THE EUKARYOTIC NUCLEUS

Organizers: Douglass Forbes and Elizabeth Blackburn

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Keynote Address

M 001 IN VITRO ASSEMBLY OF COILED BODIES IN XENOPUS EGG EXTRACT, Donna W. Bauer, Christine Murphy, Zheng'an Wu, Chung-Hsiun Herbert Wu, and Joseph G. Gall, Department of Embryology, Carnegie Institution, Baltimore, MD 21210.

When demembranated Xenopus sperm are incubated in an appropriate extract of unfertilized Xenopus eggs, they swell to form pronuclei of typical appearance. These nuclei are surrounded by a double-layered envelope with nuclear pores and an internal lamina; they import maternally derived nuclear proteins stored in the cytoplasm. Prominent in these nuclei are small inclusions ranging from <1μm to about 3μm. These and similar inclusions in cleavage nuclei have been called prenucleolar bodies. This name reflects the uncertain nature of the bodies - they resemble small nucleoli, but they occur in cells that do not transcribe rRNA (or any other RNA) and they are even present in anucleolate animals that lack the nucleolus organizer locus. Recently, Bell et al. (J.Cell Biol.**118**:1297,1992) demonstrated four nucleolar proteins in the prenucleolar bodies formed in egg extracts (fibrillarin, nucleolin, B23/NO38, and a 180 kD nucleolar protein). They were unable to demonstrate U3 snRNA, which is involved in pre-rRNA processing and is associated with fibrillarin in other cell types. Using ^{3}H-labeled antisense probes, we found clear evidence for both U3 and U8, another snRNA involved in pre-rRNA processing. Surprisingly, we were also able to demonstrate U1, U2, U4, U5, and U6, the five snRNAs involved in pre-mRNA splicing, and U7, known to be required for histone pre-mRNA 3' end formation. We found strong immunofluorescent staining with antibodies against fibrillarin, trimethylguanosine, the Sm proteins, and coilin. Antibodies against the SR splicing factors stained the nuclear contents but not the bodies themselves. These results demonstrate that "prenucleolar" bodies formed during nuclear assembly in egg extracts are, in fact, more similar to coiled bodies than to nucleoli. This system provides a unique opportunity to study the assembly of coiled bodies after experimental manipulation of the extract. We know already that truncation of the first 20 nucleotides of U1 with an antisense oligonucleotide prevents importation of U1 into the newly assembled nuclei but does not interfere with formation of the coiled bodies. Similar studies with other snRNAs and with their associated proteins should give insight into the organization of this unusual nuclear organelle that contains components from three major RNA processing systems.

The Nuclear Envelope-A Structural View

M 002 DYNAMIC PROPERTIES OF THE NUCLEAR LAMINS, Robert D. Goldman, Anne E. Goldman, Robert D. Moir and Michelle Montag-Lowy, Northwestern University Medical School, Chicago.

The nuclear lamina is a protein rich layer localized at the interface between chromatin and the inner face of the nuclear envelope membrane. It's major constituents form a complex polymer consisting of the Type V intermediate filament (IF) proteins, the A and B type nuclear lamins. Until quite recently the lamins were thought to be relatively static components of the karyoskeletal system, except for a short time period during mitosis when the nuclear envelope breaks down and reassembles. We have carried out studies aimed at determining whether the nuclear lamins possess the same dynamic properties which we have elucidated for several types of cytoskeletal IF systems during interphase. The results have yielded some surprising and intriguing results which demonstrate that the lamins are dynamic throughout the cell cycle. Our studies have involved several experimental approaches, including the direct observation of cells at all stages of the cell cycle utilizing lamin A and B type specific antibodies for indirect immunofluorescence with the confocal microscope; and the microinjection of bacterially expressed A and B type lamins (derivatized with biotin) into cultured fibroblasts. The microinjection approach was the first to reveal that the lamins might be found in the deep regions of the nuclear matrix, away from the nuclear periphery (A. Goldman *et al.* (1992), J. Cell Biol., 119: 725-735). These studies demonstrated that microinjected biotinylated lamin A becomes incorporated into the lamina in a series of discrete morphologically identifiable steps. These include the rapid formation of large nuclear foci not associated with the nuclear envelope which subsequently appear to be converted to smaller foci distributed more widely through the nuclear matrix. Within a short time, the injected lamin appears to be incorporated into the lamina. Evidence will be presented which supports the idea that the lamin foci may be processing centers involved in the post-translation modifications of lamin A which are prerequisite or coincident with its assembly into the lamina. Nuclear lamin foci are not restricted to microinjected cells, but are present in many interphase cells as distinguished by confocal indirect immunofluorescence. The nuclear foci or spots are apparent in S phase as well as in G-1 phase cells. During mid to late S-phase, we have found a striking correlation between large nuclear foci seen with lamin B antibody and DNA replication centers as revealed by co-localization with sites of BrDU incorporation or PCNA. Antibodies directed against lamins A/C do not align with these DNA replication sites. These results indicate that the nuclear lamins are dynamic elements of the nucleoplasm and that they may be involved in numerous physiological activities ranging from nuclear shape and size determination to the replication of DNA. (Supported by the NCI).

The Mechanism of Nuclear Transport: Import, Export Cytosolic Factors

M 003 THE GTP-BINDING PROTEIN RAN/TC4 IS REQUIRED FOR PROTEIN IMPORT INTO THE NUCLEUS, Mary Shannon Moore and Günter Blobel, Howard Hughes Medical Institute, The Rockefeller University, New York, NY.

Previously we reported the isolation of two cytosolic fractions (A and B) from Xenopus oocytes which are sufficient to support the import of a nuclear localization sequence (NLS) bearing substrate into the nuclei of permeabilized cells (Cell 69, 939-950, 1992). Fraction A is involved in NLS recognition and targeting to the nuclear envelope while fraction B is required for the subsequent passage of the bound substrate into the nucleus. We have found that two interacting components are required for full fraction B activity and we have purified one of these (a 25 kD polypeptide) to homogeneity. Protein sequence analysis and immunoblotting have identified this polypeptide as the Xenopus homolog of the highly abundant GTP-binding protein Ran (ras related nuclear protein)/TC4. The other component required for full B activity has an apparent molecular mass of approximately 30 kD (as determined by gel filtration chromatography) and appears to exist in the crude cytosol both in the monomeric form and in a 55 kD complex with Ran/TC4. Human recombinant Ran/TC4 (purified from E. coli) will substitute for the purified Xenopus form in the import assay while the addition of another small GTP-binding protein ($p21^{H\text{-}ras}$) has no effect. Both GMP-PNP and GDPβS are inhibitory in the import assay indicating that Ran/TC4 has to be in the GTP-bound form in order to function in nuclear import and that GTP hydrolysis is required. Since Ran/TC4 has also been implicated in RNA export (Kadowaki et al., EMBO J. 12, 2929-2937, 1993), a requirement for Ran/TC4 in protein import implies that the two processes of import and export are likely to be fundamentally similiar, with Ran/TC4 playing a key role in each.

M 004 MOVEMENT OF MACROMOLECULES IN AND OUT OF THE YEAST NUCLEUS, Pamela A. Silver[1], Mark Bossie, Jean Flach[1], Joe Vogel[1], Tim Jinks[1], Lisa Gorsch[2], Charles N. Cole[2] and Gabriel Schlenstedt[1], [1]Dana Farber Cancer Institute and Dept. of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, [2]Dartmouth University Medical School, Hanover, NH.

Transport of proteins into the yeast nucleus has properties in common with higher eukaryotes; many nuclear-destined proteins contain nuclear localization sequences (NLS) that target to them to the nucleus. Genetic and biochemical studies have been used to define components of the yeast nuclear pore complex and other factors that may be involved in the transport process. Because of the emerging genetic analysis, we have developed a reconstituted nuclear protein import reaction from the yeast *Saccharomyces cerevisiae* (1). The reaction uses semi-intact cells. In the presence of ATP and cytosol derived from yeast, mammalian cells, fly cells, or *Xenopus* oocytes, a protein containing an NLS is transported into the nucleus. The import reaction is sensitive to ATPγS, GTPγS, NEM, temperature, and antibodies to the nucleoporin Nsp1. Yeast mutants defective in nuclear transport show defects in the *in vitro* reaction. One mutant, *npl3*, encodes a protein with similarity to a family of RNA binding proteins that shuttle rapidly in and out of the nucleus (2). *Npl3* mutants also accumulate polyA RNA in the nucleus.

1. Schlenstedt, G., Hurt, E., Doye, V. and Silver, P.A. 1993. Reconstitution of nuclear protein transport with semi-intact yeast cells. J. Cell Biol 123 (In press).

2. Bossie, M.A., DeHoratius, C., Barcelo, G. and Silver, P. 1992. A mutant nuclear protein with similarity to RNA binding proteins interferes with nuclear import in yeast. Mol. Biol. Cell. 3:875-893.

Assembly and Disassembly of the Nucleus with the Cell Cycle

M 005 CELL CYCLE CONTROL OF NUCLEAR STRUCTURE AND FUNCTION, Douglass J. Forbes[1], Colin Macaulay[1], Phil Hartl[1], Joel Gottesfeld[2], Maureen Powers[1], Eva Meier[1], Brian Miller[1], Katharine Ullman[1], and Tam Dang[1], [1]Department of Biology, University of California at San Diego, La Jolla, Ca 92093-0347, [2]Scripps Research Institute, La Jolla, CA 92037.

During each cell cycle the highly complex eukaryotic nucleus must undergo a comprehensive breakdown of its component parts. The nuclear membranes, lamina, and pores dissemble. After segregation of the replicated chromosomes, the parts must be reassembled into daughter nuclei. Many of these events can be reproduced *in vitro* in cell-free extracts of Xenopus laevis eggs. In the case of the nuclear lamina and the nuclear membrane, it has been previously been shown that phosphorylation/dephosphorylation events control the disassembly and reassembly of these structures. Our laboratory has examined proteins of the nuclear pore. We find that proteins of the pore are differentially phosphorylated during interphase and mitosis. Upon analysis of the mitotic kinase involved, we find that one pore protein (p200) is a direct substrate of the master mitotic kinase cdc2/cyclin B, while a second pore protein (p62) is not a target of this kinase but of a secondary mitotic kinase. These data suggest that phosphorylation plays a role in controlling the assembly and disassembly of the nuclear pore during the cell cycle.

Mitosis involves not only the disassembly of structural elements of the nucleus, but also inactivation of nuclear function. Specifically, RNA transcription is known to be strongly repressed at mitosis *in vivo*. We have been able to reproduce this mitotic repression of transcription in cell-free Xenopus extracts using a pol III template. In analysis of the mechanism of repression, we find an inactivation of the transcriptional machinery itself at mitosis. Both cdc2 mitotic kinase and a secondary mitotic kinase can carry out this repression. Identification of the specific target of repression will be discussed.

M 006 CHARACTERIZATION OF THE INTERACTION BETWEEN CHROMATIN AND THE NUCLEAR ENVELOPE, John Newport, Rupert Pfaller, and Max Dobles, Department of Biology, University of California at San Diego, La Jolla, Ca 92093-0347.

In nuclei much of the functionally inactive heterochromatic DNA is located at the periphery of the nucleus adjacent to the nuclear envelope while active euchromatic DNA tends to locate within the nuclear interior. This observation suggests that interactions between chromatin and the nuclear envelope may play an important role in regulating chromatin activity. Using a cell-free system derived from Xenopus eggs we have been investigating the regulated interaction of chromatin with the nuclear envelope at the beginning and end of mitosis. Our results indicate that a discrete number of high affinity membrane bound receptor sites interacts with chromatin to direct reassembly of the nuclear envelope at the end of mitosis. We have also shown that the interaction between these receptor sites and chromatin is regulated by a kinase which phosphorylates the membrane receptor. Further progress towards identifying the receptor and the kinase which phosphorylates it will be discussed as well a simple model system using purified mononucleosomes and isolated membrane vesicles for investigating chromatin membrane interactions at the nuclear envelope.

M 007 VESICLE FUSION DURING NUCLEAR ENVELOPE ASSEMBLY: ROLE OF IP_3 RECEPTORS AND Ca^{2+} MOBILIZATION, Kathleen M. C. Sullivan and Katherine L. Wilson, Dept. of Cell Biology and Anatomy, The Johns Hopkins University School of Medicine, Baltimore, MD 21205.

Using *Xenopus* egg extracts, we can assay two distinct steps required for nuclear envelope assembly *in vitro*: vesicle **binding** to chromatin (which does not require cytosol), and cytosol-dependent vesicle **fusion** to form an enclosed envelope (containing pore complexes). After enclosure, the **rate of fusion** can be quantitated as a time-dependent increase in nuclear envelope surface area. We found that nuclear vesicle fusion requires mobilization of Ca^{2+} stored within the vesicle lumen. Moreover, Ca^{2+} release appears to be mediated by IP_3 receptors (IP_3R), which are ligand-gated Ca^{2+} channels that respond to the second messenger, inositol 1,4,5-trisphosphate (IP_3). IP_3 receptors classically release Ca^{2+} in response to signalling events at the plasma membrane. The evidence that IP_3 receptors may also regulate nuclear envelope assembly is as follows. (a) BAPTA, a fast Ca^{2+} buffer that suppresses local increases in cytosolic free [Ca^{2+}], inhibits fusion without changing the resting cytosolic [Ca^{2+}]. Millimolar concentrations of EGTA, a slow Ca^{2+} buffer, has no effect whatsoever. (b) Heparin, a potent antagonist of Ca^{2+} release via IP_3R, inhibits vesicle fusion in an IP_3-reversible manner (see Sullivan et al, 1993 *Cell* 73:1411-1422). (c) Fusion is inhibited by two different domain-specific IP_3R antibodies; one antibody binds the conserved C-terminus, a region critical for Ca^{2+} flux, and the other binds one or more epitopes within the large 'coupling domain'. Although these antibodies (obtained from D. Lin and W. Agnew, Dept. of Physiology) were raised against the mouse cerebellar IP_3R (type I), we have used affinity-purified antibodies as highly specific probes for IP_3R in *Xenopus* egg extracts. We are now determining whether these antibodies (and F_{ab} fragments) block Ca^{2+} flux, change the affinity of the receptor for IP_3, or have other effects on channel activity. In summary, our findings point to an unexpected role for IP_3R in regulating a late step in vesicle fusion during postmitotic nuclear assembly, and further suggest that phosphoinositide signalling may regulate nuclear vesicle fusion. We speculate that signalling and IP_3R activity (channel opening) may be coupled to the local assembly of proper attachment/fusion complexes between adjacent nuclear vesicles.

The Nuclear Matrix: Biochemistry and Molecular Biology

M 008 NuMA, AN ABUNDANT NUCLEAR PROTEIN REQUIRED FOR NORMAL CHROMOSOME SEGREGATION AND/OR NUCLEAR REASSEMBLY: D.A. Compton[1,2], P.A. Coulombe[1], and D.W. Cleveland[1] [1]Dept. of Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD 21205 and [2]Dept. of Biochemistry, Dartmouth Medical School, Hanover, N.H. 03755.

NuMA is a an abundant, 236kD intranuclear protein that plays an intrinsic role in the terminal stages of chromosome segregation and/or nuclear reassembly. The NuMA polypeptide, which consists of globular head and tail domains separated by a discontinuous 1500 amino acid coiled-coil spacer, displays the unusual property that during mitosis it distributes into each daughter cell by association with the pericentrosomal domain of the spindle apparatus. Expression of NuMA lacking its globular head domain results in cells that fail to undergo cytokinesis, but which assemble multiple small nuclei (micronuclei) in the subsequent interphase, despite the appropriate localization of the truncated NuMA to both the nucleus and spindle poles. This dominant phenotype is morphologically identical to that of the tsBN2 cell line that carries a temperature-sensitive mutation in the chromatin-binding protein RCC1. At the restrictive temperature, these cells end mitosis by forming micronuclei, concomitant with the selective exclusion of wild type NuMA from developing nuclei and the degradation of NuMA in the cytoplasm. Elevation of NuMA levels by forcing increased expression of wild-type NuMA in these mutant cells is sufficient to restore post-mitotic assembly of a single normal-sized nucleus. In cells expressing tailless NuMA, apparently normal chromosome segregation is followed unfailingly by micronucleation. Consistent with a structural role in the interphase nucleus and at the spindle poles, recombinant NuMA assembles into starfish-like oligomers from which five radial side arms project. The sum of these findings demonstrate that NuMA function is required for the terminal phases of chromosome separation and/or nuclear reassembly. Beyond this, NuMA's structure, abundance, nuclear localization and oligomerizing characteristics combine to support the view that NuMA plays an anchoring role both in the interphase nucleus and at the mitotic spindle poles.

M 009 MATRIX ASSOCIATION REGIONS (MARs) AND THE IMMUNOGLOBULIN KAPPA GENE, William T. Garrard, Julia B. George, Michael A. Hale, and Kenneth W. Trevorrow, Department of Biochemistry, The University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Boulevard, Dallas, TX 75235-9038.

The kappa light chain immunoglobulin gene locus contains a MAR adjacent to the intronic enhancer just upstream of the constant region exon (1). This MAR is required for maximal expression of a functionally-rearranged kappa gene, both in cultured plasmacytoma cells and transgenic mice (2,3). Because MARs and their nuclear matrix binding sites are evolutionarily conserved (eg. ref. 4), we have taken advantage of the tractable experimental system of the yeast *Saccharomyces cerevisiae* to identify interacting nuclear components and potential kappa MAR functions. We have previously shown that when the kappa MAR is inserted into the *GAL1* promoter, between the UAS and TATA elements, it strongly inhibits transcription of the linked gene (5). In addition, we were able to isolate temperature sensitive mutants that suppressed this inhibition (5). To explore further MAR functions in yeast, we have studied the ability of several MARs to diminish the chromosomal position effect of yeast telomeres (6). Among the MARs investigated, we find that the kappa MAR is the most effective in blocking the telomere position effect. We have also used yeast as a vehicle for deleting the kappa MAR specifically by one-step gene replacement, as a prelude for the creation of kappa MAR knockout mice. These mice should permit us to uncover the natural functions of the MAR in such events as recombination and somatic mutation. The mouse kappa locus includes several hundred variable region exons and spans some 2,000 kilobases on chromosome 6. This locus therefore provides a paradigm for the long term study of the relationship between chromatin higher order structure, cis elements and function possibly related to nuclear architecture. To identify all the MARs in this locus, as well as locus control regions (LCRs), topoisomerase II and DNase I hypersensitive sites, we have isolated 18 YAC clones that appear to represent a contig spanning the locus. We are developing novel techniques to identify VM-26 induced topoisomerase II cleavage sites throughout this locus. It is striking that VM-26 induced cleavage fits the periodicity of chromosomal loop sizes, 50-200 kb, as well as higher order multiples of these units.

References: 1.Cockerill, P.N. and Garrard, W.T. (1986) *Cell* **44**, 273. 2.Blasquez, V.C. et al. (1989) *J. Biol. Chem.* **264**, 21183. 3. Xu, M. et al. (1989) *J. Biol. Chem.* **264**, 21190. 4.Whitehurst, C. et al. (1992) *Nucleic Acids Res.* **20**, 4929. 5.Fishel, B.R. et al. (1993) Proc. Acad. Natl. Sci. USA **90**, 5623. 6.Gottschling, D.E. et al. (1990) *Cell* **63**, 751.

M 010 **SUBNUCLEAR ORGANIZATION IN YEAST: TELOMERES AND THE NUCLEAR ENVELOPE,** Francesca Palladino, Thierry Laroche, Thomas Müller*, Eric Gilson, and Susan Gasser, Swiss Institute for Experimental Cancer Research (ISREC); CH-1066 Epalinges/Lausanne, Switzerland. *ETHZ, Department of Cell Biology, CH-8093 Zürich, Switzerland.

We have examined subnuclear organization by a study of yeast telomere localization. Yeast telomeres exert a position effect both on the transcription of genes located in their close proximity and on the timing of DNA replication. Their function may be achieved through the formation of an unusual chromatin structure at telomeres and through its specific subnuclear localization.The abundant yeast nuclear protein called RAP1 (Repressor- Activator Protein 1) recognizes a 13 bp consensus present in the mating type silencers, in upstream activating regions and in the telomeric repeat sequence, $C_{1-3}A$. We show by footprinting techniques that RAP1 binds the yeast telomeric repeat as frequently as once every 18 bp in vitro, allowing up to 20 molecules of RAP1 on the average 370-bp telomeric repeat. The binding of RAP1 to its telomeric consensus creates a distinct kink in the double helix, such that the nucleotide at position 8 on the C-rich strand is highly reactive to $KMnO_4$. In addition, the binding of full-length, but not truncated forms of RAP1, causes a bend in the double helix 5' of the binding site. By scanning tunnelling microscopy we confirm the fact that full-sized RAP1 bends DNA at more than a 90^0 angle, while the DNA binding domain alone causes less than a 30^0 distortion.

Anti-RAP1 immunofluorescence shows a distinctly punctate staining in interphase yeast nuclei, located primarily, although not exclusively, at the nuclear periphery. There are 8 to 16 brightly staining RAP1-containing foci for 64 chromosomal ends in a diploid cell suggesting telomeres are grouped together. In est1$^-$ yeast cells, in which telomeric repeats shorten, the spots of RAP1 immunofluorescence reduce in intensity and number. The integrity of the yeast nuclear membrane influences the number of aggregates observed after staining with antibodies against RAP1, suggesting a role for the nuclear envelope in the maintenance of telomeric positioning.

The Silent Information Regulator genes of *S. cerevisiae*, SIR3 and SIR4, are required for transcriptional repression at the silent mating type loci and at yeast telomeres, and localize by immunofluorescence to foci at the nuclear periphery, much like those observed with antibodies recognizing the telomere-binding protein RAP1. Importantly, in both sir3 and sir4 mutant strains, telomeres lose their peripheral localization, as monitored by RAP1 immunofluorescence. This suggests a direct interaction of SIR3 and SIR4 with telomeric structures, although these two proteins are not exclusively localized at telomeres. Immunofluorescence studies using mutants of the C-terminus of RAP1 provide evidence that SIR3 and SIR4 interact with telomeres through this domain.

Chromatin and Chromosomes: Organization, Regulation of Transcription and Chromatin

M 011 CHROMATIN STRUCTURE CONTRIBUTES TO REGULATION OF EXPRESSION AT *HSP26* IN *DROSOPHILA* . S.C.R. Elgin, H. Granok, L.L. Wallrath, and Q. Lu. Washington University, Department of Biology, St. Louis, MO 63130.

Our goal is to understand the contribution to gene regulation made by the basic structure of the chromatin fiber, and to understand the role played by some specific and some general chromosomal proteins in generating that structure. While the bulk of the DNA is packaged in a nucleosome array, an active promoter must lie in a nucleosome-free region, a DNase I hypersensitive site (DH site). Creation of appropriate DH sites appears to be essential for gene activity. The *hsp26* promoter region includes two DH sites separated by a precisely positioned nucleosome. Analysis of a large number of transgenes with alterations (deletions, rearrangements, and point mutations) in the 5' regulatory region indicates that the $(CT)_n$ elements play a major role in establishing the preset chromatin structure at the *hsp26* promoter; the heat shock elements, while essential for induction of transcription, play only a minor role in establishing the chromatin structure. Similarly, a mutation in the TATA box that essentially eliminates inducible expression has only a minor effect on chromatin structure. Folding of the DNA around the nucleosome may facilitate activation by juxtaposing the two regulatory sites. The DNA of the nucleosomal region does not appear to include any regulatory elements, as it can be deleted, duplicated, or substituted with random DNA without any significant effect on chromatin structure and gene activity. However, substitution with bent DNA sequences (10 bp periodicity) results in a dramatic loss of activity, apparently due to a shift in the nucleosome positioning. Thus, in addition to the identified regulatory elements (TATA box, $(CT)_n$ elements, and heat shock elements), the wild-type chromatin structure of the promoter region is essential for regulated expression of *hsp26*. In the wild-type gene, it appears that the GAGA factor is critical in establishing that structure.

M 012 TARGETING OF SIR1 PROTEIN ESTABLISHES TRANSCRIPTIONAL SILENCING AT *HM* LOCI AND TELOMERES IN YEAST, Rolf Sternglanz[1], Cheng-ting Chien[1], Stephen Buck[2], and David Shore[2], [1]Department of Biochemistry and Cell Biology, SUNY, Stony Brook, NY 11794, [2]Department of Microbiology, College of Physicians & Surgeons of Columbia University, New York, NY 10032.

Previous studies suggest that the yeast SIR1 protein is involved in the establishment of transcriptional silencing at the *HM* mating-type loci. First, *sir1* mutations destabilize repression at the *HM* silent loci by greatly reducing the frequency of re-establishment of the repressed state. Second, overexpression of SIR1 can suppress many different mutants that are partially defective in silencing. We have now shown that a GAL4 DNA binding domain-SIR1 hybrid protein (G_{BD}-SIR1), when targeted to an *HMR* locus containing GAL4 binding sites (UAS_G), can establish silencing and bypass the requirement for the silencer element *HMR-E*. Silencing mediated by G_{BD}-SIR1 requires the trans-acting factors that normally participate in repression, namely SIR2, SIR3, SIR4 and histone H4. However, G_{BD} hybrids with SIR2, SIR3 , or SIR4 cannot establish silencing. Telomeric silencing, which does not require SIR1 and is normally unstable, is greatly improved by tethering G_{BD}-SIR1 to the telomere. These experimnets support a model in which native SIR1 protein is brougnt to the *HM* loci by proteins bound to the silencers where it acts to assure the efficient establishment of the silenced state. Telomeres appear to lack the ability to recruit SIR1 and that is why telomeric silencing is unstable. Normally the establishment of silencing requires passage through S-phase; we are investigating whether silencing mediated by targeted G_{BD}-SIR1 also requires DNA replication.

M 013 HISTONE MODULATED TRANSCRIPTION: STRUCTURAL AND DEVELOPMENTAL ASPECTS, Alan P. Wolffe, Geneviève Almouzni, Philippe Bouvet, Stefan Dimitrov, Jeffrey J. Hayes, Nicoletta Landsberger, Dmitry Pruss, and Kiyoe Ura, Laboratory of Molecular Embryology, National Institute of Child Health and Human Development, NIH, Bethesda, MD. 20892.

A chromatin environment provides many advantages to the eukaryotic transcriptional machinery in regulating gene expression. Nucleosome formation can alternately either repress (1) or potentiate transcription (2). The three dimensional folding of DNA in chromatin has an important structural and regulatory role. We have characterized the influence of individual histones and their domains on nucleosome structure, nucleosome positioning and the capacity of nucleosomes to repress transcription (3, 4, 5). These in vitro experiments have been extended to chromatin function in vivo. Replication coupled chromatin assembly is required for the general repression of basal transcription in Xenopus oocyte nuclei (6). In spite of complete chromatin assembly, certain transcription factors (eg GAL4/VP16, HSF) activate transcription from the initially repressed state. These transcription factors have two functions: the relief of repression (chromatin disruption) and the activation of the transcription process (recruitment of the basal transcription machinery). We have examined the relative importance of these two functions during vertebrate development as the structural components of chromosomes are modified. These modifications include the replacement of linker histones eg B4, H1, H1° and alterations in histone acetylation (7). Some of the consequences of removal or overexpression of the linker histones for gene expression in the developing embryo have been determined.

1. Wolffe, A.P. and Dimitrov, S. (1993). Crit. Rev. Euk. Gene Exp. 3 167-191
2. Schild, C., Claret, F.X., Wahli, W. and Wolffe, A.P. (1993). EMBO J. 12 423-433
3. Lee, D.Y., Hayes, J.J., Pruss, D. and Wolffe, A.P. (1993). Cell 72 73-84
4. Hayes, J.J. and Wolffe, A.P. (1993). Proc. Natl. Acad. Sci. USA 90 6415-6419
5. Pruss, D. and Wolffe, A.P. (1993). Biochemistry 32 6810-6814
6. Almouzni, G. and Wolffe, A.P. (1993). Genes and Development (in press)
7. Dimitrov, S., Almouzni, G., Dasso, M. and Wolffe, A.P. (1993). Develop. Biol. (in press)

DNA Replication: Basics, Relationship to Higher Order Nuclear Structure, Replication Centers

M 014 TRANSCRIPTIONAL SILENCING, DNA REPLICATION, AND THE YEAST ORIGIN RECOGNITION COMPLEX, Stephen P. Bell, Ryuji Kobayashi, and Bruce Stillman, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724-0100.

Duplication of the genome is the central event in the propagation of the cell's genetic identity. To understand the mechanisms driving eukaryotic chromosomal replication and how they are integrated into the cell cycle we have set out to identify the proteins involved in the earliest step of chromosomal DNA replication, that is the selection of sequences that will act as origins of replication. The origin recognition complex (ORC), a multi-protein complex derived from the yeast *Saccharomyces cerevisiae*, represents a strong candidate for such an activity. ORC specifically recognizes the essential ARS consensus sequence (ACS) of yeast origins of replication in an ATP dependent manner. Mutational analysis of the ACS indicates that the strength of ORC binding to a particular mutant ACS correlates well with the *in vivo* function of the same mutants. ORC is composed of six subunits and we have identified the genes encoding each of the subunits. All six genes are essential for yeast viability. Interestingly, mutations in two of the subunits have been genetically identified as regulators of transcriptional silencing at the yeast mating type loci. The predicted amino acid sequence of the largest subunit of ORC, ORC1, shows strong similarity to Sir3p, a protein required for transcriptional silencing at both the silent mating type loci and telomeres. Possible roles for ORC in linking DNA replication and the determination of transcriptional domains will be discussed. In addition, genetic evidence that ORC functions early in S phase to initiate DNA replication will be presented.

M 015 YEAST DNA POLYMERASES, Judith L. Campbell, William C. Brown, and Martin E. Budd, California Institute of Technology, Pasadena CA 91125

One of the most surprising outcomes of research on the yeast DNA polymerases is that at least three DNA polymerases appear to be essential for DNA replication -- DNA polymerases α, δ, and ε. Another surprise is their structural and functional conservation in all eukaryotes. The important question now is what is the specific function of each polymerase during replication? Do they interact and if so, how are their activities coordinated? In yeast, we are in the midst of a transition from almost purely genetic analysis to specific biochemical studies to address these questions. We have shown that DNA polymerase α is essential for the elongation stage of mitotic replication, for premeiotic DNA synthesis, and for commitment to meiotic recombination, but that it is not required for repair. We have investigated whether DNA polymerases ε and δ are required for DNA replication. Two temperature-sensitive mutations in the *POL2* gene, encoding DNA polymerase ε, have been identified and alkaline sucrose gradient analysis of DNA synthesis products in the mutant strains shows that no chromosomal-sized DNA is formed after shift of an asynchronous culture to the nonpermissive temperature. The only DNA synthesis observed is a reduced quantity of short DNA fragments. The DNA profiles of replication intermediates from these mutants are similar to those observed with DNA synthesized in mutants deficient in DNA polymerase α. We have shown that *CDC2* encodes DNA polymerase δ. Previous studies on *CDC2* mutants, suggested that there was considerable residual DNA synthesis at the nonpermissive temperature. We have reinvestigated the nature of DNA synthesis in *cdc2* mutants, which we now call *pol3*, and find that they are defective in synthesis of chromosomal-sized DNA at the restrictive temperature after release from a hydroxyurea block. In order to further define the enzymatic properties of DNA polyermase δ, the *POL3* gene, whose expression is highly toxic to bacteria in most cloning vectors, was cloned into a new T7 expression vector. Both full length and truncated forms of the protein have been expressed and characterized. Several forms of the single-subunit enzyme that have all the physical and catalytic properties, including a proof-reading and exonuclease function of the heterodimetic enzyme isolated from yeast have been characterized. The single-subunit form of the polymerase is stimulated by yeast PCNA as long as the amino terminus of the protein is intact. Thus, PCNA appears to interact directly with the core catalytic subunit, as in the case of the "corresponding" subunits in *E. coli* DNA polymerase III, the PCNA-like β subunit and the core catalytic subunit, α. By contrast, it has been proposed that mouse PCNA interacts with the 55 kDa subunit of mouse DNA polymerase δ rather than the core catalytic subunit, since a 125 kDa form of the mouse DNA polymerase does not appear to be stimulated by PCNA. Continuing our studies of DNA polymerases in yeast , we have discovered a new yeast DNA polymerase that is a member of the DNA polymerase β family. We have identified the gene encoding the polymerase and called it *POL4*. Null mutants are viable and show normal resistance to either ultraviolet irradiation or X-rays. Thus, yeast DNA polymerase β is not essential for either DNA replication or repair. *Pol4* mRNA is induced at least 30 fold during meiosis and sporulation is inefficient in *pol4Δ* diploids, suggesting an important meiotic function.

M 016 ACTIVE POLYMERASES FIXED IN REPLICATION AND TRANSCRIPTION FACTORIES. Bass Hassan, Pavel Hozàk, Rachel Errington, Dean Jackson and **Peter Cook**. *The Sir William Dunn School of Pathology, South Parks Road, Oxford OX1 3RE, UK.*

Sites of DNA and RNA synthesis, as well as sites where uv-induced damage is repaired, can be labelled after incubating synchronized and permeabilized HeLa cells with the appropriate precursors (eg biotin-dUTP, Br-UTP); then sites of incorporation can be visualized in both the light and electron microscopes using fluorescent or gold-tagged antibodies (1,2). Sites of replication and transcription are not diffusely spread throughout nuclei reflecting the distribution of euchromatin but concentrated in discrete foci or 'factories' where many polymerases act together. Both replication and transcription foci change shape during the cell cycle. For example, transcription foci aggregate at the G1/S-boundary; later they disperse and became more diffuse. Replication initiates only at transcription sites; later, when heterochromatin is replicated in enlarged foci, these are transcriptionally active.

Active polymerases, as well as both kinds of sites, remain when ~90% chromatin is removed. This implies that active polymerases do not track along the template but are fixed within the foci. Electron microscopy of pulse-labelled samples confirms that nascent DNA and RNA are extruded from discrete factories as they are made.

These results illustrate the dynamic nature of nuclear architecture and suggest:
(a) templates become active upon incorporation into factories,
(b) each factory replicates and/or transcribes ~10-50 sequences simultaneously,
(c) nascent nucleic acids are extruded as templates slide through active sites in attached enzymes,
(d) transcription may be required for the initiation of replication.

1. Hozák, P., Hassan, A.B., Jackson, D.A. and Cook, P.R. (1993). Visualization of replication factories attached to a nucleoskeleton. Cell 73, 361-373.
2. Jackson, D.A., Hassan, A.B., Errington, R.J. and Cook, P.R. (1993). Visualization of focal sites of transcription within human nuclei. EMBO J. 12, 1059-1065.

Centromeres and Telomeres

M 017 TELOMERES AND TELOMERASE, E. Blackburn, P. Cohen, D. Gilley, M. Lee and M. McEachern, Department of Microbiology and Immumology, University of California, San Francisco, CA 94143 - 0414.

The DNA of telomeres, the ends of eukaryotic chromosomes, typically consists of simple, tandemly repeated sequences characterized by a relatively G-rich strand. In the majority of telomeres in the ciliate *Tetrahymena*, the entire stretch of telomeric DNA repeats is packaged in non-nucleosomal chromatin. *In vivo* assembly of this non-nucleosomal telomeric DNA-protein complex does not require proximity to a chromosomal end. In addition, a small percentage of the telomeres are found packaged as closely packed nucleosomes in rapidly dividing cells.
The telomeric repeat sequences of several budding yeast species are more complex and divergent in composition than more typical telomeric sequences. Current efforts are directed at determining the mode of replication of the complex yeast repeat sequences and analyzing their *in vivo* functional properties. It is known that in eukaryotes with typical short repeat units, the ribonucleoprotein reverse transcriptase telomerase synthesizes the G-strand DNA. The RNA component of telomerase contains a short template specifying the telomeric DNA sequence.

We have explored the consequences of several *Tetrahymena* telomerase RNA mutations on telomere function *in vivo* and *in vitro*. Several template mutations caused novel mutated telomere sequences to be added to chromosome ends. In a strongly sequence-specific fashion, certain of these altered telomeric sequences caused nuclear division impairment and loss of short term cell vaibility. Other telomerase RNA mutations caused failure to maintain telomeric DNA, resulting ultimately in cell death. Recently, we have analyzed the effects of these telomerase RNA mutations on the *in vitro* enzymatic properties of the *in vivo* assembled mutant telomerase. Template region mutations altered the mode of action of telomerase *in vitro*. The *in vivo* consequences of the telomerase RNA mutations were not simply predictable from the effects of these mutations on the *in vitro* activity of the partially purified telomerase.

M 018 STRUCTURE-FUNCTION ANALYSIS OF THE YEAST CENTROMERE/KINETOCHORE, Kim Middleton, Weidong Jiang, and John Carbon, Department of Biological Sciences, University of California, Santa Barbara, CA 93106.

The functional centromere/kinetochore in budding yeast (*Saccharomyces cerevisiae*) consists of a DNA locus (*CEN*) 125 bp in length complexed with several proteins. Our research has concentrated on a 240 kd multisubunit protein complex (CBF3) that binds specifically to the 25 bp CDEIII locus within the centromere (1). The core CBF3 complex contains three dissimilar subunits: 110 kd, 64 kd, and 58 kd. The 110 kd subunit is specified by the essential yeast gene *CBF2/NDC10* (2). A temperature-sensitive mutation in that gene produces a pronounced chromosome segregation defect at the non-permissive temperature (3). This subunit can bind in multiple copies to the core complex in vitro (4). It appears to be localized at the spindle pole bodies and along the spindle during mitosis. The 64 kd subunit (specified by *CBF3*) contains an N-terminal zinc finger motif and a C-terminal acidic region similar to known DNA binding proteins (5), and most likely is the DNA recognition subunit. The 58 kd subunit (specified by *CTF13/CBF4*) is also an essential protein involved in chromosome segregation (6). A microtubule-based minus end-directed motor activity is associated with CBF3 preparations (7). This motor activity, when assayed by microtubule gliding on glass surfaces, can be separated from the CBF3 core complex. However, attachment of *CEN* DNA to microtubules requires the presence of both the motor fraction and the CBF3 multisubunit core complex. Thus, CBF3 in some way mediates and regulates attachment of the motor to both centromere DNA and the microtubules.

(1) J. Lechner and J. Carbon, Cell **64**, 717-25 (1991)
(2) W. Jiang, J. Lechner and J. Carbon, J. Cell Biol. **121**, 513-19 (1993)
(3) P. Goh and J. Kilmartin, J. Cell Biol. **121**, 503-12 (1993)
(4) W. Jiang and J. Carbon, Cold Spring Harbor Symp. Quant. Biol., in press.
(5) J. Lechner, Cold Spring Harbor Symp. Abstracts, June 2-9, 1993, p. 101
(6) K. Doheny, P. Sorger, A. Hyman, S. Tugenreich, F. Spencer and P. Hieter, Cell **73**, 761-74 (1993)
(7) A. Hyman, K. Middleton, M. Centola, T. Mitchison and J. Carbon, Nature **359**, 533-36 (1992)

M 019 PROTEINS OF THE CENTROMERE, William C. Earnshaw, Alastair M. Mackay, Carol A. Cooke, Ilya Goldberg, John Tomkiel and D. Mark Eckley, Dept. of Cell Biology & Anatomy, Johns Hopkins University School of Medicine, Baltimore

The INCENPs (*IN*ner *CEN*tromere *P*roteins - M_r 133 and 145 kDa) are tightly bound to chromatin until early metaphase, and arrive at the metaphase spindle plate with the chromosomes. However, they leave the chromosomes during late metaphase to become part of the spindle midzone and cleavage furrow during anaphase. We have cloned the two chicken INCENPs, and expressed them in mammalian cells. Mutational analysis has revealed a domain of 42 amino acids that is required for the transfer of these proteins from the chromosomes to the spindle at the metaphase: anaphase transition. Transfected cells in which this mutant protein is expressed have no visible phenotype. However, cells expressing various deletions of the carboxy terminal portion of the INCENPs are unable to pass through mitosis normally: these mutant proteins have dominant negative phenotypes. Two phenotypes are observed. In one, the organization of the metaphase plate is abnormal. In the other, the cells are defective in cytokinesis or lyse shortly after dividing. These observations, together with other results indicating that the INCENPs are among the earliest components to localize to the cleavage furrow (i.e. prior to myosin) suggest that these chromosome scaffold proteins have an essential role in cytokinesis.

CENP-B is a protein of the centromeric heterochromatin that binds to a 17 base pair region of α-satellite DNA (the CENP-B box). Paradoxically, both the CENP-B box and CENP-B protein are undetectible on the Y chromosome of human and mouse. To understand if another protein fulfills this role on the Y chromosome, we first conducted a statistical analysis of the sequence variability of α-satellite DNA. This yielded a surprising result. Even though the CENP-B box is the only known functional region of α-satellite DNA, this is also the most highly variable portion of the α-satellite monomer. A similar analysis of the α-satellite monomers cloned from the Y chromosome revealed that the region where the CENP-B box should be was again the most variable region of the monomer. Despite this observation, 5 of 15 monomers in the data base had an identical sequence in this region. We hypothesized that this region might be a binding site for another chromosomal protein, and have accordingly synthesized an oligonucleotide probe carrying this sequence. This probe is strongly band-shifted in nuclear extracts prepared from human and monkey cells, but not in similar extracts prepared from mouse, rat, hamster or insect cells. We are currently engaged in purification of this putative "CENP-Y" activity.

CENP-C is concentrated in the region of the inner kinetochore plate, and is a strong candidate for a component involved in assembly of the kinetochore. We have obtained evidence strongly supporting this view by microinjecting monospecific anti-CENP-C antibodies into cultured cells. The injected antibodies cause cells to delay in metaphase. Examination of kinetochores in metaphase-arrested cells by serial section electron microscopy reveals that the kinetochores in these cells retain a normal trilaminar morphology but are significantly reduced in diameter. In cells arrested for extended periods, these small kinetochores become disrupted and apparently no longer bind microtubules. These observations are consistent with an involvement of CENP-C in forming the underlying structural basis for kinetochore assembly, and suggest that CENP-C plays a critical role in both establishing and/or maintaining proper kinetochore size.

Processive Interactions with RNA (Joint)

M 020 RECODING: INVOLVEMENT OF CIS ACTING SEQUENCE ELEMENTS, Raymond F. Gesteland[1,2], Norma M. Wills[1], and John F. Atkins[2], [1]Howard Hughes Medical Institute, Salt Lake City, UT, [2]University of Utah, Human Genetics Dept., Salt Lake City, UT 84112.

Programmed alteration of genetic decoding, or "recoding", involves a site on mRNA at which the genetic code is differently enabled and a stimulatory sequence that brings the recoding event up to its appropriate level of efficiency. Often these stimulatory signals are specific folded structures in mRNA including pseudoknots, simple and complex stem loops and mRNA:rRNA interactions. Various examples of recoding will be compared and contrasted. The case of stop codon readthrough used by Moloney Murine Leukemia Virus (MMTV) to express the gag-pol polyprotein will be discussed in detail. Here a nearby, downstream pseudoknot is the crucial stimulatory signal for decoding of a UAG stop codon. Not only are the two, presumably stacked, stems crucial, but specific bases spacing the pseudoknot and the first stem and bases in loop 2 must have the right identity. This suggests either a more complex structure or specific interaction of some components with the pseudoknot.

M 021 SPHERE ORGANELLES, Rabiya S. Tuma[1,2] and Mark B. Roth[2], [1]University of Washington, Seattle and [2]Fred Hutchinson Cancer Research Center, Seattle.

Sphere organelles are nuclear structures in amphibian oocytes that are easily visible by light microscopy. These structures are up to 10 mm in diameter and have been described morphologically for decades, yet their function remains obscure. We identified a protein component of the sphere organelle, named SPH-1, which is recognized by a monoclonal antibody raised against purified Xenopus laevis oocyte nucleoplasm. SPH-1 is an 80 kD protein which is localized specifically to spheres and is undetectable elsewhere on lampbrush chromosomes or in nucleoli. We have shown using confocal microscopy that SPH-1 is localized to the cortex of sphere organelles. Furthermore, we have isolated a cDNA that can encode SPH-1. When epitope-tagged forms of SPH-1 are expressed in X. laevis oocytes the protein specifically localizes to spheres, demonstrating that the cloned cDNA encodes the sphere antigen. Comparison of the predicted amino acid sequence with sequence databases shows SPH-1 is related to p80-coilin, a protein associated with coiled bodies; coiled bodies are nuclear structures found in plant and animal cells. The sphere-specific monoclonal antibody stains X. laevis tissue culture cells in a punctate nuclear pattern, showing that spheres or sphere antigens are present in somatic cells as well as germ cells and suggesting a general and essential function for spheres in all nuclei.

M 022 RHO PROTEIN AS A "STRUCTURE-FUNCTION" MODEL OF HEXAMERIC NUCLEIC ACID HELICASES, Peter H. von Hippel, Feng Dong, Johannes Geiselmann, Steven E. Seifried, Yan Wang, and Mark C. Young. Institute of Molecular Biology and Department of Chemistry, University of Oregon, Eugene, OR 97403.

The hexameric RNA-DNA helicase rho is required to bring about release of the nascent RNA at about one-half of the transcription termination sites of *E. coli*. Although the details of the molecular process catalyzed by rho to induce transcript release are far from clear, it is the nucleic acid helicase about which we have by far the most structural and enzymatic information. As a consequence it may serve as a paradigm for the study of the many related helicases that operate in DNA replication, recombination, and repair, and perhaps in RNA splicing. In this lecture we will review the molecular biological studies of rho that suggest that this protein functions at rho-dependent operons by initially binding to the nascent transcript at a largely unstructured and cytidine residue containing "rho loading site". This binding activates the RNA-dependent ATPase activity of rho, which, in turn, fuels the preferential 5' → 3' translocation of the rho hexamer along the nascent RNA. This translocation continues until rho "catches up" with the paused elongation complex at rho-dependent termination sites. By use of its 5' → 3' RNA-DNA helicase activity, rho then separates the RNA-DNA hybrid duplex within the elongation complex and brings about termination by releasing the nascent RNA into solution. Based on available and detailed knowledge of the enzymology and structure of rho we will put forward a plausible molecular model for this process and describe recent studies that we hope will test and refine the model further. The ability to translocate along single-stranded RNA or DNA with a preferred direction may be the central feature of the function of the hexameric helicases. Accordingly we will describe a recent steady-state kinetic analysis of the dependence on the length and properties of its single-stranded DNA cofactor of the DNA-activated ATPase of the DNA replication helicase of bacteriophage T4 (gene 41 protein). These studies show that this hexameric helicase, with a structure closely resembling that of rho, does appear to move along single-stranded DNA with a preferred direction. These results will be correlated with what is known about other hexameric helicases to come up with some general mechanistic suggestions for how these entities might work in discharging their physiological functions.

Nuclear Domains and Addresses: The Nucleolus, New Intranuclear "Organelles"

M 023 NUCLEOLAR-CYTOPLASMIC TRANSPORT ON TRACKS, U. Thomas Meier[1] and Günter Blobel[2], [1]Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY 10461, [2]Howard Hughes Medical Institute, Rockefeller University, New York, NY 10021.

We previously identified and molecularly characterized Nopp140, a nuclear localization signal binding protein, and showed that it shuttled between the nucleolus and the cytoplasm on highly localized tracks. Thus, we suggested Nopp140 to be involved in nucleocytoplasmic transport of preribosomal particles out and/or ribosomal proteins into the nucleolus. To identify proteins that either provided the basis for the intranuclear tracks or that were otherwise involved in the Nopp140 transport process, Nopp140 was immunoprecipitated from rat liver nuclear extracts under nondenaturing conditions. By this approach, one major Nopp140 associated protein of 57 kD, NAP57, was identified that specifically coprecipitated with Nopp140. Internal protein sequence of NAP57 was obtained and employed to raise anti-peptide antibodies and to clone its cDNA. The cDNA deduced amino acid sequence showed NAP57 to be a novel protein that exhibited significant homology to a bacterial protein, the gene of which was located in an operon involved in protein synthesis. This indicated not only that NAP57 is evolutionary highly conserved but also linked it to the protein synthesis machinery, which was in good agreement with its proposed function in nucleocytoplasmic shuttling of ribosomal components. Immunolocalization of NAP57 by light and electron microscopy, detected it in the nucleolus and on intranuclear tracks analogously to Nopp140. Most surprisingly however, NAP57 was found along the cell surface in a pattern reminiscent of the actin-rich leading edge and of the recently described distribution of the motor protein myosin I. Indeed, the colocalization of NAP57 and myosin I was confirmed by double-immunofluorescence experiments. Taken together, these data confirmed first, that Nopp140 and NAP57 are indeed associated with each other *in vivo* (in the nucleolus and on intranuclear tracks) and suggested second, that NAP57 is involved in actin-myosin I based movement of Nopp140 (together with bound ribosomal components) in and out of the nucleus.

M 024 COORDINATION OF TRANSCRIPTION AND PRE-mRNA SPLICING WITHIN THE CELL NUCLEUS, David L. Spector[1], Raymond T. O'Keefe[1], Shelley Landon[1], and Luis F. Jiménez-García[2], [1]Cold Spring Harbor Laboratory, P.O. Box 100, Cold Spring Harbor, New York 11724 and [2]Laboratory of Electron Microscopy, Faculty of Sciences, U.N.A.M., Mexico, D.F., Mexico.

We have examined how the processes of transcription and pre-mRNA splicing are spatially coordinated within the cell nucleus. In actively transcribing cells splicing factors are localized in a speckled nuclear pattern which corresponds to both perichromatin fibrils and interchromatin granule clusters. RNA polymerase II is preferentially localized to perichromatin fibrils and is not enriched in interchromatin granule clusters. When RNA polymerase II transcription is inhibited, splicing factors redistribute and preferentially localize to interchromatin granule clusters, which become larger and more uniform in shape. Upon inhibition of RNA polymerase II transcription, connections between speckles are no longer visible. When transcription is reinitiated, the localization of splicing factors returns to its normal distribution. Furthermore, the introduction of oligonucleotides into cells, which inhibit pre-mRNA splicing *in vitro*, results in a similar reorganization of splicing factors. These data suggest that splicing factors are present at sites of active transcription (perichromatin fibrils) and at storage and/or assembly sites (interchromatin granule clusters). In order to further demonstrate that splicing factors are recruited to sites of active transcription, we introduced exogenous DNA templates into cell nuclei and found that both transcription and splicing factors shuttled from their normal distribution in the host cell to the new sites of active transcription. These findings provide *in vivo* evidence that the processes of transcription and pre-mRNA splicing are both spatially and temporally associated within the cell nucleus.

mRNA Export: General, Intranuclear Cytoskeletal Tracks for RNA Export, HIV REV Protein's Effect on Export

M 025 EXPORT OF RNA FROM THE NUCLEUS, Elisa Izaurralde, Wilbert C. Boelens, Cathérine Dargemont, Artur Jarmolowski[1], Joe D. Lewis, Isabel Palacios and Iain W. Mattaj, EMBL, Meyerhofstrasse 1, 69117 Heidelberg, Germany, [1]Present address: A. Mickiewicz University, Dept. of Biopolymer Biochemistry, ul. Fredry 10, 61-701 Poznán, Poland.

Very little is known about the mechanism of export of RNAs from the nucleus to the cytoplasm. In particular, although there is considerable indirect evidence that nuclear proteins mediate RNA export, no proteins have been identified that have a proven role in the process. We have begun to investigate this question using RNA microinjection into Xenopus oocyte nuclei as an experimental system. Various control experiments suggest that this simplified export protocol faithfully recapitulates important steps in the pathway of export of RNAs made in the oocyte nucleus following transcription of injected DNA. By comparing different classes of RNA (tRNA, 5S RNA, U snRNAs and mRNAs) we have obtained evidence that at least four distinct, class-specific factors are essential for export of the various RNAs from the nucleus. Work is in progress to identify these factors. In the case of the U snRNAs, the factor whose concentration is limiting for export preferentially recognizes RNAs that have an m^7G cap structure. A nuclear protein that is a candidate for this factor has been purified and its cDNA cloned.

M 026 KEY EVENTS IN EUKARYOTIC GENE EXPRESSION: TRANSPORT OF mRNA FROM NUCLEUS TO CYTOPLASM, Alan Tartakoff, Connie Chen, Midori Hitomi, Tatsuhiko Kadowaki,Chino Kumagai, Shuang Liang, Mutsuhito Ohno, Roger Schneiter and David Singleton, Pathology Institute, Case Western Reserve University School of Medicine, Cleveland, Ohio.

To identify factors which are important for export of mRNA from the nucleus, we have used enrichment and screening procedures to generate a corresponding family of temperature sensitive mutants of Saccharomyces cerevisiae. Some of the mutants are also cold-sensitive. The available mutants (mRNA transport or mtr mutants) are recessive and fall into 21 complementation groups. The mutants are characterized by nuclear accumulation of polyA+ RNA and progressive inhibition of protein synthesis at the restrictive temperature. Accumulated nuclear polyA+ RNA persists for hours at the restrictive temperature even if transcription is inhibited. Distinct mutants exhibit distinct patterns of accumulated polyA+ RNA within the nucleus. MTR1 codes for a homologue of the animal cell protein, RCC1, which has guanine nucleotide release activity. Like RCC1, MTR1 interacts with proteins of the RAS superfamily. We have identified yeast genes coding for two such conserved nuclear ras-like proteins (CNR1 and CNR2). Loss of CNR function also leads to nuclear accumulation of polyA+ RNA. MTR2 codes for a largely nuclear protein and the mtr2-1 mutant appears to impact much more strongly on mRNA export than on other aspects of RNA processing. MTR4 codes for a protein which includes all the motifs which characterize members of the "DEAD box" family of RNA helicases. MTR7 codes for a protein whose loss of function has dramatic consequences for the integrity of nuclear pores. MTR13 codes for a protein which includes a bipartite RNA-recognition motif and a glycine-arginine rich domain. Several of the mutants have major ultrastructural consequences and several appear also to block rRNA export. None have conspicuous effects on CRY1 mRNA splicing, per se. Supported by NIH grant No.GM46569 (A.T.).

Using Yeast Genetics to Study the Nucleus

M 027 A GENETIC AND BIOCHEMICAL STUDY OF THE NUCLEAR PORE COMPLEX IN YEAST
Eduard C. Hurt, Valérie Doye, Emmanuelle Fabre, Paola Grandi, Hildegard Tekotte and Christian Wimmer, EMBL, D-69015 Heidelberg, Germany.

To identify components that interact with the yeast nuclear pore protein NSP1 in the living cell, we screened for synthetic lethal mutants which stop cell growth in a background of mutated, but not wild type *NSP1*. Twenty-nine synthetic lethal mutants were isolated, which belong into at least nine different complementation groups. The genes of two complementation groups, *NUP116* and *NUP49*, encode novel pore proteins with many 'GLFG' repeat sequences. While NUP116 is not essential, NUP49 is required for cell viability. The carboxy-terminal domain of NUP49 has heptad repeats similar to those found in NSP1 and thus could be involved in coiled-coil protein/protein interaction. We have cloned the wild type genes of three further complementation groups and an initial characterization of their gene products will be reported.

In addition to the genetic screen, a biochemical approach was performed to identify components which physically interact with NSP1. If a Protein A-tagged NSP1 fusion protein is expressed in yeast and affinity-purified by IgG-Sepharose chromatography under non-denaturing conditions, a multimeric complex can be purified consisting of ProtA-NSP1, NUP49 and p54 (two GLFG nucleoporins), and a novel protein designated NIC96 (for Nucleoporin-Interacting Component of 96 kDa). The *NIC96* gene was cloned; it encodes a novel nuclear pore protein, but lacks repeat sequences. Interestingly, the cloned *NIC96* gene could complement two of the synthetic lethal mutants obtained in the genetic screen. Thus, biochemical and genetic data have proven that NSP1, NUP49 and NIC96 are functionally interacting and physically held together in a multimeric complex.

Conditionally lethal mutants of the various nucleoporins identified by genetic and biochemical assays have been generated and are currently analyzed for defects in nucleocytoplasmic transport of proteins and RNA.

M 028 TRANSCRIPTIONAL SILENCING AND FUNCTIONALLY DISTINCT DOMAINS OF THE YEAST NUCLEUS,
Cheryl Reifsnyder, Brandy Gazo, Mitch McVey, Joanna Lowell, Elisa Stone, Corey Nislow, and Lorraine Pillus, Molecular, Cellular and Developmental Biology, University of Colorado, Boulder.

Telomeres and specific regions of chromosome III in the budding yeast Saccharomyces are subject to transcriptional repression mediated through at least 10 proteins that have been identified by a combination of genetic and biochemical approaches. These proteins include: Rap1p, the telomere-localized protein that also functions in directly activating and repressing transcription; Nat1p and Ard1p that are subunits of an amino-terminal acetyl transferase activity; histone H4; components of the origin recognition complex; and the four *SIR* (Silent Information Regulator) proteins. Among the Sir proteins, Sir3p and Sir4p recently have been shown also to function in both the positioning and the integrity of yeast telomeres. We are pursuing these observations and are seeking to define the molecular mechanisms whereby the Sir proteins act to establish and maintain transcriptionally silenced chromatin domains. We are investigating whether the telomeric functions provided by *SIR3* and *SIR4* are distinct from those provided by other genes known to influence telomeric integrity. We are seeking to define the molecular basis for the establishment of repression through a mutant screen designed to identify genes that share establishment functions with *SIR1*. In addition to identifying genes not previously known to participate in silencing, these studies are revealing new functions for several of the previously identified silencing proteins. Finally, we are exploring the enzymatic clue that a crucial cotranslational modification of at least one of the proteins involved in silencing is important for its activity. To this end, we are attempting to define the *NAT1*/*ARD1*substrate that is important for silencing. Our recent progress on each of these topics will be discussed.

The Nuclear Envelope-A Structural View; The Mechanism of Nuclear Transport

M 100 NUCLEAR PORE PROTEIN p62 EXPRESSED IN BACULOVIRUS SYSTEM AND E. COLI, Susanne M. Bailer, Mill W. Miller, William K. Berlin, William A. Lubas, Christopher M. Starr and John A. Hanover, Laboratory of Biochemistry and Metabolism, NIDDK, National Inst. of Health, Bethesda, MD 20892

Nuclear transport of proteins can be subdivided into two major steps: binding to the nuclear periphery and ATP-dependent translocation. Translocation, but not binding, is inhibited by *in vitro* and *in vivo* addition of wheat germ agglutinin (WGA) suggesting that nuclear pore proteins modified by O-linked N-acetylglucosamine are involved in translocation. A number of rat pore glycoproteins identified recently were found to be modified by N-acetylglucosamine, the most abundant one being p62. To study the influence of p62 glycosylation on nuclear transport, recombinant rat p62 was expressed in glycosylated and nonglycosylated forms. Large amounts of glycosylated rat p62 were isolated from cells infected with Baculovirus containing the rat p62 open reading frame. Structural properties of glycosylated rat p62 have been determined and compared to those of nonglycosylated p62 expressed in E.coli. To identify and characterize possible functions of N-Acetylglucosamine addition, purified glycosylated or nonglycosylated rat p62 is added to *in vitro* Xenopus nuclear reconstitution extracts depleted of the p62 homologue. Incorporation of recombinant p62 into the nuclear pore complex will be monitored by immunofluorescence and immunoblotting. The role of p62 glycosylation in nuclear transport is analysed by following the ability of reconstituted nuclei to accumulate transport-competent NLS-peptides conjugated to phycoerythrin .

M 101 *IN VIVO* FUNCTIONAL PROTEIN - PROTEIN INTERACTION: NUCLEAR TARGETED HSP90 SHIFTS CYTOPLASMIC STEROID RECEPTOR MUTANTS INTO THE NUCLEUS, Maria-Grazia Catelli, Kwang Il Kang, Jocelyne Devin, Françoise Cadepond, Nicole Jibard, Anne Guiochon-Mantel, and Etienne-Emile Baulieu, INSERM U33, Lab. Hormones, 80 rue du Général Leclerc, 94276 Le Kremlin-Bicêtre Cedex, France

In target tissue extracts, heat shock protein hsp90 has been found associated to all unliganded steroid receptors. Modulation of important functions of these receptors, including prevention of DNA binding and optimization of transcriptional activity, has been attributed to hsp90. However no unequivocal *in vivo* demonstration of interaction between receptors and hsp90 has been presented.

We targeted chicken hsp90, a protein mainly cytoplasmic, with the nucleoplasmin nuclear localization signal (90NLS). After transfection into COS7 cells, 90NLS was found in the nucleus with specific immunofluorescence and confocal microscopy techniques. A human glucocorticosteroid receptor (hGR) mutant devoid of NLS sequence was also expressed in COS7 cells and found exclusively cytoplasmic. Coexpression of 90NLS and of the cytoplasmic hGR mutant led to complete nuclear localization of the receptor, indicating its piggy-back transport by 90NLS, and thus physical and functional interaction between the two proteins in the absence of hormone. The same nuclear localization was obtained after cotransfection of 90NLS and a cytoplasmic rabbit progesterone receptor (rPR) mutant. Finally, coexpression of wild type rPR (nuclear) and wild type hsp90 (cytoplasmic) into COS7 cells provoked partial relocalization of hsp90 into the nucleus.

These experiments lay the ground to study hsp90 as a chaperon, regulating activities of steroid receptors and possibly participating to their nuclear-cytoplasmic shuttling.

M 102 A CYTOPLASMICALLY ANCHORED NUCLEAR POTEIN INTERFERES SPECIFICALLY WITH THE IMPORT OF NUCLEAR PROTEINS BUT NOT U1snRNA

A. Dickmanns, K. van Zee, T. U. Pinkau, U. Fischer, R. Lührmann and E. Fanning

A cytoplasmically anchored mutant SV40 T antigen, FS T antigen, was shown previously to interfere specifically with the nuclear import of a heterologous nuclear protein, adenovirus 5 fiber protein, in cultured monkey cells (Schneider et al., 1988. Cell 54:117-125; van Zee et al., 1991. Mol. Cell. Biol. 11:5137-5146). More recently, we have shown that FS T antigen also interferes with the nuclear import of adenovirus E1A and a peptide-albumin conjugate bearing multiple copies of the T antigen nuclear localization signal (NLS), but not with the import of U1 snRNA. A kinetic analysis indicates that nuclear import of the albumin-peptide conjugate is inhibited only when high intracellular concentration of FS T antigen are reached. After microinjection into the cytoplasm of cultured cells, purified FS T antigen protein does not accumulate at the nuclear periphery, but rather is distributed in a punctate pattern throughout the cytoplasm. These data support a model in which cytoplasmic anchoring of FS T antigen enables the mutant protein to sequester and titrate out a cellular factor which is required for nuclear protein but not U1 snRNA import. Current work is focussed on the identification of the postulated cellular factor using an *in vitro* nuclear transport assay.

M 103 THE ROLE OF NUCLEAR LAMINS IN CELL-FREE EXTRACTS OF *XENOPUS* EGGS. Joanne A. Dyer, E. Birgit Lane and Christopher J. Hutchison. Dept. Biological Sciences, University of Dundee. DD1 4HN.

Cell division in early *Xenopus* embryos is characterised by rapid biphasic cell-cycles. Lamin L_{III} is the only lamin so far identified in early *Xenopus* embryos and its peculiar characteristics may permit rapid DNA replication. For example L_{III} is classified as a B-type lamin, but has several features in common with A-type lamins. The aim of this project is to investigate in more detail the role of lamin L_{III} in the early embryonic cell-cycle. Experiments have been designed to assess the potential of somatic lamins to function in an embryonic system and to determine whether the lamina composition influences cell-cycle progression.

Bacterially expressed human lamin A was inoculated into cell-free extracts of *Xenopus* eggs in the presence of endogenous L_{III} and demembranated sperm heads. The formation of replication competent pronuclei was judged by biotin-11-dUTP incorporation. Replication competent pronuclei were formed and a percentage of these pronuclei were both lamin A and lamin L_{III} positive. L_{III} may be immuno-depleted from extracts using the monoclonal antibody L6 5D5 bound to magnetic Dynabeads. When lamin A was inoculated into extracts depleted of L_{III}, although it was still incorporated into pronuclei, it did not assemble into a lamina-like structure. Pronuclei assembled in the presence of human lamin A, but in the absence of *Xenopus* lamin L_{III} did not initiate DNA replication.

We are currently extending these studies to bacterially expressed *Xenopus* lamin A.

M 104 EFFECT OF IRON OVERLOAD ON OXYGEN RADICAL GENERATION BY LIVER NUCLEI, Monica Galleano and Susana Puntarulo, Physical Chemistry Division, School of Pharmacy and Biochemistry, University of Buenos Aires, Argentina.

Iron overload in the liver has been associated with injury, fibrosis and cirrhosis. Previous studies have demostrated the ocurrence of lipid peroxidation in whole liver, in isolated cellular fractions in experimental models of iron overload. In this study the effect of acute mild iron overload on oxygen radical generation by isolated rat liver nuclei and on the activity of membrane-bound enzymas was evaluated. Acute iron overload was obtained by single injection of iron dextran (500 mg/kg) to male Wistar rats. Rat liver nuclei were prepared by differential centrifugation. Electron microscopyc examination was used to assess the purity and ultraestructure of the preparations. Iron levels were determined on plasma, whole liver, cellular cytosol and liver nuclei at 2h, 4h and 20h after injection. Significant increases were found in all the samples as a consequence of the treatment. Nuclear rate of iron reduction was determined using NADH or NADPH as reductants and no significant changes are detected after iron overload. However, the nuclear rate of generation of active oxygen species was decreased after 6h of iron supplementation .

	Production (nmol/min/mg prot)		
	Superoxide	Hydrogen peroxide	Formaldehyde
Control	1.02 ± 0.05	0.63 ± 0.02	0.48 ± 0.02
Treated 6h	0.51 ± 0.03	0.32 ± 0.01	0.20 ± 0.01

Membrane-bound enzymatic activities in the nuclei were measured after the treatment, both NADPH- and NADH-dependent cytochrome c reductases were slighty decreased after iron overload. Cytochrome P450 content was 0.06 ± 0.01 nmol/mg prot in control nuclei and undetectable after the treatment. The content of lipid and water soluble antioxidant were measured in isolated nuclei from control and iron overloaded rats. α-tocopherol and ß-carotene content was decreased by 40 and 83% respectively, after 6h of treatment. The nuclear glutathione content was not affected. The data presented here suggest that the cytosolic increases in the catalytic active iron could lead to inactivation of nuclear cytochrome P450 that could be responsible for the decrease in the rate of generation of active oxygen species. The significant loss in nuclear antioxidants (α–tocopherol and ß-carotene) detected simultaneously, suggest the onset of nuclear oxidative stress by cytosolic iron overload.

M 105 BIOCHEMICAL MANIPULATION OF THE NUCLEAR ENVELOPE(NE) CHARACTERISED BY FIELD EMISSION SCANNING ELECTRON MICROSCOPY(FEISEM), IMMUNOGOLD LABELLING AND WESTERN BLOTTING, Martin Goldberg, Amanda Robson, Sandra Rutherford and Terry Allen, CRC Dept. Struct Cell Biol., Paterson Institute, Christie Hosp., Manchester. M20 9BX.UK.

We have used FEISEM to study the 3D surface structure of the NE.Known NPC components have been visualised.Detail of the transporter/spokes is presented and a new model for the transport mechanism is proposed. We have studied the NPC basket in detail and shown its precise organisation on the coaxial ring. We also recently discovered a regular filamentous network, the NE lattice, attached to the baskets. These structures have been characterised further by a number of approaches. Digestion with increasing concentrations of trypsin sequentially removes structures. In particular, coaxial rings have a trypsin sensitive region. We have also correlated removal of structures with trypsin with disappearance of bands on Western blots probed with anti-nucleoporin antibodies and with lectins. These same antibodies and lectins have been used for colloidal gold labelling of isolated NEs. We have also followed the assembly/disassembly of the NE by FEISEM using cell free extracts from *Xenopus* eggs.

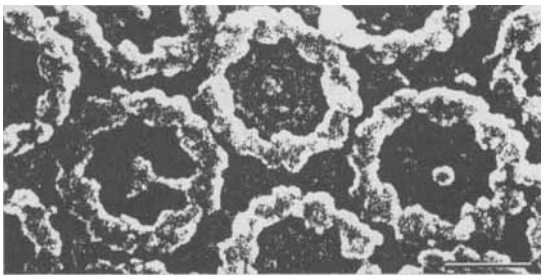

Figure. Cytoplasmic face of Xenopus oocyte NE digested with 0.1μg/ml trypsin, 15min. Bar=50nm.

M 106 IDENTIFICATION OF PROTEINS CONTAINING *N*-ACETYL-GLUCOSAMINE AT THE PLANT NUCLEAR PORE COMPLEX, Antje Heese-Peck, Valentina Kovaleva and Natasha V. Raikhel, MSU-DOE Plant Research Laboratory, Michigan State University, East Lansing, MI 48824-1312.

Nucleocytoplasmic transport occurs across the nuclear envelope through the nuclear pore complex (NPC) via a two-step process, binding and translocation. The addition of wheat germ agglutinin (WGA), a lectin which specifically binds to *N*-acetylglucosamine, inhibits the translocation step through the NPC in vertebrates, but not in yeast. This suggests that, at least in vertebrates, some NPC proteins containing O-linked *N*-acetylglucosamine (O-GlcNAc) are involved in protein nuclear import. We are interested in determining whether or not plant NPC proteins contain O-GlcNAc modification, and whether this modification is important for protein import into the plant nucleus. Several proteins of low- and high-molecular weight range were detected by protein blot analysis of isolated tobacco nuclei using WGA-alkaline phosphatase and WGA-horse radish peroxidase as probes. The WGA-binding was competed with 2 mM chitotriose, but not with 0.2 M mannose. Preliminary results showed that some of the plant proteins containing *N*-acetyl-glucosamine were extractable under high salt, but not under low salt and 2% Triton X-100 conditions indicating that they are peripheral proteins. Specific binding of WGA-conjugated colloidal gold particles (25 μg/ml) was detected by electron microscopy at the NPC of tobacco nuclei that were competed with 5 mM chitotriose. These data showed that tobacco nuclei contain protein(s) at the NPC that are modified by *N*-acetylglucosamine. Currently, we are analyzing the nature of the sugar modifications.

M 107 NUCLEAR PROTEIN TRANSPORT AND HSC70, Naoko Imamoto, Taro Tachibana, Masami Matsubae, and Yoshihiro Yoneda, Department of Anatomy, Osaka University Medical School, 2-2 Yamada-oka, Suita, Osaka 565, Japan.

We have identified 70kD heat-shock protein (hsc70) as an NLS-binding protein, and showed that anti-hsc70 antibodies inhibit nuclear import of karyophilic proteins when injected into the living cells. Using digitonin permeabilized cell free transport assay, we confirmed the requirement of hsc70 in nuclear transport. In this assay, we found that hsc70 co-localizes with karyophilic proteins during their active transport. To further investigate a role(s) of hsc70 in nuclear transport, we examined the nuclear import of native nuclear protein using digitonin-permeabilized cell assay. Although purified nucleoplasmin rapidly accumulates into the nucleus when injected into the cytoplasm of cultured mammalian cells, this protein did not accumulate into the nucleus as efficiently as SV40 T-antigen NLS-BSA conjugate in the in vitro semi-intact cell system. However, we found nucleoplasmin could be used as efficient transport substrate in this assay when it was once unfolded. We took the advantage of the unfolded nucleoplasmin to examine a role of hsc70 and to identify cytoplasmic co-factors involved in the transport of native karyophilic proteins. Fractionation of cytosol required for the in vitro assay revealed that two distinct cytoplasmic fractions apparently mediating binding step and translocation step of the transport were required for the nuclear import of unfolded nucleoplasmin. In this paper, our current results indicating the role of hsc70 in nuclear transport obtained from in vitro analysis using these fractions and unfolded nucleoplasmin will be presented.

M 108 NUCLEAR TRANSPORT OF CALPAIN IN PERMEABILIZED A431 CELL, Qin Lu and Ronald L. Mellgren, Department of Pharmacology, Medical College of Ohio, Toledo, OH 43699

The calpains are cytoplasmic proteases which require Ca^{2+} for activity. They appear to be present in virtually all animal cells. Recent investigations suggest that some DNA-associated nuclear proteins may be selectively proteolyzed by calpains under physiologic conditions. If this indeed occurs in interphase cells, cytoplasmic calpain(s) must be translocated into the nucleus. Proliferating A431 cells were treated with 20 μg digitonin/ml to permeabilize the plasmalemma, while leaving the nuclear envelope intact. Transport of FITC-labeled proteins was determined by fluorescence microscopy. Purified human μ-calpain isozyme could be fluorescein-labeled with FITC to an A_{495} : A_{280} ratio of approximately 0.3 to 0.4 with less than 20% loss of caseinolytic activity. FITC-μ-calpain could be transported into nuclei of permeabilized A431 cells in an ATP-dependent manner. Transport was observed in the presence of 1 μM free Ca^{2+} in an EGTA-Ca buffer. There was no detectable transport in the presence of 0.1 μM or 0.3 μM Ca^{2+}. A small peptide containing the SV-40 nuclear localization signal sequence substantially inhibited u-calpain transport into nuclei, indicating that u-calpain contains a functional nuclear localization signal. The calpain-specific inhibitor protein, calpastatin, did not inhibit u-calpain nuclear transport. Under the standard transport conditions, the m-calpain isozyme was poorly transported. These experiments indicate that u-calpain may be selectively transported into the nuclear compartment in response to elevated intracellular Ca^{2+} concentrations.

M 109 MONOCLONAL ANTIBODIES TO STRUCTURAL ELEMENTS OF AMPHIBIAN NUCLEAR ENVELOPES. CE Lyon,[1] CJ Hutchison,[2] C Smythe,[3] TD Allen[4] & EB Lane,[1] Dept of Anatomy &Physiology,[1] Biochemistry[3] & Biological Sciences,[2] University of Dundee, Dundee DD1 4HN, & Paterson Institute for Cancer Research,[4] Manchester M20 9BX UK.

In an attempt to clarify some of the ambiguities which still surround understanding nuclear envelope structure, further monoclonal antibodies are being generated against nuclear antigens. Hybridomas have been generated from mice immunised with *in vitro* assembled whole nuclear structures from *Xenopus* cell free extract or with karyoskeletal fractions of these nuclei. The initial screening of monoclonal antibodies was by immunofluorescence of fixed and sectioned native oocytes. Antibodies reactive with nuclear envelope and/or matrix in this primary screen were tested against the original nuclear preparations, a range of mammalian tissues, tissue cultured cells and sections of *Xenopus* skeletal muscle. Only minimal cross reactivity of antibodies with nuclei from anything other than *Xenopus* oocyte or *in vitro* formed nuclei was observed. Some of the antibodies have been successfully used for transmission and scanning electron microscopy in conjunction with gold congugated second antibodies.

Immunoblotting experiments using tissue culture supernatants on fractionated *Xenopus* oocyte extracts as antigen gave information on the distribution of the recognised antigens in the extract fractions. Some of the antibodies recognise only one membrane bound protein, whilst others recognise both membrane bound and soluble forms of the same molecular weight protein (by SDS PAGE). Initial results from immunodepletion experiments using magnetic beads coated with these antibodies suggest that at least one of the antibodies, CEL 13A, interacts with a component, that is essential for nuclear formation. This antigen is present in both the membrane and soluble fractions. Removal of the protein from both soluble and membrane fractions results in inhibition of nuclear formation although removal of the antigen from either fraction alone has a limited effect on nuclear formation.

M 110 CYTOPLASMIC MUTANT SV40 LARGE T ANTIGEN NUCLEAR LOCALISATION SIGNAL CAN BE RESCUED BY CONVERSION INTO A BIPARTITE SIGNAL

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There are two major types of Nuclear Localisation Signal, comprising of basic amino acids in either one cluster (unipartite e.g. SV40 LargeT Antigen) or two clusters separated by a spacer of ten residues (bipartite, e.g. nucleoplasmin). A mutant (cytoplasmic)unipartite SV40 Large T Antigen NLS (**PKTKRKV**) can be rescued by converting it into part of a bipartite NLS.The resulting construct can direct a pyruvate kinase fusion protein into the nuclei of transiently transfected COS cells. The mutant phenotype has been reverted by substituting two amino acids at a site distinct from the one mutated in the cytoplasmic NLS. This result is consistent with the view that bipartite NLSs are the norm and that the wild type SV40 Large T Antigen NLS (**PKKKRKV**) is an exception, perhaps because it binds its receptor unusually strongly.

These experiments and other work should give an insight into the nature of the NLS-receptor interaction. Do bipartite NLSs "loop out" the spacer segment and present one continuous basic region to a postulated receptor, or does the receptor bind an extended NLS, actually gripping the spacer and maybe interacting electrostatically with the basic clusters?

M 111 THE SMALL GTPASE RAN/TC4 IS INVOLVED IN NUCLEAR PROTEIN IMPORT, Frauke Melchior and Larry Gerace, Departments of Cell and Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037

A diverse range of cellular activities involves specific GTPases, which are thought to increase the specificity of these processes. We have used nonhydrolyzable GTP analogues to investigate a possible role of GTPases in nuclear protein import. For these studies we modified an *in vitro* nuclear import system involving digitonin-permeabilized cells supplemented with exogenous cytosol (JCB 111: 807) to allow for an ELISA based quantitative analysis. Three nonhydrolyzable analogues ($GTP\gamma S$, GMP-PNP and GMP-PCP) inhibit transport dramatically under appropriate conditions; the inhibition is specific for guanine nucleotides, as it occurres in the presence of an ATP regenerating system and can be diminished by excess GTP. The degree of inhibition varies between 0% and 95%, depending strongly on both cell and cytosol concentrations in the assay. Increasing the cell concentration decreases the inhibitory effect of $GTP\gamma S$, while increasing the cytosol concentration can increase the inhibitory effect. Using cell and cytosol concentrations which in the presence of excess $GTP\gamma S$ lead to only 50% inhibition, we tested whether additional concentrated fractions of cytosol would influence the extent of inhibition. Under these conditions the addition of a 20-30 kDa fraction of cytosol under these conditions resulted in complete inhibition by $GTP\gamma S$ without affecting the transport measured in the absence of inhibitor. The 20-30 kDa fraction is required for efficient transport, as could be shown by testing reconstituted cytosol with or without the 20-30 kDa fraction. We have examined whether one of the two small GTPases previously implicated in nuclear function (ARF and Ran/TC4) would complement reconstituted cytosol lacking the 20-30 kDa fraction. Whereas ARF has no effect, Ran/TC4 fully complements this cytosol. Moreover, Ran/TC4 renders nuclear import sensitive to inhibition by $GTP\gamma S$. These data in conjunction with previous studies on Ran/TC4 and its guanine nucleotide release protein RCC1 indicate that Ran/TC4 serves to integrate nucleocytoplasmic transport with DNA replication, RNA processing and cell cycle control.

M 112 A GLYCOPROTEIN REQUIRED FOR NUCLEAR GROWTH AND DNA REPLICATION. Maureen A. Powers and Douglass J. Forbes. Department of Biology, University of California, San Diego; La Jolla, Ca 92093-0347.
Xenopus egg extracts provide a powerful system for reconstitution of nuclei *in vitro* and analysis of nuclear transport. Such extracts contain three major N-acetylglucosaminylated proteins, p200, p97, and p60. Both p200 (Macaulay and Forbes, submitted) and p60 are components of the nuclear pore. Here, we have investigated the role of p97 in the nucleus. Affinity-purified antisera to p97 were prepared and used to immunodeplete the cytosol of Xenopus eggs. This immunodepleted cytosol was then combined with chromatin templates and a membrane fraction. Nuclei lacking p97 were reconstituted and tested for nuclear function. It was found that the p97-depleted nuclei neither grow with time nor replicate their chromosomal DNA. Replication of ssDNA templates such as M13 remain unaffected. Defects in growth and replication might be predicted to result from a defect in nuclear import. However, the p97-depleted nuclei remain competent for import. Indeed, immunolocalization experiments indicate p97 to be an intranuclear protein (Macaulay and Forbes, submitted). Thus, p97 appears to be a novel major nuclear glycoprotein that is required for nuclear growth and DNA replication.

M 113 **NUCLEAR CHANGES ASSOCIATED WITH INDUCED DIFFERENTIATION OF CAMBIAL CELLS INTO TRACHEIDS IN THE PINE, *Pinus contorta* Dougl.** Rodney A. Savidge, Department of Forest Resources, University of New Brunswick, Fredericton, NB, Canada, E3B 6C2.

In the absence of preceding cell division or cell expansion, cambial cells within dormant stems of conifers can be induced to differentiate directly into tracheary elements (*Planta* **153**:395-404, 1981). When observed by transmission electron microscopy (TEM), the cytoplasm during induced cellular differentiation has been described as highly granular with abundant polyribosomes (*J Exp Bot* **44**:395-404, 1993). Nuclear structure, however, has not been previously investigated in this experimental system. It was of interest, therefore, to compare nuclear structure between non-differentiating cambial cells and those induced to differentiate into tracheids using the TEM (glutaraldehyde, OsO_4 fixation; Ur acetate, Pb citrate staining).

Commonly observed in cells differentiating into tracheids and rarely if ever observed in non-differentiating cells were the following: 1) condensed chromatin most of which was localized near the nuclear envelope, 2) a super-abundance of nuclear ribosomes, 3) dense nucleoli, 4) several types of membrane modification, as described below. Lobing and/or deep invaginations of the nucleus also appeared to be more common in differentiating cells.

Truncations in continuity of the outer, but not the inner, nuclear membrane were frequent in differentiating cells. These breaks did not appear to be pore-associated and evidently were a result of membrane cleavage. Although the inner membrane did not exhibit similar discontinuities, variations in its transverse thickness were common. Inner and outer membranes appeared to alternate in concert between stretches having highly organized bilaminar structure to regions that were multilaminar and poorly organized. Sites of ribosome export were frequent and presented as spatially discrete and well organized membrane alterations.

Nuclear structure as described was similar from early stages of secondary-wall deposition through lignification and into early stages of protoplasmic autolysis. Nuclear changes associated with advanced autolysis were not investigated.

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M 114 PROTEIN EXPORT FROM THE NUCLEUS, Marion S. Schmidt-Zachmann[1], Catherine Dargemont[2], Lukas C. Kühn[3] and Erich A. Nigg[3]. [1]German Cancer Research Center, 69120 Heidelberg, Germany; [2]EMBL, 69117 Heidelberg, Germany; [3]ISREC, 1066 Epalinges, Switzerland.

To examine what structural features might determine the nuclear export of shuttling proteins, we investigated the effects of various mutations on the shuttling ability of nucleolin by using assays based on interspecies heterokaryons and microinjection of Xenopus oocytes. Our results indicate that nucleolin does not contain a positively acting export signal and that the rate of export is determined by structural domains that are involved in retaining the protein in the nucleolus. To further examine the role of nuclear retention in the export process two additional classes of proteins were studied: i) artifical nuclear reporter-proteins derived from cytoplasmic pyruvate kinase and ii) mutant lamin proteins, differing in their abilites to be incorporated into the preexisting lamina. We conclude that nucleocytoplasmic shuttling is a general phenomenon that concerns most nuclear proteins. Whether or not a protein scores positive in a shuttling assay may depend primarily on the extent of its interactions with intranuclear components. These findings have important implications for the proposed functions of shuttling proteins.

Schmidt-Zachmann and Nigg (1993) J. Cell Sci. 105, 799-806.
Schmidt-Zachmann et al. (1993) Cell 74, 494-504.

M 115 CHARACTERISTICS OF A DEVELOPMENTALLY REGULATED PROTEIN ASSOCIATED WITH NUCLEOCYTOPLASMIC RNA TRANSPORT. Dorothy Schumm[1], Steven Runge[2], Saroj Larroya-Runge[2], Paul Stromberg[1] and Thomas Webb[1]. Department of Medical Biochemistry, The Ohio State University, Columbus, OH 43210[1] and Department of Biology, University of Central Arkansas, Conway, AK, 72032[2]

We have identified and prepared monoclonal antibodies to a developmentally regulated 55 kD protein associated with the nucleocytoplasmic transport of RNA. During rat fetal development, this protein increases in concentration from day 9 to day 16 of gestation. It drops to non-detectable levels shortly before birth (day 20). It is non-detectable in all normal adult tissues including maternal circulation although it is present in amniotic fluid. The protein recurs in cells transformed by a number of chemical carcinogens. A similar protein has been detected in fetal tissues from mouse, cat, dog and human. A 35 kD protein is associated with RNA transport in adult tissues in rat. This protein is first detectable at 16 days fetal gestation and reaches adult levels at 5 days after birth. The adult RNA transport protein is specific for mRNA transport but shows no specificity for the nucleotide sequence of the mRNA. The fetal RNA transport protein reacts with Concanavalin A and Dolichos bifloris agglutinin indicating that the protein contains carbohydrate with a-linked D-mannose and N-acetyl-D-galactosamine. The N-terminal amino acid sequence has been determined and is unique in the Wisconsin systems data bank. Immunolocalization shows staining throughout the cytoplasm with punctate staining around the nucleus consistent with the role of this protein in RNA transport.

M 116 RIBOSOMAL PROTEIN TRAFFICKING IN YEAST, Antonius C.J. Timmers, Manita F. Feenstra, Hendrik A. Raué and Jan v. 't Riet, Department of Biochemistry and Molecular Biology, Institute for Molecular Biological Sciences, Biocentrum Amsterdam, Vrije Universiteit, De Boelelaan 1083, 1081 HV Amsterdam, The Netherlands. Exponentionally growing yeast cells have to perform the tremendous task of synthesizing 40 ribosomes per second. This implies that about every 4 seconds one ribosomal subunit per nuclear pore complex (NPC) is exported to the cytoplasm. In addition, about 80 ribosomal proteins are transported timely from the cytoplasm to the nucleus in order to assemble, together with 4 ribosomal RNAs, into preribosomal subunits, which are delivered back to the cytoplasm. Nuclear import of proteins depends on the presence of a nuclear localization signal (NLS), which often consists of a short stretch of amino acids in which arginine and lysine predominate. Although ribosomal proteins as such are small enough to pass the NPC by diffusion, a number of ribosomal proteins (L3, L25, L29, rp51) have been shown to contain specific sequences which mediate efficient nuclear import of ß-galactosidase (MW 116 kD), a protein normally excluded from the nucleus. The study of the nuclear import of L25 (MW 15.7 kD) provided indications that even for such a small protein diffusion is insufficient to support normal rates of ribosome assembly.
In order to obtain information about the presence of NLSs in the yeast ribosomal proteins S24 and S31 we have studied the subcellular distribution of fusion proteins produced by genes encoding various parts of S24 and S31 linked to *Escherichia coli* ß-galactosidase by indirect immunofluorescence microscopy. Several parts of both proteins were identified which are able to support efficient uptake of the reporter protein into the yeast nucleus. Nuclear accumulation was observed as soon as 20 minutes after induction of their synthesis by galactose. The fusion proteins did not accumulate in the nucleolus, the site of ribosome assembly. We reasoned that the presence of such a big reporter protein in comparison with the size of the ribosomal protein might prevent the normal routing of the ribosomal proteins. Therefore we epitope-tagged the yeast ribosomal proteins L25 and S31 and followed their distribution in the yeast cell. Results obtained with whole proteins and several deletion mutants will be presented during the meeting.

M 117 MEMBERS OF A NUCLEOPORIN FAMILY THAT PERTURB INTERACTIONS OF THE NUCLEAR PORE COMPLEX WITH ITS SURROUNDING NUCLEAR MEMBRANE, Susan R. Wente[1] and Günter Blobel[2], [1]Dept of Cell Biology & Physiology, Washington University School of Medicine, St. Louis, MO 63110, [2]Laboratory of Cell Biology, Howard Hughes Medical Institute, The Rockefeller University, New York, NY 10021

The mechanism of translocation through the nuclear pore complex (NPC) and the pathway of NPC biogenesis are largely undefined. We are analyzing the role of a family of nucleoporins from the yeast *Saccharyomyces cerevisiae* that share an amino terminal region of repetitive tetrapeptide 'GLFG' motifs. A subgroup of three GLFG nucleoporins, *NUP116*, *NUP100*, and *NUP63*, have sequence similarties that extend throughout their carboxy terminal regions. We previously reported that a temperature sensitive *NUP116* null mutant forms a nuclear envelope seal over the nuclear pore complex, and thereby blocks nucleocytoplasmic trafficking (Wente and Blobel, J. Cell Biol. **123**:in press, 1993). Our continued studies have found that the deletion of *NUP63* (*nup63Δ*) had only a slight effect on cell growth at all temperatures. However, by both immunofluorescence and electron microscopy, its absence resulted in a striking alteration of nuclear envelope structure and nucleoporin localization. The anti-nucleoporin staining in wild type yeast cells is distinctly punctate and localized to the nuclear periphery. In contrast, the majority of the immunofluorescence signal in *nup63Δ* cells is present in only one, or two, localizations at the nuclear periphery. Thin section electron microscopy analysis revealed unusual 'grape-like' extrusions of nuclear envelope that contain NPCs. These clusters directly correlated with the localization of anti-nucleoporin immunofluorescence signal in concentrated areas of the nuclear envelope. Such 'grape-clusters' of NPCs and the nuclear membrane have not so far been reported. Thus our previous work and these results indicate that deletion of either of two conserved GLFG proteins (Nup116p or Nup63p) gives related perturbations of nuclear envelope and NPC structure. Although neither *NUP116*, *NUP100*, nor *NUP63*, were individually required for cellular growth, haploid strains bearing any of the three pair-wise combinations of their respective null alleles were inviable. These data suggest that a functional redundancy in NPC-nuclear envelope interactions may exist between these three conserved nucleoporins.

M 118 A FORMAL DESCRIPTION OF BIOLOGICAL SYSTEMS: I. CELL STATE FUNCTION FOR A VIRTUAL CELL LINE. YAMAMOTO, S. Laboratory of Cell Biology, Aichi Cancer Center Research Institute, Chikusa-ku, Nagoya 464, JAPAN.

Computer simulation of biological system has been proved useful tools for the study of protein structure, nucleic acids and drug interactions, and so on. The next target of the computer simulation must be process analysis of complex concurrent systems. Recent progress in parallel computing gives us a way of simulating multiple parallel processes such as cell replication.

The first step of the study must be to devise the description system for the target system. The second step is to write programs for the target system, and the third step is to implement it in a computer system.

As the first step, I propose a new concept "Cell State Function (CSF)" denoted by Z( ). The Cell State Function consists a description system for the cell replication process as a function of time, with "Environment State Function (ENVSF)" denoted by ENV( ) for the external fields. $Z(cell_{ph}^{i})^{t}$ is a general expression for a virtual cell of i-th generation at time t in a phase. Here a virtual cell line cell consists of cellular components $Cc=\sum_{i=1,j=1}^{n,m} C_{cij}{}^{t}$, which are enclosed in a volume $V^{t}=f(t)=\Sigma V_{ccij}{}^{t}$ by plasma membrane, and after doubling time t_D reproduces itself through four elemental phases, G_1, S, G_2 and M under given ENV( ). By using Cell State Function, we can describe the cell replication process as a collection of expressions. The replication process of a cell in G1 phase at 10th generation enters next G1 phase is expressed as follows: $Z(cellG1^{10})^{t} \rightarrow Z(cellG1^{11})^{t+t_D}$. The contents of replication process are classified in three categories: the cell cycle dependent, independent, and the process control processes.

M 119 *IN VITRO* NUCLEAR TRANSPORT OF GLUCOCORTICOID RECEPTORS Jun Yang and Donald B. DeFranco, Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260

We have used a digitonin permeabilized cell system to analyze the *in vitro* nuclear transport of glucocorticoid receptors (GR). GRs possess two distinct nuclear localization sequences, one (i.e. NL1), whose activity is hormone-independent but usually masked in the unliganded receptor, and another (i.e. NL2), whose activity is strictly hormone dependent. Digitonin permeabilized HeLa cells, which do not contain detectable levels of GR, were incubated with cytosol prepared from the GrH2 rat hepatoma cell line, which contains relatively high levels of GR. GR was detected using an indirect immunofluorescence method. GR was not imported into permeabilized HeLa cell nuclei upon the addition of GrH2 cytosol at 30°C even in the presence of an ATP regenerating system. However, the addition to the cytosol of the synthetic glucocorticoid, dexamethasone, led to efficient nuclear import of GR into HeLa nuclei. Thus, analogous to the situation observed in many different cell types *in vivo* , nuclear import of GRs *in vitro* appears to be strictly hormone dependent. As observed for other karyophilic proteins in this system, hormone dependent nuclear import of GR was not observed in the absence of an ATP regenerating system or when the reaction was performed at 0°C. The lack of GR nuclear import at 0°C is not due to the failure of added hormone to bind to the receptor at this temperature as hormone binding to the GR was obtained in isolated cytosol at 0°C. Following the binding of hormone, GRs become activated via a process that involves its dissociation from a heteromeric complex that includes, in addition to the receptor, a dimer of the 90 kDa heat shock protein (hsp90) and other hsps. Inhibition of GR activation by the inclusion of sodium molybdate blocked the *in vitro* nuclear transport of GR. Given the fact that hsp70 appears to remain associated with GR following its activation, and the recent revelations of a role for hsp70 in nuclear transport of nucleoplasmin and the SV40 large tumor antigen (TAg), we examined whether GR nuclear import likewise required hsp70 function. 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, the GR NLS, unlike that present in TAg and other karyophilic proteins, does not appear to require hsp70 to function *in vitro* .

Assembly and Disassembly of the Nucleus with the Cell Cycle; The Nuclear Matrix

M 200 CHARACTERIZATION OF RECOMBINANT HUMAN DNA TOPOISOMERASE II.

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Chromatin structure and the topological state of DNA play a key role in many DNA transactions including DNA replication, chromosome segregation, transcription, transposition and recombination. Enzymes that alter DNA topology are termed DNA topoisomerases and have been found in all cell types. Recently it has been shown that higher eukaryotes contain two isoenzyme forms of DNA topoisomerase II. We have cloned cDNAs encoding human topoisomerase II α and β, and used these to overexpress recombinant human proteins in a yeast expression system. The recombinant proteins have been purified to homogeneity, and are enzymatically active. Investigations of the structural and mechanistic properties of these recombinant human type II topoisomerase isoenzymes will be presented.

M 201 DIFFERENTIATION OF MOUSE ERYTHROLEUKEMIA CELLS RESULTS IN SEVERAL CHANGES IN THE PROTEIN COMPOSITION OF THE NUCLEAR MATRIX.

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Nuclear Matrix proteins have been implicated in all the major nuclear processes, in an effort to identify new erythroid specific nuclear proteins and in particular new transcription factors, we have compared by electrophoresis the nuclear matrix proteins of undifferentiated Mouse Erythroleukemia cells (MEL) to the nuclear matrix proteins of HMBA-treated MEL cells (induced to differentiated along the erythroid pathway) and to various nuclear fractions.

The matrix proteins were prepared by a modification of the LIS extraction method of Mirkovitch et al (1984) using 5mM LIS and a lower DNase I concentration. After SDS-PAGE electrophoresis according to Laemmli, the proteins were visualized by Coomassie brilliant blue or silver staining and quantify with a digital densitometer. In order to obtain complete solubilization of the samples and therefore good reproducibility it was necessary to treat the nuclear matrix fraction extensively with DNase I and add high concentration of DTT (60mM) in the sample buffer. This analysis revealed that erythroid differentiation of the MEL cells resulted in a number of quantitative changes in the nuclear matrix composition. Of particular interest is a group of very high MW proteins (> 150KD) that appear to be specific of the nuclear matrix fraction and that are greatly affected by the differentiation process. Further characterization of these proteins by two dimensional electrophoresis, immunnoblots and binding assay is in progress.

In conclusion, we hypothesize that the changes in the nuclear matrix of MEL cells after HMBA induction reported here reflect the importance of the nuclear matrix in the erythroid differentiation process.

M 202 5-AZACYTIDINE INDUCTION OF MICRONUCLEI AND MITOTIC DISTURBANCES, William J. Caspary[‡], Carsten Körber*, Dietmar Schiffmann*, Diane Spencer‡ and Helga Stopper* [‡]NIEHS/NIH, RTP, NC 27709 and *Institute of Toxicology, University of Würzburg, Germany

5-Azacytidine was originally developed to treat human myelogenous leukemia. However, interest in this compound has expanded because of reports of its ability to affect cell differentiation and to alter eukaryotic gene expression. In an ongoing attempt to understand the biochemical effects of this compound, we examined the effects of 5-azacytidine on mitosis and on micronucleus formation in mammalian cells. In L5178Y mouse cells, 5-azacytidine induced micronuclei at concentrations at which we and others have already reported its mutagenicity at the *tk* locus. Using CREST staining and C-banding studies, we showed that the induced micronuclei contained mostly chromosomal fragments although some may have contained whole chromosomes. By incorporating BrdU into the DNA of SHE cells, we determined that micronuclei were induced only when the compound was added while the cells were in S phase. Microscopically visible effects due to 5-azacytidine treatment were not observed until anaphase of the mitosis following treatment or thereafter. 5-Azacytidine did not induce micronuclei via interference with formation of the metaphase chromosome arrangement in mitosis, a common mechanism leading to aneuploidy. Supravital UV microscopy revealed that chromatid bridges were observed in anaphase and, in some cases, were sustained into interphase. In the first mitosis after 5-azacytidine treatment we observed that many cells were unable to perform anaphase separation. It appears that demethylation opens the chromatin structure making the DNA susceptible to double strand breaks.

M 203 *XENOPUS* LAMIN L_{III}: ANALYSIS OF INTERPHASE AND MITOTIC FORMS. Catherine M. Crompton, Ian R. Kill and Christopher J. Hutchison. Department of Biological Sciences, The University, Dundee DD1 4HN

2-Dimensional gel and Western Blot analyses of interphase and mitotic *Xenopus* lamin L_{III} suggest that L_{III} is present as a single isoform in the mitotic extract whereas in the interphase extract, it appears as 2 or possibly 3 isoforms. This difference may be due to differential phosphorylation: mitotic forms of somatic lamins are hyperphosphorylated and it seems likely that the embryonic system also uses this strategy to solubilise its lamins at mitosis. Phosphopeptide map and phospho-amino acid analyses were carried out on ^{32}P labelled lamin L_{III} from interphase and mitotic extracts: L_{III} was isolated from extracts using monoclonal antibodies conjugated to paramagnetic Dynabeads. Our results suggest that there is one major ^{32}P labelled peptide found in the mitotic but not the interphase form of L_{III}. This peptide will be isolated and sequenced to determine where in the lamin structure this phosphorylation occurs, and which protein kinase is responsible for its phosphorylation. Phospho-amino acid analysis has been carried out on interphase L_{III}: these results suggest that interphase lamin L_{III} is only phosphorylated on serine residues.

M 204 THE FARNESYLATION DEPENDENT PROCESSING OF PRELAMIN A RESULTS IN THE SECRETION OF A PRENYLATED PEPTIDE, Marguerite B. Dalton, Kelley S. Fantle, Michael Sinensky, Eleanor Roosevelt Institute for Cancer Research, Denver, CO 80206

Lamin A is the only prenylated protein that is in direct contact with chromatin and is differentially regulated during development and in a tissue specific manner. A distinctive feature of the prenylation pathway of prelamin A is the putative endoproteolytic removal of the carboxy-terminal prenylated peptide, which should give rise to a farnesylated peptide and the nonprenylated mature lamin A. We have obtained *in vivo* results that are consistent with such an endoproteolytic mechanism. In order to assay for the prelamin A peptide we have prepared a farnesylated and carboxymethylated synthetic peptide corresponding to the carboxyl-terminal 15 amino acids of the human prelamin A. Preliminary efforts to detect the prelamin A peptide in whole cell lysates by immunoprecipitation or by Western blotting with prelamin A specific antibody have been negative. However, we have recently obtained evidence that the prelamin A peptide may be secreted from cells which express lamin A under the control of a dexamethasone inducible promoter (MMLA). We have identified radioactive material from cell culture media taken from MMLA cells which were labeled with ^{3}H-mevalonate that co-migrates with synthetic farnesylated and methylated peptide on SDS-PAGE. To further address the possibility that the peptide is secreted, cells were treated with or without dexamethasone and labeled with ^{3}H-mevalonate and the medium was sampled as a function of time for a 2 kD prenylated peptide. Confirmation of the presence of the peptide in the cell culture media was obtained by Western blotting and by its migration on reverse phase HPLC.

M 205 THE ROLE OF RCC1, A REGULATOR OF CHROMOSOME CONDENSATION, IN CELL CYCLE CONTROL, DNA REPLICATION AND NUCLEAR FORMATION: STUDIES IN *XENOPUS* EXTRACTS USING MUTANT RCC1 PROTEINS. M. Dasso[1], T. Seki[2], Y. Azuma[2], H. Seino[2], T. Ohba[2] and T. Nishimoto[2]. [1]Laboratory of Molecular Embryology, NICHD, National Institutes of Health, Bldg. 6, Rm. B1A13, Bethesda, Maryland 20892, U.S.A., and [2]Department of Molecular Biology, Graduate School of Medical Science, Kyushu University, Fukuoka 812, Japan.

RCC1 is a highly-conserved, abundant, chromatin-associated protein whose function is required to preserve a properly ordered cell cycle. Mutants with *ts* alleles of this protein advance into mitosis prematurely at the restrictive temperature, causing premature chromosome condensation and cell death. RCC1 has also been implicated in a number of other nuclear processes, including nuclear transport and RNA metabolism, and it functions enzymatically as a guanine nucleotide release protein (GNRP) for a small, *ras*-like GTPase called Ran. By immunodepletion of RCC1 from *Xenopus* egg extracts, it has been shown that RCC1 is absolutely required for DNA replication of sperm chromatin templates *in vitro*. The replication defect of RCC1-depleted *Xenopus* extracts appears to result from incorrect nuclear assembly, and it can be rescued by the addition of bacterially-expressed proteins, thus providing a convenient *in vitro* assay of RCC1's role in nuclear assembly. In our effort to gain a more detailed understanding of RCC1's function, we have selected a series of residues in the RCC1 protein for mutagenesis to alanine, based on their high degree of conservation between species or on their correspondence to sites known to have strong phenotypes *in vivo*. We have characterized the GNRP activity of bacterially-expressed mutant proteins and their affinity for Ran, as well as their ability to restore DNA replication and nuclear formation in *Xenopus* extracts. In order to examine nuclear function, we have assayed the extent of nuclear growth, the rate of nuclear import, the formation of the nuclear lamina and the formation of DNA replication foci. We find a strong correlation between the rescue of nuclear function and the GNRP activities of the mutant proteins.

M 206 IDENTIFICATION OF NOVEL REGULATORY EVENTS IN NUCLEAR ENVELOPE REASSEMBLY USING XENOPUS EGG EXTRACTS, Paul Ferrigno and Carl Smythe, MRC Protein Phosphorylation Unit, Dept of Biochemistry, Medical Sciences Institute, The University, Dundee DD1 4HN, Scotland, UK.

In higher eukaryotic cells, the onset of mitosis is characterised by dissociation of condensing chromatin from the nuclear envelope, depolymerisation of the nuclear lamins, and vesicularisation of the nuclear envelope. At the completion of mitosis, nuclear membrane vesicles (NMVs) and lamin proteins bind to the surface of condensed chromatin and initiate the processes that lead to the reassembly of an intact functional nuclear envelope. Control of dissociation and association of nuclear membranes with chromatin at the beginning and end of mitosis is clearly important for successful progress through this phase of the cell cycle.

It has been shown previously that, using a fractionated cell-free system from Xenopus laevis eggs, the binding of nuclear membrane vesicles to chromatin is modulated by a mechanism of reversible phosphorylation. In interphase, the balance in the system favours dephosphorylation and NMVs bind to chromatin. In mitosis, components of this system are phosphorylated and binding is blocked. This phosphorylation event is catalysed by a protein kinase distinct from, though probably activated by, cyclinB/ cdc2. Such a mechanism would act to prevent inappropriate nuclear reassembly during mitosis.

We have identified two discrete populations of NMVs in extracts of Xenopus laevis eggs, which differ in their response to regulation by phosphorylation. In one population, termed NMV I, phosphorylation of a membrane component is necessary, but not sufficient, to block binding of vesicles to chromatin. Inhibition of vesicle binding, such as occurs in mitosis, requires the presence of an additional novel factor present in high-speed supernatants derived from Xenopus eggs. This factor, termed NAF(for NMV Accessory Factor) is a protein.We present evidence that the inhibition occurs via a stoichiometric rather than enzymic mechanism. Immunodepletion of extracts using specific antibodies indicates that NAF is distinct from the Xenopus egg lamin Liii. Inhibition of NMV binding to chromatin is mediated by phosphorylated, but not dephosphorylated NAF. We propose that NAF is a regulatory component which is important for the control of nuclear envelope dynamics during the cell cycle.

M 207 **S-PHASE MODIFICATION OF LAMIN B FOLLOWING SERUM RESTIMULATION OF QUIESCENT HUMAN DERMAL FIBROBLASTS.**

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The nuclear lamina is a meshwork of intermediate filaments lining the nucleoplasmic face of the nuclear membrane. Nuclei lacking a lamina, formed in cell-free extracts of *Xenopus* eggs *in vitro*, are unable to replicate DNA suggesting a role for the lamina in DNA replication. To investigate the potential role of the lamina in DNA replication, we have examined properties of lamin B, a constitutive component of all nuclear laminae, in cultures of normal human dermal fibroblasts during the transition from a non-proliferating to a proliferating state. 2-dimensional Western blotting has revealed that lamin B is post-translationally modified following serum-restimulation of quiescent cultures. The modification of lamin B is contemporaneous with entry into S-phase, as demonstrated by the detection of proliferative markers in an indirect immunofluorescence assay and by estimation of the fraction of cells incorporating bromodeoxyuridine. ^{32}P-Peptide mapping has revealed the presence of a phosphorylated peptide in tryptic digests of lamin B derived from S-phase cells which is absent in samples derived from quiescent cells. Furthemore, phosphoamino acid analysis has revealed that serine is the only phosphorylated amino acid in samples derived from either quiescent or S-phase cultures. Sub-cellular fractionation experiments have demonstrated that the fraction of lamin B which is soluble in 0.6M KCl/Triton X100 extracts of quiescent, or S-phase HDF, is similar, suggesting that Lamin B solubility in this assay is unaffected by serum-restimulation. These observations establish a link between post-translational modification of lamin B and entry into S-phase.

M 208 NUCLEAR ISOFORMS OF STRUCTURAL PROTEIN 4.1, Sharon W.Krauss, Cell and Molecular Biology Department, Life Sciences Division, University of California, Lawrence Berkeley Laboratory, Berkeley, CA, 94720

The hypothesis that protein 4.1 polypeptides contribute to nuclear structure/function in diploid human fibroblasts is being tested. Protein 4.1 was first recognized as a multifunctional 80kD structural protein of the human erythrocyte plasma membrane skeleton where its protein-protein interactions are critical determinants of mechanical stability and morphology. Recent studies have shown: (a) protein 4.1 is relatively abundant in many nucleated cells, (b) 4.1 polypeptides are detected in nuclei of mammalian cells and (c) multiple 4.1 isoforms generated by alternative splicing can be tissue- and differentiation-specific.

Alternatively spliced 4.1 isoforms (30-135 kD) may have specific functions. The amino terminal end of the 80kD protein 4.1 contains binding sites for glycophorin, calmodulin and band 3 while another domain toward the C terminus contains binding sites for spectrin-actin complexes. N-terminal extensions, present in nonerythroid 4.1, may permit many other biochemical interactions. An initial survey of fibroblast 4.1 domains by PCR analysis of cDNA showed expression of an upstream AUG translation initiation site characteristic of non-erythroid cells, two splice products in the spectrin-actin binding domain and also a novel fetal fibroblast-specific band in a region encompassing the downstream 80 kD AUG initiation site along with part of the glycophorin binding domain.

By immunofluorescence, three patterns of protein 4.1 epitopes in diploid human fibroblasts are detected using a panel of IgG's against the 80kD erythroid protein 4.1 and against synthetic 4.1 peptides: (1) antibodies against 80kD 4.1 produce "speckles" in the nucleus but do not stain the cytoplasm, (2) antibodies against the C terminus and two other internal 4.1 domains localize to discrete intranuclear foci as well as cytoplasmic regions and (3) antibodies against the spectrin-actin binding domain show little nuclear binding relative to cytoplasmic staining.

Additionally, constructs encoding several cloned protein 4.1 isoform cDNAs fused to an unrelated epitope tag were transfected into fibroblasts. The tagged, expressed 135 kD 4.1 isoform was detected immunochemically in the nucleus after transient transfections. Definition of protein 4.1 isoforms present in the nucleus will provide a basis for analysis of 4.1-specific structural interactions that may be of functional significance to nucleated cells.

M 209 BINDING OF MATRIX ATTACHMENT REGIONS TO LAMIN POLYMERS INVOLVES SINGLE-STRANDED REGIONS AND THE MINOR GROOVE, M. E. Eva Ludérus*, Jan L. den Blaauwen, Oncko J. B. de Smit, and Roel van Driel, E. C. Slater Institute for Biochemical Research, University of Amsterdam,1018 TV Amsterdam, The Netherlands. *Present address, The Rockefeller University, 1230 York Avenue, New York, New York 10021-6399, U. S. A.

In eukaryotic nuclei, chromatin is partitioned into functional loop domains. These are generated by binding of defined DNA sequences, named MARs (Matrix Attachment Regions), to the nuclear matrix. We have previously identified B-type lamins as MAR binding matrix components (Ludérus et al. (1992) Cell **70**, 949-959). Here we show that also A-type lamins can bind MARs specifically, provided that they are isolated in the absence of SDS. This indicates that general features of the lamin molecule, rather than subtype-specific ones, mediate MAR binding. We studied the interaction between MARs and lamin polymers in more detail, and found that the interaction is saturable, of high affinity, and evolutionary conserved. Competition studies revealed the existence of two different types of interaction, involving different structural features of MARs: one involving the minor groove, and one involving single-stranded regions. Similar modes of binding were observed for the interaction of MARs with isolated nuclear matrices. A model is discussed for a role of MARs in stabilizing the transcriptionally active state of chromatin.

M 210 THE DYNAMICS OF THE NUCLEAR LAMINS DURING THE CELL CYCLE, Robert D. Moir, Anne E. Goldman, Michelle Montag-Lowy and Robert D. Goldman, Department of CMS Biology, Northwestern University Medical School, Chicago IL, 60657

The nuclear lamina is a fibrous network that lies adjacent to the nucleoplasmic face of the inner nuclear membrane. In mammalian cells, the lamina is assembled from at least three related proteins termed lamins A, B and C. We have been examining the process of assembly using the microinjection of derivatized lamin proteins into the cytoplasm of tissue culture cells. Each of the three lamins is rapidly transported to the nucleus when injected and becomes incorporated into the lamina at the nuclear periphery over several hours. Furthermore, the injected protein is disassembled with the endogenous lamins during the breakdown of the nuclear envelope at mitosis and is incorporated into the new lamina of the daughter cells following reassembly, suggesting the injected protein behaves as the endogenous protein. At early time points following microinjection, lamins appear as nucleoplasmic foci, although the pattern of each lamin at early time points may be different. For example, the co-injection of lamins A and C into the same cell results in different initial A and C distributions in the nucleoplasm. Nucleoplasmic lamin foci are also observed in uninjected cells in G1 using several lamin antibodies. Furthermore the distribution of A and B type lamins is different at this stage of the cell. These differences may relect the different expression and post-transitional modification patterns of these proteins. (Supported by NCI).

M 211 ARE COMPARABLE EVENTS INVOLVED IN CHROMATIN CONDENSATION DURING MITOSIS AND APOPTOSIS? Franziska Oberhammer[1], Karin Hohenegger[1], Gertraud Fröschl[1], Roman Tiefenbacher[1], Helga Stopper[2], Margit Pavelka[3]; 1 Inst. of Tumorbiology, University of Vienna, Austria; 2 Inst. of Toxicology, Würzburg, FRG; 3 Inst. of Histology-Embryology, University of Innsbruck, Austria.

Incubation of interphase nuclei in extracts prepared from mitotic cells induces chromatin condensation, solubilisation of lamins and nuclear fragmentation. The morphology of these processes is very similar to the nuclear events in apoptosis. Furthermore we could show that activation of an endonuclease is not the first trigger in apoptotic chromatin condensation (Oberhammer et al., J. Cell Science 104, 317-326, 1993). We thus investigated activation of $p34^{cdc2}$ kinase both by histone H1 kinase assay and detection of tyrosine phosphorylation by immunoblotting. A comparison of apoptotic and mitotic cells was performed in an embryonic fibroblast undergoing apoptosis a few hours after serumwithdrawal at confluence and upon arrest in mitosis with nocodazole. In contrast to mitosis, at all timepoints after serumwithdrawal no activation of $p34^{cdc2}$ could be detected. Lamin A+B got degraded in parallel with degradation of the DNA. A fragment of 43 kDa was transiently visible. The generation of this fragment could be due to activation of a nuclear calcium dependent protease (Tokes and Clawson, J. Biol. Chem. 264, 15059-15065, 1989). As observed by vital UV microscopy the onset of chromatin condensation was occurring abruptly within a few minutes. Obviously the sudden collapse of the chromatin structure was the result of detachment of the chromatin loops from the nuclear matrix, as bands of 50 kbp and multimers thereof were detectable by pulse-field gel electrophoresis (Oberhammer et al., EMBO 12, 3679-3684, 1993). Application of an improved version of in situ nick translation showed that double strand breaks started to be detectable around the bulks of condensed chromatin. For these first DNA strand breaks topoisomerases could be responsible. Recent reports of experiments investigating chromatin condensation in nuclei incubated in extracts of mitotic cells are also suggesting that chromatin condensation in this system does not need activation of $p34^{cdc2}$ kinase (Lazebnik et al., J. Cell Biology, in press).

M 212 CELLULAR DISTRIBUTION OF UV-DDB PROTEIN AND UV-DDB ACTIVITY IN PRIMATE CELLS, V.Rapic Otrin, M.Takao, M. McLenigan, A. S. Levine and M. Protic, Section on DNA Replication, Repair and Mutagenesis, National Institute of Child Health and Human Development, NIH Bethesda, MD 20892

DNA-binding proteins that recognize chemically or physically modified DNA, rather than a specific DNA sequence, are termed damaged DNA-binding (DDB) proteins. In analogy to DDB proteins in prokaryotes, DDB proteins from mammalian cells are postulated to be components of DNA repair/ lesion signaling complexes with a role in detecting damaged DNA and transferring the information to other members of DNA repair complexes and/or signal transduction pathways. We have recently identified[1], purified[2], and determined a primary sequence[3] of a 127 kDa protein (UV-DDB) from primate cells which has high affinity for UV-irradiated DNA and may be defective in xeroderma pigmentosum group E patients. UV-DDB polypeptide is a member of a novel, conserved family of proteins: Homologs of UV-DDB have been identified in slime mold and rice, and more recently in Drosophila. Mammalian cells exposed to UV light have 2-3 fold induced levels of UV-DDB activity after an initial dose-dependent inhibition[1]. To determine the mechanism of this inhibition, we have now examined cellular distribution of the UV-DDB activity and p127 in untreated and UV-treated cells. The majority of p127 is located in cytoplasm (which lacks UV-DDB activity) and the rest of the protein is in the nucleus. Within nuclei, the majority of p127 can be found in the high-salt extract, and this chromatin fraction is also the most abundant in UV-DDB activity. The UV-DDB activity and protein can also be detected in lesser amounts in the low-salt bulk chromatin and isotonic supernatant. Reproducibly, we have detected 1-5% of p127 and UV-DDB activity associated with the nuclear matrix. We obtained similar results with cells that were collected immediately after UV irradiation. However, in those cells, UV-DDB activity could not be detected in low-salt bulk chromatin while the high-salt extract showed a reduced level of the activity. At the same time, the amount of p127 did not change significantly in these nuclear fractions. Future *in situ* immunofluorescence studies, using cells at various times after UV and anti-UV-DDB antibodies, are needed to directly test our prediction that the UV-DDB complex (and p127) binds tightly to UV-damaged genomic DNA *in vivo* , and therefore its activity cannot be detected in the bulk chromatin fraction.

[1]Hirschfeld et al., Mol. Cell Biol. 10:2041, 1990; [2]Abramic' et al., J. Biol. Chem. 266:22493, 1991; [3]Takao et al., Nucl. Acids Res. 21:4111, 1993.

M 213 The Murine Homolog of the Drosophila polo Serine/Threonine Kinase is Required for DNA Synthesis and Mitotic Spindle Formation in NIH 3T3 Cells, **Mark R. Smith**[1], Ryoji Hamanaka[2], Dan L. Longo[2], and Doug K. Ferris[1], BCDP, PRI/DynCorp[1], and LLB[2], BRMP, NCI, FCRDC, Frederick, MD 21702.

An understanding of the mechanics of cell cycle regulation is critical to the development of new drugs and therapies to combat genetic disease. Much of the recent work on cell cycle control has come from lower eukaryotes such as yeast. The structure and function of these yeast genes are conserved enough that mammalian homologs have been isolated that complement yeast cell cycle mutants. Using a PCR-based method we have cloned a murine kinase similar to the Drosophila polo kinase, Plk. This kinase may directly regulate mitotic spindle assembly. Reversible protein phosphorylation by kinase and phosphatase enzymes is the basis for maintaining metabolic homeostasis and is central to cellular processes such as signal transduction, growth, differentiation, and neoplastic transformation. In this report, we describe several biological activities of the Plk kinase in fibroblast cells. Plk was shown to induce DNA synthesis, to be required for serum stimulated growth, and to play an essential role in the formation of mitotic spindles. Microinjection of Plk anti-sense RNA into serum stimulated NIH 3T3 cells resulted in cells that are synchronously blocked in prophase, they have condensed chromatin but no mitotic spindles. Injection of Plk mRNA into quiescent cells induces DNA synthesis, chromatin condensation and mitotic spindles. These results suggest that expression of Plk can override the restriction point in G_0 arrested cells and drive them into mitosis. Plk is required for cell growth and may play a role in oncogenic transformation. We are exploring relationships between Plk, oncogenes and other cell cycle kinases known to have roles in relaying signals from the exterior of the cell into the nucleus.

M 214 **STEROID RECEPTORS, TRANSCRIPTION AND NUCLEAR ARCHITECTURE**, [1]Bas van Steensel, [2]Guido Jenster, [1]Derick G. Wansink, [3]Klaus Damm, [4]E. Ronald de Kloet and [1]Roel van Driel. [1]E.C.Slater Institute, University of Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam, The Netherlands; [2]Dept. of Endocrinology and Reproduction, Erasmus University, Rotterdam, The Netherlands; [3]Max-Planck-Institut für Psychiatrie, München, Germany; [4] Center for Bio-Pharmaceutical Sciences, Leiden University, Leiden, The Netherlands.

We are investigating the functional and spatial organization of the interphase cell nucleus. We have recently shown that nascent pre-mRNA is localized in discrete domains in the nucleus, which remain present after chromatin has been removed [Wansink et al., J. Cell Biol. **122** (1993): 283-293]. Evidently, these domains are associated with the nuclear matrix. It is known that also the DNA and RNA polymerase II are bound to the nuclear matrix. It is likely that this nucleoskeleton plays an important role in transcription.

Steroid receptors are hormone-controlled transcription factors. We [Van Steensel et al., FEBS Lett **292** (1991): 229-231] and others have shown that occupied steroid receptors are bound to the nuclear matrix. To gain insight into the relationship between transcriptional control and nuclear organization, we investigated: (i) the interaction between nuclear matrix and steroid receptors and (ii) the nuclear distribution of steroid receptors.

COS cells were transfected with different deletion mutants of the glucocorticoid receptor (GR) and of the androgen receptor (AR). We find that the DNA binding domain of the GR is required for matrix association. In the AR the hormone binding domain and at least one other protein domain is involved in matrix binding. Evidently, the AR and GR bind to the nucleoskeleton through different mechanisms.

Immunofluorescent labeling shows that the GR is clustered in discrete domains in the interphase nucleus. Its spatial distribution is similar to that of nascent pre-mRNA and of RNA polymerase II. Also the GR clusters are associated with the nuclear matrix. Double labeling experiments are in progress to precisely compare the nuclear distributions of different components involved in transcription, i.e., the GR, RNA polymerase II and nascent pre-mRNA. Results give important information about organization and control of transcription in the interphase nucleus.

M 215 IN VITRO REACTIVATION OF XENOPUS ERYTHROCYTE NUCLEI, L.J. Wangh, D. DeGrace, J.A. Sanchez, P.J. Elias, K. Wiedemann, D. Lee, Department. of Biology, Brandeis University, Waltham, MA 02254

Conditions have been defined and optimized for in vitro reactivation and replication of quiescent nuclei prepared from fully differentiated frog erythrocytes. Nuclei incubated in frozen/thawed extracts from mitotically arrested and activated frog eggs swell, acquire nuclear envelopes, and initiate and complete one or more rounds of replication with a high degree of synchrony. The product of replication is very high molecular weight DNA (>2 megabase pairs), but during the process of chromosome condensation this DNA is transiently cleaved at regular intervals of about 27 kilodaltons by Type II topoisomerase. Protein synthesis in our synchronous system is required at several steps: exit from meiotic metaphase II; first round DNA replication; entry into first mitosis; and exit from first mitosis. Fluorescent microscopy using Scanalytics deconvolving software provides both optical-slice and 3D-reconstructured views of chromatin fibres in swelling and replicating nuclei.

M 216 p175, A MITOTICALLY PHOSPHORYLATED INTEGRAL MEMBRANE PROTEIN WITH CHROMATIN-BINDING PROPERTIES *IN VITRO*, Christiane Wiese, John Newport*, and Katherine Wilson, Department of Cell Biology & Anatomy, The Johns Hopkins University School of Medicine, Baltimore, MD 21205
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Nuclear envelope assembly involves the targeting of nuclear-specific vesicles to chromosomes during anaphase and telophase. To identify membrane proteins that serve as "receptors" for chromosomes during nuclear envelope assembly, we devised the following *in vitro* strategy. Vesicles isolated from *Xenopus* eggs were radioiodinated to label cytoplasmically-disposed membrane proteins. Nuclear vesicles, which bind to demembranated sperm chromatin, were isolated by pelleting the chromatin. The nuclear vesicles were then extracted with MEGA-10, a nonionic detergent, and the chromatin was repelleted. We hypothesized that any membrane proteins bound directly to chromatin would continue to pellet with chromatin after detergent extraction. Three radioiodinated integral membrane proteins with chromatin-binding activity were identified in this assay. Their estimated molecular weights were 50, 165, and 175 kD. Both p165 and p175 are significantly larger than any previously-identified membrane proteins that bind to the nuclear lamina and/or to chromatin. Protein p175 appears to be localized to nuclear membranes, as determined by indirect immunofluorescence of cultured *Xenopus* cells. Consistent with the prediction of Pfaller *et al* (*Cell* 1991, 65:209) for the behavior of a nuclear vesicle targeting protein, we find that p175 is hyperphosphorylated both in mitotic extracts, and in interphase extracts treated with okadaic acid (a phosphatase inhibitor). Phosphorylation of immunoprecipitated p175 was shown by gel shift of p175 after treatment with potato acid phosphatase, and by blotting with antibodies that recognize a phosphorylated epitope(s) on p175. Thus, three properties of p175 are consistent with a potential role in targeting nuclear vesicles to demembranated sperm chromatin during nuclear assembly: its detergent-resistent chromatin-binding behavior, its nuclear envelope localization in interphase nuclei, and its hyperphosphoryation in mitotic extracts. Molecular cloning of the p175 cDNA is now underway.

M 217 DISRUPTION OF SYNCHRONOUS PROGRESSION OF MITOTIC EVENTS BY DELAYING CHROMOSOME DECONDENSATION, Yoshihiro Yoneda and Yosuke Matsuoka, Department of Anatomy, Osaka University Medical School, 2-2 Yamada-oka, Suita, Osaka 565, Japan

In higher eukaryotic cells at the end of mitosis, chromosomes decondense, the nuclear envelope reassembles and the mitotic spindle dissociates and assembles into interphase microtubules. At mitosis, nuclear pore complexes and the filaments of the nuclear lamina disassemble. During reformation of the nucleus, the nuclear vesicles are targeted to the chromosome surface and fuse with each other.

In this study, we tried to examine whether the progression of chromosome decondensation, nuclear envelope reassembly and microtubule formation at the end of mitosis of mammalian cells occur inter-dependently in vivo by delaying chromosome decondensation. When histone H1 was injected into the mitotic rat kangaroo kidney epithelium (PtK2) cells at prometaphase, chromosome condensation was prolonged for several hours, and sister chromatid separation and cytokinesis did not occur. However, interphase microtubules reassembled and lamin B-positive structures reformed around the condensed chromosomes. Kinetic experiments showed that one type of lamin B-positive structure (stained uniformly with anti-lamin B antibodies) proceeded to the other type (peripheral lamin B staining, the normal staining pattern) competent for active nuclear protein transport. We also found that reassembly of the nuclear transport competent structure depends on the existence of chromosomes, but not on chromosome decondensation.

Chromatin and Chromosomes: Organization, Regulation of Transcription; DNA Replication: Basics, Relationship to Higher Order Nuclear Structure

M 300 ORGANISATION OF MAMMALIAN CHROMOSOMES: INTEGRATION OF STRUCTURE AND FUNCTION, Wendy Bickmore, Jeffrey Craig, Judy Fantes and Veronica van Heyningen, MRC Human Genetics Unit, Edinburgh EH4 2XU, Scotland, UK.

The ability to generate chromosome bands on mammalian chromosomes indicates that mammalian euchromatin is highly organised and compartmentalised, both structurally and functionally.

Using chromosome painting and the ability to fractionate genomic DNA on the basis of the density of CpG islands (and hence genes), we have been able to directly quantitate the different CpG island densities in different regions of the genome. CpG island densities vary between different types of chromosome band by up to several orders of magnitude, yet the variation of CpG island density within a particular type of chromosome band is small. How such an organisation is established and maintained is not known. We have shown that regions of chromosomes replicating late in S phase are all parts of the genome where CpG islands are very scarse.

We are currently using FISH to study in detail how differences in chromatin structure, attachment to the chromosome scaffold and DNA replication patterns vary as you traverse from one type of chromosome domain into another (gene dense to gene poor). These structural and functional aspects of the chromosome are also being analysed in cell lines with differing patterns of gene expression in the region under study (human 11p13-p14), and when the chromosome region is moved out of context (by chromosomal deletion, translocation and inversion) to new sites in the genome. This is of particular interest since we have genetic evidence to suggest that at least two such chromosomal anomalies result in position effects on the Pax6 gene producing the clinical disease aniridia.

M 301 THE 5' REGULATORY AND FLANKING REGION OF THE HUMAN CHOLINE ACETYLTRANSFERASE GENE IS ANCHORED TO THE NUCLEAR MATRIX. Teni Boulikas[1]*,C.F. Kong[1], Linda Xie[1], Dawn Brooks[1], Andrea Todd[2], and Maria Zannis-Hadjopoulos[2]
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Nuclear matrix-attached DNA sequences or MARs are responsible for the structuring of genomes into chromatin domains; furthernore, MARs are believed to represent the sites of initiation of DNA replication and the transcriptional enhancers of the genes bound to them. We have cloned the small fraction of nuclear DNA (1.7% of total DNA) remaining associated with the nuclear matrix of human erythroleukemia K562 cells as well as bovine thymus and liver tissues. Sequencing of about 50 different MAR clones has revealed a novel type of non-AT-rich MAR sequences containing GA-rich blocks alternating with CT-rich blocks on the same strand of DNA and highly enriched in inverted repeats. Inverted repeats are characteristic of origins of replication. A large MAR fragment of 3.8 kb was identified as the 5' flanking region including some coding region of the human choline acetyltransferase (CHAT) gene coding for a key enzyme in cholinergic neurotransmission. DpnI assays revealed that this MAR sequence can support the autonomous, extrachromosomal replication of Blue Script plasmid in transient transfection experiments and thus harbors the putative 5' origin of replication of the CHAT gene. This MAR could be involved in regulating the expression and replication of the CHAT gene during development. Evidence is presented that MARs have binding sites for protein transcription/replication factors and thus constitute a novel type of regulatory elements for genes.

M 302 ACTIVATION OF MULTIPLE PROMOTERS ON A SINGLE CHROMOSOME BY THE HUMAN β-GLOBIN LOCUS CONTROL REGION, Emery H. Bresnick and Gary Felsenfeld, Laboratory of Molecular Biology, National Institutes of Health, NIDDK, Bethesda, MD 20892

The human β-globin locus control region (LCR) is necessary for high-level and position-independent expression of globin genes in erythroid cells. The LCR consists of four erythroid cell-specific DNaseI hypersensitive sites. A variety of mechanisms have been proposed for the *cis*-activation of individual members of the β-globin gene family by the LCR located 10-50 kilobases upstream. It is not known, however, whether a given LCR can activate all developmentally appropriate globin family members on its chromosome, or whether within a given chromosome, the LCR must be committed to activating only a single gene. We have devised an experiment to distinguish between these possibilities. This takes advantage of the fact that if two genes in a cluster are transcriptionally active and their promoters, therefore, are in a conformation hypersensitive to nucleases, restriction enzymes that cleave the promoters will excise the intervening chromatin fragment. The ApaI sites on human fetal Gγ and Aγ globin promoters are accessible to cleavage in nuclei from the human erythroleukemia cell line K562, which expresses these genes, but not in HeLa cells. We show that ApaI digestion leads to excision in high yield of the fragment spanning these promoters, showing that an LCR element is capable of sharing its activating function among members of a gene cluster on a single chromosome. We are currently examining the function of minimal LCR elements and are asking if they form specific folded structures stabilized by protein-protein interactions.

M 303 ROLE OF DNA TOPOISOMERASE II (TOPO II) IN THE STRUCTURAL AND FUNCTIONAL EVOLUTION OF MITOGEN-STIMULATED LYMPHOCYTE NUCLEI, David L. Brown[1], Nathalie Chaly[2], Eugene Daev[3], Bea Valentine[1], Judy E. Little[1], Xia Chen[2], and Roy Walker[4], Departments of Biology, University of Ottawa[1] and Carleton University[2], Ottawa, Canada, K1N 6N5, and Department of Genetics, St. Petersburg State University[3], Russia, and Division of Biological Sciences, National Research Council[4], Ottawa, Canada.

To examine the role of Topo II in the mitogenic activation of mouse lymphocytes, we applied the Topo II inhibitor VM26 throughout stimulation and monitored morphological and functional parameters of lymphocyte activation. Cell viability and the increase in cell size were little affected at doses between 0.05 and 0.5 μM. DNA synthesis, however, was already significantly inhibited at 0.05 μM, with lesser inhibition of RNA synthesis. In LM autoradiographs, a smaller proportion of cells entered S phase, and each S phase cell incorporated less ^{3}H-thymidine. Immunofluorescence showed that fibrillarin, a nucleolar antigen, was reduced, though the snRNP Sm antigen and the internal component labelled by antibody pI1 were little affected. At the EM level, nucleoli were remodelled and chromatin became aggregated. With a high drug dose (5 μM), cells showed the expected high levels of apoptosis and strong inhibition in all activation parameters assayed. The data support the hypothesis that the Topo IIß isoform is involved in the very early phases of lymphocyte activation, with function of the Topo IIα isoform, more sensitive to VM26, required for progression through S phase. (Supported by MRC)

M 304 ORGANIZATION AND REPLICATION OF ADENOVIRUS DNA DURING LYTIC INFECTION, N. Chaly, X. Chen and K. Hubbard, Department of Biology, Carleton University, Ottawa, CANADA K1S 5B6

Adenoviruses (Ads) are double-stranded DNA viruses that replicate in the nucleus, assembling complex replication factories involved in both viral DNA synthesis and transcription. Ad DNA is replicated by a strand displacement mechanism in which one parental strand is copied while the other is displaced, associating with the Ad 72kDa DNA-Binding Protein (DBP) as it is released. We have used combined fluorescence in situ hybridization with Ad DNA probes and immunofluorescence labelling with antibodies to DBP and snRNPs to examine the organization of Ad ss and ds DNA in the replication factories throughout infection, and have investigated the architecture of Ad DNA replication sites by immunodetection of metabolically incorporated BrdU. The data show that Ad DNA is replicated in many small foci within each replication factory, that the foci are embedded in the surface of clear fibrillar bodies, that there is little accumulation of Ad dsDNA until these bodies have reached full size, and that DBP and snRNPs occupy mutually exclusive regions of the replication factories, with DBP restricted to areas rich in ssDNA, and snRNPs concentrated in regions comprised primarily of dsDNA. These results, in conjunction with earlier data localizing the sites of transcription within the factories, are summarized in a model in which the surface of the clear fibrillar bodies is the focus of Ad nucleic acid synthesis. (Supported by NSERCC)

M 305 THE IL3/GM-CSF LOCUS IS REGULATED BY AN INDUCIBLE CYCLOSPORIN A SENSITIVE ENHANCER, Peter.N. Cockerill, M.Frances Shannon, Andrew G. Bert, Gregory R. Ryan, Fay Jenkins, Cameron O. Osborne and Mathew A. Vadas, Division of Human Immunology, Hanson Centre for Cancer Research, Institute of Medical and Veterinary Science, Frome Road, Adelaide 5000, Australia.

Interleukin-3 (IL3) and granulocyte-macrophage colony stimulating factor (GM-CSF) are pleiotropic haemopoietic growth factors whose genes are located just 10 kb apart on human chromosome 5. These genes are co-induced in T lymphocytes upon activation of the T cell receptor, in a cyclosporin A (CsA)-sensitive fashion. GM-CSF, but not IL3, can also be induced in many other cell types such as fibroblasts by pro-inflammatory stimuli. As the promoters alone could not account for the observed activity of these genes we mapped DNase I hypersensitive (DH) sites across the locus to identify additional regulatory elements. We located an inducible DH site, 3 kilobases upstream of the GM-CSF gene, which appeared just before the onset of IL3 and GM-CSF transcription, and which was suppressed by CsA. This DH site was induced in all cell types that could be induced to express GM-CSF. The DH site functioned in T cells as a strong CsA-sensitive enhancer of both the GM-CSF and IL3 promoters, responding to protein kinase C and Ca^{2+} mediated signals. Four sites in the enhancer associate with the CsA-sensitive T cell-specific transcription factor NFAT, but differ from previously described NFAT sites present in the IL2 promoter. Three of these NFAT sites encompass AP-1 sites, and NFAT complexes bound to these sites contain AP-1. These sites retain the capacity to associate with AP-1 in the absence of NFAT. The fourth NFAT site is unique in that it can bind with high affinity to just the CsA-sensitive component of NFAT. This novel NFAT element can also be used to re-create a DH site when integrated into the genome of T cells, suggesting that the CsA sensitive component of NFAT may function as a chromatin modifying factor that normally co-operates with other transcription factors. While the inducible enhancer appears to be required for the correct control of both IL3 and GM-CSF, we have also detected four constitutive T cell specific DH sites upstream of the IL3 gene. Although these sites have no inducible enhancer function, they may prime the locus for the T cell specific expression of IL3.

M 306 HIGHER ORDER CHROMATIN STRUCTURE AND APOPTOSIS, Gerald M. Cohen, Xiao-Ming Sun, Marion MacFarlane, David G. Brown, Roger T. Snowden and David Dinsdale. MRC Toxicology Unit, Hodgkin Building, University of Leicester, P.O. Box 138, Lancaster Road, Leicester, LE1 9HN, United Kingdom.

The formation of large molecular weight fragments of DNA, 30-50 and 200-250 kilobase pairs in length, corresponding to cleavage of chromosomal domains of supercoiled loops and rosettes, precede the appearance of internucleosomal cleavage of DNA in a population of thymocytes at an early committed stage of apoptosis. These large fragments are absent in normal thymocytes and their formation is dependent on protein synthesis and associated with the condensation of chromatin abutting the nuclear membrane, which is recognised as one of the earliest ultrastructural signs of apoptosis. Subsequent cleavage of these large fragments to oligonucleosomal fragments is independent of protein synthesis. We propose that the formation of these large fragments of DNA represents a key committed step in apoptosis and it is from these large fragments that the archetypal DNA ladders associated with apoptosis are derived.

M 307 IDENTIFICATION AND CHARACTERIZATION OF A S. POMBE cdc21 HOMOLOG IN XENOPUS LAEVIS

Martine Coué, Stephen E. Kearsey* and Marcel Méchali, Institut Jacques Monod, CNRS-Université Paris 7, France; *Department of Zoology, University of Oxford, England.

The cdc21 gene originally isolated from fission yeast belongs to a family of yeast genes believed to play a role in the initiation of DNA replication. This gene family is highly conserved with members found in both human and mouse cells. We have identified and cloned a homolog of the cdc21 gene from a Xenopus oocyte library. The predicted amino acid sequence of the Xenopus protein is 46% identical to the cdc21 gene product from S. pombe and has lesser homology with other members of this family. The 3 kb Xenopus cdc21 mRNA is detectable both in oocytes and during early development. We have produced antibodies against bacterial fusion proteins containing predicted peptide sequences from the Xenopus cdc21 protein and are using these to examine both the developmental regulation of protein expression and subcellular localization pattern. These antibodies will also be used as functional probes to examine the possible involvement of the Xenopus cdc21 protein in DNA replication.

M 308 STUDIES OF LINKER HISTONE VARIANTS INDUCED DNA LINKING NUMBER CHANGE IN RECONSTITUTED MINICHROMOSOMES

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Purified histones have been assembled into a circular DNA containing 18 tandem repeats of a 207 bp nucleosome positioning sequence (Simpson et al., Cell 42:799-808) to form reconstituted minichromosomes. Addition of a H1 molecule per nucleosome core increases the linking number change by 0.1, whereas binding of H5, a linker histone associated with terminally differentiated avian blood cells increases the linking number change by 0.15. The different ability of H1 and H5 in changing DNA topology suggest specific roles of linker histone variants in chromatin organization and possibly control of gene expression.

M 309 ENHANCER-BLOCKING BY THE *su(Hw)* PROTEIN OF *DROSOPHILA* IS POSITION-DEPENDENT BUT DISTANCE-INDEPENDENT, Dale Dorsett, Bin Shen, Patrick Morcillo, Jaeseob Kim and Christina Rosen, Molecular Biology Program, Sloan-Kettering Institute for Cancer Research and Cornell University Medical College Graduate School of Medical Sciences, New York, NY 10021

Many mutations in *Drosophila* are insertions of the gypsy retrotransposon. The phenotypes of virtually all gypsy insertion alleles are suppressed by mutations in the *suppressor of Hairy-wing* [*su(Hw)*] host gene. The *su(Hw)* gene encodes a zinc finger protein (SUHW) that binds to a series of direct repeats in gypsy. When gypsy inserts into a gene control region, SUHW prevents enhancers that are promoter distal to gypsy from activating gene transcription, while enhancers that are promoter proximal to gypsy still function. The effects of SUHW occur over very long distances, and SUHW blocks a wing margin-specific enhancer in the *cut* gene from virtually any position in the 90 kilobase interval between the enhancer and promoter. Temperature-shift experiments indicate that the effects of SUHW are immediate and reversible, and that SUHW is only required when an enhancer is active to block the enhancer. Multiple domains of the SUHW protein are involved in blocking, and the sequences of the most critical domains suggest they are involved in protein-protein interactions. Although there are as yet no examples of a transcription control element in *Drosophila* that SUHW fails to block, SUHW does not block activation in yeast by a yeast activator that functions in *Drosophila*. Because yeast lack long distance activation, our working hypothesis is that SUHW does not interact or interfere with enhancer-binding proteins or the transcription activation reaction, but rather with a specialized apparatus specific to higher eukaryotes that brings distant enhancers and promoters into physical proximity of each other. Preliminary results of attempts to identify components of this apparatus will be presented.

M 310 CHROMATIN STRUCTURE AT A REPLICATION ORIGIN IN TETRAHYMENA *THERMOPHILA*,
Renata C. Gallagher, Geoffrey Kapler, Elizabeth H. Blackburn, Department of Biochemistry and Biophysics and Department of Microbiology and Immunology, University of California at San Francisco, 94143
The replication origin of the Tetrahymena rDNA mini-chromosome is one of the best localized eukaryotic non-viral origins. The origin lies within the centrally located 2 kb 5' non-transcribed spacer (5' NTS) of the 21 kb chromosome (Cech and Brehm, 1981). Mutants in the maintenance of this chromosome have base changes within the 5' NTS. DNase I footprinting of nuclei has enabled us to characterize protein-DNA contacts in this 2 kb origin region, in wild-type and maintenance mutant chromosomes. First, analysis of DNase I digested chromatin by primer extension has revealed that nucleosomes within the 5' NTS are precisely positioned, i.e. to the nucleotide. Second, results of footprints of mutant chromosomes suggest that there is an interaction between two non-nucleosomal regions of the 5' NTS that is important for wild-type chromosome maintenance. These are the Pol I promoter region *located at the extreme* 3' end of the 5' NTS, and a DNase I hypersensitive region 600 bp upstream. Two highly positioned nucleosomes between these two regions may facilitate a long-range interaction between them. Our immediate goal is to fully characterize protein-DNA contacts within wild-type and maintenance mutant chromosomes in this origin- and promoter-containing 2 kb region. We will use the results of this analysis to guide and to complement our determination of the minimal replication origin, auxilliary sequences, and trans-acting factors through other methods.

M 311 TOPOISOMERASE II CLEAVAGE SITES WITHIN THE MURINE IMMUNOGLOBULIN KAPPA LOCUS
Julia B. George and William T. Garrard; Department of Biochemistry; University of Texas-Southwestern Medical Center at Dallas; Dallas, TX 75235-9038

Topoisomerase II is modeled to be located *in vivo* at the bases of DNA loops that organize chromatin domains. Treatment of tissue culture cell lines with the topoisomerase inhibitor VM-26 results in a unique, dose dependent, bimodal distribution of DNA fragments ranging in size from 50 to 600 kb. Mapping of these sites within a developmentally regulated locus should provide insite into how, or whether, structural reorganization occurs concordantly with activation.

The mouse immunogloublin kappa locus (IgK) locus is under tight developmental regulation during the ontogeny of B-lymphocytes as evidenced by precise patterns of tissue specific transcription, replication, hypomethylation, rearrangement and somatic mutation. Work from several laboratories has estimated the size of the locus to range from 600 to 2000 kilobases. We have isolated 18 YAC clones ranging in size from 300 to 1300 kilobases which span IgK. Based on these clones, the minimum predicted size of the locus is 2000 kilobases.

We have developed a novel technique for mapping topoisomerase II cleavage sites within cloned DNA. The source of probe is genomic DNA in which cleavage at topoisomerase II associated DNA has been induced with VM-26. Cleavage sites are filled in with labelled nucleotides and the position(s) of specific hybridization are mapped on the YAC clones. Cleavage can be induced in log phase or cell cycle arrested cells. We are currently mapping the positions of topoisomerase cleavage in various B-cells and non-B-cells. These results should indicate whether structural reorganization of this exceptionally large locus occurs during the course of its activation.

M 312 NUCLEAR STRUCTURE DETERMINES THE SITES OF INITIATION OF DNA REPLICATION IN ANIMAL CELLS.
David M. Gilbert, Hiroshi Miyazawa and Melvin L. DePamphilis, Roche Institute of Molecular Biology, Roche Research Center, Nutley, N.J. 07110
In metazoan cells, DNA replication initiates predominantly at specific heritable origins of bidirectional replication (OBR). However, unlike with single-celled organisms and viruses, attempts to develop a functional assay for the genetic component of metazoan origins have failed. In *Xenopus* eggs and egg extracts, initiation of replication depends upon the assembly of DNA into a nuclear structure but does not require any specific DNA sequences, raising the question of whether origins of replication in higher eukaryotes can be genetically defined. To address this question, we have searched for conditions in which *Xenopus* eggs and egg extracts will distinguish the well defined OBR from the Chinese Hamster dihydrofolate reductase (DHFR) locus from other DNA sequences that do not contain an OBR. We have found that, when presented as naked DNA, *Xenopus* eggs and egg extracts will not preferentially initiate replication at the DHFR OBR. However, when isolated nuclei from CHO cells synchronized in G1 are incubated in *Xenopus* egg extracts, DNA replication initiates predominantly at the OBR. Thus, some structural component between the spectrum of naked DNA and whole cell nuclei dictates the specificity for initiation of DNA replication. Furthermore, the ability of *Xenopus* embryos to efficiently replicate any DNA sequence ceases abruptly at the blastula stage, coincident with dramatic changes in nuclear structure. Based on these results, we propose that nuclear structure represses non-specific initiation of replication, limiting initiation to specific "legitimate" sites, and that rapid cleavage stage *Xenopus* embryos lack this repressive nuclear structure. Thus, the function of specific origin sequences may be to prevent the general repression of replication by altering chromatin structure to create "legitimate" initiation sites. This function would be perceived only in the context of a repressive nuclear structure, explaining the difficulties that have been experienced in establishing functional genetic and biochemical assays.

M 313 A Weak Excision Mutation Blocks Developmentally-Controlled Amplification of the *Tetrahymena thermophila* rDNA Minichromosome.
Geoffrey M. Kapler and Elizabeth H. Blackburn, Department of Microbiology and Immunology, University of California, San Francisco, CA. 94143.

The abundant minichromosome encoding the ribosomal RNA genes (rDNA) of *Tetrahymena thermophila* is generated by developmentally-controlled excision, rearrangement and amplification in the newly formed somatic macronucleus (rDNA maturation). Using a classical genetic approach, we have identified mutants defective for the processing and replication of the rDNA minichromosome. The cis-acting *rmm11/6* mutation illustrates the consequences of a mutation in an early step in rDNA maturation on subsequent rDNA amplification. This mutation resides in a conserved element required for rDNA excision, the chromosome breakage sequence (Cbs) flanking the 3' end of the rDNA. The *rmm11/6* Cbs mutation only weakly affects excision of the rDNA from its germline chromosome. Surprisingly, this Cbs mutation severely blocks subsequent amplification of rDNA minichromosomes that have successfully excised from the germline, despite the separation of the mutated sequences from the rDNA upon excision. We propose that the *rmm11/6* mutation affects rDNA amplification by delaying excision of the rDNA, and that the developmental progression of the macronucleus past the period for amplification is responsible for the severe effect of this weak excision mutation on rDNA amplification. Genetic and biochemical experiments provide evidence that rDNA amplification is restricted to a defined developmental window, and suggest that the periods for rDNA excision and amplification overlap extensively. A much more defective maturation mutant, *rmm10* , is not mutated in any of the known maturation control elements, indicating that additional cis-acting determinants are essential. Based of our knowledge of rDNA maturation and replication control, we suggest that the *rmm10* mutation severely blocks excision of the rDNA from its germline chromosome.

M 314 X-INACTIVATION AND H4 ACETYLATION IN FEMALE MOUSE EMBRYONIC STEM CELLS.

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In female eukaryotic cells one X chromosome is inactivated to equate the level of X-linked gene transcription between male (XY) and female (XX) cells. The event occurs early in embryogenesis and the inactivated chromosome remains transcriptionally silent throughout subsequent cell generations. The inactive X (Xi) is late replicating, appears relatively condensed throughout interphase and has a high methylcytosine content compared to its active counterpart (Xa). In these respects Xi resembles constitutive heterochromatin. Recent studies using indirect immunofluorescence with antibodies to acetylated histone H4 have revealed that Xi is uniquely distinguished by low levels of acetylated H4 (Jeppesen & Turner Cell 74 281-289 1993). In contrast Xa, and autosomes are labelled strongly in regions coresponding to conventional R-bands. H4 in heterochromatic regions, especially those adjacent to the centromeres, is underacetylated (Jeppesen et al Chromosoma 101 322-332 1992). Female embryonic stem (ES) cells have two active X chromosomes prior to differentiation, both of which label with antibodies against acetylated H4. Induction of differentiation leads to the appearance of a pale chromosome at day 4. This event is preceded by expression of *Xist*, a gene transcibed exclusively from Xi (Kay et al Cell 72 171 1993). We are using female ES cells to investigate the order in which *Xist* expression, H4 underacetylation and cytosine methylation occur and how they contribute to transcriptional silence.

M 315 MUTATIONS AFFECTING CHROMOSOME STRUCTURE IN THE DROSOPHILA OVARY.

Linda N. Keyes and Allan C. Spradling. Department of Embryology, Carnegie Institution of Washington, 115 W. University Pkwy, Baltimore, MD 21210.

In both the nurse cells and the follicle cells of the Drosophila ovary, the switch from cellular proliferation to differentiation is accompanied by the transition to an endomitotic cell cycle. By the end of endopolyploid development the copy number of the euchromatic regions of the chromosomes in these nuclei can reach as high as 2048C. However, not all genomic sequences are present at equal levels, and the heterochromatic regions are significantly underrepresented. We are using a molecular and genetic approach to study the structure and content of chromosomes in the polyploid nuclei of the ovary.

A screen using a model minichromosome was designed to preferentially detect genes that affect the heterochromatin content of ovarian chromosomes during endopolyploid development. We have identified several mutations that abolish female fertility and cause cytologically observable changes in the nuclear morphology of the nurse cells. One of these mutations, in the gene *fs(2)cup*, causes a developmental arrest in chromosome decondensation. This gene has been cloned and encodes a novel protein; molecular and genetic data will be presented.

M 316 THE EFFECT OF SERINE SUBSTITUTIONS AT RESIDUES 37, 40, AND 42 OF H2B ON NUCLEOSOME ASSEMBLY, Ann M. Kleinschmidt and Jessica A. Gott, Department of Biology, Allegheny College, Meadville, PA 16335.

The nucleosome is one of the most highly conserved macromolecular structures in eukaryotic organisms. Within this structure are the histones H3 and H4 , showing the greatest sequence conservation, and H2A and H2B which are conserved at approximately 80 percent of the residues in the globular histone-histone interaction domains. While histone sequence conservation has long been recognized, few studies have addressed the importance of this conservation for function, especially as it pertains to H2A and H2B. During their cellular lifetime, histone proteins must participate in several dynamic processes where this sequence conservation might be important, including nuclear import, assembly into nucleosomal structures, and facilitation of passage of the transcription and replication machinery. To begin to address the function of amino acid conservation in H2B we have produced histone mutants that contain serine substitutions at residues 37, 40, and 42, sites normally occupied by tyrosine. Previous experiments have shown that iodination of these residues causes instability in the H2A-H2B dimer. We have also chosen to alter these residues because they represent a portion of the H2B that is in contact with H2A and tyrosines at these relative position in H2B have been highly conserved. The effect of substitutions at residues 37, 40, and 42 on nucleosome assembly and stability will be presented.

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M 317 CHROMATIN NUCLEAR MATRIX/SCAFFOLD ATTACHMENT REGIONS (MAR'S AND SAR'S) AND EXPRESSION OF GENOMIC MESSAGE, Ludmila B. Kokileva, Institute for Molecular Biology, Bulg. Acad. of Sci., Sofia, Bulgaria

Deblocking and derepression of selective regions of cell genome is indispensable for expression of genome encoded information. Pulsed field gel (PFG) electrophoretic study of endogenous chromatin degradation in isolated rat liver and thymocyte nuclei, and during cell apoptogenesis induced by VM26, dexamethasone or radiation revealed that common multi-step chromatin degradation represents the initial genome disassembly into two basic units of chromatin structure - 0.3 Mbp chromatin loop-domains and loop size 0.05 Mbp DNA units. This is followed by irreversible disintegration of 0.05 Mbp DNA units into oligo- and mononucleosomes. Effect of VM26, which uncouples topo II endonucleolytic activity from its ligase activity, and chromomycin A3, which binds to the GC-rich DNA sequences, strongly suggest the involvement of topo II. This indicates that genome disassembly is determined by highest order chromatin structure, most likely created, maintained and regulated by topo II through chromatin fixation to the nuclear matrix by their MAR's and SAR's. MAR's where are located topo II, promoter DNA, origins of replication, potential initial cleavage sites and which are associated with telomers are presumably the main nuclear targets in the G1 phase of cell cycle of extra- and intracellular signals. It seems the deblocking of genetic messages occurs at MAR's by 0.3 Mbp DNA bits of information. High molecular weight of DNase resistant DNA (20-25 Kbp), as judged by PFG electrophoresis, could assure the local dynamic tightening or relaxation (opening) of chromatin at MAR's. In addition, limited selective exoproteolysis of nuclei revealed the DNA fragments multiples of 0.05 Mbp DNA. DNA loops of different sizes might represent the multiples of basic 0.05 Mbp loop size DNA unit. The role of topo II might consist in deblocking and derepression of chromatin loop domains probably by modulation the extent of their attachment to MAR's, or even detachment within the limits of loop domains of loop size DNA units from their SAR's in order to generate the DNA loops of different sizes (0.05-0.25 Mbp). The possible underlying mechanisms are discussed in the light of current knowledge of interphase cell genome structure and function.

M 318 ASSOCIATION OF TOPOISOMERASE II AND CASEIN KINASE II IN A MOLECULAR COMPLEX WHICH IS CATALYTICALLY ACTIVE. Annette Kragh Larsen[&], Odile Filhol[$], Claude Cochet[$], Edmond M. Chambaz[$], and Krzysztof Bojanowski[&], [&]Institut National de la Santé et de la Recherche Médicale Unité 140, Centre National de la Recherche Scientifique Unité de Recherches Associée 147, Institut Gustave Roussy, F-94805 Villejuif Cedex, France and [$]Institut National de la Santé et de la Recherche Médicale Unité 244, BRCE, Centre d'Etudes Nucléaires 85X, F-38041 Grenoble Cedex, France

Immunoprecipitation of DNA topoisomerase II from yeast results in a preparation which contains casein kinase II; this suggests that the two proteins may associate in the intact cell. Purified recombinant topoisomerase II and casein kinase II associate to form a complex *in vitro* which is stable after topoisomerase II becomes phosphorylated by the kinase. Studies with isolated recombinant casein kinase II subunits disclosed that although the α (catalytic) subunit alone can efficiently phosphorylate topoisomerase II, the formation of a stable topoisomerase II - casein kinase II association requires the presence of the ß subunit of the kinase. Both proteins engaged in this complex retain their catalytic activities. Naturally occuring polyamines and polyanionic compounds appear to be crucial factors governing the interaction between the two proteins. Although the biological significance of a stable catalytically active topoisomerase II - casein kinase II molecular complex remains to be defined, these observations suggest the possibility of a novel mechanism regulating topoisomerase II and casein kinase II activities.

M 319 FUNCTIONAL ORGANIZATION OF THE MAMMALIAN NUCLEUS: A LINK BETWEEN CELL CYCLE REGULATION AND DNA REPLICATION

H. Leonhardt, M. C. Cardoso and B. Nadal-Ginard, Howard Hughes Medical Institute, Department of Cardiology, Children's Hospital and Department of Cellular and Molecular Physiology, Harvard Medical School, Boston, MA 02115.

DNA replication in mammalian cells occurs in discrete nuclear foci. We exploited this functional organization of mammalian nuclei to analyze the possible interaction of cell cycle proteins with these foci in vivo. Cyclin A and cdk2, but not cyclin B1 and cdc2, were specifically localized at nuclear replication foci just like replication protein PCNA or DNA Methyltransferase. This colocalization was observed throughout S-phase suggesting a direct role of cyclin A and cdk2 in the control of DNA replication (Cell 74, 979-992). A potential target of cyclin A and cdk2 is the 34 kDa subunit of replication protein A (RPA34). We could not detected RPA34 at these replication sites, which could be due to specific masking or modification of the epitope at these sites or may reflect a transient interaction. RPA34 was, however, found to associate with condensed chromosomes at M-phase, which might indicate participation of RPA34 in the licensing of DNA for replication.

The specific localization of cyclin A and cdk2 at nuclear replication foci provides a direct link between cell cycle regulation and DNA replication. We are currently studying the nature of the association of cyclin A and cdk2 with these replication foci. In case of the DNA Methyltransferase we could identify a distinct targeting sequence, which mediates this association (Cell 71, 865-873).

M 320 THE DNA POLYMERASE α - PRIMASE COMPLEX AND CELL CYCLE IN *Saccharomyces cerevisiae*,

Giovanna Lucchini[1,2], Marco Foiani[2], Marco Muzi Falconi[2], Anna Piseri[2], Marina Ferrari[2], Federica Marini[2], Giordano Liberi[2], and Paolo Plevani[2], [1]Istituto di Genetica, Università di Sassari, 07100 Sassari, and [2]Dipartimento di Genetica e Biologia dei Microrganismi, Università di Milano, 20133 Milano, Italy.

Control of eukaryotic DNA replication might involve modulation of the activity or proper assembly of key protein factors. Both initiation of DNA replication at an origin and lagging strand synthesis in eukaryotes require the four subunit DNA polymerase α-primase complex. In *Saccharomyces cerevisiae* , the pol α polypeptide is stable and is present at every stage of the cell cycle, even if the corresponding gene, *POL1*, is transiently transcribed at the G1/S phase boundary. Moreover, the level of pol α is not rate limiting, and *de novo* synthesis of pol α is not required for initiation of DNA replication within the same cell cycle. Therefore the protein can be inherited and properly used by daughter cells,and modulation of its activity, if necessary, has to take place at the post-translational level. Furthermore, the B subunit of the complex, that is tightly bound to pol α, and whose role is not yet defined, is also a stable protein and executes its essential function at an early stage of chromosomal DNA replication. Two different forms of this protein, 86 kDa and 91 kDa respectively, can be detected in yeast crude extracts, and they are differentially represented during the cell cycle. Our data suggest that cell cycle dependent post-translational modifications of the B subunit of the pol α-primase complex play a regulatory role in initiation of DNA replication.

M 321 THE ERYTHROID PROTEIN cGATA-1 FUNCTIONS WITH A STAGE-SPECIFIC FACTOR TO ACTIVATE TRANSCRIPTION OF CHROMATIN-ASSEMBLED β-GLOBIN GENES, Navid Madani, Michelle Craig Barton and Beverly M. Emerson, Regulatory Biology Laboratory, The Salk Institute, 10010 N. Torrey Pines Road, La Jolla, CA 92037

The chick ß-globin gene is developmentally regulated within erythroid cells by the interaction of multiple proteins with the promoter and 3' enhancer. These interactions are correlated with changes in chromatin structure that are characteristic of the actively expressed gene. Using in vitro chromatin assembly and transcription with staged erythroid extracts, we have determined the critical proteins required to activate expression of nucleosome reconstituted ß-globin genes. These genes contain a specialized TATA box at -30 ('GATA') through which the erythroid-restricted protein, cGATA-1, and TFIID both function to regulate different steps in ß-globin expression. We find that TBP (TATA binding protein) alone can activate transcription of ß-globin chromatin templates from promoters mutated to a canonical TATA box but is ineffective on those containing the normal -30 GATA site. The occupancy of this site by cGATA-1 also fails to generate efficient expression of ß-globin chromatin unless combined with a stage-specific protein, NF-E4, which binds to an adjacent site. However, NF-E4 does not function with TBP to derepress nucleosome assembled ß-globin genes. We propose that the developmental regulation of ß-globin expression is achieved, in part, by the requirement of an erythroid protein and a stage-specific factor, rather than TBP, to activate chromatin through a specialized TATA box.

M 322 A MULTIPROTEIN COMPLEX MEDIATES MAMMALIAN DNA REPLICATION.

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Our research interest is in the role of multiprotein complexes in mammalian cell DNA replication. We have successfully isolated and purified a multiprotein DNA replication complex from both human and murine cells. Several enzymatic and non-enzymatic proteins that fractionate with the mammalian cell DNA replication complex have been identified. In addition, the biochemical characterization of the mammalian multiprotein replication complex has revealed that the complex supports efficient DNA synthesis *in vitro* using a bi-directional replication mechanism. The human cell complex supports simian virus 40 replication *in vitro*, while the murine cell complex supports polyomavirus DNA synthesis *in vitro*. The complex has a particular and consistent size in velocity sedimentation analyses and on non-denaturing polyacrylamide gel electrophoretic analysis. Also, the complex maintains its physical integrity, and its ability to support *in vitro* DNA replication, following its treatment with a variety of agents that would disrupt non-specific association of macromolecules, suggesting that the complex is not a fortuitous aggregation of cellular components. In addition, the mammalian cell multiprotein DNA replication complex has been shown to initiate immediate DNA synthesis, and that the lag time for the initial onset of replication is reduced as the complex is purified from a cell homogenate. A model has been proposed to represent the mammalian Multiprotein DNA Replication Complex (MRC) based on the fractionation and chromatographic profiles of the individual proteins found to co-purify with the complex. The eventual full characterization of the mammalian cell DNA replication complex will provide insights into its role in DNA synthesis, and provide the information required to further our understanding of how the regulation of the activity of the complex affects overall cell proliferation.

This work was supported by awards from the NIH/NCI and the National Leukemia Association to LHM, as well as an award from the Leukemia Research Foundation to RJH.

M 323 THE UBIQUITINATION OF HISTONES IN *Dictyostelium discoideum* AX3 CHROMATIN MAY BE LINKED TO REPLICATION, TRANSCRIPTION AND STRESS, Dino C. Miano, Edwin G.E. Jahngen, Chemistry Department, University of Massachusetts at Lowell, Lowell, MA 01850

A question exists as to whether the levels of ubiquitination of histones in the slime mold *Dictyostelium discoideum* remain consistent during the inhibition of replication and heat shock stress, and decrease during the inhibition of transcription. We initiated a study to determine the level of ubiquitinated nuclear proteins from cells incubated with inhibitors of replication (aphidicolin and sodium butyrate), transcription (Actinomycin D and 5,6-dichloro-1-β-D-ribofuranosylbenz-imidazole) or from heat shock at 30°C for 8 hours. Also, cells incubated with hadacidin, an aspartic acid analog, which synchronizes by arresting the cells at the end of the G1 phase of the cell cycle were examined for ubiquitinated nuclear proteins. From these results we could ascertain the levels of ubiquitinated histones and determine if they are more predominant during the G phases of the cell cycle, where transcriptional activity is observed. These findings propose a direct link between ubiquitinated histones and transcription. The ubiquitination of nucleosome histones may cause a slight change or perhaps a repulsion within the structure to facilitate the unraveling of DNA for transcription.

M 324 IN VIVO CHROMATIN ANALYSIS: ZOOMING ON MECHANISMS FOR NUCLEOSOME POSITIONING

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Rotational and translational signals are known to be involved in nucleosome positioning although their relative contribution is still an open problem . Other factors such as boundary effects, ancillary proteins, cooperativity etc. are thought to play an additional role in nucleosome positioning. We tried to evaluate the principles that regulate chromatin organization in vivo in two different genetic systems of S.cerevisiae: a) 5S RNA gene (multicopy chromosomal); b) the ARSI-TRPI region (chromosomal and multicopy episomal) .

Therefore, we developed two new techniques that allow: a) the nuclear structure analysis directly inside viable spheroplasts; b) the unambiguous attribution of nucleosomal borders. The first one exploits the permeabilization property of the antibiotic nystatin, that creates pores in the cell membrane, promoting the entry of active enzymes, such as micrococcal nuclease and restriction enzymes . The second is a no-background assay, based on the purification and mapping of monomeric nucleosomal DNA. The results show that in the case of the 5S RNA gene rotational signals appear to be the major if not the only positioning element in vivo and in vitro . We were able indeed to change nucleosome positions in vitro and in vivo through the modification of the rotational information. In the case of the ARSI-TRPI region, at the contrary, the chromatin structure observed in vivo is partially due to the presence of bound ABFI protein that appears to behave as a physical boundary to the nucleosome deposition, thus limiting the potential of positional heterogeneity inherent to the DNA sequence and as observed in vitro.

M 325 INDISCRIMINATE SYNAPSIS IN ACHIASMATE JAPANESE BUNCHING ONION (*Allium fistulosum* L.), A.Okumus and G. Jenkins, Institute of Biological Sciences, U.W.A., Penglais, Aberystwyth, Dyfed SY23 3DD, U.K.

Following treatment with aqueous colchicine a plant of the Japanese bunching onion (*Allium fistulosum*) failed to form any chiasmata at diakinesis and metaphase I of meiosis. Ultra structural analysis of synaptonemal complexes of pollen mother cell nuclei using a whole-mount surface-spreading technique showed that the achiasmate condition is not only the result of partial asynapsis, but also the indiscriminate way in which the chromosomes form synaptonemal complexes (SCs) during meiotic prophase. Synapsis begins at any region on the chromosomes which are sufficiently close and advances along the chromosomes, with frequently switches of pairing partners forming multiple associations of heterologous chromosomes. However, chiasmata are not supported by these, with the result that 16 univalents enter metaphase I and disjoin irregularly at anaphase I. This mutant plant is completely male sterile. Consequently, the recognition of homology is an independent process and is not necessary for synaptonemal complex formation. It is hoped that this mutant will be a valuable tool for probing the molecular basis of homology.

M 326 THE 30 NM CHROMATIN FIBER AS A FLEXIBLE POLYMER. J.Y. Ostashevsky and C.S. Lange. State University of New York, Health Science Center at Brooklyn, Brooklyn, NY 11203, USA.

Our analysis of the data of van den Engh, Sachs, and Trask (*Science* **257**, 1410 (1992)) for the dependence of the mean square distance between hybridization sites ($<L_n^2>$, [μm^2]) on the genomic distance (n, [base pairs, bp]) between these sites for chromosome number 4 in G1 human fibroblast nuclei shows that $<L_n^2>$ is proportional to $n^{2\nu}$ with $\nu = 3/5$ for $n < 1$ Mbp. The ν value of 3/5 is characteristic of flexible polymer chains with excluded volume effects in dilute solutions. Since the DNA concentration in nuclei is very high (~ 5 mg/ml), and theory (*e.g.*, de Gennes, *Scaling Concepts in Polymer Physics*, Ch. 2, Cornell University Press, Ithaca, NY 1979) predicts $\nu = 1/2$ for overlapping polymers, the finding of $\nu = 3/5$ means that the chromatin fibers do not overlap in interphase nuclei. The dependence of $<L_n^2>$ on n for $n < 4$ Mbp is consistent with the model of large (~ 6 Mbp, 3 μm diameter) loops of interphase chromatin attached to nuclear membrane sites. Using the constant (*e.g.*, Widom, *Ann. Rev. Biophys. Biophys. Chem.* **18**, 365 (1989)) and variable (Williams & Langmore, *Biophys. J.* **59**, 606 (1991)) diameter fiber models, the Kuhn statistical segment of the 30 nm chromatin fiber was estimated to have a length of 196-272 nm with a corresponding DNA content of 21-37 kbp. Based on the model of Shimada and Yamakawa (*Macromolecules* **17**, 689 (1984); *Biopolymers* **27**, 657 (1988)) for circular wormlike chains, we estimated the most favorable size of the small loops of the 30 nm fiber to be 36-62 kbp with a diameter of 94-131 nm. Both the size and diameter estimates are consistent with experimental measurements from the literature: 60 kbp for average loop size (van Holde, *Chromatin*, Ch. 7, Springer-Verlag, New York, 1989) and 125 nm for the diameter (Belmont *et al.*, *Chromosome* **98**, 129 (1989)).

M 327 THE MOLECULAR INTERACTIONS OF PSORALENS WITH DNA; LOCALIZATION AND GENE INDUCTION, M. Angela Parsons and Lee A. Hadwiger, Department of Genetics and Cell Biology, Washington State University, Pullman, WA 99164

Many DNA intercalating compounds, including mitomycin C, actinomycin D and a family of psoralen compounds act on pea tissue to induce production of a group of proteins similar to those produced in the resistance response to the fungal pathogen *Fusarium solani* f. sp. *phaseoli*. Using differential screening methods with fungal induced and noninduced tissue, several cDNA's from pea were previously cloned and termed disease resistance response (DRR) genes. Due to the specific interaction of psoralen with DNA, this compound provides a tool to examine the induction of DRR genes at the molecular level. Preliminary experiments demonstrated that under inducing conditions, psoralen intercalates genomic DNA at an average of one crosslink per five kilobases.

In order to examine induction of DRR genes, two different psoralen compounds were used. The first, 4'-Aminomethyl-4,5',8-trimethylpsoralen (AMT), is able to covalently bind the DNA on both strands after irradiation with long wave UV light (366 nm) and produce a crosslink. The second, 6-Aminomethyl-4,5'-dimethylisopsoralen (AMDMIP), is only able to bind one strand of DNA, thereby producing a monoadduct. Pea pod tissue was treated with either AMT or AMDMIP at inducing concentrations (Hadwiger, *Plant Phys.*, 49:779-782 (1972)), irradiated and frozen at various time points for subsequent RNA extraction. It was determined by Northern blot analysis that the crosslinking compound (AMT) resulted in greater induction of the plant defense genes examined.

In the second set of experiments, different promoters were examined, both *in vitro* and *in vivo*, to determine the extent of crosslinking. A Southern blot method originally developed by Vos and Hanawalt (*Cell*, 50:789-799 (1987)) to examine repair in mammalian cells was utilized. Pea genomic DNA, pea pod nuclei and intact pod tissue were treated with various concentrations of psoralen and irradiated for increasing amounts of time. Genomic DNA was probed with promoters of several plant defense genes in order to evaluate the prevalence of crosslinks within differentially induced plant promoters.

Results from both sets of experiments will be presented and the interaction of psoralen with DNA will be discussed, specifically in relation to the genes involved in the disease resistance responses of plants.

M 328 DEPOSITION-RELATED HISTONE H4 ACETYLATION SITES ARE HIGHLY CONSERVED BETWEEN WIDELY DIVERGENT SPECIES Richard E. Sobel, Richard G. Cook, Anthony T. Annunziato, and C. David Allis, Department of Biology, Syracuse University, Syracuse, NY 13244 and Deparment of Immunology, Baylor College of Medicine, Houston, TX 77030 and Department of Biology, Boston College, Chestnut Hill, MA 02167

During periods corresponding to active DNA replication and chromatin assembly, newly synthesized histone H4 is deposited in a diacetylated form. This modification has been reported in a wide range of organsims ranging from protozoa to man and thus, is one of the most highly conserved features of histone acetylation. In the ciliated protozoan, *Tetrahymena*, a specific pair of lysine residues (lysines 4 and 11; Chicoine et al., 1986) has been found to undergo this modification. To investigate H4 acetylation site utilization in other species, histone acetyltransferase B (HATB) prepared from young*Drosophila* embryos was used to acetylate H4 from several species and a specific set of acetylated and unacetylated *Tetrahymena* H4 amino-terminal peptides *in vitro*. Products were microsequenced and sites of 3H-acetate incorporation were determined. In all cases, sites utilized for acetylation were remarkably non-random and corresponded to those lysines previously characterized as deposition-related in *Tetrahymena*, although a preference for acetylation at 11/12 was exhibited by the *Drosophila* HATB (DHATB) activity *in vitro*. These results strongly support the assertion that cytoplasmic HATB functions to acetylate H4 for deposition and chromatin assembly *in vivo*. Corroboratively, 3H-lysine pulse-labelled H4 isolated from *Drosophila* and HeLa cells was deblocked, microsequenced and found to exhibit the same pattern of acetylation site utilization as exhibited by 3H-lysine pulse-labelled*Tetrahymena* micro- or macronuclear H4. That the *in vivo* sites of H4 diacetyation (lysines 4/5 and 11/12) are non-random and have been completely conserved between protozoans, dipterans and humans, strongly suggests that acetylation of this specific pair of lysines in H4 plays an important functional role(s) during chromatin assembly that has yet to be understood.

M 329 THE β-PHASEOLIN GENE: A HIGHLY REGULATED PLANT GENE FLANKED 5' AND 3' BY SCAFFOLD ATTACHMENT REGIONS. Apolonia H.M. van der Geest, Gerald E. Hall, Jr.*, Steven Spiker* and Timothy C. Hall, Institute of Developmental and Molecular Biology and Department of Biology, Texas A&M University, College Station, TX 77843-3155. and *Department of Genetics, North Carolina State University. Raleigh, NC 27695.

It is now apparent that the way in which eukaryotic chromatin is organized into topologically constrained loop domains, anchored to the nuclear matrix or scaffold, affects gene expression. The DNA sequences that tightly associate with the nuclear matrix or scaffold after extraction and nuclease digestion have been called matrix or scaffold attachment regions (MARs or SARs). SARs are often found flanking highly expressed genes and may function to define the borders of active chromatin domains.

The β-phaseolin gene encodes a protein that accumulates to high levels in *Phaseolus vulgaris* seeds, and retains its spatial and temporal regulation of transcription in transgenic tobacco plants. We have identified 5' (1.1 kb) and 3' (1.2 kb) fragments flanking the coding region of this gene as SARs on the basis of their ability to bind isolated nuclear scaffolds *in vitro*. These very A/T-rich sequences contain *Drosophila* topoisomerase II, MAR, A-box and T-box consensus sequences that are characteristic of SARs. Constructs containing the phaseolin promoter fused to a reporter gene including the 5' SAR element exhibited higher expression levels and lower plant-to-plant variability in stably transformed tobacco plants than did similar constructs lacking the 5' SAR. The existence of SAR regions closely flanking each end of the β-phaseolin gene is in accordance with previous observations of increased DNase I sensitivity associated with the onset of transcription in bean and with the consistently high and position-independent activity observed in transgenic plants. These findings suggest that the native phaseolin gene is located on a small chromatin loop that functions as an active and independent domain.

M 330 DNA EXCISION REPAIR IN INTACT CHROMATIN

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We investigated UV-induced repair synthesis in intact chromatin isolated from normal human fibroblasts and several UV-sensitive human fibroblasts. The kinetics and efficiency of UV-induced excision repair have been shown to be different for various parts of the genome. Active genes are repaired faster and more efficiently than inactive genes. Within active genes the transcribed strand is repaired faster than the nontranscribed strand. RNA polymerase II activity turns out to be essential for this preferential repair of the transcribed strand. Moreover chromatin structure appears to play a role in the process of preferential repair. An enrichment of repair patches at the matrix attachment regions of chromatin loops was shown in normal and XP-C cells (both able to perform transcription coupled repair). In addition it was shown that cyclobutane pyrimidine dimers are removed preferentially from hyperacetylated chromatin.

In order to investigate in detail the relationship between chromatin structure, transcription and repair, and to isolate factors involved in these processes we adapted an in vitro system developped by D.A Jackson and P.R. Cook*). Cells are encapsulated in agarose microbeads. Subsequently 80% of the cellular proteins are extracted under physiological conditions. This results in intact chromatin which is able to perform efficient replication and transcription without added extracts or purified proteins. We show a 3-fold stimulation of ^{32}P-dAMP incorporation in chromatin by UV in 3 cell lines with normal excision repair capacity. This stimulation was absent in XP-A, XP-C and reduced in CS cells.

XP-A/XP-C= Xeroderma Pigmentosum group A/C; CS= Cockayne's syndrome.

***)J.Cell Science 1988, 90: 365-378**

M 331 ABROGATION OF CLASS I HLA EXPRESSION IN NEUROBLASTOMA ASSOCIATED WITH LONGE RANGE HYPOMETHYLATION AND LOSS OF 1P36.

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Neuroblastomas characteristically have deletions of chromosome 1p36 and abrogated class I HLA expression. Upon analysis of neuroblastoma cell lines, we found that expressed class I HLA genes are methylated, while silent HLA genes are hypomethylated. Hypomethylation was found for HLA-A and HLA-C, that are 1200 kb apart on chromosome 6p21. The modification could therefore affect higher order chromatin structures. A murine modifier of methylation has been mapped in a region homologous to human chromosome 1p36. Therefore we analyzed a possible relationship between class I HLA hypomethylation and the extend of 1p36 deletions. Our data support the notion that relatively large deletions of 1p36 are associated with hypomethylation of class I HLA genes.

M 332 CHROMOSOME PAIRING DURING MEIOSIS IN *S. CEREVISIAE*

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We have investigated chromosome pairing using *in situ* hybridization of ~30kb probes to spread yeast nuclei. This analysis detects pairing at individual chromosome loci without regard to the degree of chromosome condensation or the nature of interactions elsewhere along the chromosome, thus permitting analysis of chromosome pairing at early times and in mutants where pairing is incomplete. This analysis has revealed a significant level of association between homologous segments in cells accumulating in the G1/G0 stage prior to meiotic DNA replication. These associations disappear during the period when DNA replication is known to occur and then reappear as the cells enter prophase. The pairing signal observed before and after replication involves co-localization of homologous segments to a very specific distance, suggesting that pairing in both situations may be similar in some fundamental way. However, the reappearance of pairing after replication is dependent upon early meiosis-specific functions while premeiotic pairing is not.

A number of meiotic mutants have been investigated to determine the relationship of meiotic pairing to other meiosis-specific functions, such as double strand breaks, recombination and synaptonemal complex formation. All mutants studied so far (*mer1, hop1, rad50D, rad50S*, and *rad51dmc1*) except *spo11* are capable of pairing their chromosomes to some extent.

Significant pairing is observed using probes to different chromosomal regions, supporting the view that these interactions reflect a genome wide homology search rather than interactions between a few specific sites in the genome. One hypothesis is that pairing occurs via a paranemic rather than a plectonemic homology search. The observation of pairing in G1/G0 cells prior to meiosis is consistent with this theory, since there is no evidence that nicks or breaks in DNA are a prominent feature of chromosome metabolism at this early stage.

M 333 STRAIN-SPECIFIC METHYLATION OF A MURINE TRANSGENE OCCURS AROUND THE TIME OF BLASTOCYST IMPLANTATION AND IS ACCOMPANIED BY AN ALTERATION IN CHROMATIN STRUCTURE

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The murine locus, *Ssm-1*, on distal chromosome 4 controls the strain-specific methylation of the pHRD transgene. The inbred strain C57BL/6 carries the dominant allele of *Ssm-1*, which results in methylation of the transgene. The inbred strain DBA/2 carries the recessive allele of *Ssm-1*, which is unable to methylate the transgene. DBA/2 males carry the transgene in a completely undermethylated state in all tissues, including sperm. When a transgenic DBA/2 male mates with a non-transgenic C57BL/6 female, the resulting transgenic neonatal progeny carry the transgene in a completely methylated state. Analysis of post-implantation embryos reveals that methylation of the transgene occurs at or before day 6.5 pc. Analysis of transgenic embryonic stem (ES) cell lines derived from day 3.5 pc blastocysts reveals that *de novo* methylation of the transgene can be induced to occur upon *in vitro* differentiation of the ES lines into embryoid bodies. Expression of the transgene and its chromatin structure were analyzed from adult tissues. The transgene is expressed only when undermethylated. Chromatin structure was assessed by sensitivity of the transgene in nuclei to digestion by either DNaseI or MspI. While the transgene exhibited similar sensitivity to DNaseI in DBA/2- and C57BL/6-background nuclei, sensitivity to MspI was different. The DBA/2-background, undermethylated transgene is more accessible to MspI digestion than the C57BL/6-background, methylated transgene. These results suggest that the chromatin structure is more compacted over the methylated transgene. As the induction of methylation over the transgene can be controlled in cultured ES cells, this system allows investigation of methylation as it relates to chromatin compaction.

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Centromeres and Telomeres

M 400 IDENTIFICATION OF NEW YEAST GENES IMPORTANT FOR THE LOCALIZATION OF TELOMERES AND RAP1 PROTEIN IN YEAST NUCLEI, Judith Berman, Shinichiro Enomoto, Elaine Chamberlin and Paul Zierath, Department of Plant Biology, University of Minnesota, St. Paul, MN 55108

We have used plasmids carrying telomere DNA to identify new gene products that interact with chromosomal telomere DNA. We have identified genes *(PAX)* that, when mutated, reduce TEL+CEN-plasmid antagonism. Some, but not all, *pax* mutants influence telomere position effect and some *pax* mutants influence telomere tract length control. In wild type yeast cells, Rap1p localizes to telomeres and to a small number of loci at the nuclear periphery[1], suggesting that yeast telomeres associate with one another and with the nuclear periphery. In *sir3* and *sir4* mutants, which influcence telomere position effect[2], Rap1 antigen is mislocalized: it appears as diffuse staining within the nucleus[3]. Interestingly, Rap1p, and presumably telomere DNA, is mislocalized in all *pax* mutants studied to date. *PAX14* is a novel, non-essential gene that is highly charged and has a large region predicted to form a coiled-coil protein. Rap1p localization is diffuse and intranuclear in both *pax14-1* mutants and in mutants in which the *PAX14* gene has been deleted, suggesting that Pax14p is important for association of telomeres with each other and with the nuclear periphery. *pax1* interacts with Sir4p, is complemented by a clone carrying an N-terminally truncated *nat1* gene, and does not map to either the *SIR4* or the *NAT1* locus. We have also identified wild type genes that reduce TEL+CEN-plasmid antagonism when they are present on high copy plasmids (*ARB* genes). *ARB9* encodes a protein that has homology to DEAD box RNA helicases. In wild type cells carrying *ARB9* on a high copy plasmid Rap1p is mislocalized yet telomere position effect is not altered. While *ARB9* is not essential for spore viability, haploid *arb9Δ* cells grow very slowly, have an aberrant bud morphology, and localization of Rap1p is diffuse and intranuclear.

[1] Klein et al, (1992) J. Cell Biol. 117:935-948.
[2] Aparicio et al. (1992) Cell 66: 1-20.
[3] Palladino et al. (1993) Cell 75: in press.

M 401 STRUCTURE OF THE TELOMERE BINDING PROTEIN COMPLEXED WITH ITS SINGLE STRANDED DNA BINDING SITE Joanne M. Bevilacqua, Viloya L. Schweiker, James A. Ruggles, and Steve Schultz, Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado USA 80309

Telomeres are specialized nucleoprotein structures found at the ends of linear eukaryotic chromosomes. The telomeres of *Oxytricha nova* contain a 16 nucleotide 3' single strand extension with the sequence TTTTGGGGTTTTGGGG that is tightly bound to a heterodimeric protein referred to as the telomere binding protein (1). The α-subunit binds sequence specifically to TTTTGGGG and the β-subunit modifies the DNA binding properties of α but does not interact strongly with DNA in the absence of the α-subunit. The β-subunit is composed of two domains: a 28-kDa core (N-terminus) that interacts with the α-subunit in the presence of DNA, and a 13-kDa C-terminus that is highly charged and functions as a G-quartet chaperone (2).

We have obtained crystals of the ternary complex formed by the full-length α, the 28-kDa core of β, and a 12-mer mimic of the telomeric sequence, GGGGTTTTGGGG. These crystals diffract to at least 2.5 Å resolution and are space group $P6_{1,5}22$ with unit cell dimensions a=b=94 Å and c=427 Å. We are now determining the structure of this complex by x-ray crystallography.

Currently we have obtained heavy atom derivatives with methylmercury(II) chloride and $K_2Pt(CN)_4$. We have also grown crystals using 5-iododeoxyuracil in place of each T position in the 12-mer. We are now collecting data at high resolution on these crystals in order to solve the structure of this complex.

(1) Gray, J. T., Celander, D. W., Price, C. M., and Cech, T. R. (1991) *Cell* **67**, 807-814.
(2) Fang, G. and Cech, T. R. (1993) *Cell* **74**, 875-885.

M 402 CHARACTERIZATION OF THE STRUCTURAL DOMAINS OF YEAST RAP1 PROTEIN Ann Cassidy-Stone, Vienna Reichart, and Steve Schultz, Department of Chemistry and Biochemistry, University of Colorado, Boulder, CO 80309

The repressor activator protein 1 (RAP1) of yeast is a 92.5 kD sequence specific double strand DNA-binding protein which activates transcription, represses transcription, binds to yeast telomeres, and contributes to regulation of telomere length (1). RAP1 partitions with the nuclear scaffold in fractionation of isolated yeast nuclei and there is evidence that RAP1 mediates DNA interaction with the nuclear scaffold (2).

We have utilized limited proteolysis of RAP1 by trypsin and chymotrypsin to identify stable structural domains. Two regions are sensitive to cleavage by these enzymes to yield three stable fragments. N-terminal sequencing of these fragments showed that the cleavage sites for trypsin were at Arg-352 and Arg-606 and for chymotrypsin were at Phe-343 and Tyr -602. The three structural domains identified by these studies coincide with functional domains previously identified by deletion analysis to define an N-terminal domain with unknown function, a central DNA binding domain, and a C-terminal activation, silencing and telomere length regulation domain(3,4).

Based on these results, we have expressed in E. Coli two truncated forms of RAP1: the DNA binding domain (RAP1DBD) and the DNA binding domain plus the activation, silencing, and telomere length regulation domain (RAP1DBDTEL) and have purified both of these fragments to homogeneity.

Currently we are characterizing the DNA binding properties of RAP1, RAP1DBD, and RAP1DBDTEL. The kD for these proteins are respectively .14 nM, 6 nM, and 2.4 nM. We are also determining the precise binding site size and investigating possible cooperative interactions for the full length and both truncated forms of RAP1.

(1) Shore, David and Nasmyth, Kim (1987). *Cell* **51**, 721-732.
(2) Hoffman, Johannes F.-X., Laroche, T., Brand,A., and Gasser, S.M. (1989). *Cell* **57**, 725-737.
(3) Hardy, Christopher J., Balderes, D., and Shore, D. (1992). *Mol. Cell. Biol.* **12**, 1209-1217.
(4) Henry, Yves A.L., Chambers, A., Tsang, J.S.H., Kingsman, A.J., and Kingsman, S.M. (1990). *Nucleic Acids Res.* **18**, 2617-2623.

M 403 CHARACTERIZATION OF TELOMERIC CHROMATIN IN *TETRAHYMENA THERMOPHILA*, Philip D. Cohen and Elizabeth H. Blackburn, Department of Microbiology and Immunology, University of California, San Francisco, CA 94143

We have shown that *Tetrahymena* telomeres exist as two distinct chromatin structures. Type I is a global, non-nucleosomal chromatin structure (NNCS) which accounts for 90-97% of telomeric DNA. Type II is a periodic structure comprising 3-10% of telomeric DNA chromatin, with subunits that are, by defining criteria, nucleosome-bound telomeric repeats.

Type I telomeric chromatin limits the MNase accessibility of the whole telomeric DNA repeat tract. It is unlikely that canonical nucleosomes play any role in type I chromatin since it is at least ten times more susceptible to MNase than DNA in a positioned nucleosome core-particle. Like other telomeric NNCSs, *Tetrahymena* type I chromatin also affords protection to immediately adjacent non-telomeric sequences. We present evidence that an internal tract of telomeric DNA repeats is incorporated into type I chromatin, which suggests that proximity to an end is unnecessary for nucleation of this structure.

Type II chromatin consists of closely packed nucleosoomes. MNase, DNase I and sucrose gradient analyses demonstrate that the periodic subunits of type II chromatin are indistinguishable from canonical nucleosomes. This is first report of nucleosomes on telomeres of lower eukaryotes, while vertebrates are thought to have telomeres that are largely nucleosomal.

Although the relative proportions of these two types of chromatin are unchanged as the telomere grows, the lengthening of *Tetrahymena* telomeric DNA has effects on the structure of telomeric chromatin. Both structures respond to expansion of the telomeric repeat tract. Type I protects the growing telomere in its entirety while type II responds by the quantized expansion of the nucleosome array. This work reveals the dynamic nature of telomeric chromatin and demonstrates a previously unrecognized unity between the telomere structure of lower and higher eukaryotes.

M 404 STUDIES ON TELOMERIC DNA SEQUENCES IN SACCHAROMYCES YEASTS. Marita Cohn, Michael J. McEachern, Elizabeth H. Blackburn, Dept. of Microbiology and Immunology, UCSF, San Francisco, CA 94143-0414.

The telomeric sequences in *S. cerevisiae* show an irregular pattern of short telomeric repeats, which can be written $TG_{2-3}(TG)_{1-6}$. However, newly obtained sequence data have revealed that related budding yeasts such as *Candida albicans* have telomeric DNA that is composed of longer and identical repeat units. We have now begun to characterize the telomeres of species within the genus *Saccharomyces*. Hybridizations done using five different known yeast telomeres, at different stringencies, indicate that there are seven different telomere sequences amongst the nine *Saccharomyces* species examined. Telomeric DNA sequences were cloned from *S. exiguus* and *S. castellii* and were shown to be telomerically located by Bal31 analysis. Sequencing results show that *S. exiguus* contains the same type of telomeric repeats as *S. cerevisiae* and they show the same kind of irregularity. *S. castellii* was found to have an 8 bp telomeric repeat which is identical to half the 16 bp *C. glabrata* repeat, and furthermore highly resembles the *S. cerevisiae* sequence. A large number of *S. castellii* clones were sequenced and 76 % of the repeats were composed of the same 8 bp basic sequence. The kind of variants produced shows similarities to the irregularity found in *S. cerevisiae*, the most common type of variants being the basic 8 bp repeat followed by an additional number of extra TG dinucleotides. These findings suggest that the variants may be produced during synthesis of telomeric DNA by slippage of a putative telomerase template.

M 405 YEAST MUTANTS THAT REQUIRE THE KINETOCHORE PROTEIN Cbf1p FOR PROLIFERATION, Pamela K. Foreman and Ronald W. Davis, Department of Biochemistry, Stanford University School of Medicine, Stanford, CA 94305

Centromere binding factor 1 (Cbf1p) binds to the CDE1 region of *S. cerevisiae* centromeres. Cells that lack Cbf1p lose chromosomes at an elevated frequency and fail to properly transcribe genes involved in methionine biosynthesis. We have shown in previous studies that Cbf1p plays mechanistically distinct roles in chromosome segregation and transcriptional activation. In this study, we have isolated a number of mutants that fail to grow in the absence of Cbf1p, but proliferate in the presence of Cbf1p. Mutations that might cause this phenotype are likely to fall into one of three broad categories: 1) mutations in genes encoding components of the kinetochore, or gene products involved in kinetochore regulation or assembly, 2) mutations in gene products that are constituents of a mitotic cell cycle checkpoint responsible for detecting and responding to improperly assembled spindles, 3) mutations in transcription factors. Using separation of function alleles of the *cbf1* gene, we are currently determining which of these mutants depend on Cbf1p for its role in chromosome segregation and which depend on Cbf1p for its role in transcriptional activation. We anticipate that these mutants will lead to the identification of novel gene products involved in maintaining the high fidelity with which chromosomes are segregated.

M 406 TEMPLATE FUNCTION IN THE TELOMERASE RNA OF TETRAHYMENA David Gilley, Margaret S. Lee and Elizabeth H. Blackburn, Department of Microbiology and Immunology and Department of Biochemistry and Biophysics, Box 0414, University of California, San Francisco, San Francisco, CA 94143

The ribonucleoprotein (RNP) telomerase is an unusual reverse transcriptase responsible for the synthesis of the G-rich strand of telomeric DNA. The telomerase RNA of the ciliate Tetrahymena has been shown to contain the template for telomere repeat synthesis. We are studing the functional role of different RNA template domains in telomere formation. To achieve this we have introduced telomerase RNA genes with mutated template regions into the cell and analyzed their *in vivo* and *in vitro* effects. We have taken a new direction for the analysis of the enzymatic activity of telomerase. In this approach we analyze under *in vitro* conditions mutant telomerase RNAs assembled *in vivo* and partially purified from cells transfected with the various mutated genes. Our results indicate that all alterations within the template reduced long product formation and affect enzymatic fidelity. Reduction in the formation of long products *in vitro* cannot be simply explained by lack of or poor annealing capabilities of mutant products. Furthermore, the mutations have shown that residues 43 to 49 within the telomerase RNA are copied into DNA. Our *in vitro* studies of mutant telomerase have exposed unexpected enzymatic characteristics of telomerase and are also valuable for determining if mutant RNAs are stable and assembled *in vivo*. We found that cellular phenotypes of template residue mutations fall into three classes: early lethal, delayed lethal, and those alterations that have no effect on cellular viability. In summary, the templating region of the telomerase RNA can be separated into several functional domains: boundaries of the template directly copied during the synthesis of the G-rich strand, residues involved in annealing functions, and regions in or near the template involved in start and termination capacities.

M 407 DNA UNTWISTING ACTIVITY INDUCED BY THE YEAST RAP1 PROTEIN, IMPLICATIONS FOR TELOMERE ASSOCIATION AND RECOMBINATION, Eric Gilson[1], Thierry Laroche[2], Thomas Müller[3] and Susan Gasser[2], [1] ENS Lyon, 46 allée d'Italie, 69364, Lyon, Cedex 07, France; [2] ISREC, CH. des Boveresses, 1066 Epalinges/Lausanne, Switzerland; [3] ETH-Hönggerberg, 8093 Zürich, Switzerland.

Several lines of evidence suggest that the major telomere binding protein of yeast, RAP1, is involved in telomeric recombination. First, the binding of RAP1 at an internal site located upstream from the HIS4 gene, results in a high frequency of meiotic recombination (White et al., 1993, *Proc. Natl. Acad. Sci., USA*, 90, 6621-6625). Secondly, we have recently provided evidences that RAP1 distorts the telomeric double helix upon binding (Gilson et al., 1993, *J. Mol. Biol.*, 231, 293-310). This conformational change had characteristics typical of DNA with a single-stranded character (enhanced permanganate and DMS reactivity). We show here that RAP1 has the ability to increase the rate of oligonucleotide binding to telomeric duplex DNA. We propose that this increase is due to an untwisting activity of RAP1, as revealed by cicularization experiments and by electron microscopy of complexes between RAP1 and a circular telomeric DNA. These data strongly support the idea that RAP1 is involved in telomere association and recombination by promoting DNA-DNA pairing.

M 408 LINEAR VECTORS FOR MAMMALIAN CELLS Brenda R. Grimes, Niolette I. M^{c} Gill, Claudia Teschke and Howard J. Cooke, Chromosome Biology Section, MRC Human Genetics Unit, Western General Hospital, Crewe Road, Edinburgh EH4 2XU, Scotland, U.K.

Our aim is to isolate and define the chromosomal elements essential for the stable maintenance of linear molecules in mammalian cells with a view towards constructing a mammalian artificial chromosome. We have cloned human telomeric sequences and reintroduced them into mammalian cells and shown that they are functional since broken chromosomes which resolve are terminated by the introduced telomeres. We have now cloned human telomere repeats into YAC vectors which will enable us to test whether or not human telomeres present at the end of a YAC containing at least 100 kb of human DNA (and therefore probably also a replication origin) can be maintained as an episome when transferred to mammalian cells. In most cases reported YACs integrate into the host genome after transfer to mammalian cells strongly suggesting that yeast telomeres do not function in this background. We have constructed YAC retrofitting vectors which have human telomeres capped by yeast telomeres. These vectors can be used to retrofit any YAC 4 based YAC. The yeast telomeres can be removed prior to mammalian cell transfer using the restriction enzyme Isce I, which has an 18 bp recognition sequence so far undetected in mammalian or yeast DNA. Our centromeric retrofitting arm also has the neomycin gene for selection in mammalian cells and a suppressible yeast centromere which can allow amplification of the YAC copy number up to 100 fold, greatly facilitating YAC isolation. We have succesfully retrofitted YACs with our retrofitting constructs. The resulting YACs are amplifiable and of the predicted structure and size and we have demonstrated that the human telomeres can be protected in the yeast host. We are currently transferring Isce I digested retrofitted YACs to mouse fibroblasts using a combination of lipofection and microinjection. We are also trying to isolate YACs containing either mouse minor satellite or human alpha satellite sequences for retrofitting to try to develop a minichromosome which also has segregation functions.

M 409 AN ESSENTIAL YEAST PROTEIN, CBF5P, BINDS IN VITRO TO CENTROMERES AND MICROTUBULES. Weidong Jiang*, Kim Middleton, Hye-Joo Yoon, Claire Fouquet and John Carbon, Department of Biological Sciences, University of California, Santa Barbara, CA 93106

Yeast centromere DNA (*CEN*) affinity column chromatography has been used to purify several putative centromere/kinetochore proteins from yeast chromatin extracts. The *CBF2* gene specifying the 110 kd subunit (CBF3A) of the CDEIII DNA-binding protein complex,CBF3, is cloned. Another single yeast gene (*CBF5*) specifying one of the major low affinity *CEN*-binding proteins (p64'/CBF5p) has been cloned and shown to be essential for viability of yeast (Jiang et al., 1993. MCB 13:4884-4893). *CBF5* specifies a 55 kd highly charged protein that contains a repeating KKD/E sequence domain near the C-terminus, similar to known microtubule binding domains in MAPs 1A and 1B. CBF5p, obtained by over-expression in bacterial cells, binds microtubules in vitro, whereas C-terminal deleted proteins lacking the $(KKD/E)_n$ domain do not. Dividing yeast cells containing a C-terminal truncated *CBF5* gene, producing CBF5p containing only three copies of the KKD/E repeat, delay with replicated genomes at the G2/M phase of the cell cycle, while depletion of CBF5p arrests most cells in G1/S. However, internal deletion of the (KKD/E)10 domain has no effect on cell growth, indicating this repeat motif is not essential for CBF5p function. Over-production of CBF5p in yeast complements a temperature-sensitive mutation in the gene (*CBF2*) specifying the 110 kd subunit of the high affinity *CEN* DNA binding factor, CBF3, suggesting in vivo interaction of CBF5p and CBF2p. CBF5p is localized in the nucleolus, as determined by double immunostaining using a CBF5p-speicific peptide antibody and a nucleolus-specific antibody. Possible in vivo functions of CBF5p will be discussed. (*Present address: Myco Pharmaceuticals Inc., One Kendall Square, Bldg 300, Cambridge, MA02139)

M 410 TELOMERES IN THE GERMLINE MICRONUCLEUS OF *TETRAHYMENA THERMOPHILA* Karen E. Kirk and Elizabeth H. Blackburn, Department of Microbiology and Immunology, University of California, San Francisco, CA 94122

The ciliated protozoan *Tetrahymena thermophila* contains two nuclei which differ greatly in terms of function, chromosome content, and mechanism of division. The transcriptionally active, polygenomic macronucleus contains roughly 10^4 acentric chromosomes and divides amitotically. In contrast, the transcriptionally silent micronucleus contains a diploid number of 10 metacentric chromosomes and divides mitotically. Being the germline nucleus, the micronucleus also undergoes meiosis.

Although telomeres of the macronuclear chromosomes have been well-studied, the micronuclear telomeres were not previously identified. In the current study, we identified micronuclear telomeres by preferential sensitivity to Bal31 exonuclease and hybridization to C_4A_2 repeats, the repeat sequence of macronuclear telomeres. We have cloned six different micronuclear telomere-associated sequences and have sequenced through the telomeric repeats of 20 different clones. Our results indicate that micronuclear telomeres are distinct from those of the macronucleus in at least three respects:

1) The stretches of telomeric repeats are much longer in the micronucleus than in the macronucleus (~2.0-3.5 kb vs. ~400 bp, respectively).

2) The six cloned micronuclear telomere-associated sequences are highly homologous to one another. In addition, the telomere-associated sequence from at least one chromosome is specific to micronuclear telomeres and is not present in the macronucleus.

3) All micronuclear telomere clones contain centromere-proximal stretches of G_4T_3 repeats (up to 45 repeats) adjacent to the distal G_4T_2 repeats. Such an inner tract of homogeneous but variant repeat units has not been found in macronuclear telomeres nor in any other eukaryotic telomere.

M 411 THE MECHANISM OF INTERACTION BETWEEN CENP-B AND CENTROMERE SATELLITE DNA, Katsumi Kitagawa, Masashi Ikeno, Hiroshi Masumoto and Tuneko Okazaki, Department of Molecular Biology, School of Science, Nagoya University, Nagoya Japan 464-01

We have reported previously that one of human centromere antigen, CENP-B, binds to a 17 bp motif (CENP-B box) found in subsets of centromere specific satellite DNA families in human (alphoid DNA) and mouse (minor satellite DNA) chromosomes. The major complex made *in vitro* with CENP-B obtained from HeLa interphase cells and alphoid DNA is composed of two molecules of DNA and a CENP-B dimer (complex A). We have characterized the functional domains of CENP-B using truncated CENP-B polypeptides made in *E. coli* cells. The DNA binding domain has been delimited within the NH_2-terminal 125-amino acid region containing four potential α-helices. The dimerizing activity has been found in 59-amino acid region at the COOH terminus. These results indicate that CENP-B organizes a higher order structure in the centromere by juxtaposing two CENP-B boxes in the centromere satellite DNA through both the DNA-protein and protein-protein interactions. To correlate the CENP-B box distribution in satellite DNA to the location of centromere antigens in centromere region, the long range organization of alphoid DNA arrays in the human chromosome 21 was analyzed. Two long distinct arrays of alphoid DNA (loci α21-I (1.3 Mbp) and α21-II (1.9 Mbp)) were identified. The α-21-I locus was composed of eleven monomer higher-order repeating units with CENP-B boxes distributing evenly, whereas α21-II locus was composed of diverged alphoid repeats and contained the CENP-B box rarely. We will report the simultaneous FISH analyses and immuno-fluorescence with anticentromere sera to align the position of the centromere antigens with respect to these alphoid families.

M 412 YEAST TELOMERE REPLICATION, Victoria Lundblad, Valerie Virta-Pearlman and Thomas Lendvay, Institute for Molecular Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030.

Telomerase is an multi-subunit enzyme which is responsible for replicating the G-rich strand of the telomere. This enzyme is a novel reverse transcriptase enzyme which carries an internal RNA component responsible for templating the newly synthesized telomeric DNA. Although this enzyme acitivy was first identified ~10 years ago, the genes encoding the protein components have not yet been identified. A candidate protein component is the *EST1* gene product of yeast. Yeast cells deleted for the *EST1* gene show the phenotypes predicted for a defect in telomerase; in addition, the Est1 protein shares very limited sequence similarity with reverse transcriptases.

We are testing the hypothesis that Est1 is a component of yeast telomerase by examining the ability of the Est1 protein to bind several different nucleic acid substrates, using gel mobility shift assays. Using a recombinant protein purified to ~80% homogeneity from an *E. coli* expression system, we have demonstrated that Est1binds weakly, but with high sequence specificity, to yeast G-rich telomeric oligomers. This binding is specific for single-stranded substrates; no binding to duplex telomeric sequences is observed, consistent with the substrate specificity of telomerase. We have also shown that the Est1 protein exhibits strong, but non-specific, RNA binding: Est1 binds equally well to the*Euplotes* telomerase RNA and two other unrelated RNAs of similar size. This strong RNA binding activity is consistent with a role for Est1 as part of an RNP; the lack of specificity may be due to the fact that we have not tested the correct RNA sequence in this in vitro binding assay. We are currently examining whether Est1 is associated with an RNA (the predicted yeast telomerase RNA) in vivo.

In a parallel genetic study, we are looking for additional mutants of yeast with the same set of phenotypes as *est1* mutant strains. Using an expanded mutant screen based on the original screen that yielded *est1*-1, we have identified three new mutants which show both a telomere replication defect and a senescence phenotype. Initial mapping studies indicate that these mutants define at least two new *EST* genes. We are now in the process of repeating this mutant hunt with the goal of saturating for all genes which, when mutated, display *est1*-like phenotypes. These biochemical and genetic results suggest that we may be succeeding in defining multiple components of yeast telomerase.

M 413 ATYPICAL TELOMERIC REPEATS IN A VARIETY OF BUDDING YEASTS, Michael J. McEachern, Marita Cohn, and Elizabeth H. Blackburn, Department of Microbiology and Immunology, University of California, San Francisco, San Francisco, CA 94143

We have previously demonstrated that the telomeres in the yeast *Candida albicans* are distinct from those known in other organisms, being composed of tandem series of 23 bp repeats (M. J McEachern and J. B. Hicks, Mol. Cell Biol., *13*, 551-560). Like the telomeric repeats from other organisms, the *C. albicans* telomeric repeats are: 1) present on all *C. albicans* chromosomes; 2) preferentially sensitive to Bal31 nuclease; 3) present at the very ends of all cloned telomeric fragments; 4) present on a "healed" chromosome end. Using telomeric sequences from *C. albicans* and *S. cerevisiae* we have identified and cloned telomeres from a number of other species of related budding yeasts. In each case we have found that the telomeres were composed of numerous tandem copies of repeats; however unit repeat length varied from 8 bp to 25 bp. Although there are substantial length and sequence differences among telomeric repeats from these species, each shares a 5-6 bp region of T and G residues that is reminiscent of more typical telomeric repeats. The large telomeric repeat length of some of these yeast species makes it possible to use telomeric sequences as a probe to search for telomerase RNA genes, something that is not feasible in organisms with short telomeric repeats. Using this approach we have identified candidate telomerase RNA genes in three yeast species. Deletion of such a candidate telomerase RNA gene from the yeast *Kluyveromyces lactis* results in telomere shortening and eventual death of most cells, consistent with the possibility that the sequence contains a telomerase RNA gene. Cells that survive the initial catastrophic senescence of the culture are observed to have lengthened many or all of their telomeres by the addition of more telomeric repeats, presumably via recombination. These "regenerated" telomeres, however, are still subject to gradual shortening and cell lineages containing them will eventually display signs of senescence again.

M 414 TELOMERE SHORTENING IN RENAL CELL CARCINOMA, Christer Mehle, Börje Ljungberg, Göran Roos, Department of Pathology and Department of Urology, University Hospital, University of Umeå, S-901 87 Umeå, Sweden

Telomere shortening has been showed to be correlated to proliferative activity of the tissue. In tumor cell lines and transformed cells the length of the telomeres is stabilized by telomerase. In this study we analysed renal cell carcinoma for the occurrence of telomere shortening using the probe $(TTAGGG)_4$. Southern blots of *Hin*fI digested DNA revealed a shortening of mean telomere restriction fragment (TRF) length of 0.4 to 2.5 kilobasepairs in 2 - 3 intratumoral samples in all 10 tumors analysed. No obvious intratumoral heterogeneity was found concerning mean TRF length values. However, heterogeneity was shown by the occurrence of at least two separate peak TRF values in 7 of 10 tumors indicating the presence of different tumor cell clones. A conflicting observation was made when evaluating the intensity of the hybridisation signals, where 3 of the tumors showed an increase in hybridisation signals despite concomitant TRF reduction. We found no correlation between tumor size and calculated tumor cell divisions undergone. In 2 tumors, the calculated cell division rounds were unrealistic low compared to the tumor size. These data suggest that telomerase activation might occur in human renal cell carcinoma.

M 415 **THE TELOMERE PROTEIN HOMOLOG: A POSSIBLE TELOMERE REPLICATION FACTOR,** Carolyn Price & Wenlan Wang, Dept. Chemistry, Univ. Nebraska, Lincoln, NE 68588.

While cloning the gene encoding the telomere-binding protein from *Euplotes crassus*, we identified a gene encoding a related protein which we have named the telomere protein homolog. The telomere protein homolog shares extensive sequence identity with both the *Euplotes* and *Oxytricha* telomere-binding proteins. In all three proteins 35-36% of the amino acids are identical, while 54-56% are similar. Much of the sequence conservation maps within the N-terminal section that comprises the DNA-binding domain of the telomere-binding proteins. This finding suggests that the telomere protein homolog may have a recognition site for telomeric DNA.

To determine when during when during the *Euplotes* life cycle the telomere protein homolog gene was transcribed, we examined the abundance of homolog mRNA in vegetative cells and in cells undergoing macronuclear development. The transcript was detectable only in non-starved vegetative cells and in cells undergoing the final stages of macronuclear development. Further studies of developing cells revealed that the peak level coincided with the rounds of replication that take place towards the end of macronuclear development. This finding suggested that the homolog might be somehow involved in telomere replication. Recent experiments have provided support for this hypothesis as antibodies generated against recombinant homolog protein specifically stain the replication bands in vegetative cells. Replication bands are zones of DNA replication that pass through the macronucleus of hypotrichous ciliates.

The sequence of the homolog indicates that it is probably not a primase or a DNA polymerase, and as it is not expressed when telomeres are synthesized during macronuclear development, it is unlikely to be a component of telomerase. However, the homolog could be one of several other activities that have been implicated in telomere replication. These include an activity that determines how many T_4G_4 repeats are added to the 3' end of the daughter strand and an activity that enables telomerase to act on a blunt-ended replication intermediate.

M 416 CRYSTALS OF THE ALPHA SUBUNIT OF THE TELOMERE BINDING PROTEIN COMPLEXED WITH ITS SEQUENCE SPECIFIC SINGLE STRAND DNA BINDING SITE, James A. Ruggles and Steve Schultz, Department of Chemistry and Biochemistry, University of Colorado, Boulder, Colorado USA 80309

Telomeres from *Oxytricha nova* contain a 16 nucleotide single-stranded DNA sequence--TTTTGGGGTTTTGGGG at their 3' ends that are complexed with a heterodimeric protein, referred to as the telomere binding protein. The alpha-subunit of the telomere binding protein binds single-strand DNA sequence specifically, and the beta-subunit (which does not bind sequence specifically to single-strand DNA) modifies the DNA binding properties of the alpha-subunit. Understanding the three-dimensional structures at the ends of chromosomes will provide invaluable information about their function and provide a first example of how proteins can interact with single-stranded DNA in a sequence specific manner.

I have obtained crystals of the alpha-subunit alone, the alpha-subunit complexed with single-strand T_4G_4 DNA, and a 35 kD DNA binding domain of the alpha-subunit (identified by proteolytic digests) complexed with T_4G_4. Crystals of the alpha-subunit complexed with T_4G_4 grow quite large, up to 1 mm in each dimension, and have an octahedral morphology. These crystals diffract to at least 2.4 Å, and are space group F432 with unit cell dimensions a = b = c = 288 Å.

Currently, I am determining the structure of this alpha-subunit/T_4G_4 DNA complex by x-ray crystallography. In our efforts to obtain heavy atom derivatives of the co-crystal for phasing by isomorphous replacement, I have developed a novel approach which involves the direct mercuration of synthetic DNA oligos for subsequent co-crystallization with the protein. Synthetic oligonucleotides are being mercurated at the 5 position of specifically placed uridine or cytidine nucleotides in the synthetic oligo. This reaction occurs readily in relatively mild conditions with very little cleavage of the DNA. This approach could be of very general use for pursuing heavy atom derivatives of protein-nucleic acid complexes.

M 417 IS HYPOMETHYLATION OF CENTROMERIC SEQUENCES RELATED TO A DISTURBANCE OF KINETOCHORE FUNCTION ? Dietmar Schiffmann, Stephan Kirchner* and Helga Stopper*, Institute of Animal Physiology, Cytopathophysiology Unit, University of Rostock, 18051 Rostock, Germany; *Institute of Toxicology, University of Würzburg, 97078 Würzburg, Germany.

Some chromosomes in transformed rat cells and somatic cell hybrids fail to display kinetochores as detected by anti-kinetochore antibodies (anti-CENP-A, -B, -C, -D). Such chromosomes (K^--chromosomes) lack connection to the spindle apparatus and therefore fail to migrate normally in mitosis. Thus, K^--chromosomes may constitute a novel mechanism for the genesis of aneuploidy and the loss of tumor suppressor genes. - Our previous results suggest a correlation between the frequency of K^- chromosomes and increasing genomic instability throughout the course of neoplastic transformation in Syrian hamster embryo (SHE) cells. The lack/malfunction of kinetochore proteins was not due to a lack of centromeric DNA. Since it is known that hypomethylation induces changes in DNA structure, we now have investigated whether changes in centromeric methylation may be related to a disturbance of kinetochore protein assembly. In order to carry out a methylation analysis (Southern, Hpa II, Msp I), we have generated a SHE-specific probe (PCR using the 17 bp CENP-B-motif and a part of the mouse minor satellite consensus sequence as primers). Our results show that a stable hypomethylation occurs in a number of chemically transformed SHE cell lines. This effect was observed shortly after senescence and at various stages throughout the course of neoplastic transformation. Simultaneously, an increase in the number of dislocated K^- chromosomes was observed. These findings suggest that conformational changes in the centromeric region may cause disturbances of kinetochore function and that they occur as an early event in neoplastic cell transformation.

M 418 NOVEL ROLES FOR THE TELOMERE PROTEIN IN DNA REPLICATION, Dorothy E. Shippen[1] and Carolyn M. Price[2], Department of Biochemistry and Biophysics, Texas A&M University, College Station, Texas 77843; [2]Department of Chemistry, University of Nebraska, Lincoln, Nebraska 68588.

In the ciliate *Oxytricha* macronuclear telomeres at the ends of linear DNA molecules consist of short stretches of T_4G_4 sequence tightly bound by a non-nucleosomal protein called the telomere protein (TP). Because TP protects the DNA terminus from nucleases, this protein has been viewed as a protective cap. Telomerase, an unusual ribonucleoprotein reverse transcriptase, also interacts with the G-rich telomeric strand, facilitating the complete replication of the DNA by *de novo* synthesizes of additional telomeric repeats. In this study we investigated the *in vitro* interaction of TP and telomerase. Surprisingly, DNA bound by TP can serve as an efficient primer for telomerase. Moreover, TP-bound telomeric DNA is also accessible to AMV reverse transcriptase, and the *E. coli* Klenow fragment, but is not elongated by terminal transferase. Thus, while the DNA phosphate backbone is inaccessible, the 3' terminus and base pairing positions appear to be exposed in the protein complex. These findings suggest that removal of TP may not be required to initiate telomere replication. Paradoxically, although telomerase extends DNA bound by TP, TP dramatically reduces the number of telomeric repeats added. Dose-dependent, the inhibitory effect is observed with TP-bound DNA as well as primer DNA not bound by TP. Controls with denatured or protease-digested TP have no effect on telomerase activity nor do reactions containing a 100 fold excess of other DNA binding proteins, including histones and single-strand binding protein. The inhibitory effect of TP is specific for telomerase; Klenow reactions containing equivalent amounts of TP are unaffected. These findings suggest that the telomere protein could regulate telomere length *in vivo*. We propose a multifunctional role for TP: 1) protection of the telomeric DNA phosphate backbone against nuclease attack; 2) simultaneous presentation of the DNA bases for replication; and 3) modulation of telomerase activity.

Nuclear Domains and Addresses: The Nucleolus, New Intranuclear "Organelles"; Supra Molecular Nuclear Organelles for RNA Processing; mRNA Export

M 500 SUPPRESSION OF THE S. POMBE CELL CYCLE AND mRNA EXPORT MUTATION pim1 BY A NUCLEAR RAS-RELATED PROTEIN FROM TOMATO. Robert A. Ach and Wilhelm Gruissem, Department of Plant Biology, University of California, Berkeley, CA 94720

Ran is a 25 kD ras-related protein which is very highly conserved between humans, *S. pombe*, and *S. cerevisiae*, sharing about 80% amino acid homology. Ran forms a stable, noncovalent complex with the chromatin-associated protein RCC1, which also acts as a nucleotide exchange factor for Ran. Ran and RCC1 have been shown to be involved in a variety of nuclear processes, including mRNA export, mitotic checkpoint control, and DNA replication. In *S. pombe*, a temperature sensitive mutation in the pim1 gene, an RCC1 homolog, causes premature entry into mitosis and blocks mRNA export from the nucleus, and this mutation can be suppressed by overexpression of spi1, the *S. pombe* Ran homolog. Similarly, in *S. cerevisiae*, overexpression of either of two Ran homologs can suppress a temperature sensitive mutation in the prp20 gene, an RCC1 homolog involved in mRNA splicing, pheromone response, and mRNA export. We report here the cloning of three cDNAs from tomato encoding Ran-like proteins. The mRNAs for these proteins are expressed in all plant tissues examined, and their expression pattern does not correlate with the amount of cell division. We show that the Ran protein genes in tomato are a multigene family, and that these proteins localize to the plant cell nucleus. We also show that overexpression of a tomato Ran protein can suppress the *S. pombe* pim1 mutation, indicating that the tomato and *S. pombe* Ran proteins are functional homologs.

M 501 POSSIBLE MECHANISM OF NUCLEOLAR TRANSLOCATION AND ACTIVATION OF HIV-1 REV PROTEIN, Y. Adachi[1], T.D. Copeland[2], E. Afonina[1], M. Hatanaka[3], S. Oroszlan[2], and G.N. Pavlakis[1], [1]Human Retrovirus Section, and [2]Department of Molecular Virology & Carcinogenesis, ABL-Basic Research Program, National Cancer Institute-FCRDC, Frederick, MD 21702, and [3]Institute for Virus Research, Kyoto University, Sakyo-ku, Kyoto 606, Japan

Rev protein, the post-transcriptional regulator of human immunodeficiency virus type 1 (HIV-1) is involved in the export of unspliced or singly spliced viral mRNAs from the nucleus to the cytoplasm. This function is mediated by its nucleolar localization and sequence-specific interaction with a *cis*-acting RNA element, the Rev responsive element (RRE). An arginine-rich domain of Rev has been identified as the nucleolar targeting signal and RRE-binding domain, and shown to be important for Rev function. To study the molecular interactions between the arginine-rich domain of Rev, RRE-containing viral mRNA, and cellular proteins, we chemically synthesized a Rev peptide, Rev 34-51. This peptide binds RNA in vitro, was taken up by cells, and localized in the nucleolus. Using affinity chromatography with Rev peptide, we identified B-23 and SET as the major binding proteins. The addition of RRE led to the displacement of B-23 and SET from Rev 34-51 complex and the formation of a specific Rev peptide-RRE complex. B-23 and SET are thought to function as shuttle proteins for nucleolar and nuclear transport of ribosomal and cytoplasmic components, respectively. Our results suggest that B-23 and SET may serve as shuttle proteins for the import of Rev/Rex proteins from the cytoplasm into the nucleolus coupled to the export of RRE-containing viral mRNA. (Research sponsored in part by National Cancer Institute, DHHS, under contract NO1-CO-74101 with ABL).

M 502 THE ANALYSIS OF THE 3D POSITION OF THE EBV RNA TRACK IN THE INTERPHASE NUCLEUS OF NAMALWA CELLS.

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We have developed a staining method that allows for the simultaneous detection in intact Namalwa cells fixed in suspension, of a nuclear pore protein by immuno fluorescence and the Epstein Bar Virus RNA track (Lawrence et al., 1989) by In Situ Hybridization. This facilitates three dimensional (3D) analysis of the spatial organization of the EBV RNA-Track inside the nucleus. We applied confocal fluorescence microscopy and 3D image analysis software to analyze the position of the EBV RNA-Tracks within the nucleus.

In a sample of 47 cells the position of the tracks could be determined Over 90% of the tracks clearly touched or came very close to the nuclear periphery as determined by the nuclear pore staining. E few percent of the tracks were located more to the interior of the nucleus and for a few tracks the position was hard to determine. The angular position of the tracks was determined by estimating, on sketches of projections of the individual cells, a sphere that coincided with the nuclear periphery and a plane touching this sphere. The plane was placed where the track came closest to the interior surface of the sphere or touched it on the inside. The angle between the track and the straight line connecting the center of this sphere and the touching point of the track and the nuclear envelope was measured. The frequency of tracks pointing inward was higher than with a sample of angles calculated the same way but using randomly oriented tracks.

These results support the hypothesis (Lawrence et al 1989) that the EBV RNA-Tracks have a function in processing (Xing et al., 1993) and possibly transporting the RNA from the site of transcription in the interior of the nucleus to the nuclear envelope.

Lawrence, J. B., Singer, R.H.and Marselle L. M., (1989) Cell 57, 493-502.
Xing, Y., Johnson, C.V., Dobner, P., and Lawrence, J.B. (1993) Science 259, 1326-1330.

M 503 TRANSLATION OF INTRON-LESS RNA INJECTED INTO THE NUCLEUS OF XENOPUS OOCYTES IS BLOCKED BY FRGY2 PROTEINS.

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When *in vitro* synthesised, capped and polyadenylated CAT or LUC reporter RNAs lacking an intron, or containing a defective intron, are injected into the nucleus of *Xenopus* oocytes there is no detectable translation whereas the same RNAs are efficiently translated following injection into the cytoplasm [Braddock et al, Cell, 58, 269-279 (1989), Nature 350, 439-441 (1991)]. Fractionation experiments show that the RNAs are exported from the nucleus in a translationally repressed form. This nucleus specific translational repression pathway is mediated by the binding of phosphorylated FRGY2, a transcription factor and RNA binding protein (mRNP4) but not by other mRNP proteins e.g. mRNP2 proteins as specific antibody/RNA coinjection experiments relieve translational repression. Interestingly, RNAs containing an efficiently processed intron bypass this repression pathway. These data suggest a role for transciption factors in regulating translation and that the efficiency of splicing *per se* can influence the translational destiny of RNA. The possible involvement of this process in Xenopus development will be discussed.

M 504 **A NUCLEAR REGULATORY MECHANISM IS ENGAGED BY PREMATURE TRANSLATION TERMINATION (NONSENSE) CODONS,** *Mark S. Carter and Miles F. Wilkinson, Microbiology and Immunology Department, Vollum Institute for Advanced Biomedical Research, Oregon Health Sciences University, L220, 3181 S.W. Sam Jackson Park Rd., Portland, Oregon 97201*

A unique feature of T cell receptor-β (TCR-β) genes is that they undergo programmed mutational events as part of the normal developmental program in lymphocytes. Because this process engenders random addition or deletion of nucleotides at the junctions of the rearranged gene segments, approximately two thirds of the rearranged segments will not be in the proper translational reading frame, resulting in the generation of premature termination (nonsense) codons. We studied the effect of nonsense mutations on TCR-β transcript levels in a T cell clone (SL12.4) which possesses a TCR-β1 gene with a nonsense codon in the third exon by virture of a frameshift mutation in the second exon (the in-frame termination codon is in the final sixth exon). This cell clone accumulates TCR-β pre-mRNAs in the nucleus, but does not contain detectable levels of mature TCR-β mRNA in the cytoplasm. An unstable inhibitor protein may mediate this unusual regulation since treatment of SL12.4 cells with several different protein synthesis inhibitors, including cycloheximide (CHX), lead to the accumulation of mature TCR-β transcripts in the cytoplasm. To test directly the hypothesis that nonsense mutations cause down-regulation of TCR-β transcripts, TCR-β constructs that contain nonsense codons in exons 2,3 or 5 were stably transfected into SL12.4 cells. Little or no TCR-β transcripts were detected in the cytoplasm of cells transfected with constructs containing these nonsense mutations, unless the cells were treated with CHX. In contrast, cells transfected with an in-frame (non-mutated) TCR-β construct accumulated cytoplasmic TCR-β transcripts, regardless of CHX treatment. The down-regulation of transcripts containing nonsense mutations does not appear to be due to destabilization in the cytoplasm, based on half-life studies with the transcriptional inhibitor DRB. Instead, this regulation may occur at the level of RNA splicing since deletion of introns downstream of nonsense mutations prevented the down-regulation in transfected cells. Collectively, these results suggest that the mechanism responsible for the down-regulation of transcripts possessing nonsense mutations operates in the nucleus. This unusual mechanism may scan TCR-β transcripts for nonsense mutations before they are fully released into the cytoplasm.

M 505 FULL-LENGTH cDNA AND GENOMIC STRUCTURE OF HUMAN p80-COILIN, AN 80-kD PROTEIN ENRICHED IN NUCLEAR COILED BODIES. Edward K.L. Chan, Saeko Takano, Robert L. Ochs, and Eng M. Tan, W. M. Keck Autoimmune Disease Center, The Scripps Research Institute, La Jolla, CA 92037

Human autoantibodies recognizing distinct subnuclear domains have been reported and have proven crucial in the characterization of a structure known as the coiled body (CB). CBs are non-capsular bodies with a diameter of 0.3-1 μm and appear to be composed of coiled fibrils by electron microscopy. Human anti-CB sera recognize an 80-kD nuclear phosphoprotein that has been named p80-coilin. CBs are known to assemble and disassemble in tandem with the phases of the cell cycle. The highest number of CBs occurrs at mid to late G_1 where p80-coilin is assembled into several small CBs. In S and G_2 phases, CBs become larger and their number decreases. With human autoantibody as probe for cDNA expression cloning, we initially isolated a partial cDNA encoding p80-coilin. Recently the 5' end of the complete cDNA for p80-coilin was obtained using the 5'-RACE (rapid amplification of cDNA ends) methodology and RNAs derived from HEp-2 cells. The complete cDNA corresponded to the 2.7-kb mRNA that was detected by Northern blot analysis. The complete p80-coilin consisted of 576 amino acids and had a predicted molecular mass of 72,000. *In vitro* translation product derived from the full-length cDNA migrated slightly faster than native p80-coilin in SDS-PAGE suggesting that p80-coilin might be modified posttranslationally. During cloning for full-length p80-coilin cDNAs, a 2.2-kb clone representing a sterile transcript of a putative p80-coilin pseudogene was detected. To confirm the validity of the sequence derived from the 5'-RACE procedure, genomic DNA clones were selected from a λFIXII library derived from human placenta DNA. Two overlapping clones representing the 5' and central regions of the p80-coilin gene were selected for subcloning and sequencing. At least 7 exons were detected spanning over 23kb. Sequence analysis of exons 1 and 2 in genomic DNA clones confirmed the accuracy of the cDNA sequence derived from 5'RACE. Ongoing studies in our laboratory include the analysis of the promoter region and the chromosomal localization of the p80-coilin gene.

M 506 HIV-1 REV IS CAPABLE OF SHUTTLING BETWEEN THE NUCLEUS AND CYTOPLASM. Alan Cochrane and Nathalie Richard. Department of Microbiology and Immunology, McGill University, Montreal, Quebec, Canada, H3A-2B4.

The mechanism by which Rev facilitates the export, and consequently, the translation of the structural protein mRNAs of the human immunodeficiency virus type 1 remains undefined. Previous immunolocalization has determined that Rev is predominately in the nucleus with significant accumulation in the nucleolus, a localization consistent with the assumed site of Rev action. To determine whether the subcellular distribution is more dynamic than what is indicated by the original studies, the capacity of Rev to shuttle between the nucleus and cytoplasm was examined. It was observed that treatment of cells with DRB or actinomycin D resulted in a dramatic alteration in Rev distribution, the majority of the protein being found in the cytoplasm. Removal of the drug resulted in a rapid accumulation of Rev in the nucleus indicating that the block to nuclear import was reversible. Subsequent studies indicated that the movement of Rev into the cytoplasm was a passive process while its accumulation in the nucleus was an active one, given that only the latter displayed sensitivity to temperature. Further studies demonstrated that extensive redistribution of Rev could be attained by inhibition of RNA polymerase I alone, in marked contrast to the effect of the drug on another shuttling protein, RNP A1. Furthermore, while treatment with both high and low doses of actinomycin D resulted in equivalent redistribution of Rev within the cell, their effect on Rev function was significantly different. Addition of high doses of Rev completely blocked the Rev response, while the low dose only slightly decreased the level of induction of structural gene expression observed. Consequently, Rev activity is dependent upon RNA polymerase II activity but not RNA polymerase I. In addition, it would appear that Rev function is not dependent on either a functioning nucleolus or the extensive accumulation of the protein in the nucleus.

M 507 PROBING PRE-mRNA PROCESSING WITH THE MURINE TNFβ GENE, François Dautry, Henry Neel, Pierre Gondran and Dominique Weil, Laboratoire d'Oncologie Moléculaire, Institut Gustave Roussy, 94805 VILLEJUIF Cedex, FRANCE

The murine TNFβ gene associates a small size (2 kb), which facilitates the characterization of the transcripts, with the presence of three introns, thus providing an interesting level of complexity. We have already reported that in CTLL-2 cells, which express the TNFβ genefollowing stimulation with IL2, a majority of the transcripts are processed according to the following pathway: polyadenylation of the primary transcript and sequential removal of the three introns in a 5' to 3' order. Finally, splicing out of intron 3 is incomplete and in competition with the export to the cytoplasm of intron3-containing transcripts. Because only one intermediate product is associated with each processing step, we can - at least in principle - derive reaction rates from the relative abundance of the corresponding intermediates. This opens the possibility to investigate the regulation of pre-mRNA processing *in vivo* in the absence of metabolic inhibitors. Moreover, we have now established that the transcripts synthesized during the transfection of a genomic-like TNFβ construct in NIH 3T3 cells are processed according to the same pathway. This latter observation significantly increases the range of studies which can be carried out with the TNFβ gene, as we can now investigate the role of the cellular context, the transcriptional regulation and the structure of the primary transcript in pre-mRNA processing.

As an illustration of the potential of this experimental system, we have investigated how the number of introns present within a primary transcript influences its processing (H. N. *et al.*, Genes and Development, 1993 in press). Quantitative analysis of the precursors and products enabled us to conclude that the presence of intron 2 or introns 1 and 2 increases the splicing rate of intron 3 2.5 and 3.5 fold, respectively. Moreover, a comparable increase of intron 2 splicing rate was observed in the presence of intron 1. As these effects required functional introns, we concluded that introns cooperate for splicing *in vivo*. These results can be interpreted both within the framework of a restricted localization of the splicing machinery and in terms of spliceosome assembly. In the first case, the first intron to be spliced out would provide the appropriate localization (i. e. within a "processing center") for the subsequent splicing reactions, while in the second case one could envisioned that if the splicing machinery communicates accross the exons ("exon recognition") it can also interact within the introns thus creating the potential for a truly cooperative spliceosome assembly along the primary transcript.

M 508 1STRUCTURAL AND FUNCTIONAL ORGANIZATION OF THE YEAST NUCLEUS, D.J. Elliott and M. Rosbash, Howard Hughes Medical Institute and Department of Biology, Brandeis University, Waltham, MA 02254

We have used an *in vivo* assay based on differential RNA half lives in the nucleus and cytoplasm to examine the sub-cellular location of several pre-mRNAs. We have validated this approach by testing a synthetic pre-mRNA, the location of which can be determined by its translational status. Based on this RNA half life assay, natural yeast pre-mRNAs can be divided into two classes. Efficiently spliced pre-mRNAs are restricted to the nucleus, while inefficiently spliced pre-mRNAs leak into the cytoplasm. Cis-mutants of an efficiently spliced transcript show elevated levels of pre-mRNA and, in some cases, cytoplasmic localization, suggesting inefficient recognition by the splicing machinery. In others, pre-mRNA is still nuclear, suggesting that it is still recognized efficiently but processed (spliced) slowly. In situ hybridization to these transcripts might pinpoint the subnuclear location of splicing complexes. We have also found that super-overexpression of a pre-mRNA can lead to its inefficient splicing but not to a detectable decrease in the splicing efficiency of other cellular transcripts. This suggests that the processing pathways of different genes are spatially distinct and that all splicing components are not freely diffusible within the nucleus.

M 509 DEVELOPMENTAL REGULATION OF β TROPOMYOSIN ALTERNATIVE SPLICING M.Y. Fiszman, A.-M. Pret and L. Balvay, Department of Molecular Biology, Pasteur Institute, Paris, France

Protein isoform diversity in muscle cells is generated to a large degree by alternative splicing of precursor RNA. We have been using the β tropomyosin gene as a model system for the study of mechanisms involved in developmental- and tissue-specific splicing. In particular, two internal exons of this gene, 6A and 6B, are preferentially expressed either in non-muscle cells and myoblasts (6A) or in skeletal muscle cells and differentiated myotubes (6B). The results of both in vivo and in vitro mutational analysis have identified several sequence elements including secondary structures and polypyrimidine rich tracts that act to negatively regulate exon 6B splicing in non-muscle cells. In skeletal muscle cells, it is hypothesized that both exons 6A and 6B are accessible to the splicing machinery, but exon 6B splicing signals outcompete those of exon 6A. Recent work exploits a heterologous splicing system to identify factors that function in trans to effect the developmental switch from exon 6A to 6B splicing.

M 510 YEAST U14 RNA INTERACTIONS AND PROPERTIES OF NOVEL SNRNA GENES FROM *S.CEREVISIAE*, Maurille J. Fournier, Andrey G. Balakin, Wenqing Liang, Dmitry A. Samarsky, Max S. Corbett, Jingwei Ni, Gregory S. Schneider and Laurie Smith, Department of Biochemistry and Molecular Biology, University of Massachusetts, Amherst, MA 01003

U14 is essential for growth of *S. cerevisiae* and correct processing of 18S rRNA. Structural elements required for U14 activity and accumulation have been identified and secondary folding maps have been developed for a model U14 precursor and mature U14 RNA. New mutational data obtained for 13- and 14-base segments complementary to rRNA suggest direct interaction of U14 and rRNA. Lethal point mutations can be developed in the essential 13-base segment if the dispensable 14-base element has been replaced, whereas no effects are observed for variants containing the wild-type 14-base sequence. These results are consistent with a two-domain pairing scheme, where loss of the dispensable element creates new stringency for pairing through the required 13-base sequence. Direct binding of the latter element with RNA is further suggested by a strong G-C bias in the point mutations. Attempts to rescue pre-rRNA processing with compensatory U14-rRNA mutations are in progress. A second class of RNA-RNA interaction is suggested for an essential stem-loop domain unique to yeast U14, based on bias of point mutations in loop bases and *cs* phenotypes. U14 and a second snRNA, snR190, are derived from adjoining coding segments. Both snRNAs exhibit length heterogeneity at both ends, suggesting that each is derived by nucleolytic processing, perhaps from a common precursor. Features implicated in processing include: i) an extended 5',3' terminal stem and ii) essential, adjoining box C and box D sequence elements conserved among several snRNAs. Upwards of 40 snRNAs have been detected in extracts of purified *S. cerevisiae* nuclei. Nine new snRNA genes have been cloned and characterized, using strategies based on 3' RNA sequence data. All of the genes exist as single copy elements and none are required for cell viability. The estimated sizes of the new snRNAs are: 124, 182, 183, 188, 202, 202, 222, 258 and 386 nucleotides. While these RNAs are dispensable important roles in ribosome production or other nuclear processes can be imagined. (NIH GM19351)

M 511 TWO NOVEL SMALL NUCLEOLAR RNA U16 AND U18 ARE ENCODED INSIDE INTRONS OF THE SAME RIBOSOMAL PROTEIN GENE. Paola Fragapane, Silvia Prislei, Alessandro Michienzi, Carlo Presutti, Elisa Caffarelli and Irene Bozzoni. Centro Acidi Nucleici C.N.R., Dip. di Genetica e Biologia Molecolare, Università "La Sapienza" , Roma.

The third intron of the *X. laevis* L1 ribosomal protein gene encodes for a previously uncharacterized small nucleolar RNA called U16. This snRNA is not indipendently transcribed, instead it originates by processing of the the pre-mRNA in which is contained, besides it is associated with fibrillarin and it is localized in the nucleolus.Its sequence, localization and biosynthesis are phylogenetically conserved, in fact in the corresponding intron of the human L1 ribosomal protein gene a highly homologous region with the same characteristics is found. In addition to U16, other four introns (2,4, 7 and 8) of the same gene contains another previously uncharacterized snoRNA (U18). Similarly to U16, this snoRNA originates by specific processing of the pre-mRNA,it localizes in the nucleolus and it is associated with fibrillarin. The L1 r-protein gene seems to have a complex structure in which sequences coding for a ribosomal protein are interpose with sequence for two different sno RNAs. This peculiar gene organization is conserved in the corresponding gene of *X. tropicalis* and *H. sapiens* suggesting an important role for the conservation of this gene organization. The nucleolar localization of all these components suggests a possible common function on ribosome biosynthesis.

M 512 CHARACTERIZATION OF YEAST MUTANTS WITH DEFECTS IN NUCLEOCYTOPLASMIC TRANSPORT OF MESSENGER RNA, L.C. Gorsch[1], C.V. Heath[1], O. Li[1], D.C. Amberg[1], C.S. Copeland[2], M. Snyder[2] and C.N. Cole[1], [1]Department of Biochemistry, Dartmouth Medical School, Hanover, NH 03755, [2]Department of Biology, Yale University, New Haven CT 06510

We have isolated temperature-sensitive mutants of *Saccharomyces cerevisiae* that accumulate poly(A)+ RNA in their nuclei at the non-permissive temperature and fall into at least 10 complementation groups. We are presently cloning the genes responsible for this RNA accumulation phenotype.

The *RAT7* (**R**ibonucleic **A**cid **T**rafficking) gene encodes a novel protein of >1400 amino acids. A segment of >100 amino acids in this protein contains repeat motifs related to, but distinct from, those previously identified in both the GLFG and FXFG families of nucleoporins, suggesting that Rat7p may be a nucleoporin. Epitope tagging and immunolocalization are in progress.

We examined the distribution of nuclear pore complexes (NPCs) in the nuclear envelopes of mutant strains by immunofluorescence and EM. In strains bearing mutations in the *RAT2* or *RAT3* genes, NPCs were clustered in one or a very few regions of the nuclear envelope, rather than being evenly distributed as is the case in wild-type cells. This clustering was seen at all temperatures tested, while defects in mRNA export occured only at the non-permissive temperature. We have cloned and begun to characterize the *RAT2* and *RAT3* genes. Synthetic lethality experiments to detect overlapping functions or interactions among Rat7p, Rat2p, Rat3p and known nucleoporins are under way.

M 513 NIC96 IS A MULTI-DOMAIN PROTEIN OF THE YEAST NUCLEAR PORE COMPLEX, P. Grandi, V. Doye, H. Tekotte and E.C. Hurt, Cell Biology Program, EMBL, D-69012 Heidelberg, Germany,

Several nuclear pore complex (NPC) proteins (called nucleoporins), which reveal sequence homology with higher eukaryotic nucleoporins, have recently been identified in yeast. So far, little is known about the physical interaction between these NPC proteins. In order to start studying these interactions, we undertook a biochemical approach and affinity-purified the yeast nucleoporin NSP1 from whole cell extracts under non denaturing conditions. NSP1 was found in a complex of approximately 250 kDa with three other proteins. Two of these polypeptides were identified as GLFG nucleoporins NUP49 and p54; we have then purified and subsequently cloned the third protein of the NSP1-complex called NIC96. NIC96 is localized at the NPC as shown by indirect immunofluorescence and is essential for cell viability.

Interestingly, distinct domains can be identified within the NIC96 protein: i) the amino-terminal part contains hydrophobic heptad repeats which could be involved in protein-protein interactions probably by binding to the coiled-coil domains of NSP1/NUP49, ii) the middle domain has a continuous stretch of 20 hydrophobic amino acids that may be responsible for "pore membrane" attachment, iii) adjacent to the hydrophobic sequence a NLS that when placed in front of a reporter protein can target the fusion protein into the nucleus and iv) the 300 amino acid long carboxy-terminal domain which is not essential for cell growth, although mutations in this part can cause synthetic lethality with ts alleles of NSP1.

Moreover, transcriptional repression of *NIC96* using the regulatable *GAL10* promoter, induces cytoplasmic accumulation of NLS-containing fusion proteins such as Matα2-β-galactosidase and H2B-β-galactosidase.

In conclusion, the multi-domain organization of NIC96 may reflect several roles for this protein in NPC structure and function. We are currently investigating these functions by means of genetic and biochemical approaches.

M 514 A SMALL ELEMENT FROM THE MASON-PFIZER MONKEY VIRUS GENOME HIV-1 EXPRESSION AND REPLICATION REV-INDEPENDENT. Molly Bray [1], Robert Ernst [1], Susan Prasad[1], Kuan -Teh Jeang[2], David Rekosh[1] and Marie-Louise Hammarskjöld[1], [1] Myles H. Thaler Center for AIDS and Retrovirus Research and Department of Microbiology, University of Virginia, Charlottesville, Virginia 22908 and [2] Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, Maryland 20892

Efficient expression of Human Immunodeficiency Virus (HIV) env and gag-pol proteins usually requires co-expression of the viral rev protein and a cis-acting rev-responsive element (RRE) in the viral RNA. Rev binds to the RRE and facilitates the nuclear export of mRNA for these proteins. These mRNAs are normally retained in the nucleus in the absence of rev. It has been speculated that the RNAs may require rev for transport, either because they are incompletely spliced, and are recognized by splicing factors, or because they contain specific transport-inhibitory sequences.

Using both HIV-1 gag/pol and env vectors, we have now shown that a 154 nucleotide sequence from the 3' untranslated region of Mason-Pfizer Monkey Virus (MPMV), a type D retrovirus, eliminates the requirement for both rev and the RRE in HIV expression. This element works only in *cis* and the effect is orientation dependent. In the presence of the MPMV element, the HIV env and gag/pol mRNAs are efficiently transported out of the nucleus whether or not the RRE is present.

The MPMV element is also capable of substituting for rev in HIV-1 replication. Transfection of a rev negative proviral clone into HeLa cells gave rise to HIV virus particles that replicated with an attenuated phenotype in CD4+ lymphoid cells.

These results suggest that the MPMV element interacts with cellular factors to facilitate nucleo-cytoplasmic transport of intron containing RNA. The element is essential for MPMV replication. We are currently exploring the hypothesis that it functions to enable export of the full length MPMV RNA from the nucleus of infected cells.

M 515 A HEPATITIS B VIRUS RNA ELEMENT THAT FACILITATES CYTOPLASMIC ACCUMULATION OF SURFACE GENE TRANSCRIPTS, Z.M. Huang & T.S. B. Yen, Department of Pathology 113B, VA Medical Center, University of California, San Francisco, CA 94121.

The hepatitis B virus surface (S) gene promoter is up-regulated by two viral enhancers. Both enhancers are in an unusual position, being present in the transcribed region down-stream of the S open-reading frame. Previously, we showed that moving the enhancers to a new position up-stream of the S promoter resulted in ~4 fold weakening of this promoter. This result suggested that one or both enhancers functioned optimally only when present in the transcribed region. To test this inference, we generated a series of plasmids, in which the S transcripts were truncated at various points by insertion of a heterologous polyadenylation signal. After transfection into HuH-7 cells, the amount of S transcripts was quantitated by primer extension. The results showed that removal of enhancer II from the S transcripts resulted in ~3-4 fold decrease in steady-state transcript levels. Insertion of a second copy of enhancer II up-stream of the new polyadenylation site restored the S transcript levels to close to wild-type levels, in an orientation-dependent manner. Nuclear run-on analysis and actinomycin D treatment revealed that these changes in RNA levels were not caused by either decreased transcriptional initiation nor increased degradation. Rather, analysis of separated nuclear and cytoplasmic RNA fractions revealed that removal of enhancer II from the S transcripts led to a failure of export from the cytoplasm. Therefore, enhancer II has an additional function at the post-transcriptional level.

M 516 THE RNA SUBUNIT OF RIBONUCLEASE P RAPIDLY LOCALIZES IN THE NUCLEOLUS AFTER MICROINJECTION INTO THE NUCLEUS OF LIVING CELLS.

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The transfer RNA processing enzyme RNase P is a ribonucleoprotein, the RNA subunit of which is essential for activity. Beyond its interest as an unusual enzyme, RNase P presents an intriguing case of ribonucleoprotein assembly. Using methods for visualizing fluorescently-tagged RNAs introduced into living cells (Wang et al., PNAS **88**:7391-7395, 1991) we have investigated the localization of the RNA subunit of RNase P (termed H1 RNA) after microinjection into the nucleus of rat fibroblasts (NRK) or HeLa cells. Within 1 min. after microinjection H1 RNA became localized to each nucleolus in an array of foci reminiscent of the dense fibrillar component. Immunocytochemical experiments showed that H1 RNA colocalized with fibrillarin, a protein component of small nucleolar RNPs involved in pre-rRNA processing. Similarly, the RNA component of the ribonucleoprotein complex MRP RNase also rapidly localized in nucleoli upon microinjection into the nucleus of NRK cells, colocalizing with fibrillarin. In contrast, other small RNAs including U2 and U6 snRNAs and pre-tRNA did not show nucleolar localization. Preliminary in situ hybridization data suggest that the much of the endogeneous H1 RNA in rat fibroblast cells is localized in nucleoli, with signal also observed diffusely in the nucleoplasm and in discrete cytoplasmic structures.

We are now investigating the possibility that RNase P functions in the nucleolus or, more intriguingly, that the RNA and protein component(s) are assembled in the nucleolus and are subsequently transported elsewhere in the nucleus to function in pre-tRNA processing.

M 517 POST-TRANSCRIPTIONAL TRANSGENE-MEDIATED GENE SILENCING IN PLANTS.

Jan M.Kooter, Rik van Blokland, Pieter de Lange, Maike Stam, and Joseph N.M.Mol, Department of Genetics, Vrije Universiteit, De Boelelaan 1087, 1081 HV Amsterdam, The Netherlands.

The addition of transgenes to the genome of plants can lead to suppression of both the transgenes and endogenous homologues, which is termed co-suppression or trans-inactivation. We use the suppression of pigmentation genes in Petunia flowers as model system to study the underlying mechanism. Suppression of chalcone synthase (CHS), a key enzyme in the synthesis of floral pigments, gives rise to white flowers which is due to decreased steady-state *chs* mRNA levels. Data obtained with nuclear run-on transcription assays indicate that co-suppression occurs post-transcriptionally. Nuclear RNAs from *chs*-suppressed tissue contain almost wild-type levels of correctly 3'end processed *chs* transcripts indicating that the genes are fully transcribed but that the RNAs have under the suppressive regime a much shorter half-life. We are currently analysing the fate of the transcripts by identifying intermediates in the degradation process. It is unclear how transgenes cause such a specific suppression but neither a low nor a high transgene transcription level correlates with suppression. Since only in some transgenic plants the genes are co-suppressed, we infer that the chromosomal position and the organization of the transgenes is important for establishing an interaction between the homologous loci. We hypothesize that this interaction which is perhaps mediated by antisense RNA, disturbs the chromatin structure thereby preventing the normal flow of transcripts from the nucleus to the cytoplasm causing premature degradation.

M 518 REGULATED EXPRESSION OF A NUCLEAR DOMAIN RECOGNIZED BY MONOCLONAL ANTIBODY HIS55, Yun W. Lam*, Wim Ammerlaan*+, Wai S. O*, Frans Kroese+ and Davina Opstelten*, Departments of *Biochemistry and *Anatomy, University of Hong Kong, Hong Kong, +Department of Cell Biology and Histology, University of Groningen, Holland

Using mouse monoclonal antibody (mAb) HIS55, we have identified a nuclear antigen (ag) that exhibited an immunocytological staining pattern of discrete foci. Such foci could be detected in cells of many mammalian species including rat and man. These nuclear foci were not associated with nuclear membrane, nucleoli and mitotic chromosomes. HIS55 ag was expressed widely in many tissues but the expression level varied in a cell type-dependent manner, with the number of HIS55 nuclear foci ranging from 0 (as in gametes and neurons) to over 100 (as in megakaryocytes) and with size ranging from fine (as in cortical thymocytes) to very large (as in urethra epithelium). HIS55 ag expression level also varied amongst cells of the same lineage, as observed in embryonic development of rat and in the haemopoietic system in adult rat. Expression of HIS55 ag was sensitive to environmental stimuli, as demonstrated by the observation that HIS55 ag expression level in rat ventral prostate epithelium was significantly increased upon androgen withdrawal. Based on the subcellular and tissue distribution patterns of HIS55 ag, and the environmental control of its expression level, the foci detected by HIS55 are structures not identified previously. We suggested they play a role in the regulation of cell differentiation at the level of transcription and pre-RNA processing.

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M 519 THE SIGNALS ESSENTIAL FOR U14 snoRNA RIBOCISION ARE CONTAINED WITHIN THE INTRON-ENCODED U14 MOLECULE ITSELF, E. Stuart Maxwell, Robert Leverette, and Joyce Liu, Department of Biochemistry, North Carolina State University, Raleigh, NC 27695

U14 snoRNA is a nucleolar species required for the processing of pre-rRNA and the accumulation of mature 18S rRNA. The genes for higher eukaryotic U14 snoRNAs are located within introns of the constitutively-expressed cognate hsc70 heat shock gene. The positioning of U14 genes in introns of mouse, rat, hamster, human, Xenopus, and trout hsc70 genes has suggested an evolutionarily-conserved genomic organization for this snoRNA species in metazoan organisms. In the mouse, single U14 genes are located within hsc70 introns 5, 6, and 8. Recent work in our lab has shown that U14 snoRNA is first transcribed as part of the hsc70 pre-mRNA and then excised from the intron to produce a mature U14 snoRNA. This "ribocision" process represents a new biosynthetic pathway for an snRNA species. Such a processing scheme may metabolically link pre-mRNA transcription with pre-rRNA processing and ribosome biogenesis. We are currently investigating the sequences/structures required for ribocision of mouse U14.5 snoRNA from hsc70 pre-mRNA intron 5. Truncation experiments have shown that both upstream and downstream exons may be removed without affecting U14 processing. Indeed, the hsc70 pre-mRNA precursor may be trimmed in intron 5 to within 8 nucleotides and 17 nucleotides of the 5' and 3' termini of U14 snoRNA, respectively, without disrupting ribocision. Most recently, we have inserted an 87 nucleotide U14.5 sequence into human globin pre-mRNA intron 1 and assessed ribocision. Results demonstrate that a mature U14.5 snoRNA is accurately excised from this globin intron in the absence of any flanking mouse hsc70 exon or intron sequences. These results demonstrate that essential signals for U14.5 snoRNA ribocision are located within the primary sequence and/or folded structure of the U14 molecule itself. Current work is further defining the U14 snoRNA sequences/structures required for ribocision.

M 520 PURINE SEQUENCE ELEMENTS IN SMOOTH MUSCLE-SPECIFIC α-TROPOMYOSIN EXON 2 BIND A NOVEL 120 kD PROTEIN AND ACTIVATE 3' SPLICE SITE SELECTION, Stephen A. Mayer, Massimo Buvoli and Bernardo Nadal-Ginard, Department of Cardiology, Children's Hospital, Boston, MA 02115

Alternative splicing of α-tropomyosin mutually exclusve exons 2 and 3 is regulated by competition between the strong 3' splice site of exon 3, the default choice, and the weak 3' splice site of exon 2. In smooth muscle cells, exon 3 is repressed allowing inclusion of exon 2. We found that excess exon 2 RNA competitively inhibits in vitro splicing of both globin and tropomyosin substrates, possibly by saturating an essential splicing factor. Mutational analyses have revealed that purine rich elements in exon 2 are required for the observed competitive inhibition of splicing and that point mutations in these elements abolish splicing of exon 2 from an alternatively spliced pre-mRNA. Furthermore, the exon 2 purine element is sufficient to activate splicing of artificial substrates with either a weak pyrimidine tract or non-consensus branchpoint sequence. We have identified a ~120kD protein that binds exon 2 using Northwestern and UV crosslinking assays. Biochemical analyses indicate that this protein has not been previously characterized. Interestingly, while the 120 kD isoform is conserved among mammalian species, only a 75 kD isoform is detected in smooth muscle cell lines which activate exon 2 splicing. We will discuss whether the 120 kD protein is the essential splicing factor saturated by exon 2 RNA in the competition experiments, or if it specifically recognizes exon 2 sequences and forms a ternary complex with essential splicing factors. It is postulated that the purine sequences and their cognate trans-factors initiate spliceosome assembly. To this end we are testing whether binding of factors to the exon 2 purine element facilitates 5' as well as 3' splice site selection. We also are asking whether the purine element enhances recognition of the pyrimidine tract or if it bypasses the need for a pyrimidine tract and directly stimulates U2 snRNP binding to the branchpoint.

M 521 INVOLVEMENT OF A DISCRETE REGION OF U6 SMALL NUCLEAR RNA IN THE ACTIVE PHOSPHORYLATION/DEPHOSPHORYLATION CYCLE OF C hnRNP PROTEIN IN THE SPLICEOSOME. Sandra Mayrand and Thoru Pederson, Cell Biology Group, Worcester Foundation for Experimental Biology, Shrewsbury, MA 01545.

C hnRNP proteins bind to nascent pre-mRNA and have been implicated in spliceosome assembly. We have recently shown that the phosphorylation of these proteins regulates their binding to pre-mRNA in the spliceosome (Mayrand *et al., PNAS* 90: 7764-7768, 1993). Hyperphosphorylation of the C1 hnRNP protein inhibits binding to pre-mRNA, and subsequent dephosphorylation of the hyperphosphorylated form allows binding to pre-mRNA. Thus, binding of the C1 hnRNP protein to pre-mRNA in the spliceosome is coupled to the dynamic phosphorylation/dephosphorylation cycle of the protein. In contrast, we have not detected hyperphosphorylated forms of the A1 hnRNP protein nor a role of phosphorylation/dephosphorylation in its binding to pre-mRNA.

Intact small nuclear RNPs are not required for C hnRNP protein hyperphosphorylation in HeLa nuclear extracts. However, we have found that a discrete region of the U6 small nuclear RNA is required for dephosphorylation of C hnRNP protein. Oligodeoxynucleotide-mediated RNase H cleavage of nucleotides 78-95 of U6 RNA inhibited dephosphorylation of C hnRNP protein. This same oligo-directed degradation of U6 RNA eliminates B spliceosome complex formation as analyzed on non-denaturing gels and substantially reduces splicing of pre-mRNA in the nuclear extract. Oligo-mediated cleavage of U1, U2 and U4 small nuclear RNAs did not affect C hnRNP protein dephosphorylation, nor did degradation of other sites in U6 RNA. Thus, we have identified a unique region of U6 RNA that is linked to the dephosphorylation step of an active phosphoprotein of the spliceosome.

M 522 UPSTREAM INTRONS CAN INFLUENCE INTRON RECOGNITION IN PLANT NUCLEI, Andrew J. McCullough, Sherry Xu and Mary A. Schuler, Department of Plant Biology, University of Illinois, Urbana Illinois 61801

In mammalian nuclei, considerable experimental evidence supports an exon definition model for pre-mRNA splice site selection which states that exons, punctuated by upstream 3' splice sites and downstream 5' splice sites, constitute the initial units of recognition in spliceosome assembly. Based on our analysis of splice site selection in dicot plant nuclei, we have proposed an intron definition model for splice site recognition suggesting that 5' and 3' splice sites are selected in position-dependent manners relative to AU-rich elements spread throughout the intronic sequences. These analyses do not, however, rule out the possibility that aspects of exon definition operate in plant nuclei.

To test the exon definition model in plant nuclei, the *rbcS*3A1.+2A intron containing a 5' splice site knockout mutation, was inserted downstream of a soybean β-conglycinin intron. In single intron constructs, transcripts containing the +2U to A mutation are spliced to one of three cryptic 5' splice sites located at -57 in the 5' exon, +25 in the intron or +106 in the *rbcS*3A1 intron. The 215 nucleotide internal exon in the two intron chimeric pre-mRNA is one which the exon definition model predicts should be skipped when the downstream 5' splice site is inactivated. However, in tobacco nuclei the mutant *rbcS* intron was retained and cryptic 5' splice sites were activated. Interestingly, the cryptic exonic site at -57 was more efficiently used in the multiple intron construct than in the single intron construct while the +106 site was used at the same efficiency in both. These results suggest that splicing factors assembled on the upstream intron promote recognition of the closest functional downstream 5' splice site.

Further experiments have examined the effect of a +1 G to A knockout mutation in the second intron of a non-chimeric β-conglycinin pre-mRNA. In addition to retention of the mutant intron, three classes of spliced products were observed. These included products in which the internal exon was skipped, the mutant +1 5' splice site was ligated to one of two non canonical 3' splice sites and other products in which a non canonical intronic 5' splice site was ligated to the normal 3' splice site. The occurrence of exon skipping suggested that recognition of the upstream 3' splice site may be facilitated by interactions with factors associated with the downstream 5' splice site. Consistent with this hypothesis, strengthening the upstream 3' splice site by increasing its uridine content made its splicing independent of the 5' splice site in the downstream intron and abolished exon skipping.

These results suggest that, although splice sites are primarily intron defined in plant nuclei, interactions occurring across exons also contribute to splice site recognition.

M 523 ROLE OF hnRNP A1 STRUCTURAL DOMAINS IN mRNA SPLICING AND RNA-RNA ANNEALING, Stephen H. Munroe[1], Akila Mayeda[2] and Adrian R. Krainer[2], [1]Department of Biology, Marquette University, Milwaukee, WI 53233 and [2]Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724

A number of RNA splicing factors, including helicases and other RNA binding proteins, either promote or destabilize formation of RNA-RNA base-pairing. hnRNP A1 protein (A1), one of the most abundant proteins in the nucleus, greatly enhances the rate of RNA-RNA annealing (Munroe & Dong, PNAS 89, 895-899; refs. therein) and promotes the use of distal 5' splice sites in a concentration-dependent manner (Mayeda & Krainer, Cell 68, 365-375; Mayeda et al., MCB 13, 2993-3001). With respect to the latter activity, A1 antagonizes the activity of other splicing factors, e.g. SF2/ASF, which promote use of proximal 5' splice sites. Since base-pairing interactions play a key role in mRNA splicing, we have examined the possible relationship between the annealing and splice site selection activities of A1 and related hnRNP proteins. To compare requirements for these activities, recombinant proteins were prepared in which each of the 3 structural domains of A1 was modified or deleted. Replacement of two Phe residues in the highly conserved RNP1 motifs in either of the two RNA recognition motifs (RRMs) in the N-terminal half of A1, resulted in complete loss of the ability of A1 to direct alternative splicing. Annealing and RNA binding activities of A1 proved much less sensitive to substitutions within the RRMs. A modest decrease in RNA annealing activity was observed upon replacing conserved Phe residues in either RRM. Surprisingly, the double mutant, containing replacements for Phe residues in both RRMs, displayed nearly the same annealing activity as proteins altered in only one RRM. Deletion of the C-terminal glycine-rich domain of A1, to generate a recombinant UP1 protein which includes both RRMs, resulted in a complete loss of alternative splicing activity. The activity of UP1 and of a 12 kDa fragment that corresponds to the domain deleted in UP1, were greatly reduced with respect to both binding and annealing. However, both proteins retain significant activity. UP1 variants lacking the conserved Phe residues in one but not both RRMs retain this low level of annealing, indicating that one intact RRM is necessary and sufficient for residual annealing activity. Replacement of the entire C-terminal domain of A1 with the RS domain of SF2/ASF leads to complete restoration of annealing activity, although this hybrid protein is completely inactive in splicing (Cáceres & Krainer, unpublished). Thus the C-terminal domain of A1 (and possibly SF2/ASF) plays an important role in protein-mediated RNA-RNA annealing.However, protein-RNA contacts mediated by the conserved Phe residues in each RRM are essential for the activity of A1 in alternative mRNA splicing.

M 524 CHARACTERIZATION OF AN ARRAY OF *CIS*-ACTING ELEMENTS REGULATING MuSVts110 RETROVIRAL RNA SPLICING. Edwin C. Murphy, Jr., Jeffrey M. Touchman, Jerry R. Ainsworth, and Ian A. D'Souza. University of Texas M.D. Anderson Cancer Center. Houston, TX. 77030.

MuSVts110 RNA exhibits an unusual splicing pattern. Like most retroviruses, MuSVts110 pre-mRNA splicing is balanced, allowing the expression of the products of both unspliced and spliced viral RNAs. In addition, MuSVts110 RNA splicing exhibits a unique growth temperature-mediated restriction; temperatures of 33 C and below are permissive for splicing, while temperatures of 37 C and above are nonpermissive. Previous work has established that this thermosensitive splicing phenotype is mediated in *cis* by viral transcript features. Here we show that at least four sequence elements regulate the MuSVts110 splicing phenoype. First, a purine-rich run mapping at approximately -50 to -100 relative to the 3' splice site regulates splicing competence. This sequence contains a cluster of sequences well-matched to "exon splicing elements" (ESE) reported to positively affect ligation at the 3' splice site of exons within which they are located. The MuSVts110 ESE-like sequences are anomalous in being found in the intron, but appear to function like ESEs, since their removal abolishes splicing irrespective of the presence of other splicing signals. Second, the MuSVts110 branchpoint (BP) and polypyrimidine (PY) tract were found to be determinants of overall splicing efficiency. Wild-type MuSVts110 RNA possesses a weak BP and a short PY tract adjacent to the AG dinucleotide at the 3' splice site. Introduction of a strong BP caused MuSVts110 to proceed to virtual completion *in vivo*, thus losing any vestige of either its original balance or thermosensitivity. In *in vitro* splicing extracts, the strong BP overcame a blockade to *wt* MuSVts110 splicing at the level of splicing complex formation, and greatly facilitated exon ligation. Weakening the consensus nature of the strong BP allowed the recovery of thermosensitive splicing *in vivo*, and reinstated the blockades to splicing complex formation and exon ligation *in vitro*, arguing that these characteristics are the result of a balance between postively and negatively-acting elements. Lengthening the PY tract increased the overall efficiency of splicing *in vivo* dramatically, but was less effective than the strong BP in overriding the restriction on splicing imposed by high growth temperatures. Finally, the negative regulation of MuSVts110 RNA splicing at high growth temperatures was mapped to an approximately 100 nt sequence in E2 at least 500 nt distal to the 3' splice (the E2DE). Removal of the E2DE allowed splicing to proceed at high growth temperatures *in vivo* without losing the balance between unspliced and spliced RNA caused by introduction of the strong BP and PY tracts.

M 525 VARIANT 5S rRNA TRANSCRIPTS ARE ASSOCIATED WITH THE 60 kd Ro PROTEIN IN XENOPUS LAEVIS OOCYTE NUCLEI, Charles A. O'Brien and Sandra L. Wolin, Department of Cell Biology and Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT 06510.

Xenopus laevis oocytes synthesize and store large amounts of 5S rRNA in preparation for embryogenesis. This is accomplished by the transcription of approximately 20,000 oocyte-specific 5S rRNA genes. These oocyte 5S rRNAs are stored in a number of forms, including complexes with ribosomal protein L5 and the 5S-specific transcription factor, TFIIIA. From these particles, 5S rRNA is ultimately recruited into ribosomes.

We have recently observed that certain variant 5S rRNAs in Xenopus oocyte nuclei are found in a complex with a distinct RNA binding protein, the 60 kd Ro protein. This protein is usually found as a component of a group of small RNA-protein complexes known as Ro ribonucleoproteins (Ro RNPs). The RNA components of Ro RNPs, known as Y RNAs, are about 100 nt long. Like 5S rRNA, the Y RNAs are transcribed by RNA polymerase III. The 5S rRNAs associated with the Ro protein contain, on average, 8 additional gene-encoded nucleotides at the 3' end. In addition to being longer than mature 5S rRNA, these RNAs contain one or more point mutations compared to the consensus oocyte 5S rRNA sequence. All of these transcripts contain at least one mutation that either disrupts a stem or replaces an evolutionarily conserved nucleotide. Analysis of 5S rRNA from 80S ribosomes did not reveal the same level of sequence heterogeneity.

Our observation that all of the Ro protein associated 5S rRNAs contain mutations indicates that the 60 kd Ro protein may function as part of a quality control pathway for 5S rRNA production.

M 526 SRP1P IS A YEAST NUCLEAR PORE ASSOCIATED PROTEIN THAT PLAYS A ROLE IN NUCLEAR DIVISION/SEGREGATION, NUCLEOLAR STRUCTURE MAINTENANCE AND NUCLEAR TRANSCRIPTION ACTIVITIES, Melanie L. Oakes, Michelle Tabb, Ryoji Yano and Masayasu Nomura, Department of Biological Chemistry, University of California, Irvine, California 92717-1700

SRP1 was originally characterized as an allele-specific suppressor of temperature-sensitive mutations in the zinc-binding domain of the A190 subunit of RNA Polymerase I in *Saccharomyces cerevisiae*. The encoded protein has been localized near the nuclear pores by immunofluorescence microscopy. A system for conditional expression of *SRP1* and temperature sensitive *srp1* mutants have been utilized to demonstrate that Srp1p is essential for the maintenance of the nucleolar structure, RNA transcription and nuclear division/segregation.

The amino acid sequence of Srp1p revealed the presence of eight (degenerate) 42-amino-acid tandem repeats. These repeats have been found to be similar to the 42-amino-acid repeats in armadillo/plakoglobin/β-catenin proteins located at adhesive junction complexes of higher eukaryotes. Sequence homology to Srp1p was also found for smg p21s GDS, a protein that stimulates GDP/GTP exchange for ras p21-like GTP binding proteins. Our studies indicate that Srp1p may function in organizing and regulating specific nuclear and nucleolar structures required for transcription and nuclear division/segregation.

M 527 HIGH RESOLUTION LOCALIZATION OF THE EGF-RECEPTOR MRNA IN THE NUCLEUS, Ody C.M. Sibon, Bruno M. Humbel, Rick Wansink, Jeff Gradener,Arie J. Verkleij,, Fons F.M. Cremers, Department of Molecular Cell Biology, University of Utrecht, Padualaan 8, 3584 CH, Utrecht, The Netherlands.

We developed a high resolution pre-embedment in situ hybridization method for localizing the epidermal growth factor receptor (EGF-receptor) mRNA in the nuclei of A431 cells. Ultra small gold particles in combination with silver enhancement have been used to detect the hybrids. The mRNA of the EGF-receptor was found mainly around nucleoli. To find out if this specific localization pattern represents splicing sites, a double labeling was performed at both the light and the electron microscopical level with antibodies recognizing the essential splicing protein SC-35. Although, no exact colocalization was observed, some close contact sites were observed between the mRNA of the EGF-receptor and the SC-35 protein at the edges of the interchromatin granules. These findings support the hypothesis that splicing takes place at the edges of the interchromatin granules. The EGF-receptor genome was also localized and mainly found near the nucleoli. To test if the EGF-receptor mRNA-rich regions around the nucleoli represent transcription sites, a colocalization study was done for the EGF-receptor mRNA and transcription sites. The transcription sites were probed by incorporation of BrUTP. It was found that most of the EGF-receptor mRNA location sites do not colocalize with transcription places.

In conclusion: most of the EGF-receptor mRNA which is located around the nucleoli does not colocalize with splicing components or transcription sites. Probably the nucleolus is involved in storage or transport of the EGF-receptor mRNA from the nucleus towards the cytoplasm.

M 528 THE MRP RNA MAPS TO MITOCHONDRIA AND TO TRANSCRIPTIONALLY ACTIVE NUCLEOLI USING ULTRASTRUCTURAL IN SITU HYBRIDIZATION, Cynthia S. Smagula, James A. Richardson, William J. Parsons, Herbert K. Hagler, Kang Li and R. Sanders Williams, Departments of Medicine, Pathology and Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas 75235

In situ hybridization of 3 different biotinylated anti-sense RNA probes to mouse cardiomyocyte ultracryosections followed by immunogold detection and electron microscopic analysis shows the MRP RNA to localize to the transcriptionally active region of the nucleolus, but not to the perinucleolar condensed heterochromatin. Quantitative analysis of gold particles within electron micrographs (N=25 for each probe) was used to determine the level of RNA probe hybridized, expressed as gold particles per um^2 surface area, for each cellular subcompartment. The mitochondrial signal for each probe was entirely stable to RNAse H treatment. The corresponding RNAse H-stable nucleolar signal is comparable or somewhat higher in each case. The results indicate nucleolar assembly of an RNase MRP ribonucleoprotein complex as well as possible nucleolar function and in addition are consistent with the existence of a nucleomitochondrial transport path for the MRP RNA as well as with assembly of the RNA to form an intramitochondrial endoribonucleolytic complex. Work is in progress to determine the intracellular distribution of the MRP RNA in other tissues characterized by high levels of mitochondrial DNA replication.

M 529 PROENKEPHALIN IS LOCALISED IN THE NUCLEUS IN MULTIPLE DOMAINS AND IS REORGANISED DURING DIFFERENTIATION, GROWTH ARREST, TRANSFORMATION, AND FOLLOWING DNA DAMAGE. Barbara A. Spruce, Angelika Boettger, and Deborah Dewar. Department of Anatomy and Physiology, Medical Sciences Institute, The University, Dundee, DD1 4HN, U.K.

Neuropeptide precursors are traditionally viewed as molecules destined to be processed and released from the cell. We have recently shown that the mammalian enkephalin precursor, proenkephalin, is targeted to the nucleus as well as to secretory pathway in embryonic neural and non-neural cell types. In murine embryonic fibroblasts (Swiss 3T3 cells), a number of nuclear staining patterns are revealed with a panel of anti-proenkephalin monoclonal antibodies. Proenkephalin epitopes are selectively revealed in different subnuclear domains, one of which corresponds to interchromatin granules, indicating integration of protein presentation with nuclear substructure. Proenkephalin undergoes complex subnuclear reorganisation when fibroblasts are in transition to growth arrest, during myoblast differentiation, and when 3T3 cells become transformed. A subset of proenkephalin epitopes is promptly and transiently revealed in a fine punctate distribution when fibroblasts or myoblasts are deprived of serum or grown at high density to induce growth arrest or differentiation. The phenomenon is seen in up to 100% of the cell population which contrasts with their asynchronicity with respect to the cell cycle and degree of differentiation. This indicates that nuclear proenkephalin or the subnuclear architecture is acutely responsive to the growth environment of the cell. Other changes in the subnuclear organisation of proenkephalin occur when 3T3 cells are fully contact inhibited, when myoblasts fuse into contractile myotubes, and when 3T3 cells become transformed, in some cases accompanied by nucleocytoplasmic relocation of the molecule. As a component of studies to investigate the role of proenkephalin in cell death and survival, we have examined the response to DNA damage which also induces early subnuclear reorganisation of proenkephalin. In apoptotic (and mitotic) cells proenkephalin becomes diffusely distributed and apparently upregulated throughout the cell, but is excluded from condensed chromatin. The data as a whole, in particular the timing of subnuclear responses, suggest that proenkephalin may have a role in multiple facets of growth control, and that its function may be regulated at the level of subnuclear organisation rather than by transcriptional or translational mechanisms.

M 530 THE NUCLEO-CYTOPLASMIC TRANSPORT OF A SPECIFIC PRE-mRNP PARTICLE STUDIED WITH IMMUNO-ELECTRON MICROSCOPY, N. Visa, A. Alzhanova-Ericsson, X. Sun, T. Wurtz, B. Björkroth and B. Daneholt, Laboratory of Molecular Genetics, CMB, Karolinska Institutet, S-17177 Stockholm, Sweden

The structure and the composition of heterogeneous nuclear ribonucleoprotein (hnRNP) complexes are essential for pre-mRNA maturation and transport because post-transcriptional processes take place at the RNP level. Biochemical and structural studies have shown that hnRNP complexes display gene-specific features and, for this reason, their structure and function should be ideally studied in defined pre-mRNPs from specific genes. The Balbiani ring (BR) genes of *Chironomus tentans* have proved to be suitable for this purpose because specific pre-mRNPs, the BR particles, can be isolated from larval salivary glands. Furthermore, the BR particles can be visualized in the electron microscope (EM) so that their assembly, transport and disassembly can be directly studied *in situ*.

Two monoclonal antibodies (mAbs 4F9 and 2E4) have been used to analyze the presence of different hnRNP proteins in the BR particles at different stages of synthesis, maturation and transport. As shown by 2D-immunoblot analysis, mAb 4F9 recognized a group of acidic proteins of about 36kD. This protein family, designated *C. tentans* hrp36 (Ct-hrp36), is related to the A/B hnRNP protein group of vertebrate cells. MAb 2E4 recognized a protein of about 45kD designated Ct-hrp45, which shares high sequence homology with ASF/SF2 RNA splicing factor. Immuno-cytochemistry on cryosections of *C. tentans* salivary glands showed that both Ct-hrp36 and Ct-hrp45 become attached to the nascent BR particle during the initial formation of the RNP fiber and seem to remain in the RNP complex during intranuclear transport. However, these proteins displayed different intracellular distribution. One of them, Ct-hrp45, was clearly restricted to the nucleus of the salivary gland cells. The immuno-EM results suggested that Ct-hrp45 is stripped off the RNP particles during translocation through nuclear pores. In contrast, Ct-hpr36 appeared to be exported to the cytoplasm as a complex with mRNA. Moreover, the association of Ct-hrp36 with rough endoplasmic reticulum suggested that cytoplasmic Ct-hrp36 is bound to polysomal mRNA. Immunoblot analyses of nuclear and cytoplasmic fractions of salivary gland cells were consistent with the EM observations. Taken together, these findings support the view that some hnRNP proteins could participate in nucleo-cytoplasmic transport and might even play a role in the cytoplasm.

M 531 CYTOSTELLIN, A HIGHLY CONSERVED ~240 kD PROTEIN DISTRIBUTES TO NUCLEAR REGIONS ENRICHED WITH SPLICING PROTEINS Stephen Warren†, David Bregman†, Lei Du* and Stephen Ribisi†, Departments of Pathology† and Genetics,* Yale University School of Medicine, New Haven, CT 06510

Cytostellin (CS) is a ~240 kDa phosphoprotein found in all cells examined from human to yeast. In many types of cycling mammalian cells, over 90% of CS is intranuclear. A fraction of the intranuclear CS is distributed diffusely in the nucleoplasm, while another fraction is concentrated in nuclear regions that are enriched with multiple splicing proteins (e.g. SC35). A similar distribution is found in the amphibian germinal vesicle, where CS is concentrated in the loops of lampbrush chromosomes as well as B surpisomes. At the onset of mitosis, CS and SC35 redistribute to discrete sites ('dots') that are widely dispersed throughout the cell. They remain co-localized in these extranuclear 'dots' into G1, but all immunostainable CS is intranuclear by the beginning of S-phase. Extranuclear CS is resistant to extraction with Triton X-100, and it is therefore presumed to be associated directly or indirectly to peripheral cytokeletal structures. Despite its continuous colocalization with SC35, CS is not detected in purified spliceosomes, and it associates with a set of six unidentified proteins that are different from previously identified spliceosomal proteins. These data suggest that CS and its associated proteins form a complex that is distinct from spliceosomes, but CS's subnuclear localization suggests that it may have a role related to mRNA biogenesis. A fraction of CS molecules may be directly accessible to membrane-associated tyrosine kinases. CS was originally eluted from an anti-PiTyr immunoaffinity column charged with extracts of MDCK cells expressing elevated levels of $p60^{c\text{-}src}$. Also, CS and $p60^{c\text{-}src}$ are co-immunoprecipitated from high $p60^{c\text{-}src}$ expressing MDCK cells. Finally, the oncogenic tyrosine kinase, $p60^{v\text{-}src}$ induces a fraction of CS molecules (but not SC-35) to acummulate *outside* of the nucleus, regardless of the cell cycle stage. Isolation of cDNA clones should help us to understand CS's intranuclear function, and it hopefully will provide clues about CS's interaction with membrane-associated tyrosine kinases, such as $p60^{c\text{-}src}$.

M 532 A STABLE INTRON MAY REGULATE THE NUCLEAR-TO-CYTOPLASMIC TRANSPORT OF MATURE TRANSCRIPTS

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Mammalian introns are typically rapidly debranched and degraded following their excision from pre-mRNA. We have identified an unusual intron from the T cell receptor-β (TCR-β) gene that remains stable following excision from pre-mRNA (Nucleic Acids Res. 20:5345, 1992). This intron, IVS1$_{C\beta1}$, accumulates as a set of lariat molecules with different length tails; characteristics that imply that this intron may be sequestered in a protective nuclear microenvironment, perhaps in the splicesomal apparatus. The stable phenotype of this intron is a general property since transfected non-lymphoid cells also accumulate IVS1$_{C\beta1}$ lariats. To determine the cis-acting sequences required to engender its stability and to assess its possible functional attributes, we have made several mini-gene constructs containing IVS1$_{C\beta1}$ and stably transfected them into HeLa cells. Insertions and deletions in central regions of IVS1$_{C\beta1}$ did not abolish its stability in transfected cells - therefore its ability to escape degradation does not require internal sequences or intact secondary structure involving the entire molecule. Replacement of the 5' portion of IVS1$_{C\beta1}$ with 5' sequences from a β-globin intron also did not affect its stable phenotype. However, a chimeric intron containing the 5' portion of IVS1$_{C\beta1}$ joined to the 3' portion of a β-globin intron was no longer stable in transfected cells. Thus, the 3' terminal portion of IVS1$_{C\beta1}$ is necessary and sufficient for it to accumulate as a stable lariat molecule. Transfection experiments also revealed that this intron may regulate the transport of mature TCR-β transcripts out of the nucleus. Constructs that contained stable forms of IVS1$_{C\beta1}$ gave rise to *fully spliced* transcripts (which lack IVS1$_{C\beta1}$) that were retained in the nucleus. Control experiments showed that this unusual property was due to IVS1$_{C\beta1}$ itself and not adjacent exon sequences. The nuclear retension phenotype was also conferred to heterologous transcripts in which IVS1$_{C\beta1}$ and adjacent splicing sites were inserted. In contrast, non-stable mutant forms of IVS1$_{C\beta1}$, as well as other non-stable introns, did not confer the nuclear retension property to mature transcripts. A model is presented in which IVS1$_{C\beta1}$ is suggested to inhibit splicesomal disassembly after the completion of splicing, thus preventing the decay of this intron, as well as the transport of fully spliced transcripts out of the nucleus. Studies are in progress to determine if IVS1$_{C\beta1}$ remains bound to intact splicesomes *in vivo*.

M 533 Drs1p and Nop4p, NUCLEOLAR PROTEINS NECESSARY FOR RIBOSOME ASSEMBLY IN *SACCHAROMYCES CEREVISIAE*, John Woolford, Chao Sun, Tracy Ripmaster and Cynthia Adams, Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, Pa 15213

Most steps in the biogenesis of eukaryotic ribosomes, including transcription and processing of rRNA and association of ribosomal proteins with assembling ribosomal subunits, occur in the nucleolus. A large number of nonribosomal proteins are present in the nucleolus, including many associated with assembling ribosomal subunits. However, the structure and function of only a few of these proteins have been investigated. Our lab has taken two different approaches to search for non-ribosomal proteins involved in ribosome biogenesis: (1) We identified cold-sensitive mutants deficient in ribosome synthesis, defining seven different genes *drs1-drs7*. *DRS1* encodes a putative RNA helicase localized to the nucleolus, that is necessary for 60S ribosomal subunit assembly. A nucleolar helicase might be necessary to alter inter- or intramolecular base-pairing of rRNAs or small nucleolar RNAs (snoRNAs) during ribosome assembly. Genetic and biochemical approaches are underway to identify with which RNAs and proteins Drs1p might interact. *DRS2* encodes a putative ATPase necessary for 40S subunit assembly. (2) We used antibodies against two yeast nucleolar proteins to screen a lambda gt11 library, and identified two nucleolar protein genes. Studies thus far have focused on *NOP4*. *NOP4* is a single-copy, essential gene present on the left arm of chromosome XVI. The 71 kD Nop4p contains three classical RNA recognition motifs (RRMs) and a fourth RRM-like domain, suggesting that it binds RNA. To determine the function of Nop4p, a *GAL1::NOP4* conditional allele, was constructed. Upon termination of transcription of *NOP4* and subsequent depletion of Nop4p, the level of 60S ribosomal subunits decreases and synthesis of 25S rRNA is aborted. Thus Nop4p is necessary for 60S ribosomal subunit assembly. Currently we are using co-immunoprecipitation assays to determine whether Nop4p is associated with snoRNAs, rRNA, or other nucleolar proteins.

Late Abstracts

CHROMATIN SUBSTRUCTURE: STRUCTURAL STATES AND CONFORMATIONAL TRANSITIONS OF THE NUCLEOSOME, Gregory J. Czarnota and F.P. Ottensmeyer, Department of Medical Biophysics, University of Toronto and Ontario Cancer Institute, 500 Sherbourne St., Toronto, Ontario, Canada M4X 1K9

Nucleosome structure and changes of nucleosome conformation are important in fundamental cellular processes. Recent biochemical and genetic analyses show that nucleosomes are very active participants in gene expression, facilitating or inhibiting transcription and reflecting the physiological state of the cell[1]. Structural states and transitions for this macromolecular complex, composed of DNA wound about a heterotypic octamer of variously modified histone proteins, are recognized to occur with various post-translational modifications, gene activation and with ionic environment[1,2]. In spite of physico-biochemical studies which indicate various forms of nucleosome structure[2], all current X-ray and neutron diffraction studies have consistently resulted in only one structure suggestive of a static conformation[3,4,5]. In contrast, two-dimensional electron microscopy studies and three-dimensional reconstruction techniques have yielded a different structure[6]. The differences between EM and other ultrastructural studies have been addressed by us and resolved using spectroscopic electron microscopy and novel analyses[7] of nucleosome images in an extensive study of nucleosome structure with ionic environment. In total, we find a series of conformational states, indicative of transitions in nucleosome structure and a dynamic nature for the particle concordant with genetic and biochemical studies.

References

1. K.E. van Holde (1993) *Nature* **362**, 111-112.
2. K.E. van Holde *Chromatin* (Springer-Verlag, New York) 1989
3. T.J. Richmond *et al.* (1984) *Nature* **311**, 532-537.
4. G.A. Bently *et al.* (1984) *J. Mol. Biol.* **176**, 55-75.
5. G. Arents *et al.* (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10148-10152.
6. G. Harauz and F.P. Ottensmeyer (1984) *Science* **226**, 936-940.
7. M.Z. Zabal *et al. J. Microsc.* In Press.

NUCLEAR TRANSPORT OF U1 snRNP IN SOMATIC CELLS: DIFFERENCES IN SIGNAL REQUIREMENT COMPARED WITH *XENOPUS LAEVIS* OOCYTES, Utz Fischer[1], Jutta Heinrich[1], Karen van Zee[2,3], Ellen Fanning[2] and Reinhard Lührmann[1]. [1]Institut für Molekularbiologie und Tumorforschung Philipps-Universität Marburg, D-3550 Marburg (Germany); [2]Institut für Biochemie, Ludwig-Maximilians Universität München, D-8000 München 2 (Germany); [3] current address: Department of Horticulture Oregon State University Corvallis, OR 97331.

The signal and energy requirements for the nuclear import of U1 RNA in somatic (Vero and 3T3) cells were investigated by microinjection of both digoxygenin-labelled wild type and mutant U1 RNA molecules and *in vitro* reconstituted U1 snRNPs. U1 RNA was shown to be targeted to the nucleus by an energy-dependent process that requires the prior assembly of RNPs from the common proteins and the microinjected RNA. Competition in the cell between immunoaffinity-purified U1 snRNPs and digoxygenin-labelled U1 snRNPs reconstituted *in vitro* showed that the transport is saturable and therefore is a receptor-mediated process. The transport of a karyophilic protein under the same conditions was not affected, indicating the existence of a U snRNP-specific transport pathway in somatic cells, as already seen in the *Xenopus laevis* oocyte system. Surprisingly, the signal requirement for nuclear transport of U1 snRNP was found to differ between oocytes and somatic cells, in that the m3GpppG-cap is no longer an essential signalling component in the somatic cells. However, as shown by investigation of the transport kinetics of m3GpppG- and ApppG-capped U1 snRNPs, the m3GpppG-cap accelerates the rate of U1 snRNP import significantly. Use of a minimal U1 snRNP complex lacking stem/loops I-III demonstrated that the Sm core domain, which binds only the common proteins, is both necessary and under certain conditions also sufficient to facilitate the nuclear transport of an snRNP particle. We propose that the NLS of U1 snRNP has a modular character, and that the requirement for the m3GpppG-cap as a nuclear import signal depends on cell-specific differences in the efficiency of the transport apparatus of oocytes versus somatic cells.

IN VITRO NUCLEAR IMPORT OF snRNPS: CYTOSOLIC FACTORS MEDIATE m_3G-CAP DEPENDENCE OF U1 AND U2 snRNP TRANSPORT, Christopher Marshallsay and Reinhard Lührmann, Institut für Molekularbiologie und Tumorforschung, Philipps-Universität Marburg, D-35037 Marburg, Germany.

We have established an *in vitro* snRNP nuclear import system using digitonin permeabilised somatic cells supplemented with cytosolic extracts. As model karyophiles we have used either digoxygenin-labelled U1 snRNPs reconstituted *in vitro* or fluorescein-labelled U2 snRNPs. *In vitro* nuclear import of snRNPs requires ATP, cytosolic factors, and a nuclear localisation signal (NLS). This import occurs through nuclear pores, is saturable, and is distinct from protein transport. Nuclear import of snRNPs, in permeabilised NRK cells supplemented with somatic cell cytosol, requires the same NLS structures identified in micro-injected mammalian cells. In contrast to the situation in *Xenopus* oocytes, where the m_3G-cap and the Sm-core-domain constitute a bipartite NLS, the m_3G-cap is not essential for *in vitro* nuclear import of U1 and U2 snRNPs. Instead, assembly of the Sm-core-domain is both necessary and sufficient to mediate snRNP nuclear targeting. Interestingly, when the *in vitro* system was provided with cytosol from *Xenopus* oocytes instead of somatic cells, U1 and U2 snRNP nuclear import was m_3G-cap dependent. These results indicate that soluble cytosolic factors mediate the differential m_3G-cap dependence of U1 and U2 snRNP nuclear import in somatic cells and oocytes. We also demonstrate the existence of a soluble cytosolic factor whose interaction with the U2 snRNP m_3G-cap is both saturable and essential for U2 snRNP nuclear import in *Xenopus* oocytes.

STRUCTURAL / FUNCTIONAL ANALYSIS OF A YEAST KINETOCHORE, Kim Middleton and John Carbon, Department of Biology, University of California, Santa Barbara, CA 93106.

The centromere of *Saccharomyces cerevisiae* consists of 125bp of DNA (*CEN* DNA). The kinetochore is composed of *CEN* DNA plus associated proteins. Several of these proteins have recently been isolated, including a 240 kD complex (CBF3) that binds to an essential 25bp *CEN* region (CDEIII). The CBF3 complex consists of three proteins of 110 kD, 64 kD and 58 kD.

We have been working with highly enriched samples of the CBF3 complex (ca. 45-75% CBF3) with the aim of elucidating the function of these proteins *in vivo*. In experiments where we react *CEN* DNA linked beads with CBF3 enriched proteins we observe both *in vitro* binding of *CEN* DNA beads to microtubules, as well as an ATP dependent , minus end directed movement of the beads along microtubules. Control beads linked to mutant *CEN* DNA (a CDEIII point mutation) show neither of these activities. These results show that our protein preparations contain a motor activity that is in some way associated with the functional yeast centromere.

By using in vitro microtubule gliding as an assay we have demonstrated that it is possible to fractionate CBF3 activity (assayed by *CEN* DNA binding) away from microtubule dependent motor activity. We find however, that both CBF3 and motor fractions are necessary to mediate the binding of *CEN* DNA beads to microtubules and for subsequent motor activity.

We are presently attempting to identify the protein or proteins involved and to elucidate how CBF3 assists in these activities.

TUBULAR MEMBRANE BOUND INTRANUCLEAR STRUCTURES IN MAMMALIAN CELLS David Vaux[1], Michael Hollinshead[2] and Mark Fricker[3], [1]Sir William Dunn School of Pathology, and [3]Department of Plant Sciences, Oxford [2]European Molecular Biology Laboratory, Heidelberg, Germany

The nucleus is bounded by a pair of membranes enclosing a lumenal space continuous with the endoplasmic reticulum (ER) and bridged by nuclear pore complexes. We report a serial section EM study of deep incursions of this bounding nuclear envelope (NE) into the depths of the nucleoplasm. Tissue culture cells of human (HeLa), mouse (3T3, A431 and J774) and rat (NRK) origin were found to have characteristic patterns of multiple tubular incursions of NE into the nucleoplasm; serial sectioning confirmed their connection with the ER and the presence of nuclear pore complexes along their length confirmed their identity. Reconstruction from serial section reveals a complex branching morphology and a tendency for branches to pass close to, and often terminate near, nucleoli.

Branching intranuclear membrane bound structures were also observed by laser scanning confocal microscopy and image reconstruction after labelling cell lines and cultured primary cells with concanavalin A or *galanthus nivalis* lectin (for high mannose oligo-saccharides), an antibody to protein disulphide isomerase (for ER lumenal content), and an antibody to ER membrane proteins. These results confirmed that the tubular incursions originate from the NE/ER, and that they could be found in primary cells as well as tissue culture cell lines. The same branching structures were identified in the nucleus after labelling cytoplasm by scrape-loading of a high molecular weight fluorescent tracer, suggesting the presence of a core of cytoplasm within the tubes.

A vital double stain method using dihydroethidium to label nucleic acids and $DiOC_6$ to label ER membranes revealed dynamic tubular structures inside the nucleus with a similar number and morphology to the structures observed in fixed cells. The tubes observed by confocal microscopy under these conditions were shown to be identical to the Con A binding structures by fixation and re-labelling on the microscope stage under continuous recording.

The ubiquituous distribution of these structures, together with preliminary observations showing that they are reduced or absent in NRK cells growth arrested by contact inhibition in confluent cultures, suggest that intranuclear membrane tubes may have a significant role in nuclear function.

TRANSCRIPTIONAL ACTIVATION BY TLS - AN RNA BINDING PROTEIN INVOLVED IN A TUMOR-SPECIFIC TRANSLOCATION, Hélène Zinszner and David Ron, Departments of Medicine, Cell Biology and the Skirball Institute of Biomolecular Medicine, NYU Medical Center, New York, NY 10016.

In the last year the products of several solid tumor specific chromosomal rearrangements have been characterized at the molecular level. At least three discreet types of human tumors exhibit a common feature in which the N-terminus of a protein that normally contains an RNA-recognition motif (RRM) is fused to the DNA-binding domain of another protein. The RRM is invariably lost in the fusion. Focusing our efforts on the myxoid-liposarcoma specific fusion protein, TLS-CHOP we have found that the normal, bacterially expressed TLS can bind purified mRNA in vitro. Under normal conditions, in growing cells in culture, TLS is tightly associated with nuclear structures. However, blocking RNA-polymerase II mediated transcription leads to profound redistribution of TLS immune-reactivity in the nucleus and a significant "leakage" of the immune-reactivity to the cytosol. This result is consistent with the idea that TLS associates either directly or indirectly with a product(s) of Pol II transcription. The observation that the N-terminus of TLS, when fused to the DNA-binding domain of CHOP renders the TLS-CHOP fusion protein a transforming oncogene, led us to speculate that the N-terminus of TLS may posses transcriptional regulatory activity. We found that when fused to a heterologous DNA binding domain (of the yeast Gal4 protein) the N-terminus of TLS will powerfully activate a promoter containing Gal 4 binding sites. These results suggest the existence of a class of RNA binding proteins that normally regulate some aspect of transcription. Deregulated expression of this function by juxtaposition of the effector domain to certain heterologous DNA-binding proteins leads to cellular transformation.