPTOR IS MODIFIED BY O-LINKED N-ACETYGLUCOSAMINE

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The detailed mechanism by which gene transcription is regulated by estrogen receptor (ER) remains unknown in spite of recent studies on its receptor primary structure and function relationship. Data suggests that phosphorylation may be crucial in regulation of the ER. There are at least one tyrosine phosphorylation site and at least two consensus CMP-dependent phosphorylation sites on ER.

Another form of nuclear and cytoplasmic protein modification has been identified: O-GlcNAc. This modification consists of single monosaccharides of N-acetylglucosamine attached to serine or threonine hydroxyl groups. O-GlcNAc has been found on a wide range of nuclear and cytoplasmic proteins, notably RNA polymerase II and its transcription factors and *v-erb* A (which has extensive homology among ER and thyroid hormone receptor). Although the functional significance of O-GlcNAc modification has not been fully understood, it is implicated in playing roles in transcriptional regulation, cell activation, cell cycle regulation, and the regulation of phosphorylation and the proper assembly of multimeric protein complexes. Here we report that ER is also shown to bear O-GlcNAc based upon lectin-binding studies and in vitro galactosylation by purified galactosyltransferase. This observation not only extends the scope of this newly recognized nuclear protein modification to a member of the steroid-thyroxin-retinoid receptor gene family, but also suggests that O-GlcNAc might play a crucial role in understanding ER regulation. Supported by NIH CA42486.

K 118 ATP-DEPENDENT RECONSTITUTION OF P23 WITH OTHER PROGESTERONE RECEPTOR-ASSOCIATED PROTEINS, J.L. Johnson and D.O. Toft, Mayo Graduate School, Rochester, MN 55905.

Immune isolation of unactivated chicken progesterone receptor (PR) results in the copurification of the heat shock proteins hsp90 and hsp70, the immunophilins p54 and p50, and a recently characterized 23 kDa protein, p23. These proteins reassociate with immune isolated PR when incubated in rabbit reticulocyte lysate. In this case, p50 is replaced by its mammalian homolog p59, and an additional immunophilin, cyclophilin-40 (CyP-40) is also bound. p23 is a novel, highly conserved protein with a broad tissue and species distribution. Bacterially expressed human p23 was used to raise monoclonal antibodies to p23. These antibodies specifically immunoprecipitate p23 in complex with hsp90 in all tissues tested and they can be used to immuno-affinity isolate progesterone receptor complexes from chicken oviduct cytosol. We have used these antibodies to further examine the interaction between p23 and hsp90. Immune isolation of p23 from reticulocyte lysate results in the copurification of hsp90 and the receptor-associated protein, CyP-40. When hsp90 and CyP-40 are stripped from p23 in 0.5 M KCl, they do not spontaneously reassociate with p23 upon lysate dialysis in low-salt buffer. Effective reconstitution of the native p23:hsp90:CyP-40 complex requires elevated temperature (30°C), 3mM magnesium and ATP. Using antibodies against p54 and p59, we have found that these proteins also associate with p23:hsp90 complexes under these conditions. These findings support the ATP-dependent formation of a complex containing many of the progesterone receptor-associated proteins in the absence of receptor.

K 119 A 3-DIMENSIONAL STRUCTURAL MODEL FOR THE HORMONE BINDING DOMAIN OF THE NUCLEAR RECEPTORS. **John A. Katzenellenbogen, Donald A. Seielstad, Richard A. Goldstein, Zaida A. Luthey-Schulten, and Peter G. Wolynes, Department of Chemistry, University of Illinois, Urbana IL 61801**

While high resolution structures for the DNA-binding domains of several of the nuclear receptors are available, based on x-ray and NMR studies, no structures for the hormone binding domains (HBD) have been published. To identify a potential structure for the HBDs of the nuclear receptors, we have combined the use of a computer program for evaluating *structural* homologies with the results from biochemical experiments on ligand binding and receptor function. The computer search algorithm, which evaluates the stability of receptor HBD sequences aligned to a representative set of 83 high resolution protein crystal structures, utilizes an energy function that considers secondary structure, solvent exposure, context and pairwise interactions. The highest scoring structures for the HBDs are members of the subtilisin-like protease class, proteins having a fold with an alternating α/β structure. Further refinement of the model utilized HBD multisequence alignments and information on residues near the ligand binding site derived from affinity labeling experiments. The final model has a ligand binding site situated in the β to α cross-over region, a location typical for substrate and co-factor binding for this class of protein fold. The model accommodates the results of nearly all affinity labeling experiments and of mutational studies that have a predictable effect on ligand binding. Furthermore, residues known to affect receptor dimerization and trans-activation are located near the surface of our model. This model may prove useful in the design of further experiments to probe receptor structure-function relationships, and its relationship to forthcoming high resolution structures obtained by spectroscopic methods will provide an important evaluation of our method for protein structure prediction.

K 120 DECREASED HORMONE SENSITIVITY AND CONSTITUTIVE ACTIVITY IN THE GUINEA-PIG GLUCOCORTICOID RECEPTOR, Maria-Cristina Keightley and Peter J. Fuller, Prince Henry's Institute of Medical Research, Clayton, 3168, Australia.

The guinea-pig is glucocorticoid resistant with a glucocorticoid receptor (GR) which has a substantially lower affinity for its ligand compared to that of the mouse. We have recently cloned the guinea-pig GR, and performed expression studies comparing the human GR and a human/guinea-pig chimeric receptor containing the ligand-binding domain (LBD) of the guinea-pig GR. The guinea-pig GR LBD confers glucocorticoid resistance and, unexpectedly, also confers a high level of constitutive activity. Sequence analysis identified a number of unique amino-acid substitutions in the LBD of the guinea-pig GR. Initially, we chose to investigate a *trp*-632 substitution which occurs at a position where *cys* is absolutely conserved in other GRs, and is the site of affinity labeling by dexamethasone 21-mesylate. Reciprocal mutagenesis of *trp* for *cys*-638 in the human GR and *cys* for *trp*-632 in the guinea-pig GR revealed that *trp*-632 in the guinea-pig GR is at least partially, but not solely, responsible for the decreased hormone sensitivity and high constitutive activity of the guinea-pig GR. To delineate regions/amino-acids of the LBD which in conjunction with *trp*-632 might determine the activity of the guinea-pig GR, chimeric receptors were created where N- and C-terminal LBD regions were swapped between human and guinea-pig GRs. Dexamethasone dose response curves were obtained for each chimera. A construct containing guinea-pig N- and human C-terminal LBD showed very high constitutive activity. All constructs were treated with either testosterone, dexamethasone, ORG2058, RU486 alone, or RU486 plus dexamethasone. No difference in hormone specificity was observed, however, RU486 alone substantially reduced constitutive activity. Since the N-terminal portion of the guinea-pig LBD is associated with high constitutive activity, unique substitutions within this region are now being mutated to residues present in the human GR. In conclusion, elucidation of the molecular basis of resistance in the GR of this species may provide important insights into the structural determinants of ligand-binding and transactivation in glucocorticoid receptors.

K 121 THE LIGAND BINDING DOMAIN OF THE THYROID HORMONE RECEPTOR REQUIRES RETINOID X RECEPTOR FOR FULL TRANSCRIPTIONAL ACTIVATION FUNCTION IN YEAST, Jae Woon Lee, Mary C. McFadden, Bryan Macy, David D Moore* and Richard A. Heyman, Department of Cell Biology, Ligand Pharmaceuticals Inc., 9393 Towne Centre Dr., San Diego, CA 92127, Department of Molecular Biology*, Massachusetts General Hospital, Boston, MA 02114

Thyroid hormone receptors (TRs) regulate transcription by binding to specific DNA response elements as heterodimers with the retinoid X receptors (RXRs). To avoid influences on DNA binding and other effects from endogenous RXRs and other related receptors present in mammalian cells, we have chosen the yeast Saccharomyces cerevisiae to further examine TR function. We demonstrate that coexpression of native RXRs with otherwise weak transcriptional activator, hTRß1, increases basal and ligand-induced expression level of a ß-gal reporter controlled by upstream thyroid hormone response elements (T3RE). A chimeric protein consisting of the rat TRß1 ligand binding domain fused to the intact bacterial repressor lexA (lexATR) is also a weak transcriptional activator of a ß-gal reporter controlled by upstream lexA binding sites. However, coexpression of native RXRs or B42RXR, a chimeric RXR protein consisting of the ligand binding domain of the hRXRα fused to the B42 transcriptional activator domain, enhances the basal and ligand-induced transcriptional activity of lexATR. We conclude that, even under conditions where DNA binding affinity is not affected by heterodimerization, TR requires RXR to function as an efficient transcriptional activator in yeast.

K 122 LIGAND-INDUCED ALTERATIONS IN THE PROTEASE SENSITIVITY OF RETINOID X RECEPTOR, Mark Leid, College of Pharmacy, Oregon State University, Corvallis, Oregon 97331

Binding of 9-*cis* retinoic acid (9C-RA) by retinoid X receptor α (RXR) has been reported to enhance RXR homodimer formation and subsequent DNA binding to a specific subset of retinoid response elements [1, 2], suggesting that the receptor may undergo a ligand-dependent conformational change. Limited proteolysis and immunoblot studies were undertaken to provide biochemical evidence of a ligand-induced RXR conformational change and to identify domain(s) of the RXR protein which may undergo structural alteration upon ligand binding. Limited proteolytic digestion of 9C-RA-liganded, but not unliganded, RXR resulted in the appearance of a protease-resistant fragment with an apparent M_r of approximately 30 kDa. This protease-resistant fragment contained at least a portion of the RXR ligand binding domain (LBD) as determined by immunoblot analysis using an anti-RXR LBD monoclonal antibody. These results, which are consistent with a 9C-RA-induced RXR structural alteration involving the LBD of the receptor, are in general agreement with the findings of studies employing progesterone (PR) and retinoic acid (RAR) receptors [3]. The EC_{50} for 9C-RA induction of this protease resistant fragment was 30.8 nM, in agreement with the EC_{50} for 9C-RA enhancement of RXR homodimer DNA binding to an artificial response element consisting of a directly repeated motif (AGGTCA) spaced by one base pair (27.3 nM), suggesting that these two parameter estimates may reflect the same phenomenon, i.e., binding of 9C-RA by RXR.

Acknowledgments

I thank Ph. Kastner and P. Chambon for the RXRα construct, M.-P. Gaub, Y. Lutz and P. Chambon for the anti-RXR LBD monoclonal antibody and A. Levin for 9C-RA.

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K 123 CHARACTERIZATION OF THE LIGAND BINDING DOMAIN OF HUMAN RXRα EXPRESSED IN *E. COLI*, Li, E., Cheng, L., Norris, A., Tate, B., Grippo, J., Rosenberger, M., Departments of Medicine, Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, MO 63110, Hoffmann-LaRoche, Nutley, NJ 07110

In order to study the structural details of ligand protein interactions of human RXRa, the DEF and EF domains of the receptor were expressed as glutathione-S-transferase (GST) fusion proteins in *E. coli*. The fusion proteins were expressed at high-level representing 10-20% of the total bacterial proteins, and were affinity purified by chromatography over glutathione-agarose. The DEF and EF domains were cleaved from the fusion proteins by digestion with thrombin. The cleaved hRXRα DEF peptide but not the cleaved EF peptide exhibited tight binding to 9-*cis* RA, suggesting that the D domain was important for ligand binding. The Kd for the hRXRa DEF peptide complexed with 9-*cis* RA was $3 \pm .4$ nM and the stoichiometry of binding was 1:1.3 as determined by spectrofluorimetric titration. In contrast, the estimated Kd of the EF peptide complexed with 9-*cis* RA was estimated to be 330 nM. The Kd for the cleaved DEF peptide binding to [^{3}H]9-*cis*-retinoic acid estimated by Scatchard analysis was 5.6 nM. The molecular weights of the DEF peptide measured by sedimentation equilibrium experiments conducted both in the presence and absence of 9-*cis* RA were 33 kDa and 36 kDa respectively, indicating that the DEF peptides were predominantly in the monomeric form. However, dimers of the DEF peptides were detected (representing ~10% of the total protein) in crosslinking experiments both in the presence and absence of 9-*cis*-RA. These results indicate that the purified *E. coli*-derived DEF peptide provides a useful model system for structural analysis of the ligand-binding interactions of the RXRs.

K 124 THE ANTIANGIOGENIC EFFECTS OF RETINOIC ACID IN ORAL CARCINOGENESIS, Mark W. Lingen*, Peter J. Polverini#, and Noel P. Bouck@, Department of Pathology* and Microbiology-Immunology@, Northwestern University, Chicago, IL 60611, and Department of Pathology#, The University of Michigan, Ann Arbor, MI 48109.

Retinoic acid (RA) is clinically effective at controlling the development of second primary oral squamous cell carcinomas, but the mechanisms that underlie its chemopreventive actions are unclear. To determine if RA modulation of angiogenesis contributes to this activity, angiogenic tumor cells lines derived from the hamster buccal pouch and human oral keratinocytes were treated with all-trans or 13-cis RA (10^{-7} M) for seven days. Conditioned media (CM) were collected from treated cells, concentrated and assayed for their ability to stimulate or to inhibit angiogenesis in both in vitro *endothelial cell migration* and in vivo rat cornea assays. The treatment of hamster and human tumor cell lines with RA resulted in a switch from an angiogenic to an anti-angiogenic phenotype. In addition, RA was found to act directly on endothelial cells to inhibit their ability to migrate toward hamster tumor cell CM. We conclude that RA influences angiogenesis in two ways: (1) by causing squamous cell carcinoma cells to switch from an angiogenic to an angioinhibitory phenotype and (2) by making endothelial cells refractory to angiogenic stimuli. Both of these activities may contribute to its success as a chemopreventive agent. Supported by the NIH/NIDR Research Training Grant K15 DE00313-01.

K 125 CELL-TYPE SELETIVE GROWTH INHIBITION OF HUMAN PROSTATE CARCINOMA CELLS *IN VITRO* BY SYNTHETIC RETINOIDS, Xian Ping Lu , Marcia Dawson * and Magnus Pfahl , Cancer Research Center, La Jolla Cancer Research Foundation, 10901 N. Torrey Pines Rd., La Jolla, CA 92037, and *SRI International, Menlo Park, CA 94025

Retinoids have been recognized to play prominent roles in the chemoprevention and therapies of a variety of tumors in animal models and humans. Synthetic retinoids can also prevent chemical-carcinogen induced prostate carcinomas in the rat. However natural or synthetic retinoids have previously shown very limited effects on the growth of human prostate carcinomas *in vitro*. Here we have initiated a study on two human prostate cancer cell lines, PC-3 and DU-145. All-*trans*-retinoic acid (tRA) or 13-*cis*-RA did not significantly inhibit the proliferation of these cancer cells *in vitro* and *in vivo*. However, two synthetic retinoids showed a very specific effect on the growth of PC-3 cells at concentrations as low as 10 nM, but had no effect on DU-145 cells. On the other hand, a different synthetic retinoid showed approximately a 50% inhibitory effect on the growth of Du-145 cells at a concentration of 10 nM, but no significant effect on PC-3 cells. The mechanism underlying those effects was investigated.

K 126 ANALYSIS OF T3 INDUCED CONFORMATIONAL CHANGE IN THYROID HORMONE RECEPTORS, Laird D. Madison, Division of Endocrinology and Metabolism, Northwestern University Medical School, Chicago, IL 60611

A fundamental feature of the proteins in the steroid/thyroid/retinoic acid family of nuclear receptors is that the binding of ligand regulates their transcriptional properties. This ligand induced change in transcriptional activity is presumably the result of allosteric effects in the receptor caused by the binding of ligand. It is of interest to characterize the conformational changes occurring in the receptor since the structural features of the protein involved in these changes are likely to be involved in ligand regulated transcriptional properties. In order to develop insight into the effects of T3 binding on the thyroid receptor we have examined the conformation of the receptor with limited proteolysis. Thyroid receptors (α1 and β1) were produced in *E. coli* as radiolabeled 6x His fusion proteins using a T7 polymerase expression system and subsequently purified by metal chelate chromatography. Preincubation of the receptor with T3 resulted in an increased resistance to tryptic digestion of 29 kDa and 25 kDa fragments. Similar results were obtained with other proteases indicating that the cleavage patterns reflected a general change in receptor conformation. Analysis of the digestion patterns of deletion mutants indicated that the fragments represented the carboxy terminal region of the protein with alternate amino terminal cleavage sites in the hinge region. Limited proteolysis was also used to examine the effect of DNA binding and heterodimerization with RXRα. While there was no discernible effect on the proteolytic digestion pattern following DNA binding, preincubation of the receptor with excess RXRα protein enhanced the ligand induced stabilization of the 29 and 25 kDa fragments. These results indicate that T3 binding to the receptor alters the conformation of the carboxy terminus and specifically changes the accessibility of residues in the hinge region.

K 127 CYSTEINES IN THE RAT GLUCOCORTICOID RECEPTOR ARE NOT REQUIRED FOR MOLYBDATE STABILIZATION. Kevin J. Modarress, Pradip K. Chakraborti, Alice H. Cavanaugh, and S. Stoney Simons, Jr. LMCB/NIDDK/NIH, Bethesda, MD 20892.

The transition metal oxyanion, molybdate, stabilizes the association of the 90 kDa heat shock proteins (hsp90) with the rat glucocorticoid receptor (GR) and the steroid binding activity of the GR. Molybdate also maintains the unactivated state of receptor-steroid complexes. Immunoprecipitation studies have indicated that the smallest fragment of the GR that retains associated hsp90 and steroid binding activity is a 16 kDa steroid binding core. This was supported by the demonstration of molybdate stabilization of [^{3}H]dexamethasone (Dex) binding to a β-galactosidase fusion protein containing amino acids 540-795 of the rat GR. Thus, molybdate stabilization does not require any amino acids between positions 1 and 539. Since molybdate can form complexes with sulfhydryl groups, the role of the three cysteines within the 16 kDa steroid binding core was investigated by determining the activity of molybdate with two mutant GR receptors, C640S and C656,661S. Molybdate, as well as other oxyanions, stabilized [^{3}H]Dex binding to wild type and both mutant full length 98 kDa receptors. DNA binding studies revealed that molybdate also preserved the unactivated state of all receptors. Furthermore, the steroid binding activity of the 16 kDa fragments of both wild type and mutant receptors was maintained by molybdate. Finally, molybdate did not prevent the thiol specific reagent methyl methanethiolsulfonate from inhibiting the [^{3}H]dexamethasone 21-mesylate labeling of protein -SH groups. These results indicate that molybdate stabilization of the rat glucocorticoid receptor does not require any specific cysteine of the rat receptor.

K 128 THE EFFECT OF HSP90 TEMPERATURE-SENSITIVE MUTATIONS ON STEROID HORMONE RECEPTOR FUNCTION IN *SACCHAROMYCES CEREVISIAE* , Debra F. Nathan[1] and Susan L. Lindquist[2], [1,2]Department of Molecular Genetics and Cell Biology and [2]The Howard Hughes Medical Institute, University of Chicago, Chicago, IL 60637

In the absence of hormone, many steroid hormone receptors are found in large multiprotein complexes. One of the members of these complexes is Hsp90. *In vitro* studies have established that steroid hormone receptors are unable to bind to their hormone responsive elements in the DNA when they are bound to hsp90. After hormone binding, Hsp90 is released and DNA binding occurs. Recently, it was determined that glucocorticoid and dioxin receptors exhibit high-affinity ligand binding only when bound to Hsp90, whereas estrogen, progesterone, and androgen receptors are not dependent on Hsp90 for hormone binding activity. Several studies have demonstrated that *Saccharomyces cerevisiae* is a good model system for studying Hsp90-steroid hormone receptor interactions. The human and yeast Hsp90 proteins are functionally equivalent since the human Hsp90 is able to rescue yeast cells from the lethality that occurs when both of the endogenous Hsp90 genes are deleted. Mammalian steroid hormone receptors are active in yeast and in the absence of hormone are found bound to the yeast Hsp90 protein. In addition, yeast cells expressing very low levels of Hsp90 exhibit greatly reduced glucocorticoid and estrogen receptor function. In an effort to better understand the interactions between Hsp90 and the steroid hormone receptors, temperature-sensitive mutants of the *S. cerevisiae* Hsp90 were isolated. Two classes of Hsp90 temperature-sensitive mutants were obtained, those which exhibit reduced glucocorticoid receptor activity at all temperatures and those which exhibit wild-type glucocorticoid receptor activity at permissive temperatures and reduced glucocorticoid receptor activity at non-permissive temperatures. Three of the mutants in the latter group exhibit virtually no glucocorticoid receptor activity at the non-permissive temperature.

K 129 DEFINING THE ROLE OF THE HUMAN GLUCOCORTICOID RECEPTOR 3' UNTRANSLATED REGION IN HOMOLOGOUS DOWN REGULATION, Robert H. Oakley, Jeffrey C. Webster and John A. Cidlowski, Department of Physiology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7545

In most mammalian tissues glucocorticoid receptor (GR) expression decreases in response to homologous ligand. This process termed down regulation is an important physiological mechanism for attenuating cellular responsiveness to glucocorticoids. Our laboratory has previously demonstrated that the human GR cDNA contains regulatory signals sufficient for down regulation to occur in transiently transfected COS 1 cells. However, what role the 3' untranslated region (3'UTR) of the human GR gene and/or mRNA plays in the desensitizing process has not been determined. The 3'UTR of eukaryotic genes and mRNAs is known to be an important determinant of gene expression. It not only is a repository for motifs regulating mRNA stability and translation initiation, but for some genes it houses hormone response elements that modulate transcription. Therefore, we have investigated the function of the human GR 3'UTR during homologous down regulation. Employing southwestern blotting, we initially observed that GRs prepared from HeLa nuclei bind fragments of the human GR 3'UTR. We then constructed several expression vectors in which the entire human GR 3'UTR was linked to the human GR cDNA. These clones were transiently transfected into COS 1 cells and northern hybridization analysis revealed the expression of two messages (approximately 3.1 and 4.9 kb respectively) presumably reflecting transcription termination at alternate polyadenylation signals within the 3'UTR. In response to the synthetic glucocorticoid dexamethasone, both messages were down regulated. Work assessing the effect of the human GR 3'UTR on the extent and duration of GR mRNA and protein down regulation will be presented.

Work from this laboratory supported in part by National Institutes of Health Grant DK 32460. R.H.O. supported in part by a National Science Foundation Graduate Fellowship.

K 130 TWO DISTINCT DIMERIZATION INTERFACES DIFFERENTIALLY MODULATE TARGET GENE SPECIFICITY OF RXR HETERODIMERS, Thomas Perlmann, Pundi N. Rangarajan, Barry M. Forman, Kazuhiko Umesono, Ronald M. Evans, The Salk Institute, Gene Expression Lab, PO Box 85800, San Diego, CA 92186

A common feature of receptors for vitamin D_3 (VDR), thyroid hormone (TR) and retinoic acid (RAR) is their ability to heterodimerize with the retinoid X receptor (RXR) to form high affinity DNA binding complexes. In contrast to the steroid receptors, these heterodimeric complexes show an impressive flexibility in DNA binding, and can recognize both inverted (IR), everted (ER) and direct repeats (DRs) of a common half-site sequence. We have defined a 40 amino acid segment in the ligand binding domains of RAR and RXR that is important for selective and high affinity heterodimeric interactions. The 40 amino acid segment in RXR also promotes dimerization with TR. In contrast, both VDR and the peroxisome proliferator activated receptor (PPAR) requires additional more N-terminal RXR sequences for efficient dimerization. The dimerization occuring through the ligand binding domains produces heterodimers with flexible binding properties for IR, ER as well as DR DNA. A novel, previously defined dimerization interface in the DNA binding domains of RAR, TR and RXR promotes highly selective binding to appropriately spaced DRs. Accordingly, independent dimerization through two distinct dimerization domains imparts a dynamic DNA binding potential to the heterodimerizing receptors that both increases the diversity of the hormonal response as well as providing a restricted set of target sequences in DR DNA that ensures physiologic specificity.

K 131 THE ROLE OF HSP90 IN REGULATING STEROID RECEPTOR ACTIVITIES, Didier Picard and Jean-François Louvion, Department of Cell Biology, Sciences III, University of Geneva, 1211 Geneva 4, Switzerland

In the absence of cognate hormone, steroid receptors are inactive and complexed through their hormone binding domain with heat-shock protein 90 (HSP90). Hormone binding results in the release of HSP90 and concomitant activation of receptor functions. Even functions residing outside of the hormone binding domain may be repressed sterically by HSP90 in the absence of hormone. We have used budding yeast as a test tube to determine whether HSP90, possibly in combination with additional factors, indeed effects the inactivation of aporeceptors. Terminal and internal deletion mutants of HSP82, the yeast HSP90, were generated and tested for: (i) complementation for viability of yeast lacking HSP82; (ii) dominant negative effects on wild-type HSP82; (iii) effect on response of wild-type steroid receptors; (iv) repression of heterologous proteins fused to a hormone binding domain. As additional "mutants" we are also testing HSP90 homologues such as the E. coli htpG and the GRP94. Interestingly, an internal HSP82 mutant lacking the eukaryote-specific acidic domain is viable and shows no alteration in the steroid response. When HSP82 complexes cannot be formed because HSP82 is limiting, we observe constitutive partial activation of fusion proteins in the absence of hormone. This indicates that HSP82 is required for protein inactivation, but that an additional factor(s) may be essential for full inactivation and that its efficient removal may require the presence of HSP82. Moreover, using yeast we have also investigated the role of protein p60 which has been found in the aporeceptor-HSP90 complex; deletion of its yeast homologue STI1 failed to affect steroid receptor regulation.

K 132 DETERMINANTS N-TERMINAL TO THE "ZINC-FINGER" DOMAIN SERVE TO RESTRICT THE DNA RECOGNITION SPECIFICITY OF THE V-ERB A AND RAR PROTEINS, Martin L. Privalsky, Zeljka Smit-McBride, and Hong-wu Chen, Department of Microbiology, University of California at Davis, Davis, CA. 95616.

The v-*erb* A oncogene is a dominant negative allele of a thyroid hormone receptor gene (c-*erb* A-α) and has sustained both deletion and point mutations relative to its normal cell progenitor. As a consequence the v-*erb* A protein is unable to activate transcription in animal cells in response to hormone ligand, and is believed to act in the cancer cell as a constitutive repressor. We demonstrate here that the DNA sequence recognition properties of the v-*erb* A protein are significantly altered from those of the normal thyroid hormone receptors. This difference is manifested at the level of recognition of the DNA half-site, and is relatively independent of the spacing and orientation of the half-sites in the response element. Unexpectedly, the altered DNA recognition properties of the v-*erb* A protein are encoded, in part, by an N-terminal region not been previously implicated in receptor/DNA interactions. Deletion of the N-terminus broadens the DNA recognition specificity of the v-*erb* A protein, suggesting that the N-terminal determinants act to restrict the specificity of the wild-type v-*erb* A protein. Preliminary results indicate that similar N-terminal domains may also modulate the specificity of DNA recognition by the thyroid hormone and retinoic acid receptors. Our results suggest that unanticipated determinants within the nuclear hormone receptors may contribute to the different target gene specificity displayed by different receptor forms.

K 133 THE ROLE OF RARγ IN THE RHINO MOUSE AND RABBIT IRRITATION MODELS OF RETINOID ACTIVITY, Peter R. Reczek*, Simon Chen#, Jacek Ostrowski*, Roman Sterzycki+, Kuo-Long Yu+, Stephen J. Currier*, and Muzammil M. Mansuri+, Departments of *Molecular Biology, #Biochemical Pharmacology, Bristol Myers Squibb Pharmaceutical Research Institute, Buffalo, NY 14213 and +Central Chemistry, Wallingford, CT

Retinoic acid is known to modulate a number of biological activities. The discovery of three nuclear receptors (RARs) opened the possibility that each receptor regulates the expression of a subset of genes that controls a particular biological response.

Of the three known receptors, RARγ is the predominant form in skin. We have recently identified a number of synthetic retinoic acid analogues that are characterized by their binding and receptor mediated transcriptional activation to favor RARγ. On average, these molecules are at least 100-fold more potent activators of RARγ than either RARα or RARβ.

Several *in vivo* animal models are currently being used to assess retinoid efficacy and it's side effects. Of these, the Rhino mouse utriculi reduction assay is taken as one indicator of clinical efficacy in acne. All of the RARγ selective retinoids identified were active in this model. This response was directly related to potency in transcriptional activity (r^2=0.82). A second model, irritation on rabbit skin, directly correlated potency with RARγ transcriptional activity. Both RARα and RARβ showed no such correlation in either model.

These results indicate that RARγ can mediate utriculi reduction in the Rhino mouse as predicted by the tissue distribution of this receptor in the skin. Further, the positive correlation of receptor activity and rabbit irritation suggests a molecular mechanism for this well known side effect of the clinical use of retinoids.

K 134 INTERACTION OF THE GLUCOCORTICOSTEROID RESPONSIVE ELEMENTS (GRE) WITH THE GLUCOCORTICOSTEROID RECEPTOR (GR): BINDING PARAMETERS AND ROLE OF RECEPTOR DOMAINS. Ingrid Segard-Maurel, Nicole Jibard, Ghislaine Schweizer-Groyer, Etienne Baulieu and Françoise Cadepond. INSERM U33, 80, rue du Général Leclerc, 94276, le Kremlin-Bicêtre.

The human wild type GR, the N-terminal deleted-GR (Δ1-417), the ligand binding domain (LBD)-truncated GR (I550*) or the DNA binding domain (DBD)-deleted mutant (Δ428-490) were expressed in the baculovirus-SF9 system and labeled *in vivo* with 3H-triamcinolone acetonide. High salt extracts were subjected to gel retardation experiments by using three different ^{33}P-GREs (39-mer): the imperfect palindromic GREII from the tyrosine-amino-transferase promoter (Ip), the perfect palindromic equivalent sequence (AGAACAnnnTGTTCT) (Pp) and the corresponding half palindromic GRE (ttgctcnnnTGTTCT) (1/2p). When using Ip or Pp, we characterised specific dimeric and monomeric GR/DNA complexes with the wild type GR, the I550* mutant or the Δ1-417 mutant. We detected with palindromic GREs heterodimers that contained one monomer of the Δ1-417 mutant associated with one monomer of the I550* mutant or of the wild type GR, but not with a monomer of the DBD-deleted mutant (Δ428-490). With 1/2p, we observed predominant monomeric bands for DBD-containing GRs. However, for the wild-type GR and the Δ1-417 mutant, we also observed very small amounts of DNA-GR complexes migrating as homodimers, suggesting that some GR dimers were interacting with 1/2p when the LBD was present. These results are consistent with the dual involvement of the DBD and LBD in dimer formation. By quantifying dimeric complexes and free DNA in our experimental conditions, we found that the number of receptor binding sites for DNA of a given extract was about twice with Pp than with Ip. Whatever the nature of the GRE, the apparent equilibrium dissociation constants of the wild type GR and Δ1-417 mutant from DNA were similar (K_D~6nM), but for the LBD-truncated mutant, the affinity unchanged with Ip, was reduced three fold with Pp. These data indicate that GRE sequence may induce differences in protein-DNA and/or protein-protein contacts, thus modulating GR-DNA binding efficiency.

K 135 TIGHT ASSOCIATION OF HSP70 WITH THE HUMAN GLUCOCORTICOID RECEPTOR EXPRESSED IN THE BACULOVIRUS SYSTEM, Ganesan Srinivasan, Nutan T. Patel, and E. Brad Thompson, Department of Human Biological Chemistry and Genetics, The University of Texas Medical Branch, Galveston, TX 77555-0645

Steroid hormone receptors are known to associate with various heat shock proteins. The association of hsp90 with steroid receptors maintains the receptors in the non-activated state until interaction with their respective ligands. In addition to hsp90, progesterone receptor (PR) has also been shown to interact with hsp70. However, hsp70 appears to associate with GR only in some systems. We have investigated the interaction between and overexpressed and recombinant hGR and hsp70 in insect cells. When hGR was immunoprecipitated using an anti-GR antibody, hsp70 co-immunoprecipitated with both the non-activated and activated hGR. Hsp70 co-immunoprecipitated with hGR even when the receptor was activated *in vivo* by incubation of insect cells expressing hGR with 1 μM dexamethasone for 4 h at 27°C prior to harvest. Both the 8.35S (non-activated) and 3.8S (activated) hGR peaks were completely shifted to higher S values when reacted with either an anti-hGR antibody or an anti-hsp70 antibody. The activated hGR migrated through a Sephacryl S-400 column with a Stokes radius of 95Å. The calculated molecular weight of the activated hGR, based on sedimentation co-efficient and Stokes radius, was 154 kDa which closely matched the expected molecular weight for a hGR-hsp70 heterodimer. Activated hGR purified from a GRE (glucocorticoid response element)-Sepharose column also contained hsp70. Attempts to dissociate hsp70 from hGR using ATP and NaCl were unsuccessful. In gel retardation assays, both an anti-hGR antibody and an anti-hsp70 antibody supershifted the hGR:^{32}P-GRE complex. Our studies suggest strong association between hsp70 and recombinant hGR. The hGR overexpressed in the Baculovirus system has been shown to be ≥100-fold active than native PR or the DNA fragment of GR expressed in *E. coli*. It is possible that hsp70 has a role to play in the DNA binding and/or transcriptional activities of hGR.

K 136 POINT MUTATIONS IN THE MOUSE GLUCOCORTICOID RECEPTOR THAT AFFECT HORMONE BINDING AFFINITY AND SPECIFICITY, Michael R. Stallcup, Dagang Chen, Jon Milhon, Kulwant Kohli, Kimberly Duncan, Sunyoung Lee, and Huaming Chou, Departments of Pathology and Biochemistry, University of Southern California, Los Angeles, CA 90033

Results. A collection of point mutations that cause single amino acid substitutions in the hormone binding domain (HBD) of the mouse glucocorticoid receptor (GR) were obtained by site directed mutagenesis or from mutant cell lines that do not respond normally to hormone. By transient expression in CV1 cells, the mutant GR were assessed for their ability to activate a hormone dependent reporter gene in response to various hormone concentrations. Amino acids scattered throughout the HBD (residues 535-783 in the mouse GR) were found to be important for hormone binding. 1) At the extreme N-terminal end of the HBD, substitution of glycine for amino acids V544, I545, or V549, or substitution of alanine for L550 or D555 caused at least a 10-fold decrease in binding affinity for dexamethasone. 2) Near the extreme C-terminus of the GR, changing amino acid F780 to alanine caused a 20-fold reduced affinity for dexamethasone and a change in hormone binding specificity. 3) Of the five cysteines located in the HBD (C628, C644, C649, C671, and C742), only the last two are important for hormone binding; a 10-fold decreased affinity resulted when serine was substituted. 4) Of five randomly produced mutations that decreased hormone binding affinity (identified in mutant cell lines), two were adjacent to C671: L670 changed to F and M672 to I.

Discussion. A proteolytic fragment (amino acids 525-661 of the mouse GR) comprising the N-terminal half of the HBD binds hormone with an affinity 23-fold less than that of intact GR and with similar hormone binding specificity (Simons et al, J. Biol. Chem. 264, 14493-14497, 1989; Chakraborti et al, J. Biol. Chem. 267, 11366-11373, 1992). Thus, the N-terminal half of the GR HBD was proposed to be the core of the hormone binding function. The data presented here defines a group of amino acids at the extreme N-terminal end of the HBD that are essential for high affinity hormone binding. In addition, however, specific residues in the C-terminal half of the HBD also are important for hormone binding. Residues at three different sites on the primary sequence of the C-terminal half of the HBD were implicated: 670-672, 742, and 783. C742 is also a covalent affinity label site (Carlstedt-Duke et al, J. Biol. Chem. 263, 6842-6846, 1988). Thus, C742 apparently contributes to hormone binding through direct contact with the steroid.

K 137 HUMAN KERATINOCYTES PRODUCE TWO DIFFERENT mRNA SPECIES ENCODING THE VITAMIN D RECEPTOR, Laurie J. Sturzenbecker, Beiqian Scardaville, Claudia C. Kratzeisen, Marion Katz, and John A. McLane, Department of Preclinical Dermatology Research, Hoffmann-La Roche, Inc., Nutley, N.J. 07110.

A normal human keratinocyte cDNA library was screened at high stringency using the T47D cell VDR cDNA as probe. Eight positive plaques were identified among the $3x10^5$ screened. Five plaques were purified and their insert sequences analyzed for their capacity to serve as template for PCR amplification using VDR-specific oligonucleotides as primers. All five produced amplicons size-appropriate for VDR. The insert from one clone, BQS1, was sequenced in its entirety. The 1813 bp BQS1 insert contains the entire coding sequence of VDR, plus 91 bp of upstream- and 451 bp of downstream untranslated sequence. The sequence differs from that published for VDR (Baker, et al., 1988) by 2 base pairs, one of which is silent and the other of which results in a 3 aa truncation at the amino terminus of the polypeptide. The insert DNA from two other independently isolated plaques was sequenced and found to contain the same T to C transition.

In order to determine whether keratinocyte VDR mRNA always contains a C at this position, mRNA was isolated from primary cultures of foreskin keratinocytes, reverse transcribed, and amplified by PCR using VDR-specific primers. When the amplicon was purified and sequenced, both a C and a T were identified at this position. Thus, in human keratinocytes, there appear to be two different VDR mRNA species encoding polypeptides which differ by 3 aa at their amino termini. The possible biological implications of this finding will be discussed.

K 138 DISTINCT BINDING DETERMINANTS FOR 9-*CIS* RETINOIC ACID ARE LOCATED WITHIN AF-2 OF THE RARα, Bonnie F. Tate, Gary Allenby, Reinhold Janocha, Sonja Kazmer, Jeffrey Speck, Arthur A. Levin, and Joseph F. Grippo, Department of Toxicology and Pathology, Hoffmann-LaRoche, Inc., Nutley, NJ 07110

Retinoids exert their physiological action by interacting with two families of nuclear receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs), which regulate gene expression by forming transcriptionally active heterodimeric RAR/RXR or homodimeric RXR/RXR complexes on DNA. Retinoid receptor activity resides in several regions including DNA and ligand binding domains, a dimerization interface and both a ligand-independent (AF-1) and a ligand-dependent (AF-2) transactivation function. While 9-*cis* retinoic acid (RA) alone is the cognate ligand for the RXRs, both 9-*cis* RA and all-*trans*-RA (t-RA) compete for binding with equal affinity to the RARs. This latter observation suggests that both isomers interact with a common binding site. Here we report that RARα has two distinct, but overlapping binding sites for 9-*cis* RA and t-RA. Truncation analysis indicates that a determinant required for high affinity 9-*cis* RA binding, but not t-RA binding, resides within fifteen amino acids (404-419) of RARα. This region also defines a C-terminal boundary for AF-2, as addition of these amino acids can restore receptor-mediated gene activity to a truncated RARα lacking this region. It is interesting to speculate that binding of retinoid stereoisomers to unique sites within an RAR may function with AF-2 to cause differential activation of retinoid-responsive gene pathways.

K 139 COEXPRESSION OF THE MINERALOCORTICOID RECEPTOR AND GLUCOCORTICOID RECEPTOR : CHARACTERIZATION OF TRANSACTIVATION AND DNA-BINDING PROPERTIES, Thorsten Trapp, Rainer Rupprecht, and Florian Holsboer, Max-Planck-Institute of Psychiatry, Clinical Institute, Department of Neuroendocrinology, Kraepelinstr. 10, 80804 Munich, FRG

The physiological effects of glucocorticoids in the human brain are mediated via two types of corticosteroid receptors, the mineralocorticoid (hMR) and the glucocorticoid receptor (hGR), which bind to common response elements with distinct regulatory properties at the gene expression level. As both corticosteroid receptors are coexpressed in the hippocampus and respond to physiological glucocorticoids, we studied the functional consequence of coexpression of both receptors with regard to DNA-binding and transcriptional regulation. Transient transfection studies employing the human neuroblastoma cell line SK-N-MC and the mouse mammary tumour virus promoter fused to the luciferase gene as a reporter system revealed that cotransfection of hMR and hGR using cortisol as a common ligand in the low nanomolar range resulted in more than additive transcriptional response when compared to transfection of either hMR or hGR alone, while incubation with higher cortisol concentrations yielded a lower transcriptional activity in comparison to that of the hGR. Both hMR and hGR bound specifically to a palindromic GRE in a bandshift assay. DNA-binding was cooperative and strongly enhanced by coincubating both receptors simultaneously when compared to that obtained by either hMR or hGR alone. Our results suggest the formation of heterodimers between hMR and hGR which may be responsible for the complex transactivation patterns obtained with coexpressed corticosteroid receptors dependent on the relative expression of both receptor types and the ligand concentration.

K 140 BUTYRIC ACID ENHANCES GENE EXPRESSION REGULATED BY THE ROUS SARCOMA VIRUS PROMOTER, Douglas B. Tully, Alyson B. Scoltock, Christine M. Jewell and John A. Cidlowski, Department of Physiology, University of North Carolina, Chapel Hill, NC 27599-7545

Glucocorticoid-induced gene expression was enhanced by sodium butyrate in glucocorticoid receptor-deficient mouse L (E8.2) cells cotransfected with an expression vector (pRShGRα) containing the coding sequence for the human glucocorticoid receptor (GR) and a glucocorticoid responsive reporter plasmid (pGMCS). Furthermore, glucocorticoid-induced gene expression in transfected E8.2 cells was shown to be dependent on both the butyrate dose and duration of exposure. Subsequent experiments which examined dexamethasone (DEX) dose-response curves indicated an optimal steroid dose of 10^{-9} to 10^{-8} M DEX, but also suggested that butyrate enhancement of DEX-induced gene expression may be due to an increase in GR number. Interestingly, butyrate failed to alter glucocorticoid receptor-mediated gene expression in cells containing endogenous GR. Since GR expression from transfected pRShGRα is regulated by the RSV promoter, we next examined the possibility that butyrate was acting on the RSV promoter to enhance expression of the GR gene. Northern and Western blot experiments using COS 1 cells transfected with pRShGRα showed that both GR mRNA and protein increased following butyrate treatment. Furthermore, butyrate alone induced CAT gene expression from another RSV reporter plasmid, pRSVCAT, but not from TK CAT. These results demonstrate that transcription mediated by the RSV promoter is enhanced by sodium butyrate, suggesting that butyrate may be useful as a tool to augment expression of transfected genes regulated by the RSV promoter. Supported by NIH Grant DK32459.

K 141 Characterization of a Hormone-Responsive Orphan Nuclear Receptor

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Orphan nuclear receptors are candidate ligand-responsive transcriptional activators whose predicted hormones are being systematically sought as follows. A cDNA clone called OR2, encoding a nuclear receptor-like protein of 469 amino acids, has been isolated from a rat liver library. Its expression is confined to the liver, intestine, and kidney with lower levels also found in the adrenal cortex. The OR2 DNA binding domain is closely related to that from the Drosophila ecdysone receptor and OR2 binds ecdysone-responsive DNA elements by heterodimerizing with RXR. Introduction of plasmid DNAs encoding RXRα and an EcRE-linked CAT gene into receptor-deficient cells fashions an OR2-dependent transcription system activated by organic extracts of serum. OR2 hormone activity partitions primarily in the chloroform phase from a Bligh/Dyer extraction, supporting a lipophilic nature. OR2 activity separation by reverse phase HPLC indicates that its hydrophobicity, like steroids, is between polar ecdysone and non-polar cholesterol. These chemical features, OR2 expression in cholesterologenic tissues and its similarity to the vitamin D and ecdysone receptors which both bind sterol-like molecules, hint that the OR2 activator may also be of an oxygenated cholesterol molecular ilk. Larger quantities of the OR2 effector are being purified for its molecular structure determination. This general approach of establishing receptor ligand-dependency should be useful for evaluating the remaining orphan nuclear receptors.

Poster Session II

K 200 ALTERED GLUCOCORTICOID FEEDBACK IN THE PITUITARY OF CHRONICALLY STRESSED RATS: INVOLVEMENT OF VASOPRESSIN. Greti Aguilera, Mirza Flores and Xun Luo. Developmental Endocrinology Branch, NICHD, NIH, Bethesda, MD 20892

The mechanism of altered glucocorticoid feedback in the pituitary during chronic stress was studied by analysis of glucocorticoid receptors and the sensitivity to the inhibitory effect of glucocorticoids in pituitaries from control and rats chronically stressed by repeated daily immobilization for 14 days (RI). Preincubation of collagenase dispersed anterior pituitary cells from control rats with 10 nM corticosterone (B) for 18 hr resulted in 30% inhibition of CRH-stimulated ACTH release, whereas no effect or slight stimulation was found in cells from intact or adrenalectomized-B substituted (ADX) RI rats. Binding of [^{3}H]dexamethasone to pituitary cytosol was decreased by 30% in intact, but not in ADX rats, indicating that the failure of B to inhibit ACTH secretion in cells from RI rats was not due to glucocorticoid receptor downregulation. The inhibitory effect of stress on glucocorticoid feedback was markedly attenuated in pituitary cells from rats receiving osmotic minipump infusion of 200 ng/min of the VP antagonist [(mercapto cyclopentamethylene propionic acid)-methyltyrosine AVP] the last 7 days of immobilization. In vitro studies in cultured pituitary cells from normal rats showed that while preincubation for 18 hr with 1 nM B inhibited CRH-stimulated ACTH release by 30%, combined pretreatment with 100 nM B and 1 nM VP, completely prevented the inhibitory effect of corticosterone. The data provides strong evidence for a role of VP in the mechanism of the impaired glucocorticoid feedback observed during chronic stress. This effect in not due to changes in GR levels, but probably to VP-mediated changes in the mode of interaction of GR with its response element in the POMC gene.

K 201 PHOSPHORYLATION OF THE HUMAN OESTROGEN RECEPTOR REGULATES ITS FUNCTION, S. Ali[1], P. Pace[1], D. Metzger[2], P. Chambon[2] and R. C. Coombes[1], [1]Dept. of Medical Oncology, Charing Cross Hospital, London W6 8RF,[2]LGME-CNRS, U184 de l'INSERM, Faculte de Medicine, 11 Rue Humann, 67085 Strasbourg.

The oestrogen receptor (ER) protein can be separated into distinct domains (or functions) containing the DNA binding domain, the hormone binding domain and trans-activation domains. The N-terminal A/B region and region E contain so-called ligand-independent and -dependent trans-activation functions, respectively which act in cell- and promoter-specific manner.

We will present results which demonstrate that ER is phoshorylated upon binding oestradiol (E2). We further show that human ER (hER) is phosphorylated at several sites in different regions in the presence of E2 and that anti-oestrogens also induce ER phosphorylation, although at much lower levels than oestradiol. Phosphoamino-acid analysis shows that most of these sites are serine residues. We have mapped the N-terminal-most phosphorylation site to serine 118. Mutation of serine 118 drastically reduces *trans*-activation by hER, but has no effect on DNA or hormone binding by the ER. We also show that hER can be phosphorylated *in vitro* by at least three protein kinases involving different signal transduction pathways. These results and their importance for the regulation of ER function are discussed.

K 202 DIFFERENTIAL BINDING OF 9-CIS RA BY RAR SUBTYPES. Gary Allenby, Reinhold Janocha, Sonja Kazmer, Jeffrey Speck, Joseph F.Grippo, and Arthur A. Levin, Department of Toxicology and Pathology, Hoffmann-La Roche, Nutley, NJ 07110

Both 9-cis retinoic acid (RA) and all-trans RA (t-RA) compete for [^{3}H]-9-cis RA binding to RA receptors (RARα, β and γ) in nucleosol fractions from transiently transfected COS-1 cells with IC50s of approximately 12 and 5 nM, respectively. Curiously, 9-cis RA competes for [^{3}H]-t-RA binding to mouse RARα, β and γ with IC50s of 31, 8, and 60 nM, respectively, while t-RA itself does not exhibit such differential competition (IC50s for RARs, 5nM). A similar pattern is observed with human RARs. Displacement assays demonstrate that t-RA exhibits similar off-rates for RARα, β and γ. However, 9-cis RA is 6-fold more rapidly displaced from RARγ than from RARβ. When COS-1 cells are incubated with [^{3}H]-t-RA, [^{3}H]-9-cis RA or various mixtures of these two radioligands, HPLC analysis demonstrates that the ligands bound in nucleosol fractions from RARβ-transfected cells reflect the isomer content of the media. However in identical whole cell assays, nucleosol fractions from RARγ-transfected cells preferentially bind t-RA over 9-cis RA, consistent with the in vitro data. These binding kinetics in vitro and in whole cells suggest that there could be differences in the interactions of the receptor subtypes with the endogenous retinoic acids under physiologic conditions.

K 203 PHOSPHORYLATION OF THE HUMAN ESTROGEN RECEPTOR Steven F. Arnold, John D. Obourn and Angelo C. Notides University of Rochester School of Medicine & Dentistry, Dept. of Env. Medicine Rochester, N.Y. 14642

This laboratory has previously shown that the human estrogen receptor (hER) in MCF-7 breast carcinoma cells is a phosphoprotein and becomes hyperphosphorylated on serine residues in the presence of estradiol. We report here that the recombinant hER overproduced in insect cells (SF9) using the baculovirus expression system is also hyperphosphorylated in response to estradiol as shown by [^{32}P]-incorporation and Western blot analysis. The hyperphosphorylation of the recombinant hER is ligand-dependent at 1-2 days post baculoviral infection and is ligand-independent at 5 days post-infection. We have purified both the recombinant and native hER to homogeneity using an ERE-containing oligonucleotide attached to a Teflon matrix followed by SDS gel electrophoresis. The purified hER is chemically cleaved with cyanogen bromide and enzymatically digested with trypsin. Resolution of the CNBr and tryptic digests by HPLC reveals that all the major phosphopeptides of the recombinant and native hER are conserved. The similarity between the phosphorylation of the hER in insect cells and a human carcinoma cell line indicates that the kinases and phosphorylation sites necessary for hER activity are conserved regulatory pathways in invertebrates and vertebrates. Supported by NIH HD06707, ES01247 and ES07026.

K 204 REGULATION OF THE GLUCOCORTICOID RECEPTORS IN CHICK EMBRYONIC NEURAL RETINA, Sarah Ben-Or and Margalit Eshel, Department of Physiology, Hebrew University-Hadassah Medical School, Jerusalem, Israel.

Comparative studies on the interaction of glucocorticoid receptors (GR) of the chick embryonic neural retina with cortisol (HC) versus triamcinolone acetonide (TA) revealed remarkable kinetic differences, which were instrumental in elucidating the role of the glucocorticoid hormone (GC) in GR function. Consequent to the interaction of GR with HC or TA, in the intact neural retina in organ culture, there is a sharp loss in GC-binding activity. The reaction to HC is rapid, independent of temperature and mainly detected in the cytosol. The reaction to TA is slow, temperature-dependent and can be observed in the nucleus. The loss in binding activity induced by HC in the cold correlates with the transformation of HC-GR to a 4S complex while TA-GR is maintained as a 9S complex. The apparent correlation between the kinetics of the decrease in retention of nuclear bound TA-GR and the onset of the GC-controlled cellular specific response suggests that: a) the relative low steady state level of nuclear TA-GR, established after 5h in culture with TA, is *required* and *sufficient* for induction of the optimal response; and b) the overload of nuclear TA-GR, as distinct from the initial low level of nuclear HC-GR, impedes the expression GR function. Analysis of retinal GR from parallel cultures by Western blot, using a monoclonal antibody against rat GR, did not detect significant changes in GR protein content during 24-48h exposure to HC or TA. Autologous down-regulation of retinal GR protein was however evident *in situ*. *In ovo* expression of retinal GR down-regulation could be mimicked in retinal organ cultures by reducing the content of fetal calf serum in the culture medium.

K 205 STRUCTURE AND FUNCTION OF ANDROGEN-RECEPTOR IN ANDROGEN-INDEPENDENT MALIGNANCY. Nicholas Bruchovsky, Koichiro Akakura, Paul S. Rennie and Rob Snoek. Department of Cancer Endocrinology, B.C. Cancer Agency, Vancouver, Canada. Progression to androgen-independence may involve a change in the structure or function of the androgen receptor (AR). We examined this possibility in the Shionogi carcinoma using tumors which were either androgen-dependent (AI) or androgen-independent (AD). The AI tumors were generated in two ways: (1) male mice bearing the AD tumor were castrated to induce apoptotic regression and then maintained with no further treatment until recurrent AI growth was observed; (2) the AD tumor was transplanted into a succession of male mice, each of which was castrated when the estimated tumor weight became about 3 g; after 4-5 cycles of transplantation and castration-induced apoptosis, the tumor abruptly progressed to an AI state. DNA was obtained by reverse transcriptase-polymerase chain reaction with mRNA isolated from AD and AI tumors. After amplification of specific primer selected regions of AR cDNA, the DNA product was sequenced by the thermal cycle dideoxy DNA sequencing method. Relative levels of expression of AR-regulated mRNA for glucocorticoid receptor (GR), fibroblast growth-factor receptor (FGF-R) and TRPM-2 were compared. Sequences of AR cDNA were identical in AD and AI tumors consistent with normal structure in both. In AI tumors, replacement of androgen resulted in down-regulation of GR mRNA and up-regulation of FGF-R mRNA, indicating normal function of AR. TRPM-2 which codes for the anti-cytolytic protein, clusterin, was constitutively overexpressed in all tumors after initial androgen deprivation. Thus, androgen-independence is not due to AR failure; rather this is more closely related to the deregulated expression of TRPM-2 which occurs during tumor progression.

Supported by the M.R.C. of Canada.

K 206 SEQUENCES PRESENT WITHIN THE HUMAN ANDROGEN RECEPTOR cDNA CONFER LIGAND-INDUCIBLE RECEPTOR mRNA DOWN REGULATION, Kerry L. Burnstein, Carol A. Maiorino and Dayna J. Cameron, Department of Molecular and Cellular Pharmacology, University of Miami School of Medicine, Miami, FL 33101

Androgen receptors (AR), like other steroid receptors, are subject to regulation by their ligands and alterations in receptor levels can influence hormonal responsiveness. Androgen-inducible down regulation of AR mRNA has been documented in various rat target tissues and in certain breast and prostate cancer cell lines. We have found that a 2-4 fold androgen-mediated down regulation of AR mRNA also occurred in cell lines expressing a human AR cDNA. This effect was due to the presence of the receptor cDNA and not to androgen regulation of the CMV promoter driving the expression of the AR cDNA. Marked reduction in AR mRNA was observed by 4 hours after androgen treatment. Down regulation with the androgen methyltrienolone was dependent upon hormone concentration with a half maximal decrease between 0.01 and 0.1 nM. This concentration range was sufficient to elicit induction of an androgen-responsive reporter gene. Down regulation of AR mRNA occurred in the presence of cycloheximide suggesting that an androgen-inducible protein is not required. The clinically used AR antagonist, cyproterone acetate, promoted down regulation of AR mRNA like an agonist; whereas, the non-steroidal antiandrogen, hydroxyflutamide, did not influence AR mRNA levels. Neither antagonist completely blocked methyltrienolone-induced down regulation. To determine whether AR mRNA levels were also regulated by glucocorticoid receptors, which can bind the same DNA sequences as AR, COS 1 cells were cotransfected with human androgen and glucocorticoid receptor cDNAs. Down regulation of both androgen and glucocorticoid receptor mRNAs occurred in response to glucocorticoid treatment; in contrast, androgen administration did not affect glucocorticoid receptor mRNA levels. These results suggest that the human AR cDNA contains sequences that mediate both androgen- and glucocorticoid-inducible down regulation. Supported by DK45478.

K 207 INCREASE SENSITIVITY OF AN ALL-TRANS RETINOIC ACID RESISTANT LEUKEMIC CELL LINE TO VITAMIN D ANALOGS MAY BE DUE TO A MUTATED RETINOIC ACID RECEPTOR ALPHA, Benoit T. Doré[1], Milan R. Uskokovic[2] and Richard L. Momparler[1].

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Since drug resistance following chemotherapy with all-trans retinoic acid (ATRA) is often observed in leukemic patients, it is of interest to investigate the possible molecular mechanisms involved in the development of resistance. In this study we have isolated a clone of HL-60 leukemic cells that is resistant to ATRA (HL-60/RA). Using this model we observed that the loss of drug sensitivity was not due to a variation in the retinoic acid receptor alpha (RAR-α) mRNA expression, but may involve a point mutation in the ligand binding domain of this receptor. Since reports have shown that Vitamin D_3 can induce the differentiation of myeloid leukemic cells, we investigated the antileukemic action of different Vitamin D analogs such as 1,25-dihydroxy-16-ene-23-yne-cholecalciferol (16-23-D_3). Unexpectively, we have observed that the HL-60/RA cell line was more sensitive to the antineoplastic action of 16-23-D_3 than the parental cell line with respect to the inhibition of cell growth, the induction of differentiation and the loss of cell clonogenicity. Our hypothesis is that the increased sensitivity of the HL-60/RA cells to vitamin D analogs is due to a defective RAR-α that binds less RXR resulting in more RXR free to interact with vitamin D receptors (VDR) and enhanced its transcriptional activity. Nuclear extracts of both cell types will be used in gel retardation assays to verify the proposed hypothesis. These studies will provide new information on the antileukemic action of ATRA and vitamin D analogs and may lead to a more effective utilization of these agents in the clinical treatment of leukemia.

K 208 NOVEL NUCLEAR PML DOMAINS IN APL CELLS ARE REORGANIZED UPON RETINOID TREATMENT, Jacqueline A. Dyck*, Akira Kakizuka, Wilson H. Miller Jr.**, Gerd Maul# and Ronald M.Evans+. Howard Hughes Medical Institute+. The Salk Institute for Biological Studies, La Jolla, CA 92037#. Wistar Institute of Anatomy & Biology, Philadelphia, PA 19104**, Memorial Sloan-Kettering Cancer Center, New York, N.Y**.

Acute promyelocytic leukemia (APL) is associated with a t(15;17) translocation which fuses the retinoic acid receptor α (RARα) with PML.

Immunohistochemistry of leukemic cells performed using both anti-RARα and anti-PML antibodies demonstrated that the aberrant PML-RAR and the wild-type PML proteins are localized in the nucleus. The PML domains are distributed differently in leukemic cells than observed in non-APL cells. These domains represent a novel macromolecular organelle that includes several other nuclear proteins. Retinoic Acid treatment of APL cells triggers a reorganization of the leukemic structures to generate normal appearing PML domains. This reorganization is correlated with a loss of punctate labelling of the fusion protein and is linked to morphological differentiation of the leukemic cells. We propose that PML-RAR is a dominant negative oncoprotein that exerts its putative leukemogenic effect by acting to physically interfere with the PML domain. According to this proposal, not only is the POD (for PML oncogenic domain) a novel structure but it can be ascribed an imputed function such that its disruption leads to cellular transformation. The uniqueness of the POD suggests this may represent a new mechanism of cellular transformation.

K 209 RA AND PKA SIGNALLING PATHWAYS INTERACT AT THE LEVEL OF NUCLEAR RARS.
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Retinoids are important signalling molecules for pattern formation and cellular differentiation during vertebrate development. It is now established that the molecular effects of the RA signal are mediated through two families of retinoic acid receptors, RARs (α, β, γ and their isoforms) and RXRs (α, β and γ) which act as inducible transcriptional regulatory proteins. It is also known that cAMP treatment modifies the developmental pathway of F9 EC cells upon RA treatment. These observations prompted us to investigate the phosphorylation status of RA receptors.

"in vivo" [^{32}P] labelling of transfected Cos-1 cells has revealed that major isoforms RARα1, β2 and γ1, and RXRα proteins are phosphorylated, regardless of the presence of RA in our experimental conditions.

"in vitro" phosphorylations of RARα1 and RARγ1 are apparently different from that of RARβ2 as bacterially expressed RARα1 and RARγ1 proteins were strongly phosphorylated by the cAMP dependent protein-kinase (PKA) while RARβ2 was only weakly phosphorylated by this kinase.

We have also shown by cotransfection experiments that PKA strongly modified the ligand-dependent RA inducible transactivation by RARs of promoters containing a RARE; furthermore the magnitude of the RA response was both promoter context and cell type dependent. Mutagenesis of RARs in consensus sites for PKA phosphorylation indicated that full potentiation by PKA required phosphorylation of RARs.Whether phosphorylations of RARs and RXRs affects their DNA binding activity is under investigation.

K 210 ANDROGENS AND GLUCOCORTICOIDS SUPPRESS PROSTATE EPITHELIAL CELL GROWTH THROUGH A SHARED, RECEPTOR-MEDIATED PATHWAY, Debra Gordon and Roger Miesfeld*, Depts. of Biochemistry and Molecular & Cellular Biol.*, and Arizona Cancer Ctr., Univ. of Arizona, Tucson, AZ, 85724.
The prostate is a male-specific gland that is dependent upon androgens for normal differentiation and function. In the absence of androgens, the prostate involutes with a net loss of epithelial cells. Upon readministration of androgens, a normal population of cells is regenerated. Observations such as these have led to the widely accepted conclusion that androgens *stimulate* prostate epithelial cell growth. This view has been supported by the finding that the growth of some prostate tumors and transformed prostate cell lines are stimulated by androgens. Using SV40 T-antigen as an immortalizing agent, we have generated >100 cell lines from an enriched population of normal rat prostate epithelial cells. Given that a majority of the cells in the primary culture expressed the androgen receptor (AR), we were surprised to find that none of the resultant cell lines had functional levels of AR, even though AR mRNA could be detected by PCR amplification. Stable transfection of plasmids constituitively expressing AR cDNA into two prostate cell lines yielded considerably less than the expected number of AR^+ subclones (3.5 and 1.3% of drug-resistant subclones, respectively). Whereas, at least 30% of drug-resistant subclones from similarly transfected rat hepatoma and fibroblast cell lines expressed AR. The growth of two related AR^+ subclones of prostate origin, CA24 and CA25, was examined and found to be suppressed in 1 μM DHT. Growth inhibition was greater for CA25 than CA24, which is consistent with their relative levels of AR protein. Androgens had no effect on the AR^- parental line, C1.1, indicating that this is a receptor-mediated effect. Since these prostate cell lines express the glucocorticoid receptor (GR), we examined the effect of dexamethasone (dex) on CA25 and C1.1 growth. CA25 growth was inhibited by 1 μM dex suggesting that the growth-suppression pathway can be regulated by both AR and GR. C1.1, which has 50% less GR than CA25, was not affected by dex, indicating that there may be a threshold receptor level necessary for hormone-dependent growth regulation. Taken together, these data are consistent with the conclusion that androgens and glucocorticoids may *inhibit* growth of some prostate cells. In addition, there is a marked morphological change correlated with hormone-induced suppression of growth of these prostate cell lines which may represent a differentiation event. Studies are underway to determine the mechanism(s) underlying growth regulation by steroids in these cells.

K 211 PEPTIDE MAPPING OF THE HORMONE MODULATED SITE OF PHOSPHORYLATION IN THE HUMAN VITAMIN D RECEPTOR George M. Hilliard IV, Richard G. Cook*, Nancy L. Weigel, and J. Wesley Pike•, Depts. of Cell Biology and Immunology*, Baylor College of Medicine, Houston, TX and Biochemistry, Ligand Pharmaceuticals•, San Diego, CA.
We are interested in the phosphorylation of the human 1,25-dihydroxyvitamin D_3 receptor (VDR) in response to ligand. Our approach has been to perform peptide mapping on recombinant receptor which will identify specific *in vivo* sites within the human VDR which are modified in response to the occupation of the human receptor with its cognate ligand. This contrasts with alternative and less precise methods involving deletion or mutational analysis only, or *in vitro* phosphorylation using purifed kinases. The experimental results demonstrate that the human VDR is constitutively phosphorylated in a COS-1 cell transfection system, and the phosphorylation state of the VDR is enhanced in the presence of hormone. Amino acid analysis of the phosphorylated receptor has revealed that the human VDR is exclusively phosphorylated on serine residues. Phosphopeptide mapping has been performed using transfected, radiolabelled human VDR that is co-precipitated with pure human VDR from yeast. This full length VDR protein mixture was subjected to enzymatic and chemical cleavages, and the phosphopeptides were purified using reverse phase HPLC. The exact position of the labelled serine within the VDR was identified by protein sequencing procedures and by monitoring $[^{32}P]$phosphoserine release during manual Edman degradation. Analysis and interpretation of the results indicate that the solitary phosphorylation site identified under our conditions maps to serine 205, a region of the human VDR between the DNA binding and transactivation domains. Hormone induces this site of phosphorylation an average of 4 to 5 fold. Further studies using site-directed mutations of this site are underway to define the structural and/or functional role of hormone modulated phosphorylation observed in the human vitamin D_3 receptor.

K 212 EFFECTS OF PHOSPHORYLATION SITE MUTATIONS ON THE TRANSCRIPTIONAL ACTIVITY OF THE MOUSE GLUCOCORTICOID RECEPTOR, Paul R. Housley and Sheryl A. Mason, Department of Pharmacology, University of South Carolina School of Medicine, Columbia, SC 29208.
The mouse glucocorticoid receptor (GR) contains at least seven sites for modification by phosphorylation. We have constructed vectors containing mouse GR cDNA which express receptors mutated at some or all of the seven identified sites (ser-122, 150, 212, 220, 234, 315, and thr-159). The transactivation potential of mutant receptors has been analyzed by transient transfection into COS cells. Hormone-dependent induction of CAT activity from the MMTV-CAT reporter gene for mutant GR containing mutations in the five conserved phosphorylation sites or in all seven sites was reduced 22% relative to wild-type GR. Several stably transfected cloned cell lines were constructed which express either wild-type or mutant GR. Cells containing equal levels of wild-type or S212A/S220A/S234A mutant receptors exhibit similar levels of hormone-induced CAT activity using MMTV-CAT as the reporter gene. Cells expressing mutant GR altered at the five conserved phosphorylation sites or at all seven sites also exhibit hormone-induced CAT activity. In order to assess the effects of GR phosphorylation on other glucocorticoid-regulated promoters, dexamethasone induction of endogenous glutamine synthetase was examined. For cells containing either wild-type receptors or receptors mutated at all seven phosphorylation sites, dexamethasone treatment elicits a rapid induction of glutamine synthetase mRNA within 2 h. A marked reduction in GR mRNA levels is also observed in cells containing wild-type or mutant GR within 2 h after the addition of hormone. In the continuous presence of dexamethasone, GR mRNA levels recover within 8 h and subsequently decline. This cyclic alteration of GR mRNA levels suggests the possibility that ligand-mediated down-regulation of GR mRNA levels may be coordinated with the cell cycle.

K 213 A GENETIC SCREEN FOR FACTORS THAT MODULATE THE RESPONSE OF GLUCOCORTICOID RECEPTOR TO STEROIDS Natasha Kralli, Sean P. Bohen and Keith R. Yamamoto, Dept. of Biochemistry, University of California, San Francisco, CA 94122

The mammalian glucocorticoid receptor (GR) is functional when expressed in the yeast *S.cerevisiae*, where hormone-dependent enhancement of transcription is conferred to yeast promoters linked to glucocorticoid response elements. Surprisingly, the biological efficacies of certain hormone ligands differ in mammalian and *S.cerevisiae* cells expressing rat GR. Thus, while deacylcortivazol (DAC) and deoxycorticosterone (DOC) activate GR in both species, dexamethasone (dex) and triamcinolone acetonide (TA), strong inducers in mammalian cells, are virtually inactive in *S.cerevisiae*. These findings suggest that other factor(s) interact with the receptor and modulate ligand utilization. Presumably, subtle species-specific differences in this factor(s) yield the distinct patterns of ligand efficacy. To identify such factors, we have screened for yeast mutants that display novel hormone response profiles. We present here the characterization of a yeast mutant, *lem1*, that displays an increased GR response to dex and TA but wild-type GR response to DOC and DAC. Cloning and sequencing of the *wt LEM1* gene suggest that LEM1 is a member of the ABC (ATP-binding cassette) transporter family. Biochemical studies indicate that LEM1 exports specific steroids.

We are currently extending our screen to identify and characterize other genes that can determine hormone response. The existence of non-steroid-receptor factors that can modulate GR activity presents novel steps at which cell-type specific responses to hormone ligands can be regulated in mammalian cells.

K 214 A VARIANT GLUCOCORTICOID RECEPTOR mRNA IS EXPRESSED IN MULTIPLE MYELOMA PATIENTS.
Nancy L. Krett, Shafali Pillay, Pamela A. Moalli, Philip R. Greipp* and Steven T. Rosen, Robert H. Lurie Cancer Center and Department of Medicine, Northwestern University, Chicago, IL 60611 and *Division of Hematology, Mayo Clinic, Rochester, MN 55905.
To investigate the molecular basis of resistance to glucocorticoids, we have compared the glucocorticoid receptor (GR) in hormone sensitive and resistant cell lines derived from a patient with multiple myeloma. In the resistant cell line, we have previously identified a GR transcript with a deletion in the hormone binding domain which arises by alternative splicing. In the current study, we developed an RT-PCR assay to assess whether this aberrant GR transcript is present in the *bone marrow* of multiple myeloma patients. Using the RT-PCR assay, we have successfully identified both the wild type and variant GR transcripts in the original patient isolate which was the source of our myeloma cell lines. This indicates that the variant transcript was present in the patient and did not arise as an artifact of tissue culture. The RT-PCR products were sequenced and their identity as GR transcripts confirmed. In 5 of 5 multiple myeloma patients tested, we detected both the wild type and variant GR transcripts with higher levels of the variant GR transcript being expressed. We also detected both of the transcripts in two normal control samples, however, the level of the variant transcript was much less than that of the wild type receptor. No GR transcript was detected in a tRNA control. Based on these results, we hypothesize that the variant transcript may be present in normal tissues, however, when the expression of the variant transcript increases a resistant phenotype may be expressed.

K 215 FUNCTIONAL ANALYSIS OF ESTROGEN RECEPTOR PHOSPHORYLATION,
Hooshang Lahooti, Sophie Dauvois, Roger White and Malcolm G. Parker, Molecular Endocrinology Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, U.K.
Ligand dependent phosphorylation of the mouse estrogen receptor occurs at multiple serine residues located between residues 121 and 599. Using mutant receptors, we have shown that phosphorylation occurs in stages, as a consequence of ligand binding and following DNA binding. Phosphopeptide maps of the receptor expressed in the presence of estrogen or 4-hydroxytamoxifen are similar, suggesting that the effects of this antiestrogen on transcriptional activity are not mediated by differences in phosphorylation. To investigate the role of phosphorylation in receptor activity we have mapped the phosphorylated residues by over-expressing the receptor in Cos-1 cells in the presence of ^{32}P-orthophosphate and a number of radiolabelled amino acids and then determined the sequence of ^{32}P-labelled tryptic peptides. Three phosphorylated residues have been identified, serine 122 in the N-terminus transcriptional activation region, serine 298 near the nuclear localisation signal and serine 522 in the hormone binding domain near to a region required for estrogen binding and receptor dimerisation. Transient transfection experiments suggest that phosphorylation of serine 122 may be required for optimum transcriptional activation by the hormone binding domain and its cooperation with the N-terminal activation region. Phosphorylation also seems to be important for nuclear uptake and nucleocytoplasmic shuttling of the estrogen receptor since the phosphatase inhibitor, okadaic acid, results in the accumulation of cytoplasmic receptor. We are now investigating the role of serine 298 located near the nuclear localisation signal in this process.

K 216 **PROTEIN-KINASE C ACTIVITY IS REQUIRED FOR RETINOIC ACID-INDUCED TRANSCRIPTION**
Philippe Lefebvre, Ali Tahayato and Pierre Formstecher, CJF INSERM 92-03, Faculté de Médecine de Lille, 1, place de Verdun, 59045 Lille Cédex, France.
The major focus of this study is to explore the coupling of the protein-kinase C (PKC) and retinoids (RA) pathways. Both of these signalling pathways are known to be involved in the control of cell differentiation and proliferation and are generally considered as having opposite effects on theses processes. To decipher the functional involvement of PKC isozymes in the control of RA-induced transcription, we monitored the activity of a transiently transfected, RA-inducible reporter gene in COS cells treated with activators or inhibitors of PKCs. Activation, as well as overexpression of PKCα, did not altered the responsiveness of the reporter gene to RA. On the contrary, inhibition and depletion of cells in PKC significantly down-regulated RA responsiveness of the reporter gene. This effect was specific for RA-inducible reporter genes and could be alleviated by overexpressing PKCα. Retinoic acid receptors (RAR) extracted from cells depleted in PKC have lost their ability to bind DNA and have an altered subcellular localization. The effect of *in-vitro* phosphorylation of RARα by PKC was also investigated and caused a similar decrease in the DNA-binding activity of protein complexes containing RARα.

K 217 **CHANGES IN ESTROGEN RECEPTOR FUNCTION IN A TAMOXIFEN RESISTANT AND ICI 164,384 AND ICI 182,780 SENSITIVE ESTROGEN RECEPTOR POSITIVE HUMAN BREAST CANCER CELL LINE DERIVED FROM MCF-7 CELLS,**
Anne E. Lykkesfeldt and Mogens Madsen, Dept. of Tumor Endocrinology, Danish Cancer Society, Strandboulevarden 49, 2100 Copenhagen, Denmark.
A tamoxifen resistant cell line, MCF-7/TAM^R-1, derived from the human breast cancer cell line MCF-7 has been established after long term treatment with 10^{-6}M tamoxifen. This cell line contains estrogen receptor although the level is lower than in the parent cell line. Estradiol does not induce progesterone receptor synthesis in the MCF-7/TAM^R-1 cell line and tamoxifen has no effect on the synthesis of secreted estrogen regulated proteins in these cells, whereas tamoxifen in the parent cell line stimulates 52 kDa cathepsin D secretion, and inhibits 61 kDa antitrypsin and 66 kDa antichymotrypsin secretion. A protein with mol.wt. 42 kDa, which is down regulated by estradiol and upregulated by tamoxifen in MCF-7 cells does not seem to be regulated by tamoxifen in the MCF-7/TAM^R-1 cell line either. The steroidal antiestrogens ICI 164,384 and ICI 182,780 inhibit growth of the MCF-7/TAM^R-1 cell line and these antiestrogens exert a pure antiestrogenic effect on secreted estrogen regulated proteins both in the parent MCF-7 cells and in the MCF-7/TAM^R-1 cells. Since growth inhibition of parent MCF-7 cells by tamoxifen and by steroidal antiestrogens is associated with stimulation of a 42 kDa protein with presumed growth inhibitory function, we find it very interesting that tamoxifen does neither inhibit growth nor stimulate 42 kDa protein synthesis in the MCF-7/TAM^R-1 cells whereas the steroidal antiestrogen ICI 164,384 inhibits growth and also stimulates 42 kDa protein synthesis in these cells. These results show that tamoxifen resistance is not associated with cross resistance to steroidal antiestrogens, and the observed abnormal tamoxifen regulation of estrogen regulated proteins in MCF-7/TAM^R-1 cells may at least to some extent account for the tamoxifen resistance.

K 218 CHARACTERIZATION OF NUCLEAR TRANSPORT OF THE GLUCOCORTICOID RECEPTOR, Anuradha P. Madan and Donald B. DeFranco, Department of Biological Sciences, University of Pittsburgh, Pittsburgh PA 15260.

We previously used transient heterokaryons to monitor the internuclear migration of the glucocorticoid receptor (GR). Transient heterokaryons were made by fusion of a GR positive cell line and a GR negative cell line in the presence of hormone. The migration of GR from a GR positive to GR negative nucleus, as detected by indirect immunofluorescence, indicates that GR can shuttle between the nuclear and cytoplasmic compartments. Examination of various GR mutants provided an indication of the structural requirements of GR nucleocytoplasmic shuttling. As an example, a constitutively nuclear GR mutant that has the SV40 nuclear localization signal fused at its N-terminus exhibited impaired shuttling when tested in cells not exposed to hormone. Interestingly, this impaired shuttling was overcome in the presence of hormone and the antagonist RU486. A similar block in shuttling was observed with another GR mutant that has the nuclear localization signal of GR (NL-1) translocated to its N-terminus. Thus protein import signals appear to exert some effect on GR nuclear export implying that nuclear import and export may share some common components of the transport machinery.

K 219 CHARACTERIZATION OF THE ANTIESTROGEN RESISTANT HUMAN BREAST CANCER CELL LINE, MCF-7/TAM^R-1 WITH RESPECT TO OCCURRENCE OF VARIANT ESTROGEN RECEPTOR, *Mogens W. Madsen and Anne E. Lykkesfeldt, Department of Tumor Endocrinology, Division for Cancer Biology, The Danish Cancer Society, Strandboulevarden 49, DK-2100 Copenhagen, Denmark.* In breast cancer patients with estrogen receptor positive primary tumor approximately 60% will respond to treatment with the antiestrogen tamoxifen. Almost all patients with advanced disease will develop antiestrogen resistance after a median response time of about 2 years. In order to elucidate the mechanisms for development of antiestrogen resistance in estrogen receptor positive breast cancer cells, a tamoxifen resistant subline (MCF-7/TAM^R-1) of the human breast cancer cell line, MCF-7, has been established by long term treatment with tamoxifen. The MCF-7/TAM^R-1 subline contains estrogen receptor, although the receptor is expressed at a lower level. In contrast to the parental MCF-7 cell line, estradiol does not induce progesterone receptor synthesis in MCF-7/TAM^R-1. It has been hypothesized that antiestrogen resistance in breast cancer cells could be related to the presence of variant ER. Southern analysis did not reveal any difference between the parental cell line and MCF-7/TAM^R-1, and the estrogen receptor mRNA is expressed in both cell line as a 6.5kb transcript. By use of reverse transcriptase and PCR, we have identified 3 variant forms of ER cDNA's in MCF-7/TAM^R-1 lacking either the entire exon 2, exon 4 or exon 7, probably generated by alternative RNA splicing. Quantitative PCR analysis is in progress to elucidate whether the observed estrogen receptor splice variants are associated with the tamoxifen resistant phenotype or whether a similar variant pattern is present in the parental cell line.

K 220 PROMOTER SPECIFIC INHIBITION BY MUTANT FORMS OF RARS, Takashi Matsui and Shunsuke Sashihara, Department of Molecular Biology, University of Occupational and Environmental Health, Japan

A cDNA clone which encodes a mutant form of RAR γ_1, RAR-γ_1^M, has been isolated. RAR-γ_1^M has lost its 65 C-terminal amino acids, thus truncating the RA-binding domain. RAR γ_1^M was expressed in normal cells and tissue, although the level was very low. Since RAR-γ_1^M closely resembles the structure of v-erbA, a truncation form of thyroid hormone receptor, its function was expected to be a potent inhibitor of wild type RAR. To examine this, effects of RAR-γ_1^M on transactivation of RARE promoters by wild type RARs were analyzed in transient expression assays. The results showed that RAR-γ_1^M acts as a negative repressor of wild type RAR. Interestingly, the negative effect of RAR-γ_1^M was shown to be not only promoter specific but also cell type-specific. Taking into consideration that RAR acts as a dimeric protein, it was suggested that cell type-specific proteins interact differentially with three RAR subtypes (α, β and γ) and that each heterodimer activate a distinct class of RARE promoters in a given celltype.

K 221 INTERACTION OF N-TERMINUS AND Zn-FINGER DOMAINS OF THE V-*ERB*A AND C-*ERB*A PROTEINS IN DETERMINING THE DNA RECOGNITION SPECIFICITY, Zeljka Smit-McBride, Chi-Wai Wong and Martin L. Privalsky, Department of Microbiology, University of California at Davis, Davis, CA 95616

The v-*erb*A oncogene is a dominant negative allele of a thyroid hormone receptor gene, c-*erbA*, that differs from its normal cellular progenitor by a set of mutations and deletions. In animal cells the v-*erb*A protein is unable to bind hormone ligand and activate transcription, so it is considered to act in a cancer cell as a constitutive repressor. In yeast, though, it is a hormone inducible transcriptional activator.

We've shown in our previous work that the DNA recognition specificity of the v-*erb*A protein is significantly different from c-*erb*A. Suprisingly, region responsible maps partially to the N-terminal domain, and partially to the Zn-finger domain. Each of these regions contains two amino acids differences between v- and c-*erb*A. We report here a dissection of the effects of differing amino acids at these locations in the v-*erb*A protein, with an emphasis on studies of substitutions at the crucial P-box site.

K 222 RXR AND DIVERSE HETERODIMER PARTNERS ARE INVOLVED IN RA-MEDIATED TRANSCRIPTION IN EMBRYONAL CARCINOMA CELLS Saverio Minucci, Paul Hallenbeck[1], Jeffrey A. Medin, Dina J. Zand, Vera Nikodem[1] and Keiko Ozato, Laboratory of Molecular Growth Regulation, National Institute of Child Health and Human Development and [1]Genetics and Biochemistry Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD20892

The main effectors of Retinoic Acid (RA) action are two classes of RA receptors, termed RARs and RXRs. Recent findings indicate that RXRs form functional heterodimers with RARs and several other members of the steroid and thyroid receptor superfamily. The capacity of RXRs to form heterodimers with various partners may allow for unique cellular responses to many different stimuli.

A series of transient transfection assays was performed in P19 embryonal carcinoma cells to investigate the transcriptional activities mediated by different retinoid response elements. In the case of the RA response element from the RARß gene, a strong, RA-dependent activation of the reporter transcription was observed. This activity was almost entirely mediated by endogenous receptors, as exogenously added RXRs or RARs did not further enhance the levels of activation. In contrast, a construct containing a response element from the CRBPII gene was only weakly activated by endogenous receptors, whereas co-transfection of RXR caused a strong enhancement of the RA-dependent reporter activity. The presence of factor binding to these response elements was investigated by mobility shift assays. Using both the response elements as probes, a comparable, RA-induced binding activity was detected in P19 extracts. The presence of at least one member of the RXR family (RXRß) in these complexes was established using monoclonal antibodies against RXRß. The effect of co-transfected thyroid receptor or peroxisome-proliferator-activated receptor on the RXR-mediated activation of the CRBPII-containing reporter was investigated. Somewhat different results were obtained using NIH3T3 cells in the transient transfection assays, suggesting that additional cell specific factors may play a role in the transcriptional outcome of the interaction of this class of receptors, further emphasizing the combinatorial complexity of the system.

K 223 EVIDENCE FOR DOMINANT NEGATIVE INHIBITION OF PPARα-MEDIATED ERE ACTIVATION BY RXRβ DELETION MUTANTS Susan B. Nunez*, Hansjorg Keller∞, Jeff Medin§, Keiko Ozato§, Walter Wahli∞, James Segars*, *DEB, NICHHD, §LMGR National Institutes of Health, Bethesda, Maryland 20892, ∞Institut de Biologie animale, Universite de Lausanne 1015 Lausanne, Switzerland.

The peroxisome proliferator-activated receptor α (PPAR α) and the retinoid x receptor β (RXRβ) are nuclear hormone receptors which control gene expression through multiple response elements in the presence or absence of their respective ligands, arachidonic acid (AA) and 9-cis retinoic acid (9-cis RA). We have previously reported that estrogen responsive genes could be activated by co-transfection of both RXRβ and PPARα in the absence of the estrogen receptor (ER). To further define the role of the RXRβ in this interaction, domain-deleted constructs of the RXRβ were tested in transient transfection experiments. The deletion constructs used were: Ndel: -21 to 76 amino acid (aa), Ddel: -77 to 138 aa, and Cdel: -401 to 410 aa. Experiments were performed using an A2ERE Tk-luciferase (1µg/well) reporter and 0.5µg/well each of the expression plasmids either alone, or in combination with PPARα. Luciferase activity was determined in the absence or presence of the specific ligands, 9-cis retinoic acid (1µM) or arachindonic acid (50µM). RSV-β galactosidase (0.5µg/well) was used to control for transfection efficiency. Transfection of PPARα alone demonstrated moderate levels (2 fold) of ERE activation. Transfection of full length RXRβ, Ndel-RXRβ, or Ddel-RXRβ alone resulted in low levels of luciferase activity. Co-transfection of Ndel and PPARα showed activation comparable to results observed with full length RXRβ and PPARα (4 fold). In contrast, luciferase activity in the presence of 9-cis RA was observed to decrease by half when Ddel RXRβ was co-transfected with PPARα. Of note, transfection with Cdel-RXRβ showed luciferase activity at baseline levels in the presence or absence of ligand. Cdel co-transfection with PPARα showed no induction of luciferase activity above baseline. Both the Ddel and Cdel constructs inhibited ERE activation by PPARα; however, a dominant negative effect was observed when the Cdel construct was co-transfected with PPARα, since basal activation by PPARα was also abolished.These results strongly suggest the involvement of RXRβ in ERE activation by PPARα. Further, these data suggest a possible role for RXRβ and PPARα in the regulation of estrogen responsive genes.

K 224 HMG17-CHROMATOSOME IS AN ATP/ADP BINDING STRUCTURE SIMILAR TO ACTIN Jouko Oikarinen, Tatu Tarkka, Elina Raatikainen, Sanna Friman and Riitta-Maaria Mannermaa, Department of Medical Biochemistry, University of Oulu, Kajaanintie 52A, SF-90220 Oulu, Finland

We have previously reported that histone H1 is capable binding nucleotides such as ATP/ADP and GTP/GDP in a saturable, Mg^{2+}-dependent manner. The site of nucleotide interaction could be located using labeling with radio-active nucleotide analogs in the H1 globular domain in a ß-hairpin structure similar to the ß/γ phosphate binding loop of protein kinases.

Subsequent sequence comparison of actin and histones H2A, H2B, H3 and H4 suggested that H2A may contain an adenosine binding site, H2B a degenerated phosphate/Ca^{2+} binding site (similar to phosphate 1 of actin) and H3 a nucleotide binding site responsible for discrimination between ribose and deoxyribose. In addition, comparison of actin and HMG17 suggested that HMG17 may contain an additional phosphate/Ca^{2+} binding site (corresponding to phosphate 2 of actin). Based on sequence analysis, the previously identified H1 phosphate binding site may be suggested to correspond to phosphate 1 of actin.

Experimental evidence is put forward here to demonstrate that the reconstituted HMG17-H1-nucleosome binds ATP/ADP in a specific Ca^{2+}/Mg^{2+}-dependent manner, similarly to actin. Upon its binding, Ca/MgATP also induces polymerization of H1 in a head-to-tail manner, becomes hydrolyzed and the MgADP generated is excluded from the H1 polymer. The H3-H4 half-tetramer, H2A-H2B dimer, HMG17 and H1 may thus be suggested to form a bilobular Ca/MgATP/ADP-binding protein comparable to actin. The capability of the chromatosomes to bind Ca/MgATP, and to become consequently polymerized in an H1-dependent manner, may be involved in the organization into inactive solenoid-like chromatin structures in vivo.

We are currently studying interference of steroid receptors, e.g., with the above putative pathway of *H1/nucleosome-mediated* formation of inactive chromatin structures (repression), in order to understand the mechanisms through which they modulate template activity of DNA.

K 225 GENE-SPECIFIC RESPONSES TO OESTRADIOL AND 4OH-TAMOXIFEN IN THE HUMAN ENDOMETRIAL CARCINOMA CELL LINE, *ISHIKAWA* , G.I. Owen~, C. Emmas~, M. G. Parker* and J.O. White~,~Institute of Obstetrics and Gynaecology, Royal PostGraduate Medical School, Hammersmith Hospital, London W12 ONN. *Molecular Endocrinology Laboratory, Imperial Cancer Research Fund, PO Box 123, 44 Lincoln's Inn Fields, London WC2A 3PX

In the human endometrial cancer cell line *Ishikawa*, grown in defined medium, oestradiol and the antioestrogen 4OH-tamoxifen both act as mitogens, stimulating cell growth to similar extents. The ability of 4OH-tamoxifen to act as an agonist in regulating gene expression depended on the target gene and growth conditions. In defined medium oestrogens stimulate the expression of progesterone receptor (PR), c-myc and pS2 whereas 4OH-tamoxifen was capable of stimulating only PR expression.

In charcoal treated foetal calf serum basal expression of c-myc and pS2 was elevated compared with that observed in defined medium and neither estradiol or 4OH-tamoxifen [both 10^{-8}M] stimulated that expression. Since the agonist activity of 4OH-tamoxifen is mediated, at least in part, by TAF-1 it appears that this activation domain is functional on the PR gene promoter but not on that of c-myc or pS2.

We conclude that the response to oestradiol and 4OH-tamoxifen in the *Ishikawa* cell line is gene specific and that potential interactive factors present in serum are capable of attenuating hormone sensitivity.

K 226 ***TRANSIT TO LATE STAGES OF CELL CYCLE IS ESSENTIAL FOR STAGING A DELAYED SECONDARY RESPONSE TO GLUCOCORTICOIDS***

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Previous experiments identified novel steroid hormone responsive elements that act to clock a linked promoter for activation several hours later in a process that is blocked by the inhibitors of protein synthesis. Specifically, the rat α_{2u}-globulin (RUG) secondary GREs (sGREs) act as "delayed" transcriptional enhancers to mediate a secondary response to glucocorticoids in certain stably transfected mammalian cell lines. Further, in response to a hormonal pulse, the RUG sGREs prematurely activate a delayed secondary response and efficiently sustain them. At nucleotide level, the sGREs are nonconcensus glucocorticoid receptor (GR) binding sites containing hexanucleotides related to the GRE-like half-sites. We have proposed that in addition to GR, association/dissociation of certain nonreceptor factors with/from the sGRE are essential for the mediation of a delayed secondary response (Hess, P. and Payvar, F., [1992], *J. Biol. Chem.* **267**, 3490-3497).

The possibility that nonreceptor factors relevant to delayed hormone action might operate more optimally in some cellular contexts than others is explored in the present report. In contrast to the GRE-mediated primary responses, the RUG delayed sGREs are inactive in nonproliferating mammary cell lines. Moreover, the duration of the time lag that precedes the induction is strongly influenced by the time in cycle when the hormone is administered. Remarkably, passage to the late S/G2 stage of the cell cycle is essential for the sGRE activity. The implication of these findings for the regulation of delayed transcriptional enhancement by the sGREs will be discussed.

K 227 **EFFECT OF UNLIGANDED RAR AND UNLIGANDED CHIMERIC RECEPTORS CONTAINING THE DNA-BINDING DOMAIN OF RAR UPON TRANSCRIPTIONAL ACTIVATION FROM AN RARE.** S. M. Pemrick and J. F. Grippo, Dept of Investigative Toxicology, Hoffmann-La Roche, Inc. Nutley NJ 07110.

It is well accepted that the thyroid hormone receptor can evoke a ligand-independent inhibitory effect upon transcription by repressing the basal transcriptional level of target promoters and also by inhibiting ligand mediated RAR-dependent transactivation. Recently, it was shown that mutants of the three RAR isoforms, truncated at the c-terminal end, were converted into potent inhibitors of basal promoter activity and of transactivation mediated by wild type receptors in the presence of ligand (Damm et al (1993)PNAS 90, 2989). We decided to test the hypothesis that repression of transcriptional activity is a general property of unliganded receptors of the nuclear superfamily. Wild type RARα, RXRα, and ER receptors were studied along with chimeric receptors formed by swapping DNA and ligand-binding domains: RAR/VDR, RAR/ER, RXR/VDR, ER-RAR-ER. Among these chimeras, although each bound their appropriate ligand, only RAR/VDR induced ligand mediated transcriptional activation from an RARE. In CV-1 cells, RAR/VDR was a consistent inhibitor of basal promoter activity from the reporter gene, β(RARE)$_3$-tk-luciferase, while no inhibition of basal promotor activity was observed with ER, RXR or RXR/VDR. In the unliganded state, RAR, and chimeras derived from RAR, including RAR/VDR, RAR/ER and ER-RAR-ER, and another chimera RXR/VDR, inhibited ligand-mediated transcriptional activation on RARE. Some of these results with ER chimeras were surprising since there was no detectable binding to the response element and no strong evidence for the formation of heterodimers with RXR. It is clear, that unliganded wild-type RAR receptors, and even chimeras which are poor transcriptional activators on their own, can be negative transcriptional regulators.

K 228 AN ATYPICAL TRANSCRIPTION CONTROL MECHANISM BY THE C-ERBA/T3 RECEPTOR AND THE V-ERBA ONCOPROTEIN OF THE CAII GENE IN CHICKEN ERYTHROCYTIC CELLS.

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We have shown previously that the carbonic anhydrase II (CAII) gene is transcriptionally activated by thyroid hormone T3 in chicken erythrocytic cells and repressed by the v-erbA oncoprotein, a mutated form of the thyroid hormone receptor c-erbA. The CAII gene is up to now the only identified direct target gene of the v-erbA oncoprotein and provides then a helpful model to investigate how v-erbA alters gene expression, in relation to erythroleukemic transformation.

We used an original transient expression assay in normal chicken erythrocytic cells to analyse the action of c-erbA and v-erbA on the CAII promoter. We show that the control of the CAII promoter by c-erbA follows an atypical mechanism. c-erbA does not require RXR as a cofactor and T3 stimulation seems rather to abrogate some transcriptional inhibition. The response to T3 involves cooperation between at least two distant motifs in the promoter. These two motifs also cooperate for the binding of an erythrocytic specific nuclear factor different from c-erbA.

We propose that these two motifs are implicated in the formation of a DNA loop. *In vitro* and *in vivo* analyses will be presented that investigate chromatin structure modifications and DNA bending during T3 induction of the CAII promoter, and repression by v-erbA.

K 229 EXPRESSION OF PML-RAR DOES NOT CONFER FACTOR INDEPENDENCE TO IL-3 DEPENDENT 32D CELLS. R. L. Redner and E. A. Rush, Department of Medicine, University of Pittsburgh, and Pittsburgh Cancer Institute, Pittsburgh, PA 15213

The PML-RAR fusion protein is derived from the t(15;17) reciprocal translocation that introduces the PML gene into the retinoic acid receptor alpha locus. This is a characteristic finding in M3 leukemias, but it is unclear how the PML-RAR protein contributes to the malignant phenotype. One common mechanism for oncogenic transformation of hematopoietic cells is release from dependence on exogenous growth factor control. We sought to test the hypothesis that PML-RAR might induce factor independent growth. We generated an N2 based retrovirus that encodes the PML-RAR cDNA driven by an internal B19 parvovirus promoter. This retrovirus also contains a neomycin resistance gene driven by the Moloney LTR, which allows selection of stable transformants in the neomycin analog G418. A high titer ecotropic retroviral producer cell line was obtained by transfection of the GP&E-86 producer line, and the retroviral supernatant used to infect the interleukin-3 dependent cell line 32D. Four G418 resistant 32D clones were selected by limiting dilution in the presence of Il-3, and individuality of clones confirmed by Southern analysis of retroviral integration sites. These lines expressed high levels of PML-RAR protein as determined by Western blots of whole cell lysates. The PML-RAR transduced 32D clones were examined for Il-3 dependence along with four control clones derived from a population of 32D that had been infected with the base retrovirus not containing the B19-PML-RAR cassette. Cells were washed, and replated in medium with or without Il-3. Cells plated in medium containing Il-3 resumed logarithmic growth, whereas both PML-RAR expressing and control cells plated in medium without Il-3 uniformly died within 24 hours. We conclude that expression of PML-RAR protein does not convert 32D cells to factor independence.

K 230 CHARACTERISATION OF MUTANT RECEPTORS FROM THYROID HORMONE RESISTANCE IN YEAST

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We have identified twenty-four different mutations in the thyroid hormone β receptor (TRβ) in thyroid hormone resistance syndromes (RTH). All the mutations localised to two specific regions within the hormone binding domain such that their ability to bind ligand is impaired. Electrophoretic mobility shift assays using various thyroid response elements (TREs) show that their ability to form homodimers is variably altered whilst heterodimer formation with the retinoid X receptor (RXR) is uniformly preserved. As a result, mutant receptors exhibit impaired transactivation in transient expression assays. We wished to discriminate between the relative contributions of impaired ligand binding versus dimerization to this diminished transactivation potential. Accordingly, we expressed twenty different resistance mutants in *S. cerevisiae*, where the human TRβ has been shown to activate target genes in a ligand-independent manner. We also tested two artificial mutations in the ninth heptad repeat (L421R,L428R) which are known to affect dimerization. Initially, the mutant receptors were tested alone using reporter genes containing palindromic (TREpal) and direct repeat (DR4) TREs. We also assayed mutant receptor function on TREpal in the presence of co-expressed RXRγ. In the absence of ligand some mutants (V264D,P453A, P453T) were as active as wild type receptor, whereas others showed moderately (R338W,Δ432G), or severely (L421R,L428R) diminished constitutive activation. Interestingly, all the mutants exhibited the same pattern of relative activities on both TREs. Co-expression of RXRγ increased the absolute levels of constitutive activity, but again a similar pattern of relative activities was observed. In the presence of 10mM TRIAC significant additional ligand-dependent activation was observed for most mutants. However, two (Δ430M,Δ432G) out of four mutants which do not bind T3 were non-inducible although two (frame shift, G344E) were ligand inducible. Surprisingly, the presence of ligand also restored the activity of one of the artificial ninth heptad (L421R) mutants. We suggest that, for some mutants, decreased constitutive activity in yeast may be a result of impaired dimerization or transactivation properties. This yeast system will enable these functions to be studied in mutant receptors in isolation from variable ligand binding affinities.

K 231 ANALYSIS OF RETINOIC ACID AND THYROID HORMONE ACTIVATED TRANSCRIPTION IN *SACCHAROMYCES CEREVISIAE*

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The absence of endogenous nuclear receptors in the budding yeast *S. cerevisiae*, along with the ability to manipulate this organism genetically, makes *S. cerevisiae* an excellent system for the analysis of ligand-activated transcription by members of the nuclear receptor superfamily. Fusions between the DNA binding domain of the *S. cerevisiae* transcription factor GAL4 and ligand binding domains of retinoic acid, retinoid X, and thyroid hormone receptors function as ligand-dependent transcription factors when introduced into *S. cerevisiae*. Independent functional sub- domains involved in the activation and repression of transcription are being identified. Genetic screens to identify *trans*-acting factors involved in ligand-activated transcription will also be described.

K 232 INHIBITION OF RETINOIC ACID RECEPTOR ACTIVITY BY THE CALCIUM BINDING PROTEIN CALRETICULIN,

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Calreticulin is a calcium binding protein found in a wide variety of eukaryotic cell types. Purified calreticulin binds to an amino acid motif, KXGFFKR, in the cytoplasmic domains of integrin α subunits. A highly related sequence KXFFKR (where X is either G, A, or V) is present in the DNA binding domain of nuclear hormone receptors. Although calreticulin has been localized in the endoplasmic reticulum, it also has a nuclear targeting signal and has been shown to be present in the nucleus and nuclear matrix in some cell types. We provide evidence that calreticulin can inhibit the binding of the retinoic acid receptor/retinoid X receptor (RAR/RXR) heterodimer to its retinoic acid response element (RARE) in a gel mobility shift assay. Binding can be partially restored by addition of a KLGFFKR peptide competitor or an anti-calreticulin antibody. In P19 EC cells, overexpression of calreticulin leads to a decrease in RA-regulated gene expression. P19 cell stable transfectants overexpressing sense (cal.1) or antisense (cal.2) calreticulin cDNA were treated with RA and examined for RA regulation of a RARE-TK-luciferase reporter gene in a transient transfection assay. Cal.1 cell lines display decreased reporter gene activity relative to control P19 cells whereas an increase in luciferase activity is seen in cal.2 cell lines. To demonstrate the physiological relevance of this interaction, the ability of the P19 cell stable sense or antisense calreticulin transformants to differentiate to neuronal cell types upon RA treatment was examined. Overexpression of calreticulin (cal.1) leads to a suppression in RA induced neuronal differentiation as indicated by the dramatically decreased expression of an early neuronal marker, class III ß tubulin. Expression of this marker in the calreticulin underexpressing cell lines (cal.2) was increased relative to control cell lines. The expression of primary retinoic acid responsive genes RARß and CRABPII were examined by Northern blot analysis. In cal.1 cells, the levels of these mRNAs are decreased relative to control P19 cells while transcript levels in cal.2 cell lines are normal or slightly elevated. These results demonstrate that calreticulin antagonizes retinoic acid receptor action and suggest that calreticulin may play a modulatory role in RA mediated cell processes in vivo.

K 233 **Sp1 ANTAGONIZES TRIIODOTHYRONINE (T3) RECEPTOR-MEDIATED REGULATION OF THE HUMAN CHORIONIC SOMATOMAMMOTROPIN PROMOTER**,

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We have studied the roles of Sp1 and GHF1 in the thyroid hormone receptor (TR)-mediated induction of human chorionic somatomammotropin (hCS) promoter activity. We mutated the GHF1, Sp1, or TR binding sites on the hCS promoter, coupled the promoter to the chloramphenicol acetyltransferase (CAT) or luciferase (LUC) genes, and expressed them in deinduced GC cells in the presence and absence of T3. Mutations of the proximal (pGHF1, nts -88/-79), distal (dGHF1, nts -123/-112), and both GHF1 binding sites resulted in minimal (15-30%) reduction in T3 responsiveness. In contrast mutation of the Sp1 binding site (nts -136/-131) resulted in a 2.3-fold increase in T3 responsiveness and overexpression of Sp1 in GC cells with the CMVp.Sp1 expression vector resulted in an 11-fold increase in basal activity and 64% decrease in T3 responsiveness. DNase I footprinting of the hCS promoter with GC nuclear extract (NE) demonstrated that GHF1 binding at the dGHF1 site competes for Sp1 binding at its adjacent site. TR footprinting to the wild-type hCS TRE (TRE_WT; nts -70/-40) was not observed with GC cell extracts or extracts supplemented with *E. coli*-expressed hTRβ. However, up-mutation of the hCS TRE to the perfect direct repeat (TRE_DR4; **AGGTCA**agca**AGGTCA**) increased T3 responsiveness 3.5-fold and exhibited synergistic footprinting of the TRE_DR4 with GC NE and hTRβ. The enhanced TR binding appears to be due to an RXR/TRAP-like activity and not GHF1, since competition with a pGHF1-, TRE_WT-, or TRE_DR4-containing oligonucleotide eliminated only homologous binding site occupancy. Also, T3 increased protection of the TRE_DR with GC NE with or without added hTRβ, a finding consistent with the known ability of T3 to increase TR-RXR/TRAP heterodimer occupancy. These data indicate that alterations in T3 responsiveness that arise from mutations of the GHF1 binding sites may be due to increased Sp1 binding and associated antagonism of TR action, but not due to a requirement for GHF1 in TR-mediated induction of the hCS promoter.

K 234 EXPRESSION CLONING OF NUCLEAR RECEPTORS

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Novel bioactive retinoids and related compounds are continously identified. Their nuclear receptors, however, have exclusively been cloned by homology to previously identified nuclear receptors, and have only subsequently been identified as being specific for a particular compound by binding, transactivation studies and gel shift assays. Hence, it would be desirable to have a technique available, which would allow direct cloning of nuclear receptors, for which the ligands are known.

The powerful expression cloning technique has been employed to clone several cell surface receptors in recent years. The technique depends only on the specific binding properties of the ligand for its cognate receptor. Briefly, overexpression of pools of cDNA libraries in COS cells followed by incubation with radioligand allows detection of specific receptor clones by emulsion autoradiography.

To test the feasibility of COS cell expression cloning, we transfected COS monolayers with the retinoic acid receptor alpha (RARα) in pcDNA-I and did binding studies with tritiated retinoic acid. The results show that individual COS transfectants express in the order of 10^6 RARα, and the signal was readily detectable by emulsion autoradiography.

We will attempt to isolate the receptor for the growth factor 14-hydroxy-*retro*-retinol (14-HRR) by expression cloning, using a cDNA expression library from the 14-HRR-dependent 5/2 human B lymphoma cell line, and tritiated 14-HRR as a probe.

K 235 RECEPTOR BINDING AFFINITY AND T_3 AND 9-*CIS* RETINOIC ACID (9-*cis*RA) RESPONSIVENESS ARE DETERMINED BY THE NUMBER AND ORDER OF STRONG AND WEAK HALF-SITES IN A RESPONSE ELEMENT, Stephen R. Spindler, Walker R. Force, John B. Tillman, Carl N. Sprung, Patricia L. Mote, Department of Biochemistry, University of California, Riverside, CA 92521

T_3 response elements (TREs) consist of pairs of strong and weak (S and W), ten nucleotide, T_3 receptor (TR) monomer binding sites (half-sites). We report that the number and order of S and W half-sites in a direct repeat TRE determines whether it is preferentially bound by TR homodimers, TR-RXR heterodimers, or TR-TR accessory protein (TRAP) heterodimers. These parameters also determine whether a TRE has positive or negative effects on transcription mediated by TR, RXR and their ligands. TR homodimers bound a TRE composed of a 5' S and a 3' W half-site (SW) and one composed of the opposite arrangement, WS, equally well. TR-RXRγ heterodimers bound SW better than WS. TR-TRAP heterodimers bound WS better than SW. WS stimulated transcription in response to unliganded TR and RXR, and either ligand stimulated expression only 2-fold. SW did not respond to unliganded receptors, but T_3 strongly stimulated transcription in the presence of both TR and RXR. SS was highly T_3 responsive in the presence of TR. T_3 activated transcription mediated by SS and SW was inhibited 60% by 9-*cis*RA in the presence of coexpressed TR and RXR. Ligand independent activation of SS by TR and RXR was also inhibited by 9-*cis*RA. In contrast, WS was activated by 9-*cis*RA in the presence of coexpressed TR and RXRγ. Thus, relative half-site strength is a crucial determinant of TR homodimer and heterodimer binding, and T_3 and 9-*cis*RA responsiveness.

K 236 FLUORESCENCE SPECTROSCOPIC STUIDES OF THE THYROID HORMONE RECEPTOR USING A CYSTEINE-SPECIFIC PROBE, Michael R. Tota, Herbert H. Samuels* and Jeffrey H. Toney, Department of Mol. Pharm. Biochem., Merck Research Laboratories, Rahway, NJ, *Department of Medicine, New York University Medical Center, New York, NY

The molecular mechanism of ligand binding by steroid/thyroid hormone receptors is largely unknown. We have shown previously that purified intact cT_3R-α1 adopts distinct conformations whether free or bound to ligand or to DNA using circular dichroism (CD) spectroscopy. We are now extending these studies using fluorescence spectroscopy.

Major unanswered questions of steroid/thyroid hormone receptor activation include understanding the role of thiols in ligand binding, of ligand in DNA binding, of ligand and/or DNA in dimerization, and the orientation of the protein upon binding to DNA response elements. As a first step towards addressing these questions, we have employed a fluorescent probe, 7-diethylamino-3-(4'-maleimidylphenyl)-4-methylcoumarin (CPM). This chromophore is fluorescent only upon covalent linkage to an SH group. cT_3R-α1 is a 408 amino acid protein containing eighteen cysteines of which eight are coordinated to two zinc sites. Upon reaction with an 8-fold molar excess of CPM, cT_3R-α1 exhibits a rapid burst of fluorescence followed by a slow increase. Interestingly, the rapid phase is diminished by ~ 50% when cT_3R-α1 is allowed to bind to a 20-fold molar excess of L-3,3',5-triiodothyronine (L-T_3). One interpretation of this result is that one or more cysteines are protected from reacting with CPM when L-T_3 is docked within the ligand binding site of the receptor. Importantly, cT_3R-α1 upon reaction with CPM exhibits a rapid (<~ 1-2 min.) loss of ~ 65% binding to ^{125}I-L-T_3 relative to receptor alone, followed by a slow loss of ligand binding. This indicates that cT_3R-α1 may contain one (or more) cysteine(s) that is (are) critical for functional ligand binding. Experiments are in progress to map the site(s) of CPM binding.

K 237 RETINOIC ACID RECEPTORS IN HUMAN BREAST CANCER CELLS; INTERACTION WITH THE AP1 TRANSCRIPTION FACTOR. Bart van der Burg, Bas-jan M. van der Leede, Rivka Slager-Davidov, Siegfried W. de Laat, and Paul T. van der Saag. *Hubrecht Laboratory, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands*

Human breast tumors often are hormone-dependent at the time of detection, and regress following a therapy which interferes with estradiol (E2) action. Progression of the disease is associated with a loss of E2-dependence. Retinoic acid (RA) was found to inhibit proliferation of the hormone-dependent human breast tumor cell lines MCF7, T47D and ZR75-1, but not of the hormone-independent cell lines BT20, Hs578T, MDA-MB231 and MDA-MB468. In three of these E2-independent lines the RA insensitivity probably is due to low RA receptor (RAR) activity, which can partly be explained by low RAR expression levels. Hs578T, however, still expresses high levels RARs (in particular RARβ) that are functional with respect to RARE activation, suggesting that a different mechanism underlies its RA resistance.

The transcription factor AP1 binds to the TPA responsive element (TRE) and consists of dimers of members of the *fos* and the *jun* protooncogene families. The formation of this complex is thought to be an important event in the mitogenic cascade and during differentiation of cells, and it has been found that RA can inhibit its activity. We have found that in hormone-dependent breast tumor cells induction of proliferation by mitogens is linked to induction of c-*fos*, c-*jun* and AP1 activity. RA inhibited AP1 activity in transient transfections and gel retardations. AP1 overexpression was shown to overcome RA inhibition of AP1. In all the insensitive cell lines, including Hs578T, AP1 was constitutively active and inhibition by RA was much reduced compared to MCF7 cells. Therefore, a combination of low RA receptor expression and high AP1 expression may lead to RA resistance of human breast cancers.

K 238 ENHANCED NUCLEAR TRANSLOCATION OF THE ECDYSTEROID RECEPTOR (EcR) IN VERTEBRATE CELLS IS MEDIATED BY INTERACTION WITH ULTRASPIRACLE (USP), Martin Vögtli, Markus O. Imhof, Sandro Rusconi*, and Markus Lezzi, Institute for Cell Biology, Swiss Federal Institute of Technology, Zurich, Switzerland, and *Institute for Molecular Biology II, University Zurich-Irchel, Zurich, Switzerland

In general, untransformed nuclear hormone receptors are predominantly confined to either the nucleus or the cytoplasm of a cell. Receptors of the latter class are translocated to the nucleus upon interaction with ligand. At least for some receptors, the observed nuclear translocation might be a direct consequence of the dissociation of heat shock protein 90 from the receptor upon binding to the hormone ligand. Our studies of the use of the ecdysteroid receptor complex from insects, consisting of a heterodimer formed between EcR and USP, as a tool for target gene activation in vertebrate cells have revealed a novel mechanism of nuclear translocation of EcR that does not depend on hormone action. Expression plasmids for N-terminally epitope-tagged or for wildtype EcR and USP were transiently transfected into HeLa or CV-1 cells, either alone or in combination. The subcellular distribution of the individual receptor types was analyzed by indirect immunofluorescence staining using either a monoclonal anti-tag antibody or a polyclonal antiserum raised against a protein domain of EcR. While EcR alone was detected in both cellular compartments, nucleus and cytoplasm, USP was predominantly found in the nucleus of either host cell. Coexpression of both EcR and USP resulted in an efficient translocation of EcR to the nucleus. Thus, USP appears to trap EcR in the nucleus, presumably by forming a heterodimer. On the other hand, USP seemed to migrate from a nucleolus- and nuclear envelope-associated compartment to the chromatin in the presence of EcR. Interestingly, exogenous administration of ecdysteroids in a concentration that in a different experiment gave raise to a hormone-dependent transactivation of a target gene did not influence the subcellular distribution of the analyzed receptor types. Thus, heterodimer formation of EcR and USP not only appears to be required for high affinity binding to cognate hormone response elements (and subsequently for transactivation of an adjacent target gene) and for binding to ecdysteroid but additionally for an enhanced translocation of EcR to the nucleus.

K 239 **RXR INDEPENDENT DOMINANT NEGATIVE FUNCTION BY RAT c-erb-A (TRß$_1$ E1 BUT NOT E3 SUBDOMAIN MUTANTS.** P.G. Walfish*, Y-F Yang*, X. Zhu*, and T.R. Butt*. Samuel Lunenfeld Research Institute of Mount Sinai Hospital, Toronto, Ontario M5G 1X5, Canada*, and Research and Development, Smith, Kline, Beecham, King of Prussia, PA 19403. U.S.A.*

Although the phenomenon of dominant negative functional effects by mutant nuclear receptors has been well established, the precise molecular mechanisms remain to be elucidated. In order to determine whether dominant negative effects can occur in a non-RXR transactivation system, we have utilized the advantages of yeast which is devoid of endogenous TRs, RXRs and RARs to assess transactivating effects when rat E1 compared to E3 subdomain TRß$_1$ mutants are co-expressed with wild-type TRß$_1$ in the presence of a reporter plasmid. Triple transformants of a yeast (S.cerevisiae) strain were performed by adding a YEp56-LEU expression plasmid to our previously described bi-plasmid assays (BBRC 1991;178: 1167). The YEp46-TRP or YEp56-LEU plasmids (controls) and various combinations containing either wild TRß$_1$ or E1 subdomain TRß$_1$ mutants (D300A or Δ 286-305) (which do not heterodimerize with RXRs) or several E3 subdomain mutations (Δ 186-385, C terminal truncations T329, T419 and T429) were studied in the presence of a ß-galactosidase URA3 reporter plasmid containing 2 copies of a palindromic response element (TREp). Compared to the Yep46 and 56 TR$^-$ controls, transcriptional m ± SE unit values in the absence (presence of 1μM T3) of 136 ± 12 (128 ± 17), TRß$_1$ present simultaneously in both YEp46 and YEp56 had responses of 1,245 ± 116 (3,092 ± 295) whereas D300A or Δ 286-305 E1 mutants in both YEp46 and 56 had reduced transcriptional effects at 435 ± 24 (1578 ± 58) and 194 ± 42 (156 ± 27) respectively; while the E3 subdomain mutants: T329 at 466 ± 127, T419 at 444 ± 56 and T429 at 648 ± 42 all had reduced constitutive transcriptional activities with no significant additional responses to T3. When compared to the transcriptional activity of the two TRs in the presence or absence of T3, E1 mutants co-expressed with TRß$_1$ interfered with TRß$_1$ transcription by ≈ 40% (for D300A) and ≈ 70% (for Δ286-305) in either the presence or absence of T3, whereas all the E3 subdomain mutants failed to significantly interfere with TRß$_1$ transcription. **Conclusions: 1)** E1 subdomain mutants retain their capacity to function as dominant negatives within a homodimeric (non-RXR) constitutive transactivation system. **2)** An intact E3 "regulatory zipper" subdomain to facilitate dimerization with wild-type TRß$_1$ is essential for maintaining dominant negative effects in a non-RXR transactivation system.

K 240 UNUSUAL PROPERTIES OF A THYROID HORMONE RESPONSE ELEMENT CONTAINING AN INVERTED PALINDROMIC ARRANGEMENT, A.M. Zavacki, G.R. Williams, J.W. Harney, P.R. Larsen and G.A. Brent, Department of Cellular and Developmental Biology and Thyroid Division, Brigham and Women's Hospital, Harvard Medical School, Boston MA 02115

Thyroid hormone receptor (T3R)-accessory protein heterodimers preferentially bind to thyroid hormone response elements (TREs) with two consensus hexamer sequences (AGGTCA) arranged in a direct repeat with a 4 base pair gap (DR+4). T3R homodimers preferentially bind to TREs containing hexamers in an inverted palindromic arrangement, with a six base pair gap, as found in the chicken lysozyme silencer F2 element (F2). We, therefore, analyzed T3R binding and thyroid hormone (T3) induction of point mutations in the F2 TRE. The F2 element bound homodimers with high affinity, but had low T3 induction compared to other elements that bind homodimers with lower affinity. Mutations in either hexamer that decreased the binding of T3R homodimers correlated with decreased T3 induction. Mutations which changed the spacing between the hexamers differentially influenced homodimer binding and functional potency. Previous studies from our lab have shown that a T3R mutation (P448H) associated with generalized resistance to thyroid hormone (GRTH), loses its dominant negative properties at increased T3 concentrations on DR+4 and palindromic TREs. Unexpectedly, T3 concentrations sufficient to reverse this mutant's dominant negative behavior on these elements were ineffective in relieving inhibition of wild type receptor function on the F2 element. We are currently extending these results by studying the the interactions between mutant and wild type T3Rs on the F2 element. The discordance between strong T3R binding and weak T3 induction demonstrates that the unusual hexamer arrangement places T3R monomers in an unfavorable configuration for maximal T3-dependent transactivation. The differential T3 response of GRTH- associated T3R mutants to the F2 element suggests that this element's unusual properties could influence *in vivo* gene regulation.

Poster Session III

K 300 THE MALIC ENZYME PROMOTER CONTAINS MULTIPLE RECOGNITION SITES FOR NUCLEAR HORMONE RECEPTORS, Myriam Baes, Hilde Castelein, Peter E. Declercq, Department of Clinical Chemistry, Faculty of Pharmacy, Catholic University of Leuven, B-3000, Belgium.

Besides the induction of malic enzyme transcription by T3 through a well characterized TRE, it was demonstrated that peroxisome proliferators strongly induce malic enzyme mRNA levels. To investigate whether this induction was mediated by the PPAR, we looked for potential PPREs in the malic enzyme gene promoter. A computer search was conducted to check for DR1 repeats of AGGTCA related hexamers, a motif present in other peroxisome proliferator regulated genes. Two compatible sequences were found at positions -338/-326 and -461/-449. The -338/-326 element specifically bound PPAR/RXR heterodimers and conferred ciprofibrate responsiveness of a reporter through the TK promoter, while the -461/-449 element was not responsive. The -338/-326 element also displayed affinity for RXR homodimers. In addition, both elements strongly bound COUP-TF and HNF-4. However, preliminary results indicate that these elements are differentially regulated by these orphan receptors.

We conclude that malic enzyme expression in liver is potentially regulated by several hormone receptors and orphans through distinct regulatory elements. Moreover, the presence of a PPRE in the promoter of this lipogenic enzyme suggests a broader function for the PPAR in the regulation of lipid metabolism.

K 301 **Interaction of Members of the Steroid \Thyroid Hormone Receptor Super Family with the Basal Transcription Factor TFIIB**, Aria Baniahmad, Ilho Ha, Danny Reinberg, Sophia Y. Tsai, Ming-Jer Tsai and Bert W. O'Malley

Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030, and Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, New Jersey 08854

The mechanism of transcriptional regulation by steroid hormone receptors is largely unknown. Recent work shows that the binding of TFIIB is one of the rate limiting steps of transcriptional activation. Recombinant TFIIB has been shown to directly interact with the acidic activation domain of the herpesvirus VP16 and the adenovirus E1a proteins, suggesting that TFIIB is a target of transcriptional activation. In our laboratory, we have demonstrated that the orphan receptor COUP-TFI requires a cofactor for gene activation, which was shown to be TFIIB. Further studies revealed that COUP-TFI directly interacts with TFIIB.

Now, we have extended these studies in more detail to the human thyroid hormone receptor (hTRβ). We provide evidence that the C-terminal domain of the receptor functions as both an active silencer and a hormone dependent activator on different minimal promoters. This suggests that the transcriptional machinery is not only the target for transcriptional activation but also for transcriptional silencing. In addition, we show that hTRβ interact specifically with human TFIIB. Two domains of hTRβ interact with TFIIB. One domain is localized in the N-terminus and the other in the ligand binding domain (LBD). The interaction of the LBD is changed upon addition of thyroid hormone. Further studies identified functional subdomains within the LBD which interact with TFIIB. These data imply that TFIIB is one of the targets for transcriptional regulation for members of the steroid hormone receptor superfamily.

K 302 SPECIFIC BINDING TO VITAMIN D RESPONSE ELEMENTS OF CHICKEN INTESTINAL DNA BINDING ACTIVITY IS NOT RELATED TO THE VITAMIN D RECEPTOR, Renata Battini, Stefano Ferrari and Susanna Molinari, Istituto di Chimica Biologica, Universitâ di Modena, 41100 Modena, Italy.

In this report we confirm that the putative vitamin D response element (VDRE), located between -320 and -306 in the chicken calbindin-D_{28K} gene, is not a binding site for the vitamin D_3 receptor (VDR). In examining the ability of chicken intestinal nuclear extracts (CINE) to bind known VDREs, we observed a specific VDRE-binding activity, which is distinct from VDR. In fact, VDR-depleted CINE retains the ability to bind the rat osteocalcin VDRE. The VDRE-binding activity binds DNA with high affinity and contacts it at the same guanine residues as VDR. Its specificity in binding structural variants of the AGGTCA repeat is broader than VDR, since direct repeats spaced by 3, 4 and 5 base pairs are almost equally effective competitors when added to the probe in molar excess. Palindromic arrangements of the same motif are lower affinity competitors. The retinoid X receptor (RXR) is involved in the binding complex, since incubation of CINE with antibody to RXR results in a quantitative supershift. Antibodies to retinoic acid receptors (RARα and -β), T3 receptor (TRα) or chicken ovalbumin upstream promoter-transcription factor (COUP-TF) had no apparent effect. These data suggest that species specificity is a relevant aspect of VDR/VDRE recognition, and that a novel factor (or factors), different from VDR, might be involved in the effect of vitamin D on gene expression.

K 303 IDENTIFICATION OF RETINOIC ACID AND GLUCOCORTICOID RESPONSE ELEMENTS IN THE PROMOTER REGION OF RC3, A BRAIN-SPECIFIC GENE REGULATED IN VIVO BY THYROID HORMONE. J. Bernal, M.A. Iñiguez, B, Morte, M. Aguilera, A. Muñoz, and A. Rodriguez-Peña. Instituto Investigaciones Biomédicas, CSIC, 28029 Madrid, Spain.

Despite the important role of thyroid hormone on brain development, and the presence of T3 receptor isoforms in many brain regions, only a few genes have been shown to date to be influenced by thyroid hormone at the pretranslational level. One of these genes is RC3/neurogranin. RC3 encodes a calmodulin-binding, kinase C substrate present in the dendritic spines of discrete neuronal populations of the forebrain. Its physiological role could be related to synaptic plasticity, memory, and other processes. Neonatal, as well as adult onset hypothyroidism induced a decreased RC3 mRNA and protein in the cerebral cortex and striatum, which was reversed by T4 treatment. To define the role of thyroid hormone in the regulation of RC3 expression at the molecular level we have isolated and sequenced 2.4 kbp of genomic DNA 5' from the origin of transcription. The major features of the RC3 promoter are the absence of TATA and CAAT boxes and the presence of an initiator (Inr) sequence surrounding the cap site. By sequence analysis we identified response elements for retinoic acid (RARE) and steroid hormones (GRE). An oligonucleotide containing the RARE was able to specifically bind vaccinia-expressed RARs in vitro and to confer RA regulation to a heterologous promoter after transfection in cos-7 or neuroblastoma cells. In addition, these elements conferred regulation by RA or glucocorticoid hormones in the context of the homologous promoter, whereas T3 had no effect on RC3 promoter activity. When administered together with cotransfected RAR and GR, RA and dexamethasone had additive effects.

In conclusion, no T3RE was found within the 2.4 kbp flanking region of the RC3 gene and therefore the mode of action of T3 in vivo remains unexplained. T3 could act directly through a T3RE located in other regions of the gene still not analyzed. On the other hand, the presence of RARE and GRE suggests that T3 action could be indirect through one or both of these elements.

Supported by grants from DGICYT PM92-0025 and Fundacion Salud 2000 (Serono)

K 304 TISSUE SPECIFIC REGULATION OF FOS AND JUN BY ESTROGEN AND PROGESTERONE IN RAT UTERUS. Robert M.Bigsby and Li Aixin. Dept. of Obstetrics and Gynecology, Indiana University School of Medicine, Indianapolis, IN 46202

Uterine epithelial cell proliferation and differentiation may be related to steroidal control of c-*fos* and c-*jun* expression. This was examined by analyzing hormonal effects on mRNA levels of c-*fos*, c-*jun*, and *jun*-B in extracts made either directly from the uterine epithelium or from the uterus as a whole. Tissues were harvested from immature rats at 3 h after estradiol (E); progesterone (P) was administered 1/2 h prior to E. RNA was extracted from the luminal epithelium of one uterine horn by perfusion with a lysis/denaturation solution and from the entire contralateral horn. When whole-uterus RNA was analyzed, E increased mRNA levels for c-*fos*, c-*jun* and *jun*-B and P pretreatment reduced the E-induced increase in c-*fos* and c-*jun*. In contrast, when epithelial RNA was analyzed, E increased c-*fos* and *jun*-B but it repressed c-*jun* mRNA levels. P did not block E-induced increases in c-*fos* or *jun*-B mRNA levels in the epithelium while it did override the repressive effect of E on epithelial c-*jun*. Estrogen repression of epithelial c-*jun* expression was dose dependent, hormone specific and occurred with a half-life of 50 min., similar to the half-life of c-*jun* mRNA measured in cultured uterine cells. Thus, in the uterine epithelium, E regulates c-*fos* and c-*jun* in opposite directions, thereby causing a dramatic increase in the Fos:Jun ratio; at the same time E increases expression of *jun*-B. These changes make it likely that under E domination the predominate form of AP-1 protein in the epithelium would be a Fos:Jun-B dimer. P blockade of E-repressed c-*jun* expression allows for production of Fos:Jun, Fos:Jun-B, Jun:Jun and Jun:Jun-B dimers. Such changes would affect cell signalling mechanisms working through AP-1 and CREB. (supported by NIH HD23244)

K 305 LOSS OF RAR γ FUNCTION BY GENE DISRUPTION RESULTS IN ABERRANT HOXa-1 EXPRESSION AND DEFECTIVE CELL DIFFERENTIATION. John F. Boylan*, David Lohnes#, Reshma Taneja#, Pierre Chambon#, Lorraine J. Gudas* *Dept. of Pharmacology, Cornell University Medical College, NY, NY. #Laboratoire de Genetique Moleculaire des Eucaryotes du CNRS, Unite 184 de Biologie Moleculaire et de Genie Genetique de l'INSERM, Strasbourg, France.

Retinoic acid (RA) signal transduction is mediated through several high affinity nuclear receptors which are members of the steroid/thyroid/Vitamin D superfamily and function as transcription factors. Why multiple retinoic acid receptors exist and what gene targets are regulated by one or more of the three receptors remain compelling questions in developmental biology. Through genetic disruption of both RAR γ alleles, we have identified several of the differentiation specific genes that are regulated either directly or indirectly by RAR γ in embryonic cells. For instance, genes such as Hoxa-1 (Hox 1.6) and the extracellular matrix genes laminin B1 and collagen type IV (α1) that are RA inducible in wild type F9 teratocarcinoma stem cells are not significantly induced in the RAR γ -/- lines. In contrast, genes such as Hoxb-1 (Hox 2.9) and CRABP-II are activated by RA for a *longer* period of time in the RAR γ -/- lines compared to the wild type F9 line. Not all genes are aberrantly expressed; REX-1, RAR β and SPARC are regulated in the RAR γ -/- lines as they are in F9 wild type cells. Our results support the idea of functional differences among the three retinoic acid receptors, allowing for the complex regulation of developmental processes by retinoids.

K 306 TRANSACTIVATION BY THE PEROXISOME PROLIFERATOR ACTIVATED RECEPTOR IN YEAST REQUIRES COOPERATIVITY WITH RETINOID X RECEPTORs, John P. Capone, Sandra L. Marcus, Kenji S. Miyata, Baowei Zhang, and Richard A. Rachubinski, Department of Biochemistry, McMaster University, Hamilton, ON L8N 3Z5, CANADA

Cellular responses to peroxisome proliferators are mediated by peroxisome proliferator activated receptors (PPARs) that interact with *cis*-acting PPAR response elements (PPREs) through co-operativity with retinoid X receptors (RXRs). To investigate the mechanisms of action of PPARs, we have expressed cDNAs encoding full length rat PPAR and human RXRα in the yeast *Saccharomyces cerevisiae*. Neither receptor alone was able to transactivate expression of a ß-galactosidase reporter gene containing the PPRE from the rat hydratase-dehydrogenase gene. However, co-expression of rPPAR and RXRα resulted in transactivation, demonstrating that PPARs function in yeast and require cooperativity with RXRs. We have also exploited the yeast di-hybrid system to investigate protein-protein interaction between PPAR and RXRα. Expression of rPPAR or RXRα alone fused to the DNA binding domain of GAL4 was not sufficient to transactivate expression of a *GAL10*-ß-galactosidase reporter gene, indicating that neither of these receptors possesses an autonomous transactivation domain that functions in yeast. However, co-expression of rPPAR fused to the GAL4 DNA binding domain with RXRα fused to the GAL4 acidic activation domain resulted in activation of the reporter gene. Thus, rPPAR and RXRα heteromerize *in vivo* in yeast and this interaction is necessary for transactivation. Our findings demonstrate that at least part of the PPAR-dependent signaling pathway can be reconstituted in yeast. These strategies will allow us to identify other putative PPAR-interacting cellular factors and to study their roles in peroxisome proliferator-mediated signaling. Supported by the Heart and Stroke Foundation of Ontario.

K 307 ESTROGEN AND RETINOID RECEPTOR EXPRESSION IN NORMAL AND PRENEOPLASTIC MOUSE CERVICAL EPITHELIA. Celli, G.B., Darwiche, N. and De Luca, L.M. Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute, Bethesda, MD 20892.

Steroid hormones and retinoids are involved in the maintenance of normal differentiation and function of the cervical and vaginal epithelia. It is known that estrogen and retinoic acid (RA) exert antagonistic actions: estrogen induces keratinization of the stratified squamous ectocervical epithelium, whereas RA is essential for the maintenance of the mucus-secreting columnar epithelium of the endocervix. The two epithelia join at the squamo-columnar junction. Squamous metaplasia is considered a preneoplastic lesion and may be induced in the simple columnar epithelium by retinoid depletion. Our in situ hybridization studies show that the simple columnar epithelium expresses elevated levels of RARβ and RXRα, but fails to express the estrogen receptor (ER) transcript. Conversely, the stratified squamous epithelium expresses ER and RXRα, but not RARβ transcripts. During vitamin A deficiency, RARβ and RXRα continue to be expressed in the basal cells of squamous metaplastic foci, but are absent in suprabasal cells: this demonstrates that basal cells of squamous foci differ from those of the normal stratified epithelium and are similar to columnar cells in retinoid receptor expression. ER is highly expressed in suprabasal cells of these foci and less prominent in the basal layer. Since the squamo-columnar junction also defines the area in the cervix most subject to neoplastic transformation, our aim is to understand how receptor expression is regulated by estrogen and retinoid status, and how it may contribute to carcinogenesis and/or its prevention.

K 308 IDENTIFICATION OF cAMP RESPONSE ELEMENT AND ESSENTIAL *Cis*-ACTING ELEMENT IN HUMAN ANDROGEN RECEPTOR GENE PROMOTER, **Chang C**, Mizokami A, Yeh SY, Department of Human Oncology, University of Wisconsin Comprehensive Cancer Center, Madison, WI 53792.
Androgen and androgen receptor (AR) play an important role in sexual differentiation and prostate proliferation. To investigate AR gene transcriptional regulation, a 2.3 kb AR gene promoter region was isolated, sequenced and characterized. CAT assay and sequence homology search of AR gene promoter among human, rat, and mouse revealed some potential *cis*-acting elements including a GC box, a suppressor region and a purine-rich element. Deletion analysis and gel retardation assay using a 50 bp double-strand purine-rich element showed that this purine-rich element can bind to specific proteins in nuclear extract of LNCaP and HeLa cells and may be essential for AR gene transcription. Furthermore, to investigate the effect of cyclic AMP (cAMP) on AR gene transcription, we treated LNCaP and HeLa cells with 10 mM dibutyryl cAMP (dbcAMP) after transfection with CAT gene reporter plasmids linked to the AR gene promoter. This treatment induced several folds of CAT activity in LNCaP cells only and the induction was further confirmed at AR mRNA level by Northern blot analysis and RT-PCR assay. Deletion analysis of the AR gene promoter showed that a region between 530 bp and 380 bp upstream of AR gene transcription initiation site, which includes one potential CRE, is responsible for cAMP induction. Gel retardation analysis using this CRE (AR/CRE1) showed that AR/CRE1 can bind to specific proteins in nuclear extract of LNCaP cells which appear to form different binding complex as compared to somatostatin/CRE.

K 309 ESTROGEN REGULATION OF EGF RECEPTOR mRNA IN HUMAN BREAST CANCER CELLS, Susan A. Chrysogelos and Ronit I. Yarden, Lombardi Cancer Center, and the Department of Biochemistry and Molecular Biology, Georgetown University, Washington, DC 20007
Epidermal growth factor receptor (EGFR) expression inversely correlates with expression of estrogen receptor (ER) in breast cancer, and it is known that in an animal model (rat uterus) estrogen can transiently induce EGFR gene expression. Additionally, estrogen has been shown to downregulate expression of its own receptor in breast cancer cells, and many substances that regulate ER have been found to have the opposite effect on EGFR. In order to understand the mechanisms by which these two receptors maintain their inverse correlation, our first approach was to determine the time course of EGFR regulation by estrogen in the ER positive breast cancer cell lines MCF-7, T47D, and BT474. Total cellular RNA was isolated from cells at different time points following treatment with estradiol, and analyzed by an RNase protection assay. Quantitation of EGFR mRNA relative to an internal control, ribosomal protein 36B4, showed induction of EGFR within 30 minutes of treatment. Maximal induction of 2-3 fold was seen after 2 hours, and by 5 hours EGFR mRNA returned to the basal level. This short term effect suggests that control of EGFR induction by estrogen may be at the transcriptional level. Studies with cycloheximide confirmed that induction of EGFR by estrogen is direct and independent of protein synthesis. However, the continued increase in EGFR levels in the presence of cycloheximide (a 5 fold induction at 24 hours) may indicate involvement of an estrogen-induced repressor or nuclease as a mechanism to maintain low steady state levels of EGFR in estrogen dependent breast cancer cells. Sequencing of the EGFR promoter revealed no concensus estrogen response element. However, 3 imperfect palindromes were identified in the region between -535 and -147 relative to the major transcription start site. Currently, studies are in progress to determine if these sequences are capable of mediating estrogen induction of EGFR.

K 310 CHARACTERIZATION OF AN ANDROGEN-RESPONSIVE UNIT IN THE C3(1) GENE, F. Claessens, L. Celis, A. Devos, B.Peeters, P.De Vos, W.Heyns, G.Verhoeven and W.Rombauts Afdeling Biochemie, Katholieke Universiteit Leuven, Herestraat 49, B-3000 Leuven, BELGIUM.

The genes that code for the components C1, C2 and C3 of prostatic binding protein (PBP) are expressed under androgen control in rat ventral prostate. DNA-cellulose competition assays have demonstrated that the androgen receptor (AR) binds with high affinity immediately upstream and in the first intron of the PBP genes. Gene transfer experiments resulted in the description of an androgen response element in the first intron of the C3 genes. However, the other AR-binding gene fragments were unable to confer androgen responsiveness to a heterologous promoter.

The DNA-binding domain of the AR (AR-DBD) was expressed in *E. coli* as part of a fusion protein with protein A. By DNase I footprinting, this protein was shown to bind specific sequences in the intron of the C1 and C3 gene. Oligonucleotides containing AR-binding sequences were synthesized, and their relative affinity for the AR-DBD was compared with that of the AREs described in the promoter of the prostate-specific antigen (PSA) gene and the human glandular kallikrein (hGK) gene (Riegman *et al.* (1991) Mol. Endocr. 5, 1921-1930).

In gel retardation experiments the AR-DBD displays a 100-fold higher affinity for the AREs of *C3*, *PSA* and *hGK*, than for the other AR-binding sequences. The sequences of the AREs are very similar to the GRE consensus, and in gel retardations their relative affinities for the DNA-binding domain of the glucocorticoid receptor are identical to those for the AR-DBD. Therefore, the steroid specificity of the *in vivo* response cannot be explained by differential sequence preference of the receptors.

Several sequences near the AR-binding elements are recognized by components of nuclear extracts of rat prostate. Mutation of two such binding sites attenuate the androgen response. A third binding site is protected by a prostate-specific factor. In summary, we have demonstrated that the *C3* ARE is embedded in a complex androgen responsive unit.

K 311 IDENTIFICATION OF A NEGATIVE ELEMENT THAT MODIFIES THE ACTIVITY OF A GLUCOCORTICOID MODULATORY ELEMENT (GME), Clayton D. Collier, Hisaji Oshima and S. Stoney Simons, Jr., Steroid Hormones Section, LMCB/NIDDK, NIH, Bethesda, MD 20892
A 21 bp sequence, located 3.6 kb upstream from the transcriptional start site of the glucocorticoid inducible tyrosine aminotransferase (TAT) gene, has been identified as a glucocorticoid modulatory element (GME). One function of the GME and its associated *trans*-acting factor is to increase the activity exhibited by antiglucocorticoids, expressed as percent of maximal induction demonstrated by agonists. This increased activity was not seen initially in Fu5-5 rat liver cells transiently transfected with a hybrid construct containing 927 bps of TAT DNA (-3922 to-3050), including the GME, upstream of the chloramphenicol acetyl transferase gene under the control of a tandem glucocorticoid response element and the thymidine kinase promoter. However, deletion of a 291 bp segment of the TAT DNA caused an increase in the percent agonist activity of antiglucocorticoids. This result indicates that the deleted sequence negates GME activity. A 56 bp element was identified that shows almost full negation of GME activity. Four overlapping oligonucleotides spanning the 56 bp region were prepared and assayed. No one oligonucleotide sequence completely negated the GME activity, thus suggesting the presence of multiple elements within this 56 bp region. Co-transfection of a plasmid containing 3 tandem repeats of the 56 bp negative element partially reversed the negation of the GME by a *cis*-fused 56 bp element. This argues that the activity of the 56 bp element requires a *trans*-acting factor(s). Collectively, these results indicate that the modulation of glucocorticoid regulated TAT gene expression by the GME is further controlled, in part, by a negative element.

K 312 IDENTIFICATION OF A TRANSCRIPTIONAL SUPPRESSOR DOMAIN REQUIRED FOR ONCOGENIC ACTIVITY OF V-ERBA. Klaus Damm* and Ronald M. Evans#, *Dept. of Neuroendocrinology, Max-Planck-Institute of Psychiatry, 80804 Munich, Germany and #The Salk Institute, La Jolla, CA92037.

A novel property of the unliganded thyroid hormone receptor (TR) is its ability to suppress the basal transcription level of promoters containing appropriate binding sites. The biological significance of the suppressor function is manifested by the v-erbA oncogene product, a mutated version of the chicken TRα, which acts as a constitutive suppressor because it fails to bind hormone and does not transactivate. When coexpressed with the TR or the retinoic acid receptor, v-erbA will virtually eliminate an otherwise potent transcriptional response to the respective ligand. The mechanisms underlying v-erbA induced erythroblast transformation were determined via the analysis of two AEV derivatives. The first, AEV^{td359}, is a transformation defective mutant and the second is its revertant, AEV^{r12}, obtained after in vivo passage of AEV^{td359}. We show that the most striking difference between v-$erbA^{wt}$ and v-$erbA^{td}$ is the complete loss of v-$erbA^{td}$ to act as a suppressor of the basal transcription level. In addition, the antagonistic properties of the v-$erbA^{td}$ protein are severely impaired while the revertant v-$erbA^{r12}$ exhibits a similar or even higher activity than v-$erbA^{wt}$ in antagonizing the hormone response on palindromic and direct repeat response elements. The inactivating mutation is a single nucleotide change leading to a Pro to Arg replacement in v-$erbA^{td}$. Introducing this mutation in the context of TRα selectively inactivates the suppressor function while hormone binding or transcriptional activation properties are not affected. The mutation is located in the region linking the DNA and hormone binding domains, a region where computer algorythms predict two alpha-helical structures with the Pro serving as a potential helix breaking residue. The amino acid sequence and predicted secondary structure is conserved in all TR and also the closely related retinoic acid receptor isoforms, suggesting that the structural integrity of this *transcriptional suppressor domain* is important for the transcriptional regulatory functions of the receptors.

K 313 ***IN VIVO*** **FOOTPRINTING DEMONSTRATES THAT STEROID HORMONES ALTER THE BINDING OF A LABILE PROTEIN TO THE CHICKEN OVALBUMIN GENE. Diane M. Dean and Michel M. Sanders, Department of Biochemistry, University of Minnesota, Minneapolis, MN 55455**

The induction of the chicken ovalbumin gene by steroid hormones is abolished by inhibitors of protein synthesis such as cycloheximide, implying that a labile protein(s) is required. Because the effects of steroids map to the steroid-dependent regulatory element (SDRE; -892 to -780), this region was examined for its ability to bind labile proteins. Oligomers homologous to the SDRE and nuclear extracts from laying hens treated with cycloheximide were used in gel mobility shift analyses. The most 5' oligomer (-900 to -876) demonstrated binding of a labile protein complex. However, the binding of this protein(s) *in vitro* was not dependent on steroid treatment. *In vivo* footprinting was undertaken to ascertain whether the binding of this protein is affected by steroids *in situ*. Primary oviduct cells were treated with cycloheximide following culture in the presence or absence of estrogen and corticosterone. Dimethylsulfate was used to methylate the DNA *in situ* followed by isolation, chemical cleavage, and ligation-mediated PCR footprinting. This revealed two steroid-dependent alterations, a protection of guanidine residue -885, and a hypersensitivity of adenine residue -892. Treatment with cycloheximide abolished this *in vivo* footprint. These data demonstrate that a critical labile protein binds to the 5'-end of the SDRE, facilitating the induction of the ovalbumin gene by steroids. Thus, these results support the contention that steroid hormones regulate the ovalbumin gene via a transcriptional cascade.

K 314 ANALYSIS OF THE TRANSCRIPTION FACTORS THAT CO-OPERATIVELY INTERACT WITH THE RETINOID X RECEPTOR GAMMA DURING MYOGENEIS Dennis H. Dowhan and George E.O. Muscat. University of Queensland, Centre for Molecular Biology & Biotechnology, St Lucia, 4072 Qld, Australia.

The retinoid X receptors (RXR) heterodimerize with the vitamin D, thyroid hormone and retinoic acid receptors and function to selectively target the high affinity binding of these receptors to their cognate elements. These complexes interact with direct repeats (DR) of the 5'PuGGTCA sequence motif with spacings of 3,4 and 5 nucleotides, respectively. The retinoid X receptor family (α-,β- and γ-) can also form homodimers that bind to a number of retinoid X response elements. RXR γ is preferentially expressed in cardiac and skeletal muscle. We utilized a binding site selection assay with purified RXR γ bound to glutathione-agarose to derive the optimal binding site. The consensus sequence recovered by this protocal was the half site motif 5'AN(A/C)AAAGGTCA 3', These sequence motifs were subsequently shown to form slower migrating complexes in electro mobility shift assays[EMSA] in the presence of myogenic nuclear extracts. Oligonucleotides using the consensus sequences recovered, flanked on the 5' and 3' ends by 15 degenerate bases are currently being utilised in binding site selection assays in the presence of RXRγ and myogenic nuclear extracts to identify nuclear proteins that co-operatively interact with the RXRs in muscle.

K 315 THE HUMAN ANDROGEN RECEPTOR GENE PROMOTER, Peter W. Faber, Henri C.J. van Rooij, Helma J. Schipper, Albert O. Brinkmann and Jan Trapman, Departments of Pathology and Endocrinology and Reproduction, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.

The androgen receptor (AR) functions in the program that determines male sexual differentiation. Expression of AR is low in most tissues but elevated in tissues of the male sex organs. To characterize the elements involved in regulated AR expression we have structurally and functionally characterized the TATA-less human AR (hAR) gene promoter.

In order to define the hAR gene promoter sequences involved in utilization of the two major transcription initiation sites, AR-TIS I and AR-TIS II, COS cells were transiently transfected with hAR promoter constructs. Subsequently, RNA was isolated from the transfected cells and S1-nuclease protection assays were performed. This analysis showed that two overlapping pathways of transcription initiation are present on the hAR gene promoter. The Sp1 binding site (-45/-37) is responsible for AR-TIS II usage whereas the sequences involved in AR-TIS I usage are between position -5 and +57. Protein-DNA interactions in these regions were identified by bandshift analysis and the involvement of these proteins in transcription initiation was verified through specific mutation analysis.

To identify potential transcriptional enhancer sequences involved in AR expression DNAseI hypersensitive sites were mapped up to 30 kb upstream from the hAR gene promoter using nuclei from LNCaP prostate tumor cells. Two regions were identified this way, one at the hAR gene promoter and one in a region located far upstream from the promoter. The functionality of both regions was tested in transient transfection assays and these results will be presented.

K 316 TRANSCRIPTIONAL REGULATION OF THE HAGEMAN FACTOR XII GENE BY ESTROGENS. Antonella Farsetti*^, Silvia Misiti*°, Franca Citarella #, Angelina Felici#, Mario Andreoli°, Antonio Fantoni# & Alfredo Pontecorvi*§, *Molecular Oncogenesis Lab., CRS-IRE; °Inst. of Experimental Medicine, CNR, #Dept. of Human Biopathology and Dept. of Experimental Medicine, Univ. "La Sapienza" and §Inst. of Medical Pathology, Catholic University, Rome, Italy.

Oral administration of synthetic estrogens has been associated with an increased risk of hemostatic and thromboembolic alterations as well as with a raise in plasma levels of blood coagulation factors VII, IX and XII. Aim of this study has been to investigate the molecular mechanism by which estrogens regulate Hageman factor XII (FXII) gene expression. To investigate FXII gene transcription run-on assays were performed with isolated nuclei from liver of ovariectomized rats treated or not with β-estradiol. The relative rate of FXII gene transcription was 5-fold higher in rats treated with β-estradiol than in controls, indicating that estrogens regulate FXII gene expression *in vivo* at the transcriptional level. The molecular mechanism of this effect was investigated by studying *in vitro* estrogen action on the cloned FXII gene promoter. Chimeric genes containing the chloramphenicol acetyl transferase (CAT) coding sequence and three FXII genomic fragments with 5' endpoint at -3076, -1287 and -181 (PT-CAT) were used in a NIH3T3 transient expression system. The reporter plasmids were co-transfected with or without an expression vector for the human estrogen receptor (Heo, gift of P. Chambon) and cells cultured in the presence or absence of β-estradiol. Transfection of PT-CAT-181 resulted in a marked increase of CAT expression (≥ 10-fold) in the presence of β-estradiol (10^{-7}M), while a progressive decrease of estrogen induction appeared when longer 5' promoter regions were used, with the PT-CAT-3076 showing the minimal estrogen inducibility (~ 2-fold). Comparison of the nucleotide sequence of the FXII promoter with that of the vitellogenin gene showed the half-site motif TGACC which has been identified as crucial for estrogen receptor function.

In conclusion, our findings represent the first demonstration, at the molecular level, of estrogen modulation of the expression of a coagulation factor gene and the first evidence for the presence, in the promoter region of this class of genes, of a functional estrogen response element.

K 317 LIGAND RESPONSE PROFILES OF NUCLEAR RECEPTORS ARE MODULATED BY DNA AND RETINOID X RECEPTOR, Barry M. Forman, Thomas Perlmann and Ronald M. Evans, Gene Expression Laboratory, The Salk Institute, La Jolla, CA 92037

The Retinoid X Receptor (RXR) is a common dimerization partner for several mammalian receptors. Although dimerization increases the affinity and specificity of DNA binding, the dimerization interface that drives this reaction is embedded within the ligand binding domain. This prompted us to examine the effect of RXR dimerization on the conformation and ligand responsiveness of nuclear receptors. RXR was found to promote heterodimerization of receptors on response elements organized as direct, inverted and everted repeats. The dimerization interface required for this interaction was mapped to a small region at the C-terminus and found to promote heterodimer formation on all three types of response elements. Since the same DNA binding domain and dimerization interface is active on all three types of response elements, the ligand binding domain is likely to assume different conformations when bound to DNA half-sites that are oriented as direct, inverted and everted repeats. Indeed, our results indicate that receptor-RXR complexes bind to divergent response elements with distinct conformations. Furhtermore, these conformation differences impart differential ligand responsiveness to mammalian receptors.

K 318 HORMONAL REGULATION OF STROMELYSIN EXPRESSION, Mireille Gaire and Lynn Matrisian, Dept of Cell Biology, School of Medicine, Vanderbilt University, Nashville, TN 37232.

The endometrium is a dynamic tissue, consisting of epithelial glands and connective tissue stroma that undergoes monthly cycles in response to the fluctuation of the ovarian steroid hormone levels. The action of these hormones appears to be mediated in part by some of the locally synthesized peptide growth factors (EGF, TGF-α, TGF-β and insulin-like growth factors) and their receptors.

In concert with the morphological changes of the endometrium, we have shown a dramatic expression of a number of matrix metalloproteinases, a multigene family of enzymes degrading components of the extracellular matrix. The mRNA for the metalloproteinase matrilysin was highly expressed in the glandular epithelium during the menstrual phase, while stromelysins-1,2,3 and interstitial collagenase were expressed in the stromal component during the same phase. In addition, during the proliferative phase, matrilysin was expressed in the epithelium and stromelysins -1,3 in the stroma. None of these metalloproteinases were detected during the progesterone-dominated secretory phase.

By Northern-blot analysis, progesterone treatment of stromal cells isolated from the proliferative phase, shows a repression of stromelysin-1 mRNA. Previous characterization of the transcription elements of the rat stromelysin-1 gene have shown an AP-1 and two PEA-3 elements that are involved in the growth-factor stimulation of the gene. In addition a TGF-β inhibitory element (TIE) and two potential GREs have been localized.

In order to understand the mechanism involved in the estrogen/progesterone regulation of stromelysin, we are currently transfecting different constructs of the rat stromelysin promoter fused to the CAT reporter gene into primary endometrial cells. Similar transfections are done for comparison in HeLa cells cotransfected with the steroid receptor cDNA. Such studies will tell us if the mechanism involved in the steroid regulation of this gene requires steroid response elements or is mediated through a growth factor pathway.

K 319 MEDROXYPROGESTERONE ACETATE (MPA) MODULATES CALCYCLIN GENE EXPRESSION, Franco Ghezzo, Silvia Racca, Giuseppe Conti, Paola Badino, Francesco Di Carlo, Department of Clinical and Biological Sciences, University of Turin, Italy

The progestational agent MPA is able to inhibit growth of hormone dependent mammary tumors, in particular its effect is greatly enhanced when used in association with the aminoacid histidine in rat experimental tumors and in cell lines (CG5). Calcyclin is a cell cycle related protein with calcium binding property also recognized by a monoclonal antibody against prolactin receptor. In the present work we have observed that calcyclin gene expression is reduced in CG5 cells when MPA and histidine are added to the culture at the concentration of 10-7M, by using reverse transcription and a PCR assay with 32P-dCTP. In order to study the direct influence of the progestin on the promoter of calcyclin we have used some chimeric genes in which chloramphenicol acetyl transferase (CAT) gene was driven by various fragmentsof calcyclin promoter transfected into hamster fibroblasts to perform a transient expression assay. The cells employed (TK-ts13) were lacking in progestin receptor, their proliferative features were insensitive to MPA, and expression of endogenous calcyclin was not inhibited by the hormone, nevertheless the effect of MPA in the CAT assay was clearly appreciable: the progestin inhibited the calcyclin promoter activity and the phenomenon was potentiated by histidine. Our data show that the progestational agent MPA may inhibit calcyclin expression in hormone dependent cells and that it can interact with the promoter of the gene also in cells lacking in specific receptor during a transient expression assay showing a direct receptor indipendent interaction between hormone and gene.

K 320 EXPRESSION OF PROGESTERONE RECEPTOR A AND B PROTEINS IN HUMAN BREAST CANCER

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The ovarian hormone progesterone plays a major role in mammalian reproductive biology, in development and differentiated function of the mammary gland and the uterus, through binding to its nuclear receptor protein. The progesterone receptor (PR) is expressed as two proteins, B (116kDa) and A (83kDa), whose functional activities differ markedly in a promoter-specific and cell-specific context. We have previously found that PR B promoter activity is preferentially stimulated by oestradiol (E2) in breast cancer cells. Furthermore, recent evidence suggests that the A protein can act as a dominant repressor of the activity of the B protein. Given the emerging evidence that the ratio of the A and B proteins may determine cellular PR activity, the aim of this study was to determine whether differential promoter regulation by E2 resulted in altered PR A/B ratios in breast cancer cells and to determine the ratio of the A and B proteins detected in breast tumours. T47Dsd cells were treated for 24h with a range of E2 concentrations. PR protein levels were measured on Western blots and immunoreactivity in the A and B PR proteins was quantitated densitometrically. E2 caused a dose dependent increase in PR protein concentration which was primarily through an increase in PR B protein expression. PR A/B ratio decreased from 2.0 in control cells to 1.0 in cells treated with the maximally effective E2 concentration (1nM). PR levels were also measured in cytosol extracts of primary breast tumours using an enzyme immunoassay and A and B PR proteins were detected on Western blots of 87 PR positive cases. A novel faster migrating PR species was detected in 23 samples, which was shown by use of PR B specific antibodies to be derived from PR A. In all but 21 cases (24 %), the expression of A exceeded that of B with the mean A/B ratio for all samples being 2.5 ± 0.23 (SEM). The majority of cases (61%) had A/B ratios in the range 1-5. Interestingly, a small group of cases (15%) had very high A/B ratios, in the range 5-10. In conclusion, this study has demonstrated that the ratio of PR A and B proteins in breast cancer cells can be modulated by physiological regulators such as oestradiol and that breast tumours contain a range of A/B ratios, with a small subgroup clearly containing very high levels of PR A relative to PR B. These data suggest that differential regulation of PR proteins in breast tumours may take place and that a PR A/B ratio which deviates markedly from the mean range may indicate likely altered or aberrant response to hormonal agents.

K 321 RECONSTITUTION OF RETINOID X RECEPTOR FUNCTION AND COMBINATORIAL REGULATION OF OTHER NUCLEAR HORMONE RECEPTORS IN THE YEAST *SACCHAROMYCES CEREVISIAE*, Bonnie L. Hall, Zeljka S.-McBride, and Martin L. Privalsky, Department of Microbiology, University of California at Davis, Davis, CA 95616

The nuclear hormone receptor family of transcription factors regulates gene expression via a complex combinatorial network of interactions. Of particular interest is the ability of retinoid X receptors (RXRs) to form heterodimers with retinoic acid receptors (RARs) and thyroid hormone receptors (TRs), thereby modifying their activities. We report here that RXR, RAR, and TR function can be reconstituted in the yeast *Saccharomyces cerevisiae* and demonstrate that the combinatorial regulation seen in vertebrate cells can be reproduced in the yeast background. Using this system, we have shown that RARs respond to a wide variety of retinoid ligands but that RXRs are specific for the 9-*cis* isomer of retinoic acid. RXR enhanced the activity of RARs and TRs on a variety of hormone response elements without demonstrably altering their DNA specificity. Interestingly, the ability of RXR to potentiate gene activation by RARs and by TRs varied for different receptor isoforms.

K 322 V-erbA can act through heterodimerisation with RXR

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V-erbA was first characterised an oncogene in an avian erythroblastosis virus, which induces acute erythroid leukaemia and sarcomas. It is derived from the thyroid hormone receptor α (TR) from which it is distinguished by a number of point mutations leading to the loss of hormone binding and a transcriptional activation. Since TR heterodimerises with the retinoic acid-related X receptor (RXR) to regulate gene transcription, we have investigated whether also v-erbA interferes with RXR in the transformed avian erythroblast cell line HD3 which overexpresses retroviral v-erbA. When using palendromic (Pal0) or direct repeat elements (DR4), v-erbA from nuclear extracts of HD3 cells is found exclusively in one major complex. This complex is shifted by antibodies against RXR as well as the gag-portion of v-erbA. Together with other data from Scatchard analysis comparing the complex from HD3 cells with *in vitro* formed v-erbA/RXR heterodimers, our results indicate that v-erbA binds these TREs as a heterodimer together with RXR. In contrast to Pal0 and DR4, the inverted repeat element F2 binds v-erbA preferentially as a homodimer. To further characterise the binding specificity of TR/RXR and v-erbA/RXR, we have set up a SAAB approach. While a DR4-like element could be characterised for TR/RXR, v-erbA/RXR selected for a mixture of different elements. The nature of the different binding characteristics of v-erbA is now under further investigation in order to get a better understanding of v-erbA functions in gene transcription.

K 323 IDENTIFICATION OF GENES TRANSCRIPTIONALLY REGULATED BY THYROID HORMONE USING A MODIFICATION OF THE WHOLE GENOME PCR PROCEDURE, Teresa Iglesias, Julio Caubín*, Gabriel Márquez*, Juan Bernal, Angeles Rodriguez Peña, José Luis Barbero*, Alberto Muñoz and Angel Zaballos*, Instituto de Investigaciones Biomédicas (CSIC), E-28029 and *Departamento de Investigación, Antibióticos-Farma, E-28026, Madrid, Spain.

We have improved the whole genome PCR procedure to isolate and identify genes under direct, transcriptional regulation by thyroid hormone. First, incubation mixtures containing restricted genomic DNA and in vitro translated huTRbeta and huRXRalfa proteins were immunoprecipitated using a polyclonal anti huTRbeta. Next, coprecipitated DNA was amplified by PCR . Three such cycles of immunoprecipitation + PCR were carried out and progressive enrichment of the DNA population in TR binding sites (TRBS) was checked by competition in TRE-pal binding assays.

In order to select for fragments corresponding to genes expressed in thyroid hormone target tissues such as liver or brain, immunoprecipitated TRBS-enriched DNA was hybridized to biotinylated cDNA prepared from either mouse liver or rat brain mRNAs. DNA:cDNA hybrids were then captured, co-selected genomic DNA amplified by PCR and the resulting material cloned in pUC18 plasmid.

In the next step, clones were individually screened by two assays: presence of TRBS as determined in competition experiments and hybridization to Northern blots containing RNA from control, hypothyroid and hyperthyroid animals. A dozen of clones hybridizing to thyroid hormone-regulated genes in liver were sequenced and shown to contain a variable number of putative TRE's with distinct homologies to the consensus sequence AGGTCA nnnn AGGTCA. Several previously characterized thyroid hormone responsive liver genes together with other genes unknown to be regulated by T3 have been found. In the parallel study, only one clone has up to now been found hybridizing to a small, abundant mRNA whose expression is thyroid-dependent in the developing rat brain. The definitive characterization of TREs present in the cloned genomic DNA fragments is being made by transactivation assays.

These results confirm the validity of our approach to identify T3-regulated genes expressed in a particular tissue and should be extensive to any other transcriptional factor modulated by a ligand.

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K 324 HORMONE-MEDIATED INDUCTION OF TRANSCRIPTION FACTOR HNF-3α UPON TUMOR CELL DIFFERENTIATION, Alexander Jacob, Shalini Budhiraja, and Ronald R. Reichel, Pharmacology & Molecular Biology, The Chicago Medical School, North Chicago, IL 60064

We present evidence demonstrating that the transcription factor HNF-3α is activated during F9 teratocarcinoma cell differentiation in response to retinoic acid. According to our results, HNF-3α induction occurs at the level of transcriptional initiation. The cis-acting promoter sequences mediating the retinoic acid response are located between 0.6 and 3.7kb upstream of the HNF-3α transcriptional start site. In contrast, 600 nucleotides of HNF-3α promoter sequences are sufficient to maintain HNF-3α transcription in hepatocytes. This result suggests that HNF-3α induction in response to retinoic acid and maintenance of HNF-3α transcription in liver cells are mediated by two distinct cis-acting promoter elements. Mouse F9 cells can be differentiated into three distinct extraembryonic tissues-primitive, parietal, and visceral endoderm-and we have observed HNF-3α activation during the formation of all three tissue types. HNF-3α stimulation upon visceral endoderm differentiation is accompanied by the activation of HNF-3 target genes such as transthyretin, suggesting that HNF-3α is involved in the developmental activation of this gene. HNF-3α induction during parietal endoderm formation is lowest compared to the two other endoderm types. Only parietal endoderm differentiation requires high levels of cAMP, in addition to retinoic acid, for its induction, suggesting that cAMP suppresses HNF-3α stimulation. The creation of primitive endoderm, which constitutes a precursor for both parietal and visceral endoderm, is an extremely early event during mammalian development. The activation of HNF-3α in primitive endoderm points towards a role for the factor in crucial determination processes that occur early during development.

K 325 GENETIC INTERACTION BETWEEN HSP82 AND YDJ1 IN *SACCHAROMYCES CEREVISIAE* ; GLUCOCORTICOID RECEPTOR ACTIVITY IN YDJ1 MUTANTS, Yoko Kimura,[1,2] Ichiro Yahara[2], and Susan Lindquist[1,3] [1]Department of Molecular Genetics and Cell Biology and [3]The Howard Hughes Medical Institute, The University of Chicago, Chicago, IL 60637; [2]Department of Cell Biology, The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan

The 90-kDa stress protein, HSP90, form complexes with variety of proteins including steroid hormone receptors (SHR). HSP90 plays an important role in regulating SHR activity by potentiating SHR transcription once hormone binds to the receptor. To seek new target proteins with which HSP90 interacts, we have screened mutagenized temperature-sensitive *hsp82* strains of *Saccharomyces cerevisiae* for synthetic lethal mutations. We isolated a single mutation, designated as *slh1-1*, that synergistically causes cell lethality when combined with *hsp82-4*. Segregants carrying *slh1-1* with a wild-type *HSP82* gene exhibited a temperature-sensitive phenotype. We isolated two genes which suppress the synthetic lethality. One isolated from a centromere plasmid yeast library was identified as the *YDJ1/MAS5* gene that encodes a yeast homologue of the *Escherichia coli* DnaJ protein, and the other isolated from a multi-copy plasmid library was *SIS1* that also endodes another homologue of DnaJ. We found that the *slh1-1* mutation maps to *YDJ1* gene. These results suggest that HSP90 and DnaJ homologue proteins are functionally related. To investigate this possibility, we examined the effects of *ydj1* mutation on the level of GR activity. In *ydj1* mutants, GR activity was much higher than that of a control strain. We are now attempting to determine how the *ydj1* mutation exerts this effect.

K 326 THYROID HORMONE RESPONSE ELEMENTS IN THE HEPATIC GLUCOKINASE PROMOTOR

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The glucokinase (GK) (EC 2.7.1.2.) is one of the key enzymes of hepatic glucose utilization. Transcription of the GK gene is stimulated by insulin, while glucogon leads to its repression. In addition to these hormones triiodothyronine (T3) plays an important permissive role in the insulin-mediated induction of the gene.

We cloned a 1,4 kb fragment of the GK promotor/enhancer region to identify thyroid hormone response elements (TREs). Transactivation experiments in Hep G2 cells showed, that this promotor/enhancer fragment is sufficient to induce T3-dependent reporter gene activation. To resolve the involved DNA binding sites, we employed an avidin-biotin-complex-DNA-binding assay (ABCD assay) and gel retardation, as well as the methylation interference assay. Oligonucleotides representing putative TREs from the GK promotor/enhancer region were compared with established TREs from rat growth hormone, malic enzyme and an optimal element (DR4) for binding to the thyroid hormone receptor (TR) and heterodimers formed by TR and the retionic acid related X receptor (RXR).

Two of the putative GK-TREs (-712 to -727 and -896 to -911) bound TR/RXR heterodimers with high affinity, but no homodimeric complex of TR. The specifity of the binding to these elements was confirmed in competion experiments using the optimal element (DR4). The contact points for TR/RXR binding were determined by methylation interference, and demonstrated that both elements are of the direct repeat type, spaced by 4 nucleotides.

From our data we conclude that T3 induces GK gene expression in the liver through the binding of TR/RXR heterodimers to specific TREs in the GK promotor.

K 327 IDENTIFICATION OF ESTROGEN RECEPTOR ASSOCIATED PROTEINS, Deborah A. Lannigan, Robin Abramson and Sara. M. Bush, Department of Zoology, University of Vermont, Burlington, VT 05405

The mechanism by which hormone-activated estrogen receptor activates RNA polymerase II is unknown. There is evidence to suggest that accessory proteins transduce the transcriptional activation signal from the estrogen receptor to RNA polymerase II. To identify proteins which associate with the estrogen receptor fusion proteins consisting of glutathione-S-transferase (GST) and either the N- or C-terminal domains of the estrogen receptor were expressed in *E. coli*. The C-terminal fusion protein bound estradiol with a stoichiometry greater than 45% and with a binding affinity similar to that which has been reported for the holoreceptor. Furthermore, the C-terminal fusion protein behaved similarly to the holoreceptor in competition experiments using either tamoxifen or estradiol to compete for [^{3}H]estradiol binding. These data suggest that the C-terminal fusion protein has the same structural conformation as the holoreceptor, and therefore can associate with other proteins in a manner similar to the holoreceptor. Nuclear extracts from either [^{35}S] methionine-labelled COS or HeLa cells were incubated with either immobilized GST-estrogen receptor fusion proteins or GST alone. Two novel proteins, with molecular weights of approximately 75,000 daltons and 175,000 daltons, were identified which associated with the C-terminal fusion protein only when it was bound by estradiol, but not when it was unliganded or bound by the antiestrogen, tamoxifen. Additional estrogen receptor associated proteins were identified which bound specifically with either the N- or the unliganded C-terminal fusion protein, but not with H-Ras, a protein unrelated to the estrogen receptor.

K 328 HORMONAL REGULATION AND CELL-TYPE SPECIFICITY OF THE HUMAN AdoMetDC GENE PROMOTER

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The human S-adenosylmethionine decarboxylase (AdoMetDC) gene encodes a keyenzyme in polyamine biosynthesis. AdoMetDC gene can be considered a housekeeping gene since its activity has been found in almost all mammalian tissues. However, AdoMetDC gene is highly inducible and the AdoMetDC mRNA and protein concentration are regulated in a temporal and tissue-specific manner by multiple stimuli, such as steroid hormones. For example, AdoMetDC mRNA and protein levels are androgen-inducible in rodent accessory sex organs, with the maximum levels of mRNA expression being reached within 24-48 h of steroid administration. Furthermore, AdoMetDC enzyme activity and the level of polyamines are much higher in rapidly proliferating cells than in nonproliferating cells.

We previously isolated and sequenced a 3158 bp DNA fragment of the human AdoMetDC promoter (Marić et al., (1992) JBC 267: 18915-18923). In order to indetify potential cell-type specific *cis*-acting elements in AdoMetDC promoter, we have generated serious of deletion mutants of the AdoMetDC promoter (-3158/, -2596/, -2149/, -1538/, -1098/, -688/, -440/, -208/, -96/, -45/+22). A transient transfection assay involving AdoMetDC deletion mutants/chloramphenicol acetyltransferase (CAT) reporter gene and the CV-1 and MCF-7 cell lines were used to quantitate promoter function. We found an androgen responsive element (ARE) at position (-63 to -48) in the proximal promoter. Using baculovirus expressed androgen receptor (AR), we could detect AR-DNA specific complex *in vitro*. Furthermore, we tested inducibility of this promoter region using human AR expression vector in CV-1 and HepG2 cell lines. We can demonstrate a cell-specific differences in AR induction of the proximal promoter of AdoMetDC gene. Further studies are required to determine the presence of non-receptor proteins that preferentially alter the transactivation functions of the AR.

K 329 **ANDROGEN RECEPTOR SPECIFICITY OF A COMPLEX RESPONSE ELEMENT IN THE RAT 20 kDa PROTEIN GENE.** K.B. Marschke, S.G.A. Power, K.-C. Ho, E.M. Wilson and F.S. French, The Laboratories for Reproductive Biology, University of North Carolina, Chapel Hill, NC 27599

A 130 base pair DNA sequence (D2) from intron 1 of the androgen regulated 20 kDa protein gene expressed in rat ventral prostate functions in a reporter vector, D2-tkCAT, as an androgen, but not a glucocorticoid, response element in CV1 cells, a human prostate carcinoma cell line, PC3, and Hela cells cotransfected with either an androgen (AR) or glucocorticoid (GR) receptor expression vector. The level of R1881-induced AR transactivation with D2-tkCAT in CV1 cells (10-12 fold induction) was equivalent to that attained by AR and GR with the simple consensus-like androgen and glucocorticoid response element of the C3 subunit gene of rat prostatein (C3-ARE/GRE). In gel mobility shift assays using purified receptor DNA binding domain polypeptides overexpressed in *E. coli*, D2 bound AR 2-fold greater than GR, however, the binding of both was 4-8-fold less than their equivalent binding to C3-ARE/GRE. Subfragments of D2 consisting of the 5' (D2-3) and the 3' (D2-7) halves bound AR DNA binding domain, however neither subfragment functioned independently as an androgen response element in CV1 cells. Methylation interference assays with D2 identified binding sites in both the D2-3 and D2-7 subfragments. Neither AR binding site resembles the consensus ARE/GRE, however, the site in D2-3 overlaps a potential recognition site for AP-1. A receptor chimera containing the GR N-terminal domain linked to the AR DNA- and steroid-binding domains was less effective than intact AR as a R1881-induced transactivator of D2-tkCAT. The reverse chimera, AR N-terminal domain with GR DNA- and steroid-binding domains, like intact GR was not a Dex-induced transactivator of D2-tkCAT. Our results suggest that the D2 complex response element is specifically regulated by androgen through selective binding of AR to non-consensus elements and potential interactions of other transcription factors with specific AR domains. (Supported by the NICHD Center for Population Research)

K 330 A NOVEL RESPONSE ELEMENT INVOLVED IN THE TISSUE-SPECIFIC EXPRESSION OF HUMAN α2-MACROGLOBULIN BINDS ORPHAN MEMBERS OF THE STEROID RECEPTOR SUPER-FAMILY, Gert MATTHIJS, Myriam BAES* and Peter MARYNEN. Center for Human Genetics and *Laboratory for Clinical Chemistry, K.U. LEUVEN, B-3000 LEUVEN, BELGIUM

Alpha2-macroglobulin (α_2M) is a wide-spectrum proteinase-inhibitor showing a unique mechanism of proteinase-trapping. In rat, α_2M is an acute phase reactant. In man, α_2M synthesis is not turned off after birth and plasma α_2M is constitutively synthesized in the liver. HepG2 cells and fetal lung fibroblasts (LF) have been shown to produce α_2M.

We have shown that 240 bp 5' of the transcription start site contain all the necessary information for efficient and cell-specific expression of the gene in HepG2 and LF. The promoter remains silent in skin fibroblasts (SF). In DNAsel footprint experiments five regions bind nuclear factors from both expressing and non-expressing cells. One of these footprints, FPII (-143 to -106), is far more prominent with extracts from HepG2 and LF. This region encompasses 3 hexamer repeats: a central, canonical half-site (TGACCT) as a part of a PR4 at the 5' end and an ER1 at the 3' end of the footprint. In mobility shift assays, the entire element binds nuclear factors which are present in the order : HepG2 > LF > > SF. We have begun to characterize the nuclear factor(s) that bind to this element. In vitro, recombinant COUP-TF1/Ear3 and HNF-4 but not RXRα, RARα nor PPAR bind to the FPII oligomer. When combined, RXRα and RARα bind weakly. Oligomers for HNF-4, ARP-1 and COUP-TF1/Ear3 compete for FPII binding and show a similar cell-specific gel retardation pattern. HNF-4 and COUP-TF1/Ear3 are thus candidates for the tissue-specific regulation of the human α_2M gene but HNF-4 is absent in the complex retarded by the FPII oligomer, as shown by supershift experiments. A preliminary dissection of the element shows that a synthetic ER1 competes somewhat better than a PR4 for binding but they do not show the same mobility shift pattern as the entire FPII oligomer. In these experiments, PR4 and ER1 did not bind efficiently to the recombinant proteins.

We conclude that COUP-TF1/Ear3 preferentially binds to this response element and may have a stimulatory role in the context of the α_2M promoter. We cannot exclude that other, uncharacterized orphan receptors act through this novel binding site, which is important for the cell type-specific expression of the gene.

K 331 t-RETINOIC ACID SYNERGISES WITH PROTEIN KINASE C TO STIMULATE TISSUE PLASMINOGEN ACTIVATOR PRODUCTION: EVIDENCE FOR TRANSCRIPTIONAL ACTIVATION VIA TWO DISTINCT REGIONS ON THE PROMOTER, Rheem D. Medh and Eugene G. Levin, Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA-92037.

The effects of trans retinoic acid (t-RA) on tissue plasminogen activator (tPA) secretion was studied in HeLa-S_3 cells and human umbilical vein endothelial cells (huvecs). t-RA stimulated tPA production in a dose dependent manner, with maximum secretion of 4-5 times the basal levels seen at 40 nM and 40 uM t-RA in HeLa and huvecs respectively. In view of recent reports suggesting that t-RA and protein kinase C are mutually antagonistic in their effects on various genes, we also evaluated the effects of t-RA on PMA induced tPA production. t-RA potentiated the stimulation of tPA production by PMA up to 3.4 times the additive effect. This synergism was found to be independent of cAMP levels, although cAMP has also been shown to independently potentiate PMA induced tPA production.

We have generated several tPA promoter -luciferase reporter constructs to study the transcriptional regulation of the promoter by t-RA. In HeLa cells, the full length promoter (1635bp) was capable of inducing luciferase activity 3.5 to 4 times the basal level by both t-RA (10 uM) and PMA (10 nM) independently. Cells cotreated with t-RA and PMA showed a 12.5 fold increase in luciferase activity, suggesting that the synergism occurred at least partially at the transcriptional level. Using various deletion mutants of the tPA promoter in transient transfections, we have identified two t-RA responsive regions. One is within 200 bases upstream the transcription start site, and the other is downstream, in the first untranslated exon.

K 332 CAN LIGANDS OF APPROPRIATE HORMONE RECEPTORS BE USED AS ANTI-VIRAL DRUGS?

Janet Mertz, Fengrong Zuo, Steven Wiley, and Richard Kraus. McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, WI 53706, USA

Members of the steroid-thyroid hormone receptor superfamily have previously been shown to affect expression of some retroviruses (e.g., MuMTV) and lentiviruses (e.g., HIV). We have recently discovered they can affect expression of the late genes of DNA viruses as well (Wiley et al., Genes & Devel., in press).

Expression of DNA viruses is regulated during latency or the early phase of the lytic cycle of infection, with only the early genes being expressed at significant levels. After induction or during the late phase, expression of the late genes predominates. We have identified binding sites for members of the steroid-thyroid receptor superfamily (e.g.'s, hERR1, COUP-TF1, TR2) in the late promoter of the primate DNA tumor virus SV40. Addition of a purified fraction from HeLa cells containing hERR1 as a major component or recombinant COUP-TF1 to a cell-free transcription system specifically represses transcription from the SV40 late promoter. These receptor binding sites function to repress transcription from the SV40 late promoter at early times after infection; repression is relieved at late times by replication-dependent titration of the receptors. Overexpression of COUP-TF1 inhibits the early-to-late switch in SV40 gene expression even in the presence of viral DNA replication. *In vitro* order of addition experiments indicate that these receptors repress transcription by preventing formation of preinitiation complexes. Other viruses including BKV, human adenovirus 2, and human hepatitis B virus probably also contain binding sites in their late promoters for members of this receptor superfamily. Thus, hormones modulate expression of the late genes of many viruses via these receptor binding sites. We speculate that appropriate ligands for these receptors may be usable as anti-viral drugs.

K 333 CALRETICULIN BINDS TO THE GLUCOCORTICOID RECEPTOR AND MODULATES GLUCOCORTICOID-SENSITIVE GENE EXPRESSION, Marek Michalak, Kimberly Burns, Brenda Duggan, Erci A. Atkinson, Konrad S. Famulski, Mona Nemer*, R. Chris Bleackley, Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada; *Clinical Research Institute of Montreal, Montreal, Quebec, Canada

Calreticulin, a major Ca binding protein, has been reported to bind to a synthetic peptide KLGFFKR (Biochemistry **30**, 9859, 1991), and an almost identical amino acid sequence is found between the two "Zinc-fingers" in the DNA binding domain of a family of nuclear receptors. Could calreticulin interaction with the DNA binding domain of GR modulate its function *in vivo*? In order to test this hypothesis, interactions between calreticulin and the DNA binding domain of glucocorticoid receptor (GR) were examined using affinity chromatography and DNA mobility shift assays. The DNA binding domain of GR interacted with a synthetic glucocorticoid response element (GRE) in a mobility shift assay. This interaction was completely abolished in the presence of calreticulin. The interaction between the GR and calreticulin was further demonstrated by the calreticulin-affinity chromatography and confined to the N-terminus of the protein and the DNA binding domain of GR.

To assess the *in vivo* significance of the interaction between calreticulin and GR, mouse L fibroblasts were transfected with a plasmid encoding GRE-luciferase reporter gene and asked whether cotransfection with a calreticulin expression vector (pSVL-CRT) would modulate GR-mediated activation of luciferase. In the presence of a control plasmid (pSVL) luciferase activity increased 20-fold upon addition of dexamethasone (DEX). When pSVL-CRT was included in the transfection the DEX-sensitive increase in reporter gene expression was reduced by 85%. We conclude that calreticulin may interact with the GR within the cell and thereby blocks binding of the receptor to DNA. This suggests that the expression of endogenous genes modulated by the GR could be affected by the levels of calreticulin within the cell. Mouse L fibroblasts cell lines were established which express reduced and elevated amounts of calreticulin. Experiments using these cell lines further suggest that calreticulin may be involved in influencing the genetic programs that are controlled by steroid hormones.

supported by MRC & HSFA

K 334 PROGESTERONE RECEPTOR BINDS TO THE UPSTREAM FLANKING REGION OF THE HUMAN C-MYC GENE. **Michael R. Moore and Renee N. Gentry, Department of Biochemistry and Molecular Biology, Marshall University School of Medicine, Huntington, WV 25704**

Our previous reports have shown that progestins stimulate growth of the human breast cancer cell line T47D in tissue culture under our conditions. Our data from experiments to probe the mechanism of progestin regulation of growth suggest that expression of mRNA for the growth-associated protooncogene c-myc is stimulated by progestins at very early times. Consistent with this early stimulation, computer search has indicated a putative progesterone responsive element (PRE) with 93% homology to the consensus PRE sequence 2072 base pairs upstream of the first transcription start site. We are investigating an oligodeoxynucleotide of 35 base pairs containing this putative PRE, flanked by a synthetic Bg1 II site at the 5' end and a synthetic Bam HI site at the 3' end. We have found that the human progesterone receptor, both expressed in the baculovirus system and from the human breast cancer cell line T47D, binds to this fragment in a specific manner in gel shift assays. Further, progesterone receptor in crude extracts from T47D human breast cancer cells binds better than that from the insect cell-baculovirus system, suggesting the presence of a factor(s) that stimulates (in breast cancer cells) or inhibits (in insect cells) the specific binding of progesterone receptor to the DNA fragment. Additionally, monoclonal antibody to the progesterone receptor causes a supershift of the fragment, confirming that the progesterone receptor is indeed binding to this putative PRE in the upstream flanking region of the human c-myc protooncogene.

K 335 Regulation of myogenic helix loop helix gene transcription by thyroid hormone: Identification of the thyroid hormone and retinoid X receptor heterodimeric binding site. *George Muscat[1], Russell Griggs[1], Eric N. Olson [2]and Michael Downes[1].* [1]University of Queensland, Centre for Molecular Biology, St Lucia, 4072, QLD., AUSTRALIA. [2]University of Texas, M.D. Anderson Cancer Centre, Department of Biochemistry and Molecular Biology, Box 117, 1515 Holcombe Boulevard, Houston, Texas 77030; USA

Thyroid Hormones are major determinants of skeletal muscle differentiation. We have identified a thyroid hormone response element (TRE) in the promoter of the mouse myogenin (MM) gene, a muscle specific, helic loop helix(HLH) transcription factor. The myogenin gene belongs to the MyoD family of hierarchical regulatory HLH proteins that direct the fate of pluripotential embryonic cells, inhibit mitosis and trans-activate muscle specific genes involved in terminal differentiation and contraction.

This MM TRE conferred appropriate hormonal regulation to an enhancerless SV40 promoter. In vitro DNA binding studies showed that the thyroid hormone receptor(TRα) formed a heterodimeric complex with the retinoid X receptor(RXRα) on the MM TRE that was specifically competed by classical TREs and not by other response elements. Analyses of this heterodimer with a battery of steroid hormone response elements indicated that the complex was efficiently competed by direct repeats with a 4 or 5 nucleotide gap as predicted by the tandem direct repeat model (3-4-5 rule). Mutagenesis of the MM TRE indicated that a direct repeat of the **AGGTCA** binding motif and a 4 nucleotide gap was necessary for efficient binding to the TRα/RXRα heterodimeric complex. In conclusion this is the first identification and characterization of a hormonal response element in the muscle specific, helix loop helix family, that directly interacts with TR and RXR. These results provide a molecular basis for the positive regulation of myogenic differentiation by thyroid hormones

K 336 POTENTIAL ROLE FOR CALMODULIN IN HORMONE DEPENDENT ACTIVATION OF THE GLUCOCORTICOID RECEPTOR, Yang-Min Ning and Edwin R. Sánchez, Department of Pharmacology, Medical College of Ohio, Toledo, OH 43699

In the signaling cascade of membrane-bound receptors, calmodulin (CaM) plays an important role. However, little is known about the involvement of CaM in the activation process of intracellular steroid receptors. Recent studies have indicated that hsp90 can bind CaM, and that hsp56 contains a putative CaM binding sequence. Both hsp90 and hsp56 have been identified as the components of untransformed steroid receptor complexes. These facts have led to the speculation that CaM may be involved in the regulation of steroid receptors. To test this idea, we have assessed the effects of CaM antagonists on hormone-dependent, glucocorticoid receptor (GR)-mediated transcription enhancement activity. In mouse L929 cells stably-transfected with an MMTV-CAT reporter plasmid (LMCAT cells), pre-treatment (but not post-treatment) with phenoxybenzamine (POBA) resulted in a concentration-dependent inhibition of dexamethasone (Dex)-induced CAT gene expression. Trifluoperazine (TFP) and N-(6-aminohexyl)-5-chloro-1-naphtalenesulfonamide (W7) also inhibited Dex-induced CAT gene expression, whereas N-(6-aminohexyl)-5-hydro-1-naphtalenesulfonamide (W5), a CaM antagonist that is less potent than W7, was less inhibitory than W7. To determine if the inhibition of Dex-induced gene expression was the result of a non-specific effect on gene transcription and protein synthesis by CaM antagonists, L929 cells stably- transfected with the constitutively expressing pSV2CAT reporter (LSV2CAT cells) were subjected to similar treatments. Except for TFP, treatment of LSV2CAT cells with POBA, W7 and W5 did not result in a decrease of CAT expression. In the case of TFP, the inhibitory effect was not as great in the LSV2CAT cells as compared to the LMCAT cells. An examination of the effects of POBA on GR nuclear translocation revealed a concentration-dependent decrease in Dex-induced nuclear localization of the GR. By use of a DNA-cellulose binding assay, GR that accumulated in the cytosol under these conditions was found to be in the untransformed state. Moreover, POBA decreased GR hormone-binding capacity in a manner that was consistent with its inhibitory effects on GR translocation and transactivation. Although the precise molecular mechanisms underlying these observations are not yet clear, the results provide the first pharmacological evidence that CaM may be involved in hormone-dependent activation of the glucocorticoid receptor. In general, this study implies that CaM may play an important role in the translocation and transactivation functions of steroid receptors.

K 337 PROMOTER ELEMENTS INVOLVED IN EITHER BASAL OR ESTROGEN-INDUCED TRANSCRIPTION OF THE SMALL HEAT SHOCK PROTEIN HSP27 IN HUMAN BREAST CANCER CELLS, Oesterreich S., Weber L., Hickey E., and Fuqua S.A.W., The University of Texas Health Science Center, San Antonio, Texas 78284, and The University of Nevada, Reno, Nevada 89557.

The small molecular weight heat shock protein hsp27 is known to be induced by environmental stress and certain steroids in human breast cancer cells. Because we have now shown that its expression is involved in cellular proliferation, and response to chemotherapeutic agents such as doxorubicin in hsp27-transfected breast cancer cells, we are now interested in examining the regulatory sequences which control hsp27 transcription. We hope to identify either regulatory transcription factors, or specific promoter regions which could be clinically targeted to interfere with hsp27 expression.

Sequencing a 1.2 kb region of the hsp27 promoter revealed multiple consensus TATA-, SP1-, AP2, and CAAT-sites, in addition to the known heat shock responsive element (HSE). Furthermore, there are several putative hormone responsive elements, such as glucocorticoid and estrogen responsive elements within this region. Analysis of this region by DNaseI footprint and gel-retardation analyses has revealed that many of these elements are involved in regulation of hsp27 transcription, dependent on the hormonal environment. Basal hsp27 transcription appears to be mainly driven by elements within the most proximal promoter region, with higher levels of transcriptional activity seen in ER-positive, as compared to ER-negative breast cancer cells. A consensus ERE was not identified within this proximal region, however there are two 1/2 ERE sites which appear to bind a novel protein in gel-retardation analyses. We have not yet determined the relationship between this novel protein, ER, or other steroid receptor members in these cells. We have also identified a negative regulatory element in the hsp27 promoter, and are investigating its use as a target for inhibition of hsp27 expression.

Conclusion: The hsp27 promoter has a compound structure with features of housekeeping genes (SP1 sites), regulated genes (TATA box), and hormone-responsive genes which may serve as future targets for intervention.

K 338 Regulation of Phosphoenolpyruvate Carboxykinase Gene Expression by Thyroid Hormone. Edwards A. Park, David C. Jerden and Suleiman W. Bahouth. Department of Pharmacology, University of Tennessee, Memphis, TN 38116

Thyroid hormone (T3) has profound effects on the metabolism of glucose and lipids in the liver. Phosphoenolpyruvate carboxykinase (PEPCK) catalyzes the key initiating step in gluconeogenesis and is regulated by hormones, including T3, cAMP, glucocorticoids and insulin primarily at the transcriptional level. T3 modulates PEPCK gene expression through at least two mechanisms. First, T3 stimulates PEPCK transcription directly through a TRE in the promoter. Secondly, T3 greatly potentiates the induction by glucagon (cAMP), which is the major inducer of PEPCK gene expression. Using PEPCK-CAT vectors in transient transfections into HepG2 cells, we have identified a TRE in the PEPCK promoter. We have shown that this element binds the T3 receptor. The T3 stimulation of PEPCK transcription also involves a liver-specific C/EBP binding site which is located 80 base pairs 3' to the TRE. Mutation of the C/EBP binding site abolishes the T3 induction of PEPCK transcription despite the fact that the TRE is intact. In our transient transfections, T3 and cAMP synergistically stimulate transcription from the PEPCK gene. This synergistic induction involves three elements on the promoter- the TRE, the C/EBP binding site and a cAMP responsive element (CRE) which binds CREB. Our results suggest that there is cross-talk between proteins involved in the T3 and cAMP induction of PEPCK transcription. This work was supported by the American Heart Association.

K 339 TWO ACTIVE REPRESSION DOMAINS ARE PRESENT IN THE ORPHAN RECEPTOR ARP-1, Bernhard Paulweber*, Gertrude Achatz*, Bertram Hölzl*, Friedrich Sandhofer*. *General Hospital Salzburg, Austria

ARP-1, a member of the nuclear receptor superfamily, represses promoter activity of various human apoliprotein genes. It has been proposed that ARP-1 represses transcriptional activity by competing for DNA binding with activator proteins, such as HNF-4. Recent studies from our laboratory, however, provided strong evidence, that ARP-1 is also operating as an active repressor protein, since overexpression of ARP-1 resulted in repression of transcriptional activation promoted by HNF-3, even in the absence of a binding site for ARP-1. To map the domain(s) required for this active repressor effect of the protein we tested a series of ARP-1 variants, either truncated at their NH2- or COOH- terminus or harbouring internal deletions, for their effect upon transcriptional activity of the apo-B promoter. A strong repressor domain was identified in the carboxy terminus of the protein (AA 395 to 414). The amino terminus of the protein (AA 1-78) was found to contain an additional weak repressor domain. As suggested by the results of internal deletions presence of the DNA binding domain is required for the repressor effect. To ensure nuclear localization of the variant proteins a nuclear translocation signal from the SV40 large T antigen was attached to the NH2-terminus of all the variant proteins. Interestingly this modification completely abolished binding of the variant proteins and of the wild type protein to the ARP-1 recognition site in the apo-B promoter (-86 to -62), without affecting the repressor effect of ARP-1 upon transcriptional activity of the apo-B promoter. This result strongly suggests that binding of ARP-1 to its recognition site in the apo-B promoter is not required for its negative effect upon transcriptional activity. The repressor effect of ARP-1 is most likely the result of a protein-protein interaction between ARP-1 and a second protein, either a transcriptional activator or coactivator protein or a general transcription factor, such as TFII B. Interaction with a general component as the mechanism underlying the repressor effect seems unlikely, since overexpression of ARP-1 did not affect transcriptional activity promoted by two other activator proteins, namely Sp1 or ATF.

K 340 TRβ MUTANTS ASSOCIATED WITH THE GENERALIZED THYROID HORMONE RESISTANCE ARE DEFECTIVE IN THE LIGAND-SENSITIVE REPRESSION FUNCTION. F. Javier Piedrafita, Maria A. Ortiz and Magnus Pfahl., La Jolla Cancer Research Foundation, La Jolla, CA 92037.

TR homodimers bind with high affinity to TREs containing two half-sites (AGGTCA) arranged as inverted palindromes (InvPal) with 4, 5, or 6 bp spacer. TR homodimer binding to some of these elements is more stable than TR-RXR heterodimer binding. However, the TR homodimers are highly sensitive to low concentrations of thyroid hormone. When the InvPal sequences are located 3' of the TATA box, TR homodimers act as very strong repressors in the absence of ligand and this repression is released by addition of T3. Naturally ocurring TRβ mutants associated with the generalized resistance to T3 are defective in this ligand-sensitive repression function. Seven mutantions, located in both "hot spot" regions of the ligand binding domain of TRβ kindreds GH, ED, S, NN, OK, PV, and QW, have been studied. Although all of them bind to different TREs as heterodimers with RXR as well as the wild type receptor, their homodimer binding capabilities to InvPal are different. Kindred GH binds InvPal-6 very weakly as homodimer, but kindred PV forms very strong homodimers with that DNA sequence. Kindreds S and PV, that have been reported not to bind T3, are not responsive to ligand in our "repression" system and their homodimer binding is not disrupted by T3. Other kindreds required higher concentrations of T3 to inhibit homodimer binding. Accordingly, higher concentrations of ligand, as compared to the wild type protein receptor, are required to release the repression by these TRβ mutants. The significance of these findings for different TR response pathways will be discussed.

K 341 COUP-TF1 BINDS TO THE PEROXISOME PROLIFERATOR-RESPONSIVE ELEMENTS OF THE RAT ACYL-CoA OXIDASE AND HYDRATASE-DEHYDROGENASE GENES AND ANTAGONIZES PEROXISOME PROLIFERATOR-MEDIATED SIGNALING, Rachubinski, R.A., Zhang, B., Miyata, K.S., Marcus, S.L., and Capone, J.P., Department of Biochemistry, McMaster University, Hamilton, ON L8N 3Z5, CANADA

Peroxisome proliferators are a family of diverse xenobiotic compounds that includes hypolipidemic agents, herbicides, and plasticizers. These compounds activate transcription of a subset of nuclear genes, including genes coding for the enzymes of the peroxisomal β-oxidation pathway. Chronic elevation of the activities of these enzymes can lead to hepatocarcinogenesis. Induction of the genes encoding fatty acyl-CoA oxidase (AOx) and hydratase-dehydrogenase (HD), the first and second enzymes of the pathway, is mediated through the action of peroxisome proliferator-activated receptors (PPARs) that bind to upstream peroxisome proliferator-responsive elements (PPREs) through heterodimerization with retinoid X receptors. The AOx PPRE consists of two direct repeats related to the nuclear hormone core consensus motif TGACCT. These repeats are separated by one nucleotide. The HD PPRE consists of three direct related repeats separated by two nucleotides and one nucleotide, respectively. We have shown that COUP-TF1 binds to both the AOx and HD PPREs *in vitro* and that HNF-4, another nuclear hormone receptor that is enriched in liver, binds to the AOx PPRE *in vitro*. We have also shown that COUP-TF1 can antagonize peroxisome proliferator-mediated signaling *in vivo* from both the AOx and HD PPREs, suggesting that COUP-TFs play a role in the regulation of peroxisome proliferator-responsive genes. Supported by the Heart and Stroke Foundation of Ontario.

K 342 TWO HORMONE RESPONSIVE ELEMENTS, ESSENTIAL FOR ANDROGEN INDUCTION OF THE PROBASIN GENE, BIND ANDROGEN RECEPTORS IN A COOPERATIVE, MUTUALLY DEPENDENT FASHION, Paul S. Rennie, Susan Kasper^, Nicholas Bruchovsky, Robert J. Matusik^, Robert Snoek, Patricia C. Sheppard^ and Helen Cheng, Dept. of Cancer Endocrinology, B.C. Cancer Agency, Vancouver, B.C., Canada and ^Dept. of Physiology, University of Manitoba, Winnipeg, Man., Canada

Using deletion mapping and DNase I footprinting, we previously identified two androgen responsive elements (AREs) that are essential for androgen induction of the probasin gene; ARE-1, whose sequence resembles a glucocorticoid responsive element, and ARE-2, which has a relatively unique sequence. The binding of androgen receptors to these AREs was further characterized by measuring the binding affinity (Kd) to each and by determining whether occupancy of either ARE with androgen receptors influenced binding to the other. Peptides encompassing the DNA-binding domain of the androgen receptor were expressed in *E. coli*, affinity-purified, and then used in band-shift assays with synthetic oligomers containing either ARE-1 or ARE-2 sequences. Scatchard analyses indicated that androgen receptors bound to ARE-1 and ARE-2 with Kds of 20.0 nM and 6.7 nM respectively. Consistent with the higher affinity of ARE-2 sites, in DNase I footprint experiments, regions of the probasin DNA containing ARE-2 but not ARE-1, footprint at a lower threshold concentration of receptor than that required in reciprocal experiments where only regions of the probasin gene with ARE-1 sequences are present. However, with probasin DNA containing both AREs, the relative intensity of the footprints for ARE-1 and ARE-2 were equivalent; suggesting a cooperative interaction between the sites. This cooperative effect was substantiated in footprint experiments with probasin DNA containing mutations in either ARE-1 or ARE-2. A point mutation in ARE-1, which reduced androgen induction by 95%, blocked binding of androgen receptors to both ARE-1 and ARE-2. Similarly, a point mutation in ARE-2 prevented receptor binding to both AREs. Thus while androgen receptors have an higher affinity for ARE-2 than ARE-1, in the probasin gene the binding of androgen receptors to these AREs occurs in a cooperative, mutually dependent fashion. (Supported by MRC of Canada)

K 343 ACTIVE AND INACTIVE GLUCOCORTICOID RECEPTOR MUTANTS, Sandro Rusconi; Rainer Lanz; Martin Hug, Stefan Wieland; Manuela Höfferer; Stefan Schneider; Patrick Rigoni; Mauro Gola; *Institut für Molekularbiologie II der Universität, Winterthurerstrasse 190, 8057 Zürich, Switzerland*

1) The rat GR cDNA contains a CAG repeat encoding a stretch of glutamine residues toward the amino terminus. The stretch of Gln is not evolutionarily conserved suggesting that it does not significantly contribute to the activity of the GR. However, by altering the reading frame of the CAG repeat we have obtained a variant (GR.Ala) that maintains hormone and DNA binding capacity, while having lost the majority of its trans-activation potential. This variant behaves as a trans-dominant negative (TDN) molecule, since it is able to strongly and specifically interfere with the action of the wild type GR. We present now experiments aimed to an understanding of the reasons of the surprising desactivation of the trans-acting domains of the GR. The results suggest that a transient interaction with ER membranes (in *statu nascendi*) may be responsible for the loss of transactivation. We have also linked the module encoding oligo Ala to other transcription factors and found that the effect is not general but factor-specific. We are expressing TDN GR.Ala mutants in different cell lines hoping that they will interfere with the activation of chromosomal genes that are known to be normally GR regulated. These assays are performed with stable transformation or with transient assays. For the latter we have developed an assay that allows the selection of transiently expressing cells. These are intermediate steps to assess the possibility of using these TDN mutants for tissue-specific interference of GR mediated response in transgenic animals.

2) The structure of the GR DBD:DNA complex has been solved by X-ray crystallography (*Nature*, **352**, 497-505.) and the structure of the free DBD has been determined by NMR (*Science*, **249**, 157-160.).We have focussed our attention to a residue (Ile, rat map 484) that is very conserved in the receptor super-family and has a very peculiar orientation in the DNA:GR complex for its side chain is oriented toward the solvent rather than buried in a hydrophobic core. Substitution of this residue with all the possible alternatives indicates that Ile484 is involved in both DNA binding and in transactivation. We believe that this dual property is consistent with the idea of a major rearrangement that occurs when the DBD is interacting with the specific DNA target site. The rearrangement can be easily deduced by comparing crystal and NMR data and involves the "loop of the second Zn finger that flips by almost 180 degrees during DNA binding. This rearrangement (that we call "finger flip-flop" FFF) may have have important consequences for the structure of the other portions of the GR.

K 344 REGULATION OF THE RAT GROWTH HORMONE GENE BY PIT-1 AND THE THYROID HORMONE RECEPTOR

Fred Schaufele, Ishtiaq Qadri, Rosemarie Forde, John Baxter and Brian West, Metabolic Research Unit, University of California, San Francisco CA 94143-0540

To understand the coordinate regulation of the rGH promoter by the pituitary-specific transcription factor, Pit-1, and numerous more tissue-general factors, we have been investigating the effect of the binding of each factor on the activities of both the other factors and the basal transcription apparatus. We have concentrated on the individual and coordinate properties of the thyroid hormone receptor, TR, and Pit-1 which we have previously shown to synergistically activate the rGH promoter. We report that Pit-1 and TR physically associate in vitro with the same spectrum of basal transcription factors thus far tested. Pit-1 and TR also bind to each other through domains conserved in other homeobox factors and steroid hormone receptors. A mutation in Pit-1 is described that specifically inhibits the TR-synergistic activation of the rGH promoter without affecting the activity of Pit-1 in isolation. This synergism-specific activity appears to be associated with the ability of Pit-1 to bind two as of yet uncharacterized factors present in pituitary cell extracts.

K 345 A NOVEL ANDROGEN-DEPENDENT GENE FAMILY CODING FOR CYSTEINE-RICH SECRETORY PROTEINS (CRISPs), Wolf-Dieter Schleuning, Bernard Haendler, Jörn Krätzschmar and Uta Schwidetzky, Research Laboratories of Schering AG, D-13342 Berlin, Germany

Acidic epididymal glycoprotein (AEG) was originally described in the rat as a sperm surface protein secreted in abundance by the epididymis. We present evidence that the gene for CRISP-1, the mouse counterpart of AEG, is expressed in the salivary gland as well as in the epididymis, albeit to a lower level. We were further able to identify a new, salivary gland-specific transcript showing 77% identity to CRISP-1 at the deduced amino acid level. This new protein was named CRISP-3. In addition, both proteins display strong sequence identity to the recently described testis-specific product Tpx-1/CRISP-2. The most striking structural feature of the three CRISPs is the presence of 14 entirely conserved cysteine residues in their C-terminal moieties.

CRISP-1 transcripts were mainly detected in the epididymis and, in less abundance, in the male but not in the female salivary gland. The synthesis of *CRISP-3* transcripts appeared to be restricted to the salivary gland, with a higher level in males. Castration experiments revealed that the expression of both the *CRISP-1* and *-3* genes was regulated by androgens, although not in the same way. Whereas no *CRISP-1* expression was noted any longer seven days after the operation, *CRISP-3* expression was still detectable even after fourteen days. These results suggest the existence of specific *cis* and *trans* elements involved in the expression regulation of these genes.

Genomic clones for CRISP-1 and -3 were isolated to help us understand this regulation. Both genes share a common exon/intron distribution with the first exon coding for the 5'-untranslated region and an unusually large intron between exons 6 and 7. The transcription start point, embedded in the canonical consensus motif 5'-PyPyCAPyPyPyPyPy-3', was identified by primer extension. It is located 27 bp downstream from the centre of a classical TATA box. A DNA sequence identity of 84 %, slightly less than that found for the exon regions, was found for the already sequenced promoter regions of the *CRISP-1* and *-3* genes.

K 346 THE EFFECTS OF AGONISTS AND ANTAGONISTS ON DOWN-REGULATION OF GLUCOCORTICOID RECEPTORS IN A RAT COLONIC ADENOCARCINOMA CELL LINE, Thomas J. Schmidt and Andrew S. Meyer, Department of Physiology and Biophysics, The University of Iowa, Iowa City, IA, 52242

The relative abilities of a potent glucocorticoid receptor (GR) agonist (RU 28362), a weak GR agonist (aldosterone) and a potent GR antagonist (RU 38486) to promote in vivo activation and subsequent down-regulation of GR mRNA and protein levels were compared using the rat colonic PROb cell line. In vivo activation of the GR promoted by 1μM of these different ligands was evaluated in terms of their abilities to deplete cytoplasmic GR protein (consequence of nuclear translocation) after a brief (30 min.) incubation. Western immunoblots with the anti-GR monoclonal antibody BUGR2 demonstrated that treatment with RU 28362 resulted in nearly complete depletion of the cytoplasmic GR, while treatment with aldosterone promoted partial (approximately 50%) depletion. The antagonist RU 38486 depleted the cytoplasmic GR more effectively than aldosterone, but less effectively than RU 28362. Treatment of confluent monolayers with RU 28362 significantly down-regulated both GR mRNA and total cellular GR protein levels. Aldosterone treatment transiently down-regulated GR mRNA levels and also decreased total cellular GR protein levels. Although treatment with RU 38486 did not result in detectable down-regulation of GR mRNA levels, the antagonist very effectively down-regulated total cellular GR protein levels after 18 hours. Collectively these data demonstrate that agonists capable of promoting in vivo activation of GR can subsequently down-regulate receptors at both the mRNA and protein levels. Likewise a pure antagonist which is also capable of promoting activation can dramatically down-regulate GR protein levels, presumably via enhanced degradation, without affecting transcription of the GR gene. Thus target tissue sensitivity to glucocorticoids can potentially be decreased via two mechanisms: agonist-induced repression of GR gene transcription resulting in reduced GR mRNA and protein levels; and/or a ligand-induced reduction in the half-life of the GR protein that occurs independently of transcriptional effects.

K 347 EFFECTS OF PROGESTAGENS ON CELL GROWTH OF HUMAN BREAST TUMOR CELLS. Willem G.E.J. Schoonen, Jan W.H. Joosten, and Helenius J. Kloosterboer, Department of Endocrinology, Organon International B.V., P.O. Box 20, 5340 BH Oss, The Netherlands.

Two types of progestagens, e.g. C_{21}-steroids (Org 2058, medroxyprogesterone acetate, R5020, progesterone) and C_{19}-nortestosterone derived progestagens (3-keto-desogestrel, levonorgestrel, gestodene, norethisterone, Org 30659) were studied for their effect on cell growth of two human breast tumor T47D cell lines of different origin, i.e. from ATCC and Sutherland*. We investigated the effect of estradiol (E_2) and the progestagens at several concentrations alone and of E_2 (10^{-10} M) in combination with the progestagens at several dose levels. Alone, C_{21}-steroids and C_{19}-nortestosterone derived progestagens did not stimulate cell growth in both cell types with exception of C_{19}-nortestosterone derivatives in the ATCC cell line at high (10^{-6} M) concentrations. Estradiol (E_2) showed maximal stimulation at 10^{-10} M in both cell types. With the E_2/progestagen combinations, all tested progestagens did not affect the estrogen-induced growth in the T47D cells from ATCC, whereas these progestagens inhibited cellular proliferation completely in the T47D cells from Sutherland* at doses between 10^{-10} and 10^{-8} M.

The involvement of estrogen and progesterone receptors in the observed growth stimulation was studied by using specific antihormones. In ATCC cells the growth stimulation at high concentrations of C_{19}-nortestosterone derivatives alone and E_2 could be blocked by antiestrogens, like 4-hydroxytamoxifen and ICI 164.384, but not by antiprogestagens, such as RU 38486 and Org 31710. In T47D cells from Sutherland* the estrogen-induced cell proliferation was blocked very efficiently by both antiestrogens and antiprogestagens. In the latter experiments the antiprogestagens appeared to be 10-fold more potent than the antiestrogens. Since antiprogestagens block already the E_2 stimulated growth these compounds are not able to counteract the growth inhibiting effect of progestagens on E_2-stimulation.

In conclusion, our studies show that dependent on the type of permanent cell line used quite different effects were found with progestagens and antiprogestagens on E_2-stimulated growth. Especially, the antiestrogenic effects of antiprogestagens supports the idea that besides progestagens and antiestrogens also this class of antihormones may be used in the treatment and or prevention of certain breast tumors.

*Sutherland et al. Cancer Res. 48: 5054-5091 1988

K 348 CELL-TYPE SPECIFIC REGULATION OF ORNITHINE AMINOTRANSFERASE GENE EXPRESSION BY THYROID HORMONE AND RETINOIDS, James D. Shull[1], Karen L. Pennington[1], James A. Gurr[2], and A. Catharine Ross[3], [1]Eppley Institute, University of Nebraska Medical Center, Omaha, NE 68198; Departments of Biochemistry, [2]Temple University, and [3]Medical College of Pennsylvania, Philadelphia, PA

In the rat, the gene encoding ornithine aminotransferase (OAT) is expressed to varying levels in all tissues and is subject to cell-type specific regulation by numerous hormones including estradiol (E2) and thyroid hormone (T3). To examine this regulation at the molecular level, we cloned and characterized the OAT gene. Two clusters of AGGTCA-like motifs were identified near the promoter that appear to be associated with DNAse hypersensitive sites. As the estrogen (ER), thyroid hormone (T3R), retinoic acid (RAR), and retinoid X (RXR) receptors, among others, appear to function through response elements based upon the AGGTCA motif, we are investigating interactions between E2, T3, and retinoids in regulating OAT gene expression. Herein we report that OAT mRNA in the liver was reduced by 70% in animals maintained on a retinoid deficient diet, and was partially restored within 18 hours of injection of retinoic acid (RA). Interestingly, the effect of RA was more pronounced in retinoid deficient animals made hypothyroid by propylthiouracil (PTU), suggesting possible interactions between T3R and one or more retinoid receptors. In the kidney, OAT mRNA was neither decreased in retinoid deficient animals nor increased in response to RA injection, whereas T3 increased OAT mRNA in the kidney of both retinoid deficient and PTU treated animals. Cotransfection of RXRα or RXRβ into kidney cell lines enhanced basal expression of an OAT/CAT reporter construct; addition of RA (10 μM) inhibited OAT/CAT expression in an RXR-dependent manner. Cotransfection of RXRγ markedly inhibited basal OAT/CAT expression. Together these data indicate that retinoids play a physiological role in regulating OAT gene expression and suggest that the OAT gene will be an informative model for examining interactions between different members of the nuclear receptor superfamily.

K 349 DIFFERENTIAL ACTIVATION OF TRANSIENTLY-TRANSFECTED AND STABLY-INTEGRATED MMTV TEMPLATES BY TRANSIENTLY-TRANSFECTED GLUCOCORTICOID AND PROGESTERONE RECEPTORS, Catharine L. Smith, Ronald Wolford, and Gordon L. Hager, Laboratory of Molecular Virology, National Institutes of Health, Bethesda, MD 20892

Both glucocorticoid (GR) and progesterone (PR) receptors induce transcription from the MMTV promoter. We recently reported that transiently-transfected PR cannot activate a stably-replicating MMTV template with an ordered chromatin structure, whereas it can activate a transiently-transfected MMTV-luciferase template with a disorganized nucleoprotein structure (Smith, C.L. et al., PNAS, in press). These results imply either (1) that the transiently-transfected PR cannot productively interact with the MMTV template when it adopts an ordered nucleosomal structure, or (2) that additional requirements (factors/posttranslational modifications) are necessary for the transfected receptor to activate the chromatin template. These requirements may not be fulfilled during the short term of the transient experiment. Long term expression of the PR in stably-transfected cell lines does result in full activation of the stably-replicating MMTV template in the presence of progestins.

We have now extended these studies to examine the action of transiently-transfected GR and various receptor chimeras on the two MMTV templates, using magnetic bead sorting technology to obtain cell populations enriched for transfected cells. We find that our previous observations extend to these receptors also, implying that transiently-transfected receptors are generally defective for activation of the MMTV template with an ordered nucleoprotein structure. These results lead us to propose that there are receptor processing events or receptor-associated accessory factors that are uniquely necessary for productive interactions of the GR and PR with the MMTV promoter in its native nucleoprotein conformation.

K 350 REGULATION OF THE K#14 KERATIN GENE BY THYROID HORMONE AND RETINOIC ACID.

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Transcription of the keratin genes is affected by thyroid hormone (T3) and retinoic acid (RA) receptors. We have analyzed in detail the regulation of the basal-cell specific keratin K#14 and found that it is downregulated by both receptors in the presence of their respective ligands, but that it is upregulated by the unliganded T3 receptor. Using a mutant T3 receptor that lacks the DNA binding domain we showed no effect on K#14 expression. Thus direct binding to the DNA is necessary for regulation. On the other hand, the V-erb-A mutant which lost the ability to bind T3, caused induction of the K#14 expression. Thus, the unliganded T3 receptor is a constitutive inducer. Using gel-shift experiments, we identified the RA/T3 receptor binding site at position -95. It contains 5 TREpal-like half sites in various relative positions. Using site-specific mutagenesis we confirmed that the -95 region is necessary for regulation since mutations in that region abolish the regulation. However, when introduced to the MMTV promoter this region is no longer affecting transcription. Interestingly, addition of 50bp from the K#14 DNA restored the downregulation in MMTV promoter. This 50bp segment was shown to be an enhancer. Therefore, regulation of the K#14 keratin gene by nuclear receptors for RA and T3 proceeds by regulating the nearby enhancer.

K 351 DIFFERENTIAL DIMERIZATION PROPERTIES OF MUTANT β1 THYROID HORMONE RECEPTORS AND VARIABLE DOMINANT NEGATIVE ACTIVITY IN MAN, S.J. Usala, E. Hao, J.B. Menke, M.E. Geffner, J.M. Hershman, H.H. Samuels and D.K. Ways, East Carolina University School of Medicine, Greenville, NC 27858, UCLA School of Medicine, Los Angeles, CA 90024, New York University Medical Center, NY 10016

The S receptor (deletion of THR332) is a potent dominant negative protein cloned from a kindred with generalized resistance to thyroid hormones. The G-H receptor (ARG311HIS) has reduced dominant negative function and was found in both normal individuals and in a patient with severe pituitary resistance to thyroid hormone. We have investigated the mechanism responsible for the difference in receptor phenotypes by analyzing the binding of S and G-H receptors to TREs with gel-shift. Wild-type human c-erbAβ1 (WT), S, and G-H receptors were synthesized in reticulocyte lysate, reacted with DR+4, IR (TREpal), IR+5, or F2 (lysozyme gene silencer element), and the products analyzed by gel-shift. G-H homodimerization was greatly reduced with DR+4 and F2 compared to WT, and formed predominantly monomeric complexes. The G-H receptor was able to form heterodimeric DR+4 complexes with TRAP factors from Cos-7 and HeLa cells and with human RXRα synthesized in reticulocyte lysate, but the level of heterodimeric complex was reduced 2-3-fold compared to WT when TRAP was limiting. In contrast to WT and G-H, the S receptor formed almost exclusively a homodimeric complex with DR+4. Heterodimeric S formation with DR+4 was slightly increased compared to WT. Although IR+5 was non-dimer permissive for WT and G-H, homodimeric complexes were visualized with S. In the presence of thyroid hormone, WT (but not G-H) bound as a homodimer with IR+5. Qualitatively similar results were seen with IR. In conclusion, the G-H mutation predominantly reduced homodimerization, although perturbation of heterodimerization was also detected. The S mutation augmented dimerization activities. These results indicate that the dimerization properties of the mutant receptors are important in modulating the level of thyroid hormone resistance in man.

K 352 RXRα AND RARβ ACTIVATE TRANSCRIPTION THROUGH DIFFERENT MECHANISMS IN A CELL-SPECIFIC MANNER, Paul T. van der Saag and Gert E. Folkers, Hubrecht Laboratory, Netherlands Institute for Developmental Biology, Utrecht, the Netherlands.

We have shown previously (Folkers et al., Mol. Endocr. **7** (1993), 616) that RARβ2 contains two transactivation domains: AF-1 located in the A domain acts as a constitutive activator, and AF-2 in the ligand-binding domain functioning in a ligand-dependent fashion. Both activating functions display cell-specificity, for which there is presently no explanation. In the RARβ2 promoter we have identified a CRE-like sequence upstream from the RARE which contributes to induction of RARβ2 in vitro (Kruyt et al., NAR **20** (1992), 6393). The role of adenoviral 13S E1A as a co-activator of RARβ2 induction by retinoic acid can be partially explained by our findings that E1A uses this CRE-like element as a target sequence (Kruyt et al., Mol. Endocr. **7** (1993), 604) and that cellular E1A-like activity can function in a similar way.

We now have investigated the activity of RARβ and RXRα AF-1 and AF-2 coupled to GAL4 DNA-binding domain on two different promoters (E1B-tata and tk) each carrying one or five GAL-binding sites. From these experiments we conclude that AF-1 and AF-2 function by different mechanisms as AF-1 acts synergistically only on the E1B-tata reporter containing five GAL-binding sites. Transfection of these AF constructs into various cell lines indicated that both regions display differential cell-specific activation. With respect to AF-2 these results could be explained by the presence of viral E1A or cellular E1A-like activity in 293 and P19EC cells, respectively. Cotransfection of E1A constructs showed that the activating function of RARβ AF-2 was enhanced by 13S E1A, while no enhancement was observed for RXRα AF-2. These results demonstrate that co-activators are important in retinoid-mediated signalling pathways and that E1A (-like) co-activators activate RARs and RXRs differentially.

K 353 RETINOID X RECEPTOR α MEDIATES THE TRANSCRIPTION OF α-FETOPROTEIN GENE INDUCED BY RETINOIC ACID, Yu-Jui Y. Wan, Theresa Pan and Tsung-Chieh J. Wu, Department of Pathology, Harbor-UCLA Medical Center, Torrance, CA 90509. Department of OB/GYN, UCLA Medical Center, Los Angeles, CA 90024.

We have previously demonstrated that all-trans-retinoic acid (t-RA) exerted similar effect as glucocorticoid analogue dexamethasone in inducing the differentiation of rat hepatoma McA-RH8994 cells. Both t-RA and dexamethasone increased the α-fetoprotein (AFP) and albumin gene expression in McA-RH8994 cells, and resulted in differentiated fetal hepatocyte phenotype. In the present study, we further characterized the molecular mechanism involved in the induction of AFP mRNA by retinoic acid (RA) in McA-RH8994 cell line. Various doses of both t-RA and 9-cis-RA were used to treat the cells; then northern blot hybridization was performed to quantitate the levels of AFP mRNA by densitometry. The results demonstrated that 10^{-7}M of 9-cis-RA induced the AFP mRNA amount threefold after three days of treatment. In contrast, 10^{-7}M of t-RA was ineffective in inducing AFP mRNA within three days. For all three doses tested (10^{-7}, 10^{-6}, and 10^{-5}M), 9-cis-RA was more effective than t-RA. High concentration of t-RA (10^{-5}M) was needed to significantly induce the AFP mRNA in the McA-RH8994 cells. This data suggest that activation of retinoid x receptor (RXR) is crucial for the regulatory process. To further analyze the role of each type of RXRs in this regulatory process, the entire 7.3 Kb regulatory region of AFP gene was fused with chloramphenicol acetyltransferase (CAT) gene and used for co-transfection. Overexpression of RXRα alone in McA-RH8994 cells increased the fold of induction in CAT activity by RA. In addition, the half life of AFP mRNA was measured in RA treated and untreated McA-RH8994 cells. RA did not change the half life of AFP mRNA. Conclusion: 1) RA induces AFP mRNA by increasing the transcription of the AFP gene; 2) RXRα, which is liver specific, participates in the regulation of AFP gene transcription.

K 354 GENE ACTIVATON BY ANTIESTROGENS VIA A COMPLEX OF ESTROGEN RECEPTOR AND AP-1, Paul Webb, Gabriela N. Lopez and Peter J. Kushner, Metabolic Research Unit, University of California, San Francisco, CA 94143

Antiestrogens, such as tamoxifen and ICI 164,384, are usually pure antagonists when tested on classical palindromic estrogen response elements (EREs). Nevertheless, antiestrogens sometimes exhibit potent, puzzling, and unexplained estrogen-like effects on transcripton. Tamoxifen, for example, has potent estrogenic activity on growth and gene transcription in endometrial cells.

Here, we show that the human collagenase gene promoter exhibits an estrogen response in transfected mammalian cell lines mediated by the AP-1 site at -70 b.p. from the start of transcription. The response can be as strong as that mediated by an ERE and can occur at physiological levels of receptor in endometrial cell lines This "indirect" estrogen response pathway may, therefore, be widespread. Remarkably, antiestrogens are agonists at the collagenase AP-1 site, whereas they lack agonist activity when tested on a promoter in which the AP-1 site has been replaced by an ERE. The agonist activity of antiestrogens does not require the ER DNA binding domain but does require both the N-terminal and C-terminal domains. Transfection of F9 cells indicates that the indirect estrogen response requires Jun and Fos proteins as well as estrogen receptor (ER). However, the Jun-ER ratio, but not the Fos-ER ratio, is critical to the response. Moreover, either full length or C-terminal domain of the ER binds Jun, but not Fos, protein in solution. These observations suggest that the indirect response may occur when ER "piggybacks" on Jun/Fos bound to AP-1 sites. In support of this model we find that VP16-ER fusions are more potent than wild type ER as activators at AP-1 sites, parallel to their action at an ERE.

K 355 STRUCTURAL AND FUNCTIONAL ANALYSIS OF THE MOUSE GENOMIC LOCUS ENCODING THE ß2 THYROID HORMONE RECEPTOR, William M. Wood, Janet M. Dowding, Virginia D. Sarapura, Bryan R. Haugen and E. Chester Ridgway, Department of Medicine, U.C.H.S.C., Denver, CO 80262.

Generation of the ß2 isoform of the thyroid hormone receptor (TRß2) appears to be derived from the TRß gene locus by alternate promoter usage and differential splicing of a unique amino-terminal region to the same DNA and ligand binding domain exons utilized by the TRß1 isoform. Expression of the TRß2 isoform is mainly restricted to the anterior pituitary gland where it is present in all hormone-secreting cell types. T3 down-regulates TRß2 mRNA levels in rat pituitary-derived GH3 cells and mouse TtT-97 thyrotropic tumors. These unique expression and regulatory properties prompted us to clone and characterize the genomic locus encoding the TRß2 isoform. Screening of mouse genomic libraries yielded several overlapping clones which contained all of the TRß2 coding exons. The unique TRß2 amino-terminal region was contained within a single exon separated from the DNA binding domain exons by at least 25 kb. The common TRß exon sizes and location of the intron/exon junctions were perfectly conserved when compared to the human TRß1 and TRα genes. The position of the most upstream in frame AUG in the unique mouse TRß2 exon (designated +1) was 117 nts downstream of that reported for a rat cDNA isolated from GH3 cells. RNase protection assays performed with TtT-97 tumor RNA identified a major transcriptional site 71 bp upstream of the AUG codon although other minor protected bands mapping both upstream and downstream of this were also evident. This major start site as well as two of the downstream sites were substantiated by anchored PCR analysis. When a fragment containing 465 bp upstream of the AUG codon and extending to +40 was fused to a luciferase reporter and transfected into several cell types its activity as a promoter reflected the endogenous expression of TRß2 mRNA (GH3 cells, 9000; TtT-97, 4000; and αTSH cells, 400 light units (L.U.) relative to $1x10^6$ L.U. for an RSV promoter construct. αTSH cells are a thyrotrope-derived cell line that expresses very low levels of TRß2 transcripts. The promoter activity in GH3 cells was decreased to 20% of control levels by treatment with T3 demonstrating that T3 regulation *in vivo* is in part reflected at the level of transcription. 5' deletion studies reveal that no decrease in promoter activity was observed by truncation to position -204 but removal of a further 120 bp which deletes a consensus Pit-1 binding site (TTTATTCAT) reduces basal expression in GH3 cells by 90%. In conclusion, we have cloned the murine gene locus encoding the pituitary-specific TRß2 isoform and have demonstrated that a 465 bp region immediately upstream of the translation initiation codon contains the transcriptional signals responsible for pituitary cell type expression and T3 regulation.

K 356 THE FUNCTIONAL AND PHARMACOLOGICAL ECDYSONE RECEPTOR IS THE PRODUCT OF ECR AND ULTRASPIRACLE

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The biological activity of the insect molting hormone, ecdysone, is manifested through a hormonally regulated transcriptional cascade associated with chromosomal puffing. These events are thought to be initiated upon binding of hormones to a nuclear ecdysone receptor complex, however, a direct association of the receptor with the puff has yet to be established. The cloned ecdysone receptor (EcR) is by itself incapable of high affinity DNA binding or transcriptional activation. Rather, these activities are dependent on heterodimer formation with ultraspiracle (USP) the insect homologue of vertebrate RXR. Here we show that native EcR and USP are co-localized on ecdysone responsive loci of polytene chromosomes. Moreover, we demonstrate that natural ecdysones selectively promote physical association between EcR and USP and conversely, that high affinity hormone binding requires both EcR and USP. This unusual interdependence between receptor heterodimerization and ligand binding is novel for nuclear receptor and redefines the ecdysone receptor as a dynamic complex whose activity is determined by combinatorial interactions among subunits and ligand.

K 357 ALTERATION OF RETINOID RESPONSES IN HUMAN LUNG CANCER CELL LINES. Xiao-kun Zhang and Magnus Pfahl. Cancer Research Center, La Jolla Cancer Research Foundation, 10901 N. Torrey Pines Rd., La Jolla, CA 92037.

Responses to retinoids are mainly mediated by their nuclear receptors, the retinoic acid receptors (RARs) and retinoid X receptors (RXRs). In many human lung cancer cell lines, the RARβ gene is not expressed and/or its expression does not respond to RA treatment. The effect of RA on RARβ gene expression is largely controlled by a RA response element (βRARE) in RARβ gene promoter. We have investigated the possible molecular mechanisms that account for the abnormal RARβ gene expression in human lung cancer cell lines. Our transfection results demonstrate that endogenous retinoid receptors are functional. However, the transfected RARβ promoter displayed impaired activity in some of the RARβ-negative lung cancer cell lines. The impaired RARβ promoter activity may be due to the loss or defect of protein factors required for RARβ promoter activation as revealed by gel retardation assays. Other mechanisms could also account for the loss of RARβ gene expression. In one of the RARβ-negative cell lines, the cotransfected RARβ promoter could be activated by RA. In addition, the nuclear proteins prepared from this cell line exhibited a normal RARβ promoter binding pattern. Thus, failure of expressing RARβ in this cell line may be due to mutations present in the endogenous RARβ promoter, that affect retinoid receptor activities. Together, different mechanisms exist that are responsible for the loss of RARβ gene expression in human lung cancer cell lines.

Poster Session IV

K 400 EXPRESSION AND DNA - BINDING OF A NOVEL MEMBER OF THE STEROID/THYROID SUPER-FAMILY OF HORMONE RECEPTORS, Rainer Apfel and Magnus Pfahl, La Jolla Cancer Research Foundation, La Jolla, CA 92037

We have identified and characterized a new orphan member of the nuclear hormone receptor superfamily, called RLD-1, that is predominantly expressed in spleen, pituary gland and fat-tissue. RLD-1 selectively binds and transactivates a retinoic acid response element which consists of direct repeats related to the consensus sequence AGGTCA. RLD-1 binds to these elements as a heterodimer with the 9-cis-retinoic acid receptor RXR.

Transactivation of this type of retinoic response element does not require the presence of all-trans retinoic acid, 9-cis-retinoic acid, Thyroin-hormone T-3, or any other exogenously added ligand tested so far. We propose that RLD-1 may play an important role in the complex network of the nuclear hormone receptor superfamily.

K 401 A SYSTEMATIC ANALYTICAL CHEMISTRY/ CELL ASSAY APPROACH TO ISOLATE ACTIVATORS OF ORPHAN NUCLEAR RECEPTORS FROM BIOLOGICAL EXTRACTS : CHARACTERIZATION OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR ACTIVATORS IN PLASMA, Carol D. Banner, Martin Göttlicher, Eva Widmark, Jan Sjövall, Joseph J. Rafter and Jan-Åke Gustafsson, Department of Medical Nutrition, Karolinska Institute, Huddinge University Hospital F60, NOVUM, 141 86 Huddinge, Sweden

Using a novel combination of analytical chemical and molecular biological techniques, lipophilic components of human plasma separated according to their physico-chemical properties were screened for their ability to activate the rat peroxisome proliferator activated receptor (rPPAR). Activation of a rPPAR/glucocorticoid receptor chimera stably expressed in CHO cells by fractions in the initial screening guided further subfractionation. Characterisation of an active subfraction by gas chromatography alone and in combination with mass spectrometry (GC/MS), indicated the presence of free fatty acids. Individual active components in this mixture were isolated by a final fractionation using high performance liquid chromatography (HPLC). GC/MS analyses of HPLC fractions able to activate the chimeric receptor identified palmitic acid, oleic acid, linoleic acid and arachidonic acid as endogenous activators of rPPAR. No other activators were identified. This approach is able to specifically extract and identify endogenous activators of PPAR from a complex biological extract and as such may be valuable in the identification of activators of other orphan receptors in the steroid hormone receptor superfamily.

K 402 RZRS: A NEW FAMILY OF RETINOID RELATED ORPHAN RECEPTORS THAT FUNCTION AS BOTH MONOMERS AND DIMERS, Michael Becker-André, Carsten Carlberg,* and John F. DeLamarter, Glaxo Institute for Molecular Biology, CH-1228 Plan-les-Ouates, Genève, Switzerland, and (*) Clinique de Dermatologie, Hôpital Cantonal Universitaire, CH-1211 Genève 14, Switzerland.

Members of the superfamily of nuclear receptors share the greatest homology in their DNA binding domains. We have used reverse transcription-polymerase chain reaction (RT-PCR) and highly degenerate primers based on the amino acid sequence of the zinc finger motif of known nuclear receptors to identify novel members of the family. Starting with rat brain RNA we have isolated an orphan receptor which we call RZRβ. The sequence of its near full-length cDNA shows high similarity to RZRα, a receptor we recently identified from human umbilical vein endothelial cells (HUVECs). These RZR subtypes represent members of a new family of orphan nuclear receptors that most likely regulate specific gene expression. Sequence comparison with other known nuclear receptors reveals high similarity for both RZR subtypes to retinoic acid (RA) and retinoid X receptors (RARs and RXRs). By Northern blot analyses we found RZRβ mRNA only in rat brain, whereas RZRα is expressed in many tissues. We show here that the RZRs bind as monomers to natural retinoid response elements formed by $(^{A}/_{G})$GGTCA half sites. However, a T-residue in the -1 position of this motif greatly enhances the DNA binding affinity of RZRs, while the -2 position has no influence. We show that RZRs can bind as hmodimers on response elements formed by palindromes, inverted palindromes or direct repeats of two TAGGTCA half sites. Interestingly, these response elements display dramatically reduced affinity for RAR-RXR heterodimers. Thus the 5'flanking sequences of hexameric half-sites appears to be crucial to direct the affinity of several nuclear receptors. On monomeric as well as on dimeric binding sites RZRs show constitutive transactivational activity that, however, can be enhanced by unidentified components of fetal calf serum.

K 403 DROSOPHILA ECDYSONE RECEPTOR MUTATIONS REVEAL FUNCTIONAL DIFFERENCES BETWEEN RECEPTOR ISOFORMS, Michael Bender [1], William S. Talbot, Scot D. Munroe and David S. Hogness, Department of Developmental Biology and Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305
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In Drosophila, the steroid hormone ecdysone triggers the larval to adult metamorphosis, a complex process in which many tissues in the animal die while others differentiate to form the adult structures. The ecdysone receptor (EcR) gene encodes three ecdysone receptor isoforms (EcR-A, EcR-B1 and EcR-B2) with common DNA and hormone binding domains but distinct N-terminal sequences. Through the use of two promoters, EcR proteins are expressed with different tissue-specific and developmental profiles, suggesting that alternative EcR protein forms may contribute to distinct tissue-specific or developmental responses to ecdysone.

To assign functions to the different EcR proteins, we have isolated a large number of EcR mutants and molecularly mapped many of these using PCR and denaturing gradient gel electrophoresis. Thus far we have obtained mutants which inactivate all EcR isoforms as well as mutants which inactivate only EcR-B1. The different phenotypes of these two classes of EcR mutants establish distinct temporal requirements for alternate EcR proteins and support a model in which different EcR proteins contribute to distinct tissue-specific responses to ecdysone. In addition, the panel of EcR mutants which map to the EcR DNA binding and hormone binding domains provide a basis for the biochemical dissection of EcR functions (See poster by Arbeitman et al.).

The question now arises as to whether the different EcR proteins are themselves functionally distinct or whether the discrete developmental functions revealed by EcR mutants might derive solely from different expression patterns of the EcR isoforms. Recently, we have begun to address this question by using transgenes which express different EcR isoforms to rescue EcR-B1 mutants. The results of experiments assaying rescue of viability and rescue of EcR target gene expression in these mutants will be presented.

K 404 RETINOIC ACID RECEPTORS AND LIMB DEVELOPMENT, David E. Cash,[1] T. Michael Underhill,[1] Cheryl B. Bock,[2] Klaus Schughart,[3] and Elwood Linney.[1]
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A major aim of developmental biology is to understand the molecular networks that regulate morphogenesis and differentiation in developing embryos. The limb has been used as a model system to study the mechanisms that lead to pattern formation, and extensive classic embryological experiments on vertebrate limb buds have identified many of the cell interactions involved. Recent experiments have begun to elucidate the molecular basis of some of these cell interactions, and indicate that retinoids, the collective term for derivatives of vitamin A, may be involved in the process of limb morphogenesis. The discovery that retinoic acid (RA) acts through a family of nuclear receptors provided insight into how RA may regulate events which take place during limb development. There are three types of nuclear receptors for RA in mammals, RARα, RARβ and RAR γ, and although all three are differentially expressed in the developing murine limb, their individual roles in pattern formation remains unclear.

We have examined the *in vivo* activity of a mutant RARα that exhibits weak constitutive transactivation properties in transient expression assays in the mouse P19 embryonal carcinoma cell line. When this constitutively active RARα construct is targeted to the developing limbs in transgenic mice it creates a novel inductive field, with concomitant changes in pattern formation, as manifested by congenital limb malformations. The use of a constitutively active RAR to study the role of retinoids in limb development has the advantage of allowing one to examine the role that an individual receptor may play when activated by ligand. To that end, we are currently analyzing changes in the expression patterns of putative downstream RA-responsive genes by whole-mount *in situ* techniques. Additionally, we are examining the *in vivo* activity of other constitutively active retinoid receptors.

K 405 HORMONE-INDEPENDENT ACTIVATION BY A NOVEL MURINE ORPHAN RECEPTOR, Hueng-Sik Choi, Wongi Seol, Tod Gulick, Devendranath Simha and David D. Moore, Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114

We have isolated clones encoding two isoforms of a new orphan receptor, called CM49, from a mouse liver cDNA library. The amino acid sequence of one isoform, CM49A, is very similar to that of MB67/CAR, another orphan receptor isolated in this laboratory over its full length. CM49B differs from CM49A by a 105 nucleotide deletion within the putative ligand binding or E domain and encodes a truncated protein. Like MB67, CM49 mRNA is predominantly expressed in the liver. Also like MB67, CM49A activates reporter constructs containing the DR5 retinoic acid response element from the RARβ2 promoter (βRARE), in the absence of added retinoids or any other exogeneously added ligands. CM49A also activates the intact RARβ2 promoter via the RARE. As expected, CM49B shows neither effect. Among a series of reporters with synthetic direct repeats from DR0 to DR6, only DR4 was activated by CM49A. In contrast to the stimulatory effect observed with MB67, the activation of both the βRARE and the synthetic DR4 is strongly inhibited by cotransfection with RXR.

K 406 THE LOCAL SITES OF RETINOID MEDIATED GENE ACTIVATION ARE COINCIDENT WITH RETINOID RECEPTORS AND RETINOID BINDING PROTEINS IN THE DEVELOPING MOUSE SPINAL CORD, Melissa C. Colbert, Anthony-Samuel LaMantia and Elwood Linney, Dept of Microbiology and Neurobiology, Duke University Medical Center, Durham, NC, 27710

The relationship between the sources of retinoic acid (RA) in embryos, the molecules that transduce retinoid signals, and their targets are not well understood. Previously, we have demonstrated that distinct domains of retinoid-activated transgene expression in the developing mouse spinal cord are related to local sources of RA. Here we show that proteins involved in RA signaling and homeostasis like the RA receptor β(RAR β), cellular retinol binding protein I(CRBPI), and cellular RA binding protein II(CRABP II) are also preferentially expressed at these sites. In contrast, the distribution of other components of this regulatory pathway, such as CRABP I, other RARs, and the retinoid X receptors (RXRs) may be less restricted. Retinoid signaling in the embryonic day (E)13 mouse spinal cord is localized primarily to the alar regions of the cervical and lumbar, but not thoracic or sacral cord, based upon studies in transgenic indicator mice. To examine the distribution of regulatory proteins which may affect the response to-or regional availability of-RA, we have performed *in situ* hybridization on whole E13 spinal cords. mRNA for RARβ is localized to the alar region of the cervical and lumbar cord; in addition, CRBP I & CRABP II show enhanced expression in basal portion of these domains. The mRNA for CRABP I is uniformly distributed along the length of the spinal cord. Finally we have examined the distribution of RAR α, as well as RXRs α and β to determine if receptor pair specificity may be significant. Regional localization of RAR-β, CRBPI, and CRABP II, which are themselves RA regulated, may serve to restrict the field of action of RA in the developing spinal cord. This restriction may play a role in the establishment of cervical and lumbar enlargements in the spinal sensory and motor columns. Region specific regulation via localized RA sources in the cervical and lumbar cord may influence the differentiation and maintenance of neurons related to the developing limbs. In contrast, RA, RAR-β and CRABP II are diminished in the thoracic region, which is devoid of limbs and the site of enhanced neuronal cell death at this time. Therefore, local retinoid induction in the central nervous system may be important in the establishment and maintenance of patterned connections related to peripheral targets as well as acting as a general signal for neuronal differentiation.

K 407 ANALYSIS OF THE EXPRESSION PATTERN OF A NOVEL ORPHAN MEMBER OF THE NUCLEAR RECEPTOR SUPERFAMILY, Austin J. Cooney, Fang Chen, Yaolin Wang and Bert O'Malley, Department of Cell Biology, Baylor College of Medicine, 1 Baylor Plaza, Houston, TX 77030.

Nuclear receptors are a superfamily of ligand activated transcription factors. Each of these receptors have significant homology to each other in the DNA binding domain (DBD) and and to a lesser extent in the ligand binding domains (LBD). Based on the high homology in the DNA binding domain cloning strategies were devised in which an ever expanding subfamily of receptoroids have been cloned. These novel members of the nuclear receptor superfamily were termed orphan receptors as their functions and putative ligands are unknown. Here we describe the cloning of a novel orphan receptor which is quite distinct from previously cloned members of the superfamily. An adult mouse heart cDNA library was screened under low stringency conditions using a DNA fragment encoding the DBD of the mouse homologue of hNUC1 as a probe. One of the identified clones was found to encode a novel nuclear receptor DBD. Alignment of the amino acid sequence with the other family members showed that it was most closely related to the retinoic acid receptor RAR and a recently published orphan receptor RZR, whose closest relative is also RAR. However, it only shares 60% and 59% amino acid identity with RZR and RAR in the DBD respectively, indicating that it is not closely related to previously cloned members. In contrast, in the LBD this orphan receptor is more closely related to RXR than to either RAR or RZR. Northern analysis identified two messages one of 2.4 Kb and a larger one of 7.5 Kb. The two messages could be related at the RNA processing level due to alternate splicing, alernative initiation or may encode two closely related factors. Analysis of the tissue distribution of the mRNA showed that it is most highly expressed in the testis relative to other tissues and may be considered a testis specific receptor. Expression of the gene is also detected in the ovaries, liver and kidney. From this expression pattern its high level expression in steroidogenic tissues suggest it may play a role in steroidogenesis in addition to other functions.

K 408 THE SEA URCHIN ORPHAN RECEPTOR SpCOUP-TF IS AN ESSENTIAL GENE EXCLUSIVELY EXPRESSED IN THE EMBRYONIC ECTODERM, Constantin N. Flytzanis, Siu-Ming Chan and Naidi Xu, Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030

Injection of SpCOUP-TF antisense oligonucleotides in fertilized eggs of the sea urchin *S purpuratus* results in developmental arrest of the ensuing embryos before hatching. The observed effect depends upon the concentration and the sequence specificity of the oligonucleotides. This result suggests an essential embryonic function for this orphan receptor.

The embryonic localization of SpCOUP-TF mRNA was studied by whole mount *in situ* hybridization. The transcripts are exclusively detected in the ectoderm of the gastrula and pluteus stage embryos and the presumptive ectoderm of the blastula stage. Furthermore, localization is restricted in the embryonic lineage of the aboral ectoderm. The localization and blastomeric distribution of the maternal SpCOUP-TF mRNA and protein will be discussed.

K 409 ISOFORM-SPECIFIC N-TERMINAL DOMAINS DICTATE DNA-BINDING PROPERTIES OF RORα, A NOVEL FAMILY OF ORPHAN NUCLEAR RECEPTOR, Vincent Giguère, Mark Tini, Grace Flock and Gail Otulakowski, Department of Molecular and Medical Genetics, University of Toronto, and Research Institute, Hospital for Sick Children, Toronto, Canada M5G 1X8

Three isoforms of a novel member of the steroid hormone nuclear receptor superfamily related to the retinoic acid receptors have been identified. The three isoforms, referred to as RORα1, RORα2 and RORα3 share common DNA- and putative ligand-binding domains but are characterized by distinct N-terminal domains generated by alternative RNA processing. An exon encoding a functionally important subregion of the N-terminal domain of the RORα2 isoform resides on the opposite strand of a cytochrome C processed pseudogene. Binding site selection using *in vitro* synthesized proteins reveals that the RORα1 and RORα2 isoforms bind DNA as monomers to hormone response elements composed of a 6 bp AT-rich sequence preceeding a half-site core motif PuGGTCA (RORE). However, RORα1 and RORα2 display different binding specificities: RORα1 binds to and constitutively activates transcription from a large subset of ROREs whereas RORα2 recognizes ROREs with strict specificity and displays weaker transcriptional activity. The differential DNA binding activity of each isoform maps to their respective N-terminal domains. While truncation of the N-terminal domain diminishes the ability of RORα1 to bind DNA, a similar deletion relaxes RORα2 binding specificity to that displayed by RORα1. Remarkably, tranfer of the entire N-terminal region of RORα1 or N-terminal deletion of RORα2 confers RORE-binding specificities to the thyroid hormone receptor. These results demonstrate that the N-terminal domain and the zinc finger region work in concert to confer high affinity and specific DNA-binding properties to the ROR isoforms and suggest a novel strategy to control DNA-binding activity of nuclear receptors.

K 410 FATTY ACIDS REGULATE MITOCHONDRIAL HMG-COA SYNTHASE GENE EXPRESSION MEDIATED BY PPAR.

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Hepatic ketogenesis produce important fuels for the brain and peripheral tissues during episodes of starvation or sustained exercise. Ketone bodies can also act as fuels for the developing brain and lactating mammary gland. For these reasons complete understanding of how this metabolic process is controlled is of great importance. It has been suggested that mitochondrial HMG-CoA synthase is, with carnitine palmitoyltransferase I, another important site of control of ketogenesis in different metabolic situations. It is known that fat feeding increases ketogenesis. There are also evidences of the regulation of mitochondrial HMG-CoA synthase activity by fatty acids. Fatty diet in rats increases HMG-CoA synthase mRNA levels and fatty acids added to HepG2 cells increases mitochondrial HMG-CoA synthase-CAT quimeric gene transcription. The combination of these processes results in a dual control of ketogenesis by increasing the amount and the activity of the enzime.

It has been shown that fatty acids can regulate gene expression mediated by a member of the steroid nuclear receptor superfamily termed Peroxisome Proliferator Activated Receptor (PPAR). Recent results have demonstrated that PPAR can bind to a specific DNA sequence (Peroxisome Proliferator Response Element, PPRE) located upstream of the rat Acyl CoA oxidase gene and that this element is also present in the 5' region of other peroxisomal β-oxidation related genes.

This report shows that mitochondrial HMG-CoA synthase gene promoter contains a responsive element to PPAR that has been precisely localized. In HepG2 cells the response to that receptor is increased by the addition of exogenous ligand. In addition, it is shown that the activation of mitochondrial HMG-CoA synthase gene expression by fatty acids could be mediated by this receptor. In conclusion PPAR is related with lipids metabolism not only by inducing β-oxidation genes, but increasing another pathway of fatty acids disposal as it is ketogenesis.

K 411 THE ISOLATION AND CHARACTERIZATION OF MINOR, A NOVEL MITOGEN-INDUCIBLE NUCLEAR ORPHAN RECEPTOR, Cyrus V. Hedvat and Steven G. Irving, Department of Pathology, Georgetown University School of Medicine, Wash., D.C. 20007

We have sequenced an immediate early gene isolated from a mitogen-induced human T cell cDNA library. The open reading frame encodes a protein of 586 amino acids which, by sequence homology, is a previously undescribed member of the steroid receptor superfamily. MINOR (mitogen-inducible nuclear orphan receptor) is most similar to nur77/NGFI-B and NURR-1/RNR-1 which are considered orphan members of the family since they have no known ligands. The DNA binding domains of these proteins are highly conserved, with considerably less similarity in the N-, and C-termini. Expression of MINOR is not detected in resting T cells; however, expression is stimulated in response to mitogens such as PHA, PMA, or calcium ionophore. Likewise, MINOR is expressed in fibroblasts only upon serum stimulation.

In addition, based on DNA binding specificity, we show that MINOR binds to the same sequence as NGFI-B, and thus can be grouped into a subclass of receptors including NGFI-B and SF-1 that bind to DNA as monomers. The binding site for these receptors contains a half ERE with nucleotides 5' of the half site being important to specificity. Given the divergence of putative transcriptional activation and other domains among the members of this subclass of receptors, the inducible and possible cell type specific expression of this class of regulatory proteins may play an important role in specific gene regulation by proteins which recognize identical elements.

K 412 IDENTIFICATION OF A NEW ORPHAN RECEPTOR THAT IS SELECTIVELY EXPRESSED IN THE MEIOTIC STAGE OF SPERMATOGENESIS.

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Members of the retinoic acid/steroid/thyroid hormone receptor superfamily share the highest degree of homology in their DNA binding domains. Highly degenerate PCR primers were designed based on the conserved amino acid sequences of the DNA binding domains of members of retinoid receptors (RARs and RXRs). Using these degenerate primers PCR was performed with lymphoblastoma cDNA as template in order to discover new members of this receptor superfamily. Fragments of 13 different nuclear receptors (NRs) were identified in a single PCR experiment. Among these NRs, 12 represented known receptors and one encoded the DNA binding domain of a new orphan receptor, which we named TAK1. Using anchor PCR method, we obtained a 600bp fragment of TAK1. A full-length cDNA of TAK1 was obtained by screening a human testis cDNA library with the 600 bp TAK1 fragment TAK1 encodes a 2.8kb mRNA that is selectively and highly expressed in the testis. In situ hybridization studies using sections of mouse testis revealed that TAK1 is selectively expressed in primary spermatocytes, and not in other germ or somatic cells, suggesting a primary role for this putative transcription factor in the regulation of gene expression in spermatogenesis and in particular in meiosis.

K 413 IDENTIFICATION OF RXR HOMOLOGS IN *DIROFILARIA IMMITIS* AND *C. ELEGANS*, David M. Hough, Jennifer Richer, and Claude V. Maina. New England Biolabs, Beverly MA 01915.

We are studying the genetic regulation of molting in the nematodes *C. elegans* and *D. immitis* through the use of ecdysone and its receptor, EcR (see abstract by Richer *et al.*). Drosophila EcR is a member of the nuclear hormone receptor (NHR) superfamily (Koelle *et al.* 1991, *Cell* **67**:59-77) and requires the presence of ecdysone and another protein ultraspiracle, *usp*, to activate transcription (Yao *et al.* 1992, *Cell* **71**:63-72). *usp*, another member of the NHR superfamily, is a homolog to the vertebrate retinoid x receptor (Oro*et al.* 1990, *Nature* **347**:298-301). To determine if a nematode EcR behaves as does the Drosophila EcR, we have initiated a search for nematode *usp* homologs.

We have used the Drosophila *usp* to search for *D. immitis* and *C. elegans* homologs by PCR with degenerate primers to the DNA binding domain. Using genomic DNA from the two organisms as a template, we have amplified 2 fragments from *C. elegans* (*crf-6* and *crf-7*), and 1 from *D. immitis* (*dirf-4*) ranging in size from 137 to 190 bps. The predicted amino acid sequence for each of the 3 shows significant similarity to the NHR superfamily. The sequence of *dirf-4* shows 93% similarity at the amino acid level to Drosophila *usp*. While *crf-4* shows less similarity to *usp* (68%), we are pursuing it further because it shows cross hybridization to *dirf-4* and most matches in a TFASTA search of Genbank/EMBL are to RXR sequences.

Neither of the *C. elegans* NHR's have been described previously and they have been mapped to the genome. *crf-6* maps to chromosome III and cosmid DF12. *crf-7* shows hybridization to 2 non-contiguous regions of the genome, strong hybridization to chromosome II and weak hybridization to chromosome IV. We have obtained a cDNA for *crf-6* from a screen of a mixed stage cDNA library. The *D. immitis* RXR homolog is the first to be described for a nematode and we are presently screening an adult female *D. immitis* cDNA library for a cDNA copy of this gene. Once cDNA's are obtained, the biochemistry and genetic regulation of these nematode RXR homologs will be studied.

K 414 A novel estrogen responsive gene, efp, isolated by genomic binding-site cloning encodes a RING finger protein,

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Estrogen receptor(ER)-binding fragments were isolated from human genomic DNA utilizing a recombinant ER protein. Using one of these fragments as probe we have identified a novel estrogen responsive gene which encodes a putative zinc finger protein. It has a RING finger motif present in a new family of apparent DNA-binding proteins and is designated estrogen responsive finger protein (efp). The efp cDNA contains a consensus estrogen responsive element at the 3' untranslated region that can act as a downstream estrogen-dependent enhancer. Moreover, the efp is regulated by estrogen as demonstrated at both the mRNA and the protein level in ER positive cells derived from mammary gland. These data suggest that the efp may represent an estrogen responsive transcription factor which mediates phenotypic expression of the diverse estrogen action. Thus, the genomic biding-site cloning may be applicable for isolation of the target genes of other transcription factors.

K 415 THE DISTRIBUTION OF RARγ mRNA IN NORMAL AND RETINOIC ACID TREATED TAILBUD XENOPUS EMBRYOS, Elizabeth Jones, Darrin Smith, Robert Old, Derek Gatherer and Surinder Bhamra, Department of Biological Sciences, Warwick University, Coventry, CV4 7AL, England.

Exogenously supplied retinoic acid has diverse effects on many cell types at different stages of early Xenopus development. The presence of retinoic acid, and retinoic acid receptors (RAR's; ligand-modulated transcription factors of the nuclear receptor superfamily) in early Xenopus embryos, points to retinoic acid and its receptors as regulators of normal development. We have analysed the distribution of the RARγ mRNA in tailbud Xeniopus embryos by wholemount *in situ* hybridisation. RARγ mRNA is expressed in anterior and posterior domains, which correspond to the sites of retinoic acid teratogenesis when applied from neurula stages. The anterior domain of the RARγ mRNA expression is predominantly in the visceral arches.

K 416 THE EAR-2 NUCLEAR ORPHAN RECEPTOR IS OVER-EXPRESSED IN HUMAN OVARY, BREAST AND COLON TUMOR CELL LINES, Raymond E. Jones, Michelle M. Hanobik, David M. Kiefer, Elizabeth M. McAvoy, Kathleen M. Haskell, Allen Oliff, and Deborah Defeo-Jones, Department of Cancer Research, Merck Research Laboratories, West Point, PA. 19486

We set out to determine if any of the nuclear orphan receptors are aberrantly expressed in transformed human cell lines. We performed Northern blot analysis on normal lung, bladder, colon, breast, ovary, prostate, pancreas and stomach tissues as well as a human melanocyte cell line. These results were compared to Northern blots on several human tumor cell lines derived from each of these tissue types. Specific human DNA sequences for the following nuclear orphan receptors were utilized as probes: ERR1, ERR2, EAR2, EAR3, NGF1-B, PPAR, TR2 and TR2-11. The results show that EAR2 was consistently and uniquely overexpressed in ovary, breast and colon tumor cell lines. Further characterization of EAR2 expression in adult human tissues showed that EAR2 was maximally expressed in placenta, liver and skeletal muscle; moderately expressed in heart and pancreas, and weakly expressed in brain, lung, and kidney tissues. EAR2 specific expression was unique among the nuclear orphan receptors tested demonstrating that EAR2 mRNA must be regulated differently in each tissue type.

EAR2 was transiently transfected into Chinese hamster ovary (CHO) cells along with various steroid/retinoid response elements. This CHO cell line was shown to contain active vitamin D_3, retinoic acid and estrogen receptors. The results show that EAR2 strongly inhibited expression from the retinoic acid direct repeat 2, 3, 5 response elements while having no effect on the estrogen response element. A cell based screen was established for EAR2 activity on a direct repeat element in CHO cells. Approximately 2500 steroid, thyroid and retinoid like compounds have been assayed for EAR2 modulating activity. To date no compound with EAR2 regulating properties has been identified.

K 417 ANALYSIS OF GENE EXPRESSION TRIGGERED BY T_3 DURING AMPHIBIAN METAMORPHOSIS USING TISSUE CULTURED CELLS, Akira Kanamori and Donald D. Brown, Department of Embryology, Carnegie Institution of Washington, 115 W. University Parkway, Baltimore, MD 21210

We have identified more than 20 T_3 up-regulated genes in Xenopus laevis through extensive screening of subtractive libraries. To our surprise, more than half of the up-regulated genes, including both direct and secondary response genes, are regulated by T_3 in several tissue cultured cell lines in a manner similar to their regulation in the tadpole. Thyroid hormone receptor ß (TRβ) and a zinc finger protein (J1) are among several putative transcription factors included in the direct response genes, which may be responsible for regulation of secondary response genes. We have analyzed cascades of gene expression triggered by T_3 using cultured cells, where expression of both exogenous and endogenous genes is relatively easily manipulated. Stablely transformed cell lines have been prepared, in which TRα, TRβ , or J1 cDNAs are in either sense or antisense orientation. Expression of these cDNAs are controlled by a bacterial tetracycline repressor-VP16 fusion transactivator (Gossen and Bujard, PNAS 89, 5547-51, 1992). We are examining effects of the transgenes on expression of endogenous T_3 regulated genes. Two main question asked are 1) whether the direct response genes are differentially regulated by TRα or TRβ, and 2) what roles TRβ and J1 play on regulation of secondary response genes. We will present these data as well as some biochemical characterization of J1 protein.

K 418 **SELECTIVITY AND PROMISCUITY OF TARGET ENHANCERS FOR DISTINCT CLASSES OF NUCLEAR RECEPTORS.**

Shigeaki Kato[1], Haruna Sasaki[1], Miyuki Suzawa[1], Shoichi Masusige[1], Laszlo Tora[2], Hinrich Gronemeyer[2], and Pierre Chambon[2]

[1]Department of Agriculture, Tokyo University of Agricurture, Tokyo, Japan and [2]Labolatoire de Géné tique Molé culaire des Eucaryotes du CNRS, Unité 184 de INSERM, Institut de Chimie Biologique, Strasbourg, France

We found a novel type of estrogen response element(ERE) in the chicken ovalbumin gene promoter(S. Kato et al., Cell, 68, 731, 1992). This ERE(OV-ERE) is composed of four directly repeated AGGTCA motifs, which are separated from each other by more than 100 bp. Moreover, we showed that the two widely spaced AGGTCA motifs are bound by estrogen receptor(ER) homodimers.

In the present study, we systematically investigated whether two direct repeated AGGTCA motifs with different spacers might be target enhancers for ER, retinoid receptors(RAR and RXR), vitamin D receptor(VDR) and thyroid hormone receptor(TR). Transient transfection assays using the CAT reporter plasmids containing two AGGTCA motifs in front of rabbit β-globin promoter and expression vectors for these receptors revealed ligand- and receptor-specific transactivation of distinct elements, as long as spacers didn't exceed 5 bp. However, promiscous target element recognition by several receptors was observed when the two AGGTCA motifs separated more than 10 bp. The binding selectivity to these motifs by ER, RAR, RXR, VDR and TR has been studied using in vitro DNA binding assays(gel-shift and DNA bend assays).

K 419 SYNERGISTIC INDUCTION OF GENE 33 EXPRESSION BY RETINOIC ACID AND INSULIN, Terrie A. Kent, Joseph L. Messina and Joseph P. Stein, Departments of Pharmacology and Physiology, SUNY Health Science Center, Syracuse, NY 13210

Insulin is a key hormone that regulates blood glucose concentrations and is required for the long term health and maintenance of the microvasculature of nerve, kidney, eye, and heart. Retinoic acid, a derivative of vitamin A, exerts profound effects on the development and homeostatic properties of many endodermally, mesodermally, and ectodermally derived tissues. In order to investigate the regulatory interplay between these two disparate hormone pathways, we have examined the expression of gene 33 in rat H4IIE hepatoma cells. It was previously demonstrated that insulin increases both mRNA levels and the transcription rate of gene 33 (Endocrinology 123: 366-372, 1988). By using both Northern analyses and nuclear run-on studies, we have shown that retinoic acid also increases gene 33 mRNA levels 19-fold and the transcription rate 6-fold in serum-deprived H4IIE cells. In addition, retinoic acid and insulin added together have a synergistic effect such that gene 33 mRNA levels are increased 55-fold and the rate of transcription is increased 19-fold. To our knowledge, this is the first example of a synergistic interaction of insulin and retinoic acid on the induction of a specific gene. Two possible retinoic acid response elements have been identified in the 5' flanking region of gene 33 based upon sequence analysis. A putative retinoic acid response element (RARE) is located at -1.12 kb upstream from the transcription initiation site of gene 33, and a putative retinoid X response element (RXRE) is located at -1.4 kb. Incubation of either element with H4IIE cell extracts, or *in vitro* synthesized retinoid receptors (RARα, RXRα, or RXRγ) elicits an electrophoretic mobility shift, which can be competed for by the corresponding unlabeled consensus response elements. These experiments, coupled with *in vitro* transcription assays and transfection experiments using gene 33/CAT and gene 33/luciferase constructs, should lead to the identification of both retinoic acid and insulin response elements, which is the first step towards an understanding of the mechanism by which retinoic acid and insulin work together to produce a synergistic induction of gene 33.

K 420 PROGERSTERONE RECEPTOR MEDIATED TRANSACTIVATION IN CELL-FREE TRANSCRIPTION SYSTEMS.

Michael Klotzbücher, Verena Ulber, and Ludger Klein-Hitpass. Institut für Zellbiologie (Tumorforschung), Universitätsklinikum Essen, 45122 Essen, Germany.

The human progesterone receptor (hPR) belongs to the family of ligand-inducible nuclear receptors which activate transcription through binding to progesterone elements. Cotransfection studies have revealed that hPR contains a constitutive (AF1) and hormone-inducible (AF2) transactivation function located in the amino- and carboxy-terminal parts of the protein, respectively. To analyze the mechanism of transcriptional activation in vitro, we have overexpressed His-tagged full-length hPR as well as truncated receptor lacking the hormone binding domain in the baculovirus system. The Ni^{2+}-NTA affinity purified proteins, including full length hPR, bound to DNA in a sequence-specific, but hormone-independent, manner. Compared to full-length hPR, the mutant lacking the carboxy-terminal hormone binding domain showed lower DNA-binding activity in the bandshift assay, suggesting that carboxy-terminal sequences contribute to stable interaction with DNA. In an in vitro system based on crude rat liver nuclear extract full length hPR proved to be a more potent transactivator than the carboxy-terminally truncated receptor, indicating that the activation function present in the hormone binding domain (AF2) contributes to the overall stimulation seen. The activity of the full-length hPR could not be modulated by ligands, suggesting that AF2 is constitutively active in this system. Using a transcription system reconstituted from partially purified rat liver basal transcription factors and recombinant human factors, we have determined the factors required for transactivation in vitro by the full-length and the truncated receptor.

K 421 MOLECULAR ANALYSIS OF A NOVEL STEROID RECEPTOR HOMOLOG IN DROSOPHILA, Tatyana Kozlova, George Tzertzinis and Fotis C. Kafatos, Department of Cellular and Developmental Biology, Harvard University, Cambridge, MA 02138

We have isolated a new member of the steroid receptor superfamily in *Drosophila*. This *Drosophila* protein TD4 is most homologous to rat NGFIB orphan receptor, an early response protein, with 85 % identity in the DNA - binding (C) domain and 50 % identity in the ligand - binding (E) domain. There is no significant homology between the two proteins in the activation (A/B) domain. Northern blot analysis reveals 2 transcripts 4 kb and 5 kb expressed in late embryogenesis (15 hours post egg laying) along with a 2 kb transcript present throughout embryogenesis. Induction experiments in Schneider 2 cells were performed in order to detect if TD4 transcription is affected by mitogens. The 2 kb and 4 kb messages are detected in S2 cells prior to induction, 4 kb transcript being at a very low level. FCS (fetal calf serum) and TPA had no effect on the TD4 expression, although 4 kb RNA, corresponding to TD4, was accumulating in the presense of cycloheximide as did dfra RNA.

K 422 LOCAL ACTIVATION OF RETINOID DEPENDENT GENE EXPRESSION IN THE MAMMALIAN CENTRAL NERVOUS SYSTEM. A.-S. LaMantia*, M. C. Colbert†, and E. Linney†. *Depts. of Neurobiology and Microbiology, Duke University Medical Center, Durham, NC 27710

We have asked if *in vivo* patterns of retinoid mediated gene expression reflect localized sources of retinoids, rather than pre-existing patterns of retinoid receptors. Mouse embryos that carry a retinoic acid(RA) response element (RARE)/β-galactosidase reporter transgene show two distinct patterns of RA-dependent gene expression during midgestation: domains in the cervical and lumbar, but not thoracic or sacral spinal cord, and in the ventrolateral forebrain and olfactory placode. Similarly, molecules that participate in RA signaling—retinoic acid receptors (RARs) and cellular retinoic acid binding proteins(CRABPs and CRBPs)—are localized in patterns complimentary to the domains of transgene expression in the spinal cord and forebrain, based upon data from *in situ* hybridization studies. When cultured on monolayers of L-cells stably transfected with the RARE promoter/reporter construct, alar regions of the cervical and lumbar spinal cord induce transgene activity; alar regions of the thoracic and sacral cord, as well as basal regions and floorplate throughout the entire cord do not. Similarly, explants of the mesoderm between the olfactory placode and ventrolateral forebrain activate transgene expression; adjacent tissues do not. Thus, RA is apparently available from local sources to activate subsets of RARs and mediate local gene expression in the developing central nervous system. The addition of exogenous RA(10^{-7}M) to explanted whole spinal cords from transgenic RA indicator embryos results in ectopic transgene expression in thoracic and sacral cord. Similarly, expression in the forebrain is expanded to include the entire forebrain up to the mesencephalic/ diencephalic junction. Patterns of expression of RARβ and CRAPBII are also expanded in response to exposure to exogenous RA. These results suggest that normal patterns of RA-mediated gene activation in the developing mammalian brain reflect the local activation of subsets of widely distributed retinoid receptors by discretely localized sources of RA. This local RA signaling may participate in the regional identification of neurons in domains of RA induced gene expression. In contrast, ectopic activation of retinoid mediated gene expression via normally inactive RARs may lead to morphogenetic anomalies and alterations of neuronal differentiation and connectivity in the brains of embryos exposed to teratogenizing amounts of RA.

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K 423 THE C. ELEGANS daf-12 GENE ENCODES A MEMBER OF THE STEROID/THYROID HORMONE RECEPTOR SUPERFAMILY AND INTERACTS WITH daf-2 MUTATIONS TO DETERMINE LIFE SPAN, Pamela L. Larsen, Wen-Hui Yeh, Patrice S. Albert, and Donald L. Riddle, Division of Biological Sciences, University of Missouri, Columbia, MO 65211

The dauer larva is a developmentally arrested, non-feeding dispersal stage that has been described as non-aging (Klass and Hirsh, Nature 260:523, 1976), and daf-12 may regulate the genes that enable efficient life maintenance. The daf-12 gene specifies what we believe to be the last step in the signal transduction process that regulates dauer larva development. Whereas daf-12 activity is required for dauer larva morphogenesis, daf-2 is required for normal non-dauer development. The daf-12 gene has been cloned by transposon-tagging, and been found to encode a member of the steroid/thyroid hormone receptor superfamily that is most similar to the human vitamin D and retinoic acid receptors. Efforts to clone daf-2 are in progress.

The daf-2 and daf-12 genes interact both in dauer larva formation and in determination of adult life span. When analyzing dauer formation in double mutants, some daf-2; daf-12 allelic combinations display the daf-12 (dauer-defective) phenotype, but other allelic combinations display a non-dauer, larval-arrest phenotype. The daf-2 mutations increase life span. Furthermore, three of the double mutant combinations that have the novel arrest phenotype enhance the already increased daf-2 life span, whereas double mutants displaying the daf-12 phenotype do not. Thus, daf-2 and daf-12 show allele-specific interactions affecting both larval development and adult life span. There are a variety of exciting hypotheses concerning how the gene products may interact to control the growth/arrest decision and the life limiting role of the wild-type daf-2 gene product. One allele of daf-2 is temperature-sensitive for the life span increase, and temperature-shift experiments indicate that the temperature sensitive period extends into late life.

The current hypothesis is that the daf-12 receptor acts as a transcriptional activator for genes involved in dauer larva morphogenesis in response to a dauer-inducing hormone, the synthesis of which is inhibited by the action of the daf-1 and daf-4 receptor kinases (Georgi et al., Cell 61:635, 1990; Estevez et al., Nature 365, in press). Molecular experiments underway include sequence analysis of daf-12 mutants, and isolation of target genes. Among these may be genes determining life span.

K 424 **CONVERGENCE OF PEROXISOME PROLIFERATOR AND RETINOID SIGNALLING PATHWAYS REGULATES PREADIPOCYTE DIFFERENTIATION, PROLIFERATION, AND SURVIVAL.** Mitchell A. Lazar and Ajay Chawla, Departments of Medicine and Genetics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104.

3T3-L1 preadipoctyes provide a useful model for studying fat cell differentiation. Retinoic acid (RA), which promotes differentiation in a variety of systems, causes no morphological change in the preadipocyte, but inhibits adipose conversion. This effect is only observed when RA is added within 24h, correlating with the expression of RARγ1, which disappears from the cells after this time. Furthermore, we have found that activators of peroxisome proliferator activated receptor (PPAR), including clofibrate and pirinixic acid (WY-14,643), are sufficient to induce differentiation to an adipocyte phenotype that is morphologically and molecularly similar to that induced by standard conditions (fetal calf serum dexamethasone, isobutylmethyxanthine, and insulin). Two PPARs are induced during this process along with two retinoid X receptors (RXRs), which have been shown to be heterodimer partners required for high affinity DNA binding by PPAR. Indeed, RXRα is an early response gene, induced within 4h of exposure to differentiating conditions. Since fatty acids activate PPAR, preadipoctyes were incubated in serum depleted of lipids. This treatment converts the cells to a novel phenotype characterized by morphological elongation and increased proliferation. These changes can be prevented and reversed by PPAR activators. In contrast, treatment of these cells with RA induces cell death by apoptosis. Thus, preadipocyte differentiation and survival are regulated by peroxisome proliferator and retinoid signalling pathways.

K 425 CLONING AND EXPRESSION OF THREE MEMBERS OF STEROID/THYROID/RETINOIC ACID RECEPTOR SUPER FAMILY FROM SPRUCE BUDWORM, SUBBA REDDY PALLI, CANADIAN FOREST SERVICE, FOREST PEST MANAGEMENT INSTITUTE, SAULT ST. MARIE, ONTARIO, CANADA.

Degenerate oligonucleotides designed on the basis of conserved amino acid sequences in the DNA and ligand binding regions of the members of the steroid/thyroid/retinioc acid receptor superfamily were used in RNA-PCR to isolate three cDNA fragments, CHR1, CHR2 and CHR3 from *Choristoneura*. Comparison of deduced amino acid sequences of these cDNA fragments with the members of the steroid hormone receptor superfamily showed that CHR1 is most closely related to ecdysone receptor and hence renamed as CECR. CHR2 is most related to E75, and CHR3 is related to MHR3. CECR detects a 6 kb transcript present in the embryos of *Choristoneura* during 57 to 71% of their development. CHR2 binds to a 2.5 kb transcript appearing at 57% of embryonic development and lasting until 71% of development. CHR3 recognizes two transcripts, a 4.5 kb transcript present at 57% of development and a 3.8 kb present at 71% of development.

FPMI-CF-70 cell line from *Choristoneura* responds to 20-hydroxyecdysone (20HE, $4X10^{-6}$ M) in the medium by producing cytoplasmic extensions and clumping of cells within 24 hrs. Both clumping and extensions reach a peak between 96-120 hrs after which the cells begin to deteriorate. At this concentration 20HE induces the expression of both CHR2 and CHR3 reaching maximum in 6-12 hrs followed by a decline in 24 hrs. Based on the sequence similarity, RNA size and expression pattern and ecdysone inducibility, we conclude that these three cDNA fragments cloned from spruce budworm are regions of EcR (CECR), E75 (CHR2) and MHR3 (CHR3) like genes.

K 426 MOSQUITO ECDYSTEROID RECEPTOR: ANALYSIS OF THE cDNA AND TRASNCRIPTION DURING EGG MATURATION, Alexander S. Raikhel, Wen-Long Cho and Marianna Z. Kapitskaya, Department of Entomology and Program in Genetics, Michigan State University, East Lansing MI 48824

An insect steroid hormone, 20-hydroxyecdysone (20E), plays an important role in regulating egg maturation in mosquitoes. To better understand its role, we cloned the cDNA coding for the putative ecdysteroid receptor (MqEcR) from the mosquito, *Aedes aegypti*. The 4,158 bp MqEcR cDNA has an open reading frame of 675 amino acids with 10 potential glycosylation sites and a putative phosphorylation polyserine domain. The MqEcR has a DNA binding domain with two zinc fingers and a ligand binding domain characteristic of members of the steroid hormone receptor superfamily. These MqEcR domains share 97% and 87% identities with the respective domains of the *Drosophila* ecdysterone receptor (DEcR). Potential nuclear targeting and dimerization signals are also present in the MqEcR sequence and similar to those of DEcR. There are three MqEcR transcripts of 11 kb, 6 kb and 4.2 kb in adult mosquitoes. Six and 4.2 kb mRNAs are predominantly expressed in female mosquitoes during vitellogenesis. The A/B region of the 4.2 kb isoform of the MqEcR shares 42% similarity with that of DEcR-B1 isoform and has no similarity with the A/B region of DEcR-A or DEcR-B2. The F region, located at the carboxy-terminal of the MqEcR, has only 19% similarity with the corresponding region of DEcR. In both the fat body and ovaries of the female mosquito, the MqEcR mRNA peaks during previtellogenic period and after the onset of vitellogenesis. Surprisingly, the concentration of MqEcR mRNA in the ovary is considerably higher than in the fat body.

K 427 ECDYSONE RECEPTOR AND OTHER NHR'S IN NEMATODES. Jennifer Richer, Dave M. Hough and Claude V. Maina, New England Biolabs, Beverly, MA 01915

Filariasis is a parasitic disease which afflicts over 300 million people worldwide resulting in lymphoedema, elephantiasis, or blindness. The disease is caused by several species of parasitic nematodes. A related disease, dog heartworm, is caused by *Dirofilaria immitis*. The superficial similarity between the development of insects and nematodes through a series of larval molts to the adult, has led to the theory that nematode molting may also be controlled by steroid hormone(s). Ecdysone has been reported to be present in *D. immitis* and other helminths and has been shown to stimulate production of microfilaria and molting *in vitro* as reviewed by Barker and Rees (1990, *Parasitol Today* **6**: 384-387). However, attempts to establish a biosynthetic pathway for ecdysteroids in nematodes have been unsuccessful. We have utilized the Drosophila EcR (Koelle *et al.* 1991, *Cell* **67**:59-77) to search for *D. immitis* and *C. elegans* homologs using several approaches, the most fruitful being PCR with various degenerate primers in the DNA binding domain. PCR products were generated from genomic DNA of *D. immitis* and *C. elegans* and were subsequently cloned into pUC19 and sequenced. We obtained a *D. immitis* DNA fragment of 473 bps which upon sequencing shows 90% similarity at the amino acid level to the Drosophila EcR. Two other DNA fragments from *D. immitis* and 3 from *C. elegans* were also generated. All 5 show significant similarity to the NHR superfamily at the amino acid level, although no striking similarity to any one particular member. In addition, using another set of degenerate primers, we amplified a *D. immitis* DNA fragment which shows 98% similarity to *C. elegans daf-12*. None of the 3 *C. elegans* NHR's have been described previously. We have mapped them to the genome and have obtained cDNA's for each from a screen of a mixed stage cDNA library. The 4 *D. immitis* NHR's are the first to be described for any parasitic nematode. Once we obtain full length cDNA for the *D. immitis* EcR we will begin to study the functional role of these receptors in nematode development.

K 428 MUTATIONAL ANALYSIS OF THE DIMERIZATION PROPERTIES OF THE ORPHAN RECEPTOR COUP-TF. Gilles Salbert and Magnus Pfahl, La Jolla Cancer Research Foundation, 10901 North Torrey Pines Road, La Jolla, CA 92037.

COUP-TF is an orphan receptor belonging to the nuclear receptor superfamily. Recent investigations proved the repressor functions of this orphan receptor, showing that it is capable of restricting different hormonal pathways mostly by competition for the DNA recognition sites of RXR homodimers and RXR/RAR, RXR/TR, and RXR/VDR heterodimers. Like for RXRs, the COUP-TF ligand-binding domain is highly conserved from Drosophila (Seven up) to human. However, this receptor is able to form very stable homodimers and differs in this respect from RXRα which requires 9-*cis* retinoic acid for strong homodimeric interactions. A sequence comparison between RXRs and COUP-TF revealed that the C-terminal ends of the ligand-binding domains (from the ninth heptad repeat to the carboxy-terminus of the proteins) were highly similar. This homology prompted us to mutate COUP-TF residues that differed from the RXRs residues.

We will present the results of this mutational analysis in terms of dimerization and repression properties of the COUP-TF mutants. Interestingly, one of the COUP-TF mutants, harboring modified residues in a very short region crucial for RXR homodimerization, showed a greatly reduced homodimerization activity correlated with a decreased repression of RXR/RAR transcriptional activation.

Our study point out a region, outside the heptad repeat dimerization interface defined previously, that is important for the homodimerization properties of a certain class of nuclear receptors.

K 429 A THIRD TRANSACTIVATION FUNCTION OF PROGESTERONE RECEPTORS UNIQUE TO THE B ISOFORM, Carol A. Sartorius, Mary Y. Melville, Lin Tung, Glenn S. Takimoto and Kathryn B. Horwitz, Department of Medicine and The Molecular Biology Program, University of Colorado Health Sciences Center, Denver, CO 80262.

Human progesterone receptors (hPR) occur naturally as two isoforms -- termed hPR_A (94kD) and hPR_B (120kD) – which exhibit functional differences that are promoter and cell specific. To study the transcriptional properties of the N-terminal 165 amino acids that are unique to hPR_B, termed the B unique segment (BUS), we constructed an expression vector linking BUS to the DNA binding domain and nuclear localization signal of hPR (BUS-DBD-NLS). When extracts prepared from HeLa cells transiently transfected with BUS-DBD-NLS were analyzed by SDS-PAGE, the expressed protein retained the triplet band structure characteristic of full length B-receptors, indicating that BUS-DBD-NLS was appropriately phosphorylated and contained sites responsible for the appearance of the triplet band. Transient transfection studies using a PRE_2-TATA-CAT promoter-reporter, showed that BUS-DBD-NLS is a strong transactivator, stimulating CAT to levels comparable to those of full-length hPR_B. This transcription was promoter specific, and did not occur with MMTV-CAT. Moreover, BUS-DBD-NLS-induced transcription occurred only when BUS was bound to DNA since no transactivation was obtained with BUS-NLS lacking the DBD, or with the DNA binding mutant, $BUS\text{-}DBD_{cys}$-NLS. Consistent with this finding, BUS-DBD-NLS was able to bind a progesterone response element (PRE) in an *in vitro* gel mobility shift assay, either in the presence of concentrated nuclear extract or upon addition of a BUS-specific antibody. The DBD alone had no intrinsic activation function, since a DBD-NLS fragment was transcriptionally inactive. We conclude from these studies that hPR_B contains a third, independent transactivation function (TAF-3) located in the BUS fragment. TAF-3 is missing in hPR_A explaining the functional differences between the two PR isoforms. TAF-3 is promoter-specific and only active when bound to a PRE through its own DBD.

K 430 ISOLATION AND CHARACTERIZATION OF PROTEINS THAT INTERACT SPECIFICALLY WITH RXR, THE RETINOID X RECEPTOR, Wongi Seol, Hueng-Sik Choi and David D. Moore, Department of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114

To help to elucidate the central role of RXRs *in vivo*, a yeast genetic system was used to isolate cDNAs encoding proteins that specifically interact with human RXRβ. Among a number of clones isolated, three encoding RXR interacting proteins (RIPs) were selected for further study. RIP14 and RIP15 are novel orphan members of the receptor superfamily that are presumably new RXR heterodimer partners. RIP13 is a novel protein that is of interest because it also interacts specifically with the thyroid hormone receptor (TR), the retinoic acid receptor (RAR), the peroxisome proliferator activated receptor (PPAR) and two other orphans cloned in this lab. Sequence comparison of RIP14 and RIP15 to other receptors indicates that the former is most closely related to TR and the latter to RAR.

K 431 THYROID HORMONE-DEPENDENT REGULATION OF GENES INVOLVED IN AMPHIBIAN METAMORPHOSIS. Y.-B. Shi, W.P. Hayes*, D. Patterton, M. Puzianowska-Kuznicki and M. Ranjan. Lab. of Mol. Embryol. and * Develop. Neurobiol. NICHD/NIH, Bethesda, MD. 20892

Amphibian metamorphosis is initiated by and totally dependent on thyroid hormone (TH). This process affects every tissue in a tadpole. Of particular interest is the remodeling of the intestine from a simple tubular organ comprising predominantly a single layer of primary epithelium into a complex structure with multiple epithelial folds. Both specific cell death of the primary epithelium and selective proliferation and differentiation of the cells of the secondary (adult) epithelium, muscles, and connective tissues. To understand how TH regulates this apprantly apposite processes in a single organ, we have characterized in detail many of the TH response genes isolated previously in the intestine of *Xenopus laevis* (Y.-B. Shi and D.D. Brown, 1993, J.B.C. in press). We will present data on their identification and regulation by TH as well as cell type-specific expression in relation to metamorphosis.

Among the TH response genes are the two thyroid hormone receptor β genes (TRβ). The TRβ genes respond to TH directly since their regulation is independent of new protein synthesis. Each of the TRβ genes has two promoters and only RNAs derived from one of the promoters are up-regulated by TH (Y.-B. Shi and D.D. Brown, 1992, J.B.C. 267, 733-738; A. Kanamori and D.D. Brown, 1992, J.B.C. 267, 739-745). We have cloned the TH-inducible promoter and studied its regulation by TH in a *Xenopus* tissue culture cell. A putative thyroid hormone response element (TRE) has been identified. The characteristics of this first known amphibian TRE will be compared to those found in the well-studied mammalian genes.

K 432 MOLECULAR CLONING AND BIOCHEMICAL STUDIES OF A NOVEL UBIQUITOUS NUCLEAR RECEPTOR, Ching Song, John Kokontis, Richard A. Hiipakka, and Shutsung Liao, Ben May Institute and the Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL 60637

Nuclear receptors are a growing family of transcription factors that include receptors for steroids, thyroid hormone, vitamin D, cis and trans retinoic acids, fatty acids (or other peroxisomal proliferators), and so called orphan receptors, members of this family with no known ligand. We have found a new nuclear receptor that appears to be ubiquitously expressed in many human and rat organs. This receptor was named Ubiquitous Receptor (UR). The molecular weight of rUR, calculated from its amino acid sequence deduced from the rUR cDNA sequence, is 49,448 daltons. UR synthesized in vitro by transcription/translation of rUR cDNA and analyzed by SDS-PAGE has a molecular weight of 55,000 daltons. Polyclonal antibodies against UR were produced by immunizing rabbits with fusion proteins or peptides containing UR sequences. These anti-UR antibodies were used for immunocytochemical localization of UR in various organs. UR is predominantly localized in the cell nuclei of epithelial cells in many organs. Northern analysis of UR mRNA also showed that UR is expressed in many organs including brain, heart, liver, kidney, ovary, vagina, prostate and testis. The amino acid sequence homology between rUR and other known receptors is lower than 60% in the DNA-binding domain and less than 30% in the ligand-binding domain. A comparison of the DNA-binding domains of various nuclear receptors shows that UR diverged from other receptors very early in evolution. We have studied the binding of UR with other nuclear receptors on various oligonucleotides representing different hormone response elements by gel mobility-shift assay and also investigated the effects of coexpression of UR and other receptors on reporter (CAT) gene expression. These studies indicate that UR can form heterodimers with other receptors to modulate gene transcription.

K 433 **IDENTIFICATION AND CLONING OF GENOMIC TARGETS OF THE VITAMIN D3 RECEPTOR.** **Zalman Suldan and Leonard Freedman, Cell Biology and Genetics Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10021.**

Vitamin D has been shown to have clear regulatory effects on many systems in vivo. The Vitamin D3 receptor (VDR) interacts with the hormone and mediates the hormone's effects through DNA binding. This receptor belongs to the superfamily of steroid/nuclear hormone receptors which regulate transcription, either positively or negatively, in a ligand-dependent manner and probably also in a response element-dependent manner. Our knowledge, however, of how the receptor acts and on which systems it acts is severely limited by the relatively few number of gene-associated response elements known to be bound by the receptor.

We are using a method for isolating gene-associated genomic fragments which contain strong binding sites for VDR. Genomic DNA is sheared to a convenient size and PCR linkers are ligated to both ends. The DNA is incubated in batch with VDR and its heterodimeric partner, RXRα, in conditions identical to those used in gel shift assays. RXRα is made as a GST fusion protein and is immobilized on glutathione agarose beads. As a result, RXRα has the advantage of precipitating easily by a simple centrifugation. The heterodimeric-protein-bead complex is washed and the bound DNA is recovered. This DNA is amplified using PCR and is used to repeat the binding site selection. This cycle is repeated several times, each time with increasing amounts of "unlinked" competitor DNA.

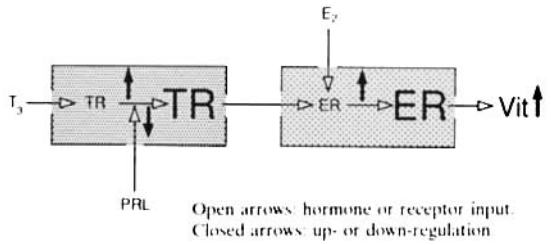
After the final selection, the DNA fragments recovered are cloned and at this point, two tactics are taken depending on the size of the fragments. Smaller fragments will be sequenced directly and then analyzed for Vitamin D response element homology and for the hallmark signs of either an enhancer region or a promoter region. Larger fragments will be subtracted against mRNA from a cell line induced with 1,25 dihydroxyvitamin D3 and those fragments which hybridize will be examined further. In addition, all fragments will be tested for their ability to confer Vitamin D responsiveness to a promoter driving luciferase expression, either in groups to be narrowed down, or singly. Those fragments which appear to be gene-associated will be used as primers to clone candidate full-length genes which will be further analyzed for response element structure and gene product function.

K 434 MOSAIC ANALYSIS OF *ULTRASPIRACLE*, A *Drosophila* RXR HOMOLOG, SUGGESTS MULTIPLE ROLES IN IMAGINAL DISC DEVELOPMENT, James D. Sutherland, Martin Shea, and Fotis C. Kafatos, Department of Cellular and Developmental Biology, Harvard University, Cambridge, MA 02138

The *ultraspiracle* gene (*usp*) in *Drosophila* encodes a member of the nuclear receptor superfamily. This orphan receptor resembles vertebrate RXRs both in sequence and ability to heterodimerize with other nuclear receptors. Because *usp* homozygotes die during larval development, roles in later stages (pupation, oogenesis, etc.) must be explored by other means. We have used two FLP-recombinase-mediated techniques to create genetic mosaics in flies. The first has been recently described by Xu and Rubin (Development, 117:1223-1237 [1993]) and relies on mitotic recombination of *usp*$^-$ and *usp*$^+$ chromosomes. The second approach relies on excision of a *usp*$^+$ cassette from a *usp*$^-$/*usp*$^-$ genetic background. Both result in patches of mutant tissue as determined by molecular markers (loss of MYC epitope or loss of bacterial lacZ, respectively). Both also result in imaginal disc phenotypes in mosaic flies. These include: loss or reduction of legs, wings, and halteres; deformed legs and cleft thoraces with varying severity; abnormal outgrowths of imaginal tissue; possibly abnormal fusion or overgrowth of eye discs (a single "cyclops" fly was observed). Ongoing work will determine the affected stages of imaginal disc development and pattern of *usp* expression in discs.

K 435 AUTO- AND CROSS-REGULATION OF EXPRESSION OF STEROID/THYROID HORMONE RECEPTOR GENES, Jamshed R. Tata, Elida M. Rabelo, Irma Machuca, Betty S. Baker and Graeme Esslemont, Laboratory of Developmental Biochemistry, National Institute for Medical Research, Mill Hill, London NW7 1AA, UK.

There is increasing evidence of autoinduction of several nuclear receptors, particularly during development (Tata *et al.*, *J. Steroid Biochem. Mol. Biol.* **46**, 105-119, 1993). We shall present data on the autoinduction of estrogen (ER) and thyroid hormone receptor (TR) α and β genes in different tissues of whole larval and adult *Xenopus*, organ and primary cell cultures and cell lines. Triiodothyronine (T_3) rapidly potentiates the activation by estrogen (E_2) of the silent vitellogenin (Vit) genes in tadpole and adult male hepatocytes. This is accompanied by the autoinduction of TRα and β mRNAs and the cross-induction of ER transcripts. Prolactin (PRL), which inhibits natural and T_3-induced metamorphosis, almost completely abolishes the autoinduction of TRα and β mRNAs, as well as the potentiation by T_3 of the autoinduction of ER and activation of Vit genes by E_2. The expression of *Xenopus* retinoid receptors RARα and γ and RXR α and γ was not similarly auto- or cross-regulated, nor did retinoic acid significantly modify the expression of TR and ER genes in larval or adult *Xenopus* cells. The model below summarizes the interplay between T_3, E_2 and PRL and emphasizes nuclear receptor amplification by the process of induction by its own ligand. Although the wider significance of receptor autoinduction in general is not fully understood, its relevance to morphogenesis and cell death during amphibian metamorphosis will be briefly discussed.



Open arrows: hormone or receptor input.
Closed arrows: up- or down-regulation

K 436 CLONING AND CHARACTERIZATION OF GENES EXPRESSED DURING XENOPUS LAEVIS METAMORPHOSIS, Joyce Tay and Mark Danielsen, Department of Biochemistry and Molecular Biology, Georgetown University Medical School, 3900 Reservoir Rd., N.W., Washington, DC 20007

We are studying gene expression during metamorphosis of Xenopus laevis. This is being approached in three ways a) isolation of genes that are expressed during metamorphosis (Xenopus expressed sequence tags [xESTs]), b) isolation and characterization of genes that are either induced or repressed during metamorphosis, c) isolation and characterization of genes that we expect to be involved in the control of metamorphosis (e.g. thyroid hormone receptors, corticoid receptors, retinoid receptors). The PCR differential display technique is being used to analyze and clone genes that are differentially expressed during metamorphosis. Of particular interest is one mRNA that is polyadenylated at the beginning of metamorphosis but appears to loose its polyadenylation as metamorphosis progresses.

K 437 A NOVEL ORPHAN NUCLEAR RECEPTOR MODULATES 9-CIS RETINOIC ACID SIGNALLING PATHWAYS Michèle Teboul, Eva Widmark, Carol D. Banner, Qiao Li and Jan-Åke Gustafsson. Department of Medical Nutrition, Karolinska Institute, Huddinge Hospital F60, and Center for Biotechnology NOVUM, S-141 86 Huddinge, Sweden

Nuclear receptors represent a large family of transcription factors that mediate complex effects on development, growth and homeostasis by selective modulation of gene expression via interactions with small hydrophobic ligands such as steroids and thyroid hormones, vitamin D and retinoids. This family also contains members referred to as orphan nuclear receptors for which no ligands have been identified.

We have cloned a novel member of the nuclear receptor superfamily by low stringency screening of a rat liver library with a mixture of synthetic oligonucleotide probes derived from known steroid receptors. This orphan receptor clone (OR) shows limited homology to known members of the nuclear receptor superfamily. However, the amino acid sequence at the base of the first DNA-binding zinc finger allows the classification of OR into the retinoic acid / thyroid hormone receptor subfamily . The members of this subfamily bind to a DNA motif formed by a repetition of the hexameric motif AGGTCA. Discrimination between target elements is achieved through different arrangaments and spacing of this motif. Most of the members of this subfamily require retinoid X receptor (RXR) for efficient binding to their respective target DNA sequences. Using gel shift experiments, we have shown that OR interacts with RXR and that this complex binds to a direct repeat of two hexameric motifs spaced by 4 nucleotides (DR4). This DR4 motif is characteristic of a thyroid hormone response element. To study the in vivo interaction between OR and RXR, we have used transient transfection experiments utilizing a cell line which expresses endogenous RXR. For the reporter construct, we have used the alkaline phosphatase gene under the control a DR4 containing promoter. We have found that cotransfection of OR is necessary for the induction of the reporter gene by 9-cis retinoic acid (the ligand of RXR). We are currently using this transactivation system for screening through a broad spectrum of potential activators for OR.

K 438 TEMPORAL COORDINATION OF REGULATORY GENE EXPRESSION BY THE STEROID HORMONE ECDYSONE, Carl S. Thummel, Howard Hughes Medical Institute, Department of Human Genetics, Bldg. 533, University of Utah, Salt Lake City, Utah 84112

Drosophila metamorphosis provides an ideal model system for unravelling the molecular mechanisms of steroid hormone function. Several successive pulses of the steroid 20-hydroxyecdysone (referred to here as ecdysone) trigger puparium formation and the onset of a complex series of morphogenetic transformations resulting in the formation of the adult fly. Ecdysone exerts its effects on development by triggering a two-step genetic regulatory hierarchy, visible as waves of transcription puffs in the giant salivary gland polytene chromosomes. A small set of <10 early puffs are directly induced by the hormone. These puffs encode regulatory proteins, one or more of which repress early gene expression and induce >100 late secondary-response puffs. Molecular characterization of three early puffs has revealed that they contain long complex genes that encode families of related DNA binding proteins. Each early transcript is induced directly by ecdysone in a precise temporal order, dictated by the length of its transcription unit and the threshold ecdysone concentration required for promoter activation. Furthermore, genetic and molecular experiments have demonstrated that the prior expression of some early genes is essential for the appropriate subsequent ecdysone-induction of other early genes, defining a network of cross-regulatory interactions. We have also identified several members of the nuclear hormone receptor superfamily that are controlled by ecdysone at the onset of metamorphosis. This raises to seven the number of these genes that appear to play a role in these regulatory hierarchies; *EcR*, *usp* (these two genes encode the ecdysone receptor heterodimer), *E75*, *E78*, *DHR3*, *DHR39*, and *βFTZ-F1*. Most recently, we have used a PCR-based molecular screen to identify three other nuclear receptor superfamily members that are expressed at the onset of metamorphosis. At least one of these genes is rapidly induced by ecdysone in late larvae. We will discuss our efforts to better define the regulation and function of these receptor superfamily members using both molecular and genetic approaches. The growing number of nuclear hormone receptor superfamily members that appear to be transducing the ecdysone signal during *Drosophila* metamorphosis provides an ideal opportunity to define the function of these proteins in the context of an intact developing animal.

K 439 DNA-BINDING AND TRANSCRIPTIONAL ACTIVATION PROPERTIES OF BmCF1, A *usp* HOMOLOG FROM ***B. mori*** George Tzertzinis and Fotis C. Kafatos Dept. of Cellular and Developmental Biology,Harvard University, Cambridge MA, 02138.

BmCF1 is a member of the nuclear receptor family with greater similarity to the subfamily of vertebrate RXRs. It is closely related by sequence similarity of the DNA-binding and ligand domains to the *Drosophila ultraspiracle* (*usp*), a gene required maternally and zygotically for development . *usp* is implicated in metamorphosis and ecdysone response by virtue of its association with ecdysone receptors.

Bacterially produced full length BmCF1 protein fails to bind DNA sites consisting of variations of the AGGTCA half site motif whereas C-terminally truncated protein binds these elements with varying affinities. The full length protein is able to heterodimerize readily with thyroid receptor from rat and thus to bind with high affinity to the palindromic s15 element TAGGTCACGTAAATGTCCAC and other elements such as the EcRE from *hsp27*. A conserved sequence near the C-terminus is required for heterodimerization.

Cotransfection of BmCF1 in *Drosophila* Schneider cells with a concatamerized s15-CAT reporter gene causes a dramatic transcriptional activation which is further enhanced by cotransfection of Drosophila ecdysone receptor. These results suggest that BmCF1 is transcriptionally actve in *Drosophila* cells and an appropriate partner of the ecdysone receptor.

K 440 INVOLVEMENT OF C-JUN IN THE CONTROL OF GLUCOCORTICOID RECEPTOR TRANSCRIPTIONAL ACTIVITY DURING DEVELOPMENT OF CHICKEN RETINAL TISSUE, Lily Vardimon, Yehudit Berko-Flint and Gil Levkovitz, Department of Biochemistry, The George S. Wise Faculty of Life Sciences, Tel-Aviv University, Tel-Aviv, 69978 Israel.

Transcriptional activity of the glucocorticoid receptor in embryonic chicken retinal tissue increases dramatically with development although the quantity of the receptor molecules does not alter greatly with age. Glucocorticoids can induce a marked increase in transcription of the endogenous glutamine synthetase gene and transiently transfected CAT constructs that are controlled by minimal consensus GRE promoters in the retina on embryonic day 11 (E11), but not on day 6 (E6). This study examines the possible involvement of c-Jun in the developmental control of glucocorticoid receptor activity. The level of c-Jun expression was found to be high in E6 retina and to decline with age. Treatment with 12-O-tetradecanoylphorbol-13-acetate (TPA) caused an increase in c-Jun expression in E11 retina and a decline in the inducibility of both the endogenous glutamine synthetase gene and the transiently transfected CAT constructs pDG46TCO and pGS2.1CAT, that are controlled by a minimal consensus GRE promoter and the glutamine synthetase promoter, respectively. CAT induction could also be suppressed by introduction of the c-Jun expression vector, pRSV-c-jun, into E11 retina, but not by introduction of pRSV-c-fos, pRSV-jun B or pRSV -jun D. The effect of c-Jun was dose dependent and could be reversed by overexpression of GR. Overexpression of Jun D could, however, relieve the c-Jun evoked repression and restore the ability of the glucocorticoid receptor to induce gene expression in response to glucocorticoids. Transfection of the Jun D expression vector into E6 retina caused a fivefold increase in the hormone-dependent expression of pDG46TCO. The effect of Jun D could be reversed by overexpression of c-Jun. These results suggest that high levels of c-Jun expression might be important for repression of glucocorticoid receptor activity at early embryonic ages.

K 441 DNA BINDING SPECIFICITY OF ZINC-FINGER TYPE NUCLEAR RECEPTORS, Joel Yang Zhou, Kazuhiko Umesono & Ronald M. Evans, Gene Expression Lab, Salk Institute, La Jolla, CA 92037

Employing a PCR-assisted selection assay, we identified and optimized the DNA binding sites for RAR, TR, v-erbA, RXR, Ecdysone receptor(EcR), Ultraspirical(Usp), and/or their heterodimeric combinations. All those receptors share a similar P-box and bind to the same "AGGTCA" core sequence, however, there are striking differences in the half site orientation, spacing and flanking sequence preference. Currently, we are focusing on the structural signals in the receptors that determine the DNA binding specificity. Those signals will be tested in mutant receptors, and further applied in establishing assay system devoid of endogenous receptor interference.

K 500 FUNCTIONAL PROPERTIES OF THYROID HORMONE RECEPTOR MUTATIONS IN RESISTANCE SYNDROMES.
M.Adams, T.N.Collingwood, Y.Tone, C.H.Matthews, V.K.K.Chatterjee. Department of Medicine, Addenbrooke's Hospital, Hills Rd, Cambridge CB2 2QQ, U.K.
Forty cases of thyroid hormone resistance syndromes (RTH) with elevated thyroid hormone levels and unsuppressed pituitary TSH secretion were analysed. Affected individuals were heterozygous for mutations in the thyroid hormone receptor β gene, including twenty-one different point mutations, two in-frame deletions and a single frame shift insertion. All the mutations localised to the hormone binding domain such that sequences mediating dimerization (e.g.the ninth heptad repeat), were not involved.We have characterised the properties of twenty different mutant receptors.Using transfection assays we examined T3 dose-dependent repression of the human TSHα promoter and activation of reporter genes containing palindromic (TREp), direct repeat (malic enzyme, MAL), or everted repeat (lysozyme, F2) thyroid response elements. In each case, the mutant receptors exhibited either right shifted or flat activation and repression profiles, consonant with the degree of impairment in ligand binding. Some mutants (R438H,P453A,P453S,P453H,P453T) were fully active on TSHα and TREp but unable to maximally transactivate on MAL. Mutant receptor proteins also inhibited wild type receptor action in a dominant negative manner when co-expressed. The magnitude of this inhibition was variable and also dependent on response element configuration. The inhibition could be overcome by increasing ligand concentrations, except for mutants (G344E, Δ430M, Δ432G and Frame Shift), which were unable to bind T3. DNA binding was tested by electrophoretic mobility shift assays using TREp, MAL and F2 elements. Homodimer formation on TREp and F2 elements was detected but markedly impaired for some mutants (R316H,R338W,R429Q).The addition of T3 led to decreased homodimer formation except for the non-hormone binding mutants. In contrast, all mutant receptors formed heterodimers with the retinoid X receptor (RXR) on TREp, MAL and F2 and these complexes were unaffected by the presence of either ligand. Artificial mutations in the ninth heptad (L421R, L428R) markedly impaired the dominant negative action of natural receptor mutants and their ability to form heterodimers. Conclusions: a) All receptor mutants associated with RTH exert a dominant negative effect on the TSHα promoter, generating the biochemical abnormality; b)The dominant negative effects of mutant receptors are variable and related to their transactivation and dimerization potential.These observations could account for the clustering of resistance mutations and may provide a basis for the variable clinical phenotype in this disorder.

K 501 COMPARTMENTALIZATION OF GLUCOCORTICOID RECEPTOR EXPRESSION DURING EMBRYONIC DEVELOPMENT OF CHICKEN RETINA, Iris Ben-Dror *, Rena Gorovits*, Lyle E. Fox*, Hannes M. Westphal# and Lily Vardimon*, *Department of Biochemistry, The George S. Wise Faculty of Life Sciences, Tel Aviv University, Tel Aviv 69978, Israel, and #Institut für Molekularbiologie und Tumorforschung, Philipps Universitat, 3550 Marburg, Germany.

Expression of glutamine synthetase in the neural retina of chicken embryo is an example of a glucocorticoid-inducible gene activity that is cell-type-specific and developmentally controlled. Glutamine synthetase induction in the retina is regulated at the transcriptional level, is always confined to Müller glia cells and can be only induced at mid or late but not at early embryonic ages. Analysis of the glucocorticoid receptor (GR) revealed that the transcription activity of GR increases markedly during development although the level of the GR protein does not greatly change [Ben-Dror et al., (1993) Proc. Natl. Acad. Sci. 90: 1117-1121]. This apparent discrepancy was investigated by examination of the pattern of GR expression in undifferentiated early retina and in retina of mid-developmental ages that consists primarily of differentiated cells. Two GR isoforms, of 90 and 95kDa, were found to be expressed in both differentiated and undifferentiated retina cells, in similar total amounts, but at different relative quantities: in the undifferentiated retina the level of the 90kDa isoform was higher than that of the 95kDa receptor, while in the differentiated tissue the 95kDa receptor was higher. These differences diminished, however, in the presence of cortisol, which induced changes in isoform quantities such that the 95kDa isoform became the predominant receptor protein at both stages. Immunohistochemical analysis revealed a marked difference in receptor localization: in the undifferentiated retina GR was expressed in virtually all cells, while in the differentiated tissue GR expression was restricted to Müller glial cells. The latter represent about 20% of the cells in the tissue and are the only cells to express the endogenous glutamine synthetase gene in response to glucocorticoids. Because the total amount of the receptor protein did not alter with age, its restriction to Müller glia implies a cell-type specific increase in receptor expression. Compartmentalization of receptor expression might be part of a mechanism that modulates receptor activity in the developing retina tissue and controls the temporal and cell-type specific induction of glutamine synthetase.

K 502 **ANALYSIS OF T3 AND T4 ANALOGUES FOR GENE REGULATION BY TR HETERODIMERS AND TR HOMODIMERS,** Igor Bendik and Magnus Pfahl, La Jolla Cancer Research Foundation, 10901 North Torrey Pines Road, La Jolla, CA 92037
The thyroid hormones 3,5,3'-triiodo-L-thyronine (T3) and 3,5,3',5'-tetraiodo-L-thyronine (T4) regulate a large variety of biological processes in all higher vertebrates, comprising development, growth, metabolism and homeostasis. Two different mammalian nuclear thyroid hormone receptors (TRs), thyroid hormone receptor α (TRα) and β (TRβ), are able to bind these hormones and regulate responding genes. TRs can bind to specific thyroid hormone response elements (TREs) as heterodimers with the retinoid X receptors (RXRs) and act as transcriptional enhancers (Zhang and Pfahl; Trends Endocrinol Metab, vol. 4:156-162, 1993). As TR homodimers they bind a distinct set of sequences and function as ligand sensitive repressors (Piedrafita et al.; submitted, 1993).
In our study we have analyzed different T3 and T4 analogues for their ability to enhance or repress TR-mediated gene regulation. We have addressed the question whether these compounds can discriminate between the TR-RXR heterodimeric and TR-TR homodimeric response pathways. The compounds were analyzed on natural response elements using transient transfections of CV-1 cells. The results of these studies will be presented.

K 503 ISOLATION OF ENDOGENOUS THYROID HORMONE RECEPTOR BINDING SITES IN GH4 CELLS. J. Bigler and R.N. Eisenman, Fred Hutchinson Cancer Research Center, 1124 Columbia Street, Seattle, WA 98104

In order to obtain a better understanding of thyroid hormone biology, we attempted to isolate genes directly regulated by T3 by means of their T3 receptor (TR) binding site. TR-DNA complexes were eluted from GH4 nuclei digested with a restriction enzyme and then immunoprecipitated. The recovered DNA was cloned and screened for TR binding sites. Positive clones were then tested for enrichment in the immunoprecipitated DNA fraction compared to preclear DNA. One of the positive clones, 122, was enriched approx. 10-fold and was characterized in more detail. It contains three TR binding sites as determined by footprinting. Two of these sites are located upstream of the promoter and the third one downstream. The binding sites have an affinity for TR similar to TREpal but show no obvious sequence homology to previously identified TREs. In transient transactivation assays this clone mediates activation by TR in the absence of T3 and neither activation nor repression in the presence of T3. This represents a mode of TR action not previously described.

Clone 122 hybridizes to at least three different mRNAs of approx. 4, 6, and 9 kb resp., isolated from GH4 cells. These mRNAs are upregulated in cells starved for T3 compared to cells grown in the presence of hormone. Thus endogenous mRNAs are regulated in the same manner as a reporter construct under the control of clone 122 sequences.

These data indicate that this approach allows us to isolate genomic DNA sequences that represent biologically relevant TR binding sites.

K 504 **IDENTIFICATION AND CLONING OF PROTEINS WHICH BIND ESTROGEN RECEPTOR CONTINGENT ON THE PRESENCE OF LIGAND.** Myles Brown, Heather MacKay, Sophia Cariati, Emily Marden, Glover W. Martin, and Shlomit Halachmi, Department of Medicine, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA 02115

Estrogen receptor (ER) contains two transcriptional transactivation domains, AF-1 and AF-2. AF-1 activity is constitutive when the receptor is bound to DNA. In contrast, the AF-2 domain contains the hormone binding domain (HBD) and is active only in the presence of hormone, suggesting that the AF-2 domain regulates estrogen dependent transcription. To study the function of AF-2, we expressed the ER hormone binding domain as a glutathione s-transferase (GST) fusion protein in *E. coli* and used it as an affinity reagent to identify associated proteins. Metabolically labeled cell extracts were incubated with immobilized GST-HBD protein in the presence or absence of estradiol. A 160 kDa protein bound HBD specifically in the presence of estradiol or diethylstilbestrol (DES) but not in the presence of the antiestrogens 4-OH tamoxifen, ICI 164,384, or ICI 182,780. Furthermore, estradiol induced binding was inhibited by an equimolar concentration of 4-OH tamoxifen. Mutants of ER that preserve hormone binding but reduce hormone-dependent transactivation lose the ability to bind p160 even when assayed with increased estradiol concentrations. Significantly, both RAR-β and RXR-α also bind p160 dependent on their specific ligands. We have used ^{32}P-labeled GST-HBD fusion protein as probe on western blots and have found that p160 binds directly to ER in a ligand dependent manner. In addition we have identified a second protein also present in nuclear extracts, p140, that has similar properties. To identify cDNA clones of p140 and p160 we have used ^{32}P-labeled GST-HBD to probe a human B-cell cDNA λgt11 expression library. We have identified a candidate p140 clone through estradiol dependent binding of the fusion protein probe. The cloned protein expressed either as a β-galactosidase or GST fusion is bound by ^{32}P-labeled GST-HBD in a hormone dependent manner. Antibodies raised against the cloned protein recognize p140, but not p160. Cloning of p140 and p160 will allow us to define the role played by these putative adaptors in the pathway of ligand dependent transactivation.

K 505 THE EFFECTS OF ESTROGEN AND PROGESTERONE ON VITELLOGENIN mRNA LEVELS IN THE PAINTED TURTLE, CHRYSEMYS PICTA, Callard, I.P.[1], Charnock-Jones, S.D.[2], Malloy, K.D.[1], [1]Department of Biology, Boston University, Boston, MA 02215 and [2]Department of Obstetrics and Gynecology, University of Cambridge, UK

Considerable homology exists between the lipid-rich vertebrate vitellogenins (vtg) and apolipoprotein B, and we have demonstrated dual control of reptilian vtg gene expression by estrogen (E; up-regulation) and progesterone (P; down regulation). For these reasons we are using the painted turtle, Chrysemys picta, as a model for the hormonal regulation of synthesis of apolipoproteins of importance in the genesis of atherosclerosis. Here we will present our initial studies using two partial (1.2 kb) cDNA clones encoding turtle vtg obtained by using a differential screening technique. Sequences of these clones had 73% homology to vtg cDNA sequences of Xenopus and Gallus. The clones were used as a probe in Northern blots to quantify vtg mRNA levels in livers of pre-ovulatory females treated with E (0.5mg/kg/d, 8 d), P (5 mg/kg/d, 8 d) or a combination (E: 8 d, P: last 3 d), untreated post-ovulatory females, and untreated males. The hormone treatments resulted in clear-cut differences in Vtg mRNA levels which support important regulatory effects of the steroids at the genomic level. Data on changes in hepatic ER mRNA obtained by using a homologous ER cDNA clone are anticipated to clarify the effect of P on E-induced vitellogenesis.

Supported by NIH RR RO1 06633 to IPC.

K 506 INDUCTION OF NERVE GROWTH FACTOR BY 1,25 DIHYDROXYVITAMIN D3 IN THE BRAIN AND IN MOUSE L929 CELLS, Susan Carswell, Heide M. Wilcox, Kristin Clopton-Hartpence, Ellen M. Brown, Elaine Robbins and Michael S. Saporito, Cephalon, Inc., West Chester, PA 19380

Nerve growth factor (NGF) has shown therapeutic potential for Alzheimer's disease. However, it cannot cross the blood-brain barrier. An alternative strategy is to up-regulate endogenous levels of NGF with small molecules that can enter the central nervous system. Because 1,25 dihydroxyvitamin D3 (1,25 D3) was reported to be a potent inducer of NGF in mouse fibroblast L929 cell by Wion et al., we examined this compound for activity in the brain. 1,25 D3 was injected intraventricularly and NGF mRNA levels in various brain regions were measured. An approximate 2-fold induction was observed in the frontal cortex, hippocampus and basal forebrain, regions which contain the cells that selectively degenerate in Alzheimer's disease and which NGF has been shown to rescue. Time course studies performed in L cells demonstrated that NGF induction by a single application of 1,25 D3 persisted for up to 96 h, which suggested that in vivo induction by 1,25 D3 might be sustainable during its chronic administration. This proved to be the case when 1,25 D3 was infused continuously into the brain for up to 14 days. Both NGF and NGF mRNA levels were increased over vehicle-treated controls. NGF mRNA levels were also increased in the brain when 1,25 D3 was administered peripherally, suggesting that this compound can cross the blood-brain barrier. RNA stability studies using actinomycin D in L cells suggested that the observed increase in NGF mRNA is not attributable to the stabilization of this mRNA by 1,25 D3. Data will also be presented from reporter gene transfection experiments and nuclear run-on analyses in progress examining the transactivation of the NGF promoter by 1,25 D3.

K 507 ESTRADIOL INCREASES AND ANTIESTROGENS ANTAGONIZE THE GROWTH FACTOR-INDUCED AP-1 ACTIVITY IN MCF7 BREAST CANCER CELLS WITHOUT AFFECTING c-*fos* AND c-*jun* SYNTHESIS, Dany Chalbos, Alexandre Philips, Florence Galtier and Henri Rochefort, INSERM U148, 60 rue de Navacelles, Montpellier, 34090, France

In estrogen receptor (ER) positive human breast cancer cells, antiestrogens inhibit the mitogenic effect of growth factors, in the absence of estrogens. As, AP-1 activity is one of the first nuclear events following growth factor receptor activation, we studied the effects of estrogens and antiestrogens on growth factor-induced AP-1 activity using transient transfection of the AP-1 responsive gene (AP-1)4-TK-CAT into MCF7 cells. The growth factor-induced AP-1 response was increased by estradiol and inhibited by antiestrogens in conditions where growth factor-induced c-*fos* and c-*jun* mRNA levels were unchanged by hormone and anti-hormone treatments. The same regulations were obtained when the AP-1 response was directly induced by co-transfection of c-*fos* and c-*jun* expression vectors. Co-transfection of the wild-type estrogen receptor HEGO amplified both effects. Antiestrogens inhibited AP-1 activity in conditions where they had no effect on basal ERE-mediated activity levels, whereas estradiol was as efficient in stimulating both activities. Moreover, the relative efficacy of the two antiestrogens, OH-Tamoxifen and ICI 164,384 in inhibiting these two activities was different, OH-Tamoxifen was more efficient in inhibiting ERE-mediated activity, whereas ICI 164,384 was more efficient in trans-repressing AP-1 mediated activity.

Modulations of AP-1 activity by ER ligands were measured in other cell lines. Whereas estradiol also enhanced AP-1 activity in T47D and ZR75.1 human breast cancer cells, it strongly inhibited its activity in Ishikawa endometrial cancer cells, in ER negative MDA-MB231 breast cancer cells and NIH-3T3 fibroblasts which were co-transfected with HEGO. The mechanism of this cell specificity is under investigation.

We conclude that ER regulates not only ERE-mediated transcription but also the growth factor action on AP-1 activity and that modulations of AP-1 activity are both ligand- and cell-dependent.

K 508 THE v-erb A ONCOGENE REPRESSES THE ACTIONS OF BOTH RXR AND RAR, BUT BY DISTINCT MECHANISMS, Hong-wu Chen and Martin L. Privalsky, Department of Microbiology, University of California at Davis, Davis, CA 95616

The v-erb A oncogene is an aberrant derivative of the thyroid hormone receptor. We report here a dissection of the mechanism of action of v-Erb A oncoprotein as a dominant negative inhibitor of other nuclear hormone receptors. Using transient transfection in culture cells and gel electrophoresis mobility shift assay, we demonstrate that v-Erb A interferes with thyroid hormone receptor (TR) and trans-retinoic acid receptors (RAR) at the level of the DNA, by binding to the corresponding response elements; heterodimerization of v-Erb A protein with these receptors does not play an observable role in repression. In contrast, however, v-Erb A does efficiently form a heterodimer with the RXR class of nuclear hormone receptors; complex formation enhances the DNA binding activities of v-Erb A but dramatically interferes with the ability of the RXR component to activate gene expression. Our results indicate that the v-erb A oncogene can exert multiple effects on the regulatory network defined by the nuclear hormone receptors.

K 509 GENERAL TRANSCRIPTION FACTORS AND LENS SPECIFIC EXPRESSION OF THE MIP GENE. Ana B. Chepelinsky, Chiaki Ohtaka-Maruyama and Xiaoyan Wang. Laboratory of Molecular and Developmental Biology, National Eye Institute, National Institutes of Health, Bethesda, MD 20892.

MIP, the major intrinsic protein of the lens fiber membrane, is specifically expressed in the ocular lens fibers. MIP belongs to an ancient family of transmembrane channel proteins and may play a role in regulating the interfiber space. MIP gene expression is temporal and spatially regulated during development.

We are studying the *cis* regulatory elements responsible for the lens specificity and developmental regulation of the MIP gene. We found that a DNA fragment containing 253 bp of 5' flanking sequence and 42 bp of exon one of the human MIP gene fused to the reporter gene chloramphenicol acetyltransferase (CAT) gene directs CAT gene expression to lens cells in transient assays.

Several motifs, known to bind transcription factors in other genes, are present in the 5' flanking sequence of the MIP gene. To elucidate whether those motifs are involved in the regulation of MIP gene expression we are studying their interaction with several transcripton factors. A DNA fragment corresponding to the human MIP 5' flanking sequence -313/+71 binds Sp1 and AP2 transcription factors *in vitro* at regions containing the Sp1 and AP2 consensus sequence, respectively, when analyzed by DNA footprinting and gel mobility shift assays. Purified Sp1 and AP2 activate the *in vitro* transcription of the MIP promoter in Drosophila nuclear extracts. We found that the initiation site of transcription of the human MIP gene was the same *in vivo* and *in vitro*. These results suggest that at least Sp1 and AP2 are involved in the regulation of transcription of the MIP gene.

K 510 ANDROGEN REGULATED EXPRESSION OF THE PROSTATE SPECIFIC ANTIGEN PROMOTER IN LNCaP PROSTATE CELLS. Kitty, B.J.M. Cleutjens, Conny C.E.M. van Eekelen, Remko J. Vlietstra, Peter H.J. Riegman, Hetty, A.G.M. van der Korput and Jan Trapman, Department of Pathology, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.

Prostate-specific Antigen (PSA) is a kallikrein-like serine protease, which is exclusively synthesized by the epithelial cells of the human prostate. Expression of PSA is androgen regulated. The promoter of the PSA gene contains at -170 the sequence AGAACAgcaAGTGCT, which is closely related to the HRE (hormone response element) consensus sequence. This sequence can bind the androgen receptor and acts as an androgen response element (ARE), as shown by deletion mapping and mutational analysis with PSA promoter constructs transfected in LNCaP cells. Co-transfection with an androgen receptor expression plasmid increased the activity of the PSA promoter. Similary, the glucocorticoid receptor was also able to stimulate PSA promoter activity. Transfection experiments of LNCaP further showed that sequences upstream of the ARE are necessary for maximal androgen inducibility. Detailed analysis of the upstream region by exonuclease III deletion mapping revealed that a small segment of 20 bp was essential for cooperativity with the ARE. A minimal PSA promoter construct, to which three copies of this segment are linked , showed a strong (600-fold), hormone induced, activity of the promoter. The effect of this segment on PSA promoter activity as measured in LNCaP cells was at least ten-fold higher than in Hela cells, indicating (prostate) cell specificity. Specific protein-DNA interactions with this fragment were observed in footprint and gel retardation assays.

K 511 ANDROGENS HAVE PHYSIOLOGIC EFFECTS ON BONE AND CARTILAGE BUT DO NOT STIMULATE TRANSCRIPTION FROM AN ANDROGEN RESPONSE ELEMENT. Frank S. Czerwiec, Jiin-Jia Liaw, Kerry L. Bumstein, and Robert M. Grumbles. Depts. of Medicine and Molecular & Cellular Pharmacology, University of Miami School of Medicine, and GRECC, VA Medical Center, Miami, FL 33131.

Despite evidence for androgen action and androgen receptor presence in bone and cartilage, no androgen receptor-dependent effects on specific gene transcription have been reported. We investigated parameters representing proliferation (DNA synthesis) and differentiated function (proteoglycan synthesis) in primary cultures of rat epiphyseal chondrocytes and transcriptional activation with mouse mammary tumor virus (MMTV)-CAT reporter plasmid transfected in chondrocytes and various bone cells. Nanomolar amounts of androgens (DHT and R1881) stimulated DNA synthesis (40 ± 3% over control) and proteoglycan synthesis (20 ± 2% over control). Whole cell binding of ^{3}H-R1881 in primary chondrocytes showed the presence of approximately 5800 high-affinity binding sites per cell (Kd ~ 0.3 nM). A rat calvarial-derived chondrocyte cell line (RCJ3.1C5) showed similar androgen binding properties. The primary cells and RCJ 3.1C5 cells were transfected with MMTV-CAT and assayed for CAT activity after stimulation with R1881 ($5x10^{-9}$M) or dexamethasone (10^{-7}M) for 40-72 hours. Despite significant activation by dexamethasone (10-20-fold), R1881 failed to elicit a response above control levels. SaOS-2 and MG-63 osteoblast-like cell lines were also unresponsive to androgens but responded after cotransfection with a human AR cDNA expression vector. These preliminary results suggest: 1) Androgen target genes may be differentially controlled by androgen receptor number or availability. 2) Androgen responses in bone may be mediated through an androgen receptor- or androgen response element-independent mechanism. 3) Glucocorticoids can activate transcriptional gene expression in bone. Efforts to determine the effects of varying receptor numbers on the transcriptional and physiologic responses of these bone cells are in progress. Supported by: NIH-HD 07129; NIH-DK 07346; T32HL07188

K 512 ALTERATIONS IN RETINOID RECEPTOR EXPRESSION DURING MOUSE SKIN CARCINOGENESIS. Darwiche, N., Celli, G.B., Yuspa, S.H. and De Luca, L.M. Laboratory of Cellular Carcinogenesis and Tumor Promotion, National Cancer Institute, Bethesda, MD 20892.

The two stage system of mouse skin carcinogenesis causes the formation of benign tumors (papillomas) and of malignant carcinomas. Retinoic acid (RA) has been shown to inhibit papilloma formation when applied topically, and to block the conversion step from papillomas to carcinomas when given in pharmacological doses in the diet. We are interested in determining RAR and RXR gene expression in normal skin, papillomas and carcinomas, since these receptors are probably involved in the control of normal differentiation and carcinogenesis in these tissues. The different stages of malignant progression are identified in each tumor by specific keratin mRNA and protein expression. In situ hybridization analysis shows that RARα (isoforms 1 and 2) transcripts are expressed at the basal and suprabasal levels in normal epidermis. However, RARα expression is downmodulated in papillomas and almost absent in carcinomas. In contrast, RARγ (isoforms 1 and 2) is expressed in all these tissues, while RARβ (isoforms 2 and 3) is absent. RXRα transcripts are mostly localized in basal cells in normal epidermis, and expression increases in papillomas and carcinomas as the number of undifferentiated cells is also increased. RXRβ is expressed at low levels in all these tissues, while RXRγ is absent. We conclude that RARα and RXRα seem to be regulated in opposite directions during skin tumor progression.

K 513 STRUCTURE AND REGULATION OF THE GENE ENCODING THE 637 kDa PRECURSOR FOR THE PROLINE-RICH POLYPEPTIDES (PRPs). N. De Clercq*, K. Hemschoote, W. Rombauts and B. Peeters. *Laboratory of Biochemistry, Faculty of Medicine, Campus Gasthuisberg, Catholic University of Louvain, B-3000 Leuven, Belgium. *N.D.C. is now at the Institute of Obstetrics and Gynaecology, Royal Postgraduate Medical School, Hammersmith Hospital, DuCane Road W12 0NN London UK.*

In the rat ventral prostate androgens control the synthesis of a heterogeneous group of 38-residue proline-rich polypeptides (PRPs) which are proteolytic sub fragments of a 637 kDa precursor. To understand the mechanism by which these PRPs are generated and how the expression of the encoding gene is androgen regulated we have characterised the PRP gene (De Clercq *et al.*, 1992). From the analysis of a genomic- and 20 overlapping cDNA-fragments the highly repetitive structure of the mRNA (18653 nt) was established. The predicted protein has a signal peptide followed by three domains of 956, 830 and 3914 residues. Domain B comprises 7 tandem repeats of 122 amino acids and domain C displays 39 units of 100 aa in which the PRP sequence is embedded. In the 5' upstream region (5097 bp) of this remarkable PPR precursor gene with no introns, consensus promoter elements (TATA box or GC-rich region) are missing. Instead, a potential binding site (GGGCGG) for the Sp1 transcription factor is separated by 47 nt from a putative 'Initiator' element. This conformation is known to form an alternative to classic promoter elements. The combination of an 'Initiator' element with a Sp1-binding site is also found in different androgen regulated genes and has been shown to be active as a promoter. In addition a GGATTCTtct-TGTTCT motif, resembling the distal GRE of the hormone responsive unit located in the LTR of MMTV, is present in the vicinity of the Sp1 binding site. A part of this potential regulatory sequenceis also found in the androgen response element (ARE) described in the functional receptor-binding fragment of the C3(1) gene of Prostatic Binding Protein (PBP) (Claessens *et al.*, 1989). Further upstream, two well known structural elements frequently observed in non-protein coding genomic DNA were detected. First two *Alu* type III elements showing all the characteristic of a third class of rodent *Alu*-repeats were found displaying a 95% similarity to elements found in other rat genes. Another structural element is the simple repeat displaying the sequence (CA)n, which is found in many other genes, and occurs twice in the 5' upstream region of the PRP gene. Claessens *et al.*, 1989, *Biochem. Biophys. Res. Commun.* **164**, 833-840. De Clercq *et al.*, 1992, *J. Biol. Chem.*, **267**, 9884-9894.

K 514 THE NF-κB AND SP1 MOTIFS OF THE HIV-1 LTR FUNCTION AS NOVEL THYROID HORMONE RESPONSE ELEMENTS, Vandana Desai-Yajnik and Herbert H. Samuels, Departments of Medicine and Pharmacology, New York University Medical Center, New York, NY 10016

We report that thyroid hormone (T3) receptor (T3R) can activate the HIV-1 long terminal repeat (LTR). Purified chick T3R-α1 (cT3R-α1) binds as monomers and homodimers to a region in the LTR (-104/-75) which contains two tandem NF-κB binding sites and to a region (-80/-45) which contains three Sp1 binding sites. In contrast, human retinoic acid receptor-α (RAR-α) or mouse retinoid X receptor-β (RXR-β) do not bind to these elements. However, RXR-β binds to these elements as heterodimers with cT3R-α1 and to a lesser extent with RAR-α. Gel mobility shift assays also revealed that purified NF-κB p50/65 or p50/50 can bind to one but not both NF-κB sites simultaneously. Although the binding sites for p50/65, p50/50, and T3R, or Sp1 and T3R overlap, their binding is mutually exclusive. With RXR-β, in reaction, the major complex is the RXR-β/cT3R-α1 heterodimer. The NF-κB region of the LTR as well as the NF-κB elements from the κ light chain enhancer both function as thyroid hormone response elements (TREs) when linked to a heterologous promoter. The TREs in the HIV-1 NF-κB sites appear to be organized as a direct repeat with an 8 or 10 base pair gap between the half-sites. Mutations within the NF-κB motifs which eliminate binding of cT3R-α1 also abolish stimulation by T3, indicating that cT3R-α1 binding to the Sp1 region does not independently mediate activation by T3. The Sp1 region, however, is converted to a functionally strong TRE by the viral *tat* factor. These studies indicate that the HIV-1 LTR contains both *tat*-dependent and *tat*-independent TREs and reveal the potential for T3R to modulate other genes containing NF-κB- and Sp1-like elements. Furthermore, they indicate the importance of other transcription factors in determining whether certain T3R DNA binding sequences can function as an active TRE.

K 515 Retinoic acid induced transcriptional activation of the RARß gene accompanies in vivo enhancer occupancy in EC cells, Anup Dey, Saverio Minucci and Keiko Ozato, Laboratory of Molecular Growth Regulation National Institute of Child Health and Human Development, Bethesda, MD 20892

The retinoic acid receptor ß gene (RARß) is one of the immediate early genes that are induced to transcribe following retinoic acid (RA) treatment in embryonal carcinoma (EC) cells. RAREß, is composed of a direct repeat of the GTTCA motif present in the upstream region, is shown to bind to a heterodimer of RAR and RXR, and is implicated to play a major role in RA mediated transcriptional activation of this gene. In vivo footprinting has been performed to determine the factor occupancy of upstream regulatory sites in the endogenous RARß gene in murine P19 EC cells. We show that the RARE of the RARß gene is occupied following RA treatment of the EC cells. This is in contrast to in vitro mobility shift data where receptor binding to the element occurs irrespective of RA. The in vivo binding became undetectable when RA is withdrawn from the culture. In addition to the protection at the RARE, G residues upstream from the RARE and downstream near the putative initiator like sites show RA induced protection in vivo. P19 clones carrying a deletion mutant at the DNA binding domain of RXR-ß shows no footprinting at the RARE element, indicating a role of RXRß in the transcriptional regulation of this gene. Functional assay with the mutations at the promoter elements suggests a role of the RARE and an upstream site in the retinoic acid induced ßRAR gene expression in P19 cells.

K 516 Role of IL-1, IL-6 and corticosteroids in the regulation of the Alpha-1-Acid Glycoprotein gene and the α, ß and ∂ isoforms of the C/EBP family of transcription factors *in vivo*.

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We have examined the Interleukin-1ß (IL-1ß) and Interleukin-6 (IL-6) stimulated expression of three different isoforms of the C/EBP family, a, ß and ∂, in liver of normal and adrenalectomized (ADX) Male CD-COBS rats and compared it to the expression of the positive acute phase gene AGP (α-1-Acid Glycoprotein). C/EBPα mRNA level was not affected by IL-1ß and IL-6 in liver of treated animals, while translational regulation of a 30 kD form of the C/EBPα protein was observed. C/EBPß mRNA expression was dramatically induced by IL-1ß in both normal and ADX rats. As a consequence, a high induction of the C/EBPß protein was also detectable.
IL-1ß was also effective in inducing C/EBP∂. Interestingly, C/EBP∂ mRNA induction was strongly enhanced in ADX compared to that in normal rats. The pattern of protein synthesis reflect that of mRNA since C/EBP∂ was only visible in IL-1ß treated rats, but not as pronounced as that of the corresponding mRNA. The hormone modulation of the C/EBPß and ∂ factors partially correlated to changes in AGP expression. In fact, AGP like C/EBPß and C/EBP∂ was highly induced by IL-1ß and to a lesser extent by IL-6 in normal rats. Contrary to what we observed for C/EBP∂, IL-1ß induced expression of AGP was inhibited in ADX rats. Interestingly the lack of endogenous corticosteroids (CS) never caused a decrease in the expression of the C/EBP genes.

K 517 ANALYSIS OF PML FUNCTION VIA SYNTHETIC CHIMERAS WITH THE ESTROGEN AND GLUCOCORTICOID RECEPTORS,

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The t(15;17) translocation is exclusively associated with acute promyelocytic leukemia. It fuses the retinoic receptor α (RARα) and a novel nuclear protein (PML). Administration of high doses of retinoic acid to APL patients overcomes, for a time, the ongogenic effect and restores myelocytic differentiation. PML is part of a newly recognized family of zinc-finger proteins including transcription factors, DNA modifiers, and oncoproteins. Immunofluorescence studies using polyclonal PML antibodies have demonstrated that the protein is concentrated in defined domains within the nucleus and that the nuclear localization properties of the fusion protein are altered compared to the wild type RARα. In an attempt to understand the function of PML and how PML-RAR contributes to the leukemic phenotype we have previously shown that the fusion protein modifies the AP-1 activity (Doucas, V. et al., PNAS in press, Kakizuka, A. et al., unpublished data). In view of the association between AP-1 activity and PML function we replaced the RARα in the fusion protein with the coding sequences of the human estrogen receptor (PML-ER) or the glucocorticoid receptor (PML-GR). We asked the question whether the presence of the PML in fusion with these wild type nuclear receptors modifies, in the same way as for PML-RARα, their transcriptional activities vis à vis AP-1 and whether it alters their nuclear localization properties.

K 518 Interactions of Chicken Ovalbumin Upstream Promoter-Transcription Factor (COUP-TF) with thyroid hormone response elements in the MyoD family and contractile muscle gene promoters. *Michael Downes, Shane Rea and George Muscat.* University of Queensland, Centre for Molecular Biology, St Lucia , 4072, QLD., AUSTRALIA.

Functional thyroid hormone response elements (TREs) have recently been characterized in promoter elements of certain members of the MyoD family[1], and in contractile protein[2] genes. MyoD proteins are key regulators of skeletal myogensis. Thyroid hormone (T3) and Retinoic acid (RA) have also been implicated in the regulation of this process. The consensus TRE sequence is comprised of a directly repeated six nucleotide core motif (AGGTCA) with a four base pair spacing (DR-4). In skeletal muscle, thyroid hormone and retinoid X receptors (TRs and RXRs), as well as COUP-TF, are expressed at abundant levels.TR, RXR and COUP-TF are members of the steroid/thyroid hormone receptor superfamily. TR and RXR form functional heterodimers on DR-4 motifs. COUP-TF binds to all DR's from 1 to 8 with varying affinity.

In this study we investigated whether COUP-TF modulates the binding of TR and RXR's to TRE's in the muscle-specific MyoD and contractile gene promoters. Our findings indicate that *in vitro*, COUP-TF bind as a dimeric complex to these response elements in a sequence-specific fashion. Furthermore, COUP-TF inhibits monomeric and dimeric TR binding to the TRE's found in these genes. We are currently evaluating the effects of COUP-TF on the formation of the TR/RXR heterodimeric complex to these myogenic TRE's. Our main aim in these studies is to determine whether COUP-TF acts to modulate T3 and RA regulation of the muscle differentiation process.

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2. G.E.O. Muscat, R. Griggs, M. Downes, J. Emery. Characterization of the thyroid hormone reponse element in the skeletal alpha actin gene: Negative regulation of T3 receptor binding by the retinoid X receptor. Cell Growth and Differentiation **Vol. 4, 269-279, April** (1993).

K 519 NEGATIVE REGULATION OF THE CHICKEN OVALBUMIN GENE. Sarah A. Ehlen Haecker, Karl R. Sensenbaugh, and Michel M. Sanders, Department of Biochemistry, University of Minnesota, Minneapolis, MN 55455

Regulation of eukaryotic gene transcription is often controlled by protein interactions at both positive and negative DNA elements. Two such domains were identified in the chicken ovalbumin(Ov) gene, a steroid-dependent regulatory element (SDRE, -892 to -780) and a negative regulatory element (NRE, -308 to -87). The SDRE is essential for the regulation of the Ov gene by steroid hormones, while the NRE appears to actively repress the gene in the absence of steroids. Deletional mutagenesis of the NRE identified three negative domains (-308 to -256, -239 to -220, and -174 to -132) that repressed transcription. The internal domain, -239 to -220, has been previously characterized as a silencer. To ascertain whether the other three negative domains function independently to repress gene transcription, oligomers to the sequences were ligated in the foward and reverse orientations relative to the CAT reporter plasmid driven by the thymidine kinase (TK) promoter. These plasmids were transfected into primary oviduct cell cultures, and transcriptional activity of the promoters was determined by CAT assay. The DNA fragment from -280 to -252(D) was able to repress the TK promoter about 3-fold in the foward orientation but did not significantly suppress CAT activity when fused in the reverse orientation. The DNA fragment, -175 to -134(E) showed about a 50% inhibition of CAT activity in the foward orientation while the fragment from -134 to -87(F) repressed CAT activity about 3-fold, irrespective of orientation. Gel mobility shift assays revealed that fragments D and E bound specific protein(s) from oviduct nuclear extracts as well as nuclear extract from other chick tissues. Interestingly, fragment F bound specific protein(s) from oviduct but did not significantly bind protein from other tissues. Thus, the regulation of the ovalbumin promoter is under complex control that involves multiple positive and at least four negative regulatory domains.

K 520 AN ANDROGEN RECEPTOR (AR) GENE MUTATION IN AN APPARENTLY NORMAL MALE WITH PROSTATE CANCER, Bronwen A. J. Evans, Claire E. Daniells, James Green, Maureen E. Harper and Keith Griffiths, Depts. of Child Health and Tenovus Cancer Research Centre, UWCM, Heath Park, Cardiff and Dept. of Urology Royal Gwent Hospital, Newport, UK

The androgen receptor (AR) is the principal component of the androgen-response apparatus within cells that are targets of androgen action. Mutations at the X-linked AR locus cause AR deficiency or dysfunction and thereby various degrees and patterns of androgen resistance. Over the past few years there have been major advances in our understanding of the aetiology and pathogenesis of the androgen insensitivity syndrome (AIS), but it is clear that the type of AR binding observed in genital skin fibroblasts does not correlate with the phenotype of the affected individual or with the nature of the AR mutation. Furthermore, it seems possible that mutations of the AR gene may be involved in the pathogenesis of some prostate cancers, especially in the development of resistance to various forms of endocrine therapy. During a screening programme for AR gene mutations in prostate tumours by PCR-SSCP analysis we have found one sample with a point mutation within the F exon of the steroid binding domain (C to G at nucleotide 3423). This is predicted to cause an amino acid substitution (glutamine to glutamic acid) at amino acid 798. It also introduces a Taq 1 restriction site. This mutation at nucleotide 3423 was also found to be present in DNA isolated from peripheral blood lymphocytes obtained from the same individual. The subject is an elderly man, married with no children and phenotypically normal. A change in amino acid 798 has not previously been described in normal or AIS individuals. We are currently recreating the mutant protein so that we may study its binding characteristics and its ability to switch on reporter genes in an in vitro transactivation system.

Supported by a Merck Sharp & Dohme Medical School Research Grant.

K 521 TOPICAL RETINOIC ACID IN HUMAN SKIN IN VIVO INDUCES EXPRESSION OF CELLULAR RETINOL BINDING PROTEIN I. Gary J. Fisher, Ambati P. Reddy, Subhash Datta, William Dunkle, Sujatha Venkatapuram, John J. Voorhees, Department of Dermatology, University of Michigan, Ann Arbor, MI 48109.

Retinoic acid (RA) binds to and activates nuclear receptors, which bind to specific DNA sequences (Retinoic Acid Response Elements) in the promoter region of target genes, thereby regulating their transcription. We have investigated expression and regulation by RA, of the RARE-containing gene, cellular retinol binding protein I (CRBPI), which binds ROL in the cell cytoplasm, and is implicated in the metabolism of vitamin A (retinol, ROL). RA cream (0.1%), sodium lauryl sulfate (SLS, 2% a non-specific irritant control), and vehicle were applied under occlusion to human subjects for 6 hours, 24 hours, and 4 days. Epidermal biopsies were obtained from treated areas, and extracted for total RNA and soluble protein. Northern blots probed revealed CRBPI mRNA (1kb) was induced 3.9 fold ($p<0.005$, $n=8$) at 24 hours, and 3.6 fold ($p<0.0.001$, $n=8$) at 4 days, compared to vehicle. CRPBI mRNA was not induced, however, 6 hours after RA treatment ($n=6$). There was no difference in CRBPI mRNA between vehicle and SLS-treated skin at any time point. CRBPI protein was also elevated 2.4 fold ($p<0.05$, $n=6$) and 2.5 fold ($p<0.05$, $n=6$) following 24 hours and 4 days, respectively, as determined by charcoal separation [^{3}H]ROL binding assay and Western analysis. These data indicate: 1) that topical RA modulates gene expression *in human skin in vivo*, and 2) induction of CRBPI by RA may act to negatively regulate conversion of endogenous ROL to RA through sequestration of ROL and enhancement of ROL esterification.

K 522 IDENTIFICATION OF ESTROGEN RECEPTOR ASSOCIATED PROTEINS BY SCREENING A HIGH-EXPRESSION HeLa LIBRARY, Sara C. Folta and Deborah A. Lannigan, Department of Zoology, University of Vermont, Burlington, VT 05405

There is evidence to suggest that accessory proteins transduce the transcriptional activation signal from the estrogen receptor to RNA polymerase II. In order to identify proteins which may be involved in a complex which mediates the estrogen response, a fusion protein containing the C-terminus (hormone binding domain) of the estrogen receptor, glutathione S-transferase (GST), and a short target region for heart muscle kinase was made and expressed in *E. coli*. The purified fusion protein was ^{32}P labeled using the catalytic subunit of heart muscle kinase, and this was used to probe a HeLa library in EXlox. This system uses a T7 promoter and has high protein expression when induced with IPTG. Approximately half a million plaques were screened in the presence of estradiol, resulting in four positive clones. These clones also bind in the absence of estradiol and in the presence of tamoxifen, but do not bind to the N-terminus of the estrogen receptor. We are in the process of sequencing these four positive clones which interact specifically with the C-terminus of the estrogen receptor.

K 523 MULTIPLE FUNCTIONS AND CONFIGURATIONS OF NUCLEAR RECEPTORS ON A PROMOTER REGULATING TERMINAL DIFFERENTIATION, Gerhart Graupner and Magnus Pfahl, Cancer Center, La Jolla Cancer Research Foundation, 10901 N. Torrey Pines Rd., La Jolla, CA 92037

Recently, it has been shown that retinoic acid receptors and thyroid hormone receptors preferentially form heterodimers with the retinoid X receptor RXR. In that configuration, they can bind tightly to those cognate response elements which consist of direct repeats of the half-site AGGTCA, set in characteristic spacing that largely defines receptor selectivity. A modulating influence of the promoter context on binding strength has been demonstrated (Nagpal et al, 1992). However, few natural promoters have been analyzed that contain complex hormone response elements regulated by more than one receptor type.

Here, we investigate an 82-bp promoter sequence from the cardiac α -myosin heavy chain gene. By means of Northern blots from developing mice and transient transfections, we demonstrate strong responsiveness to retinoid receptor X, in addition to the weaker responsiveness to retinoic acid receptor previously described by us and the well- known regulation by thyroid hormone receptor. Further delineation of the interaction sites leads to a model for the receptor binding configuration and "crosstalk" between the receptors. We propose that natural promoters may differ from isolated response elements not only through modulation of receptor binding strength, but also by selection of the receptor configuration.

K 524 THE TRANSCRIPTIONAL AND PROLIFERATIVE EFFECTS OF PROGESTIN AGONISTS, ANTAGONISTS AND cAMP ON PR-NEGATIVE T47D CELLS STABLY TRANSFECTED WITH EITHER hPRA OR hPRB. Steve D. Groshong, Carol A. Sartorius, Roger L. Powell, Louise A. Miller, Twila A. Jackson and Kathryn B. Horwitz, Department of Medicine and The Molecular Biology Program, University of Colorado Health Sciences Center, Denver, CO 80262.

In order to produce stably-transfected T47D breast cancer cell sublines expressing either the human progesterone receptor A-isoform (hPRA) or the B-isoform (hPRB), but not both, a progesterone receptor (PR) negative cell line was generated. Monoclonal sublines of PR-positive T47D cells were flow-cytometrically screened for very low PR levels, and one such subline, T47D-Y, was found to be absolutely PR negative by ligand-binding assay, RT-PCR, Western blot analysis, and transcription assays using a PR-sensitive promoter. The T47D-Y subline was then stably transfected with a plasmid containing either the hPRA or hPRB cDNA sequence to generate cell lines termed T47D-YA and T47D-YB, respectively. When these cell lines were transiently transfected with a MMTV-CAT reporter construct, the progesterone agonist R5020 induced CAT transcription in both cell lines, and the antagonist RU486 was transcriptionally inactive. In the presence of 1mM 8BrcAMP, however, RU486 induced an agonist-like increase of transcription only in the T47D-YB cells. Furthermore, this antagonist-to-agonist transcriptional switch with the *hPRB*-containing cells was paralleled by their proliferative responses to the hormones. By flow-cytometric analysis of the cell cycle, wild-type T47D cells had a characteristic transient increase in the percent S-phase in the population approximately 16 hours after the addition of the agonist R5020. This S-phase peak was also seen in both the T47D-YA and -YB cells upon addition of R5020 with or without 8BrcAMP. In the presence of RU486 and 8BrcAMP, T47D-YB cells demonstrated an agonist-like increase in percent S-phase within the population. We have therefore demonstrated using a novel model system that transcription data previously generated by transient transfection models remain valid and translatable to the more physiologic setting of a stable transfection system in breast cancer cells. They show furthermore that the cAMP-induced agonist-like effects of the progesterone antagonist RU486 are restricted to the B-isoform, as assessed by transcription and growth analyses.

K 525 DEVELOPMENT OF OSTEOBLAST CELL LINES FROM TRANSGENIC MICE CONTAINING BONE MORPHOGENIC PROTEIN 2 (BMP2) PROMOTER-T-ANTIGEN CONSTRUCTS: ANALYSIS OF BMP 2 RETINOIC ACID AND $1,25(OH)_2$ VITAMIN D RESPONSE REGIONS IN THE BMP 2, PROMOTER IN THE CONTEXT OF CHROMATIN STRUCTURE. Harris SE, Ghosh-Choudhury N, Harris MA, Mundy GR, Windle J[2]. University of Texas Health Science Center, Department of Medicine, Division of Endocrinology, San Antonio, TX 78284; [2]Cancer Therapy and Research Center, San Antonio, TX 78284

Using primary cultures of fetal rat calvaria osteoblast and various BMP 2 promoter-gene constructs we have mapped retinoic acid, $1,25(OH)_2$ Vitamin D_3, rBMP 2 and p53 response regions by transient transfection assays. To further our studies, a BMP 2 - Promoter (-2736/+111)-T-antigen plasmid was used to develop transgenic mice carrying this transgene. From the calvariae of these mice we isolated osteoblasts and subcloned various cell lines. One of these lines, BMP 2-TAG 3, is capable of undergoing a complex differentiation "program" with unique spatial and temporal expression patterns of a variety of bone markers. The BMP 2-Tag 3 cells ultimately form in vitro bone or mineralized nodules with many of the characteristics of bone formed in whole embryo calvariae. The process occurs over a 20 day period. Recombinant BMP 2 greatly accelerates this differentiation process. Retinoic acid slows the differentiation process but results in well-organized mineralized nodules. BMP 2-Tag 3 cells are also response to $1,25(OH)_2$Vitamin$_3$ by exhibiting increase osteocalcin expression. Using these BMP 2-Tag 3 osteoblasts we have now stably transfected various length BMP 2-promoter-luciferase constructs such that the BMP 2-promoter-reporter gene is integrated into the chromatin structure. Clonal BMP 2-Tag 3/BMP 2-Luc osteoblasts will be utilized to study both *equilibrium* and *non-equilibrium* states of the promoter over short and long time intervals during bone cell differentiation in the presence and absence of rBMP2, retinoic acid and $1,25(OH)_2$ Vitamin D_3.

K 526 FURTHER CHARACTERIZATION OF TRE1 IN THE CARDIAC SARCOPLASMIC RETICULUM Ca^{2+} ATPase (SERCA2) GENE: DETERMINATION OF THE POLARITY OF TR-RXR HETERODIMERS, Ron Hartong[1], Riki Kurokawa[1], Chris Glass[1], Mitch Lazar[2], and Wolfgang Dillmann[1], Div of Endocrinology, Univ of California, San Diego, CA; Endocrine Div, University of Pennsylvania, Philadelphia, PA 19104[2].

The SERCA2 protein lowers cytosolic $[Ca^{2+}]$, causing cardiac relaxation. Transcription of the SERCA2 gene is responsive to T_3 in the rat heart, and we recently mapped 3 separate TREs within 500 nt 5′ to the transcription start site, using transient transfections. These TREs have different spacing and orientation of the halfsites. The present study focused on TRE1 which contains a "classical" DR+4 motif. In band shift assays, TRE1 binds bacterially expressed rat TRα as a monomer; homodimerization is not observed, but TRα does form a heterodimer with bacterially expressed rat RXRβ. Methylation interference footprints showed that the TRα monomer specifically interacts with the 3′ halfsite, and that the heterodimer interacts with both halfsites. Independent experiments were performed to further assess the polarity of the heterodimer. P box mutants of TRβ and RXRα were generated by site-directed mutagenesis, replacing the P box with the glucocorticoid receptor P box. Bandshift assays were done using TRE1 in which either the upstream or downstream halfsite consisted of a GRE halfsite as probes for binding by various combinations of wild-type and mutant receptor proteins. These experiments showed that RXR binds 5′ to the TR in TRE1. In addition, we observed that RXR is still capable of cooperative enhancement of TR binding when the 5′ halfsite of the element consists of a GRE halfsite; conversely, the RXR P box mutant is still capable of cooperative interaction with the TR, even when the 5′ halfsite of the element consists of a TRE halfsite. In conclusion: 1) RXR binds 5′ to the TR in a naturally occurring TRE with a DR+4 motif; 2) the P box and the 6 nt core halfsite motifs are not the sole determinants of receptor-DNA interactions.

K 527 THE COMBINATION OF STEEL FACTOR AND GM-CSF UPREGULATES AP-1 AND BLOCKS APOPTOSIS INDUCED BY RETINOIC ACID IN A HUMAN GROWTH FACTOR-DEPENDENT CELL LINE, Masato Horie and Hal E. Broxmeyer, Walther Oncology Center, Indiana University School of Medicine, Indianapolis, IN 46202-5121

The effects of hematopoietic growth factors were examined on the cellular action of retinoic acid (RA) using the human factor-dependent cell line, M07e. Treatment of cells with Steel factor (SLF) plus granulocyte-macrophage colony-stimulating factor (GM-CSF) synergistically stimulated cell proliferation compared to that with each factor alone. This synergism was even greater in the presence of RA than in its absence. Treatment of cells with RA resulted in apoptotic cell death associated with internucleosomal DNA fragmentation in the presence of either SLF or GM-CSF. RA-induced apoptosis and DNA fragmentation were completely blocked by treating cells with SLF plus GM-CSF. Northern analysis showed that the inhibition of RA effects on M07e cells by SLF- plus GM-CSF-treatment occurred without modulation of expression of retinoic acid receptor (RAR) α gene. Furthermore, a higher amount of AP-1 complex was detected by electrophoretic mobility shift assays in a nuclear extract prepared from cells treated with SLF plus GM-CSF compared to those treated with each factor alone, while the level of RAR complex remained similar in cells treated with SLF and/or GM-CSF. The combination of SLF and GM-CSF enhanced the level of Fos-Jun heterodimers more efficiently than that of Jun-Jun homodimers. These data suggest an interaction in signaling pathways among different types of receptors through the AP-1 complex.

K 528 REGULATION OF GLUCOCORTICOID RECEPTOR FUNCTION DURING THE CELL CYCLE IN MAMMALIAN CELLS, Shu-chi Hsu, Ming Qi and Donald B. DeFranco, Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260

Various activities of the glucocorticoid receptor (GR) protein such as transactivation, nuclear translocation, and phosphorylation were examined during different stages of the cell cycle in mouse L cell fibroblasts. Glucocorticoid-dependent transactivation of the mouse mammary tumor virus promoter was observed in G_0 and S phase synchronized L cells, but not in G_2 synchronized cells. G_2 effects were selective on the glucocorticoid hormone signal transduction pathway, since glucocorticoid but not heavy metal induction of the endogenous *Metallothionein-1* gene was also impaired in G_2 synchronized cells. The failure of glucocorticoid to induce gene expression in G_2 synchronized cells was not due to inhibition of hormone-dependent nuclear import of GR, but was associated with an inefficient nuclear retention of receptors in dexamethasone treated cells. In contrast, GRs bound by the glucocorticoid antagonist RU486 are efficiently retained within nuclei of G_2 synchronized cells. Inefficient nuclear retention was observed for both dexamethasone and RU486-bound GRs in L cells that actively progress through G_2 following release from S phase arrest. During G_2, trapped cytoplasmic recycled GRs preferentially localize at the nuclear periphery in some cells, suggesting that energy-dependent translocation of receptors through the nuclear pore may be impaired. Finally, site-specific alterations in GR phosphorylation were observed in G_2 synchronized cells suggesting that cell cycle regulation of specific protein kinases and phosphatases could influence nuclear retention, recycling and transactivation activity of the GR.

K 529 PURIFICATION OF A FACTOR THAT ENHANCES SPECIFIC DNA BINDING OF ANDROGEN AND GLUCOCORTICOID RECEPTORS, Stuart R. Kupfer, Jiann-an Tan, Elizabeth M. Wilson, and Frank S. French, Laboratories for Reproductive Biology, University of North Carolina, Chapel Hill, NC 27599

Protein-protein interactions are common among transcriptional activators and may have important consequences for gene regulation by steroid hormone receptors. Using the mobility shift assay we have identified a factor that enhances specific DNA binding of truncated rat androgen (AR) and glucocorticoid (GR) receptors expressed in *E. coli* by 25-fold and 6-fold, respectively, through the formation of heteromeric complexes. This factor, designated receptor accessory factor, or RAF, also potentiates DNA binding of full-length human GR expressed in baculovirus. RAF is temperature- and trypsin-sensitive and by gel filtration has a predicted molecular mass of 130 kDa. RAF is present in both nuclear and whole cell extracts of a variety of cultured mammalian cells, including human cervical carcinoma (HeLa), green monkey kidney (CV1), mouse Leydig tumor (MA10), and adenovirus-transformed human kidney (293) cells. Analysis of truncated rat ARs indicates that a region in the NH_2-terminal domain (amino acids 460-520) is required for RAF to enhance AR-DNA binding. RAF enhancement of AR-DNA binding requires androgen response element DNA, however, RAF alone does not bind the androgen response element. RAF activity is recovered from an androgen response element DNA affinity column only in the presence of AR. RAF has been purified to homogeneity from Hela cells by anion exchange chromatography, DNA affinity chromatography, and SDS-PAGE and correlates with a molecular mass of 110 kDa. Since RAF is amino terminally blocked, we are attempting to obtain internal amino acid sequence by cyanogen bromide cleavage and Edman degradation. The interaction of RAF with AR and GR suggests that RAF might influence the ability of these steroid receptors to activate transcription. (Supported by the NICHD Center for Population Research)

K 530 A NEW RETINOID ANTAGONIST INHIBITS THE HIV-1 PROMOTER, Mi-Ock Lee*, Peter D. Hobbs¶, Xiao-kun Zhang*, Marcia I. Dawson¶ and Magnus Pfahl*, * Cancer Center, La Jolla Cancer Research Foundation, La Jolla, CA 92037, ¶ Life Sciences Division, SRI International, Menlo Park, CA 94025

Retinoids regulate a broad range of biological processes and affect cell growth and differentiation of many cell types, including the immune system. Recently, it was reported that human immunodeficienty virus type 1 (HIV-1) expression in macrophages is enhanced by retinoic acid (RA). Retinoid signals are mediated by the retinoic acid receptors (RARs) and retinoid X receptors (RXRs) that bind to specific retinoic acid responsive elements (RAREs) in the promoter region of susceptible genes. Here, we define a novel type of retinoic acid response element (RARE) in the long terminal repeat (LTR) region that allows HIV-1 activation by homo- and heterodimeric retinoid receptors. We show that the COUP orphan receptors and a new retinoid antagonist are potent inhibitors of the retinoid response, suggesting a novel approach for the inhibition of HIV replication.

K 531 TRANSCRIPTIONAL SYNERGISM BETWEEN THE VITAMIN D_3 RECEPTOR AND OTHER NON-RECEPTOR TRANSCRIPTION FACTORS, **Min Liu and Leonard Freedman, Cell Biology & Genetics Program, Memorial Sloan-Kettering Cancer Center, New York, NY**

Tissue-specific and developmental patterns of eukaryotic gene expression are complex processes; profound alterations in these patterns could conceivably result from relatively small changes in the concentrations and/or combinations of trans-acting factors. Synergistic interactions between different classes of transcription factors bound to distinct sites within a promoter/enhancer region are examples of how this potentially can occur. Hormone response elements (HREs), recogntion sites for steroid/nuclear receptors, are sometimes found in promoter regions organized as multiple copies or are tightly clustered among binding sites for other trans-acting factors. For example, in the promoter of the vitamin D_3-regulated human osteocalcin gene (hOC), a CCAAT box is overlapped by two AP-1 sites, one GRE lies within the TATA box, and a vitamin D response element (VDRE) appears to be a composite site consisting of perhaps four half-sites and containing one AP-1 site within it. Various interactions between all the trans-acting factors binding to these sites appear to contribute to stage/tissue specific expression of the hOC gene.

In order to systematically examine the potential interactions between one such factor, the ligand-inducible vitamin D_3 receptor (VDR), and other, non-receptor tanscription factors, we have constructed a series of reporter plasmids (E1b TATA box driving the firefly luciferase gene) containing one copy of the mouse osteopontin (mSpp-1) VDRE, consisting of two direct repeats spaced by three base pairs, and one binding site for the transcription factors SP1, NF-1, OTF-1, or AP-1. We also generated reporters under the control of two copies of the hOC or mSpp-1 VDREs. The various reporters were used to transiently transfect HeLa cells in the presence and absence of 1, 25 dihydroxyvitamin D_3. Our results show that VDR transactivates 20 times more strongly from two VDREs than from one, indicating that VDR synergizes with itself. VDR also synergizes with the other, non-receptor factors, since we observe a 6-12-fold degree of synergistic induction of luciferase activity following vitamin D_3 addition, depending on the particular factor. The functional basis for the transcriptional synergism appears to be at the level of cooperative DNA binding, at least for VDR-VDR and VDR-OTF-1, as demonstrated in vitro by gel mobilty shift assays using purified factors. Consistent with this, we show that the minimal requirement for transcriptional synergism in vivo by VDR is its DNA-binding domain.

K 532 THE LEUCINE ZIPPERS OF C-FOS AND C-JUN: A NEW TOOL FOR THE FORMATION OF PURE HETERODIMERS BETWEEN THE TWO PROGESTERONE RECEPTOR ISOFORMS, Mohamed K. Mohamed, Alicia R. Hovland, Lin Tung, Glenn S. Takimoto and Kathryn B. Horwitz, Department of Medicine and The Molecular Biology Program, University of Colorado Health Sciences Center, Division of Endocrinology, Denver, CO 80262.

Human progesterone receptors (hPR) exist as two isoforms: 120 kDa B-receptors and N-terminally truncated 94 kDa A-receptors. In human tissues, the two receptor isoforms are expressed in approximately equimolar amounts. Since both homo- and heterodimers can form between the A and B isoforms, three possible classes of receptor dimers (A:A, A:B, B:B), in the ratio of 25:50:25, respectively, can bind to a progestin response element (PRE). To study the transcriptional phenotype of pure A:B heterodimers uncontaminated by homodimers, we used the ability of Fos and Jun to form pure heterodimers through their 40 aa leucine zippers (*zip*). Chimeric constructs were made linking the *zip* of either c-*fos* or c-*jun* to the C-terminus of hPRB or hPRA (hPR-*zip*) to produce A-Fos, B-Fos, A-Jun, or B-Jun. Gel mobility shift assays show that in the presence of the antiprogestin, RU486, only pure heterodimers form between A-Fos/B-Jun or A-Jun/B-Fos, and bind a PRE. Thus, the endogenous dimerization domain of PR is bypassed in the presence of antagonists. By contrast, with agonists, not only PR-Jun/Fos heterodimers but also PR-Jun/Jun homodimers form. We speculate that in these chimeric receptors the endogenous dimerization domain remains functional in the presence of agonists. This domain is being mapped. Interestingly, the pure PR-*zip* heterodimers, unlike wild type receptors, bind a PRE in the absence of hormone. Transcriptional analyses with several promoter-reporter constructs show that antagonist occupied B-*zip* homodimers stimulate transcription, but A-*zip* homodimers do not. Pure A:B *zip* heterodimers have the transcriptional phenotype of A-*zip* homodimers. Thus, in the heterodimers, A-receptors are dominant over B-receptors. Despite the fact that the hormone-free PR-*zip* dimers bind to DNA, they are transcriptionally inactive, suggesting that PR dimerization and DNA binding are necessary but not sufficient to activate transcription.

K 533 A POTENTIAL RECEPTOR FOR $1,25(OH)_2D_3$ IN INTESTINAL EPITHELIAL PLASMALEMMA: RELATIONSHIP TO NON-NUCLEAR EFFECTS I. Nemere and A.W. Norman, Dept. Biochem. Univ. CA, Riverside CA 92521

The seco-steroid hormone 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] has been demonstrated to have non-nuclear effects including the rapid, hormonal stimulation of intestinal calcium transport (transcaltachia). This report presents evidence for a receptor for $1,25(OH)_2D_3$ located in the basal-lateral membrane (BLM-VDR) of chick intestinal epithelium, which may mediate transcaltachic responses. The protein requires detergent levels above critical micelle concentration for solubilization and exhibits saturable binding (K_D=0.72 x 10^{-10} M, B_{max}=0.24 pmols/mg protein). In addition, saturable binding was observed for [^{3}H]-$24,25(OH)_2D_3$ at physiologically relevant levels (K_D=19 x 10^{-9} M, B_{max}=2.4 pmols/mg protein) to a component apparently distinct from the $1,25(OH)_2D_3$ binding protein. A further 150-fold purification of the $1,25(OH)_2D_3$ BLM-VDR, relative to solubilized BLM, was achieved by anion exchange chromatography. Vitamin D-deficiency resulted in reduced or absent specific [^{3}H]-$1,25(OH)_2D_3$ binding in post-nuclear membranes, solubilized BLM, and partially purified fractions from chromatography, relative to corresponding fractions from normal, vitamin D-replete chicks. The BLM-VDR also exhibited down-regulation of specific [^{3}H]-$1,25(OH)_2D_3$ binding: vascular perfusion of normal duodena for 15 min with 650 pM $1,25(OH)_2D_3$ abolished specific binding in BLM fractions, relative to corresponding fractions from duodena perfused with control medium. The combined results support the existence of a plasmalemmal $1,25(OH)_2D_3$ receptor and suggest it is a prime candidate for mediating transcaltachia.

K 534 DIFFERENTIAL REGULATION OF RABBIT, RODENT AND HUMAN PROGESTERONE RECEPTOR GENES THROUGH AN INTRAGENIC ENHANCER REGION. Alan Robinson, Mary Wu, and Geoffrey L. Greene, Ben May Institute, The University of Chicago, Chicago, IL 60637.

Female sex steroids control the growth of hormone responsive tissues and cancers through the regulation of specific gene networks. A useful model system for studying the molecular basis of this activity is the human progesterone receptor (hPR) gene, which is transcriptionally regulated by estrogens, progestins, and antagonists via their cognate receptors in cultured human breast cancer cells. To better define some of the underlying mechanisms, genomic DNA and T47D cDNA clones encompassing the entire translated portion of hPR mRNA and 6.3 kb of upstream hPR DNA were isolated, sequenced. The promoter region for the larger B isoform of hPR contains binding sites for several general transcription factors. However, it lacks a classical TATA box and does not appear to contain any recognizable ERE or PRE sites in the region from -711 to +1. A long GC-rich untranslated region (744 bp) precedes the initiation codon (ATG_B) for the larger B form of hPR. A potential composite response element that contains an imperfect ERE is located in the intragenic region that includes the ATG_B codon at +744. To determine whether this region comprises a functional ERE, transfection experiments were performed with reporter plasmids that contained appropriate segments of the hPR gene linked to CAT cDNA through the heterologous thymidine kinase (tk) or collagenase (coll) promoter, as well as the natural hPR promoter(s). Initial studies with several hPR-tk-CAT reporters indicate that a weak estrogen responsive element exists within the +731/+761 region of the hPR gene, which includes the ATG_B codon at +744 bp. A 31-bp oligonucleotide containing this sequence bound hER with about 50-fold reduced affinity when compared to the *xenopus* vitellogenin A2 gene ERE. Notably, progestins inhibited the estrogenic induction of the "ERE" located at +744, presumably via the progesterone receptor present in MCF-7 cells. However, an ERE-tk-CAT reporter containing the perfect palindromic vitellogenin A2 ERE was unaffected by progestins in these cells. When this region was mutated to represent either the mouse or rabbit PR DNA sequences, moderate to strong induction of the equivalent coll-CAT reporters was observed in the presence of estrogens, which correlated well with the relative binding affinities of this region for ER *in vitro*. Since the rodent, rabbit, and human PR genes are regulated similarly by estrogens and progestins *in vivo*, it is likely that other *cis* elements play a role in PR gene regulation. We are currently trying to determine the role of an overlapping AP1-like site as well as proximal SP1 sites as modulators of estrogen and progestin mediated activation and repression of hPR gene expression in MCF-7 cells.

K 535 STEROID RECEPTOR MEDIATED EFFECTS OF NEUROACTIVE STEROIDS, Rainer Rupprecht, Johannes M.H.M. Reul, Thorsten Trapp, Bas van Steensel and Florian Holsboer, Max-Planck-Institute of Psychiatry, Clinical Institute, Department of Neuroendocrinology, Kraepelinstr. 2-10, 8000 München 40, FRG

Several 3α-hydroxy steroids accumulate in the brain after local synthesis or after metabolization of steroids that are provided from the adrenals. As this may occur independently from peripheral sources these steroids are called neurosteroids. The 3α-hydroxy ring A-reduced pregnane steroids allopregnanolone and tetrahydrodeoxycorticosterone are believed not to interact with intracellular receptors but enhance γ-aminobutyric acid (GABA)-mediated chloride currents.

The present study shows that these neuroactive steroids can regulate gene expression via the progesterone receptor (PR). Although they do not bind to the PR of either species, they confer an exclusively nuclear localization of the PR. However, the induction of DNA-binding and transcriptional activation of the PR requires intracellular oxidation of the neurosteroids into PR-active 5α-pregnane steroids.

Thus, in physiological concentrations, these neurosteroids regulate neuronal function through their concurrent influence on transmitter-gated ion channels and gene expression. These findings extend the concept of a "cross-talk" between membrane and nuclear hormone effects and provide a new lead for the therapeutic application of these steroids in neurology and psychiatry.

K 536 RECONSTITUTION OF THYROID HORMONE RECEPTOR AND RETINOIC ACID RECEPTOR FUNCTION IN THE FISSION YEAST *Schizosaccharomyces pombe*, Stephen Sande and Marin L Privalsky, Department of Microbiology, University of California, Davis, Davis CA 95616

Investigations into the molecular mechanisms of action of the nuclear hormone receptors would benefit from the development of a model system amenable to biochemical and genetic manipulation, but lacking endogenous receptors or potential metabolic interconversions of hormone ligands. The fission yeast, *Schizosaccharomyces pombe*, has proven to be a useful model biological system for the functional characterization of many higher eukaryotic processes and, unlike vertebrate or insect cell culture systems, *S. pombe*, provide a relatively well defined background that lacks known nuclear receptors. We report here a study of the properties of several vertebrate nuclear hormone receptors by employing *S. pombe*. We have expressed and characterized the function of retinoid (RARs and RXRs) and thyroid hormone receptor, as well as the oncogenic v-erb A derivative, in *S. pombe* and were able to demonstrate high levels of receptor function and strong responsiveness to hormone ligand. In addition, we were able to determine the DNA recognition properties of these receptors. We conclude that the *S. pombe* system is a particularly useful one for characterizing the actions of these nuclear hormone receptors.

K 537 AN N-TERMINALLY TRUNCATED PROGESTERONE RECEPTOR BINDS [^{3}H]PROGESTERONE NON-COOPERATIVELY. D.F. Skafar, L. Zhao, A. Golembiewski and N. Tchopev, Department of Physiology, Wayne State University School of Medicine, Detroit, MI 48201

We have expressed and characterized a 43 kDal N-terminally truncated bovine progesterone receptor containing both the DNA-binding domain and the hormone-binding domain in *E. coli* using the pET expression system. The protein reacted with a polyclonal antibody against the first zinc finger of the DNA-binding domain, indicating that the reading frame was correct. We then characterized the DNA and hormone-binding properties of the truncated receptor. The expressed truncated receptor bound specifically to a PRE-containing oligonucleotide in a gel-shift assay. The results from competition binding experiments showed that the expressed truncated receptor bound progesterone and R5020; it did not bind estradiol, cortisol, testosterone or dihydrotestosterone. Saturation binding assays were then used to determine the affinity of the truncated receptor for progesterone and its Hill coefficient of binding. The truncated receptor bound [^{3}H]progesterone with a K_d of 12.6±3.3 nM and a Hill coefficient of 0.91±0.11 at receptor concentrations between 1 and 12 nM (n=11). The corresponding Scatchard plots of the data were linear. Therefore, the truncated receptor did not exhibit positive cooperativity. Moreover, there was no dependence of the Hill coefficient on receptor concentration. These results contrast with previous studies of the bovine progesterone receptor in uterine cytosol, in which the receptor exhibited a maximum Hill coefficient for [^{3}H]progesterone binding of 1.2-1.4 at receptor concentrations greater than 8 nM. Several factors could account for this difference. First and most interestingly, the N-terminus of the receptor could be required for cooperative binding and ligand-induced conformational changes. Second, post-translational modifications of the bacterially-produced protein could be absent or different than in the native protein. Finally, the dimerization of the truncated protein could be altered. Further experiments are required to determine the best explanation for the observed elimination of cooperativity. Supported by NSF grant IBN-9104857 (to DFS).

K 538 REGULATION OF CHOLESTEROL 7α-HYDROXYLASE (CYP7) EXPRESSION BY BEZAFIBRATE AND CHOLIC ACID: DIFFERENTIAL EFFECTS IN NORMAL AND HYPOPHYSECTOMIZED RATS, Scott S. Sundseth, Melissa K. Foshee, Jo S. Zulkoski, and Roy L. Hawke, Wellcome Research Laboratories, Burroughs Wellcome Co, Research Triangle Park, NC 27709

The fibrates are a large class of compounds that share a common phenoxyisobutyric acid core structure and exhibit hypolipidemic and hypocholesterolemic activity. One side-effect historically associated with the therapeutic use of fibrates is an increased lithogenic index caused by supersaturation of the bile with free cholesterol. To examine the effect of fibrates on cholesterol 7α-hydroxylase (CYP7) expression, the rate-limiting step in both cholesterol metabolism and bile acid biosynthesis, rats were fed diets containing either bezafibrate, a potent fibrate compound used to treat hypertriglyceridemia, or cholic acid, a bile acid that decreases CYP7 expression via a feedback repression mechanism, or a combination of bezafibrate and cholic acid. Hepatic cholesterol 7α-hydroxylase activity and CYP7 mRNA levels were decreased with either bezafibrate (54%) or cholic acid (62%) feeding. Treatment with both bezafibrate and cholic acid exerted an additive effect and suppressed enzyme activity and mRNA to nearly undetectable levels. However in hypophysectomized rats, bezafibrate treatment had no effect on CYP7 expression. In contrast, cholic acid downregulated CYP7 activity and mRNA levels in both normal and hypophysectomized animals. These results indicate that fibrates may cause choleolithostatic disease by decreasing cholesterol metabolism to more polar, readily excreted bile acids, resulting in cholesterol supersaturation of a reduced bile acid pool These experiments also demonstrate that fibrate down-regulation of CYP7 expression requires a hormonal cofactor, but bile acid feedback repression of CYP7 is independent of an intact hypothalamic-pituitary axis and circulating hormones.

K 539 DOWN-REGULATION OF RARβ IN MAMMARY CARCINOMA CELL LINES AND ITS UP-REGULATION IN SENESCING NORMAL MAMMARY EPITHELIAL CELLS, Karen Swisshelm, Karen Ryan, Xinhua Lee, Hui C. Tsou, Monica Peacocke, and Ruth Sager, Department of Pathology, University of Washington, Seattle, WA; Division of Cancer Genetics, Dana-Farber Cancer Institute; and Department of Dermatology, New England Medical Center, Boston, MA

We have investigated the expression of the retinoic acid receptor genes (RARα, β, γ; RXRβ) in normal, senescing, and tumorigenic human mammary epithelial cells (HMECs). We find that RARα and γ as well as RXRβ are variably expressed in both normal and tumor cells, but that most tumor cells show a loss of RARβ expression. RARβ gene expression is induced both by retinoic acid and by fenretinide [N-(4-hydroxyphenyl)] in normal cells, but tumor cells fail to respond to either. Paradoxically, both normal and tumor cells can transactivate an exogenous beta-retinoic acid responsive element (βRARE) as demonstrated by reporter gene assays (CAT and luciferase). Oligonucleotide mobility shift assays with the βRARE show a single discrete complex in normal cells, whereas tumor cells exhibit a heterogeneous set of larger complexes. Reporter gene assays with extended promoter regions indicate the presence of negative regulatory elements and/or factor binding sites that reside between -1500 and the RARE located at -59, and that the promoter is clearly down-regulated in MCF-7 tumor cells. Senescent HMECs exhibit increased RARβ expression with serial passage. These results support the hypothesis that RARβ transcription is down-regulated in tumor cells compared with normal HMECs, and up-regulated in senescence.

K 540 A THYROID HORMONE/RETINOIC ACID (T3/RA) RESPONSE ELEMENT IN THE MYELOBLASTIN PROMOTER, Jianye Teng, Paola Ballerini, Veronique Barbu and Yvon E. Cayre, Department of Microbiology/Immunologie, Cancer Institute, Thomas Jefferson University, Philadelphia, PA, 19107, and CHU Saint Antoine, Paris, France.
Myeloblastin (mbn) is a serine protease involved in the control of growth and differentiation of human leukemic cells. In the promyelocyte-like human leukemia cell line HL-60, this protease is down-regulated during retinoic acid (RA)-induced differentiation.
We have constructed a genomic library using the HL-60 human leukemic cell line as a source of DNA. A 735 bp fragment corresponding to the sequence uptream the ATG was subcloned and further sequenced. A putative response elements for RARα, RE-2, a novel palindromic sequence, was selected according to homologies with other response elements. This sequence was tested in electrophoresis mobility gel shift assays using in vitro-transcribed and tranlated RARα and RXRα incubated with the labelled RE-2 oligomer. The RE-2 sequence was specifically shifted after binding to the RARα/RXRα heterodimeric complex. The shifted band had the same position and intensity as when the heterodimeric complex was linked to a positive control synthetic RARE oligomer, DR5G. The RE-2 sequence was able to compete with DR5G for binding to the RARα/RXRα. Because of a high sequence homology between RE-2 and a RXR-associated palindromic thyroid hormone/RA (T3/RA) response element, we have explored whether the two sequences were able to compete with each other. Gel shift assays have confirmed that the two sequences were cross-reacting showing that the RE-2 DNA-binding site is indeed a combined T3/RA transactivation domain. As with the T3/RA, the estrogen response element was able to compete with RE-2. We will discuss the implication of these findings as related to the presence of t(15;17) in NB4 cells.

K 541 DETECTION OF ANDROGEN RECEPTOR GENE MUTATIONS IN AIS PATIENTS BY SEMI-AUTOMATED DNA SEQUENCE ANALYSIS, Mark N. Patterson, Helen R. Davies and Ieuan A. Hughes, Department of Paediatrics, University of Cambridge.
The androgen insensitivity syndrome (AIS) is a disorder of male sexual differentiation caused by defects in the androgen receptor. Over 100 mutations have been reported in cases of complete and partial androgen insensitivity. The majority of these are point mutations distributed throughout the receptor coding sequence. Screening of the complete androgen receptor gene is therefore necessary to identify the mutation in new AIS cases and to provide information for genetic counselling of affected families.
We have been investigating semi-automated sequencing (Applied Biosystems Model 373A) as a rapid and sensitive method for screening the androgen receptor gene in suspected cases of AIS. The gene is amplified exon by exon, from genomic DNA prepared from blood, and gel-purified PCR fragments are sequenced by taq cycle sequencing using fluorescent dye terminators. Using this approach, it is possible to sequence the complete receptor coding sequence on one sequencing gel. Recently, we have identified 2 novel ligand binding domain mutations by this method in patients with CAIS: leu907phe and leu881val. Neither of these mutations affects residues which are highly conserved in receptors, and both amino acid changes are relatively conservative. It will therefore be necessary to express these mutant receptors *in vitro* to confirm their effects on receptor function. However, the first mutation is 12 amino acids from the receptor C-terminus and, consistent with its location in the ligand binding domain, is associated with dramatically reduced levels of androgen binding activity in genital skin fibroblasts from the patient. This is the most C-terminal mutation reported in a CAIS patient and suggests the importance of this region in receptor function. Binding data is not available for the second mutation. Both mutations also create new restriction sites (BglII and ScrFI, respectively), which has provided a straightforward test for carrier detection in other family members.

Late Abstracts

AMIODARONE-INDUCED HYPERCHOLESTEROLAEMIA: DOES AMIODARONE INTERFERE WITH THYROID HORMONE-DEPENDENT GENE EXPRESSION ?, Onno Bakker, Cisca Hudig, Mieke van Beeren and Wilmar M. Wiersinga, Department of Endocrinology, Academic Medical Centre, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands
Amiodarone (AM), a potent antiarrythmic drug, causes a dose-dependent increase of serum cholesterol in patients and experimental animals, resembling the hypercholesterolaemia of hypothyroidism. We therefore conducted experiments to evaluate if the mechanism underlying the hypercholesterolaemia during amiodarone treatment is similar to that of hypothyroidism. Rats were given AM (10 mg/100g BW) or PTU (4 mg/100g BW, to induce hypothyroidism) for 14 days with pair-fed animals (PFC) serving as controls. Both treatments resulted in a decrease of thyroid hormone (T3) plasma levels. They also resulted in a rise in cholesterol in both the AM and PTU treated groups (PFC, 1.9±0.3 mM; AM, 3.0±0.2 mM; PTU, 2.3±0.4 mM). The cholesterol increase consisted of a rise in both LDL and HDL cholesterol. To get an insight into the mechanism of the cholesterol rise we studied the mRNAs of three key proteins in cholesterol metabolism (HMG-CoA reductase, cholesterol 7α-hydroxylase and the LDL receptor), all of which are more or less dependent on T3 . Rat liver poly(A+) RNA was hybridised with specific probes for these genes, using C/EBP as a control. We found that the mRNA for the LDL receptor was lowered (to 50%) in both the AM and PTU groups, whereas the other two were not influenced by either treatment.
To further elucidate the mechanism of action of amiodarone we studied the effect of AM and its major metabolite desethylamiodarone (DEA) on the binding of T3 to the rat β1 receptor. The receptor was expressed in a bacterial system, partially purified and incubated with [^{125}I]T3, with or without AM and DEA up to 10^{-4} M, for 2h at 22°C in a buffer containing 0.05% Triton X-100 to solubilise the drugs. We found that DEA but not AM was able to interfere with the binding of T3 to the β1 receptor protein. Scatchard analysis in the presence of different concentrations of DEA and double reciprocal plots, indicated that DEA acts as a parabolic non-competitive inhibitor of T3 binding, suggesting that the effect gets stronger as concentrations increase. This is supported by *in vivo* studies in humans were the rise in cholesterol is related to the cumulative dose of AM.
Conclusion: AM treatment and hypothyroidsim are associated with a decrease in the LDL-receptor mRNA , which may be responsible for he rise in cholesterol observed in both cases. As the expression of LDL-receptor is T3-dependent, this decrease may be explained by the interference of DEA with T3 binding to its receptor protein, possibly resulting in an inactive receptor complex that doesn't dimerise or bind DNA. Studying the mechanism of action of AM may yield important insights in thyroid hormone receptor action.

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. We have previously demonstrated that partially purified recombinant xER derepresses and enhances *in vitro* transcription from the ERE containing vitellogenin B1 promoter in a *Xenopus laevis* male liver nuclear extract. For *in vitro* transcription and DNA binding studies, we purified the full length receptor to homogeneity using a DNA-affinity column. We discovered 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 cell extracts. We present evidence, that the high-affinity interaction of the xER with its cognate response element *in vitro* requires an additional protein factor(s). This factor did not appear to be involve in direct interaction between xER and the ERE. Here, we describe purification and characterization of this receptor binding stimulatory activity (RBSA) and we provide evidence that this activity is involved in the phosphorylation of xER. This RBSA factor may represent a novel element in the complex modulation of the steroid receptor-DNA binding process.

LIGAND-INDUCED CONFORMATIONAL CHANGES IN RARs, RXRα AND VDR ANALYZED BY PROTEASE MAPPING, Siegfried Keidel, Peter LeMotte, and Christian Apfel, Pharma Division, Preclinical Research, Department of Dermatology, F. Hoffmann-La Roche Ltd, 4002 Basel, Switzerland

Retinoids exert their effects on cell differentiation and proliferation through two families of nuclear receptors, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). In a limited proteolytic digestion assay, complexation of RARα, RARβ, RARγ, RXRα or the related vitamin D_3 receptor (VDR) with their natural ligands resulted in the resistance of protein fragments to proteolytic digestion. This suggests ligand-induced conformational changes and could mean that these are a general feature in the hormonal activation of vitamin D_3 and retinoid receptors. Recently the synthetic retinoid Ro 41-5253 was identified as a selective RARα antagonist. As demonstrated by gel-retardation assays, Ro 41-5253 does not influence the RARα/RXRα heterodimerization and DNA binding. An excess of antagonist over RA is able to change the digestion pattern from the one characteristic of agonists to a different one that is characteristic of antagonists. This suggests that antagonists compete with agonists for binding to RARα and may induce a different structural alteration. This RARα conformation seems to allow RARα/RXRα binding to DNA but not the activation of target genes. Protease mapping using C-terminally truncated receptors revealed that the proposed conformational changes mainly occur in the DE regions of RARα.

RECEPTOR -SELECTIVE RETINOID INDUCED CHANGES IN GENE EXPRESSION DURING DIFFERENTIATION AND APOPTOSIS IN HUMAN MYELOID LEUKEMIA CELL LINES (HL-60)

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The human myeloid leukemia cell line (HL-60) serves as a useful model for studying myeloid differentiation and apoptotic cell death. Retinoids induce differentiation towards mature neutrophil granulocytes which subsequently undergo apoptosis. We have investigated the expression of regulatory (c-myc, bcl-2) and effector (tissue transglutaminase) genes during these processes. The biological effect of retinoids are mediated by at least two families of retinoid receptors , the Retinoic Acid Receptors (RAR's) that bind all trans retinoic acid and the Retinoid X Receptors (RXR's) that bind 9-cis retinoic acid. Little is known of the relative contribution that each class makes to the biological effects of retinoids in vivo. To examine this issue we have used natural (all-trans retinoic acid, 9-cis retinoic acid) and synthetic retinoids (TTNPB and 3-methyl TTNPB) that differ in their affinity for RAR's and RXR's. We have used three HL-60 cell lines , HL-60 ATCC cells undergo retinoic acid-induced differentiation whereas HL-60 CDM-1 and HL-60R cells retinoid-induced differentiation resistant. In HL-60 ATCC cells, compounds that activate both RAR's and RXR's can activate cellular differentiation coupled to downregulation of c-myc and bcl-2 and upregulation of tissue transglutaminase expression. In HL-60 CDM-1 cells retinoids such as TTNPB that selectively activate RAR's neither induce tissue transglutaminase induction nor cellular differentiation or death , compounds that activate RXR's (9-cis retinoic acid, 3met-TTNPB) induce the expression of tissue transglutaminase and apoptotic cell death. These studies suggest that RAR's appear to be critically involved in the induction of cellular differentiation whereas RXR's appear to play an important role in the induction of apoptotic cell death.

ESTRADIOL INCREASES AND ANTIESTROGENS ANTAGONIZE THE GROWTH FACTOR-INDUCED AP-1 ACTIVITY IN MCF7 BREAST CANCER CELLS WITHOUT AFFECTING *c-fos* AND *c-jun* SYNTHESIS, Dany Chalbos, Alexandre Philips, Florence Galtier and Henri Rochefort, INSERM U148, 60 rue de Navacelles, Montpellier, 34090, France

In estrogen receptor (ER) positive human breast cancer cells, antiestrogens inhibit the mitogenic effect of growth factors, in the absence of estrogens. As, AP-1 activity is one of the first nuclear events following growth factor receptor activation, we studied the effects of estrogens and antiestrogens on growth factor-induced AP-1 activity using transient transfection of the AP-1 responsive gene (AP-1)4-TK-CAT into MCF7 cells. The growth factor-induced AP-1 response was increased by estradiol and inhibited by antiestrogens in conditions where growth factor-induced *c-fos* and *c-jun* mRNA levels were unchanged by hormone and anti-hormone treatments. The same regulations were obtained when the AP-1 response was directly induced by co-transfection of *c-fos* and *c-jun* expression vectors. Co-transfection of the wild-type estrogen receptor HEGO amplified both effects. Antiestrogens inhibited AP-1 activity in conditions where they had no effect on basal ERE-mediated activity levels, whereas estradiol was as efficient in stimulating both activities. Moreover, the relative efficacy of the two antiestrogens, OH-Tamoxifen and ICI 164,384 in inhibiting these two activities was different, OH-Tamoxifen was more efficient in inhibiting ERE-mediated activity, whereas ICI 164,384 was more efficient in trans-repressing AP-1 mediated activity.

Modulations of AP-1 activity by ER ligands were measured in other cell lines. Whereas estradiol also enhanced AP-1 activity in T47D and ZR75.1 human breast cancer cells, it strongly inhibited its activity in Ishikawa endometrial cancer cells, in ER negative MDA-MB231 breast cancer cells and NIH-3T3 fibroblasts which were co-transfected with HEGO. The mechanism of this cell specificity is under investigation.

We conclude that ER regulates not only ERE-mediated transcription but also the growth factor action on AP-1 activity and that modulations of AP-1 activity are both ligand- and cell-dependent.

INTERACTION OF THYROID HORMONE AND RETINOID X RECEPTORS WITH A NEW MEMBER OF A FAMILY OF TRANSCRIPTIONAL REGULATORS. Fergus Ryan, Jae Woon Lee and David D. Moore. Dept. of Molecular Biology, Mass General Hospital, Boston MA 02114.

We have used a genetic interaction trap to select cDNAs encoding a novel protein that interacts with the ß1 isoform of the thyroid hormone receptor (TRß1). The protein, called TRIP1 (for thyroid hormone receptor interacting protein 1), belongs to a family of nuclear transcription regulators that includes the yeast SUG1, a specific co-activator of GAL4 function, and the human HIV TAT-interacting proteins TBP1 and MSS1. In yeast, TRIP1 specifically interacts with both TRß1 and the ß isoform of the retinoid X receptor (RXRß) in a ligand dependent manner. In mammalian cells, TRIP1 specifically represses both thyroid hormone induced transactivation directed by TRß1 and the 9-cis -retinoic acid-induced transcription directed by RXRß. TRIP1 over-expressed in E Coli has been demonstrated to be an ATPase of comparable specific activity to that of other members of this family. Preliminary evidence from immune precipitations suggests a physical interaction between baterially produced, or *in vitro* transcribed and translated, TRIP1 and TRß1, in the presence of a mammalian cell extract. This suggests that TRIP1 may be part of a protein complex involved in transcripional regulation.

A NOVEL CIS ELEMENT WHICH MEDIATES LIGAND-INDEPENDENT ACTIVATION BY c-ErbA: IMPLICATIONS FOR HORMONAL REGULATION, Fahri Saatcioglu, Tiliang Deng, and Michael Karin, Department of Pharmacology, School of Medicine, University of California, San Diego, San Diego, CA 92093-0636

A new type of hormone response element (HRE) is described. Unlike classical HREs, this element, RSV-T3RE (found in RSV LTR), mediates strong activation by the c-ErbAα thyroid hormone (T3) receptor in the absence of T3 and addition of T3 reverses this response. Whereas both c-ErbAα and v-ErbA are potent ligand-independent activators through the RSV-T3RE, c-ErbAβ is not. The RSV-T3RE is recognized and activated by either c-ErbAα homodimers or c-ErbAα/RXR homodimers. Ligand-independent activation by c-ErbAα depends on a unique N-terminal activation domain, while the C-terminal activation domain is not absolutely required. Ligand-dependent activation on the other hand, requires the C-terminal but not the N-terminal activation domain. Upon binding to the RSV-T3RE, c-ErbAα assumes a different conformation than when bound to a classical T3RE. c-ErbAα is therefore capable of selective deployment of activation domains, dictated by both the HRE with which it interacts and T3 binding.

TRANSFECTION OF RARα cDNA IN RETINOID-RESISTANT ESTROGEN RECEPTOR NEGATIVE HUMAN BREAST CARCINOMA CELLS RESULTS IN SENSITIVITY TO GROWTH INHIBITION BY RETINOIDS, M.S. Sheikh, Z-M. Shao and J.A. Fontana, University of Maryland Cancer Center and the V. A. Medical Center, Baltimore, MD 21201.

Numerous studies have shown that estrogen receptor (ER)-positive human breast carcinoma (HBC) cells are growth inhibited by retinoids while the ER-negative cells are resistant. We and others have shown that ER-positive cells express higher levels of RARα mRNA than the ER-negative counterparts. We have recently found that the expression of functional ERs in the ER-negative MDA-MB-231 cells resulted in cells expressing higher RARα mRNA and exhibiting sensitivity to growth inhibition by retinoic acid (RA). These findings suggested that adequate levels of RARα may be required for retinoid inhibition of HBC growth. To test this hypothesis 2 ER-negative HBC cell lines namely MDA-MB-231 and MDA-MB-468, were transfected with the expression vector carrying the RARα cDNA under a SV40 promoter. RARα transfectants expressing differential RARα mRNA levels were further characterized. Treatment with 1μM RA of RARα transfectants expressing higher RARα mRNA levels resulted in 50% growth inhibition on day 9. RAR specific retinoid TTAB (1μM) was more potent and its growth inhibitory effects ranged from 65% inhibition (468) to complete growth block (231). Mock transfected or nontransfected cells did not show growth inhibition by these agents suggesting that the growth inhibitory effects were due to RARα transfection. Transient transfection of the reporter vector carrying βRARE revealed higher RA-induced CAT activity in the RARα transfectants over that observed in mock transfected or nontransfected cells. Our results thus suggest that adequate RARα levels are important for retinoid actions in HBC cells and RARα levels may determine which HBC patient would respond to retinoid therapy.

RETINOIC ACID MODULATES RELEASE OF EPSTEIN-BARR VIRUS FROM LATENCY, Nirupama Deshmane Sista[1], Kimberly Sampson[1], and J.S. Pagano[1,2,3]. UNC Lineberger Comprehensive Cancer Center[1] and Departments of Medicine[2] and Microbiology[3], University of North Carolina at Chapel Hill, NC 27599

Epstein-Barr virus (EBV) requires differentiated mucosal epithelial cells for its replication, and can also cause latent infection and immortalization of lymphocytes. The synthesis of EBV replicative antigens can be induced in latently infected lymphoid cell lines by phorbol esters such as TPA, which induces expression of the BZLF1 (Z) immediate-early protein. Z, a member of the b-Zip family, triggers viral reactivation. Some years earlier, we showed that viral reactivation by TPA is inhibited by retinoic acid, but the mechanism was unknown. Retinoic acid receptors (RARs) negatively regulate the AP1 transcription factor. Since the BZLF1 (Z) protein has homology to *c-fos*, RARs could also potentially down-regulate transactivation by Z.

Here we demonstrate the role of retinoic acid receptors, RARα and RXRα, in the regulation of a prototypic early promoter, BMRF1, which directs the transcription of an EBV early replicative gene product, Early Antigen-Diffuse (EA-D). The Z protein transactivates the BMRF1 promoter through an AP1-binding site. We have shown that RARα and RXRα abrogate activation of the BMRF1 promoter by Z. In addition the Z protein can act reciprocally to inhibit RARα and RXRα-induced activation of an autoregulated cellular promoter for the RARβ gene through a non-DNA binding mechanism. RXRα prevents Z from binding to the AP1 site in the BMRF1 promoter, and, reciprocally, Z inhibits RARα from binding to its retinoic acid response element (RARE) in the RARβ promoter. Furthermore, a direct interaction between the two proteins can be demonstrated *in vitro* using glutathione-S-transferase fusion proteins. Point mutations in the dimerization domain of Z prevent its binding to a GST-RXRα fusion protein *in vitro*. These mutants are unable to down-regulate RXRα-mediated transactivation in transfected cells. Interestingly, the transactivation and repressor domains of Z are distinct. We have also mapped the domain in RXRα that is required for interaction with Z. These results indicate that a 'cross-coupling' or direct interaction between Z (the viral protein) and RARα and RXRα (cellular proteins) can modulate the reactivation of latent EBV infection. The results lead to the possibility that the cell differentiation state mediated by RARs can affect viral reactivation, and also viral infection itself may affect the cell differentiation state controlled by RARs.

PROTEIN KINASE C IN ANDROGEN-SENSITIVE AND INSENSITIVE PROSTATIC TUMOUR CELLS: EFFECTS ON CELL GROWTH AND GENE EXPRESSION, Pirkko Vihko and Pirkko Henttu, Biocenter and Department of Clinical Chemistry, University of Oulu, FIN-90220 Oulu, Finland

Unstimulated DU-145 and PC-3 prostatic cells, which are androgen-insensitive, contained 4-5.5-fold more protein kinase C activity that was sensitive to Ca-ions and phospholipid than did the androgen-sensitive LNCaP cells. TPA-activation increased the amount of PKC activity about 2.2-fold in LNCaP cells within 3 hours, but slightly decreased the activity in DU-145 and PC-3 cells, to 60 and 81% of the control, respectively. TPA-treatment did not affect the growth of PC-3 cells, but slightly decreased the growth of DU-145 cells during the first three days of treatment. In contrast, treatment of LNCaP cells with TPA resulted in the death of approximately 50% of cells within 2 days. TPA had only minor effects on the expression level of the immediate early genes c-Fos and C-Jun in PC-3 cells, whereas in DU-145 and LNCaP cells the amounts of these mRNAs were up-regulated within 3 hours after stimulation. Interestingly, the amount of c-Myc mRNA was up-regulated by TPA in DU-145 and PC-3 cells but down-regulated in LNCaP cells. Activation of PKC in LNCaP cells resulted in the down-regulation of the steady-state levels of mRNAs coding for androgen receptor and two prostatic marker proteins: prostatic acid phosphatase (PAP) and prostate-specific antigen (PSA). Furthermore, in LNCaP cells that contained TPA-activated PKC androgen-regulation of these mRNAs was abrogated. However, the androgenic up-regulation of PSA and down-regulation of PAP mRNA did not require the presence of active PKC as was demonstrated by LNCaP cells that were depleted of PKC with a 2-day treatment of TPA. In contrast, the effect of androgens on the human androgen receptor mRNA was inversely correlated to the activity of PKC. These results suggest that commonly used prostatic cell lines LNCaP, DU-145 and PC-3 differ in respect to the activity and effects of PKC. Lack of prostate-specific gene expression may partly result from the high basal level of PKC activity found in DU-145 and PC-3 cells. Furthermore, PKC activation interferes with androgen-regulated gene expression.

ACTIVITY OF THE GLUCOCORTICOID RECEPTOR DURING DERMAL FIBROSARCOMA FORMATION,

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Mice harboring the bovine papillomavirus type I (BPV-1) genome develop skin tumors in a pathway comprised of at least three stages: mild and aggressive fibromatoses and dermal fibrosarcoma. In derivative cell lines, the proto-oncogenes junB and c-jun are induced in the aggressive fibromatosis and fibrosarcoma, in contrast to junD and fos, which remain constant. These proto-oncogenes encode bZIP gene regulatory proteins that have been shown to interact with another family of transcription factors, the zinc finger steroid and thyroid hormone receptors. Furthermore, steroid hormones, such as glucocorticoids, influence cell growth and differentiation in various cell types. Therefore, we were interested in the potential role of steroid hormone receptors (and in particular of the glucocorticoid receptor, GR) during this multistep tumorigenesis process.

Regulation of gene expression by the endogenous GR was studied by transient transfection assays using several response elements linked to a reporter gene. Western blot analysis showed that the level of endogenous GR is similar in all three stages of fibrosarcoma progression. However, the ligand dependent activity of GR, both through simple and composite GREs, was more than ten fold higher in the later stage, dermal fibrosarcoma, than in either of the other stages, thus providing a distinction between the aggressive fibromatosis and the tumor cells. The striking increase in the activity of GR at this critical stage suggests that the receptor may be specifically localised or modified, thus affecting its ability to interact with AP-1 or other cellular factors, and that the activated GR may play a direct role in tumorigenesis.

RETINOIC ACID REPRESSES OCT-3/4 GENE EXPRESSION THROUGH SEVERAL RETINOIC ACID RESPONSIVE ELEMENTS LOCATED IN THE PROMOTER-ENHANCER REGION,

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The Oct-3/4 gene product which belongs to the POU family of transcription factors is expressed in the earliest stages of embryogenesis and repressed in subsequent stages. Retinoic acid (RA) - induced differentiation of embryonal carcinoma (EC) cells is accompanied by decrease expression of the Oct-3/4 gene. Previous findings show that sequences in the Oct-3/4 enhancer region (designated RARE1) are targets for RA-mediated repression. Our present results demonstrate conclusively that the TATA-less Oct-3/4 promoter is also a target for RA-induced repression. We identified a novel cis element in the Oct-3/4 promoter that harbors a putative Sp1 binding site and a RA responsive element (designated RAREoct), which are juxtaposed to one another. Protein binding to the Sp1 site is independent of protein binding to the RAREoct sequence. Unlike the RARE1 situated in the Oct-3/4 enhancer which does not contain a typical RAR recognition site, the RAREoct identified in this study consists of the directly repeated motif, that exhibits extensive homology to RARE sequences located in RA responsive genes. Moreover, the RAREoct shows different DNA binding characteristics and DNase I footprint patterns with nuclear proteins isolated from undifferentiated versus RA-differentiated EC cells. This suggests that the RAREoct element binds different nuclear proteins in RA-treated and untreated EC cells. It preferentially binds the RARβ-RXR hetrodimers as well as the COUP/EAR-3 and ARP-1 orphanan receptors.

Using site directed mutagenesis we show that the RAREoct contributes to the transcriptional activation of Oct-3/4 promoter in P19 cells, and most interestingly, mediates the RA-induced repression in RA-differentiated EC cells. Thus, the RAREoct element could be one of the points of integration of several signalling pathways influencing Oct-3/4 expression. In accordance with the suggestion that suppression of Oct-3/4 expression is a crucial step during embryogenesis, the Oct-3/4 upstream region contains multiple targets for RA induced repression probably to ensure accurate and prompt repression of Oct-3/4 expression. It is possible that these repressors are differentially used at specific stages of development in response to various signals.