# STEROID/THYROID RECEPTOR GENE SUPER FAMILY

*Organizers: John Cidlowski, Bert O' Malley and Herbert Samuels*

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## *Plenary Sessions*

Page



## *Poster Sessions*

*Plenary Lecture*

**L 001** RETINOID RECEPTORS IN DEVELOPMENT AND DISEASE, Ronald M. Evans[1], Akira Kakizuka, Steve Kliewer David Mangelsdorf, and Kazuhiko Umesono[1], Howard Hughes Medical Institute[1], The Salk Institute, La Jolla, CA 92037.

The cellular responses to RA are mediated by two families of transcription factors, which include the RA receptors (RARs) and the retinoid X receptors (RXRs). Although both RAR and RXR respond specifically to RA, they differe substantially from one another in primary structure and ligand specificity. A major question raised by the discovery of two retinoid-responsive systems is whether their functions are independent, interactive, or redundant. One approach to answer this question is to determine whether they share common or distinct downstream target genes. In regard to target sequences we have recently described properties of direct repeats (DRs) of the half-site AGGTCA as hormone response elements. According to our results, spacing of the half-site by 3, 4, or 5 nucleotides determines specificty of response for vitamin D3, thyroid hormone and retinoic acid receptors, respectively. This so-called "3-4-5" rule suggests a simple physiologic code exists in which half-site spacing plays a critical role in achieving selective hormonal response. As part of these studies, we have also identified that the RXR, but not the RAR, is able to activate through a direct repeat spaced by one nucleotide. In contrast, both RAR and RXR are able to commonly activate through a DR with a spacing of 5. Evidence that RXR heterodimers modulate the RA response will be presented.

Finally we will discuss the isolation and characterization of a fusion product produced as a consequence of a t(15;17) translocation characteristic of human acute promyelocytic leukemia. This translocation which occurs in the retinoic acid receptor gene generates a unique mRNA which encodes a fusion protein between the retinoic acid receptor alpha (RARα) and a myeloid gene product called PML. Structural analysis reveals that the PML protein is a member of newly recognized protein family that includes a variety of putative transcription factors as well as the recombination-activating gene product (RAG-1). The abberant PML-RAR fusion product, while typically retinoic acid responsive, displays both cell type and promoter specific differences from the wild type RARα. Because patients with APL be induced into remission with high dose RA therapy, we propose that the non-liganded PML-RAR is a new class of dominant negative oncogene product.

*Receptor Phosphorylation*

**L 002** REGULATION OF HUMAN PROGESTERONE RECEPTORS (hPR) BY PHOSPHORYLATION, Kathryn B.Horwitz, Glenn S.Takimoto Lin Tung, Carol A. Sartorius, Department of Medicine, University of Colorado Health Sciences Center, Denver, CO 80262

Human progesterone receptors are basally phosphorylated at multiple serine residues, and hyperphosphorylated following agonist or antagonist binding. To test the function of hormone-dependent phosphorylation, two classes of site directed mutants were constructed in cDNA expression vectors encoding the $hPR_A$ (94kD) and $hPR_B$ (120kD) isoforms. *Class I*, designed to test the function of hPR phosphorylation on DNA binding, are mutated in the first zinc finger of the DNA binding domain and either abolish or alter the specificity of PR binding to cognate progesterone response elements (PREs). *Class II*, designed to test the function of phosphorylation on transcriptional activation, are ser to ala substitution mutants of 9 single-point or serine clusters in the N-terminal domain, and 1 ser to ala substitution in the hinge region of $hPR_A$. Additional mutants include ser to glu revertants. Mutant and wild-type cDNA expression vectors were transfected into PR-deficient COS-1 and HeLa cells and tested for progestin dependent transcriptional activation of PRE linked CAT reporters containing either simple or complex promoters; for phosphorylation by immunoblot analysis and [$^{32}$P]orthophosphate incorporation; and for DNA binding by gel retardation assay. Failure to bind DNA (*class I* mutants) diminished but did not abolish phosphorylation, suggesting that hormone-dependent phosphorylation consists of DNA-independent and DNA-dependent stages. Analysis of *class II* mutants identified at least 3 sites, $ser_{190}$ ($M_1$) in the N-terminal domain, a ser cluster upstream of the DNA binding domain ($M_9$), and $ser_{689}$ ($M_H$) in the hinge region, important for promoter and cell-specific progestin-stimulated transcription. Depending on the promoter and cell type tested, transcription by these mutants was reduced as much as 60%. The revertants had wild-type activity. 8 Br-cAMP did not hyperphosphorylate hPR but superinduced PRE-dependent transcription whether wild-type or $M_1$ or $M_H$ mutant receptors were present, suggesting that 8 Br-cAMP acts through an hPR phosphorylation-independent pathway. DISCUSSION: 1) Analysis of hPR phosphorylation function is complex and varies with the promoter and cell being studied. 2) Multiple sites in hPR are phosphorylated sequentially in at least a three-stage progressive cascade. 3) hPR phosphorylation influences both DNA binding and transcription. 4) PRE activation by cAMP is independent of hPR phosphorylation.

**L 003** PHOSPHORYLATION OF GLUCOCORTICOID RECEPTORS. Jack E. Bodwell[1], Eduardo Ortí[1], Li-Ming Hu[1], Jiong-Ming Hu[1], James M. Coull[2], Darryl J.C. Pappin[2], and Allan Munck[1], [1]Department of Physiology, Dartmouth Medical School, Hanover, NH, [2]Milligen/Biosearch, Burlington, MA.

Glucocorticoid receptors in WEHI-7 mouse thymoma are basally phosphorylated in the absence of hormone. After binding of hormone most of the receptors, whether cytoplasmic or nuclear-bound, become hyperphosphorylated; a small fraction (~5%) of nuclear-bound receptors that are unextractable with salt solutions (but extractable with SDS) are relatively dephosphorylated. In the absence of ATP, with or without hormone, almost all the receptors accumulate in the nucleus in a salt-unextractable form, the "null" receptor, that cannot bind hormone. Most of these phenomena can be reproduced with mouse glucocorticoid receptors that are overexpressed in CHO (*WCL2*) cells.

Kinetic and other studies suggest that in hormone-treated cells the receptors are reutilized, traversing an energy-dependent cycle that may involve phosphorylation and dephosphorylation. It is possible that such cycling takes place even in absence of hormone. Time course measurements of the rate of hyperphosphorylation after addition of hormone show that hyperphosphorylation begins after the hormone-receptor complex is activated and Hsp90 has dissociated. The substrate for hyperphosphorylation is therefore the activated hormone-receptor complex. This complex appears to be in rapid equilibrium between cytosolic and nuclear-bound salt-extractable forms, so it is unclear whether hyperphosphorylation takes place before or after nuclear binding. *Unliganded receptors and nonactivated hormone-receptor complexes* eventually also become hyperphosphorylated.

A model has been proposed that incorporates these observations in an energy-dependent receptor cycle. The cycle requires ATP both to phosphorylate the receptors and to reconstitute receptor-Hsp90 (and other Hsps) complexes from activated receptors after hormone dissociation. *Unextractable receptors* are assumed to be formed from extractable nuclear-bound receptors. On a short-term basis, all receptors are recycled to unliganded receptors after hormone dissociation. Over longer periods, degradation and synthesis of receptors can play a role.

Phosphorylated sites on the mouse glucocorticoid receptor have been located by phosphopeptide mapping and sequencing of tryptic peptides from $^{32}$P-labeled receptors. HPLC phosphopeptide maps of receptors from WEHI-7 and WCL2 cells were found to be almost identical, so most of the analyses were done with WCL2 cells. All seven sites identified so far are in the N-terminal domain, on serines 122, 150, 212, 220, 234 and 315, and on threonine 159. Except for threonine 159 and serine 315, which have no homolog in the human receptor, all these sites and their surrounding sequences are conserved in the rat and human receptor. All but serine 315 are within transactivation domains identified in human or rat receptors, and serines 212, 220 and 234 are in a highly acidic region that in the mouse receptor reduces nonspecific DNA binding and is required for full transcriptional activity. Serines 212, 220 and 234 and threonine 159 are in proline directed or $p34^{cdc2}$ kinase consensus sequences. Serine 122 is in a casein kinase II sequence. Site directed mutagenesis is being performed at these sites, singly and collectively, to determine their influence on receptor function.

L 004 PHOSPHORYLATIONS OF THE PROGESTERONE RECEPTORS: STRUCTURAL AND FUNCTIONAL CONSEQUENCES, William T. Schrader[1] Nancy L. Weigel[1], Milan K. Bagchi[1], Tim H. Carter[2], Larry A. Denner[1], Ming-Jer Tsai[1], Sophia Y. Tsai[1] and Bert W. O'Malley[1], [1]Department of Cell Biology, Baylor College Medicine, Houston, TX 77030 and [2]St. Johns University, Jamaica, NY 11439.

The chicken progesterone receptor (PR) is phosphorylated in vivo in response to hormone treatment resulting in reduced mobility on SDS gels. We have previously identified three phosphorylation sites, two of which show enhanced phosphorylation in response to progesterone and one of which is phosphorylated only in response to hormone. The progesterone receptor lacking the hormone-dependent phosphorylation is active in an in vitro transcription assay. Since the assay contains HeLa nuclear extract, we have now examined these reactions to determine whether the receptor was phosphorylated during the in vitro transcription assay. We find that the receptor is rapidly and extensively phosphorylated in this assay. Based on incorporation of [$^{32}$P] and changes in mobility on SDS gels, at least two sites are phosphorylated. The phosphorylation requires the presence of double-stranded DNA, suggesting that the DNA-dependent kinase (DNA-PK) previously purified from HeLa cells is responsible for the phosphorylation. Phosphorylation of purified PR with authentic purified DNA-PK shows the same change in mobility on SDS gels. Phosphopeptide mapping indicates that there are at least three phosphopeptides which each contain phosphoserine. These data suggest that phosphorylation following hormone administration may be a two step procedure with the first round of phosphorylation being hormone dependent followed by a subsequent DNA-dependent phosphorylation(s) to give the fully active receptor.

Human PR is also phosphorylated, but with some differences. We demonstrated previously that progesterone is required to induce binding of PR to its DNA progesterone response element (PRE). This receptor also stimulates cell-free RNA synthesis in a hormone-dependent manner. Hormone-mediated activation of PR requires more than simply removal of associated heat shock proteins. We find that treatment with hormone leads rapidly to multiple phosphorylations of both the A and B forms of human PR using HeLa nuclear extract. The kinase is present in the transcription extracts but, unlike chick PR, fails to phosphorylate receptor significantly in the absence of hormone and DNA. PR phosphorylation efficiency is enhanced in the presence of PREs, indicating that ligand-induced binding of PR to its cognate DNA response element makes it a preferred substrate for the kinase. Phosphorylation is rapid and extensive.

*Plenary Lecture*

L 005 CELLULAR MECHANISMS FOR ACTIVATION OF STEROID RECEPTOR SUPERFAMILY MEMBERS. Bert W. O'Malley, Deptartment of Cell Biology, Baylor College of Medicine, Houston, Texas 77030.

Progesterone acts by combining with a specific receptor in target cells. Authentic progesterone causes dissociation of heat shock proteins from the nascent receptor and converts the receptor into an allosteric form capable of dimerizing and binding to DNA. It is thought that a key series of phosphorylation steps occur to convert the receptor to a "transcriptionally active" form. This activated progesterone-receptor complex then binds tightly to a 15 bp recognition sequence (PRE) located at the 5'-end of target genes. The receptor then recruits and stabilizes general transcription factors at target gene promoters (TATA box) and causes RNA polymerase II to repetitively initiate transcription at the adjacent gene. During the course of progestin action, new mRNAs are generated which code for the cellular proteins required for the physiologic actions of progesterone. Compounds such as RU-486 fail to provide a receptor conformation which induces efficient gene expression, thereby acting as antihormones. In recent studies, we have discovered that many but not all members of the steroid receptor superfamily can be activated by a hormone-independent mechanism. This occurs subsequent to activation events at plasma membrane receptors which promote signal transduction via cAMP/kinase pathways. The pathway cross-talk occurs at the level of nuclear receptor phosphorylation. This surprising result generates a number of important new considerations as to how activation of this steroid/thyroid/vitamin superfamily relates to target organ physiology.

*Natural Mutations in Receptor Genes*

L 006 ESTROGEN RECEPTOR "OUTLAW" VARIANTS IN CLINICAL BREAST CANCER, William L. McGuire, and Suzanne A.W. Fuqua, University of Texas Health Science Center, Department of Medicine/Oncology, San Antonio, Texas.

The presence of ER in a primary breast tumor is a good indicator of both disease free survival and benefit from adjuvant hormonal therapy. However its usefulness is hindered by the fact that 40 to 50% of ER+ tumors will not respond to therapies directed thru ER. To determine whether variant ERs exist which may play a role in treatment response, we have examined the structure of tumor ER by dideoxysequence analysis of polymerase chain amplified cDNA. Several neutral polymorphisms were found. When sequence alterations were found, they were always coincident with wild type transcripts perhaps reflecting tumor heterogeneity. All ER+ tumors expressed low levels of a transcript missing exon 3 resulting in the deletion of the second zinc finger necessary for DNA binding. One tumor contained a 6 bp insertion within the DNA binding domain resulting in the introduction of 2 amino acids (Asn Arg) and an A to G transition (Lys to Arg substitution) within the hinge domain. The functional significance of these variants is currently under study. Mutations within the DNA binding domain were not common; alterations within this region may not play a major role in ER dysfunction. We have identified alternate splicing within the 3' region of ER within the hormone binding and hormone-dependent transactivation domain which are associated with specific receptor phenotypes. Certain ER-/PgR+ tumors contained low levels of wild type ER in addition to elevated levels of an alternatively spliced ER transcript missing exon 5. This truncated ER product is transcriptionally active and hormone-independent in transactivation assays. ER+/PgR- tumors contained elevated levels of an alternatively spliced transcript lacking exon 7 resulting in a truncation within the transactivation domain. This ER variant is unable to function as a transcriptional inducer of estrogen-responsive genes and may be involved in ER dysfunction in tumors. Our results suggest that the frequent occurrence of alternate splicing may represent a deregulation of splicing mechanisms within certain breast tumors. Conclusion: Variant ER transcripts exist in human breast tumors which may be involved in variable response to therapies directed at the ER.

## *Receptor Heat Shock Protein Interactions*

**L 007** STRUCTURE AND FUNCTION OF HETEROOLIGOMERIC FORMS OF STEROID RECEPTORS, Etienne-Emile Baulieu, Lab. Hormones, Faculté de Medecine de Bicêtre, Université Paris-Sud, INSERM U33, 94275 Bicêtre Cedex, France.

Since the original description of "8-9S", hsp90-containing, heterooligomeric forms of steroid hormone receptors, much work has been designed in order to describe receptor associated, non-steroid binding proteins, their mode of interaction and the function of the complexes formed.

Recent findings in our laboratory include the following:

1) p59, a protein found in 8-9S steroid receptors in rabbit and other mammals, has been cloned (M.C. Lebeau, N. Massol et al., submitted), and the complete sequence will be presented. This protein interacts with hsp90 and not with the steroid binding receptor itself.

2) Hsp90 interacts with two of the main domains of the steroid receptors (work with estrogen receptor ER (1) and with glucocorticosteroid receptor GR) (2): a) The DNA binding domain (DBD), and more specifically with the positively charged C-terminal region of this domain which includes the nuclear localization signal "NL1". b) The ligand binding domain (LBD), in apparently three regions, none of them being sufficient and indispensable by itself.

It is proposed that region A of hsp90 (putatively two α-helices separated by proline, with preponderance of negatively charged amino acids aligned in a so-called DNA-like conformation (3) interacts with the DBD charged segment, being thus responsible for lack of DNA binding of hsp90-receptor complexes. It is also suggested that the apparently multipoint attachement of hsp90 to LBD is related to the release of the heat-shock protein following hormone binding.

3) Hsp90 in vitro can dissociate the binding of ER to its cognate hormone responsive element (M. Sabbah, G. Redeuilh et al., in preparation). The binding equilibrium between receptor, DNA and hsp90 suggests a physiological role for the heat shock protein in the control of hormone regulated transcription.

4) New evidence has been obtained for different conformationnal changes of the progestin (R5020) or antiprogestin (RU486) bound, rabbit progesterone the progestin (R5020) or antiprogestin (RU486) bound, rabbit progesterone receptor PR (M. Renoir et al., in preparation). The results indicate differential effects of SH reagents on ligand binding and receptor forms (heterooligomeric 8-9S vs dimeric 6S or monomeric 4S).

5) Two opposite mechanisms for antihormone activity have been observed at the level of heterooligomeric receptor stability: a) The already described stabilizing effect of RU486 upon GR and PR 8-9S structure; b) A destabilizing activity of spironolactone and other antimineralocorticosteroids on the 8-9S mineralocorticosteroid receptor (MR) (4), which, combined to rapid dissociation of the ligand, may be involved in the antihormonal mode of action.

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**L 008** INTERACTION OF hsp 70 AND hsp 90 WITH HUMAN PROGESTERONE RECEPTOR, Dean P. Edwards, Patricia A. Estes, Sergio Oñate, Angelo DeMarzo, Candace A. Beck, and Steven K. Nordeen, Pathology Department, University of Colorado Health Sciences Center, Denver, Colorado 80262.

Human progesterone receptors (PR) in their inactive cytosolic form associate with at least two heat shock proteins, hsp 90 and hsp 70. Hsp 90 binding to PR *in vitro* is stabilized but not dependent on molybdate and the following treatments effectively dissociate hsp 90; 0.4 M NaCl, ATP/$Mn^{++}$ (1 hr 4°C) or warming. We have not been able to demonstrate an effect of hormone on hsp 90 dissociation independent of other treatments. Hsp 70 binding to PR is relatively insensitive to these same treatments, thus hsp 90 dissociation leaves PR-hsp 70 interactions relatively intact. ATP-dependent dissociation of PR from hsp 70 can be achieved by immobilizing the PR-complex to anti-PR MAb-resins and then treating with ATP or by partial purification of PR on ATP-agarose. The fact that hsp 70 association is retained in the absence of hsp 90, but the reverse does not occur, suggests that hsp 90 binding to PR may be dependent on hsp 70. To attempt to determine the function of hsp 90 in the cytosol PR complex we have prepared receptors to contain different proportions of associated hsp 90. The ability to form PR dimers in solution was found to correlate with the extent of hsp 90 dissociation, suggesting that the function of hsp 90 is to prevent PR monomers from dimerization. It was also observed that hormone-free PR completely dissociated from hsp 90 was virtually unable to bind to specific DNA in a gel-mobility shift assay. DNA binding required addition of progestin *in vitro*. This indicates that PR stripped of hsp 90 is not constitutively active and that hormone binding is required, independent of hsp 90 dissociation, to induce or maintain a conformation necessary for PR recognition of specific DNA. Since hsp 70 remained associated with activated PR preparations that were capable of binding to specific DNA we investigated whether hsp 70 might directly participate in DNA binding or function as a molecular chaperone to maintain PR in an active conformation required for DNA binding. No hsp 70 was detectable in specific PR-DNA complexes on gel-shift assays and dissociation of hsp 70 from activated PR preparations neither inhibited nor enhanced DNA binding activity. These results indicate that hsp 70 is not involved directly or indirectly in PR-DNA binding implying that it functions at an earlier step in receptor activation pathways, perhaps to facilitate binding and release of hsp 90 in the inactive complex. Since PR associates with hsps under normal cellular conditions, and hsps appear to play an important regulatory role, we questioned whether heat shock would alter cellular PR-hsp interactions and receptor function. Heat shock and a related chemical stress (sodium arsenite) were found to alter the composition of newly synthesized complexes and to enhance PR activity *in vivo* as measured by PR-mediated induction of target gene transcription. Possible mechanisms underlying heat shock effects on receptor activity will be discussed.

**L 009** RECONSTITUTION OF GLUCOCORTICOID RECEPTOR AND pp60$^{src}$ HETEROCOMPLEXES WITH HEAT SHOCK PROTEINS, William B. Pratt, Kevin A. Hutchison, Lawrence C. Scherrer, Louis P. Stancato, Michael J. Czar, Barbara K. Brott and Richard Jove, The University of Michigan Medical School, Ann Arbor, MI 48109-0626.

Glucocorticoid receptors that are translated in rabbit reticulocyte lysate become bound to hsp90 at the termination of their translation (1). Recently, it was shown that incubation of immunopurified, unliganded chicken progesterone receptor (PR) with rabbit reticulocyte lysate results in formation of a PR-hsp90 complex (2). Hormone-free glucocorticoid receptors (GR) that are immunoadsorbed to protein A-Sepharose or bound to DNA-cellulose become associated with hsp90 when they are incubated with reticulocyte lysate (3). Binding to hsp90 is a temperature-dependent and ATP-dependent process and the heteromeric complex that is formed contains hsp70 as well as hsp90. Reconstitution of the GR-hsp90 complex is accompanied by repression of DNA-binding activity and reactivation of the high-affinity steroid-binding conformation of the receptor. DNA-bound receptors incubated with reticulocyte lysate are released from DNA in the process of being reassociated with hsp90. It is proposed that the receptor is unfolded by the action of hsp70 and that hsp90 becomes bound either simultaneously or immediately thereafter. As hsp90 and hsp70 are known to exist in a multiprotein complex (4,5), we favor the former model in which hsp90 and hsp70 act together in unfolding the receptor and stabilizing the unfolded state (6).

We have now shown (Hutchison, Brott, De Leon, Perdew, Jove, and Pratt, submitted manuscript) that reticulocyte lysate reconstitutes the multiprotein complex between pp60$^{src}$, hsp90 and p50. The reconstitution is time-dependent, temperature-dependent and ATP-dependent, and the heterocomplex that is formed also contains hsp70. Like the steroid receptor-hsp90 complexes, the pp60$^{src}$-hsp90 complex is stabilized by molybdate, vanadate and tungstate. The presence of p50 in the complex is also stabilized by molybdate, consistent with the proposal (5) that p50 is bound to hsp90.

Currently, our laboratory is focusing on resolving the components of the reconstitution system from rabbit reticulocyte lysate. We find that the reconstitution activity can be precipitated with ammonium sulfate, but the dialyzed ammonium sulfate precipitate alone has only a limited ability to reconstitute the GR-hsp90 complex and steroid binding activity. Both activities are markedly increased by readdition of small molecular weight components of the reticulocyte lysate, and the partially purified reconstitution system is completely dependent upon the presence of an energy generating system (Supported by NIH grants DK31573 and CA28010).

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L 010 IN VITRO ASSEMBLY OF PROGESTERONE RECEPTOR WITH HEAT SHOCK PROTEINS AND RECEPTOR ACTIVATION, David Toft[1], David Smith[2], William Sullivan[1], Jill Johnson[1], and Robert Schumacher[1], Miguel Marrero[1], [1]Mayo Medical School, Rochester, [2]Memphis State University, Memphis.

To better understand assembly mechanisms of progestrone receptor (PR) complexes, we have developed a cell-free system for studying PR interactions with the 90 and 70 kDa heat shock proteins (hsp90 and hsp70), and we have used this system to examine requirements for hsp90 binding to PR. Purified chick PR, free of hsp90 and immobilized on an antibody affinity resin, will rebind hsp90 in rabbit reticulocyte lysate when several conditions are met. These include: 1) absence of progesterone, 2) elevated temperature (30°C), 3) presence of ATP, and 4) presence of $Mg^{++}$. These conditions also cause the binding of hsp70 and a receptor-associated 23 kDa protein, p23. We have obtained maximal hsp90 binding to receptor when lysate is supplemented with 3 mM $MgCl_2$ and an ATP regenerating system. ATP depletion of lysate by dialysis or by enzymatic means blocks hsp90 binding to PR and destabilizes existing complexes. Addition to lysate of a monoclonal antibody against hsp70 inhibits hsp90 binding to PR and destabilizes pre-formed complexes indicating that hsp70 and ATP are needed to form and to maintain hsp90 complexes. Much hsp90 in cellular extracts is complexed with hsp70 and a 60 kDa protein, p60. The appearance of p60 with PR complexes under some limiting conditions suggests that it may function in intermediate stages of receptor complex formation. Thus, receptor complex formation may be a multi-step process involving ATP and other cellular factors. Hormone-dependent dissociation of reconstituted receptor complexes was also examined. The addition of progesterone to the reticulocyte lysate promotes a time and temperature dependent dissociation of hsp90, hsp70, and p23 from the receptor. This dissociation event is also ATP-dependent and is optimal in the presence of an ATP regenerating system. These results illustrate the utility of this cell-free system for studying the biochemical events and molecular interactions involved in receptor assembly and activation.

*Receptor DNA Interaction*

L 011 **INTERPLAY OF TRANSCRIPTION FACTORS ON THE MMTV PROMOTER,** Thomas Preiss, Jörg Bartsch, Mathias Truss, Emily P. Slater and Miguel Beato, Institut für Molekularbiologie und Tumorforschung, Philipps Universität, D-3550 Marburg, F. R. Germany

Steroid hormones modulate the expression of a variety of genes by influencing the activity of their intracellular receptors that can either bind to hormone responsive elements (HRE) on the DNA or interact with other transcription factors. The outcome of these interactions can be induction or repression of specific genes. The MMTV promoter, that responds to glucocorticoids and progestins, as well as to androgens and mineralocorticoids, contains a complex HRE that in chromatin is covered by a phased nucleosome. Hormone treatment leads to alterations in chromatin structure that make the HRE region more accessible to digestion by DNaseI and permit binding of transcription factors, including nuclear factor I (NFI), to the MMTV promoter. NFI acts as a basal transcription factor on the MMTV promoter and does not cooperate but rather competes with the hormone receptors in terms of binding to free DNA *in vitro*. In chromatin, the precise positioning of the DNA double helix on the surface of the histone octamer precludes binding of NFI to its cognate sequence while still allowing recognition of the HRE by the hormone receptors. The changes in chromatin structure that follow receptor binding enable NFI binding and subsequent formation of a stable transcription complex. However, neither receptor nor NFI binding are sufficient to completely remove the nucleosome from the MMTV promoter, as a 10 base pairs pattern of DNaseI digestion is seen over even after hormone induction.

In addition to the NFI binding site other elements are also important for efficient transcription *in vivo*. In particular, two degenerated octamer motifs located immediately upstream of the TATA box are recognized by the ubiquitous transcription factor OTF-1 and influence the extent of induction. Under cell-free conditions, mutations in these motifs do not change basal transcription but completely abolish the stimulatory effect the receptor. Both glucocorticoid and progesterone receptors, when bound to the HRE, facilitate binding of OTF-1 to the two octamer motifs and activate transcription from the MMTV promoter *in vitro*. Recombinant NFI and OTF-1 can be used to complement nuclear extracts from HeLa cells that have been depleted of the corresponding factors. NFI does not appear to interfere nor to cooperate with OTF-1 in terms of MMTV promotor utilization. This *in vitro* assay has been used to map the functional domains of the various transcription factors required for MMTV promoter function.

Thus, steroid hormones, acting through the same receptor protein, can activate transcription by at least two different molecular mechanisms: chromatin derepression by nucleosome alteration and DNA binding cooperation with other transcription factors. We do not know whether these two induction pathways are mutually exclusive or whether they can occur simultaneously on the same template.

L 012 THE INTERACTION OF CONSTITUTIVE AND TISSUE-SPECIFIC TRANSCRIPTION FACTORS WITH POSITIONED NUCLEOSOMES. Gordon Hager, Trevor Archer, Catharine Smith, Ken Carison, Emery Bresnick, Sam John, and Philippe Lefebvre. Lab of Molecular Virology, National Cancer Institute, NIH, Bethesda MD 20892.

DNA sequences of the MMTV LTR are positioned in vivo on a phased array of six nucleosomes (A-F). Hormone activation of the MMTV promoter leads to modulation of nucleosome B structure in vivo, manifested as a region of DNA hypersensitive to nucleolytic reagents, and recruitment of transcription factors into an initiation complex. Analysis of nucleosome position using PCR amplification indicates that the nucleosomes are positioned at high resolution, and that the hypersensitivity is asymmetrically localized to the 3' side of nucleosome B and into the A-B linker region. NF1, a component of the MMTV initiation complex, is excluded from uninduced stable chromatin but binds constitutively to transiently introduced DNA. Hormone induction also leads to H1 depletion from the A-B region in stable chromatin, whereas the core histone complement of this region is unchanged. We find that a disomic structure composed of the A and B nucleosomes can be reconstituted in vitro, with the octamer cores accurately positioned, and that NF1 is excluded from this disomic structure[1]. The glucocorticoid receptor, in contrast, can bind to the disome. Thus, two potential mechanisms exist for the exclusion of NF1, either (1) exclusion by nucleosome positioning, or (2) exclusion by a higher-order, H1-dependent, chromatin structure. Modulation of the uninduced chromatin structure is necessary during transcription activation to permit binding of the initiation complex. These results indicate that a chromatin template containing specifically positioned nucleosomes is an active participant in transcriptional activation, and that modulation of this template structure is one feature of steroid hormone action.

Activation of cellular oncogenes by MMTV occurs by proviral insertion. We have identified a regulatory element in the viral regulatory sequences that is responsible for cell-specific viral transcription, and presumably also for tissue-specific oncogene activation[2]. This element, located at the 5' end of the LTR, acts as a cell-specific, positive enhancer region, and has been shown to contain binding sequences for at least two proteins, mp5 and mp4, both of which also appear to be limited in distribution to mammary cells. This element is also active in human mammary cells, and factors that bind to this region are uniquely present in human cell lines of mammary orgin. These observations suggest that MMTV protooncogene activation is mediated by a tissue-specific enhancer, which can constitutively activate a target promoter, but also function synergistically with the HRE of MMTV.

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**L 013** CORRELATIONS BETWEEN THYROID HORMONE RECEPTOR DNA BINDING AND TRANSCRIPTIONAL ACTIVATION IN THYROID HORMONE RESPONSIVE PROMOTERS, Gregory A. Brent[1], Graham R. Williams[1], Barry M. Forman[3], Herbert H. Samuels[2], David D. Moore[2], and P. Reed Larsen[1], [1]Thyroid Division and Howard Hughes Medical Institute, Brigham and Women's Hospital, [2]Department of Molecular Biology, Massachusetts General Hospital, Harvard Medical School, Boston, and [3]Division of Molecular Endocrinology, Departments of Pharmacology and Medicine, New York University Medical Center, New York.

A hexameric binding domain AGGTC/AA has been identified as the idealized binding sequence for thyroid hormone receptors. However, there is considerable variation in the arrangement of these hexamers in the promoters of thyroid hormone responsive genes with respect to spacing and orientation as well as in the nucleotide match to the idealized sequence. These genes contain direct repeats, inverted (palindromic) arrangements and inverse palindromic orientations or combinations of the above. Since at least two adjacent core sequences are required for a positive transcriptional response, $T_3$ receptor homo- or receptor-protein heterodimers are presumably formed. The availability of bacterially expressed purified chicken $T_3$ receptor $\alpha$ has permitted correlations between receptor binding and transcriptional activation by thyroid hormone. Such studies are necessary to understand the role of hexamer spacing and arrangement as well as the slight deviations from the cannonical hexameric element on the response of a given gene to thyroid hormone in model systems. We have evaluated the $T_3$REs of the $\alpha$ myosin heavy chain (rMHC) and malic enzyme (rME). The rMHC response element is analogous to the rGH promoter and contains 3 half-sites. Mutations in the B (central) domain of this response element cause marked decreases in both function and dimer binding. There is an excellent correlation between the fraction of receptor bound as dimer and $T_3$ induction using selective mutations based on the predicted contact points for the $T_3$ receptor. The malic enzyme TRE contains a direct repeat with a 4 base pair separation. Mutations which cause functional impairment also inhibit receptor binding as dimers. While there is a general correlation between the ratio of receptor to DNA required for dimer formation and the transcriptional response conferred by different $T_3$REs, this correlation is not as close as is that between function and dimer binding in mutations within a given $T_3$RE. This suggests that sequences within and surrounding the hexamers have an important role in modulating the responsiveness of the wild type promoter to $T_3$.

**L 014** IDENTIFICATION OF A CONSERVED REGION REQUIRED FOR HORMONE DEPENDENT TRANSCRIPTIONAL ACTIVATION BY STEROID HORMONE RECEPTORS. Malcolm G. Parker, Paul Danielian, and Roger White. Molecular Endocrinology Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX, U.K.

Oestrogen receptors function as transcription factors when the binding of hormone causes dissociation of receptor from an inactive complex containing hsp90 to allow receptor dimerisation, high affinity DNA binding and transcriptional activation. The regions of the receptor responsible for DNA binding and hormone binding are conserved at both the structural and functional level within members of the nuclear receptor family. We considered the possibility that certain transcriptional activation functions might also be conserved. In the oestrogen receptor, transcriptional activity depends on two transcriptional activation functions, TAF-1 in the N-terminal domain and TAF-2 in the hormone binding domain. We have now found that ligand-dependent transcriptional activity, TAF-2, requires a region of the hormone binding domain which is conserved among many nuclear receptors. Point mutagenesis of conserved hydrophobic and charged residues significantly reduced transcriptional activation but had no effect on steroid or DNA binding. In addition, the mutation of the hydrophobic residues abolished the ability of TAF-2 to synergise with TAF-1. Mutation of the corresponding residues in the glucocorticoid receptor also abolished transcriptional activation in a similar fashion to that for the oestrogen receptor indicating that the function of these residues is conserved. The importance of the conserved region is consistent with the observation that its deletion is responsible for the lack of transcriptional activation by v-erb A. We therefore propose that the conserved region may be essential for ligand dependent transcriptional activation not only by steroid receptors but also by retinoic acid and thyroid hormone receptors.

*Receptor and Cell Biology*

**L 015** ESTROGEN RECEPTORS BEHAVE IN INTACT CELLS AS MONOMERIC PROTEINS AND NOT AS HOMODIMERS. Jack Gorski, Departments of Biochemistry and Animal Sciences, University of Wisconsin-Madison, Madison, WI 53706

Steroid receptors are widely proclaimed to function as homodimers. Notides was the first to show that cytosolic estrogen receptors behaved as dimers in vitro, binding estrogens in a cooperative manner. Our group and others have confirmed these observations. However, estrogen receptors in intact cells are nuclear proteins, probably bound to a nuclear component even in the absence of estrogen binding. We have reexamined equilibrium estrogen binding to its receptor in intact cells and found that such binding appears to be monomolecular with no evidence of cooperativity. Extreme care was taken to assess the levels of free steroid in the media to ensure that estimates of affinity at low concentrations were accurate. Cultured rat uterine cells that respond to estrogen by increases in the accumulation of progesterone receptors were used in these studies. If a homodimer model is involved in estrogen receptor function, it is predicted that the response to estrogen is modified when receptor occupancy is 50%. Some homodimeric receptors would have one occupied site and one unoccupied site (if each site has equal affinity for the estrogen, then 50% of the receptors would be in this state). What would be the activity of such homodimer with both an unoccupied and an occupied site? Similarly, what would be the activity of a homodimer with one site occupied with an estrogen and one site occupied with an antiestrogen? Dose response studies in intact cells indicate that the response to mixtures of estrogens and antiestrogens behaves as if each receptor site is an independent entity with no evidence that a two-site receptor is involved. Older data have clearly shown that the occupied estrogen receptor is differentially extracted from the nucleus as compared to the unoccupied receptor. Examination of such data also fails to indicate any evidence of a dimer being involved in the acquisition of the tight nuclear binding state. The behavior of the estrogen receptor in the intact cell is best explained by a monomeric model or by a heterodimer model in which the partner molecule is not rate limiting in estrogen response systems.

**L 016** DEVELOPMENTAL, HORMONAL AND SPATIAL EXPRESSION OF STEROID/THYROID HORMONE RECEPTOR GENES DURING AMPHIBIAN METAMORPHOSIS. Jamshed R. Tata, Betty S. Baker and Elida Rabelo, Laboratory of Developmental Biochemistry, MRC National Institute for Medical Research, London NW7 1AA, U.K.

This review will largely deal with how prolactin (PRL) prevents the rapid auto-induction by thyroid hormone (TH) of genes encoding both the α and β isoforms of its own receptor (TRα and β) during *Xenopus* metamorphosis. It will also briefly describe the induction of estrogen receptor (ER) transcripts by triiodothyronine ($T_3$) during metamorphosis and the consequent precocious activation of vitellogenin genes.

Abundant TR mRNA is detected in stage 1 and 2 oocytes in *Xenopus*. It is also found in small amounts in embryos and early larval stages. An abrupt and substantial accumulation of TRα and β mRNA begins at stage 44, when the early tadpole is known to acquire responsiveness to TH[1]. The accumulation reaches a maximum until metamorphic climax and then declines rapidly in adult tissues. *In situ* hybridization shows that the brain, spinal cord, limb bud, intestinal epithelium, and tail are major sites of localization of TR mRNA[2]. Administration of exogenous $T_3$ to *Xenopus* tadpoles at developmental stages 50-52, i.e. before endogenous THs are secreted, causes a massive and rapid induction of TR mRNA particularly noticeable for TRβ (within 4 hr)[3]. This auto-induction can be considered as 'early' gene activation. In contrast, the slow activation requiring 2-3 days of the 63 kDa keratin and albumin genes in whole stage 50-54 tadpoles and organ cultures of tails can be considered as the response of 'late' $T_3$-inducible genes.

Exogenous PRL inhibits normal and TH-induced metamorphosis, both in intact tadpoles and in cultures of tissues programmed for morphogenesis and cell death[4]. In an attempt to explain the anti-metamorphic or juvenilizing action of PRL, we have examined its effect on early and late gene responses of tadpole tissues to $T_3$. PRL abolished both the rapid auto-induction of TRα and β mRNAs and the late activation of 63 kDa keratin and albumin genes.

At the onset of natural metamorphosis PRL levels drop as TH levels increase simultaneously. In a model based on auto-induction of TR to explain the juvenilizing action of PRL in metamorphosis, the first action of TH would be to act on the very small amount of TR, possibly of maternal origin, to enhance the formation of its own mRNA and protein, assuming that TR gene promoters ('early' genes) have very high affinity sites for TR. The rapid amplification of TR, caused by the increasing secretion of TH accompanying the release from the inhibition by PRL of TR auto-induction, would result in the activation of the more slowly responding 'late' genes in different tissues, such as those encoding adult hemoglobin, albumin and 63 kDa keratin. The *de novo* appearance of the latter constitutes the acquisition of the adult phenotype as metamorphosis reaches its climax.

During late metamorphosis (stages 60 onwards), $T_3$ induces *de novo* ER mRNA, exclusively in the liver. The consequence of this induction of a different member of the steroid/thyroid hormone receptor family is the precocious activation of the silent vitellogenin genes in tadpole and froglet liver if estrogen ($E_2$) is present along with $T_3$. Normally $E_2$ synthesis, acquisition of ER and vitellogenesis occur late in adult life.

The hormonal and spatial control of expression of nuclear hormone receptor genes is therefore an important factor determining the timing and progression of post-embryonic developmental processes.

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## *Plenary Lecture*

**L 017** STRUCTURE AND FUNCTIONAL INTERACTIONS OF GENERAL INITIATION FACTORS, REGULATORY FACTORS AND COFACTORS. Robert G. Roeder, Ananda Roy, Micheal Meisterernst, Philippe Pognonec, Yan Luo, and Hiroshi Fujii, Laboratory of Biochemistry and Molecular Biology, The Rockefeller University, New York, NY 10021.

Continued studies of the purification and characterization of general factors, which are ubitquitous and commonly required by most class II genes, have implicated factors IIA, IIB, IID, IIE, IIF, IIG, and II-I in transcription initiation from minimal (core) promoters by RNA polymerase II. Amongst these factors, TFIIA and TFII-I are functionally interchangeable and form alternate preinitiation complexes on the adenovirus ML promoter, which suggests the possibility of selective responses to different regulatory factors. Unlike TFIIA, TFII-I binds stably to a number of initiator elements, but, like TFIIA, interacts cooperatively with TFIID. TFII-I also has been shown to interact cooperatively with the upstream activator USF (a helix-loop-helix protein), suggesting a novel mechanism for communication between regulatory factors and the general transcriptional machinery. The cloning of TFII-I has revealed structural relationships with helix-loop-helix proteins.

Studies of the AdML and HIV promoters have identified a novel cofactor (USA) that is required for physiological levels of promoter induction in purified systems (with general factors) by USF (AdML), Sp1 (HIV) and NFkB (HIV). The action of USA involves both a large net increase in promoter activity in conjunction with the activator and a repression of activator-independent activity. Fractionation and mechanistic studies indicate the involvement of both a negative cofactor (NC1) which competes with TFIIA for binding to TFIID, leading to basal repression, and a positive cofactor (PC1) which, with the activator, reverses the action of NC1 and effects a large positive promoter response. These results, along with the identification of other negative and positive factors interacting with TFIID, suggest novel promoter regulatory mechanisms that differ from previous models involving simple adaptors. Although the action of USF appears general, the possibility of activator-specific components has not been ruled out.

Related studies have revealed that the ubiquitous Oct1 and the lymphoid-restricted Oct2 are indistinguishable with respect to their intrinsic abilities to activate immunoglobulin (Ig), H2B, or snRNA promoters but that they interact with additional factors in a promoter-specific way to enhance transcription by the common factors. The isolation and characterization of a novel B cell-specific accessory factor (BAF) that specifically and markedly enhances Ig promoter activity via Oct1 or Oct2 suggests the existence of a new class of tissue specific cofactors/coactivators that may play a major role in the determination of promoter specificity and activity in vivo.

## *Receptor Biochemistry*

**L 018** ON THE MECHANISM OF DNA BINDING BY NUCLEAR RECEPTORS, Leonard P. Freedman[1], Min Liu[1], Terri L. Towers[1], Ben F. Luisi[2], Wexin Xu[2], Z. Otwinowski[2], Keith R. Yamamoto[3], and Paul B. Sigler[2], [1]Cell Biology & Genetics Program, Sloan-Kettering Institute, New York, NY 10021, [2]Department of Molecular Biophysics & Biochemistry and HHMI, Yale University, New Haven, CT 06511, and [3]Department of Biochemistry & Biophysics, UCSF, San Francsico, CA 94143-0448.

Structural and functional approaches have been used to study the DNA binding domain of two nuclear receptors. This domain is a discrete, short region with two $Zn^{2+}$ binding sites, each forming a peptide "finger". The metal is tetrahedrally coordinated by four cysteine sulfurs; this coordination is essential for specific DNA binding and protein folding. The three-dimensional structure of the glucocorticoid receptor (GR) DNA binding domain bound to a glucocorticoid response element (GRE) has recently been determined by x-ray crystallography (1). The amino-terminal finger associates with the phosphate backbone of the DNA, and an a-helical region immediately C-terminal to the first finger interacts with specific bases in the major groove. Although in solution the protein is monomeric, it dimerizes head-to-head upon DNA binding through reciprocal interactions of a region within the carboxy-terminal finger. The nature of the dimer interface explains why protein binding to a GRE has been observed to be cooperative, and why typical GREs consist of two half-sites oriented as inverted repeats separated by three base pairs.

In contrast to the GR paradigm, receptors such as those for vitamin D3, thyroid hormone, and retinoic acid (VDR, TR, and RAR, respectively) appear to bind to cognate response elements organized as direct repeats, with half-site spacings of at least three to five nucleotides. The preference these receptors have for direct repeats implies that their DNA binding domains may utilize different stratagies from those that GR uses to recognize and bind the inverted repeats. We have overexpressed and purified the vitamin $D_3$ receptor DNA binding domain, and begun to characterize its DNA binding properties. We find that the VDR derivative binds strongly and specifically to direct repeats constituting a vitamin D response element (VDRE) from the mouse osteopontin (Spp-1) promoter region. Unlike GR, VDR DNA binding domain appears to bind two half-sites noncooperatively, without the free energy contribution of dimerization seen when the GR DNA binding domain associates with a GRE. This is consistent with the notion that VDR binds to direct repeats in a head-to-tail configuration, as suggested by the orientation of the half-sites. A series of synthetic binding sites similar to the Spp-1 VDRE were made that differed only in the half-site spacing between the two direct repeats (DR + 3, 4, 5). Remarkably, even the minimal VDR DNA binding domain appears to be influenced by half-site spacing, as has been previously shown for full-length VDR, TR, and RAR (the "3, 4, 5, rule"), in that it only binds as a dimer to a DR+3. Thus although the protein most likely binds in a head-to-tail configuration, these results infer that monomers of the vitamin $D_3$ DNA binding domain make decisive contacts with one another as they bind two optimally spaced VDRE half-sites. The nature of those contacts are currently being investigated.

(1) B.F. Luisi et al. (1991) *Nature* 352, 497-505.

**L 019** THE HUMAN GLUCOCORTICOID RECEPTOR: STRUCTURE/FUNCTION RELATIONSHIPS IN GENE CONTROL AND CELL VIABILITY. E. B. Thompson, G. Srinivasan, B. H. Johnson, L. V. Nazareth, R. Thulasi, and R. D. Stith*, Dept. of Human Biological Chemistry & Genetics, University of Texas Medical Branch, Galveston, TX 77550 and *Dept. of Physiology & Biophysics, University of Oklahoma, Oklahoma City, OK 73190.

Glucocorticoids (GCs) kill certain leukemic lymphoblasts by direct interaction with malignant cells that contain functional intracellular glucocorticoid receptors (GRs). Normal lymphoid cells' activity and viability can also be modulated by GCs indirectly, via their modulation of a variety of lymphokines. To what extent such modulation applies to GCs' effects on leukemias is not clear. In clones of CEM cells (derived from a human acute lymphoblastic leukemia) we find that lymphokines interleukin-2 (IL-2), IL-1, IL-6, and interferon afford no protection against G-induced cell lysis, although these lymphokines have been reported to protect normal lymphoid cells (Gillis, *et al.*, J.Immunol.123:1624-31,1979; Almawi, et al., J.Immunol.146:3523-27,1991). Our CEM clones therefore provide a model in which the direct actions of GCs responsible for cell death can be studied. By transfecting a CEM subclone resistant to GCs with an expression vector containing holoGR, we have shown that one can restore GC sensitivity (Harbour, et al., J.Steroid Biochem.35:1-9,1990). GRs act as transcription factors by binding to quasi-palindromic, cis-acting heptameric sequences (GREs) in or near regulated genes. Other important properties of GRs are their ability to dimerize, to form noncovalent protein and RNA heteropolymers, and to serve as substrates for site-specific phosphorylations. GRs have been mapped extensively for their ability to stimulate gene transcription, and "domains" responsible for site-specific DNA binding, dimerization, protein:protein interactions, transcriptional trans-activation, etc., have been defined.

How GC:GR complexes work to kill cells is not known. By transfecting GC-resistant CEM cells with expression vectors containing various mutant GR genes, we are mapping the GR for the structural elements necessary for lethality. Our data indicate that the portion of the GR specifying interaction with GREs is essential, but that little else of the GR protein is required. Deletion of the steroid binding domain (SBD) results in a 100% active, ligand-independent, constitutive GR for stimulation of lethal response. The same mutants in transcription-enhancement assays were constitutive but had only 1 -10% activity compared to holoGR plus steroid. The limit GR mutant so far found effective in killing GR-deficient CEM clone ICR27 coded for amino acids 1-9, 386-532 of the holoreceptor and was therefore lacking all known transcription-enhancing, steroid-binding, nuclear localization, and non-GR protein-binding sequences. Among the more interesting mutants is 465*. This gene has not only lost the SBD, but also has a frame-shift mutation that disrupts most of the carboxy-terminal "zinc finger" of the critical GRE-binding domain. We are now producing this protein in the baculovirus system to determine whether it can bind to GRE sequences and whether it is active in a GRE-driven in vitro transcription system.

These results suggest that the critical property of the GR in killing cells is not induction of specific genes. However, GRE-occupying GR fragments could interfere with transcription of genes essential for cell viability. A candidate for such genes in CEM cells is c-myc. This gene is rapidly suppressed in G-sensitive clones exposed to dexamethasone. Introducing c-myc on vectors that are not down-regulated by GCs protects cells that otherwise are lysed by the steroids. Preventing expression of c-myc by antisense oligonucleotides also kills the cells.

We conclude that GR fragments may act to kill CEM cells by suppressing genes critical for cell viability, and that c-myc may be such a gene.

**L 020** STRUCTURAL STUDIES OF THE THYROID HORMONE RECEPTOR AND THE ANDROGEN RECEPTOR AS A THERAPEUTIC TARGET, Jeffrey H. Toney[1], Ling Wu[1], Gautam Sanyal[2], Barry M. Forman[3], Jaibi Zhu[3], Michael J. McPhaul[4], Ann E. Summerfield[1], Samuel Wong[1], and Herbert H. Samuels[3], [1]Merck, Sharp & Dohme Research Laboratories (MSDRL), Department of Biochemistry, Rahway, NJ, [2]MSDRL, Pharmaceutical Research, West Point, PA, [3]New York University Medical Center, Department of Medicine, New York, NY, [4]The University of Texas Southwestern Medical Center, Department of Biochemistry, Dallas, TX

The thyroid hormone receptor ($T_3R$) and androgen receptor (AR) are both members of a superfamily of ligand-responsive transcription factors that play a critical role in development and differentiation. As a first step towards activation of transcription, these receptors are thought to undergo conformational changes upon ligand binding which then potentiate DNA binding. This paper will present the first physical chemical data to support this idea in the case of the $T_3R$. Chicken $T_3R\,\alpha 1$ ($cT_3R$-$\alpha 1$) has been expressed in *Escherichia coli* (*E. coli*) using a vector containing a promoter for T7 RNA polymerase. The resultant receptor was found to be fully functional in specific DNA binding to a thyroid hormone response element (TRE)-containing oligonucleotide as well as in binding to $^{125}$I-$T_3$. Functional $cT_3R$-$\alpha 1$ was purified to homogeneity and studied by circular dichroism (CD). Hormone-free receptor was found to possess high $\alpha$-helical content. Addition of a cognate ligand L-3,5,3'-triiodothyroacetic acid (triac) increased stability of receptor conformation against thermal denaturation as monitored by CD. The human AR has also been expressed in functional form in both Chinese hamster ovary cell stable transfectants ($K_d$ = 0.11 nM for dihydrotestosterone) and in *E. coli*. The expressed hAR can be used for screening assays to monitor interaction of compounds with the receptor. Emerging structure-activity relationships will be presented. Potential application of hAR antagonists to the management of prostatic carcinoma will be discussed.

*Control of Receptor Gene Expression*

**L 021** REGULATION OF GLUCOCORTICOID RECEPTOR GENE AND PROTEIN EXPRESSION: John A. Cidlowski, Kerry L. Burnstein, Christine M. Jewell, Victoria Allgood, Corinne Silva, Deborah Bellingham, Douglas Tully and Madhabananda Sar, Department of Physiology, Cell Biology and Anatomy and the Lineberger Cancer Center Cell Biology Program, University of North Carolina at Chapel Hill, N.C. 27599.

Glucocorticoid receptors (GR) are ligand-dependent transcription factors that are subject to down regulation by their cognate ligand; however, the mechanisms mediating this response are not fully understood. Studies from several laboratories including our own have shown that down regulation of the glucocorticoid receptor by ligand occurs at the level of both GR gene expression and GR protein metabolism. To evaluate the mechanisms mediating the down regulation response, we have studied GR regulation in both transiently and stably transfected cells. Our studies show that the hGR cDNA, devoid of 5' and 3' flanking sequences, contains regulatory elements sufficient to elicit down regulation of both hGR mRNA and protein in response to glucocorticoids. Interestingly, this autoregulation also occurs when the GR is occupied by the glucocorticoid antagonist RU486. Run-on transcription analysis of the transfected hGR gene shows that the GR has a pronounced inhibitory effect on the transcription of the hGR gene and a smaller effect on hGR mRNA stability. Autoregulation of GR gene expression by GR requires both DNA and steroid binding functions of the GR. Mapping studies reveal that the intragenic regulatory sequences crucial for receptor autoregulation by ligand reside in the DNA and steroid binding domains of the hGR cDNA. We have used receptor immunohistochemistry to gain insight into the mechanism of glucocorticoid dependent down regulation of the GR protein. These studies indicate that GR is exported from nuclei in the presence of hormone and that GR protein degradation is a cytoplasmic event. Finally, we provide evidence that down regulation of GR is a component of the process by which cells attenuate their response to ligand induced signal transduction. Supported by DK 32460.

L 022 ESTROGEN AND PROGESTIN ACTION IN BREAST CANCER CELLS AND ALTERNATE PATHWAYS OF RECEPTOR ACTIVATION, Saverio Bettuzzi*, Alan Robinson#, Robin Fuchs-Young# and Geoffrey L. Greene#. *Universita di Modena, Istituto Chimica Biologica, 41100 Modena, Italy, #University of Chicago, Ben May Institute, Chicago, Illinois 60637

Female sex steroids control the growth and progression of hormone responsive cancers through the coordinate regulation of specific gene networks. A useful model system for studying the molecular basis of this activity is cultured human breast cancer cells, in which estrogens and progestins control proliferation and other specialized cell activities through their cognate receptor proteins. To better understand some of the underlying mechanisms, we have isolated, sequenced, and expressed, both *in vitro* and *in vivo*, human ER and PR cDNAs, and studied receptor structure and activity in various cells. An unexpected observation was the sensitivity of CHO cells stably transfected with hER to estrogens. In cells expressing high levels of hER, estrogens were cytotoxic. The partial antagonist hydroxytamoxifen was equally toxic, whereas the complete antagonist ICI-164 was not. It is still not clear whether some form of ER-mediated squelching is occurring, or whether induction or suppression of a gene(s) involved in replication might be occurring, but it appears that when some nontarget cells express ER constitutively, estrogens are growth inhibitory. In regard to mechanisms of target gene regulation, it is clear that estrogens induce rapid increased levels of phosphorylation of hER in CHO-ER cells. Preliminary data suggest that serine residues are involved. Recent experiments have shown a synergistic action of estradiol and cAMP on the induction of various ERE-tk-CAT reporter plasmids in Hela, CHO, T47D and MCF-7 cells that express either recombinant or natural hER. This response has both hormone dependent and independent components. However, the presence of hER is absolutely required. What is not yet clear is whether hER is being directly phosphorylated in response to cAMP, or whether hER is interacting with another protein whose activity is stimulated by cAMP. What is intriguing about this phenomenon is that it suggests an alternate pathway for regulating the transcriptional activity of steroid receptors. Because the PR gene is a target for estrogens, progestins and their antagonists, human genomic DNA containing approximately 7 kb of 5' untranslated flanking sequence has been isolated, sequenced, and used to create CAT reporter plasmids for studying the regulation of hPR expression. A transcription start site and promoter region for the full length B form of hPR have been identified. Although we have been unable thus far to demonstrate direct estrogen or progestin effects on hPR-CAT reporters, several potential ER and PR binding sites, as well as other regulatory elements, have been found in the 3 kb region preceeding the hPR promotor.

L 023 ESTROGEN RECEPTOR AND PROGESTERONE RECEPTOR REGULATION: LIGAND DISCRIMINATION AND MODULATION OF RECEPTOR ACTIVITY, Benita S. Katzenellenbogen, Susan M. Aronica, Hyeseong Cho, William L. Kraus, Pascale LeGoff, Farzad Pakdel, Joseph C. Reese, Cynthia H. Wooge and Carol K. Wrenn, University of Illinois, Department of Physiology & Biophysics, Urbana, IL 61801.

Affinity labeling studies with covalently attaching estrogens (Es) and antiestrogens (AEs) and the use of site directed mutants have identified cys 530 and cys 381 as sites of covalent labeling by aziridine analogs of Es and AEs. Site-directed mutagenesis of select amino acids in these regions of the ER hormone-binding domain, and random chemical mutagenesis of the ER cDNA with screening in yeast, followed by assessment of interesting mutants in mammalian cells, have provided information about regions of the receptor important in ligand binding and in discrimination between Es and AEs. Of note, some mutants which fail to respond to Es still respond to AEs and vice versa, and some mutants are even more sensitive to low concentrations of estrogen than is wild type receptor. Changes of specific amino acids near cys 530 result in changes in binding affinity of receptor for one category of ligands (Es) but not for another (AEs), resulting in a greatly reduced potency of Es in transactivation and a greatly enhanced sensitivity to AEs in suppression of E-stimulated transcription, suggesting that these amino acids near cys 530 are involved in receptor discrimination between Es and AEs. Studies on ER regulation of progesterone receptor biosynthesis and ER regulation of transfected estrogen response element-CAT reporter plasmids indicate that some growth factors such as IGF-1 and agents that increase intracellular cAMP influence the ability of the ER to stimulate transcription. In addition, protein kinase inhibitors and antiestrogens suppress the increases evoked by cAMP, IGF-1 and estrogen indicating the involvement of the ER and phosphorylation pathways. Direct phosphorylation studies show that many of these agents do alter the magnitude of ER phosphorylation, although the role of phosphorylation in transcription regulation is not clear. Our studies provide evidence for multifactor regulation of ER bioactivity and are beginning to provide a detailed picture of the ER hormone binding domain and amino acids important in ligand binding and discrimination between different categories of agonist and antagonist ligands.

*In Vitro Transcription Regulation By Receptors*

L 024 MOLECULAR ANALYSIS OF THE NEONATAL SURVIVAL LOCUS *alf/hsdr-1*: FAILURE TO ACTIVATE GLUCOCORTICOID-DEPENDENT GENE EXPRESSION IN LIVER AND KIDNEY, Günther Schütz, Gavin Kelsey, Siegfried Ruppert, and Andreas Schedl, Institute of Cell and Tumor Biology, German Cancer Research Center, 6900 Heidelberg, FRG.

Mice homozygous for a subset of the chromosomal deletions at the albino locus die shortly after birth. Lethality is associated with biochemical and ultrastructural lesions in hepatocytes and tubular cells of the kidney. The most profound biochemical abnormality is failure of hormonal induction of several mRNAs in the liver, including mRNAs encoding enzymes of gluconeogenesis and the urea cycle. By exploiting a set of genetically characterized deletions around the *alf/hsdr-1* locus we have confined *alf/hsdr-1* to a 300 kb segment. Molecular access to the minimal region was subsequently gained by chromosomal jumping and walking. Within the genetically defined region for *alf/hsdr-1* we isolated a transcript coding for fumarylacetoacetate hydrolase (FAH), an enzyme involved in tyrosine metabolism. FAH deficiency is the primary defect in human tyrosinemia type I, a disease associated with severe liver failure leading to lethality in the infant. Analysis of its temporal and spatial expression strongly suggests that FAH is encoded at *alf/hsdr-1*. Moreover, we have been able to phenocopy the *alf/hsdr-1* mutant phenotype in primary hepatocytes by experimentally mimicking the FAH deficient state. Expression of FAH in transgenic mutant mice restores gene expression suggesting that deficiency of this enzyme is the primary cause for the failure to activate glucocorticoid-dependent genes. The basis for the impairment of glucocorticoid receptor mediated induction in liver and kidney is presently elucidated.

**L 025** ESTROGEN RECEPTOR REGULATION OF GENE TRANSCRIPTION AND mRNA STABILITY, David J. Shapiro, Ann M. Nardulli Hong Xing, Jongsook K. Ahn, David A. Nielsen and Robin Dodson, Department of Biochemistry, 430 RAL, University of Illinois, 1209 West California St., Urbana, IL 61801

Estrogen induces both an increase of several thousand fold in the rate of hepatic vitellogenin gene transcription and a >20 fold increase in the cytoplasmic stability of vitellogenin mRNA.

To analyze the mechanism of estrogen regulation of gene transcription we are using synthetic estrogen regulated promoters, and wild type and mutant forms of the Xenopus laevis estrogen receptor (ER). The role of DNA topology in the interaction between ER, the estrogen response element, and the transcription apparatus, will be discussed. We are also using HeLa cell transcription extracts and a fractionated yeast transcription extract to study the role of the hormone ligand, and to examine early events in transcription activation.

The interaction of the DNA binding domain of the estrogen receptor and ER-regulated promoters has been examined in vivo and by investigating transcription regulation by the purified, bacterially expressed, protein. The DNA binding domain activates transcription from vitellogenin derived promoters. The monomeric DNA binding domain is able to function as a weak dominant negative mutant on synthetic ERE-containing promoters. A single estrogen receptor mutant can therefore serve as both an activator and a repressor of transcription on different estrogen regulated promoters.

A transfection system using a homologous Xenopus liver cell line, a minivitellogenin mRNA lacking most of the protein coding region, and the Xenopus estrogen receptor has been used to examine the regulation of vitellogenin mRNA stability. The extraordinary stability of vitellogenin mRNA in estrogen stimulated liver cells appears to derive from both tissue-specific mRNA stabilization, and stabilization of the mRNA by the estradiol-ER complex. A novel mRNA sequence important in 3'-end formation and polyadenylation, has been identified, as has an mRNA segment essential for estrogen stabilization of vitellogenin mRNA.

**L 026** ROLE OF LIGAND IN ACTIVATION OF PROGESTERONE RECEPTOR AND CHARACTERIZATION OF COUP-TRANSCRIPTION FACTORS, M.-J. Tsai, M.K. Bagchi, G.F. Allan, Austin Cooney, S.Y. Tsai and B.W. O'Malley, Baylor College of Medicine, Houston, TX, USA 77030.

Steroid receptor mediated stimulation of target gene expression has been demonstrated to be dependent on hormone *in vivo* and *in vitro*. It is known that ligand-free progesterone receptor (PR) is functionally inactive, associated with numerous heat shock proteins. It has been proposed that ligand-induced receptor activation involves removal of such receptor-associated proteins. To examine if release of the receptor-associated proteins is sufficient for activation of the progesterone receptor, we have isolated the ligand-free receptor from human mammary carcinoma (T47D) cells, free of hsp90, hsp70, and hsp56 proteins following several column chromatographic steps. The receptor prepared in this way was then used in DNA binding and cell-free transcription assays. We demonstrated for the first time that dissociation of heat shock proteins is insufficient for the activation of receptor function. The purified receptor still requires hormone for high affinity binding to a hormone response element (PRE) and to trans-activate a PRE-containing target promoter in a cell free transcription system. These results led us to propose that an additional hormone-mediated activation event must precede target gene activation in the cell. We next examined the possibilities that hormone induces an allosteric change in receptor conformation and/or a covalent modification of the receptor via phosphorylation. Using limited protease digestion and Western analysis, we have shown that a hormone dependent conformational change and phosphorylation of the receptor occur prior to the activation of target template.

In addition to studying the role of hormone in activation of progesterone receptor, we have characterized another subfamily of the receptor superfamily, COUP-transcription factors (COUP-TFs). Members of this subfamily bind to many promoter elements and are important for the expression of those genes. Examination of binding sequences indicated that they are either a direct repeat or an inverted repeat of the GGTCA motif. However, it was demonstrated earlier that COUP-TFs exist in solution as stable dimers. These results raise an interesting question about how a stable dimer can bind to both direct and inverted repeat sequences. Experiments have been carried out to distinguish different possible mechanisms and will be discussed.

**L 027** CONTROL OF THE PEROXISOMAL β-OXIDATION PATHWAY BY A NOVEL FAMILY OF NUCLEAR HORMONE RECEPTORS, Walter Wahli[1], Hansjörg Keller[1], Grigorios Krey[1], Françoise Givel[1], Gerd Helftenbein[2], Christine Dreyer[2], [1]Institut de Biologie animale, Université de Lausanne, CH-1015 Lausanne, Switzerland, [2]Max-Planck-Institut für Entwicklungsbiologie, D-7400 Tübingen, Germany.

Three novel members of the *Xenopus* nuclear hormone receptor superfamily have been cloned. Analysis of their structure indicated that they are closely related to each other and similar to the group of receptors that include those for thyroid hormones, retinoids and vitamin $D_3$. A differential expression of these receptors has been found in *Xenopus*. Transcripts of two of them (xPPAR-1 and xPPAR-2) are detected during oogenesis, in embryonic and larval stages and are present in all adult organs tested. The third one (xPPAR-3) is prominent only in fat body, kidney and liver. Transcriptional activity of all three receptors is regulated by agents causing peroxisome proliferation and carcinogenesis in rodent liver. Thus, we propose that together with a recently cloned mouse receptor, they form the new class of peroxisome proliferator activated receptors (PPAR). In transient co-transfection experiments, all three *Xenopus* receptors stimulated the promoter of the acyl-CoA oxidase gene that encodes the key enzyme of the peroxisomal fatty acid β-oxidation spiral, which is strongly stimulated by peroxisome proliferators *in vivo*. Therefore, PPARs may be essential control elements of the metabolism of fatty acids. Furthermore, they may be involved in morphogenesis and in liver tumor promotion by endogenous and xenobiotic agents causing peroxisome proliferation.

*Receptors and Development*

**L 028** AMPHIBIAN METAMORPHOSIS - THYROID HORMONE INDUCED CHANGES IN GENE EXPRESSION, Donald D. Brown, Leonard Buckbinder, Brian Elicieri, Akira Kanamori, Yun-Bo Shi, Kenneth Vernick, Zhou Wang, Department of Embryology, Carnegie Institution of Washington, 115 W. University Parkway, Baltimore, MD 21210.

Thyroid hormone (TH) is the biological determinant for amphibian metamorphosis. We have devised a "gene expression screen" which enables us to estimate the number of TH-induced up- and down-regulated genes in a given tissue. This subtractive library method has been applied to tail resorption, limb growth, intestinal change, brain change and epithelial cell transformation. The first stage of this research has been to clone and identify TH-regulated genes in these tissues, analyze their tissue specificity and the kinetics of their induction. We will report on the state of our progress. This research is supported by an NIH grant and a grant from the Lucille P. Markey Charitable Trust.

**L 029** POTENTIAL ROLE OF THE β1–THYROID HORMONE RECEPTOR IN T3-DEPENDENT AND T3-INDEPENDENT DEVELOPMENT OF NEONATAL RAT BRAIN. Jack H. Oppenheimer, Kevin A.Strait, Lanling Zou, and Harold L. Schwartz Section of Endocrinology and Metabolism, Department of Medicine, University of Minnesota, Minneapolis MN 55455

We have recently begun to explore the molecular basis of thyroid hormone-stimulated events in the neonatal rat brain. Northern analysis combined with RNAs protection studies have shown that in brain there is a striking 40-fold increase in the expression of the β1 thyroid hormone receptor (TR-β1) from gestational day 19 to postnatal day 10 whereas the level of the other thyroid hormone-binding receptor, TR-α1, undergoes only a transient 3-fold increase. The rise in TR-β1 coincides with the rise in brain triiodothyronine (T3) and immediately precedes the structural changes induced by the hormone. All regions of the brain studied show a rise in TR-β1 mRNA. This is not dependent on the thyroidal state. Immunoprecipitation of T3 binding activity with a specific IgG to TR–β1 showed a marked increase in TR β1-protein. Immunohistochemical studies pointed to a strong β1 signal in cerebellar Purkinje cells, a widely recognized target in the developmental actions of thyroid hormone. Three mRNAs preferentially expressed in Purkinje cells coding for the IP3 receptor, calbindin, and PCP2 rose within the first 10 neonatal days and reached near maximal levels by day 15-20. Pups rendered hypothyroid by addition of.025% methimazole to the drinking water of the nursing dams markedly slowed the rise of these mRNAs. The same maximal values were attained but only after a considerable delay. The kinetics of mRNA accretion in methimazole-treated rats were normalized by injection of 0.1 μg T3/d. Studies were initiated to define the mechanism underlying the rise in PCP2 mRNA. A construct (Construct A) consisting of the 408 bp 5' flanking DNA of PCP2 and 430 bp corresponding to the 5' untranslated sequence of the mRNA were transiently transfected into CHO cells. Cotransfection of TR-β1 resulted in a 6-fold increase in the β galactosidase reporter expression even in the absence of T3. An additional 40% increase in reporter gene expression occurred with the addition of $10^{-7}$M T3 ($p<.01$). Cotransfection of TRα1 was without effect either in the presence or absence of T3. As expected, when a construct containing an inverted repeat TRE linked to a MTV promoter and a CAT reporter (Construct B) was co-transfected either with TR-α1 or TR-β1, reporter gene expression increased 7-10-fold but only in the presence of T3. Further evidence for a TR–β1 specific regulatory element in Construct A is based on the finding that deletion of bp -408 to -205 from the 5' terminus of construct A results in a construct (Construct C) which shows approximately 50% fall in TR-β1-stimulated regulation. Ligation of the deleted segment to a MTV promoter and a CAT reporter results in chimeric construct (Construct D) which is upregulated (4 to 6 x) in a TR–β1 specific T3 independent manner. Since Construct C continues to show substantial TR-β1 regulation we deduce that an additional TR-β1 response element must be located either in the remaining flanking DNA or in the 5' untranslated portions of the gene incorporated into Construct A. Thus, TR-β1 per se may both support a rise in the level of the Purkinje cell mRNAs in the absence of T3 and mediate the accelerated rate of their accretion in the presence of the hormone. Given the multiplicity of interacting factors involved in late brain development synchronous coordination of such events may be essential in establishing normal structure and function. The role of thyroid hormone could be to assure an appropriate rate of expression of critical genes to meet the specifications of the developmental schedule.

*Receptor Mediated Positive and Negative Regulation*

**L 030** PEPCK GENE TRANSCRIPTION: INTEGRATION OF HORMONAL, DEVELOPMENTAL AND TISSUE SPECIFIC REGULATORY SIGNALS THROUGH A COMPLEX DNA CONTROL DOMAIN, John A. Mitchell[1], Enyu Imai[2], Richard O'Brien[1], Robert L. Hall[1] and Daryl Granner[1], Vanderbilt University School of Medicine, Nashville, Tennessee and [2]Osaka University Medical School, Osaka, Japan.

Transcription of the phosphoenolpyruvate carboxykinase (PEPCK) gene is stimulated by glucocorticoids, retinoic acid and cyclic AMP and is dominantly inhibited by insulin and phorbol esters. The glucocorticoid effect is mediated by a complex regulatory unit that consists of (5' to 3') two accessory factors elements (AF1 and AF2) and two glucocorticoid receptor binding sites. Using a fusion gene that consists of a consensus glucocorticoid response element ligated to the thymidine kinase promoter and the chloramphenicol acetyltransferase reporter gene (pGRETK), we show that AF1 and AF2 both function in a heterologous context to augment the glucocorticoid response. In this reporter system mutation of bases -451 to -446 (TGACCT) render the AF1 element incapable of supporting this accessory factor activity. Other studies have shown that this core sequence is required for the retinoic acid response, HNF-4 binding, and the developmental expression of the gene. In this heterologous system mutation of bases -416 to -407 (TGGTGTTTTG) within AF2 results in the loss of accessory factor activity. This same sequence mediates insulin and phorbol ester effects on PEPCK gene transcription. thus, the AF1 and AF2 elements have multiple functions. They are required for the glucocorticoid response, serve as response elements for several other hormones, and mediate other apparently diverse functions. These elements collectively constitute a complex metabolic control domain through which positive and negative signals provide an integrated response of the gene that encodes the rate-limiting enzyme in gluconeogenesis.

**L 031** TRANSCRIPTIONAL REPRESSION OF THE THYROID STIMULATING HORMONE α GENE BY THYROID HORMONE,

J. Larry Jameson, Laird D. Madison,Shoumen Datta, V. Krishna K. Chatterjee, and Takashi Nagaya, Thyroid Unit, Massachusetts General Hospital, Boston, MA 02114

Thyroid stimulating hormone (TSH) regulates thyroid hormone (T3) secretion from the thyroid gland. T3, in turn, inhibits TSH secretion from the pituitary gland, providing a feedback mechanism to control the circulating level of T3. In vivo,T3 inhibits the TSH α and ß subunit genes at the transcriptional level. We used transient expression assays in thyroid hormone receptor (TR) deficient JEG3 cells to define the promoter sequences in the TSH α gene that are required for negative regulation by T3. In the absence of added T3, cotransfected TR did not affect the basal activity of the α promoter; 80% inhibition of promoter activity occurred after the addition of hormone. Thyroid hormone receptor mutants that contain alterations in DNA binding domain or in the hormone binding domain prevented transcriptional repression, indicating that receptor binding to DNA and hormone activation are critical for negative regulation by the TR. The region of the α promoter that confers T3 mediated repression was localized between -195 and +1 bp. This region contains at least two receptor binding sites. One site (nTRE1) overlaps enhancer sequences between -195 and -97 bp; the other site (nTRE2) is located in the proximal promoter (-22 to -7 bp) between the TATA box and the transcriptional start site. Mutagenesis of either site was insufficient to eliminate repression, indicating that these elements function independently. Each nTRE conferred repression when linked to heterologous sequences, although the position of the nTREs with respect to enhancer and promoter sequences was critical for their activity. Cell free transcription systems were also used to study transcriptional repression by the TR. Receptor deficient HeLa extracts were supplemented with baculovirus produced TR. Addition of TR conferred transcriptional activation (5-fold) of a positively regulated template (TREpMLP) and repression of the negatively regulated α promoter (50-70%). Transcription from a control MLP promoter was unaffected by added TR and the activity of the receptor was blocked by receptor antisera or by excess TRE binding sites. Unlike the transient expression assays, T3 was not required for activation or repression in the in vitro transcription sysem. Although transcriptional stimulation of TREpMLP was optimal when TR and HeLa extracts were added concomitantly, transcriptional repression by the TR was seen only when the receptor was pre-incubated with the α promoter template, suggesting that the order in which transcription factors bind to DNA templates may be an important determinant of transcriptional control by the TR.

*Poster Session I*

**L 100** RETINOIC ACID RECEPTOR $\alpha$, $\beta$, AND $\gamma$ FORMS IN VARIOUS RAT TISSUES DURING AGING, Masarrat Ali, Jeff Brock, Charles D. Gulley, and Wayne V. Vedeckis, Gene Expression Lab., Pennington Biomedical Res. Ctr., 6400 Perkins Rd., Baton Rouge, Louisiana 70808, U. S. A.

Retinoic acid (RA) affects the growth and differentiation of many normal and tumor cells of ectodermal, mesodermal, and endodermal origins. RA, like steroid hormones, acts via binding to at least three nuclear retinoic acid receptors (RAR $\alpha$, $\beta$, and $\gamma$) that mainly differ in the C-terminal ligand-binding domain. Since the three RAR mRNAs are differentially expressed and autoregulated by RA in various cells and tissues, it is believed that each RAR may have tissue specific functions. Most of these studies have been performed by measuring specific RAR mRNAs using cloned cDNAs. We have recently produced RAR subtype-specific polyclonal and monoclonal antibodies using synthetic peptides corresponding to the C-terminal RAR subtype sequences. In this study, we report the relative levels of the three receptors in some major rat tissues (brain, heart, liver, kidney, eye, skin, spleen, and skeletal muscle) during aging (1-week, 12-month, and 18-month old rats). The RARs were examined in high salt nuclear extracts of various tissues by Western blotting using RAR subtype-specific polyclonal antibodies. RAR$\alpha$ was abundant in the heart, liver, and muscle and did not significantly change during aging. With aging, it registered a decline in the kidney and a significant increase in the eye and brain. The spleen and skin showed low levels in most samples. RAR$\beta$ levels in the heart, liver, and kidney showed a pattern similar to that for RAR$\alpha$. As opposed to RAR$\alpha$, RAR$\beta$ declined in the 18-month old brain when compared to the young tissues. RAR$\beta$ was found in significant amounts in the eyes and spleen only in 12-months of age. It remianed at low levels in the skin and muscle in 1-week old tissues and it subsequently declined to undetectable levels. Interestingly, RAR$\gamma$ was found at the highest level in the eye, and it was also elevated in the skin and spleen. It declined in all three tissues to low or undetectable levels at 18-month when compared to 12-month of age. RAR$\gamma$ was not detected in most other tissues at all ages. These data suggest that the three RAR proteins are independently expressed and regulated in various tissues during normal development and aging.

**L 101** **INTERACTIONS BETWEEN RETINOIC ACID AND THYROID HORMONE RECEPTORS.** Macy Au-Fliegner and Herbert H. Samuels, Depts. of Medicine and Pharmacology, New York University School of Medicine, New York, N.Y. 10016.

Retinoic acid receptor (RAR) and thyroid hormone receptor (c-erbA) activate transcription from a variety of response elements consisting of inverted or direct repeats of the motif AGGTCA. In addition to forming homodimers, these receptors are capable of forming heterodimers with each other and with other nuclear proteins. Deletion mapping studies have indicated that conserved heptad repeats in the carboxy terminal ligand binding domain are important for dimerization. In an effort to determine if dimerization activity is linked to the ability to activate transcription effectively, we have constructed carboxy terminal deletion mutants as well as point mutants in a conserved heptad region of each receptor. Dimerization activity has been assessed in a gel shift assay using a gapless inverted repeat of the AGGTCA motif and purified receptors. Mutation of the first leucine in the c-erbA heptad abolishes heterodimer formation with RAR but not homodimer formation in gel shift assays. Transcriptional activity has been assessed by transient transfection of HeLa cells using an MTV-CAT reporter with the same inverted repeat sequence inserted at position -88 relative to the transcription start site. Cotransfection of a vector encoding wild-type c-erbA into HeLa cells inhibits the RA-dependent activation of the reporter by RAR, presumably by the formation of inactive heterodimers. The mutated c-erbA, however, does not act as a dominant negative when cotransfected with RAR in HeLa cells, supporting the idea that dominant negative activity is due to heterodimer formation. Although the mutant has a lower affinity for T3, it efficiently activates the inverted repeat MTV-CAT reporter. Prelimary evidence suggests the analogous RAR mutation behaves in a similar fashion. Our results indicate that a highly conserved heptad mediates heterodimer formation but not homodimer formation. This suggests that the function of homodimers is distinct from that of heterodimers.

**L 102** EFFECTS OF HORMONE AND CELLULAR MODULATORS OF PROTEIN PHOSPHORYLATION ON HUMAN PROGESTERONE RECEPTORS, Candace Beck, Nancy L. Weigel[†] and Dean P. Edwards, Pathology Dept., Univ. Colo. Hlth. Sci. Ctr., Denver, CO 80262, and [†]Cell Biology Dept., Baylor College of Medicine, Houston, TX 77030

Human progesterone receptors (PR) in T47D breast cancer cells exist as two different sized proteins, PR-A (94 kDa) and PR-B (120 kDa). Progestin addition to cells (*in vivo*) causes a 2 fold increase in total phosphorylation and an increase in apparent molecular weight of both PR-A and PR-B on SDS gels. Time course experiments show that hormone stimulation of PR phosphorylation is a two-step process, the first step involves a rapid increase in total [$^{32}$P]labeling that takes place prior to the more slowly occurring phosphorylation that is responsible for PR upshift on SDS-gels. The effects of cellular modulators of protein phosphorylation on PR-mediated target gene transcription were examined. Treatment with 8-Br cAMP (activator of cAMP-dependent protein kinases) or okadaic acid (protein phosphatase 1 and 2A inhibitor) did not stimulate target gene expression in the absence of progestin. When added together with progestin either compound augmented PR-mediated target gene transcription by 3-4 fold. The cyclic nucleotide dependent protein kinase inhibitor, H8, completely blocked target gene responsiveness to hormone. When all three compounds were tested for effects on PR itself, none of the treatments altered hormone or DNA binding activities or cellular concentrations of PR. Moreover, they did not alter transcription from a control reporter gene, pSV2-CAT, indicating that these agents were affecting PR-mediated processes directly and not acting on general transcription. Effects on PR phosphorylation were assessed by measuring [$^{32}$P]labeling of PR *in vivo*. None of these treatments substantially affected total [$^{32}$P]labeling or the phosphorylations responsible for PR upshifts on SDS-gels. The present result indicate that these agents modulate PR transcriptional activity either through phosphorylation of another protein intimately involved in PR-mediated transcription or through modification of a key site(s) in PR other than the site responsible for upshifts on SDS-gels and not measurable as a change in total PR phosphorylation.

**L 103** REGULATION OF KERATIN GENES BY NUCLEAR RECEPTORS, M. Tomic, D. Yang, I.M. Freedberg, and M. Blumenberg. Departments of Dermatology and Biochemistry, NYU Med. Ctr., NY, NY 10016.

Keratins constitute a large multiprotein family. They form the intermediate filament networks in epithelial cells. Expression of various keratins is cell-type specific, which is at times used in cancer diagnosis. Separate from the cell type specific regulation of keratin expression, various hormones and vitamins also determine the levels of keratin synthesis. To analyze the effects of hormones and vitamins on keratin gene expression, we have prepared DNA constructs in which promoters of keratin genes drive expression of CAT reporter gene, and co-transfected them with vectors expressing nuclear receptors into various cell types. We find that thyroid hormone and its receptor suppress all keratin gene promoters tested. Retinoic acid and its receptor suppress transcription only of the keratin genes expressed in stratified epithelia, but not of others. Vitamin D3 and its receptor had no effect on any of the keratin genes tested. We found no cell-specific differences in the actions of nuclear receptors on keratin promoters. None of the keratin gene sequences tested contain consensus TRE palindrome, but instead the nuclear receptor responsive elements apparently consist of clusters of imperfect half-palindromic sites in head-to-head, head-to-tail and tail-to-tail configurations, with various numbers of spacer nucleotides separating the half sites. Preliminary experiments indicate that estrogen and progesterone receptors also selectively regulate some of the keratin genes, but not others.

**L 104 LIGAND DEPENDENT BINDING OF A 160 kDa PROTEIN TO THE ESTROGEN RECEPTOR,**

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The details of the mechanism by which the estrogen receptor (ER) functions as a hormone dependent transcription factor are only partially known. The receptor contains two transactivation domains termed TAF-1 and TAF-2. TAF-1 is a constitutive activation domain dependent only on DNA binding. TAF-2 contains the hormone binding domain and is dependent on the presence of estradiol for function. Thus the mechanism of action of TAF-2 is critical to the hormone dependent action of the ER. In order to approach this problem, the ER hormone binding domain (HBD) was expressed as a glutathione s-transferase (GST) fusion protein in *E. coli* and used as an affinity reagent to identify associated proteins. Radiolabeled cell extracts were incubated *in vitro* with the GST-HBD fusion protein immobilized on glutathione beads in the presence or absence of estradiol. A 160 kDa protein (p160) was identified that bound specifically to the GST-HBD fusion protein only in the presence of estradiol. Neither 4-hydroxytamoxifen (4-OHT) nor ICI 164,384 stimulated p160 binding. In addition, estradiol induced binding of p160 to the GST-HBD fusion was blocked by the addition of equimolar concentrations of 4-OHT. Finally, mutations in the ER HBD altered the ability of the receptor to bind p160. These characteristics of p160 binding are consistent with it playing a role in the pathway of estradiol dependent transactivation.

**L 105 SEQUENCES INVOLVED IN THE REGULATION OF THE RAT PLASMINOGEN ACTIVATOR INHIBITOR (PAI-1) GENE: EVIDENCE FOR BOTH SIMPLE AND COMPLEX GLUCOCORTICOID RESPONSE ELEMENTS,**

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Glucocorticoids regulate tissue-type plasminogen activator (tPA) activity in HTC rat hepatoma cells primarily by modulating plasminogen activator-inhibitor (PAI-1) gene expression. To investigate the molecular mechanisms underlying this regulation, we have cloned the rat PAI-1 gene from an HTC genomic library. Multiple putative glucocorticoid response elements (GREs) were identified within 2.4 kb of 5' flanking sequence. Analyses of a series of hybrid genes, containing various portions of the 5' flanking region of the rat PAI-1 gene fused to the chloramphenicol acetyl transferase reporter gene, transfected into HTC rat hepatoma cells localized the region involved in the transcriptional regulation by glucocorticoids to between -1230 and -760. This region contains a sequence that is >90% identical to the consensus GRE 15-mer. The sequence binds purified glucocorticoid receptor DNA binding domain protein (GR-DBD), as demonstrated by both gel mobility shift assays and DNAse I footprinting. Point mutations of this GRE within the context of the rat PAI-1 promoter reduced the glucocorticoid induction by 60-75% when analyzed by transfection of HTC cells and abolished the binding of GR-DBD in gel mobility shift assays, confirming that this GRE mediates the glucocorticoid response of the rat PAI-1 gene in HTC cells.

In HepG2 cells, a human hepatoma cell line, neither the endogenous PAI-1 gene nor the transfected rat PAI-1 promoter is induced by glucocorticoids. However, when it is co-transfected with a glucocorticoid receptor expression vector, the rat PAI-1 promoter is induced 10-fold. Two regions are responsible for this induction: the GRE at -1196 and a second region between 760 and 270 bp upstream of the start site of transcription. This area contains >200 bp which are >80% identical with a region in the human gene that is involved in the glucocorticoid regulation of that gene, but does not contain a putative GRE 15-mer. Since this region is not active in response to glucocorticoids when the rat PAI-1 promoter is transfected into HTC cells, the underlying mechanism may be more complex, involving not only the glucocorticoid receptor, but other auxiliary transacting protein(s), not present in HTC cells.

**L 106 *cAMP ACTIVATION OF THE MOUSE ANDROGEN* RECEPTOR AND REPRESSION OF $P450_{17\alpha}$ GENE EXPRESSION BY ANDROGENS,**

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We have previously reported that testosterone represses cAMP-induced expression of cytochrome $P450_{17\alpha}$-hydroxylase/$C_{17-20}$ lyase ($P450_{17\alpha}$) in primary cultures of mouse Leydig cells via an androgen receptor mediated mechanism. To examine whether the repression of $P450_{17\alpha}$ by androgens occurs at the level of the gene, MA-10 tumor Leydig cells were first examined for the presence of endogenous androgen receptors. Cells were transiently transfected with a thymidine kinase (tk) CAT plasmid containing a trimer of a hormone-response-element from the mouse Slp gene, which serves as an androgen dependent enhancer, 3x(HRE)tkCAT (a gift from D.M. Robins) and treated for 12 h with 2 μM dihydrotestosterone (DHT). DHT treatment did not increase CAT activity relative to control, indicating that MA-10 cells do not contain androgen receptors. In contrast, cotransfection of 3x(HRE)tkCAT with a mouse androgen receptor expression vector, mAR, (a gift from D.J. Tindall) followed by treatment with DHT resulted in a 3-4 fold increase in CAT activity. To investigate the effect of androgens on the expression of $P450_{17\alpha}$, a plasmid containing 4.5 kb of 5' flanking region linked to the CAT reporter gene was cotransfected with the mAR and cells were treated with cAMP or cAMP plus DHT. Increasing amounts of the mAR caused a dosed dependent decrease in cAMP-induced expression of CAT activity. DHT resulted only in a small further decrease in CAT activity (~25%), suggesting that cAMP by itself activated the mAR. To test this hypothesis, the 3x(HRE)tkCAT was cotransfected with the mAR into MA-10 cells that were treated with 500 μM cAMP, 2μM DHT or cAMP plus DHT. Treatment with cAMP shows a 4-fold increase in CAT activity similar to the increase observed when cells were treated with DHT alone or with cAMP plus DHT. These data demonstrate that 1) repression of cAMP-induced expression of $P450_{17\alpha}$, mediated by the androgen receptor, occurs at the level of the gene; 2) cAMP as well as DHT can activate the androgen receptor. Supported by NIH grants HD-08358 and HD-17916.

**L 107 A NON-PALINDROMIC $T_3$RE MEDIATES NEGATIVE TRANSCRIPTIONAL REGULATION BY $T_3$,**

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We have recently identified a $nT_3RE$ in the rTSH gene that mediates negative regulation by $T_3$. The $nT_3RE$, CGCCAGTGCAAAGTAAG, contains a 6 bp sequence that appears as a half-site in the rGH $T_3RE$. To determine whether the $nT_3RE$ is active in a heterologous system, we constructed chimaera containing the $nT_3RE$ and the viral TK-promoter. The suppressive effects of $T_3$ (5-fold) were mediated by a single copy of the $nT_3RE$ inserted either 5' (-125, -50, -26) or 3' (+11) relative to the transcriptional start site (TSS); thus dispelling the idea that the motif acted by physically blocking transcription. To test whether the $nT_3RE$ is active in a cell-free transcription assay, we inserted the motif in front of a DNA fragment spanning -441 to -2 of a $T_3$-responsive gene, S14, from rat liver. Transcriptional activity of the template containing the $nT_3RE$ was 5-fold more active in extracts from the livers of hypo- compared to hyperthyroid animals. Conversely, the same S14 template lacking the $nT_3RE$ was 3-fold more active in extracts from hyper- compared to hypothyroid rats. To determine whether the $nT_3RE$ binds to a $T_3R$, we further examined the transcriptional elements that bind to the rTSH $nT_3RE$ by gel mobility shift assay and southwestern hybridization. Multiple nuclear protein complexes are observed with $GH_3$ nuclear extracts, one of which co-migrated with the only complex arising frum a $T_3R$ synthesized in vitro. Additionally, southwestern analysis of $GH_3$ nuclear extracts reveadled binding of the $nT_3RE$ to a protein with a Mr identical to that of a $T_3R$ (48kd) as well as 2 other proteins (90,36kd). In summary, rTSH $nT_3RE$ containing half-site of the consensus rGH-$T_3RE$ imparts negative regulation by $T_3$ is bound by $T_3R$.

L 108 ACTIVATION AND REPRESSION OF TRANSCRIPTION BY GAL4-THYROID HORMONE RECEPTOR CHIMAERAS,
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As with other members of the nuclear receptor superfamily, the thyroid hormone receptors consist of an N-terminal A/B domain, a central C domain which mediates DNA binding and carboxyterminal D/E/F domains encoding ligand binding and dimerisation functions. We have assayed the properties of these domains in the chicken α1 and human β1 (hTRβ1) receptor proteins by coupling them to the DNA binding domain (αα1-147) of the yeast transcription factor GAL4 and co-expressing them in a receptor deficient (JEG-3) cell line with a reporter gene (UASTKCAT), containing two copies of a GAL4 binding site. In the absence of ligand, the native receptor is known to repress basal gene transcription and the fusion proteins exhibit the same effect. A construct containing the A/B/C domains is weakly inhibitory whereas the D/E/F domains possess marked inhibitory activity (80%) comparable to the full length receptor protein. Further deletion analyses indicate that the D domain is important for this inhibitory effect. In the presence of thyroid hormone (T3), the A/B/C domains are unable to mediate transactivation. However fusions containing the D/E/F domains of either receptor isoform confer a concentration dependent increase in gene expression with a maximal (1000 fold) effect in the presence of 1nM T3. Deletion of a carboxyterminal nine amino acid (Δ 448-456) motif in hTRβ1 that is predicted to form an amphipathic helix, leads to a complete loss of activation. Mutation of a single acidic residue within this helix (Glu 455 to Ala) is not deleterious but mutation of an additional acidic residue (Glu 452 to Ala) severely impairs transactivation (30fold). We have also expressed these chimaeric proteins under the control of the alcohol dehydrogenase promoter in a yeast strain (GGY1:171) which contains a GAL4 responsive LacZ reporter stably integrated at the URA3 locus. Whereas full length GAL4 is active in this system (2400U of β galactosidase activity), fusions containing either full length β receptor or A/B/C or D/E/F domains are inactive, both in the absence of ligand and in the presence of 1μM T3 or TRIAC. We conclude that the D/E/F domains of the α and β isoforms of the thyroid hormone receptor are capable of mediating both basal repression and ligand dependent transactivation functions in a mammalian cell system.

L 109 PHOSPHORYLATION REGULATES THYROID HORMONE RECEPTOR-MEDIATED TRANSCRIPTION,
Sheue-yann Cheng, Kwang-huei Lin, and Kiyoto Ashizawa, Laboratory of Molecular Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD 20892
We have recently shown that human placental thyroid hormone nuclear receptor (h-TRβ1) expressed and purified from *E. coli* can be phosphorylated by the endogenous kinase from several cultured cells. The *in vitro* phosphorylation of h-TRβ1 led to a 2-fold increase in the binding of receptor to 3,3',5-triiodo-L-thyronine ($T_3$) and a 2-3-fold increase in the binding of receptor to the $T_3$ response element of rat growth hormone gene. Furthermore, phosphorylation confers the ability of h-TRβ1 to bind to the receptor binding nuclear proteins. The present study evaluated the function of phosphorylation in the regulation of transcription mediated by h-TRβ1 *in vivo*. Monkey COS-1 cells which are functionally deficient in response to $T_3$ was transfected with a h-TRβ1 expression plasmid (pCLC51). The expressed h-TRβ1 is a phosphoprotein as indicated by immunoprecipitation of [$^{32}$P]-labeled cellular lysate with a monoclonal antibody (J52) against h-TRβ1. Serine and threonine are the phosphorylated amino acids. No phosphotyrosine was detected. Phosphorylation of h-TRβ1 was increased by 4 and 20-fold after treating cells with 0.05 and 0.25 μM of okadaic acid, respectively. The synthesis of h-TRβ1 was evaluated by metabolically labeling the cells with [$^{35}$S]-methionine followed by immunoprecipitation with J52. In the presence of 0.25 μM okadaic acid, the synthesis of h-TRβ1 was not significantly increased. These results indicate that the increase in the phosphorylation of h-TRβ1 in the presence of okadaic acid was not due to the increase in the synthesis of h-TRβ1. COS-1 cells were co-transfected with pCLC51 and a reporter gene which contained the palindromic $T_3$ response elements upstream of the chloramphenicol acetyltransferase (CAT) gene (pTK28mult, kindly provided by D. Moore). Treating cells with 0.05 and 0.25 μM of okadaic acid resulted in the increase of the CAT activity by 2- and 3-fold, respectively. But okadaic acid did not stimulate basal transcription by pTK28mult in the absence of transfected h-TRβ1. These results indicate that phosphorylation stimulates the transcriptional activity of h-TRβ1. Thus, phosphorylation plays a regulatory role in the gene activation of h-TRβ1.

L 110 NUCLEIC ACID SEQUENCE SIMILARITIES OF THE ESTROGEN RECEPTOR GENE IN BREAST TUMOUR AND OSTEOSARCOMA CELLS,
Stephanie A. De Grandis, Susan Sibley, Joshua Owolabi, Yiping Zhang and Rick Bozzato, Bone and Research Development Programme, Allelix Biopharmaceutials, Mississauga, Ontario, Canada.
The detection of estrogen receptor (ER) in human osteoblast-like cells( Eriksen et al., Science 241:84) and avian osteoclasts (Oursler et al., PNAS 88:6613) using biochemical or in vivo assays indicates that estrogen can act directly on bone cells by a receptor-mediated mechanism. We examined whether the nucleotide sequence of the ER mRNA species from the human osteosarcoma cell lines Saos-2 and HOS-TE85 was similar to the nucleotide sequence determined from the ER mRNA species from the breast tumour cell line MCF-7. Total RNA was isolated from the three cell lines using guanidium thiocyanate cell lysis with cesium trifluoroacetate ultracentrifugation. A reverse transcriptase PCR kit was used to produce cDNA and three primer pairs targeting the DNA and ligand binding region of the breast tumour ER gene were used to amplify both osteoblast-like and breast tumour ER cDNA. Products of identical molecular size were obtained using either Saos-2 or MCF-7 cDNA. Human osteosarcoma HOS-TE85 cDNA could also be used to generate the same PCR products. The DNA sequence of the Saos-2 products was nearly identical to the breast tumour ER sequence with the exception that there was a single base change at Val-400 [GTG] to Gly [GGG]. The same base change has been detected from a human leukocyte genomic library (Ponglikmongkol et al., EMBO 7:3385) and therefore represents the wild-type sequence. These data support the hypothesis that ER exists in osteoblast-like cells and validates the similarities of the DNA and ligand binding domains of the ER in breast and bone cells.

L 111 DNA ELEMENTS MEDIATING RETINOIC ACID ACTIVATION AND THYROID HORMONE REPRESSION OF THE HUMAN ALCOHOL DEHYDROGENASE GENE *ADH3*
Gregg Duester, Cancer Research Center, La Jolla Cancer Research Foundation, 10901 N. Torrey Pines Road, La Jolla, CA 92037.
Mammalian alcohol dehydrogenase (ADH) catalyzes the oxidation of retinol (the alcohol form of vitamin A) to form retinaldehyde which is the rate-limiting step in the synthesis of retinoic acid, a known transcriptional regulatory molecule. There exists an ADH gene family, and we are interested in studying the regulation of mammalian ADH genes in order to understand the regulation of retinoic acid synthesis during development. The human class I ADH gene family includes three members (*ADH1*, *ADH2*, and *ADH3*) which display differential gene expression. Deletion mapping experiments identified a region in the *ADH3* promoter located between -328 and -270 bp which confers retinoic acid activation; this region is missing in the *ADH1* and *ADH2* genes. This region was demonstrated to confer retinoic acid responsiveness on the *ADH1* and *ADH2* genes in heterologous promoter fusions. In a 34 bp stretch from -318 to -285 bp, the *ADH3* retinoic acid response element (RARE) contains three direct TGACC repeats which constitute the critical nucleotides of RAREs present in other genes. The laminin RARE also contains three TGACC repeats, but the retinoic acid receptor β (RARβ) RARE has only two such repeats. We have dissected the *ADH3* RARE and determined that receptor binding as well as transactivation were dependent upon only the two downstream TGACC motifs. Thus, the *ADH3* RARE, like the previously characterized RARβ RARE has a requirement for two directly repeated TGACC motifs. Cotransfection assays were utilized in which various receptor expression vectors were introduced into cells with the reporter gene. The *ADH3* RARE and the RARβ RARE were transactivated preferentially by RARβ; RARα, RARγ and RXR were weak transactivators. Since ADH and RARβ are both involved in transduction of the retinoic acid signal, the former in retinoic acid synthesis and the latter in binding retinoic acid to regulate transcription, it makes physiological sense to have feedback mechanisms utilizing a common RARE and common receptor controlling both.

ADH is also known to be repressed by thyroid hormone which exerts its effects through response elements containing TGACC motifs. We have identified a negative thyroid hormone response element in the *ADH3* promoter which lies near to the *ADH3* RARE or overlaps with it. In the presence of thyroid hormone and its receptor this element may interfere with the normal functioning of the *ADH3* RARE.

L 112 THE ABERRANT FUSION PROTEIN, PML-RAR, FOUND IN ACUTE PROMYELOCYTIC LEUKEMIA (APL) PATIENTS LOCALIZES IN THE CELL DIFFERENTLY THAN EITHER WILD TYPE RAR OR WILD TYPE PML-1, Jacqueline Dyck, Akira Kakizuka, and Ronald M. Evans, Gene Expression Laboratory, The Salk Institute, San Diego, CA 92186-5800.

The juxtaposition of two sequences of DNA encoding the amino terminal portion of PML-1 (for "promyelocytes") and the carboxy terminal portion of RARα (retinoic acid receptor α) is the consequence of the chromosomal transolcation t(15;17) associated with APL. The expression of the resulting chimeric protein is the first suggestion of the involvement of a retinoid receptor in a neoplastic process. Immunohistochemical procedures were applied in order to visualize the wild type PML-1 and RARα as well as the PML-1 fusion protein in transiently transfected COS-7 cells. Our results demonstrated a unique pattern of distribution for each type of protein. The characterization of aberrant localization of PML-RAR may provide important clues leading to the elucidation of the molecular events involved in the pathogenesis of APL

L 113 ORGANIZATION OF CORE DNA BINDING-SITES DETERMINES THE HOMO- AND HETERO-DIMERIZATION POTENTIAL OF THYROID HORMONE RECEPTOR, RETINIOIC ACID RECEPTOR AND COUP-TF, Barry M. Forman and Herbert H. Samuels, Departments of Pharmacology and Medicine, New York University School of Medicine, New York, NY 10016.

We have previously shown that non-DNA binding mutants of the thyroid hormone receptor ($T_3R$) act as dominant-negative inhibitors of wild-type $T_3R$ and retinoic acid receptor (RAR). Dominant inhibition required a leucine zipper-like dimerization motif that is conserved among $T_3Rs$ and RARs.

In order to directly study the dimerization properties of these receptors, the chicken $T_3R$-α1 and human RAR-α1 were expressed in *E. coli* and purified to homogeneity. Both receptors were monomers in solution and bound DNA and ligand with appropriate affinity and specificity. Homo- and hetero-dimerization was examined by measuring cooperative binding to DNA elements arranged as inverted repeats (IR), direct repeats (DR) or indirect repeats (InR) with various nucleotide gaps (+n). Both receptors bound as monomers to a six nucleotide core binding-site (5'AGGTCA3'). For $T_3R$, homo-dimers formed on IR and DR+5 but not on IR+3, IR+5, IR+10 or DR. Although $T_3R$ dimers are known to form on InR+6, no dimerization was detected on InR. For RAR, dimers formed on IR, IR+3, IR+5, DR and DR+5 but not IR+10. $T_3R$-RAR or $T_3R$-TRAP ($T_3R$ associated protein) hetero-dimers formed only IR and DR+5. The dopamine responsive orphan receptor COUP-TF bound these elements with the following affinity: DR+1> DR+5> IR>>>IR+3> single core binding site=0. No interactions were detected between COUP-TF and $T_3R$. These results indicate that given the appropriate spacing, $T_3R$, RAR and COUP-TF can dimerize in head-to-head (IR), head-to-tail (DR) and tail-to-tail orientations (InR), suggesting that transcriptional specificity is modulated by core-site architecture.

L 114 DOMINANT-POSITIVE AND DOMINANT-NEGATIVE ESTROGEN RECEPTOR VARIANTS IN HUMAN BREAST CANCER, Suzanne A.W. Fuqua, D. Craig Allred, C. Kent Osborne, Geoffrey L. Greene, Bert O'Malley, Donald McDonnell, and William L. McGuire, University of Texas Health Science Center, San Antonio, TX 78284, Ben May Laboratories, Chicago, IL 60637, and Baylor College of Medicine, Houston, TX 77030

Breast tumors containing estrogen receptor (ER) and progesterone receptor (PgR) have a higher objective response to endocrine therapy compared to those tumors lacking these receptors. But the correlations are imperfect. Some ER+ tumors behave as if they are ER-, and fail endocrine therapy. We hypothesize that these tumors contain an ER which is unable to transactivate estrogen-responsive genes. Such tumors would be functionally receptor-negative. We have detected such a variant, truncated within the transactivation domain, which is abundant in some ER+/PgR- tumors. This variant inhibits the binding of wild type ER to its cognate response element in gel-retardation assays, and interferes in a dominant-negative manner with normal ER function in a yeast expression vector co-transformation system. It is also known that some ER- tumors behave as if they are ER+. We hypothesize that these tumors contain a dominant-positive ER which is unable to bind hormone, but still capable of transactivation of genes such as PgR. We have identified such a transcriptionally-active variant in ER-/PgR+ tumors. This variant directs low level constitutive activation of an estrogen-responsive construct in yeast cells. We have placed both of these variants under control of a metallothionein promoter and have transfected them into MCF-7 breast cancer cells. Induction of the dominant-positive ER variant with cadmium results in increased growth of MCF-7 transfectants in soft agar as compared to nontransfected cells; estrogen and tamoxifen does not affect the growth of this variant in vitro.

Conclusion: Both dominant-negative and positive ER variants exist in human breast tumors. Experiments are in progress to determine whether expression of these variants results in hormone-independent growth in vivo.

L 115 SCREENING FOR ACTIVATORS OF NOVEL MEMBERS IN THE NUCLEAR RECEPTOR SUPERFAMILY, Martin Göttlicher, Eva Widmark and Jan-Åke Gustafsson, Department of Medical Nutrition, Karolinska Institute, Huddinge Hospital F60, and Center for Biotechnology, NOVUM, S-141 86 Huddinge, Sweden

Recently, a number of genes with striking similarity to the known steroid type receptors have been identified. The conserved structure of a putative ligand binding domain gave rise to the hypothesis that there exist high affinity ligands for at least some of these so-called orphan receptors. However, low molecular weight activators have been identified for only very few of them.

We now have cloned the homologous gene from rat to the murine peroxisome proliferator activated receptor (PPAR) (Issemann and Green (1990) *Nature*, **347**, 645-650). In order to search for physiologically occuring activators or putative ligands to that receptor we integrated the gene for a secreted form of placental alkaline phosphatase under the control of the MMTV-promoter into the genome of CHO cells. In these reporter cells we stably expressed either the full length glucocorticoid receptor (GR) or a chimera resembling the N-terminal and DNA-binding domains of GR and the putative ligand binding domain of PPAR. Consistent with limited responsiveness of CHO cells to glucocorticoids, dexamethasone induced the reporter 4-6 fold in cells expressing no additional receptor or the PPAR chimera. Overexpression of GR increased this response to over 100-fold. In cells containing the PPAR chimera the known peroxisome proliferators WY14,643 and clofibric acid induce the reporter gene activity 50- and 20-fold. Significant induction occurs at 1, 10 and 100 μM of WY14,643, nafenopin or clofibric acid, respectively. Maximum induction by those compounds appears limited by their solubility in cell culture medium.

Since the reporter gene activity can easily be detected in a colorimetric assay from 2 $cm^2$ cultures, the described transactivation sytstem appears feasible for screening through a broad spectrum of potential activators of PPAR and, in principal, for other orphan receptors. The application on the search for physiologically occuring activators for PPAR will be discussed.

**L 116** MECHANISM OF ENHANCEMENT OF THYROID HORMONE RECEPTOR DEPENDENT TRANSCRIPTIONAL ACTIVATION AND LIGAND-BINDING BY H-2RIIBP, Paul L. Hallenbeck, M. S. Marks*, R. E. Lippoldt, M. Phyillaier, K. Ozato* and V. Nikodem, Genetics and Biochemistry, NIDDK, and *Laboratory of Developmental and Molecular Immunity, NICH, NIH, Bethesda, MD 20892. Recently we reported that thyroid hormone (TH) receptor (THR) dependent TH and TH response element (TRE) binding and TH mediated transcriptional induction could be increased by addition or coexpression of H-2RIIBP, a non-TH binding member of the steroid hormone receptor superfamily. The enhancing activity of H-2RIIBP was suggested to be related to its ability to form more stable heterodimers with THR than THR formed with itself. Here we report that H-2RIIBP, containing a deletion in its dimerization domain, was incapable of dimerizing with THR and unable to increase ligand binding to THR or transcriptional induction. This demonstrated that the dimerization domain of H-2RIIBP was essential for its enhancing activity. Deletion of the DNA binding domain of H-2RIIBP also abolished the ability of H-2RIIBP to increase THR and TH dependent transcription but did form heterodimers with THR in solution. However, those heterodimers were unable to bind the malic enzyme-TRE. Mutations constructed within either half-site of the malic enzyme-TRE eliminated the binding of the heterodimer and THR homodimer to this TRE and abolished TH dependent transcription. These data indicate that the binding of the heterodimer to the TRE, with each DNA binding domain contacting a half-site of the TRE, was essential for the transcriptional enhancement of H-2RIIBP and supports a model in which the ability of H-2RIIBP to form stable heterodimers in solution with THR is partially responsible for its enhancing activity.

**L 117** STUDIES ON THE HORMONE-INDEPENDENT ACTIVITY OF c-ERBAs, Elizabeth Helmer, Bruce M. Raaka and Herbert H. Samuels, Departments of Medicine and Pharmacology, New York University Medical Center, New York , NY 10016.

Thyroid hormone receptors (c-erbAs) are thought to activate transcription by binding to a thyroid response element (TRE) in the promoter region of a regulated gene. While c-erbA proteins bind to TRE sequences in the absence of thyroid hormone, transcriptional activation generally appears to require occupancy of the receptor with hormone. However, chick c-erbA-α1was previously found by this laboratory to activate transcription from certain promoters in a hormone-independent fashion when introduced at high levels of expression into rat pituitary tumor GH4C1 cells by transient transfection. Analysis of carboxy terminal deletion mutants indicated that a conserved heptad repeat region within the ligand binding domain which may play a role in homo- and heterodimeric interactions of c-erbA may be important for this hormone-independent activity. We have extended these original observations by showing that rat c-erbA-α1 is also a constitutive activator of transcription when expressed at high levels in cells comparable to the levels achieved with chick c-erbA-α1. In contrast, both receptors are fully hormone-dependent in HeLa cells. Furthermore, our results indicate that the presence of a high affinity TRE in the promoter may not be required for constitutive activation by these two receptors. However, because c-erbA constructs which lack the DNA binding domain do not exhibit constitutive transcriptional activation, binding of c-erbA to lower affinity DNA binding sequences not previously defined as TREs may be required. O'Donnell et al. (Mol. Endocrinol. 5, 94-99, 1991) have recently found that several mutants with single amino acid changes in a highly conserved 20 amino acid region of the c-erbA family impair the interaction of human c-erbA-β1 with a particular thyroid receptor associated protein (TRAP). Interestingly, we have found that two analogous mutations, L234S and N244A, introduced individually into chick c-erbA-α1 abolish constitutive activation in GH4C1 cells but appear to maintain normal hormone-dependent transactivation in HeLa cells. Our study thus suggests that both the heptad repeat dimerization domain and the conserved 20 amino acid domain may be necessary for the hormone-independent activities of rat and chick c-erbA-α1 on various promoter constructs in GH4C1 cells.

**L 118** RECONSTITUTION OF GLUCOCORTICOID RECEPTORS WITH HEAT SHOCK PROTEINS IN RETICULOCYTE LYSATE REQUIRES ATP AND HEAT-STABLE FACTORS, Kevin A. Hutchison, Michael J. Czar, Lawrence C. Scherrer, and William B. Pratt, The University of Michigan Medical School, Ann Arbor, MI 48109-0626.

We have recently shown that rabbit reticulocyte lysate causes the reassociation of hsp90 and the association of hsp70 with both the glucocorticoid receptor (GR) (Scherrer, L.C., Dalman, F.C., Massa, E., Meshinchi, S., and Pratt, W.B. (1990) J. Biol. Chem. 265, 21397-21400) and pp60$^{src}$ (Hutchison, K.A., Brott, B.B., De Leon, J.H., Perdew, G.H., Jove, R., and Pratt, W.B., submitted). Reconstitution of GR is time, temperature, and energy dependent, and is accompanied by a return of the steroid-binding activity and a repression of the DNA-binding activity of the receptor. With pp60$^{src}$, in addition to the reassociation of hsp90 and the association of hsp70, lysate causes the 50 kDa phosphoprotein component of the native multiprotein complex to become reassociated with pp60$^{src}$. Both the GR and pp60$^{src}$ native and reconstituted hsp90 complexes are stabilized by sodium molybdate.

We have begun to identify components of the reticulocyte lysate necessary to cause reassociation of heat shock proteins with the GR. Reconstitution of GR-hsp90 complexes in reticulocyte lysate requires lysate proteins, an ATP-regenerating system, and a heat-stable, dialyzeable, small molecular weight fraction of lysate. This dialyzeable fraction enhances hsp90 reassociation and the return of steroid binding in a saturable, dose-dependent manner and can be replaced with a cocktail of sodium molybdate and reduced thioredoxin. Ammonium sulfate fractionation has been used to examine lysate proteins necessary for reconstitution. Proteins that precipitate between 30-50% of saturation cause association of hsp70 with the GR, but material soluble at 50% of saturation has little ability to cause association of either hsp70 or hsp90 with the GR. Combination of the 30-50% cut and the 50% supernatant together restores full activity to the reticulocyte lysate and allows for reconstitution of hsp90 association and steroid binding activity of the GR.

(Supported by NIH grant DK31573)

**L 119** DEXAMETHASONE NEGATIVELY REGULATES THE ACTIVITY OF A DIHYDROFOLATE REDUCTASE/ GLUCOCORTICOID RECEPTOR CHIMERIC PROTEIN, Israel, D. I. and Kaufman, R. J., Department of Molecular and Cellular Genetics, Genetics Institute, Cambridge MA, 02140

We have made a chimeric protein consisting of the entire murine dihydrofolate reductase (DHFR) protein with a carboxy terminal extension encompassing amino acids 493-795 of the rat glucocorticoid receptor (GR). The chimeric protein (DHFR/GR) contains functional DHFR activity, as measured by the ability to transform DHFR-deficient CHO cells to a DHFR positive phenotype. The DHFR/GR protein binds [3H]-dexamethasone with a similar affinity as wild-type GR. Selection of stable CHO transformants in increasing methotrexate results in amplification of DHFR/GR. Addition of dexamethasone results in a decrease in DHFR activity in the chimeric protein, as measured by colony formation in methotrexate, binding of fluoresceinated methotrexate, and directly by an enzymatic assay for DHFR. The half-maximal response for decreasing DHFR activity occurs at 3-10nM dexamethasone, similar to the responsiveness of wild-type GR to dexamethasone. The half-life of the chimeric protein is approximately 3 hours and is not changed by the addition of dexamethasone to cells. The subcellular location of DHFR/GR was determined in intact cells incubated with fluoresceinated methotrexate. In the absence of dexamethasone, the chimeric protein is primarily cytoplasmic. Addition of dexamethasone results in the translocation of DHFR/GR into the nucleus.

These results demonstrate that glucocorticoids negatively effect the function of DHFR activity in the chimeric protein. This contrasts with other chimeric steroid receptor proteins, such as E1A/GR (1), myc/ER (2), and FOS/ER or FOS/GR (3), in which protein function is positively effected by steroid hormones. Therefore, the generalization that the hormone binding domain of steroid receptors act as global inhibitors of protein function, possibly by virtue of its interaction with hsp90, does not apply to DHFR/GR.

1) Picard et. al. *Cell* 54:1073-1080 (1988)
2) Eilers et. al. *Nature* 340:66-68 (1989)
3) Superti-Furga et. al. *PNAS USA* 88:5114-5118 (1991)

L 120 THE REGULATION OF THYROID HORMONE RECEPTOR β GENES BY THYROID HORMONE IN Xenopus laevis. Akira Kanamori and Donald D. Brown, Department of Embryology, Carnegie Institution of Washington, 115 W. University Parkway, Baltimore, MD 21210

In tadpoles and cultured cells of Xenopus laevis, the mRNA level of the two thyroid hormone receptor ß (TRβ) genes is up-regulated by exogenous thyroid hormone (TH). The increased mRNA can be detected in 8 h after the addition of TH. Quantitative PCR assays show that up-regulation of transcripts of the 5' most exon is detectable 2-3 h before that of full-length TRß mRNA, strongly suggesting that up-regulation is under transcriptional control. The TH-induced up-regulation in cultured cells is inhibited by cycloheximide when measured either by the PCR quantitation of the 5' most exon transcripts or by the level of full-length TRβ mRNA. From this we conclude that in cultured cells protein synthesis is required for up-regulation of TRß mRNA. However, the up-regulation of TRß mRNA by TH in tadpoles is only partially inhibited by protein synthesis inhibitors. A survey of "thyroid response" genes in the literature reveals that the reported change in gene expression is, in most cases, sensitive to protein synthesis inhibition. If the prior induction of a crucial and unidentified factor is required, the regulation of TH-response genes, including Xenopus laevis TRβ genes, is more complicated than suggested by co-transfection assays that only take into account the binary reaction between TR and a putative thyroid hormone response element.

L 121 CHARACTERIZATION AND DIFFERENTIAL EXPRESSION OF THREE NUCLEAR HORMONE RECEPTOR-LIKE PROTEINS ACTIVATED BY PEROXISOME PROLIFERATORS, H.J. Keller[1], G. Krey[1], F. Givel[1], G. Helftenbein[2], C. Dreyer[2], and W. Wahli[1], 1. Institut de Biologie animale, Université de Lausanne, CH-1015 Lausanne, Switzerland, 2. Max Planck Institut für Entwicklungsbiologie, Tübingen, Germany

Continued exposure to hypolipidemic drugs such as clofibrate and industrial plastizisers such as diethylhexylphtalate leads to peroxisome proliferation and subsequent carcinogenesis in rodent liver. Since these carcinogens fail to damage DNA directly, it has been postulated that the observed stimulation of genes encoding fatty acid ß- and Ω-oxidation enzymes and the concommitant increase of production of reactive oxygen species like $H_2O_2$ might be responsible for hepatocarcinogenesis. We describe here three novel nuclear hormone receptors of *Xenopus laevis* (xPPAR-1,-2, and -3), which are activated by chemicals causing peroxisome proliferation (peroxisome proliferators) and, which regulate the peroxisomal ß-oxidation of fatty acid. The receptors were cloned by low stringency screening of cDNA libraries with DNA probes comprising the conserved DNA binding domain of nuclear hormone receptors. On the basis of their domain arrangement and the sequence of the P-box (CEGCKG), they belong to the group of nuclear hormone receptors including thyroid hormone, retinoid, and vitamin D receptors. Since the cDNAs of xPPAR-1 and xPPAR-2 were isolated from an oocyte library, whereas the cDNA of xPPAR-3 was isolated from a liver library, we have investigated the expression of all three mRNAs in oocytes, embryos and several adult organs. xPPAR-1 and xPPAR-2 are expressed throughout oogenesis, embryogenesis and in all adult organs tested. During oogenesis and embryogenesis xPPAR-2 is the most abundant of the three xPPAR mRNA species and xPPAR-3 could not be detected. xPPAR-3 is mainly transcribed in fat body and kidney and very little in liver. We have been able to demonstrate in transient co-transfection experiments that all three receptors activated the acyl-CoA oxidase (ACO) gene, which encodes the key enzyme of the peroxisomal fatty acid ß-oxidation spiral and, which is induced by peroxisome proliferators in *Xenopus in vivo* (Ciolek and Dauça, 1991). This induction is dependent upon the presence of peroxisome proliferators. Furthermore, we have localized the regulatory sequence in the ACO gene promoter to the region -553 to -578, where a protein binding site was found by footprint analysis (Osumi et al., 1991). In conclusion, PPARs may be essential control elements of fatty acid metabolism and may be responsible for peroxisome proliferator induced liver carcinogenesis.

L 122 GLUCOCORTICOID RECEPTOR PHOSPHORYLATION IN YEAST. M. Krstic, M.J. Garabedian, and K.R. Yamamoto, Dept. of Biochemistry, UCSF.

The glucocorticoid receptor is known to be a phosphoprotein, and specific sites of serine and threonine phosphorylation in hormone-treated mouse and hamster cells have been reported. However, the possible functions of these modifications have not been determined. As one approach to such an analysis, we are studying phosphorylation of rat glucocorticoid receptor sequences expressed in *Saccharomyces cerevisiae*, in which receptor action is similar to that seen in animal cells. We have found that the receptor in yeast is phosphorylated in the absence of hormone, and that agonist binding is accompanied by additional phosphorylation. These hormone-dependent phosphorylation events produced a reduction in the electrophoretic mobility of the receptor which was reversed by alkaline phosphatase treatment; interestingly, the magnitude of the mobility change correlated with agonist potency. Tryptic peptide maps of receptor metabolically labelled with $^{32}P$ in yeast and mammalian cells were qualitatively similar, except the relative labeling intensity of certain peptides differed in the two species and at least one yeast-specific peptide was observed. Labeling studies with receptor deletion mutants in yeast indicated that most of the receptor phosphates reside within an N-terminal region that includes a transcriptional activation domain, and that the others lie within or close to the zinc finger region, which contains activities for DNA binding, transcriptional regulation, receptor dimerization and contact with nonreceptor proteins. These two segments of the receptor encompass the phosphorylation sites described in mammalian cells, and include ten potential sites for the Ser-Pro-directed family of protein kinases. Mutants that alter these sites are being constructed to test their effects on receptor function.

L 123 **ISOLATION AND CHARACTERIZATION OF THE 5'-FLANKING REGION OF THE MOUSE ANDROGEN RECEPTOR GENE.** M. Vijay Kumar, Michael E. Grossmann, Mark E. Leo, Evan A. Jones, and Donald J. Tindall, Departments of Urology and Biochemistry & Molecular Biology, Mayo Clinic/Foundation, Rochester, MN 55905.

It has been known for some time that androgens regulate their own receptor mRNA levels in target tissues. In order to understand further the mechanism of this regulation, we have isolated and characterized the 5'-flanking region of the mouse androgen receptor (mAR) gene. An EMBL3 mouse genomic library was screened with an EcoRI-AflII fragment of the mAR cDNA. A clone of about 1.5 kb was isolated, which is located immediately upstream of the ATG translation start site of the mAR cDNA. Analysis of the DNA sequence indicated neither TATA nor CAAT boxes. However, in addition to a GC box, a homopurine stretch of multiple GGGGA sequences is present. Further upstream of the homopurine stretch, an Ap1 binding site has been identified. A putative androgen response element is also present, which may be involved in autoregulation of the gene. About 400 bp upstream of the ATG site, we have found a functional cyclic AMP response element. To further analyze the 5'-flanking region, mutants of 200, 400, 600 and 800 bp deletions from the 5'-end were constructed using the PCR technique, cloned into a chloramphenicol acetyltransferase (CAT) pBLCAT3 reporter plasmid and transfected into QT6 cells. The full length construct expressed CAT activity 22X compared to the vector alone, whereas the mutants containing 200, 400, 600, and 800 bp deletions were 27X, 18X, 11X, and 2.5X, respectively. These results indicate that promoter activity is located between -1100 bp and -900 bp upstream of the ATG start site. Moreover, the 5'-flanking region of the AR gene consists of many regulatory sequences which may be involved in its regulation, including autoregulation by androgen. We are currently analyzing the functional contributions of these elements. (Supported by NIH grant CA 32387 and HD 09140.)

L 124 EXPRESSION OF THE AVIDIN AND ITS RELATED avr3 GENE IN CHICKEN MACROPHAGES, Tarja Kunnas[1], Pekka Lappalainen[2], Mika Wallén[1], Timo Joensuu[1] and Markku S. Kulomaa[1,2], [1]Univ. Tampere, Dept. Biomed. Sci., Tampere, and [2]Univ. Jyväskylä, Dept. Biology, Jyväskylä, Finland.
Molecular cloning has indicated existence of multiple avidin related genes avr1-avr5 with high homology to the chicken avidin gene (see Keinänen et al.). Besides well-known progesterone receptor mediated induction of avidin in the oviduct, it is also induced in most chicken tissues in connection with inflammation and infections. To ascertain whether the avrs are expressed due to bacterial infection, hybridization analysis and RT-PCR methods were used. The results indicated the presence of avidin mRNA in the oviduct and intestine after E. coli infection (RNA hybridization analysis). The "inflammation avidin" was found mainly be encoded by the avidin gene, but the avr3 was also expressed at a low level in both tissues (RT-PCR and sequencing). Immunohistochemical studies suggested that macrophage-like cells might be responsible for synthesis of the "inflammation avidin" in these tissues. By an enzyme immunoassay, a chicken macrophage cell-line HD-11 was found to express and secrete avidin which again was mainly encoded by the avidin gene but also by the avr3. In conclusion: (1) The "inflammation avidin" is mainly encoded by the avidin gene suggesting a multifactorial regulatory region. (2) The macrophage cell-line HD-11 provides an interesting cell system for the studies of progesterone receptor and inflammation mediated avidin gene expression. (3) The avr3 gene is expressed at a low level in the oviduct and intestine as well as in the HD-11 cells. It remains to be seen whether the avr3 is also expressed in the oviduct by progesterone?

L 125 THE LIMITS OF THE CELLULAR CAPACITY TO MEDIATE AN ESTROGEN RESPONSE, Peter J. Kushner, Garbriela N. Lopez, Geoffrey Green, John D. Baxter and Paul Webb, Metabolic Research Unit, University of California, San Francisco, CA 94143
While steroid response is generally restricted by the availability of steroid receptors, the theoretical limits of the response are not known. We have constructed a series of cell lines that stably express the estrogen receptor (ER) at levels up to 5,000,000 per cell and employed these cells to explore the limits of the estrogen response. Several reporter genes with estrogen response elements upstream of the herpes tk promoter showed typical saturation kinetics with increasing ER. Maximum response was 10 times that seen in cell lines with receptor titers comparable to physiological levels. Half maximal responses required 500,000 receptors per cell, and cells with 5,000,000 ERs showed greater than 90% maximum induction. Estradiol dose response studies indicated that the receptors are limiting below 500,000 ERs per cell, but at higher ER titres there are "spare" receptors. In contrast, the widely used reporter pA2-CAT, which has 200 base pairs of Xenopus vitellogenin DNA between the response element and the promoter, showed squelching at ER levels beyond 500,000 per cell. Cell lines that expressed ER above this level activated pA2-CAT with a distorted hormone dependence, where saturating ligand concentrations were inhibitory. All reporters displayed squelching when the ER was provided by transient transfection at a level that we judge is 20 million per cell by extrapolation from the behavior of stable cell lines. These findings suggest that saturation of the cellular capacity to mediate an estrogen response and ER dependent squelching occur at receptor titres well above those encountered in nature. If current models of steroid hormone action are correct, the findings also imply that estrogen response elements are occupied to very small extents under normal conditions.

L 126 DIFFERENTIAL REGULATION OF HUMAN PROGESTERONE RECEPTOR A- AND B-MEDIATED TRANSACTIVATION BY PHOSPHORYLATION, Syed Kazmi, Vito Visconti, Richard Plante, Audrey Phillips*, Do Won Hahn*, Robert Capetola* and Catherine Lau, The R.W. Johnson Pharmaceutical Research Institute, Toronto, Canada; *The R.W. Johnson Pharmaceutical Research Institute, Raritan, N.J.
Phosphorylation plays a major role in the transcriptional activity of chicken progesterone A and B receptor isoforms. Cyclic AMP, given in the absence of progesterone, can mimic progesterone-dependent, receptor-mediated transcription. Since human progesterone receptors are also suggested to be phosphoproteins, the present study was designed to investigate the relative roles of the kinase-mediated signal transduction pathways in the regulation of receptor activity. Unlike chicken receptors, no significant ligand-independent transcription activity was observed with either 8-bromo cyclic AMP or phorbol ester (PMA) in the absence of progesterone. However, activators of both kinases synergized with R5020 to yield amplified CAT activity, driven by hPRa and hPRb receptors. Interestingly, the effect of cyclic AMP or okadaic acid appeared to be much more pronounced on hPRa receptor-mediated transcription, as compared to hPRb. The cyclic AMP-mediated differential regulation was observed with both simple (PRE2-tkCAT) and complex (MMTV-CAT) promoters. PMA-induced protein kinase C, on the other hand, did not discriminate between hPRa and hPRb-mediated CAT activity. Observations with inhibitors of protein kinase A and C, as well as co-transfection of hPRa and hPRb further suggested the preferential activation of hPRa by cyclic AMP. The physiological implications of the differential activation of the two progesterone receptor isoforms and subsequent target gene regulation will be discussed.

L 127 **THE THYROID HORMONE RECEPTOR (TR) VARIANT, c-erbAα2, AND TRα1 HAVE DIFFERENT DNA-BINDING AND HETEROMERIZATION PROPERTIES, Mitchell A. Lazar, Deborah Katz, and Thomas J. Berrodin, Departments of Medicine and Human Genetics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104.**
TRs mediate the regulation of gene transcription by thyroid hormone (T3). c-erbAα2, a C-terminal TR variant which does not bind T3, is a dominant inhibitor of T3 action. When synthesized in *E. Coli*, α2 formed two DNA-binding complexes which migrated more slowly than the corresponding monomeric and homodimeric forms of TRα1, consistent with the larger size of α2. However, α2 bound DNA more weakly than TRα1 and failed to form heteromeric complexes in the presence of nuclear proteins which interact with TRs. α2 also did not bind DNA as a heterodimer with TRα1. We also studied C-terminal mutants synthesized in reticulocyte lysates. Deletion of the last 20 of the 122 unique amino acids (aa) of α2 increased its DNA binding to approximately that of TRα1, indicating that the C-terminus of α2 is an inhibitory domain. This α2 mutant (α2ΔC) was still unable to heterodimerize with nuclear proteins, as were C-terminal deletion mutants of TRα1. We hypothesized that fusing the 40 unique aa of the TRα1 C-terminus to the end of α2ΔC would allow it to heterodimerize. However, neither this α2/α1 chimera nor one containing the last 70 aa of TRα1 heterodimerized with nuclear proteins. Yet, addition of the last 100 or 150 aa of TRα1 to the end of α2ΔC did allow heterodimerization. Thus, α2 cannot heterodimerize with TR-binding proteins because it lacks a transferrable C-terminal structure present in TRα1. The dominant negative activity of α2 is not likely to be due to heterodimerization with proteins which enhance TR binding to DNA, but could still be due to competition with the TR either for DNA binding or for other limiting transcription factors.

L 128 CHARACTERIZATION OF A NEO TRANSGENE WHICH IS TRANSCRIPTIONALLY DOWN-REGULATED BY GLUCOCORTICOIDS, Stephen S. Lippman, John R. Bracamontes, Jacqueline Howard-Bedford, Department of Medicine, Division of Endocrinology, University of Arkansas for Medical Sciences, Little Rock, AR 72205

We have developed an enhancer-trap strategy to clone additional genes that are down-regulated by glucocorticoids in AtT-20 cells not identified by previous methods which depended on information about the protein expressed by each gene. This strategy uses the plasmid, pA10-NEO, which contains the selectable marker gene, amino-glycoside 3' phosphotransferase II, coupled to an enhancerless SV40 promoter. When transfected, this gene is only effectively expressed when integrated near an active enhancer. In a stable, transformed clone of AtT-20 cells (IDG8) transfected with pA10-NEO, NEO mRNA level is down-regulated by dexamethasone. When IDG8 cells were grown in DME/F12 medium with ethanol vehicle or $10^{-6}$ M dexamethasone for 4 hours and nuclei isolated for *in vitro* run-on transcriptional assay, dexamethasone treatment decreased transcription of the NEO gene to 54±5.6% (SEM, n=3 separate experiments) of the level measured in control nuclei. Southern analysis of IDG8 genomic DNA digested with multiple restriction enzymes has shown it to contain a single inserted copy of NEO DNA. IDG8 genomic DNA was partially digested with MBO I and ligated into EMBL3 phage to construct a genomic library. Phage clones containing the NEO gene and approximately 15 kilobases of both 5' and 3' flanking genomic DNA were identified and sub-cloned into plasmid pUC18 for sequencing. A restriction map of genomic DNA adjacent to the inserted NEO gene has been generated. A genomic library of DNA from wild-type AtT-20 cells has also been made and phage whose DNA hybridized to genomic sequences adjacent to the NEO gene in the IDG8 cells has been identified. The characterization of these cloned sequences will allow direct analysis of the mechanism of glucocorticoid-down-regulation of this transgene.

L 129 INTERACTION OF THE $\tau_1$ TRANSACTIVATION DOMAIN OF THE HUMAN GLUCOCORTICOID RECEPTOR WITH THE TRANSCRIPTIONAL APPARATUS, Iain J. McEwan, Anthony P.H. Wright, Karin Dahlman-Wright, Jan Carlstedt-Duke and Jan-Åke Gustafsson, Center for Biotechnology and Department of Medical Nutrition, Karolinska Institute, NOVUM, Huddinge University Hospital, Huddinge S-141 57, Sweden.

The glucocorticoid receptor (GR) is a member of a large family of ligand dependent sequence-specific transcription factors, that includes receptors for other steroid hormones, thyroid hormones, vitamin $D_3$ and retinoic acid. After the binding of it's cognate ligand and DNA response element the subsequent events whereby the GR effects the rate of target gene expresssion remain unclear. However, protein-protein interactions are likely to play a major part in this process. We have studied the role of protein-protein interactions in the mechanism of action of the $\tau_1$ transactivation domain, using recombinant receptor proteins expressed in bacteria and yeast cells. Over expression of the $\tau_1$ domain in yeast cells resulted in an inhibition of reporter gene activity (squelching) and severe impairment of cell growth. Kinetic studies revealed that the effect on cell growth occurred subsequently to the effect on gene expression. Analysis of different promoter constructs suggested that the $\tau_1$ transactivation domain contacted directly a factor(s) required for the activity of a basal promoter. The implications of these studies for GR action together with results from *in vitro* studies will be discussed.

L 130 RESISTANCE TO GLUCOCORTICOIDS IN MULTIPLE MYELOMA: TRANSIENT EXPRESSION OF A C-TERMINALLY TRUNCATED GR mRNA, Moalli PA, Pillay S, Cidlowski JA, Krett NL, and Rosen ST, Northwestern University, Chicago, IL 60611 and University of North Carolina, Chapel Hill, NC 27599

Glucocorticoids are successfully used in the treatment of multiple myeloma, however, patients ultimately develop resistance to their therapeutic effects. To address this issue, we have been studying the effects of the glucocorticoid dexamethasone (DEX) on hormone sensitive and resistant clones (MM.1S and MM.1R, respectively) of a human myeloma line. MM.1S is highly sensitive to the cytolytic effects of glucocorticoids previously observed in T-cell malignancies. This response is effectively blocked by the glucocorticoid receptor (GR) binding antagonist, RU486. MM.1S synthesizes the full length 7.1 kb GR mRNA and approximately 50,0000 ligand binding GR per cell. In contrast, MM.1R maintains 90% viable in high levels of dex ($10^{-5}$M), expresses the 7.1 kb GR transcript at low levels and has little hormone binding activity. During its early passages in culture, MM.1R expresses a variant GR mRNA of 5.5 kb that has a deletion in its 3' end including a significant portion of the hormone binding domain. With time, the expression of the aberrant transcript is repressed resulting in a "receptorless" phenotype. No discernible differences in the genomic GR sequences of MM.1S and MM.1R are detectable by Southern analysis. Mobility shift assays using a synthetic glucocorticoid responsive element (GRE) have demonstrated the presence of a faster migrating GRE-protein complex in extracts isolated from MM.1R as compared to MM.1S. However, the protein has not yet been detected by Western analysis. Currently, our efforts are focused on cloning the cDNA for the aberrant GR so that functional assays can be performed.

L 131 **FREQUENT ALLELES OF THE HUMAN VITAMIN D RECEPTOR GENE ARE FUNCTIONALLY DISTINCT.** **N.A. Morrison*, R. Yeoman, P.J. Kelly and J.A. Eisman.**

Garvan Institute of Medical Research. St. Vincent's Hospital. Sydney 2010 Australia

The vitamin D receptor is the central regulator of the vitamin D endocrine system. Mutations which have a profound effect on the receptor result in clinical disorders of calcium homeostasis and bone formation. Little is known of more subtle effects of receptor genetic variation. Osteocalcin is a bone protein regulated by the vitamin D receptor and osteocalcin which appears in serum is monitored as an index of bone formation rate. Twin studies have shown a strong component of genetic control on serum osteocalcin levels as well as bone mass. However, paradoxically, osteocalcin levels are negatively correlated with bone mass.

We hypothesized that if functionally significant genetic variation exists in a trans-acting factor gene (such as that of the vitamin D receptor), this should be detected by a correlation of alleles of the gene with the product of a target gene regulated by the factor in question. The vitamin D receptor is the most powerful known regulator of the osteocalcin gene, acting directly through a vitamin D responsive enhancer in the osteocalcin promoter. This model was applied to the relationship between the osteocalcin gene and the vitamin D receptor by correlating genetic variation in the vitamin D receptor gene with osteocalcin serum concentrations.

We describe here that frequent RFLPs which define human vitamin D receptor alleles fulfil the requirement as markers for functionally distinct receptor genes in that they are highly correlated with either high or low serum concentrations of osteocalcin in normal subjects. This genotypic classification essentially splits the normal range: the mean (±SD) osteocalcin values of genotype subgroups are homozygote A 17.3±7.2 ng/ml, heterozygote 9.0±4.6 ng/ml and homozygote B 7.7±4.7 ng/ml. Since the osteocalcin gene is on chromosome 1 and the vitamin D receptor gene is on chromosome 12 the only mechanism by which increased levels of serum osteocalcin can be correlated with vitamin D receptor gene RFLPs is through different functionality of the vitamin D receptor alleles. This example of functionally different naturally occurring alleles of a trans-acting factor provides the paradigm for other genes of the steroid receptor superfamily and their relationship to physiological variation and homeostasis as well as diseases of multifactorial etiology, such as osteoporosis.

L 132 RETINOIC ACID DEPENDENT TRANS-ACTIVATION OF MAJOR HISTOCOMPATIBILITY COMPLEX CLASS I PROMOTERS BY A NUCLEAR HORMONE RECEPTOR H-2RIIBP IN UNDIFFERENTIATED EMBRYONAL CARCINOMA CELLS

Toshi Nagata, James H. Segars, Ben-Zion Levi, and Keiko Ozato. Laboratory of Developmental and Molecular Immunity, Natinal Institute of Child Health and Human Development, N.I.H. Bethesda, Maryland 20892

H-2RIIBP is a member of the nuclear hormone receptor superfamily that binds to the region II enhancer of major histocompatibility complex (MHC) class I genes. The role of H-2RIIBP in developmental regulation of MHC class I genes has been studied in undifferentiated embryonal carcinoma N-Tera2 cells. Transient co-transfection of the expressible H-2RIIBP plasmid enhanced MHC class I promoter activity in cells briefly treated with retinoic acid. Retinoic acid concentrations required for H-2RIIBP trans-activation overlapped with those required for induction of morphological differentiation and expression of endogenous MHC class I genes. This trans-activation was mediated by region II, as a heterologous tk promoter driven by region II also increased its activity when co-transfected with H-2RIIBP. Deletion of the bulk of the DNA binding domain or the ligand binding domain of H-2RIIBP, but not of the N-terminal domain abolished trans-activation, indicating that the former two domains are both critical for the enhancement. This trans-activation exhibited a strict cell-type restriction. As observed in other cell lines, N-Tera2 cells that had undergone differentiation failed to elicit trand-activation, suggesting that H-2RIIBP acts in concert with a co-factor expressed in undifferentiated N-Tera2 cells, which requires retinoic acid for its function.

L 133 CELL SIGNALLING AND THE MODULATION OF GLUCOCORTICOID RECEPTOR ACTIVITY, Steven K. Nordeen, Marissa L. Moyer and Betty J. Bona, Department of Pathology and Program in Molecular Biology, University of Colorado Health Sciences Center, Denver, CO 80262

Recent data from a number of laboratories has indicated that glucocorticoid receptor function is modulated directly or indirectly by both cytoplasmic and nuclear oncogenes. Additional evidence indicates that the activity of the chicken progesterone receptor can be modulated post-transcriptionally, perhaps at the level of phosphorylation. We have begun to investigate the ability of conditions that activate or repress different cell signalling pathways to modulate the transcriptional activity of the glucocorticoid receptor. A sensitive reporter system has been constructed using a breast carcinoma cell line possessing a stably integrated MMTV-luciferase reporter gene and expressing constitutively high levels of glucocorticoid receptor. Treatment of this cell line with activators of protein kinase A, protein kinase C, cell stress, or inhibitors of protein phosphatase 1, protein phosphatase 2A, or protein synthesis rapidly (within 4 hours) increase the glucocorticoid response of the MMTV-luciferase reporter. No effect is seen in the absence of hormone. Current experiments are directed to early events shared by these very different modulators in hopes of defining the mechanism which culminates in altered receptor activity.

L 134 **GLUCOCORTICOID REPRESSION OF TRANSCRIPTION VIA A NEGATIVE GLUCOCORTICOID RESPONSE ELEMENT INVOLVES INTERPLAY BETWEEN THE GLUCOCORTICOID RECEPTOR AND TWO DNA-BINDING NUCLEAR FACTORS.**

Sam Okret and William Cairns. Department of Medical Nutrition, Karolinska Institute, Huddinge University Hospital F60, Novum, S-141 86 Huddinge, Sweden.

We have studied the mechanism of repression of gene transcription by glucocorticoids using a negative glucocorticoid response element from the bovine prolactin gene. This element increased basal transcription from a heterologous promoter in mouse Hepa 1c1c7 cells, which could then be repressed by dexamethasone. This data suggested that a constitutively acting positive transcription factor(s) interacted with this element. Gel retardation analysis with nuclear extracts demonstrated the presence of two specific protein-DNA complexes. One of the complexes was shown to be dependent on octamer factor Oct-1. The other factor (XTF) was found to bind to the same region of the DNA element as the glucocorticoid receptor. Further gel retardation experiments showed that the binding of Oct-1 to the prolactin element was dependent on XTF and at least in part due to a direct protein-protein interaction. This was further supported by the appearance of the Oct-1 dependent complex with sigmoidal kinetics. Mutation of the Oct-1 binding site in the prolactin element slightly reduced the basal level of transcription without affecting the ability of the GR to repress. The glucocorticoid receptor dimer was also shown to bind specifically to this element in gel retardation experiments. Surprisingly however, incubation with the bacterially expressed isolated DNA binding domain of the receptor resulted in the formation of a monomeric protein-DNA complex, suggesting that in contrast to activation, only one of the GR moities in the full length GR dimer determines the specificity. We postulate that the binding of the glucocorticoid receptor to this element interferes with the binding and/or activity of the nuclear factors resulting in a reduction of transcription.

L 135 MODULATION OF TRANSCRIPTION FACTOR ACTIVITY BY A DISTANT STEROID MODULATORY ELEMENT,

Hisaji Oshima and S. Stoney Simons, Jr., Steroid Hormones Section, NIDDK/LMCB, National Institutes of Health, Bethesda, MD 20892

Variations in the biological agonist activity of antisteroids, as determined by their percent agonist activity, is a well-known but poorly understood phenomenon. For example, in tyrosine aminotransferase (TAT) induction by the antiglucocorticoid dexamethasone 21-mesylate in Fu5-5 rat hepatoma cells, the percent agonist activity is higher than that of infected MMTV and varies with (i.e., is modulated by) the density of cultured cells. A 21 bp segment at ~-3.6Kb of the rat TAT gene has now been isolated which confers high percentages of glucocorticoid receptor-mediated agonist activity to heterologous genes and promoters in transient transfections. Clustered base substitutions identified the 4 base sequence (CGTC) as being crucial for the biological activity. Furthermore, in cells at different densities, this sequence can modulate the induction activity of antiglucocorticoids (percent agonist activity) with a heterologous gene in a manner unlike that of other reported *cis*-elements but identical to that seen for the endogenous TAT gene. We therefore call our element a glucocorticoid modulatory element (GME). Gel-shift data showed the specific binding of a *trans*-acting factor to this GME, the sequence of which was different in competitive gel shift assays from related *cis*-sequences for other reported *trans*-acting factors. A model incorporating this new element (GME) is advanced which can explain the known features of TAT induction and may be generally applicable for the mechanism of action of other steroid hormones.

**L 136** MUTATIONS OF THE ANDROGEN RECEPTOR GENE IN PATIENTS WITH PARTIAL ANDROGEN INSENSITIVITY
M.N. Patterson, J.A. Batch, B.A.J. Evans*, I.A. Hughes, Department of Paediatrics, University of Cambridge and * Department of Child Health, University of Wales College of Medicine, Cardiff, United Kingdom.

The androgen insensitivity syndrome (AIS) is a group of X-linked disorders comprising a spectrum of genital phenotypes in 46XY subjects. The phenotypes range from complete AIS (normal female appearance) to partial AIS (including ambiguous genitalia, perineoscrotal hypospadias and micropenis). Mutations of the androgen receptor (AR) gene have been described in both complete and some forms of partial AIS.

We have identified AR gene mutations in subjects from two unrelated PAIS families. Brothers in the first family (AB and MB) both presented with severe perineoscrotal hypospadias, bilateral cryptorchidism and micropenis. In contrast, brothers in the second family (TS and MS) had isolated hypospadias, with normally descended testes and penile size. Biochemical studies in both families demonstrated normal androgen synthesis. Androgen binding assays from all four subjects demonstrated reduced binding affinity and thermolability, suggesting a defect in the androgen receptor. We have combined PCR amplification of AR exons and single strand conformation polymorphism to screen for point mutations within the AR gene. Mutations were then defined precisely by DNA sequencing of the relevant gene segment. We have found single base AR gene mutations in both families. In AB and MB the mutation changes amino acid 855 from arginine to histidine, and in TS and MS isoleucine 869 is changed to methionine. These mutations presumably give rise to the observed abnormality in androgen binding. However, the difference in the clinical phenotypes between the two families remains unexplained. One possibility is that the mutation at position 855 has a more profound effect on overall AR function, thus giving rise to the more severe phenotype in AB and MB. We plan to test this hypothesis by expressing the mutant ARs in COS cells and examining their ability to transactivate a reporter construct. Complex perineoscrotal hypospadias and familial isolated hypospadias represent different parts of the spectrum of PAIS. We have demonstrated that both these abnormalities may be explained by mutations of the androgen receptor gene.

**L 137** TRIIODOTHYRONINE (T3) ALTERS *IN VITRO* RECOGNITION OF TARGET DNA ELEMENTS BY T3 RECEPTORS (TRs), Ralff C.J. Ribeiro, Peter Kushner, James Apprileti, Brian L. West and John D. Baxter, Metabolic Research Unit, University of California, San Francisco, CA 94143.

The effects of T3 are mediated by nuclear receptors that bind to specific DNA sequences termed TREs. In contrast to steroid receptors the TRs are found tightly bound to chromatin in the absence of T3 and previous studies suggested that T3 treatment does not stimulate *in vitro* TR-DNA binding. We used gel shift assays to demonstrate the binding pattern and effects of T3 on binding of TRs from several sources to a variety of TREs. Human beta TRs produced in bacteria, yeast and in reticulocyte lysate system bind to oligos containing the palindromic motif 5'GGTCA-TGACC (frog vit A2-3bp gap and the TREpal-no gap) as both monomers and dimers, to a chicken embryonic myosin (EM1) containing one perfect half-site 5'TGACC as monomers and to a mutated version of EM1 (EM1pal-inverted palindrome) 5'TGACC-GGTCA as dimers. T3, independent of DNA structure, greatly increased the monomer-DNA interaction but had diverse effects on the dimer-DNA interaction. T3 had minor or positive effects on dimer-DNA interaction of vit A2 and TREpal respectively, and negative effects on dimer-DNA interaction of TREs with opposite orientation of half-sites. These effects were much more pronounced with TRs partially purified from E. coli. Saturation experiments suggest that the T3 augmentation of DNA binding was due to an increment of the number of TRs that are able to bind DNA and not by increasing the affinity of TR-DNA interaction. Furthermore, T3 increased the gel mobility of monomer-DNA interactions implying that ligand binding may induce conformational changes on them. The positive effects of T3 on the monomer-DNA configuration and the fact that naturally occuring TREs are not palindromic suggest that T3 might generate at the DNA binding level monomeric forms of TR that may favor heterodimerization with other proteins. These findings together with the influence of spacing and orientation of half-sites on T3 effects on dimer-DNA interaction can expand the capability of T3 receptors to regulate transcription.

**L 138** STRUCTURE - FUNCTION RELATIONSHIP OF HUMAN ANDROGEN RECEPTORS, MUTATED IN THE STEROID BINDING DOMAIN, Carrie Ris-Stalpers, Hetty van der Korput, Hans-Udo Schweikert, Len Pinsky, Jan Trapman and Albert O. Brinkmann, Departments of Endocrinology & Reproduction and Pathology, Erasmus University, Rotterdam, The Netherlands, University of Bonn, Germany, McGill University, Montreal, Canada.

In three unrelated families with the complete form of androgen insensitivity we have identified two different single nucleotide alterations in codon 686 of the androgen receptor gene. An aspartic acid is substituted for a histidine as a result of a G→C mutation, whereas a G→A mutation leads to an aspartic acid → asparagine mutation. Both mutant androgen receptors were generated in vitro and expressed in Cos and HeLa cells. The receptor protein with the aspartic acid → histidine mutation displays an androgen binding capacity 15-20% compared to the wild-type receptor, whereas the aspartic acid → asparagine mutation has normal androgen binding capacity, but a rapidly dissociating ligand-receptor complex. These binding characteristics mimic those found in genital skin fibroblasts of the patients. Transcriptional activation in HeLa cells was strongly diminished by both mutant receptors and was only partially restored using a 100-fold higher concentration of ligand compared with wild-type receptor. Amino acid 686 of the human androgen receptor appears essential for normal androgen receptor function and is clearly implicated in the process of ligand binding. Whether, in addition to hormone binding, amino acid residue 686 is directly involved in transcriptional activation, is currently under investigation.

**L 139** TRANSACTIVATION BY THE GLUCOCORTICOID RECEPTOR, Sandro Rusconi; Rainer Lanz; Stefan Wieland; Franca Baggi; Tiziano Tallone; *Institut für Molekularbiologie II der Universität, Winterthurerstrasse 190, 8057 Zürich, Switzerland.*

We have studied the behavior of wild type or mutated glucocorticoid receptor (GR) by expressing GR-cDNAs along with appropriate reporter genes and other non-ubiquitous trans-activators in mammalian cells.
**(1)** We have analyzed the effect of all possible substitutions of the evolutionary ultra-conserved Cys500 residue, either alone or in combination with other specific point-mutants. While our results are generally compatible with the recently published 3-dimensional structure of the GR-DNA complex, some of our data are in open conflict with other publications. So far, we have been unable to separate the weak transactivation function contained within the Zn-finger region from DNA binding.
**(2)** We have observed that in presence of relatively small amounts of GR, the action of ectopically expressed lymphoid-specific Oct2A is significantly impaired. Control experiments demonstrate that the interference is not caused at the level of target DNA binding and that the competition is not mutual. We speculate that the GR-mediated inhibition of Oct2A action is due to titration of one or more rate-limiting co-activator(s) (Wieland *et al.*, 1991, EMBO J. 10, pp. 1513-2521).
**(3)** Systematic studies on artificial promoters and enhancers in which the idealized GREs (palindromic GREs) have been separated by different distances (from 24 to 96 bp, center-to-center) demonstrate that the intact or carboxy-truncated GR (i.e., depleted of hormone binding domain, HBD) can be distinguished in their cooperative stimulation. Our results are consistent with the idea that chromatin structure is involved in the process of cooperativity and may explain the size of the repeat length of some strong viral enhancers. The distance required for cooperativity maximum seems to be influenced by the presence of the HBD, which so far had never been suggested to play a role in the target recognition process.
**(4)** The rat GR contains a stretch of repeated glutamine residues within its major activation domain and this feature is shared by many other transcription factors. We have initiated a systematic study on the role of monotonous aminoacid repeats in the GR and other factors involved in transcription regulation. We can show that in the rat GR there are different effects depending on the frame by which the repeated segment is translated. Furthermore, our data suggest that there are tissue-specific sets of factors belonging to the repeat-containing class and we have devised a cloning strategy to better characterize them.

L 140 MODULATION OF ESTROGEN RESPONSIVE GENE ACTIVATION BY THE DNA BINDING-PROTEIN, H2RIIBP, James H. Segars, Mickey S. Marks, Paul H. Driggers, Steven Hirschfeld, Walter Wahli, and Keiko Ozato, Laboratory of Developmental and Molecular Immunity, NICHD, NIH, Bethesda, MD 20892, and Institut de Biologie Animale, Universite de Lausanne, Lausanne, Switzerland

H2RIIBP is a member of the nuclear receptor superfamily which recognizes several different hormone response elements, including estrogen response elements [ERE]. We have studied the ability of H2RIIBP to modulate estrogen-responsive gene transcription. Cotransfection experiments employing a vitellogenin A-2 ERE-tk-CAT reporter in the cell line MCF-7 showed that addition of an expression vector for H2RIIBP resulted in a dose dependent reduction in transcription. Specificity of the effect upon the ERE was demonstrated using related reporter constructs such as GRE-tk-CAT. Cotransfection of the A-2 ERE-tk-CAT reporter into the estrogen-receptor negative cell line, MDA-MB-231, with expression vectors for H2RIIBP and estrogen receptor (HEO) confirmed the specificity of inhibition. The inhibitory effect upon estrogen-dependent transcription was analysed further using domain deletion constructs of H2RIIBP in the same cotransfection system. Methylation interference experiments employing recombinant H2RIIBP demonstrated that binding to the A-2 ERE occurs via recognition of nucleotides similar to those recognized by the estrogen receptor. Using monoclonal antibodies directed against H2RIIBP in gel-shift analysis of nontransfected MCF-7 cell extracts, we observed an ERE binding activity which could be specifically super-shifted. These studies suggest that H2RIIBP is present endogenously in MCF-7 cells and is capable of inhibiting estrogen-responsive transcription by a specific interaction with an ERE.

L 141 A GLUCOCORTICOID RECEPTOR MUTANT WITH DISRUPTION OF THE SECOND ZINC-FINGER MOTIF BINDS TO THE GLUCOCORTICOID RESPONSE ELEMENT, Ganesan Srinivasan and E. Brad Thompson, Department of Human Biological Chemistry and Genetics, The University of Texas Medical Branch, Galveston, TX 77550.

A mutant human glucocorticoid receptor (465*) which carries a frame-shift mutation at position 465 that disrupts the second zinc-finger motif in the DNA binding domain was very active in inducing lysis of transfected glucocorticoid resistant ICR-27 cells (J Biol Chem 266:12976-12980, 1991). The 465* mutant was expressed in the Baculovirus Expression Vector System. A partially purified 465* was capable of retarding the mobility of a $^{32}$P-labelled 23-mer glucocorticoid response element (GRE) from the tyrosine amino transferase gene. Polyclonal anti-human GR ($AhuGR_{150-175}$) blocked the retardation of $^{32}$P-GRE suggesting that the retardation was due to 465* and that receptor-receptor interactions involving the tau-1 region stabilize the receptor-DNA complex despite the disruption of the second zinc-finger motif. Our results suggest that the 465* induced cell lysis may result from negative regulation, whereby the 465* binds to DNA and blocks transcription of gene(s) vital for maintaining cell viability.

L 142 MECHANISMS IN TRANS-ACTIVATION BY RETINOIC ACID RECEPTORS, Henk Stunnenberg, EMBL, Meyerhofstrasse 1, 6900 Heidelberg, FRG.

Retinoic acid receptor (RAR) and thyroid hormone receptor (T3R) are thought t ' 'nd as (homo/hetero)dimers to a T3 responsive element (T3REpa mprised of inverted repeats of the half-site motif GGTCA. However A responsive element (βRARE) was previously identified in the promoter of the RARβ2 gene which contains two direct repeats of the motif GTTCA spaced by a six nucleotide gap. Surprisingly, the GTTCA motifs rearranged to form a palindrome do not confer RA responsiveness to a heterologous promoter. Furthermore, no significant level of *trans*-activation is detected by ligand-activated RAR through the Moloney Murine Leukaemia Virus T3RE, which comprises two direct repeats of the sequence GGTCA/C spaced by a five nucleotide gap. Similarly, T3R does not induce gene expression through the βRARE. Through mutational analysis and co-transfection assays we have established the preference of T3R to *trans*-activate through direct repeats spaced by a 5 nucleotide gap as opposed to a 6 nucleotide gap. In contrast, RAR appears to be more flexible with respect to spacing requirements between repeats. Interestingly, although some elements mediate either RA or T3 induction, changing a single nucleotide in the MoMLV T3RE with a 5 nucleotide spacing creates a promiscuous RA/T3 responsive element.

The close apposition between the RARE and the TATA box is striking in comparison with other natural response elements for RA which so far have been located further upstream from the transcription start site. We therefore investigated whether RAR and TFIID functionally interact in trans-activation of the RAR-β2 promoter and which domain of TFIID is required for this presumed interaction. It has been postulated that the N-terminal domain of human and Drosophila TFIID is required for upstream transcription factor dependent trans-activation in vitro. We show that human TFIID and retinoic acid receptor (RAR) cooperate to activate the RARβ2 promoter in vivo when expressed in an EC cell line. Functional cooperativity by RAR and TFIID is strictly dependent on the presence of retinoic acid. The analysis of human TFIID mutants demonstrates that the core domain suffices to mediate trans-activation of the RAR-β2 promoter by ligand activated receptor. Drosophila but not yeast TFIID can substitute for human TFIID in trans-activation. Finally, we show that the functional cooperativity between the TATA box and RARE binding proteins renders the RAR-β2 promoter highly sensitive to subtle changes in RA and/or RAR levels.

L 143 **REPRESSION OF TGFβ1 AUTOREGULATION BY RETINOIC ACID AND NUCLEAR RECEPTORS**

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Oncogenesis can result from the amplification of growth factor expression, such as of transforming growth factor (TGF), or from the uncontrolled expression of oncogenes. It has been shown that TGFβ1 positively autoregulates its expression by inducing the expression of *c-jun*, which in turn transactivates TGFβ promoter through its AP-1 binding site. Realizing the involvement of retinoic acid (RA) in oncogenesis through possible regulation of the cross talk between TGFβ and AP-1 systems, we have investigated the effects of RA with retinoic acid receptor (RAR) in TGFβ-induced expression of TGFβ1 promoter. TGFβ1 is transcribed from two major promoters which are separated by 271 nucleotides. Both promoters have been shown to respond to autoregulation by TGFβ1. To investigate the effect of RA on the expression of TGFβ1, we have used the TGFβ1-CAT reporter plasmid in transfection of the hepatoblast HepG2 cell line. Cotransfection experiments indicated that TGFβ1 autoregulation is repressed by RARα and RARβ in an RA-dependent fashion. To see if repression of TGFβ1 by RARs results from RAR binding to TGFβ1 promoter we performed gel retardation analysis of TGFβ1 promoter with *in vitro* synthesized RARs. The results showed that RARs do not bind to TGFβ1 promoter. This suggests that RAR repression of TGFβ1 expression is not likely to involve competition of binding to DNA , but rather due to other mechanisms likely to involve protein-protein interaction. The mechanism by which RARs and other related nuclear receptors represses TGFβ1 autoregulation will be presented.

L 144 **INHIBITION OF ESTROGEN RECEPTOR ACTIVITY BY THE TUMOR PROMOTER TPA: A MOLECULAR ANALYSIS**

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Cell proliferation and phenotype of cells from female reproductive tissues are regulated by estrogens. It is therefore important to understand how estrogen action can be modulated. It has recently been reported that certain nuclear receptors can antagonize the tumor promoter TPA by direct interaction with the transcription factor AP-1, and that the AP-1 constituents cJun and cFos can inhibit receptor activity. This mutual antagonism appears to be based on direct protein-protein interaction. In the human breast cancer cell line MCF-7, TPA leads to growth arrest and altered cell morphology. We have investigated here whether in MCF-7 cells and other cell lines AP-1 and ER can inhibit each other's activity. We find that TPA or the AP-1 components cJun and cFos can inhibit $E_2$ dependent estrogen receptor activity in most cell lines investigated. In addition, ER mRNA is rapidly downregulated in MCF-7 cells. Gel retardation experiments show that ER DNA binding is inhibited in vitro by cJun protein while ER can also inhibit cJun DNA binding. However, in vivo we do not observe inhibition of AP-1 activity by ER in the here investigated cell lines. To the contrary, we observed an enhancing effect at low ER concentrations on AP-1. Together our data suggests a new regulatory pathway by which ER activity can be modulated by AP-1. Several mechanisms including ER-AP-1 protein interaction appear to be involved.

L 145 AN ARG TO HIS MUTATION IN CODON 311 OF C-ERBAβ RESULTS IN DEFECTIVE T3-BINDING ACTIVITY BUT DOES NOT NECESSARILY CONFER RESISTANCE TO THYROID HORMONES, S.J. Usala, J.B. Menke, J.M. Abdallah, T. Eaton, E.H. Hao, M.E. Geffner, A.J. Dulgeroff, N.Ross, J.M. Hershman, East Carolina University School of Medicine, Greenville, N.C. 27858; UCLA School of Medicine, Los Angeles, CA 90024

We have recently reported a point mutation, a guanine to adenine at nucleotide 1232, in the c-erbAβ receptor gene in a 17 year-old female with selective pituitary resistance to thyroid hormones (Usala et al., Thyroid, V.1, suppl. 1, 199l, S-89). This patient was hyperthyroid with very high serum $T_4$ and $T_3$ levels and inappropriately normal TSH. The adenine-1232 base substitution was shown to be a mutation by screening 94 random alleles. The parents of this patient have now been examined for the adenine-1232 mutation; the mother does not harbor this mutation, but the father does carry the mutant allele. Amazingly, the father has normal TSH and normal thyroid hormone levels (TSH=0.88 mU/l, normals 0.32 -5.0; free thyroxine = 1.43 ng/dl, normals 0.71 - 1.85; T3=150 ng/dl, normals 80-200 ng/dl). Furthermore the father has no symptoms of hypothyroidism. A mutant β-receptor cDNA, GH, with the adenine-1232 base was recreated and the receptor synthesized with reticulocyte lysate. The T3-binding affinity of receptor GH was markedly defective with $K_a$~ 5 x $10^8$ $M^{-1}$ compared to that of wild-type β-receptor with $K_a$~ 5 x $10^{10}$ $M^{-1}$. This perturbation in function contrasts with the ARG to HIS mutation in codon 315 of Kindred CL with generalized resistance to thyroid hormones where the mutant receptor T3-binding affinity was only reduced two-fold (Cugini et al., J.C.E.M., in press). It is presently unclear what role the adenine-1232 mutation may have in pituitary resistance to thyroid hormones. Interestingly, a novel c-erbAβ mutation has been identified in the ligand-binding domain, outside of canonical dimerization domains, which does not appear to be sufficient for a dominant negative function in man.

L 146 BINDING STUDIES OF THYROID HORMONE RECEPTOR RESPONSE ELEMENTS (T3REs) WITH VARYING SPACING BETWEEN THE HALF-SITES (AGGTCA). Gunilla Wahlström, Björn Vennström. Department of Molecular Biology, CMB, Karolinska Institute, Stockholm, Sweden.

Thyroid hormones (T3, T4) produce diverse physiological and developmental effects. This control is mediated by the thyroid hormone receptors (TRs) which belong to a superfamily of nuclear hormone receptors also comprising the receptors for glucocorticoids, oestrogen, retinoic acid and vitamin D3. The thyroid homone receptors regulate transcription of target genes through specific response elements (T3REs). The T3REs behave as transcriptional enhancers which function independently of position and orientation but are dependent upon the presence or absence of ligand. They contain a consensus motif AGGTCA which occurs as palindromic or direct repeats. We have determined the sequence and structural requirements of different T3REs with regard to binding of receptors as monomers and dimers. Modified oligonucleotides were synthesized and assayed in band shift experiments with HeLa nuclear cell extracts containing TRs expressed from vaccinia virus vectors. In a first series of experiments the properties of a perfect palindrome (TRE pal) are being compared with those of a half-site and palindromic or direct repeats containing various numbers of intervening nucleotides. Our present data show that TR alpha binds a half-site weakly, whereas direct repeats were bound at least as efficiently as TRE pal. The complexes formed are dependent upon spacing between the response element. For instance, two nucleotides between the half-sites results in TR binding as a monomer, whereas an increased spacing (3-6 nt.) results in receptor binding as both monomers and dimers. Response elements allowing receptor dimerization show a faster off-rate and, in addition, the off-rate is increased many fold in $Mg^{2+}$ containing buffers. The data suggest that thyroid hormone receptor - DNA interactions are strongly dependent on the spacing of half-sites when these occurs as direct repeats. We propose that receptor dimerisation on DNA is excluded by steric hinderance when the distance is short. Dissociation constants (kD) for receptor/DNA complex formation are in accordance with the off-rate analyses. Finally, we are correlating the in vitro receptor binding properties of the different T3REs with their ability to mediate transcriptional activation of T3RE-CAT reporter gene constructs.

L 147 PHOSPHORYLATION IN THE REGULATION OF NGFI-B, Christine A. Weaver, Ragnhild E. Paulsen and Jeffrey D. Milbrandt Washington University School of Medicine Departments of Pathology and Internal Medicine, St. Louis, Mo. 63110.

NGFI-B is a nuclear receptor which is induced in PC12 cells (and other cultured mammmalian cells) as part of the early response to growth factor stimulation and membrane depolarization. We have previously demonstrated that NGFI-B is multiply phosphorylated and that the predominant phosphorylated form varies depending on the inducing stimulus. Here, we report initial results of mapping studies to localize the phosphorylation in NGFI-B. By assessing the degree of phosphatase-sensitive MW heterogeneity on SDS PAGE in an estrogen receptor-NGFI-B chimera as well as in deletion mutants of NGFI-B, we have determined that most of the phosphorylation in the receptor occurs in the amino-terminus (A/B region) near the activation domain. In addition, results of pulse-chase and subcellular localization studies on N-terminal deletion mutants suggest that phosphorylation in this region may regulate receptor turnover and nuclear association.

L 148 CLONING OF FOUR COMPLEMENTARY DNAs OF THE RETINOIC ACID/THYROID RECEPTOR GENE FAMILY FROM NEURULATING *AMBYSTOMA MEXICANUM* EMBRYOS, Liz Wirtanen, Ursula Busse, Johane Guay and Carl Séguin, Centre de recherche en cancérologie de l'université Laval, l'Hôtel-Dieu de Québec, Québec, Canada.

Retinoic acid (RA) is involved in morphogenesis of the nervous system as patterning or morphogen agent. The effects of RA are mediated through nuclear RA receptors (RARs) that act as trans-acting factors which regulates the transcription of specific sets of genes. To study the effect of this class of molecules on the development of the nervous system, we screened a cDNA library from the amphibian *Ambystoma mexicanum* (axolotl) stage 18 neurulae with a fragment corresponding to the conserved DNA-binding domain of the RARδ of *Notophtalmus viridescens*. We have found that at least three different classes of nuclear receptor-encoding genes are expressed during neurulation in the axolotl. One (aRARγ) codes for the γ subclass. Another (aRARα), which probably corresponds to a partial clone since the ligand binding domain is missing, codes for the α subclass. The third class, comprising two different isolates (aRXR-1 and aRXR-2), encodes for putative receptors that have not been described previously. Of the nuclear RARs described previously, the deduced amino acid sequence of aRARγ most closely ressembles that of the $\gamma_B$ subtype of RAR found in mice and humans. Sequence analyses revealed that aRAR$\gamma_B$ and mouse RAR$\gamma_B$ show 58% amino acid identity in domain A, between 95% and 100% in domains B, C and E, but only 31% in domain F. *Notophtalmus* RARδ (which corresponds probably to a $\gamma_A$ subtype) and aRAR$\gamma_B$ are only 21% identical in domain A, 98% to 100% in domains B, C, D and E and 67% in F. aRAR$\gamma_B$ is expressed in both neuroectodermal and non-neuroectodermal tissues during neurulation. The RXR clones are very conserved in their DNA-binding domain with other members of the superfamily of steroid/thyroid receptors but they have no significant homology in the ligand binding domain. aRXR-1 transcripts are present in both neuroectodermal and non-neuroectodermal tissues during neurulation. Supported by the M.R.C. and the Spina Bifida Association of Canada.

*Poster Session II*

L 200 NUCLEAR FACTOR 1 IS INVOLVED IN VITAMIN $B_6$ MODULATION OF STEROID HORMONE RECEPTOR-MEDIATED GENE EXPRESSION, Victoria E. Allgood and John A. Cidlowski, Department of Physiology, University of North Carolina, Chapel Hill, NC 27599.

Glucocorticoid-induced gene expression mediated through the complex mouse mammary tumor virus (MMTV) promoter is inversely modulated by alterations in intracellular vitamin $B_6$ concentration. We have used a series of reporter plasmids with progressively less complex promoters to investigate the mechanism through which vitamin $B_6$ affects steroid receptor-mediated gene expression. We show that the level of glucocorticoid-induced gene expression from simple promoters containing only hormone response elements and a TATA sequence is quantitatively similar to that observed with the complex MMTV promoter, demonstrating that the receptor binds to its response element and activates transcription efficiently in the absence of other promoter elements which are present in the natural MMTV promoter. However, the level of expression mediated through the simple promoters was not affected by alterations in intracellular vitamin $B_6$ concentration, indicating that the vitamin does not influence either binding of the glucocorticoid receptor to DNA or activation of transcription from the hormone response element. In contrast, modulation of hormone-induced gene expression was restored upon inclusion of a binding site for the transcription factor NF1 within the hormone-responsive promoter; glucocorticoid-induced gene expression was reduced by 40% under conditions of elevated intracellular vitamin $B_6$ concentration and enhanced by 110% in mild vitamin deficiency. Analogous effects were observed with estrogen receptor-induced gene expression when an NF1 binding site was introduced into an estrogen-responsive promoter derived from the vitellogenin A2 gene. NF1-mediated constitutive transcription was not affected by alterations in vitamin concentration, indicating that the modulatory response is not mediated solely through the NF1 protein. In addition, the modulatory effect of the vitamin did not require strict positioning of or spacing between the receptor and NF1 binding sites. These observations are consistent with the notion that multiple transcription factors are involved in mediating the transcriptional response to hormone, and support the hypothesis that vitamin $B_6$ modulates steroid hormone-mediated gene expression through its influence on a functional or cooperative interaction between steroid hormone receptors and the transcription factor NF1. Supported by DK 32459

L 201 BINDING OF THYROID HORMONE RECEPTORS AS DIMERS TO DIRECT REPEAT RESPONSE ELEMENTS IS PREVENTED BY THYROID HORMONES. Monika L. Andersson, Kristina Nordström and Björn Vennström, Department of Molecular Biology, CMB, Karolinska Institute, Stockholm, Sweden.

Thyroid hormone receptors (TR) can either enhance or repress transcription depending on the presence or absence of ligand, whereas the v-erbA oncogene product, homologous to TRα but unable to bind ligand, functions as a trans-dominant repressor of TRα. This raised the question whether DNA binding is affected by thyroid hormone (T3), and therefore we investigated the effect of hormone on receptor/DNA interaction. The binding of TRα or TRβ (-/+ T3) to oligonucleotides with the consensus half site AGGTCA arranged in a palindromic or inverted or directly repeated configuration was assayed in band shift gels. These studies showed that, depending on the spacing between the half sites, either one or two TR molecules bound to the oligonucleotides. T3 abolished the binding of 2 TRs to the same DNA element when the half sites were present as direct or inverted repeats but not as palindromes. In heterodimer formation with receptors that do not bind T3, such as v-erbA or retinoic acid receptors, T3 abolished 50-70% of the heterodimerization using the direct or inverted repeat elements. Furthermore, monomer (and dimer) complexes migrated faster when bound T3, with the exception of those containing v-erbA protein and no other T3 binding protein. The data suggest that binding of T3 to homodimeric TRs complexed to DNA causes a conformational change allowing only one TR molecule to bind response elements with half sites arranged as direct or inverted repeats.

**L 202** DNA-binding properties and transactivation potential of wild-type and mutant ErbAα, J. Bigler, D. Tellier*, and R. N. Eisenman, Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA 98104 and *Department of Biochemistry, University of Washington, Seattle, WA 98195

Thyroid hormone receptor is a regulator of transcription, which can activate or repress transcription in a ligand-dependent manner. We studied the DNA-binding properties of wild-type and mutant chicken ErbAα p46 and p40 using co-immunoprecipitations and gel mobility shift assays. ErbA p46 was N-terminally tagged with a c-myc-derived sequence to provide an epitope for a monoclonal antibody. In co-immunoprecipitations using a mix of tagged and untagged ErbA proteins we show that ErbA dimers are only formed in the presence of a TRE. Mutants which have an intact DNA-binding domain but are defective for DNA-binding are also unable to form dimers. Results from gel mobility shift assays support the notion that ErbA exists in solution as monomers and forms a stable complex only after binding to DNA.

Since the mutations that we introduced into the C-terminus of the ErbA proteins influenced their DNA-binding properties, we assessed these changes quantitatively by determining the affinities of these proteins for the F2-TRE and $TRE_{pal}$. The mutants were then introduced into an eukaryotic expression vector and their transcriptional activation potential determined in transient transfections using constructs containing the F2-TRE or TREpal linked to the CAT gene. Results will be shown.

**L 203** VITAMIN D RESPONSE ELEMENTS AS DIRECT OR INVERTED REPEATS OF A PENTAMER MOTIF,

Carsten Carlberg, Igor Bendik and Willi Hunziker, Department PRTB, F. Hoffmann-La Roche LTD, CH-4002 Basel, Switzerland

Vitamin D receptors as well as retinoic acid- and steroid hormone -receptors act as ligand modulated transcription factor by binding to specific DNA sequences referred to as response elements. The retinoic acid response element identified in the retinoic acid receptor β promoter consists of a direct repeat of the motif GTTCA spaced by 6 nucleotides. An exchange of two nucleotides at position 2 and 4 of this motif to GGTGA renderes the element responsive to vitamin D. This shows that retinoic acid and vitamin D response elements are closely related and furthermore, that GGTGA is a good vitamin D response motif. Alterations in the spacing between the two motifs show that a maximal vitamin D response is obtained with a spacing of 6 or 7 nucleotides between the two motifs. Interestingly, the motif also confers a similar vitamin D inducibility when present as inverted repeat with no spacing. The data show that a vitamin D response element can be composed of two copies of a response motif arranged either as direct or as inverted repeat suggesting that the vitamin D receptor binds to the element as a dimer. The fact that the distance between the motifs is critical and different for the direct and the inverted repeat further suggests that the receptor may dimerize in a tandem and in a head to head complex on the directly and the inversely repeated motif, respectively.

**L 204** PROGESTERONE RECEPTORS BOUND TO RU 486 HAVE ENHANCED DIMER STABILITY AND PARTICIPATE IN A HETERODIMER WITH AN AGONIST-BOUND RECEPTOR, Angelo M. De Marzo, Steven K. Nordeen and Dean P. Edwards, Department of Pathology, University of Colorado Health Sciences Center, Denver, CO 80262

We demonstrate that the progestin antagonist, RU 486, promotes more efficient dimerization in solution of the human progesterone receptor (PR) than the agonist R5020. Using T47D breast cancer cells we show that this occurs when hormones are added *in vivo* to whole cells or *in vitro* to nuclear extracts. The more efficient dimerization correlates with an enhanced specific DNA binding activity of RU 486-bound receptors. We suggest, therefore, that one possible mode of RU 486 action is the failure of stabilized dimers to exchange and interact with other nuclear factors. Failure of the antagonist-receptor complex to interact appropriately with other nuclear factors is also inferred from two lines of evidence that suggest that RU 486 promotes a more compact or spherical ligand-receptor complex than that produced by R5020. Using the gel mobility shift assay with either endogenous T47D PR or baculovirus expressed A and B receptor isoforms, we also show that a PR subunit (PR-A) bound with RU 486 is capable of dimerizing with a PR subunit (PR-B) bound with R5020. We further demonstrate, using an immune coisolation assay, that these PR dimers occupied by heterologous ligands also form in solution. Thus, RU 486 action can potentially be mediated in a dominant-negative fashion by formation of inactive heterodimers. We propose that differences in dimer stability, altered PR conformation, and the ability to form inactive heterodimers all contribute to the antagonistic activity of RU 486.

**L 205** DNA SEQUENCE-SPECIFIC BINDING OF THE Ah RECEPTOR TO A DIOXIN RESPONSIVE TRANSCRIPTIONAL ENHANCER, Michael S.Denison and Eveline F. Yao, Department of Biochemistry, Mich. State Univ., East Lansing, NJ 48824.

Exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD, dioxin) and related compounds results in numerous species- and tissue-specific toxic and biological responses, including induction of microsomal cytochrome P-450IA1. The induction of cytochrome P450IA1 is mediated by the Ah receptor (AhR), to which TCDD binds saturably and with high affinity. Mechanistically, induction of P450IA1 is similar to that described for steroid hormones and their receptors. Following ligand (TCDD) binding, hsp90 dissociates from the AhR and the liganded AhR complex acquires the ability to bind to DNA with high affinity. The binding of transformed AhR to a specific DNA sequence, a dioxin responsive enhancer (DRE), adjacent to the CYPIA1 gene results in an increase in its rate of transcription. Deletion and DNA-binding analysis studies have revealed that the 5'-flanking region of the murine CYPIA1 gene contains at least 5 DREs to which transformed AhR can specifically bind. Sequence comparison has revealed that functional DRE sequences contain invariant core sequence of 5'-TNGCGTG-3', as well as several variable bases flanking the core. We have utilized gel retardation analysis to characterize the binding of transformed TCDD:AhR complexes to DRE-containing oliognucleotides. Liganded AhR binds specifically and with high affinity (Kd ~ 1nM) to double-stranded DRE oligomers and with low affinity to nonspecific DNA. Utilizing mutant DRE oligomers we have identified those nucleotides important for formation of the high affinity TCDD:AhR:DRE complex and have derived a putative TCDD:AhR DRE-binding consensus of GCGTGNNA/TNNNG/C. Nucleotide-specific binding of transformed TCDD:AhR to the DRE was ligand- and tissue-independent. Although the AhR is similar to steroid hormone receptors our results demonstrate that the AhR interacts with DNA in a distinctly different manner and may represent another class of ligand-dependent DNA regulatory proteins.

L 206 FUNCTIONAL AND IN VITRO BINDING STUDIES OF TR α AND β TO THE NATIVE TREs OF MALIC ENZYME AND MYELIN BASIC PROTEIN GENES.Beatrice Desvergne, Antonnella Farsetti, Josef Lazar and Vera Nikodem. National Institute of Health, NIDDK, Bethesda, MD 20892.

To elucidate differences in the functional properties of the two forms of thyroid hormone receptor (TRα or TRβ ), we previously identified and characterized the thyroid hormone response element (TRE) in two T3 responsive genes: , Malic Enzyme (ME), and Myelin Basic Protein (MBP). These genes differ in their tissue and developmental expression, ME is a housekeeping gene involved in lipid metabolism, MBP a brain specific and developmentally regulated gene. Their promoters are structurally dissimilar ( ME is GC rich, MBP contains a TATA and CCAAT-like sequence). Futhermore, the corresponding TREs contain different features.Mutation studies of MBP-TRE pointed out the importance of a palindromic-like sequence while ME-TRE encompasses 2 half sites organized as a direct repeat.

Transfection studies using either ME 352-CAT or MBP 256-CAT and RSV-TRα or TRβ expression vectors showed that ME-TRE functions more efficiently in the presence of TRα (α/β> 1.5) while MBP-TRE is more responsive to the TRβ (β/α> 4.5) This preference depends on the DNA binding domain (DBD) of the receptor since, in the case of MBP-CAT, a chimeric receptor $\beta_{DBD}/\alpha_{LBD}$ ( L: ligand) was a more efficient T3 dependent activator than $\alpha_{DBD}/\beta_{LBD}$,while ME-CAT no longer showed any significant preference for either chimeric receptor. Using in vitro translated (i.v.t.) receptors and gel shift assays, the α receptor forms two specific bands with MBP-TRE (designated as a putative dimer and monomer), while the TRβ forms a strong single specific band (putative dimer). However the ME-TRE forms only a single band with either i.v.t.receptor.

While the direct correlation between these binding studies and the functional differences described above is still unclear, it suggests that two thyroid hormone receptors might regulate different sets of genes, depending on the structure of TREs as well as promoter sequences. TRα and TRβ are highly related to each other but they do not have redundant functions. It is very likely that differences in TR α and β binding patterns as well as different chromatin environment contribute to the functional properties of these two receptors on these genes.

L 207 A GROWTH FACTOR-INDUCED GENE PRODUCT PRODUCED IN A BACULOVIRUS EXPRESSION SYSTEM IS A NUCLEAR ZINC FINGER PROTEIN THAT BINDS TO DNA, J. Albert Fernandez-Pol, Dennis J. Klos, Paul D. Hamilton, and Vera M. Schuette, Laboratory of Molecular Oncology, DVA Medical Center, and Department of Medicine, St. Louis University, St. Louis, MO 63106.

We have recently identified by cDNA cloning a gene, denoted MPS-1, that is activated in various cultured human cells by transforming growth factor beta (TGFβ) in combination with purified growth factors or serum. MPS-1 was found to have a nucleotide sequence that predicts a 10-kDal protein with homology to transcriptional regulatory proteins. This clone contains one "zinc finger" domain similar to those present in *Xenopus laevis* TFIIIA and other DNA-binding proteins. The protein product of this gene was produced in the insect cell line *Spodoptera frugiperda* (Sf9) using the baculovirus expression system *Autographa californica* nuclear polyhedrosis virus (AcNPV). When a cloned MPS-1 cDNA sequence was inserted into the AcNPV viral genome downstream from the promoter of the polyhedrin gene, a polypeptide with an apparent molecular weight of approximately 10,000 was observed in the nuclei of infected Sf9 cells. This protein was not detected in Sf9 cells infected with AcNPV-MPS-1-Del, a vector in which the MPS-1 gene was deleted. Antibodies were raised against the MPS-1 protein and used to detect MPS-1 in various cultured cell types. Characterization of the MPS-1 protein extracted from Sf9 infected cells showed that (1) the MPS-1 protein is produced at high levels in this host-vector system; (2) the protein accumulates in the nucleus; and (3) it is tightly bound to DNA. These results support the hypothesis that the MPS-1 protein, which may be a potentially important mediator of cellular proliferative responses to various growth factors, is a transcription regulatory protein that acts by binding specifically to DNA.

L 208 ON THE MECHANISM OF DNA BINDING BY NUCLEAR RECEPTORS Leonard P. Freedman, Cell Biology & Genetics Program, Sloan-Kettering Institute, New York, NY 10021.

Structural and functional approaches have been used to study the DNA binding domain of two nuclear receptors. This discrete, short region has two Zn binding sites, each forming a peptide "finger". The metal is tetrahedrally coordinated by four cysteine sulfurs, and this coordination is essential for specific DNA binding and protein folding. The three-dimensional structure of the glucocorticoid receptor (GR) DNA binding domain bound to a glucocorticoid response element (GRE) has recently been determined by x-ray crystallography [Luisi et al (1991) Nature 352, 497-505]. The amino terminal finger associates with the phosphate backbone of the DNA, and an α-helical region immediately C-terminal to the first finger interacts with specific bases in the major groove. Although in solution the protein is monomeric, it dimerizes upon DNA binding through reciprocal interactions of a region within the carboxy-terminal finger. The nature of the dimer interface explains why binding to a GRE has been observed to be cooperative, and why typical GREs consist of two half-sites oriented as inverted repeats separated by three base pairs.

In contrast to the GR paradigm, receptors such as those for vitamin D3, thyroid hormone, and retinoic acid appear to bind to cognate response elements organized as direct repeats, with half-site spacings from at least three to five nucleotides. The preference these receptors have for direct repeats implies that their DNA binding domains may utilize different stratagies from those described for GR to recognize and bind hormone response elements. We have overexpressed and purified the vitamin $D_3$ receptor DNA binding domain (VDRF), and begun to characterize its DNA binding properties. We find that the VDRF protein binds strongly and specifically to direct repeats constituting a vitamin D response element (VDRE) from the mouse osteopontin (Spp-1) promoter region, but weakly to the human osteocalcin VDRE. Unlike GR, VDRF appears to bind half-sites noncooperatively, without the free energy contribution of dimerization seen when the GR DNA binding domain associates with a GRE. By comparing and contrasting the DNA binding properties of VDR and GR, we suggest a model for how receptors that prefer direct repeats differ in their binding strategy from those that recognize inverted repeats.

L 209 CRYSTALLOGRAPHIC ANALYSIS OF THE COMPLEX BETWEEN THYROID HORMONE RECEPTOR DNA-BINDING DOMAINS AND THEIR DNA TARGETS. Daniel T. Gewirth & Paul B. Sigler, Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT 06511.

We have constructed a mutational variant of the glucocorticoid hormone receptor's DNA-binding domain which has previously (1) been shown to convert the wild-type protein's target specificity to that of the thyroid hormone receptor. Crystals of this mutational variant, called GRTR3, have been obtained in complex with a variety of DNA targets which mimic palindromic thyroid response elements (TREs). While functional TREs have been shown to contain two hexanucleotide TRE half sites separated by up to six base pairs of DNA 'spacer', we have focused our attention in particular on those TREs which contain zero base pairs of DNA spacer between the two TRE half sites. Several different crystal forms of these complexes have been obtained which diffract to at least 3 Å resolution. In addition, we have obtained co-crystals of the GRTR3 protein in complex with a modified glucocorticoid response element (GRE), which also contains zero base pairs of DNA 'spacer' between the two palindromic GRE half sites, and thus constitutes a fully non-specific DNA-protein complex. Analysis of this structure should be useful in determining the nature of the specific interaction between hormone receptors and their DNA targets. We have also recently received clones of the wild-type human thyroid hormone receptor DNA binding domains and work is in progress crystallizing these in complex with a variety of TREs.

(1) Danielson, M. *et al., Cell* **57**, 1131-1138 (1989); Mader, S. *et al., Nature* **338**, 271-274 (1989); Umesono, K. & Evans, R., *Cell* **57**, 1139-1146 (1989); Green, S., *et al. EMBO J.* **7**, 3037-3044 (1988).

L 210 FUNCTIONAL INTERACTION BETWEEN THE TWO ZINC FINGER DOMAINS OF THE v-*erb* A ONCOPROTEIN, Bonnie L. Hall, Beverly G. Bonde, Catherine Judelson and Martin L. Privalsky, Department of Microbiology, University of California Davis, Davis, CA 95616 The v-*erb* A oncogene of avian erythroblastosis virus is a mutated and virally transduced copy of a host cell gene encoding a thyroid hormone receptor. The protein expressed by the v-*erb* A oncogene binds to DNA and acts as a dominant negative inhibitor of both the thyroid hormone receptor and the closely related retinoic acid receptor. The v-*erb* A protein has sustained two amino acid alterations within its DNA binding domain relative to that of c-*erb* A, one of which, at serine 61, is known to be important for v-*erb* A function in the neoplastic cell. We report here that the second alteration, at threonine 78, also plays an important, although more indirect role: altering the sequence at threonine 78 such that is resembles that of c-*erb* A can act as an intragenic suppressor, and can partially restore function to a v-*erb* A protein rendered defective due to a mutation at position 61. Threonine 78 lies within the D-box of the v-*erb* A protein, a region thought to mediate receptor-receptor dimerizations, and is not in physical proximity to the serine at position 61. It therefore appears that an indirect interaction occurs between these two sites and that this interaction is crucial for v-*erb* A function.

L 211 *This abstract was inadvertently placed in the wrong meeting and can be found in the Cell Biology of Virus Entry, Replication and Pathogenesis meeting on page 147.*

L 212 HOMOGENEOUS ISOLATION OF OVEREXPRESSED HUMAN VITAMIN D RECEPTOR AND CHARACTERIZATION OF VITAMIN D RESPONSE ELEMENT INTERACTIONS, Paul N. MacDonald, Michael A. Galligan, Carol A. Haussler, and Mark R. Haussler, Department of Biochemistry, The University of Arizona, Tucson, AZ 85724
Human vitamin D receptor (VDR) has been overexpressed through recombinant baculovirus-mediated infection of *Spodoptera frugiperda* ovarian cells (MacDonald, P.N., *et al.* (1991) *J. Biol. Chem.* **266,** 18808). The recombinant receptor was purified by sequential chromatography on blue dextran-Sepharose, DNA-cellulose, DEAE-Sephadex, and hydroxylapatite columns. This protocol achieved >95% purification of the VDR as assessed by silver staining of SDS-PAGE gels. When examined in gel mobility shift assays, the purified VDR was capable of sequence-specific interaction with a vitamin D response element (VDRE) from the rat osteocalcin gene. Moreover, incubation of a nuclear extract obtained from CV-1 cells with the purified baculovirus-expressed VDR led to the appearance of an additional VDR-dependent complex that was distinct from the complex formed with pure VDR. Similar studies with nuclear extracts obtained from different cell lines provided evidence for multiple, cell-specific nuclear proteins that participate in VDR:VDRE interactions. Interestingly, chase experiments with unlabelled VDRE demonstrated that the complex formed with purified VDR dissociated more rapidly than the complex resulting from the VDR/CV-1 mixture. These observations indicate that purified VDR by itself recognizes and binds a VDRE in a sequence-specific manner and also binds its element in concert with other nuclear proteins. These unidentified receptor auxiliary factors may be required for stabilization of the VDR:VDRE complex. Thus, we report both the purification of functional VDR to near homogeneity and preliminary evidence of one or more nuclear, cell-specific receptor auxiliary factors that participate in high affinity VDR:VDRE interactions.

L 213 LIGAND-DEPENDENT AND INDEPENDENT ACTIVATION OF NORMAL AND MUTANT ANDROGEN RECEPTORS.
M.J. McPhaul and J.-P. Deslypere, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas 75235-8857.
Several steroid hormone receptors, particularly the progesterone receptor, can be activated by a cAMP-dependent mechanism. This activation requires a functional receptor molecule but does not require ligand. Under physiological conditions the androgen receptor is activated by testosterone or dihydrotestosterone. The availability of cDNAs encoding normal and mutant androgen receptors permitted us to examine the capacity of the ligand-independent pathway to activate the androgen receptor. Normal androgen receptor can be activated either by forskolin or 8-bromo cyclic AMP. The use of specific inhibitors suggest that this pathway is initiated by protein kinases and is terminated by protein phosphatases. Some mutant androgen receptors (caused by premature termination codons and amino acid substitutions) predicted for several patients with complete testicular feminization that are severely impaired in hormone-dependent activation retain full activity in assays of ligand-independent activation, but a normal DNA-binding domain is essential to achieve maximal cAMP-stimulated activation. Interestingly, anti-androgens (such as flutamide) do not block the ligand-independent pathway and may, in fact, amplify the activating effects of cAMP. These results suggest that the activation of the androgen receptor by cAMP acts at a late step in the pathway of androgen action and that many mutant receptors for patients with complete testicular feminization are blocked at earlier points in this pathway (either inability to dissociate from associated proteins or inability to associate with accessory proteins). The phenotype of complete androgen resistance in these patients suggests that this ligand-independent pathway is not operative in classic androgen target tissues.

L 214 BINDING OF THE ESTROGEN RECEPTOR TO A SINGLE STRAND OF THE ESTROGEN RESPONSE ELEMENT DEPENDS ON THE CENTRAL NUCLEOTIDE, Ranjan Mukherjee, Laboratoire de Genetique Moleculaire des Eucaryotes du CNRS, Unite 184 de Biologie Moleculaire et de Genie Genetique de l'INSERM, Institut de Chimie Biologique, Faculte de Medecine, 11 Rue Humann, 67085 Strasbourg Cedex, France. Present address: The Du Pont Merck Pharmaceutical Company, Experimental Station, P.O. Box 80328; Wilmington, Delaware, U.S.A.

The human estrogen receptor hER activates gene transcription by binding to cognate palindromic sequences called estrogen responsive elements (ERE). We used the gel retardation assay and oligonucleotides containing the ERE from the Xenopus

vitellogenin gene 5' AGGTCACAGTGACCT 3' to
3' TCCAGTGTCACTGGA 5'

study the interaction of the hER with the ERE. We observed that the hER bound to the double stranded ERE and to the single strand of the ERE that has T in the center with nearly equal affinity, but not to the strand which has A in the center. Inverting the two central nucleotides changed the strand specificity. Binding of the hER to the single strand is extremely sensitive to temperature. Specific recognition of one of the two strands of the ERE may be involved in the binding of the ER to the ERE.

L 215 GLUCOCORTICOID RECEPTORS FACILITATE NUCLEOTIDE EXCHANGE IN HISTONE H1.

Jouko Oikarinen, Natalja Yli-Mäyry, Tatu Tarkka and Riitta-Maaria Mannermaa, Collagen Research Unit, Biocenter and Department of Medical Biochemistry, University of Oulu, Kajaanintie 52A, SF-90220 Oulu, Finland

There is a large family of nuclear receptors which dimerize and bind to specific DNA elements upon recognition of a ligand, and are thought to produce changes in the chromatin structure along responsive genes as a part of their action. These receptors may either inhibit or activate transcription, the exact mechanisms of neither of the actions being elucidated. We have recently demonstrated that histone H1 is a nucleotide-binding protein with similar properties to GTPases, and suggested that the mechanism of action for nuclear receptors may be analogous to that for GTPase-coupled receptors on the plasma membrane (Oikarinen, BBRC 176, 343-348, 1991).

Histone H1 binds to the hinge region of nucleosomes, and this interaction is regulated by nucleotides (Nilsson et al., submitted). Any specificity for the nucleotide sugar or base cannot be demonstrated. Nucleoside diphosphates such as GDP stabilize the binding and nucleoside triphosphates such as GTP inhibit (Mannermaa et al., submitted). The inhibitory effect results most likely from a conformational change brought about by the γ phosphate, which eliminates recognition of a specific DNA sequence by the globular domain of H1 and allows binding through the C-terminal tail to AT-rich regions (Oikarinen et al., Neurosci. Lett., in press). It has previously been demonstrated that H1 really interacts with chromatin in a different manner along actively transcribed genes when compared with those not as efficiently expressed.

We report here identification of two regions in glucocorticoid receptor which interact with histones H3 and H4 upon binding of the receptor to a regulatory nucleosome, and facilitate GDP/GTP exchange in H1. We suggest therefore that nuclear receptors may produce dissociation of H1 from the chromatosome by modulating its sequence-specific interaction with DNA. H1 may thus be a mediator of the nuclear receptor action (Oikarinen, FEBS Lett., in press).

L 216 HSP90 AND THE DOMINANT ACTIVATION AND INACTIVATION OF HETEROLOGOUS PROTEINS BY THE HORMONE BINDING DOMAIN OF STEROID RECEPTORS, Didier Picard, Pierre-André Briand, Cathy Fankhauser, Biserka Havaux, Peter K. Jackson§, Jean-François Louvion, Marc Worek, Département de Biologie Cellulaire, Université de Genève, 1211 Genève 4, Switzerland, and §Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143-0448

The hormone binding domains of the glucocorticoid and estrogen receptors contain a protein inactivation function which is neutralized by the addition of hormone. The heat-shock protein HSP90 - associated with the hormone binding domain in a hormone-reversible fashion - may be responsible for keeping several functions of unliganded receptors off. Thus, the primary function of the hormone binding domain may be to provide a hormone-reversible HSP90 binding site. As such it can also subject a variety of heterologous proteins to hormonal control by dominant hormone-reversible inactivation. This finding is now further substantiated: (i) At least 3 of the 5 vertebrate steroid receptors (ER, GR, and MR), in contrast to the ecdysone receptor, contain an inactivation function in the hormone binding domain. (ii) Even a protein tyrosine kinase (activated c-abl), unrelated to transcription factors, becomes hormone-dependent by fusion to a hormone binding domain. Hybrid proteins have also highlighted the importance of the hormone-dependent dimerization function associated with the hormone binding domain. In fact, wild-type c-abl fused to a hormone binding domain can be dominantly activated as an oncogene in a hormone-dependent fashion, possibly due to activation of the tyrosine kinase by hormonally induced dimerization. We are currently analysing the role of HSP90 and another component of the complex, a 59 kD protein, genetically in yeast. Preliminary evidence suggests that only dominant activation may be reconstructed in yeast. We are exploring a genetic yeast system to investigate protein-protein interactions with respect to both HSP90 function in yeast in particular and receptor-HSP90 interaction and receptor dimerization in general.

L 217 DIFFERENTIAL DNA-BINDING ABILITIES OF ESTROGEN RECEPTOR OCCUPIED WITH TWO CLASSES OF ANTIESTROGENS: STUDIES USING HUMAN ESTROGEN RECEPTOR OVEREXPRESSED IN MAMMALIAN CELLS., Joseph C. Reese and Benita S. Katzenellenbogen, Department of Physiology and Biophysics, University of Illinois, Urbana IL 61801

We have developed a transient transfection system using the Cytomegalovirus (CMV) promoter to express the human estrogen receptor (ER) at very high levels in COS-1 cells and have used it to study the interaction of agonist and antagonist receptor complexes with estrogen response element (ERE) DNA. ER can be expressed to levels of 20-40 pmol/mg or 0.2-0.3% of total soluble protein and all of the soluble receptor is capable of binding hormone. The ER binds estradiol with high affinity (Kd 0.2 nM), and is indistinguishable from native ER in that the receptor is capable of recognizing its cognate DNA response element with high affinity, and of transactivating a transgene in an estradiol-dependent manner. Gel mobility shift assays reveal interesting ligand-dependent differences in the binding of receptor complexes to ERE DNA. Receptors occupied by estradiol or the type I antiestrogen trans-hydroxytamoxifen bind to DNA response elements when exposed to the ligand *in vitro* or *in vivo*. Likewise, receptors exposed to the type II antiestrogen ICI 164,384 *in vitro* bind to ERE DNA. However, when receptor exposure to ICI 164,384 is carried out *in vivo*, the ER-ICI 164,384 complexes do not bind to ERE DNA, or do so only weakly. This effect is not reversed by subsequent incubation with estradiol *in vitro*, but is rapidly reversible by *in vivo* estradiol exposure of intact COS-1 cells. This suggests there may be some cellular process involved in the mechanism of antagonism by the pure antiestrogen ICI 164,384, which is not observed in cell-free extracts.

**L 218** MULTIPLE COMPONENTS OF AN ANDROGEN DEPENDENT ENHANCER, Diane M. Robins, Adam Adler, Arno Scheller and Cameron Scarlett, Department of Human Genetics, University of Michigan, Ann Arbor, MI 48109.

The mouse sex-limited protein (Slp) gene arose from a duplicated complement C4 gene that has acquired dependence on androgen for expression. The hormone responsive enhancer resides 2 kb upstream of Slp within the 5' LTR of an ancient inserted provirus. This provides a well-delimited model for defining critical elements of androgen regulation and for determining how hormonal specificity is exerted for receptors that bind similar DNA sequences.

A 160 bp DNA fragment was shown to confer androgen response to a reporter gene in transient transfection assays in cells with endogenous or cotransfected androgen receptor (AR). An element that is necessary, but not sufficient, for induction is a consensus glucocorticoid (hormone) response element (HRE). AR binds this sequence, albeit weakly, in vitro. Internal deletions and clustered point mutations have defined accessory sequences within the enhancer that greatly augment induction, suggesting that cooperative interactions allow strong response to androgen. Two degenerate HREs adjacent to the consensus sequence do not function independently, but become significant in conjunction with other elements to confer sensitivity to receptor concentration. Although oligomerized HREs can be activated by cotransfected AR, glucocorticoid or progesterone receptors, some enhancer constructs show marked selectivity in response to different receptors. Thus additional factors are not only required for response of the enhancer, but also may ensure specificity of AR activation in vivo.

**L 219** EFFECTS OF SITE-DIRECTED MUTATIONS IN THE RECOGNITION HELIX OF HUMAN c-*erb*A ß ON DNA BINDING AND TRANSCRIPTIONAL REGULATION.

Paul J. Romaniuk, Colleen Nelson and Jon Faris, Dept. of Biochemistry and Microbiology, University of Victoria, Victoria, B.C. Canada V8W 3P6

The thyroid hormone receptor (TR) binds to cognate DNA sequences (thyroid response elements, TRE) and in the presence of hormone modulates transcription from TRE-linked genes. The binding of TR to DNA occurs *via* a DNA binding domain (DBD) that contains two zinc fingers. Crystal structure data for a glucocorticoid receptor DBD-DNA complex reveal that an α-helix extending from the knuckle of the first finger into the linker region forms base-specific contacts with the DNA. We have created a series of alanine substitution mutations in the corresponding "recognition" α-helix of human TRß to identify amino acid residues important for DNA binding and transcriptional regulation. Alanine substitutions at those residues which are conserved in all steroid and thyroid receptors disrupt DNA binding as measured in a gel shift assay. Amino acids $E_{120}$, $G_{121}$ and $G_{124}$ have been shown previously to be involved in discrimination of TREs from other hormone response elements. Of these three critical residues, only alanine substitution at $E_{120}$ disrupted DNA binding. Alanine substitutions at non-conserved, non-discriminator positions did not discernibly affect DNA binding activity.

The ability of mutant TRß receptors to activate transcription in the presence of $T_3$ was assayed in COS cells cotransfected with plasmids expressing mutant TRß along with a CAT reporter plasmid. All alanine substitution mutants which did not affect DNA binding activated transcription at wild type levels, with the exception of one. A mutant TRß with an alanine substituted for $G_{121}$ failed to activate transcription, but retained wild type DNA binding affinity. Mutations which disrupted DNA binding did not activate transcription, and exhibited a dominant negative effect on wild type TRß. This dominant negative effect most likely results from the formation of mutant-wild type TR heterodimers which are unable to form stable complexes with the DNA. The effects of additional substitutions at the three discriminator positions are currently being investigated.

**L 220** STUDIES ON HEAT SHOCK MEDIATED NUCLEAR TRANSLOCATION OF THE HORMONE-FREE GLUCOCORTICOID RECEPTOR, Edwin R. Sanchez, Ping Shen, Suju Zhong and Michael J. Greene, Department of Pharmacology, Medical College of Ohio, Toledo, Ohio 43699.

We have recently reported that both heat (43° C) and chemical stress (sodium arsenite) will cause the unliganded glucocorticoid receptor (GR) to become tightly bound within the nuclear fraction of L929 and WCL2 cells (Sanchez, E.R., *J. Biol. Chem.* 267, in press). In order to determine if this nuclear localization is the result of an "unmasking" of the DNA-binding domain, we have fractionated cells expressing two distinct GR functional mutations. In cells expressing GR which cannot bind DNA, the GR does not translocate to the nucleus in response to heat shock. This suggests that stress-induced GR nuclear binding requires a functional DNA-binding domain. Surprisingly, in cells expressing GR which cannot bind hormone, the majority of the GR is already present within the nuclear fraction in the absence of any treatment. This suggests that mutations within the steroid-binding domain can result in spontaneous nuclear translocation, perhaps as a result of destabilization of the cytosolic receptor complex.

Attempts by us to block stress-induced translocation of the GR by incubating cells with sodium molybdate or RU486 have not been successful. However, both molybdate and RU486 do not block hormone-driven tranlocation in our system, and RU486 by itself will cause at least some GR to translocate. In recycling experiments designed to determine the rate at which GR exits the nucleus in response to cessation of heat shock, we find that the majority of GR disappears from the nuclear fraction and reappears in the cytosolic fraction within two (2) hours of recovery. Experiments are underway, with protein synthesis inhibitors and with hormone-treated cells, to determine if this pattern is indeed the result of receptor recyling.

**L 221** HSP70, UNLIKE HSP90, IS NOT A SUBUNIT OF THE RAT HEPATIC GLUCOCORTICOID RECEPTOR COMPLEX (GR), Thomas J. Schmidt and Edward E. Diehl, Department of Physiology and Biophysics, The University of Iowa, Iowa City, IA 52242.

The constitutively expressed form of the 70 kDa heat shock protein (hsp70) has been shown to be involved in numerous cellular processes, including maintenance of specific proteins in an unfolded state favorable for translocation across intracellular membranes. Our experiments have addressed whether hsp70, like hsp90, is specifically associated with the rat hepatic GR. Both the unactivated and thermally activated forms of cytosolic [$^3$H]triamcinolone acetonide-bound GR were immunopurified with the anti-receptor monoclonal antibody BUGR2 and protein A-Sepharose, and subjected to SDS-PAGE followed by transfer to Immobilon sheets. The resulting Western blots were subsequently incubated with a monospecific polyclonal antibody directed against a C-terminal peptide of hsp70, which also cross reacts with hsp90. A prominent hsp90 band was noted only in the unactivated form of the GR, while an hsp70 band was detected in both forms. Neither hsp was present if a non-specific mouse IgG or protein A-Sepharose alone were substituted for BUGR2. However, when identical immunopurified samples were subjected to SDS-PAGE and then stained with Coomassie Blue or silver nitrate, the hsp90 band was approximately 50-fold more abundant than hsp70. We have demonstrated that Coomassie Blue is a very quantitative stain for hsp70 and hsp90, since purified samples of each protein stain equivalently. Although we cannot explain the apparent discrepancy between Western blots and stained gels, we feel that the latter technique is more reliable for heat shock protein quantitation and suggests that the stoichiometry of the hsp70-GR association is considerably less than 1:1. Additional Western blots performed on unactivated GR purified by a three-step biochemical scheme have revealed a very minor hsp70 band when compared to hsp90. These data obtained with highly purified unactivated GR also indicate that hsp70 is not associated with endogenous GR. These results differ from the previously described association of hsp70 with chick oviduct progesterone receptors and overexpressed mouse GR.

L 222 A NUCLEAR PROTEIN IS REQUIRED FOR THYROID HORMONE RECEPTOR BINDING TO AN INHIBITORY HALF-SITE IN THE EPIDERMAL GROWTH FACTOR RECEPTOR PROMOTER, Karol L. Thompson, June B. Santon, Lee B. Shephard, Gordon M. Walton, Gordon N. Gill, Department of Medicine, Division of Endocrinology and Metabolism, University of California, San Diego, La Jolla, CA 92093-0650

The epidermal growth factor receptor promoter is negatively regulated by thyroid hormone and retinoic acid. This regulation can be mapped to a 36 bp GC-rich region of the promoter (EGFR P/E) that functions autonomously as a promoter and as an enhancer when placed in front of the thymidine kinase gene TATA element. Thyroid hormone receptor ($T_3R$) binds directly with high affinity to this element only in the presence of a nuclear protein. Through ion exchange chromatography and gel filtration of Hela nuclear extract, this activity was identified as a 67 kDa protein. p67 did not bind to DNA alone but greatly augmented $T_3R$ binding to the EGFR P/E in gel mobility shift and DNA precipitation assays. When combined with p67, the $T_3R$ migrated as a larger complex on the DNA. Chemical crosslinking identified this complex as a heterodimer between $T_3R$ and p67. $T_3R$-p67 binds to a 7 bp site in the EGFR P/E (GGGACTC) that has weak homology to a consensus thyroid hormone response element (TRE) half-site. Thus, on this element, $T_3R$-p67 heterodimers contact the DNA primarily on a single TRE half-site.

L 223 CLONING OF BMFTZ-F1, A MEMBER OF NUCLEAR HORMONE RECEPTOR SUPERFAMILY IN THE SILKWORM, BOMBYX MORI AND COMPARISON WITH FTZ-F1, A DROSOPHILA HOMOLOGUE OF BMFTZ-F1, Hitoshi Ueda[1], Guancheng Sun[2], Susumu Hirose[2], [1]Genetic Stock Research Center and [2]DNA Research Center, National Institute of Genetics, 411 Mishima, Shizuoka-ken, Japan.

FTZ-F1 is a sequence specific DNA binding factor which binds 280 bp upstream of the Drosophila homeobox segmentation gene, fushi tarazu, and supposed to be a positive regulator of the ftz gene. FTZ-F1 is regulated qualitatively and quantitatively during the development of Drosophila, suggesting that FTZ-F1 is a developmentally important gene. Analysis of cDNA for FTZ-F1 revealed that it is a member of the nuclear hormone receptor superfamily. BmFTZ-F1 was found as a silkworm, Bombyx mori, factor which binds to the FTZ-F1 binding site. BmFTZ-F1 is present in embryos and silk glands of the silkworm and is regulated during the development of the silkworm. Comparison between BmFTZ-F1 and FTZ-F1 revealed that BmFTZ-F1 recognizes exactly the same sequence with FTZ-F1 and they share the same chromatographic behav-iors, suggesting that BmFTZ-F1 is a silkworm factor corresponding to Drosophila FTZ-F1. We have purified BmFTZ-F1 and determined partial amino acid sequence of trypsin digests of this factor. We have also cloned cDNA for the factor using the information of these amino acid sequences and homology with FTZ-F1 and its nucleotide sequence has been determined. Predicted amino acid sequences of FTZ-F1 and BmFTZ-F1 share highly conserved regions in the Zn finger, the ligand binding domain and the region between them. Deletion analysis of the conserved regions of these proteins revealed the presence of a novel domain which facilitates of target sequence.

L 224 ANTI-ANDROGENS AND THE MUTATED ANDROGEN RECEPTOR OF LNCaP CELLS: DIFFERENTIAL EFFECTS ON BINDING AFFINITY, HEAT SHOCK PROTEIN INTERACTION AND TRANSCRIPTION ACTIVATION, Jos Veldscholte, Cor A. Berrevoets, Albert O. Brinkmann, J. Anton Grootegoed and Eppo Mulder, Department of Endocrinology & Reproduction, Erasmus University Rotterdam, P.O. Box 1738, 3000 DR Rotterdam, The Netherlands.

LNCaP prostate tumor cells contain an androgen receptor (AR) which shows an altered steroid specificity, due to a point mutation in the steroid binding domain (codon 868, Thr to Ala). Estrogens, progestagens and the anti-androgens cyproterone acetate, hydroxyflutamide and nilutamide stimulate LNCaP cell growth rate through the AR. In contrast to the other anti-androgens, 'Casodex'* does not stimulate growth rate of LNCaP cells but inhibits R1881- induced growth. Both the positive and negative effects of anti-androgens on growth rate are paralleled by effects on transcription activation, observed in a HeLa cell co-transfection system with an androgen regulated reporter gene construct (GRE-tk-CAT) and the mutant receptor.

The AR is associated with heat-shock proteins (hsp90, hsp70 and hsp56), as shown by immunoblotting, after immunoprecipitation of the AR from LNCaP cell cytosol. Incubation of LNCaP cells with R1881 (10 nM) or the anti-androgen hydroxyflutamide results in dissociation of the AR-heat-shock-protein complex. This dissociation is paralleled by transformation to the tight nuclear binding form of the AR. In contrast, 'Casodex' does not induce a release of heat-shock proteins and does not increase nuclear binding and inhibits the transformation process induced by R1881.

From these results, we propose that anti-androgens show differential effects on receptor transformation and transcription activation. In LNCaP cells, 'Casodex' affects steps before DNA binding occurs. In contrast, other anti-androgens including hydroxyflutamide, induce receptor transformation to the DNA binding state and permit interaction of the receptor with the transcription machinery.

* 'Casodex' is a trade mark, the property of Imperial Chemical Industries PLC.

L 225 STRUCTURAL REQUIREMENTS FOR HIGH AFFINITY MONOMER/DNA BINDING BY THE ORPHAN RECEPTOR NGFI-B, Thomas E. Wilson, Ragnhild E. Paulsen, Kerstien A. Padgett, Mark Johnston and Jeffrey Milbrandt, Division of Laboratory Medicine, Depts. of Pathology and Internal Medicine, Washington University School of Medicine, St. Louis, MO 63110

NGFI-B is an orphan member of the steroid/thyroid receptor superfamily that is rapidly and transiently induced in rat pheochromocytoma cells by nerve growth factor (NGF). This pattern of induction identifies NGFI-B as an early response protein, a feature unique among this receptor superfamily, and suggests that NGFI-B may mediate the cellular response to NGF. To address this hypothesis we are characterizing the DNA binding specificity and identifying genomic targets of this protein. We developed a novel yeast selection system that detects targets for DNA binding proteins by virtue of their presence upstream of the selectable HIS3 reporter gene. The NGFI-B binding site (NBRE) identified by this method is similar to the sites recognized by other members of the steroid/thyroid receptor superfamily because it contains the estrogen receptor half-site AGGTCA, but it is distinct because it lacks repeated elements and strictly requires two A nucleotides immediately 5' to the half-site. The requirement for only one half-site suggests that NGFI-B binds to the NBRE as a monomer, a hypothesis supported by the lack of heterodimer formation between full-length and truncated NGFI-B. The affinity of the NGFI-B/NBRE interaction is in the range seen for steroid hormone receptors, however. Domain swapping between NGFI-B and the orphan receptor H-2RIIBP has identified residues downstream of the NGFI-B zinc fingers that confer monomer-specific NBRE binding to H-2RIIBP. We hypothesize that these residues make base-specific contacts to the A nucleotides upstream of the half-site in the NBRE, and that these contacts provide the binding energy necessary to stabilize the monomer/DNA complex.

L 226 INTERACTION OF THYROID HORMONE RECEPTORS WITH NUCLEAR PROTEINS

*Xiao-kun Zhang, Paul Bui-Vinh Tran, Ken N. Wills, Birgit Hoffmann, Gerhart Graupner, Matthias Husmann, Thomas Hermann, and Magnus Pfahl.* La Jolla Cancer Research Foundation, 10901 N. Torrey Pines Rd., La Jolla, CA 92037 USA

Many essential biological pathways, including cell growth, development, and metabolism, are regulated by thyroid hormones (THs). TH action is mediated by intracellular receptors that belong to a large family of ligand dependent transcription factors, including the steroid hormone and retinoic acid receptors. So far it has been assumed that thyroid hormone receptors (TRs) regulate gene transcription only through the classical protein-DNA interaction mechanism. Here we provide evidence for a regulatory pathway that allows cross-talk between TRs and the signal transduction pathway used by many growth factors, oncogenes and tumor promoters. In transient transfection studies we observe that the oncogenes, cJun and cFos inhibit thyroid hormone receptor activities, while TRs inhibit induction of the cFos promoter and repress AP-1 site dependent gene activation. A truncated TR that lacks only 17 amino acids from the carboxyterminus can no longer antagonize AP-1 activity. The cross-regulation between TRs and the signal transduction pathway appears to be based on the ability of TRs to inhibit DNA binding of the transcription factor AP-1 in the presence of thyroid hormones. The constituents of AP-1, cJun and cFos, vice versa, can inhibit TR induced gene activation *in vivo* and cJun inhibits TR DNA binding *in vitro*. To further study the molecular mechanism of TR action, the interaction of TRs with other nuclear protein(s) was analyzed. Nuclear factor from several cell lines, including CV-1, F9, and GC cells interacts with TRα receptor to form a larger molecular weight complex as determined by gel retardation assay. This factor could not be detected in HeLatk⁻ cells, where TRα does not activate a TRE containing reporter gene. The nuclear factor is heat sensitive and does not bind to TRE itself but can interact with TRα in the absence of DNA. Deletion analysis demonstrates that the Leucine Zipper like sequence located in the LBD of TRα is involved in this interaction. The characterization of such a related nuclear protein will be presented.

L 227 ANDROGEN RESISTANCE ARISING FROM AN N-TERMINAL TRUNCATION OF THE ANDROGEN RECEPTOR: PRENATAL DIAGNOSIS IN A FAMILY.

S. Zoppi, M. Marcelli, M.D. Harbison, C.M. Wilson, J.E. Griffin, J.D. Wilson, and M.J. McPhaul, Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, Texas 75235-8857, and Department of Pediatrics, Cornell University Medical College, New York, New York 10021.

Mutations causing androgen resistance frequently alter the structure of the hormone- and DNA-binding domains of the human androgen receptor (AR). By contrast, mutations that affect only the N-terminal domain of the AR have not yet been reported to cause androgen resistance in humans. In the present study we have characterized the molecular defect causing androgen resistance in two 46,XY siblings (patients 776 and 782) affected by complete testicular feminization. Binding studies in cultured genital skin fibroblasts have shown a reduced Bmax (8 fmol/mg/protein, normal >15 fmol/mg protein), increased dissociation rate of the ligand from the receptor, normal upregulation and normal thermostability. Sucrose gradient centrifugation detected a peak of specific DHT binding that migrated with a sedimentation coefficient of 8S. Despite the measurable amount of ligand binding in 776 and 782 genital skin fibroblasts, no immunoreactive AR was detected in immunoblots using an anti-N-terminal antibody, suggesting an abnormality of the amino terminus of the AR in this family. This hypothesis has been confirmed by sequence analysis of the androgen receptor that detects a point mutation CAG→TAG (Gln→Stop) at nucleotide 340. In vitro mutagenesis studies suggest that the phenotype is caused by the synthesis of a reduced quantity of AR that lacks a normal amino terminus. The definition of the AR gene mutation in this family permitted the 'tracking' of this mutant allele within the family and was used to ascertain antenatally whether the affected allele was present in a 9 week 46,XY fetus, sibling of the affected proband. These studies indicate the presence of the normal androgen receptor allele in the fetus at risk. The expectation of normal male development was confirmed by the presence of a normal phallus by ultrasonography at 24 weeks.

*Poster Session III*

L 300A METHOD FOR CHARACTERISATION OF ENDOGENOUS LIGANDS TO ORPHAN RECEPTORS BELONGING TO THE STEROID HORMONE RECEPTOR SUPERFAMILY,

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In our work, identification of ligands to orphan receptors is being pursued using an analytical chemical approach. Initial studies involved development of the method for isolation of a known ligand from tissue extracts based upon its interaction with a known member of the steroid hormone superfamily. An analytical method is described whereby progesterone is isolated from pregnancy plasma on the basis of the high affinity and specificity of progesterone receptor for its ligand. Partially purified progesterone receptor ligand-binding domain expressed as a protein A fusion protein in Escherichia coli, is incubated with a neutral steroid fraction obtained by extraction and ion exchange chromatography of human late pregnancy plasma. The incubated sample is passed through two Lipidex 1000 (lipophilic gel) beds. The first, at 4°C, separates the specific ligand-fusion protein complex from non-specifically bound and unbound compounds and the second, at 40°C, separates the specific ligand from the protein. Elution of the second bed with methanol yields a fraction containing specific ligand that can be characterised by gas chromatography -mass spectrometry.

This methodology may be valuable for identification of endogenous ligands to orphan receptors of the steroid hormone receptor superfamily.

L 301 SEQUENCES PRESENT WITHIN HUMAN GLUCOCORTICOID, ANDROGEN AND ESTROGEN RECEPTOR cDNAs CONFER LIGAND-INDUCIBLE RECEPTOR mRNA DOWN REGULATION,

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Glucocorticoid receptors, like other classes of steroid receptors, are susceptible to regulation by their cognate hormone. The human glucocorticoid receptor (hGR) cDNA was found to contain sequences sufficient for glucocorticoid-mediated receptor down regulation (Burnstein et al. (1990) J. Biol. Chem. 265:7284). The existence of such intragenic regulatory sequences was based on studies using COS 1 cells transfected with an hGR cDNA linked to the Rous Sarcoma Virus promoter, which is glucocorticoid-insensitive. To determine whether ligand-induced reduction in transfected hGR mRNA levels was due to effects on transcription of the hGR cDNA or to alterations in hGR mRNA turnover, nuclear run-on assays and RNA stability studies were performed. Glucocorticoid treatment resulted in an ~2-fold decrease in the transcription of the hGR cDNA. Glucocorticoids also elicited a modest decrease in hGR mRNA half-life in transfected cells treated with the transcriptional inhibitor actinomycin D. Therefore, glucocorticoid regulation of the expression of the hGR cDNA appears to occur at both transcriptional and post-transcriptional levels. To examine whether the expression of other steroid receptor cDNAs might be influenced by ligand, cDNAs which encode the human androgen and estrogen receptors were individually transfected into COS 1 cells. Androgen treatment of cells expressing the transfected human androgen receptor cDNA resulted in down regulation of androgen receptor mRNA. Similarly, in COS 1 cells transfected with the human estrogen receptor cDNA, estradiol administration caused a reduction in the level of estrogen receptor mRNA. These findings indicate that the human androgen and estrogen receptor cDNAs, similar to the hGR cDNA, contain intragenic sequences that are involved in hormone-mediated steroid receptor mRNA regulation. The majority of this work was conducted at the University of North Carolina and supported by DK32460.

L 302 ROLE OF CYSTEINES-640, -656, AND -661 IN STEROID BINDING TO RAT GLUCOCORTICOID RECEPTORS, Pradip K. Chakraborti*, Michael J. Garabedian†, Keith R. Yamamoto†, and and S. Stoney Simons, Jr*., * Steroid Hormones Section, LMCB/NIDDK, NIH, Bethesda, MD 20892 and †Department of Biochemistry, University of California, San Fransisco, CA 94143.

The involvement of a vicinal dithiol group in steroid binding to the glucocorticoid receptor was initially deduced from experiments with the thiol specific reagent methyl methanethiolsulphonate (MMTS) and confirmed by our recent studies with the vicinal dithiol specific reagent sodium arsenite. We have recently suggested that the vicinal dithiols were two of the three cysteines (Cys-640, -656, and -661 of the rat receptor) present in the 16 kDa trypsin fragment of the receptor, which is now found to have the same specificity of binding as the intact 98 kDa receptor. To definitively identify the vicinal dithiol group, a total of 5 point mutants (cysteine-to-serine for each cysteine and cysteine-to-glycine for Cys-656) were prepared, including one double point mutant. Unexpectedly, each point mutant and the double point mutant still bound steroid, demonstrating that no one cysteine is essential for steroid binding. However, Cys-656 was required for the receptor to be covalently labeled by [$^3$H]dexamethasone 21-mesylate. An analysis of the effects of arsenite and MMTS pre-incubation on the steroid binding capacity of the receptors revealed both that Cys-656 and -661 were the vicinal dithiols reacting with arsenite and that any two of the three thiols could form an intramolecular disulfide after treatment with low concentrations of MMTS. These data support a model for the steroid binding cavity that involves all three thiols in a flexible cleft.

L 303 CELL-SPECIFIC REGULATION OF THE HUMAN PROGESTERONE RECEPTOR PROMOTERS.

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The progesterone receptor (PR) in the mammalian uterus and in human breast cancer cells is known to be regulated by oestrogens, progestins and retinoids by mechanisms which include modulation of PR gene transcription and receptor protein half-life. The existence of multiple promoters and transcripts for this receptor suggests that transcriptional regulation of PR is likely to be complex. It also raises the question of whether there are cell-specific differences in this process. Accordingly, the expression and oestrogen regulation of each PR gene promoter was examined using transfection experiments in human breast cancer cells (T-47D) and Chinese hamster ovary (CHO) cells. These cells were compared because T-47D cells are known progestin-responsive cells whereas CHO cells lack endogenous PR. The A or B PR promoters linked to sequences coding for chloramphenicol acetyltransferase (CAT) were transiently co-transfected into T-47D or CHO cell monolayers, using the calcium phosphate precipitation method, with expression vectors coding for the human oestrogen receptor and bacterial β-galactosidase. Transfected cells were treated with 17β-oestradiol (10 nM) or vehicle and cultures continued for 44 h at which time cells were harvested and lysates prepared. CAT activity was determined using a non-chromatographic method and β-galactosidase was measured to monitor transfection efficiency. Basal CAT activity in both T-47D and CHO cells transfected with the B promoter was low but was inducible 5-8 fold in cells treated with oestrogen. This concordance in the behaviour of the B promoter in T-47D and CHO cells was not observed for the A promoter. In CHO cells CAT activity under the control of the A promoter was significant in vehicle-treated cells and was not augmented further by oestrogen treatment. In T-47D cells by contrast, no CAT activity from the A promoter was detectable either in the basal state or after oestrogen exposure. In summary, the basal level of activity from the B promoter was similar in both cells and oestrogen induction to a similar extent was observed. However, there were marked differences in the basal expression of the A promoter in T-47D and CHO cells. Whether this reflects the dependence of the PR promoters on cell-specific transcription factors or whether other mechanisms are responsible is presently under investigation.

L 304 INDIRECT TRANSCRIPTIONAL ACTIVATION BY PHENOBARBITAL BLOCKED BY ANTIGLUCOCORTICOIDS, L. CORCOS*, P. SHAW#, M. WEISS**, M. ADESNIK# AND N. DRINKWATER*, *MC ARDLE LABORATORY FOR CANCER RESEARCH, MADISON, WI 53711, #NYU MEDICAL CENTER, NEW YORK, NY 10016, **PASTEUR INSTITUTE, PARIS, 75015.

Induction of liver-specific cytochrome P450 genes by phenobarbital(PB) and dexamethasone(DEX) has been studied in rat hepatoma cells of the Fao cell line. In these cells, accumulation of cytochrome P450IIC6 mRNA was induced more than 10 fold upon exposure to 2mM PB or 1μM DEX. Addition of both inducers at their optimal dose did not lead to any further induction; sub-optimal doses provoked additive effects. Induction by either compound resulted from an indirect transcriptional activation since it was blocked by alpha-amanitin and cycloheximide. Moreover, induction by PB or DEX was fully prevented by co-exposure to 20μM RU38486. These data are consistent with a model where PB and DEX act to induce expression of the cytochrome P450IIC6 gene via a common pathway, possibly involving a steroid receptor. In support of this hypothesis, PB was able to increase the affinity of the glucocorticoid receptor for the binding to DEX *in vitro*. In the rat *in vivo*, PB induces expression of the P450IIC6 gene whereas DEX is inefficient. However, both PB and DEX have been shown to be able to induce an increase in the accumulation of P450IIC6 mRNA in mouse liver (Meehan, RR et al, Biochem. J. (1988) **254**, 789-797). With the aim of extending the observations made with hepatoma cells to an *in vivo* model, we initiated a comprehensive analysis of the parameters of induction by PB and DEX in mice. Time course and dose-response analyses showed that the level of accumulation (4 fold increase over control) of P450IIC6 mRNA was almost maximal 4 hours after administration of 10 mg/kg PB in female mice. However, we did not observe any significant effect of DEX up to 18 hours after injection, even at a 100 mg/kg dose, in any of six inbred mouse strains. In agreement with the previous report, both PB and DEX were able to induce significant increases in the accumulation of P450IIB1/2 mRNA, in all of the mouse strains examined. Co-injection of optimal doses of both inducers did not lead to any further increase in the response. While PB, but not DEX, induced the P450IIC6 gene *in vivo*, both compounds acted as agonists to induce expression of P450IIB1/2 genes. These findings argue for the involvment of PB and DEX in related pathways.

L 305 REGIONS OF THE GLUCOCORTICOID RECEPTOR INVOLVED IN HORMONE BINDING SPECIFICITY, Mark Danielsen, Xiayuan Liang, and Jacqueline Jonklaas, Department of Biochemistry and Molecular Biology, Georgetown University Medical School, 3900 Reservoir Rd., N.W., Washington, DC 20007.

Deletion analysis of the mouse glucocorticoid receptor has shown that the hormone binding site lies within the C-terminal half of the receptor. Characterization of hormone binding deficient receptors from mouse lymphoma cells resistant to glucocorticoids has revealed that residues throughout this region are required for high affinity binding of glucocorticoids. The hormone binding domain of the androgen receptor is highly homologous to the equivalent domain from the glucocorticoid receptor, yet they bind steroids with different specificity. To determine which amino acids in the hormone binding domains of these two receptors are functionally homologous, and which are involved in determining the specificity of hormone binding, we have subjected the glucocorticoid receptor to homolog scanning mutagenesis. Regions of the glucocorticoid receptor hormone binding domain (approximately 15 amino acids each time) were replaced systematically by the homologous sequence from the androgen receptor. 15 out of 19 of these fusions result in receptors that can activate transcription in a glucocorticoid dependent manner. The other four constructs are inactive with both glucocorticoids and androgens. The data indicate that specificity may be encoded in some combination of these four functionally non-homologous regions. We are testing this hypothesis by constructing glucocorticoid-androgen receptor fusion proteins that have two or more of these possible specificity regions from the androgen receptor replacing the equivalent regions of the glucocorticoid receptor.

**L 306 INTERACTION OF RETINOIDS WITH WILD-TYPE RETINOIC ACID NUCLEAR RECEPTOR ISOFORMS**, Marcia I. Dawson,[1] Peter D. Hobbs,[1] Tina S. Berger,[2] Robert B. Stein,[2] and Richard A. Heyman,[2] [1]Bio-Organic Chemistry Laboratory, 333 Ravenswood Ave., SRI International, Menlo Park, CA 94025, and [2]Ligand Pharmaceuticals Inc., 9393 Towne Center Drive, San Diego, CA 92121

All-*trans*-retinoic acid (RA) and a series of conformationally restricted aromatic retinoids were examined for their ability to interact with the three wild-type retinoic acid receptor (RAR) isoforms and then activate gene expression. A sensitive and reproducible *cis-trans* transfection assay was used in which cultured CV-1 cells were cotransfected with each RAR expression vector and the firefly luciferase reporter gene coupled to a retinoid-responsive promoter. The intensity of light emitted on addition of luciferin and ATP to the lysed cells was found to be directly proportional to the level of gene transcriptional activation by the complex formed from the applied retinoid and the RAR. Transcriptional activation results obtained with this assay will be compared with reported results and our data using cotransfection assays with hybrid gene constructs in which the RA-binding domain of the RAR was coupled to a steroid receptor DNA-binding domain. In these studies, either the luciferase gene or the chloramphenicol acetyltransferase gene coupled to a retinoid-responsive promoter was employed as the reporter.

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**L 307** COMPLETE AND PARTIAL ANDROGEN INSENSITIVITY SYNDROMES DUE TO POINT MUTATIONS IN THE AR GENE Alessandra De Bellis, Charmian A.Quigley, Malcolm V.Lane, Elizabeth M. Wilson, Frank S.French The Laboratories for Reproductive Biology, University of North Carolina at Chapel Hill, NC 27599

Androgen receptor (AR) gene mutations have been identified in patients affected by the androgen insensitivity syndrome (AIS), an X-chromosome linked disorder in which the phenotype of affected individuals ranges from minimally affected males (partial AIS, PAIS) to 46, XY phenotypic females (complete AIS, CAIS). Analysis of the AR gene in two subjects with complete and two with partial AIS revealed no major deletion. Point mutations were detected by screening each exon individually using denaturing gradient gel electrophoresis (DGGE). Exon DNA was amplified by the polymerase chain reaction (PCR). One of the two unrelated patients had a G to A substitution in exon F, changing Arg to His at amino acid (aa) 774. In the other CAIS patient an A to G base mutation changed aa 864 from Asp to Gly in exon G. Both these exons encode part of the AR steroid binding domain. In one PAIS patient androgen binding affinity in cultured genital skin fibroblasts was decreased (7 fold < wild type). A single G to A mutation changed Arg to His at aa 840 in exon G. Androgen binding affinity was normal in genital skin fibroblasts of the other PAIS who had a T to G substitution, changing Leu to Arg at aa 616 in exon C near the C terminal of the 2nd zinc finger. Functional effects of these base changes were studied by introducing the point mutations into cDNAs encoding the normal human AR, and analyzing AR binding or androgen induced transcriptional activation after transfection of the cDNAs into COS or CV1 cells. CAIS in these subjects was explained by the inability of AR to form a functional AR-steroid complex , and to normally activate gene transcription. Mutant ARs of the PAIS subjects demonstrated decreased, but not absolute absence of biological function in the one case resulting from abnormal androgen binding and in the other presumably DNA binding. In both cases AR function is consistent with the observed phenotype. In summary this study indicates an increasingly complex picture of the molecular heterogeneity in AIS, and shows that nucleotide substitutions interfere with the role of AR in regulating gene transcription.

**L 308** GLUCOCORTICOID- AND CAMP-INDUCED APOPTOSIS IN LYMPHOCYTES: GENETIC EVIDENCE THAT INDEPENDENT PATHWAYS SHARE DISTAL EVENTS, Diane R. Dowd and Roger L. Miesfeld, Department of Biochemistry and Arizona Cancer Center, University of Arizona, Tucson, Arizona 85724

WEHI7.2 murine lymphocytes are subject to apoptotic death upon treatment with glucocorticoids or agents which elevate intracellular cAMP levels. These two pathways to death are mediated by the glucocorticoid receptor (GR) and protein kinase A (PKA), respectively. We report the isolation and characterization of a novel WEHI7.2 variant cell line, WR256, which was selected for growth in the presence of $10^{-6}$ M dexamethasone and arose at a frequency of ~$10^{-10}$. WR256 was determined to express functional GR which can mediate glucocorticoid/GR dependent gene transcription and inhibition of proliferation, events which are associated with apoptotic cell death. To determine if WR256 was sensitive to cAMP, the cells were treated with 8-Br cAMP or forskolin. Surprisingly, WR256 was found to be cAMP-resistant despite normal levels of PKA. In addition, WR256 did not exhibit a decrease in mitochondrial activity associated with apoptosis. These results suggest that WR256 has a unique defect in a step of the apoptotic cascade which is shared by glucocorticoid- and cAMP-mediated cell death. This apoptosis defective variant may prove very useful in deciphering the complex pathway(s) that leads to cell death.

**L 309** THE DNA-BINDING CAPABILITIES OF ISOLATED MEMBRANE GLUCORTICOID RECEPTOR: A FURTHER INDICATION OF STRUCTURAL HOMOLOGY WITH THE INTRACELLULAR RECEPTOR. Bahiru Gametchu, Cheryl S. Watson, and Tim Labecki. Dept. of Pediatrics, Medical Coll. of Wisconsin, Milwaukee, WI 54226 and Dept. of Human Biol. Chem. & Genetics, Univ. of Texas Med. Br., Galveston TX 77550 (CSW).

We have previously described a membrane-resident form of the glucocorticoid receptor (mGR) in S-49 mouse lymphoma cells. Our recent work has been aimed at demonstrating the role of the plasma membrane-associated GR in mediating lymphocytolytic effects of glucocorticoids. The only structural difference that has been noted to date between intracellular GR (iGR) and mGR, is a molecular size variation on denaturing polyacrylamide gels further identified by immunoblot and sucrose density gradient (SDG) analyses with the anti-rat GR monoclonal antibodies BuGR-1 and -2. The mGR is frequently larger in size and has multiple, large-molecular-size variants. We now show the similarities between the DNA binding regions of the iGR and the mGR via binding of calf-thymus or specific glucocorticoid response element (GRE) DNA to either receptor. SDGs were used to show a DNA-mediated shift to a higher density when either iGR or detergent-extracted mGR was labeled with $^{3}$H-dexamethasone and then incubated with calf thymus DNA before centrifugation. Characterization of a stringently-immunoselected, mGR-negative, cell population showed that GR from these cells was truncated and was much less efficient at shifting an end-labeled GRE in a SDG. In addition, these GRs lacked the BuGR-2 epitope. In gel mobility shift assays, iGR bound to end-labeled GRE oligonucleotides. Similarly, detergent-extracted mGR bound to the same GRE but produced a protein-DNA complex which was more retarded in mobility than the iGR/GRE complex. In summary, mGR shares both the structure and functional capabilities of the iGR DNA binding region as well as other structural similarities, suggesting that the primary modification responsible for placement in the cell membrane is minimal.

## L 310 RECEPTOR-MEDIATED PEROXISOME PROLIFERATOR ACTION

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Peroxisome proliferators (PP) are a diverse group of rodent carcinogens that include hypolipidemic drugs, plasticizers and herbicides (1). We have reported previously (2) the cloning of a member of the nuclear hormone receptor superfamily and, through the use of chimeric receptors, discovered that it could be activated by PPs. The receptor is therefore termed the PP activated receptor (PPAR). More recently, three *Xenopus* nuclear hormone receptors have been described that are closely related to the mouse PPAR that can all be activated by PPs (3). Therefore a family of PPARs that could mediate the action of some hypolipidemic drugs may exist in many species including mouse and man.

The most widely used marker of PP action is the peroxisomal β-oxidation enzyme acyl CoA oxidase (ACO). We now demonstrate that PPAR recognizes a specific PP response element (PPRE) located in the ACO gene promoter and that the response is dependent upon the presence of receptor and the addition of the PP Wy 14,643. These data therefore support a model in which the mechanism of PP action is mediated by PPAR in a manner similar to that of steroid hormone action.

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## L 311 DIFFERENTIAL BINDING OF PROGESTERONE IN GRAVID AND NON-GRAVID UTERINE HORNS FROM THE SAME RAT.

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Progesterone exerts its effects in the uterus through a primary reaction with specific receptors for the hormone. The plasma glycoprotein corticosteroid-binding globulin (CBG) may also mediate the action of progesterone in the uterus. In a study of progesterone-binding proteins in uterine cytosols from pregnant rats, we found four animals in which one uterine horn was gravid and the other was not. Cytosols were prepared from the uteri and progesterone-binding proteins analyzed quantitatively by Scatchard analysis, and qualitatively using agarose flat-bed isoelectric focussing at 4°C. Binding studies were carried out by incubating aliquots of cytosols with a range of concentrations of progesterone and a tracer amount of the tritiated hormone, for 2h at 0°C, prior to separating bound from free ligand by rapid passage through minicolumns of SEPHADEX LH20 at 0°C., and measurement of eluted bound radioactivity by scintillation spectrometry. Non-gravid horns possessed $3.42 \pm 0.46 \times 10^{11}$ binding sites per mg cytosol protein, while gravid horns possessed $8.43 \pm 0.91 \times 10^{11}$. These results differed significantly at the 1% level (Mann-Whitney U-test). The molar dissociation constant ($K_d$) was unchanged, at about 50nM. Isoelectric focussing of [$^3$H]progesterone-labelled cytosol from gravid horns yielded a peak of pI 6.3, and a more basic peak, both of which were absent from cytosols from non-gravid horns. The peaks observed were different from those obtained after electrofocussing CBG. Thus, both the abundance and nature of uterine progesterone-binding proteins may change during gestation in the rat.

## L 312 PRODUCTION OF RECEPTOR POSITIVE, DEXAMETHASONE RESISTANT MUTANTS BY INSERTION OF A SELECTABLE GENE.

Robert W. Harrison, Stephen S. Lippman, William J. Hendry, Richard VerHoeven, Ewa Koslowska, Edward Nzambi, Fiona Azabuike and Avis Hall, Division of Endocrinology, University of Arkansas for Medical Sciences, Little Rock, AR 72205.

The AtT-20/IDG8 cell line contains the gene for neomycin phosphotransferase under negative glucocorticoid control so that it grows in medium containing dexamethasone or G418 but is growth-arrested in medium containing both agents. Fourteen AtT-20/IDG8 clones with defective glucocorticoid responses, termed AtT-20/EMS1-14, were produced by chemical mutagenesis of AtT-20/IDG8 cells and subsequent growth in medium containing dexamethasone and G418. Northern blotting confirmed that the dexamethasone-induced, down-regulation of neomycin phosphotransferase, present in the parent AtT-20/IDG8 line, was lost in the AtT-20/EMS lines. In 10 of the cell lines, Northern blots showed that dexamethasone regulation of POMC mRNA was retained. Dexamethasone treatment failed to reduce POMC mRNA levels in four lines, indicating that the altered regulation involved a defective general factor such as the glucocorticoid receptor. However, receptor abundance, size, steroid binding and nuclear binding ability in these four clones was identical to the parent AtT-20/IDG8 line. These results illustrate the utility of an important, new, flexible, genetic analysis paradigm that is generally applicable to transcription regulators. Lastly, as contrasted with response-deficient lines isolated from lymphoma and thymoma cell lines, none of the AtT-20/EMS clones appear to have a defective glucocorticoid receptor, suggesting that other aspects of the response mechanism will be accessible to genetic analysis by this approach.

## L 313 ENDOGENOUS GLUCOCORTICOID RECEPTOR LEVELS ARE REDUCED BY THE Ha-RAS ONCOGENE.

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Expression of activated human Ha-ras has been reported to repress transcription from the glucocorticoid-dependent MMTV-LTR promoter (Jaggi *et al.*, EMBO J. 5, 2609-2616, 1986). To determine if the Ha-ras p21 protein alters the activity of the glucocorticoid receptor (GR), we examined parent NIH 3T3 cells and a subline stably transformed with Ha-ras genomic sequences. Compared to parent 3T3 cells, 3T3/ras cells contain 20-25% of the cytosolic glucocorticoid specific binding capacity, total immunoreactive GR protein, and GR mRNA. Dexamethasone decreased the GR mRNA level in 3T3 cells by >50% in 4 h, while the GR mRNA level in 3T3/ras cells was not reduced. Attenuation of endogenous GR levels by Ha-ras is not restricted to NIH 3T3 cells, as transfection of L929 cells with Ha-ras resulted in a 50% reduction of GR specific binding capacity. Following transfection with MMTV-CAT, 3T3/ras cells had very little glucocorticoid-inducible CAT activity, whereas 3T3 cells exhibited appreciable induction of CAT. When GR cDNA under control of the SV40 early promoter (SV2Wrec) was co-transfected into 3T3/ras cells, they exhibited glucocorticoid-inducible CAT activity. GR specific binding capacity was not reduced in CHO cells stably transfected with SV2Wrec after transfection with Ha-ras. Co-transfection of SV2Wrec and Ha-ras into COS-1 cells also resulted in no reduction in GR levels compared to SV2Wrec alone. These results suggest that the presence of activated ras p21 results in a marked reduction in the steady state level of GR mRNA expressed from the endogenous GR gene. This effect may be dependent on the particular set of nuclear transcription factors regulating endogenous GR gene expression, as GR levels are not reduced by Ha-ras in cells expressing GR cDNA from a heterologous promoter. (Supported by ACS IN-107 and BRSG)

L 314 **FUNCTIONAL DOMAINS OF THE HUMAN ANDROGEN RECEPTOR**

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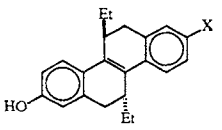
Androgens mediate their function through the intracellular androgen receptor (AR), which belongs to a large family of ligand activated nuclear transcription factors. In an ongoing study on the molecular mechanism of androgen action, we are interested in defining the relationship between the structural domains and their various functions in the human AR. By expression of several human AR deletion mutants in COS-1 and HeLa cells we have mapped domains in the AR involved in hormone binding, trans-activation and nuclear import.
Immunoblots of wild type (wt) and AR mutants, expressed in COS cells, revealed a doublet appearance of all AR proteins. One exception was an AR lacking a.a. 51 to 211 that migrated as a single protein band, most likely due to deleted phosphorylation sites. The steroid binding domain is located in the last C-terminal 250 a.a.. Large and small deletions in this region abolished hormone binding. Even a truncated AR lacking the last 12 a.a. was unable to bind androgen.
The N-terminal domain of the AR was found to be essential for transactivation. Receptors lacking parts of this domain were transcriptionally inactive.
The unliganded wt AR expressed in COS cells was distributed over nucleus and cytoplasm. Hormone directed all ARs to the nucleus. An AR mutant lacking the highly conserved SV40-like nuclear localization signal, was almost exclusively cytoplasmic in the absence of hormone. A ligand induced nuclear import of this mutant indicates the presence of a second, hormone dependent nuclear targeting mechanism.

L 315 INHERENTLY FLUORESCENT LIGANDS FOR THE ESTROGEN RECEPTOR. John A. Katzenellenbogen, Kwang-Jin Hwang, and Kathryn E. Carlson, Department of Chemistry, University of Illinois, Urbana, IL 61801.

Fluorescent ligands for the estrogen receptor (ER) could be used to measure the kinetics of ligand association and dissociation from ER and for quantitating ER levels in cells. The latter application could be of particular benefit for assessing both the content and distribution (i.e., heterogeneity) of ER levels in breast tumor cells, perhaps to achieve an improved correlation with response to hormone therapy. Because the level of ER in cells is low (ca. 5,000-20,000 sites per cell), the fluorescent probe for ER needs to have high sensitivity (high molar absorptivity and fluorescence quantum yield), and its emission needs to be at long wavelengths to avoid the background from cell autofluorescence. While many non-steroidal estrogens embody a stilbene structure, commonly present in highly fluorescent molecules, the additional substituents needed for ER binding twist the system out of planarity, disrupting the through conjugation of the chromophore. We have synthesized a series of tetrahydrochrysenes that embody a stilbene system rigidified by the addition of two extra rings. Additional substituents provide the bulk and lipophilicity and phenolic OH needed for good ER binding, and a series of electron accepting substituents provide long wavelength emission. These molecules have an affinity for ER that is 5-40% relative to estradiol, and their binding to ER can be monitored by fluorescence.




| X | RBA | $\lambda^{ex}$ | $\lambda^{em}$ (nm) | (EtOH) |
|---|---|---|---|---|
| -OH | 126 | 337 | 382 | |
| $-COCH_3$ | 40 | 380 | 535 | |
| $-CO_2CH_3$ | 24 | 364 | 482 | |
| $-CONH_2$ | 15 | 350 | 474 | |
| $-C\equiv N$ | 16 | 366 | 462 | |
| $-NO_2$ | 5 | 414 | 665 | ($CH_3CN$) |

($E_2$ = 100)

L 316 **Influence of Ligand Structure on Estrogen Receptor Transactivation** Hirao Kohno and Kenneth S. Korach, Lab Reprod Dev Tox, NIEHS/NIH, P.O. Box 12233, Res Tri Park, NC 27709

Previously, we have demonstrated that mouse estrogen receptor (ER) exhibits a 10-20 fold stereochemical binding preference for a specific stereogenic center and group on a stilbestrol estrogen ligand (Indenestrol-A). The ER binds the IA-S enantiomer (ENT) with the same high affinity as DES, while the IA-R ENT is weaker. The stereogenic center and stereochemical methyl group confers high affinity binding which is 3-fold greater than estradiol binding. Biochemical response studies show that the IA-R ENT has very weak biological activity as judged by stimulation of uterine DNA synthesis and ODC activity. To elucidate the possible site and mechanism for this stereochemical recognition a series of unique site directed mutations in the ER were made C-terminal to the dimerization region in the ligand binding domain between Met-521 and Val-538. Mouse ER was expressed in yeast and the transactivation activity monitored with a co-transfected reporter gene, $VitA_2$-ERE-CYC1-βgal. Wild type ER showed a 10-fold hormonal response difference between the IA ENTs similar to that seen in uterine tissue. However, the relationship of receptor binding did not correlate directly with stimulation since IA-S was 6-8 fold lower in activity than DES, although they both have the same binding affinity. Mutation of Met-526 to Gly resulted in weak receptor activity. Mutations at Met-521 or His-528 to Gly reduced stereochemical preference to 4.5-fold with a similar maximal stimulation as estradiol. Mutations at Leu-529 also reduced the preference to 4-fold, but resulted in only a 30% stimulation. When Met-532 was mutated to Gly there was a dramatic increase in ENT preference to greater than 60-fold suggesting some involvement of the stereochemical group with this site on the receptor protein. Dose response curves with mutant ER, but not wild type, showed an indication of cooperativity. The increased stereochemical action and cooperativity of the mutant receptor was not due to a change in ligand binding affinity preference, but resulted from a change in the transactivation activity of the particular ligand receptor complex. Use of these structurally altered ligands suggests that ligand structure influences not only receptor binding and dimerization, but also has an influence on the overall conformational structure of the receptor complex towards transactivation.

L 317 EVOLUTION OF THE NUCLEAR RECEPTOR GENE SUPERFAMILY, Vincent LAUDET, Catherine HÄNNI, Jean COLL, François CATZEFLIS+ and Dominique STEHELIN, INSERM U186/CNRS URA 1160, Institut Pasteur, 1 Rue Calmette, 59019 LILLE Cedex, FRANCE and +Laboratoire de Paléontologie, Institut des Sciences de l'Evolution (UA 327 CNRS), U.S.T.L., Place Eugène Bataillon, 34095 MONTPELLIER Cedex, FRANCE

Nuclear receptor genes represent a large family of genes encoding receptors for various hydrophobic ligands such as steroids, vitamin D, retinoic acid and thyroid hormones. This family also contains genes encoding putative receptors for unknown ligands. Nuclear receptor genes products are composed of several domains important for transcriptional activation, DNA binding (C domain), hormone-binding and dimerization (E domain). It is not known if these genes have evolved through gene duplication from a common ancestor or if their different domains came from different independent sources.

To test these possibilities we have constructed and compared the phylogenic trees derived from two different domains of 30 nuclear receptor genes. The tree built from the DNA binding C domain clearly shows a common progeny of all nuclear receptors, which can be grouped into three subfamilies : *(i)* thyroid hormone and retinoic acid receptors, *(ii)* steroid hormone receptors and *(iii)* orphan receptors.

The tree constructed from the central part of E domain implicated in transcriptional regulation and dimerization shows the same distribution in 3 subfamilies but 2 groups of receptors are placed in a different position as compared to the C domain tree : *(i)* the Drosophila knirps family genes have acquired very different E domains during evolution, and *(ii)* the vitamin D receptor, as well as the NGF1B genes, seem to have DNA binding and hormone binding domains belonging to different classes. These data suggest a complex evolutionary history for nuclear receptor genes in which gene duplication events and swapping between domains of different origins took place.

In addition we show that the three subfamilies of nuclear receptors emerged at an early stage during evolution before the arthropods/vertebrates split which took place 500 millions years ago.

L 318 FUNCTIONAL ANALYSIS OF MUTANT ESTROGEN RECEPTORS, AND CELLULAR HETEROGENEITY IN T47Dco BREAST CANCER CELLS, Kimberly K. Leslie, Kathryn B. Horwitz, Department of OB/GYN, Division of Maternal-Fetal Medicine, and Department of Internal Medicine, Division of Endocrinology, University of Colorado Health Sciences Center, Denver, CO 80262. Estrogen receptor (ER) positive breast cancers initially respond to hormone therapy but invariably progress to a resistant state. The breast cancer cell line T47Dco is a model for such stage IV breast cancer. It is a polymorphic line, composed of multiple cell populations which demonstrate the presence of mutant ERs by cloning and sequencing techniques. Dual parameter flow cytometry shows in addition, extensive heterogeneity in mitotic indices and progesterone receptor (PR) levels among cell subpopulations. We cloned and sequenced three mutant ER from two independent T47Dco cDNA libraries. The first mutant (DBD) had two thymidine residues inserted into the DNA binding domain which results in a frame shift and a protein truncated after amino acid 250. The second mutant (HBD) has a single guanine deletion which also results in a frame shift and a protein truncated after amino acid 417 in the hormone binding domain. The third mutant has an in-frame 462 bp deletion from the end of the DNA binding domain through the beginning of the hormone binding domain. These mutants have now been recreated in an expression vector (HEGO-pSG5, obtained from P. Chambon) using site-directed mutagenesis, and the mutant ERs tested for their ability to bind to DNA, to dimerize with wild-type ER, and to activate gene transcription. The molecular heterogeneity reflected in the variant ERs may arise from extensive cellular heterogeneity in these cells. We demonstrate that subpopulations may be PR-negative, PR-poor or PR-rich. Clonal derivatives are growth inhibited or stimulated by estradiol and tamoxifen. Chronic tamoxifen treatment suppresses growth and PR in a majority of cells in one subline, but paradoxically stimulates growth and PR in a subpopulation of the same cells. We postulate that tamoxifen resistance which characterizes 50% of ER-positive breast cancers results from the actions of mutant ER in tumor-cell subpopulations that are constitutively active, or that confer agonist effects on tamoxifen.

L 319 H2RIIBP, A MEMBER OF THE NUCLEAR HORMONE RECEPTOR SUPERFAMILY, FORMS HETERODIMERS WITH THYROID HORMONE AND RETINOIC ACID RECEPTORS, RESULTING IN ENHANCED DNA BINDING AND TRANSCRIPTIONAL ACTIVITY, MS Marks, PL Hallenbeck, T Nagata, JS Segars, E Appella, V Nikodem, and K Ozato, Laboratory of Developmental and Molecular Immunity, NICHD, NIH, Bethesda, MD 20892. H2RIIBP is a RA-responsive transcriptional activator that binds to the RII enhancer of major histocompatibility complex (MHC) class I genes. Using chemical cross-linking analysis, we determined the oligomerization state of H2RIIBP both in the absence and presence of target DNA. We show that H2RIIBP monomers, which are predominant in solution, weakly associate to form homodimers when bound to target DNA. More importantly, H2RIIBP forms heterodimers with thyroid hormone (T3) receptor $\alpha$ (T3R$\alpha$) and retinoic acid (RA) receptor $\alpha$ (RAR$\alpha$). Heterodimer formation did not require DNA and was stable in the absence of cross-linking reagents. Heterodimerization with either receptor resulted in greatly enhanced binding to several DNA response elements. DNA binding was also qualitatively altered, as judged by methylation interference patterns of H2RIIBP homodimers and H2RIIBP/T3R$\alpha$ heterodimers. Dimerization was mediated by a subdomain of the ligand binding domain that is homologous to regions of T3R$\alpha$ and RAR$\alpha$ previously shown to be necessary for association with each other and with other nuclear proteins. The interaction of either receptor with H2RIIBP differed both qualitatively and quantitatively from their interaction with each other. In cotransfection assays, H2RIIBP synergistically enhanced the ligand-dependent transcriptional activation of the MHC class I gene promoter by T3R$\alpha$ and RAR$\alpha$ in a human embryonal cell line. While RA was required for transcriptional activation by H2RIIBP alone, RA had no effect on activity when H2RIIBP and T3R$\alpha$ were co-transfected. Furthermore, H2RIIBP was capable of forming RII-binding heterodimers with a novel nuclear factor induced in the embryonal cell line by RA treatment. These data suggest that H2RIIBP may be one type of co-activator of RARs and T3Rs, similar to the TRAP activity described by others. They also suggest that H2RIIBP does not bind to RA or RA derivatives, but rather responds to RA by forming heterodimers with RA-induced cellular factors.

L 320 STRUCTURE/FUNCTION STUDIES OF UBIQUITIN/ERBA ALPHA FUSION PROTEINS EXPRESSED IN E. COLI, Charles F. McTiernan, Kristine A. Goodrich, Benedict Kim, Heart and Hypertension Research, Cleveland Clinic Foundation, Cleveland OH 44195. Interaction of erbA $\alpha$1 (a nuclear thyroid hormone receptor) with ligand (T3) causes a structural change in the erbA protein, modifying it's interactions with other nuclear regulatory proteins and nucleic acid residues, and leading to alterations in gene expression, cell growth and differentiation. Since knowledge of the molecular configuration of the ligand binding domain (LBD) of erbA proteins is important to understanding how T3-binding modifies gene expression, the goal of these studies is to define the erbA amino acid residues intimately involved in contacting the regulatory ligand T3. While a broad region of the rat erbA $\alpha$1 protein (amino acid residues 122-410) has been suggested to be involved in forming the LBD, the manner in which particular residues contribute to T3-binding and formation of the LBD remains unknown. To study the interaction of erbA $\alpha$ with T3, we have created and expressed ubiquitin(UBQ)/rat erbA $\alpha$1 fusion proteins in E. coli. Using previously described vectors, we have created two UBQ/erbA fusion proteins containing either erbA $\alpha$1 residues 1-410 (full length, pUerbA$\alpha$) or residues 122-410 (pULBD). Scatchard analysis of cleared bacterial lysates expressing either construct indicates a $K_d$ for T3 of 15-45 pM (rat liver nuclear extract is 62 pM under similar analysis). Soluble extracts bind 60 (pULBD) to 450 (pUerbA$\alpha$) fm T3/mg protein. Guanidinium extracts from inclusion bodies bind 1600 (pULBD) to 250 (pUerbA$\alpha$) fm T3/ mg protein. Competition studies (using pULBD extracts) of $^{125}$I-T3 binding in the presence T3 analogues show IC50s similar to that previously reported for erbA $\alpha$ proteins (TRIAC 0.09 nM, T3 0.5 nM, T4 1.8 nM, rT3 85 nM). pUerbA$\alpha$ proteins of the expected size (58 kd) are detected after Western blot analysis with antibodies against UBQ or erbA $\alpha$ (raised against a peptide encoding residues 17-33). pULBD proteins of the expected size are recognized by anti-UBQ antibody (44 kd). Proteins of the same size detected by these antibodies can be UV cross-linked to $^{125}$I-T4. Current studies are directed at identifying tryptic- and CNBr-generated peptides cross-linked to T4, and at the functional consequence of altering defined amino acid sequences in the erbA LBD.

L 321 CHARACTERIZATION OF THE LIGANDED AND UNLIGANDED HUMAN ESTROGEN RECEPTOR OVER-EXPRESSED IN THE BACULOVIRUS EXPRESSION VECTOR SYSTEM, John D. Obourn and Angelo C Notides, Department of Biophysics and Environmental Health Sciences Center, University of Rochester School of Medicine and Dentistry, Rochester, New York 14642 We have prepared and characterized the liganded and unliganded recombinant estrogen receptor (hER) that was over-expressed in a baculovirus expression vector system. The estrogen receptor cDNA (HE0) was corrected to the wild type using a specific oligonucleotide to change the val mutation to a gly at amino acid 400. The recombinant hER is full length and is specifically recognized in a western blot assay by a polyclonal antibody directed against calf ER as well as two polyclonal antibodies directed against peptides from the amino terminus and hinge region of the human estrogen receptor. Using the gel mobility shift assay we determined that the recombinant hER bound specifically to an estrogen response element (ERE). The recombinant estrogen receptor bound the ERE with high affinity in both the liganded and unligand state independent of the prescence of $Mg^{2+}$. The recombinant estrogen receptor bound estrogens specifically and bound ICI 164,384, a steroid that may prevent dimerization of the hER. The ICI 164,384 altered the mobility and banding pattern of the recombinant estrogen receptor in the gel shift assay. These data may provide information as to how the receptor conformation effects ERE binding. Ethylation interference and thymidine interference footprints of the recombinant hER were generated on the perfect ERE contained in the chicken vitellogenin (cVit)-11 gene. These footprints provide information on how recombinant human estrogen receptor interacts with the phosphate backbone of the DNA as well as specific nucleotide residues contained in and around a perfect ERE. This recombinant human estrogen receptor expressed in a baculoviral expression system will provide sufficient quantity and purity of receptor for detailed studies of receptor-DNA interaction.

**L 322** LOCALIZATION OF COUP-TF mRNA EXPRESSION IN MOUSE BRAIN BY *IN SITU* HYBRIDIZATION OF A COUP-TF cRNA PROBE, Angelica Oviedo*, Orla M Conneely, Bert W O'Malley and Miles Herkenham, Section on Functional Neuroanatomy, National Institute of Mental Health, Bethesda, MD 20892, and Baylor College of Medicine, Houston, TX 77030
*Howard Hughes Medical Institute-National Institutes of Health Research Scholar

The Chicken Ovalbumin Upstream Promoter Transcription Factor (COUP-TF) family of genes belongs to the group of orphan receptors, which are receptor-like proteins that have no known ligands. It has recently been shown that hCOUP-TF can be activated by physiological concentrations of dopamine, even though dopamine is not a hCOUP ligand. In order to help elucidate the function of COUP-TF in the brain, we used *in situ* hybridization and autoradiography to localize the expression of COUP-TF in mouse brain. The results showed that there was specific hybridization of a full-length (1.5 kb) hCOUP-TF cRNA probe in the following structures: olfactory bulb, neocortex, piriform cortex, hippocampus, thalamus, amygdala, cerebellum, pia-arachnoid, and ventricular lining. Since hCOUP-TF can be activated by dopamine, it is remarkable that there was no specific hybridization in the major dopaminergic systems of the brain, notably the striatum and substantia nigra. These experiments were done with a full length cRNA probe; therefore, there may have been non-specific hybridization to other members of the COUP-TF gene family. Further experiments are planned with shorter probes, in order to rule out this possibility.

**L 323** NATURALLY-OCCURRING STRUCTURAL DEFECTS IN THE ANDROGEN RECEPTOR GENE Charmian A. Quigley, Jorge A. Simental, Keith B. Marschke, Dennis B. Lubahn, Elizabeth M. Wilson, Frank S. French. The Laboratories for Reproductive Biology, Univ. of North Carolina at Chapel Hill, NC 27599.

The quintessential example of steroid hormone resistance is the androgen insensitivity syndrome (AIS). This X-linked disorder encompasses a heterogeneous group of defects in the androgen receptor (AR) which produce varying degrees of defective masculinization in 46,XY individuals with testes. To determine the prevalence of gene deletions underlying abnormalities of the AR we examined the AR gene in 16 families with complete AIS. Polymerase chain reaction (PCR) and Southern blot analysis revealed deletion of the entire AR gene in affected members of one family. Other than complete lack of sexual hair, and of Wolffian and Müllerian structures, these individuals had normal female physical and intellectual development. In another family, supranormal levels of high-affinity androgen binding were found in genital skin fibroblasts despite complete clinical resistance to androgen. PCR and Southern blot analysis revealed an in-frame deletion of exon C which encodes the second zinc finger of the AR DNA-binding domain. When recreated *in vitro* and examined in a cotransfection assay this zinc-finger-deficient AR failed to activate transcription of an androgen-responsive reporter gene. Further studies in cultured genital skin fibroblasts revealed normal nuclear localization of AR by immunocytochemistry but a marked reduction in DNA-binding affinity of the mutant AR. This abnormality of receptor-DNA interaction likely underlies its lack of transcriptional activity. In one further family, PCR analysis revealed a deletion of exon B, which encodes the first zinc finger of the AR DNA-binding domain. In contrast to the exon C deletion however, this deletion does disrupt the reading-frame and further studies are in progress to determine its effect upon expression of the AR gene. Our studies indicate that major structural defects account for a minority of AR gene mutations, however these defects provide important correlations between molecular structure and sex phenotype. The second zinc-finger deletion highlights the functional dependence of the AR upon this region *in vivo* and complete deletion of the AR gene defines the null phenotype of AIS.

**L 324** CHARACTERIZATION OF TRANSACTIVATION PROPERTIES AND FUNCTIONAL DOMAINS OF THE MINERALOCORTICOID RECEPTOR. Rainer Rupprecht, Dietmar Spengler, Florian Holsboer and Klaus Damm Dept. of Neuroendocrinology, Max-Planck-Institute for Psychiatry, Kraepelinstr. 10, 8000 Munich 40, Germany.

Adrenal corticosteroids have a major influence on maintaining central nervous system homeostasis, cell proliferation and differentiation. The actions exerted by these hormones are mainly mediated by two types of corticosteroid receptors, the mineralocorticoid (MR) and glucocorticoid (GR) receptors. As a result of sequence similarity in the ligand binding domain (57% identity) hMR and hGR are responsive to physiological corticosteroids and only few synthetic ligands can discriminate selectively between the two receptors. Because of the near identity in the DNA binding domain both receptors can also interact with similar target DNA sequences. To improve our understanding of hMR and hGR function, the ability of both receptors to mediate biological responses to adrenal corticosteroids and to synthetic ligands was examined in the human neuroblastoma cell line SK-N-MC. Transient transfection studies employing a MMTV-Luciferase reporter gene were used to monitor the transactivation properties of wild-type and hMR/hGR hybrid receptors. The analysis of these chimeras revealed that the trans-activation potential of the hMR appears to be partitioned equally between amino-terminus, DNA- and ligand-binding domain. In particular, the hMR amino terminus is lacking the strong trans-activation function present in the equivalent hGR domain. However, in addition to determining activation efficiency this region appears to be involved in the differential hormone response of the receptors. Sensitivity and efficacy for various hormones are changed in hybrid receptors, indicating that specific hormonal response patterns of steroid receptors may be affected by interactions between the ligand-binding domain and amino terminal sequences.

**L 325** DIMERIZATION CONSTANT OF THE RU486-BOUND-PROGESTERONE RECEPTOR. D.F. Skafar, Dept. of Physiology, Wayne State University School of Medicine, Detroit, MI 48201.

Receptor dimerization is part of the pathway for gene activation by steroid hormones, yet few quantitative studies of dimerization have been performed. The positive cooperative binding of RU486 to the calf uterine progesterone receptor (PgR) was therefore used to measure the dimerization constant of the RU486-bound receptor. At a PgR concentration of 4 nM, the Hill coefficient was 1.38; at a PgR concentration of 1 nM, the Hill coefficient was 0.96. The Scatchard plots also changed shape from curved to linear as the PgR concentration was reduced. The PgR in cytosol which was diluted with TDE buffer bound [$^3$H]RU486 with the same Hill coefficient as PgR in cytosol which was diluted with heat-treated cytosol [1.07 ± 0.03 vs. 1.03 ± 0.03 respectively (n=4)]. This indicated the Hill coefficient depended on the PgR concentration, not the total protein concentration. These results were consistent with the PgR being in a dimeric state at high concentrations and a monomeric state at low concentrations. The dimerization constant of the RU486-bound PgR was then determined using nonlinear regression analysis of the dependence of the Hill coefficient on the PgR concentration. The limiting value of the Hill coefficient at high PgR concentrations was 1.38 ± 0.01. The limiting value of the Hill coefficient at low PgR concentrations was 1.05 ± 0.01. The dimerization constant was 2.57 ± 0.01 nM, which is lower than that reported for the progesterone-bound receptor, 7 nM [Skafar, D.F. (1991) Biochemistry 30, 6148]. This supports observations that the RU486-bound receptor dimerizes more readily than the progesterone-bound receptor. Furthermore, this quantitative approach facilitates making comparisons among different classes of receptors and will aid in studying the effects of other ligands on dimerization. RU486 and [$^3$H]RU486 were gifts of Roussel-Uclaf. Supported by NSF grants DCB-8716044 and DCB-9104857.

L 326 COMPARISON OF THE TRANSACTIVATION AND DOMINANT NEGATIVE ACTIVITIES OF SEVERAL MUTANT THYROID HORMONE RECEPTORS FROM PATIENTS WITH RESISTANCE TO THYROID HORMONES. F. Su, S.J. Usala*, J.B. Menke*, M.E. Geffner#, J.M. Hershman#, & H.H. Samuels, New York Univ., *East Carolina Univ., and #UCLA Schools of Medicine, New York, NY 10016, Greenville, NC 27858, and Los Angeles CA 90024

Generalized resistance to thyroid hormones (GRTH) results from a variety of distinct mutations localized in the thyroid hormone (T3) binding domain of the human c-erbA-beta thyroid hormone receptor (T3R) gene. We have studied two mutant T3Rs (CL and S) from patient kindreds with GRTH and a third mutant (GH) from a patient with apparent selective pituitary resistance to T3. Mutants CL and GH are arginine to histidine changes at positions 315 and 311, respectively, while mutant S is a deletion of threonine 332. Mutant CL binds T3 with about 2-fold lower affinity than wild type T3R and mutants S and GH bind T3 with about 1000- and 100-fold lower affinity than wild type, respectively. In transient transfection assays using T3R-deficient HeLa cells, mutant CL activated a T3-responsive reporter construct, TREir-MTV-CAT, but maximal activation required higher levels of T3 than wild type T3R. Mutants S and GH did not activate CAT expression from this reporter. GRTH is believed to result from a dominant negative inactivation of wild type T3R by formation of heterodimers with mutant receptors. The dominant negative activity of mutants CL, S and GH against wild type T3R was assessed in HeLa cells by cotransfection. Mutant CL showed dominant negative activity only at concentrations of T3 less than 10 nM. Mutant S was dominant negative at all hormone concentrations. Mutant GH was not dominant negative even when transfected into HeLa cells in 5-fold excess over wild type T3R, consistent with the failure to observe GRTH in the patient carrying this mutation. We are currently purifying these mutants expressed in E. coli and will assess receptor dimer formation on DNA in a gel retardation assay. If dimerization is required for dominant negative activity, mutants CL and S are expected to form dimers while mutant GH is not.

L 327 RETINOIC ACID DECREASES NUCLEAR T3 RECEPTOR/C-ERB A EXPRESSION AND INHIBITS AN EARLY STEP OF ADIPOSE DIFFERENTIATION IN THE PREADIPOCYTE CELL LINE OB 17. J. Torresani, M. Teboul, J. Bismuth, J. Gharbi-Chihi, A. Valette*, J. Bonne. INSERM U 38 and *U 260, Biochimie Médicale, Faculté de Médecine, 13385 Marseille Cédex 5 France.

In the murine preadipocyte cell line Ob 17, T3 is necessary at an early step of adipose differentiation for the expression of late markers (i.e malic enzyme ME, glycero-3-phosphate dehydrogenase GPDH) and not necessary for the expression of lipoprotein lipase (LPL) which emerges early at confluence. These cells contain nuclear T3 receptors (T3R) which number is regulated, up- during differentiation, down- by T3. We demonstrated that these T3R mainly belong to c-erb A gene products of the α type (immunodetection, mRNA analyses). In another preadipocyte cell line, 3T3-L1, retinoic acid (RA) was reported as inhibiting differentiation and the emergence of GPDH. RA action is mediated by nuclear receptors (RAR) which belong to the same gene superfamily as the T3, vitamin D3, steroid receptors. The existence of interactions between T3R and RAR at the level of proteins and respective DNA response elements, has been proposed. RA action on the Ob 17 cell line development and on T3R expression was studied. The Ob 17 cells were grown in DMEM-10 % FCS, supplemented or not at confluence with insulin (17 nM) ± T3 (1.5 nM) and/or RA (1 nM-10 μM). RA at 1-10 μM totally inhibited cell differentiation and both late markers (GPDH, ME) and LPL expression. T3 sensitized the cells to RA, the ED50 for GPDH inhibition being shifted from 500 to 3 nM RA when 1.5 nM T3 was present. RA also brought about a marked decrease of T3R cell concentration (80% at 10 μM) without changing the Ka for T3. T3R decrease was concentration-dependent (ED 50 200 nM RA), fast (1/2 max after 7h i.e less than T3R 1/2-life estimated at ~13h after cycloheximide addition), reversible, amplified by T3 and detected at all stages of cell development with a higher sensitivity at confluence. RA markedly reduced the expression of c-erb Aα mRNA transcripts detected with a rat c-erb A α cDNA probe containing the entire coding sequence or a shorter PCR-designed mouse c-erb Aα cDNA probe lacking the conserved DNA-binding domain. Other PCR-designed mouse cDNA probes for c-erb A subtypes indicated that RA down-modulated all the detected RNA species ($\alpha_1$, $\beta_1$, $\alpha_2$, Rev-α). These results show that in Ob 17 preadipocytes : 1/RA inhibits T3R expression at both post- and pre-translational levels this involving both c-erb Aα and β subtypes ; 2/ RA impairs adipose differentiation at an early step and in a T3-sensitive manner. A decreased level of T3R at an early step could play a role in this inhibitory action of RA.

L 328 **RAR β and Cellular Senescence. X Lee, HC Tsou, SP Si and M Peacocke, Department of Dermatology New England Medical Center, Boston, MA 02111.**

Vitamin A and its derivatives (RA) have profound effects on human skin. RA, like steroid hormones, mediates certain of its effects via nuclear receptors. Four nuclear receptors for RA have been cloned, RAR α, RAR β, RAR γ and RXR α. They are all members of a multi-gene family which includes steroid and thyroid hormone receptors. Each receptor has five structural domains with D domain responsible for DNA binding and the E domain for ligand binding. We have previously demonstrated that human dermal fibroblasts express high basal levels of RAR α and RAR γ, but not RAR β and that the mRNA of RAR β and RAR γ are rapidly induced by 1 uM RA. Because retinoids are felt to play a role in aging skin, we explored the effect of cellular aging on the expression of RAR α, RAR β and RAR γ by using dermal fibroblasts harvested from inner arm biopsies of healthy young and old adults. We first generated growth curves from these donors. By Northern analysis, we showed no difference in the expression and regulation of RAR α and RAR γ between donors of different age. However we note a striking increase in the ability of 1 uM RA to induce RAR β mRNA in the senescent fibroblasts. Our preliminary studies have also suggested that RAR β protein is upregulated in these slow growing cells. This suggests that this gene and protein may have a role in the senescent cell, although what the function is unknown. This finding is made more intriguing by the association of RAR β with programmed cell death of fibroblasts during development. Thus, RAR β may be involved in a program of cell death in fibroblasts and potentially serve as a biomarker of skin aging.

L 329 ESTROGEN INHIBITS the GROWTH of IMMORTAL HUMAN MAMMARY EPITHELIAL CELLS EXPRESSING a RECOMBINANT ESTROGEN RECEPTOR, Deborah A. Zajchowski[1] and Ruth Sager[2], [1]Department of Cell Biology and Immunology, Berlex Biosciences, Alameda, CA 94501; [2]Division of Cancer Genetics, Dana Farber Cancer Institute, Boston MA 02115.

Estrogen is mitogenic for the epithelial cells of the normal mammary gland as well as most estrogen receptor (ER) containing breast carcinomas. In normal tissue, estrogenic stimulation of growth and differentiation is carefully controlled. This contrasts markedly with the unchecked proliferation of estrogen-dependent breast cancer cells. Comparative analyses of the effects of estrogen on normal and tumor cells have been hampered by the inability to obtain homogeneous cultures of normal mammary epithelial cells which express estrogen receptors in culture. To this end, we have introduced the ER cDNA into immortal human mammary epithelial cells and isolated stable transfectants which express high levels of ER (500 to 1000 fmoles/mg protein). Instead of the expected enhanced proliferation in response to estrogen administration, the growth of these ER-transfected cells was inhibited by estrogen (at concentrations of >1.0 nM). The control transfectants and parental cells exhibited no response to estrogen concentrations as high as 1 μM. FACS analysis of the cell cycle distribution of estrogen-treated cells showed that the proportion of cells in the G1 phase of the cycle was increased 20% within 24 hrs of treatment. These data suggest that estrogen is increasing the duration of G1 transit. Since the ER is an estrogen-inducible transcription factor, it is of interest to determine whether any of the known estrogen-regulated genes are modulated in these cells. Consistent with its growth-inhibitory effects, estrogen decreased the steady state c-myc mRNA levels. Yet, paradoxically, estrogen augmented the levels of transforming growth factor α and 52 kDa cathepsin D mRNAs. No effects were noted on the amounts of erbB2 or EGF receptor mRNA following estrogen treatment. The physiological significance as well as potential mechanisms for estrogen's inhibitory effects will be discussed.

*Poster Session IV*

**L 400** MOLECULAR CLONING AND EXPRESSION OF HUMAN CELLULAR RETINOIC ACID BINDING PROTEIN (CRABP) GENES, Anders Åström, Ulrika Pettersson, James T. Elder and John J. Voorhees, Department of Dermatology, University of Michigan, Ann Arbor, MI, 48109

Cellular retinoic acid binding proteins (CRABP) are low molecular weight proteins whose precise function remains unknown. To investigate the role of CRABP in human skin we have cloned the human CRABP-I and CRABP-II cDNAs. We have found that the CRABP-II mRNA is expressed in human skin and is induced (16-fold) by application of 0.1% retinoic acid cream *in vivo* for 16h. CRABP-I message was on the other hand undetectable and not induced by retinoic acid in human skin. Expression of CRABP-II, but not CRABP-I transcripts was also markedly increased (15-fold) by retinoic acid treatment of skin fibroblasts *in vitro*. However, CRABP-II mRNA was not inducible by retinoic acid in lung fibroblasts, demonstrating cell-specific regulation of this gene. Human CRABP-II, but not CRABP-I mRNA was increased by agents known to induce keratinocyte differentiation *in vitro*. Wether retinoic acid induction of the CRABP-II gene occurs at the level of transcription and wether this regulation is mediated by the retinoic acid receptors or RXRs is currently under investigation.

**L 401** THE BETA2 THYROID HORMONE RECEPTOR IS EXPRESSED IN TISSUES OUTSIDE THE PITUITARY DURING RAT DEVELOPMENT, David J. Bradley, Howard C. Towle and W.Scott Young, III, National Institute of Mental Health, Bethesda, MD 20892 and Department of Biochemistry, University of Minnesota, Minneapolis, MN 55455.

Numerous abnormalities, including mental retardation, movement disorders and deafness, are associated with thyroid hormone deficiency during human development. To identify specific anatomic targets of thyroid hormone action in the developing mammalian nervous system, we examined thyroid hormone receptor gene expression in the developing rat by hybridization histochemistry. $^{35}S$-labelled cRNA probes were generated from divergent sequences of rat alpha1, alpha2, beta1 and beta2 *c-erbA* cDNAs. These probes were hybridized to rat nervous system tissue sections collected from 12 developmental stages ranging from embryonic day 11.5 to 9 weeks postnatally. Surprisingly, β2 thyroid hormone receptor transcripts, previously described as being pituitary-specific in adult rats, were detected in the developing rat brain. Highest levels of β2 transcripts were found in the striatum, subiculum and CA1 region of the hippocampal formation. To confirm these results, RNA from developing rat brain was reverse transcribed and the resulting cDNAs amplified by PCR using β2-specific primers. PCR products of expected sizes were produced. The presence of three (i.e. α1, β1 as well as β2) rather than two functional thyroid hormone receptor mRNAs in the developing CNS suggests a previously unrecognized level of complexity in thyroid hormone regulation of brain development.

**L 402** A STEROID/THYROID HORMONE RESPONSIVE ELEMENT IN THE HIV-1 LTR.

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Expression of the integrated proviral genome of HIV-1 is regulated by cellular transcription factors interacting with the 5' long terminal repeat (LTR). We have previously described the characterization of a novel transcription factor binding site within the LTR of HIV-1 (site B) which has striking homology to the hormone responsive elements or HRES (1). These elements are the specific target for the binding of steroid/thyroid hormone receptors and confer hormone inducible expression to genes. The expression of several retroviruses can be shown to be hormonally regulated, operating through HRES within the LTRs of the proviral genome. Our studies indicate that the HRE-like element in HIV-1 can bind recombinant human thyroid hormone, estrogen or retinoic acid receptors. The significance of such binding was analysed by transient expression of the CAT gene in constructs driven by the minimal promoter of HSV thymidine kinase linked to multimers of site B. Such constructs impart hormone inducible expression of CAT. Furthermore, in the human myeloid progenitor cell line HL60, site B confers retinoic acid responsiveness upon the HIV-1 LTR. This is the first report of an HRE within the 5' LTR of HIV-1.

(1) Orchard, K., Perkins, N., Chapman, C., Harris, J., Emery, V., Goodwin, G., Latchman, D. and Collins, M. (1990) J. Virol. *64*, 3234-3239.

**L 403** INHIBITION OF RETINOIC ACID AND THYROID HORMONE RESPONSE BY DOMINANT NEGATIVE RECEPTOR MUTANTS.

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The diverse effects of retinoids and thyroid hormone on the development, growth and homeostasis of vertebrate organisms are mediated in part by three distinct isoforms of retinoic acid receptors (RARs) and two isoforms of thyroid hormone receptors (TRs). These proteins regulate patterns of gene expression in target tissues. One approach to study the distinct effects of retinoic acid and thyroid hormone in cells is to subvert this activity of endogenous receptors by the expression of dominant negative receptor derivatives. Using site directed mutagenesis a series of mutant RAR and TRs was created and shown to exhibit distinct phenotypes in the regulation of gene expression. Single point mutations can change receptor properties dramatically and create potent negative transcriptional regulators that block wildtype receptor function. Some of these mutants, but not the wildtype RARs, interact with the transcription machinery to lower the basal activity of target promoters. These inhibitory functions are remarkably similar to those of the product of the v-erbA oncogene and support a model whereby mutant TRs and RARs may contribute to oncogenesis. The analysis of a biologically inactive mutant of v-erbA, exhibiting a single amino acid replacement which is responsible for its defectiveness, demonstrated that these negative regulatory properties are directly linked to the transformation potential of v-erbA. In the context of the TR, this mutation, a Pro to Arg change, does not interfere with either recognition of the response element, hormone binding or transcriptional activation. However, this mutated amino acid severely and specifically affects the negative regulatory functions of the TR and v-erbA and eliminates the ability of v-erbA to act as a dominant negative inhibitor of TR and RAR function.

L 404 HORMONAL AND CONSTITUITIVE INHIBITION OF HUMAN BETA MYOSIN HEAVY CHAIN (βMHC) EXPRESSION IN THE MYOCARDIUM.

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3,5,3'-triiodo-L-thyronine ($T_3$) strongly influences the myosin isoforms in the myocardium to increase the relative amount of the αMHC isoform and decrease the amount of the βMHC isoform. The βMHC gene is expressed both in the myocardium and in skeletal muscle. We have studied $T_3$ regulation of the βMHC human promoter region, fused to the CAT gene, in rat fetal heart cells. In heart cells, $T_3$ inhibited the expression of human β-MHC constructs with an apparent $EC_{50}$ of $1x10^{-9}$M. A number of cis-acting elements were identified: 1) a proximal $T_3$ response element (TRE); 2) a strong positive element located between -273/-298 that caused constitutive expression and that was not regulated by $T_3$; 3) a $T_3$-independent silencer region located -305/-330; 4) possibly additional regulatory elements including another TRE. Gel shift and DNase footprinting determined that several regions could interact with $T_3$ receptor protein. The regions that appear responsible for $T_3$ regulation do not adhere to any known $T_3$ consensus region and possibly $T_3$ receptors may bind in combination with another trans-factors. Interaction of various trans-acting factors with these cis-elements may account for the pattern of expression observed in vivo.

L 405 TRANSCRIPTIONAL REGULATION AT THE ANDROGEN RECEPTOR GENE PROMOTER, Peter W. Faber[1], Henri C.J. van Rooij[1], Helma Schipper[1], Albert O. Brinkmann[2] and Jan Trapman[1]. Departments of Pathology[1] and of Endocrinology and Reproduction[2], Erasmus University, P.O. Box 1738, 3000 DR, Rotterdam, The Netherlands.

Transcription at the human and mouse androgen (hAR/mAR) receptor gene promoters initiates at two well defined positions in a 13 bp region. Structurally, the proximal promoter region lacks the canonical TATA/CCAAT boxes and is not particularly GC-rich. Potentially regulatory elements consist of a consensus Sp1-binding site around -45 and a long homopurine stretch from -60 to -120. The distal promoter region up to -1000 is devoid of well known enhancer sequences.

In transfection assays, using CAT reporter constructs, the minimum requirements for promoter function in T47D cells were defined. By deletion mapping it was found that a fragment starting at -63 contained minimal promoter activity. This activity could be slightly elevated (five-fold) by adding upstream sequence up to 9kb. In addition, it was observed that 5'-UTR sequences played a major role in determining this basal level CAT-activity. In a parallel set of transfection experiments the minimum sequence requirements for the usage of the two transcription initiation sites were determined and it was found that in the -63 fragment both sites were used. This led to the investigation of the role of the Sp1-consensus sequence by deletion and mutation analysis. Interestingly, it was found that although this sequence is absolutely essential for transcription initiation at position +12 it is of no importance for initiation at position +1. These findings might provide a mechanism for a differential regulation of AR gene expression in different tissues or during embryogenesis.

L 406 **HUMAN EPIDERMAL TRANSGLUTAMINASE IS ELEVATED *IN VIVO* AND DECREASED IN KERATINOCYTES *IN VITRO* BY RETINOIC ACID TREATMENT.** GJ Fisher, CEM Griffiths, A Reddy, J Elder, A Astrom, D Rosenthal, K Leach, T Wang, S Yuspa, and JJ Voorhees. Department of Dermatology, University of Michigan, Ann Arbor, MI, & National Cancer Institute, Bethesda, MD.

Epidermal transglutaminase (TGase K) catalyzes covalent linking of loricrin and involucrin to form cross-linked envelopes, an essential component of the protective barrier of human skin. In normal skin, TGase K is expressed in the epidermis in the outermost layer of keratinocytes (KC), and its expression is a marker of KC terminal differentiation. Since retinoic acid (RA) is an important physiological regulator of skin growth and differentiation, we have investigated the modulation of TGase K by retinoic acid *in vivo* and *in vitro*. RA (0.1% cream) or its vehicle was applied to human skin under occlusion for 4 days, and biopsies of the treated skin were obtained. Normal human KC were grown in serum free media and treated with RA (1μM) for 4 days. Following treatments, TGase K activity was measured, in skin and KC samples, by its ability to catalyze the incorporation of [$^3$H]putresine into casein. TGase K protein was localized in sectioned skin biopsies and cultured KC by immunostaining. The cDNA for TGase K was cloned from a human KC λgt11 library and used as a probe to determine TGase K mRNA levels by Northern analysis. TGase K activity was elevated 2.7-fold in Ra, compared to vehicle-treated skin ($p<0.005$, $n=12$). This increase in enzyme activity was accompanied by a small, statistically insignificant, increase in TGase K mRNA levels (78% increase, $p=0.14$, $n=9$). In vehicle-treated skin, TGase K immunostaining was limited to a thin band just beneath the outer later of KC, but was significantly expanded throughout the epidermis in RA-treated skin ($p<0.001$, $n=18$). The TGase K substrate involucrin displayed a similar pattern of expression, by immunostainig, as TGase K in vehicle and RA-treated skin. In contrast, TGase K activity was reduced 80% ($p<0.01$, $n=4$) in RA vs vehicle-treated cultured KC *in vitro*. This reduction in TGase K activity correlated with decreased TGase K protein, as determined by immunostaining. These results demonstrate that RA treatment *in vivo*, in contrast to *in vitro*, leads to increased TGase K and involucrin expression in human KC. The difference between the effect of RA on TGase K expression *in vivo* and *in vitro* indicates that responses of KC to RA may be modulated by the cellular milieu.

L 407 DYNAMIC INTERPLAY BETWEEN THE GLUCOCORTICOID RECEPTOR AND A LIVER-SPECIFIC FACTOR DURING TRANSCRIPTIONAL ACTIVATION OF THE RAT TYROSINE AMINOTRANSFERASE GENE

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The tyrosine aminotransferase (TAT) gene is expressed specifically in liver cells where its transcription is increased by glucocorticoid hormones. Two remote glucocorticoid responsive units (GRUs) interact cooperatively to promote full induction of the TAT gene (1). These two GRUs function in a cell-type specific way (2, 3). They are constituted of multiple contiguous and overlapping binding sites for three trans-acting factors: the glucocorticoid receptor (GR), C/EBP (and the C/EBP-like family) and a liver-specific factor: HNF5 (3). One of the GR-binding site is also an HNF5-binding site and these two factors cannot interact simultaneously with that site (4).

*In vivo* footprinting analysis of the two GRUs reveal that: 1) the interaction of the GR with DNA does not last long enough to be detectable, 2) before maximal GR-mediated activation of transcription is achieved (10 min.), one of the GR-binding site is occupied by HNF5 and not by the GR, 3) the interaction of HNF5 with its target sites appears to require beforehand an alteration of the chromatin structure which is mediated by the activated GR over only one of the two GRUs (4).

Based on these observations, we propose a hit-and-run mechanism for GR-mediated activation of transcription of the TAT gene which involves GR-dependant alteration of chromatin structure.

1. Grange *et al* (1989) Nucl. Acid Res. **17**, 8695-8709.
2. Grange *et al* (1989) Exptl. Cell Res. **180**, 220-233.
3. Grange *et al* (1991) Nucl. Acid Res. **19**, 131-139.
4. Rigaud *et al* (1991) Cell *in press*

**L 408** 6'-SUBSTITUTED NAPHTHALENE-2-CARBOXYLIC ACID ANALOGS, A NEW CLASS OF RETINOIC ACID RECEPTOR SUBTYPE-SPECIFIC LIGANDS

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Retinoic acid (RA) has been recognized as a pivotal regulatory compound in cell differentiation,proliferation and malignant transformation. These effects are mediated by nuclear receptors (RARα, RARβ, RARγ) which are members of the steroid/thyroid receptor family and show tissue-specific expression patterns during embryogenesis and in adult life. The RARs are encoded by three different genes from which several isoforms can be derived : these isoforms appear to be regulated by tissue-specific expression as well. RA analogs (retinoids) have long been employed to gain insight into molecular parameters governing such seemingly unrelated properties as chemoprevention of tumorigenesis, and malformation. It is from these studies proving differentiation in the absence of cytoplasmic retinoic acid binding protein (CRABP) and a lack of correlation between differentiation and retinoid CRABP binding that the initial concept of cytoplasmic proteins as the mediators of RA activity was replaced by the now established mechanism of action through nuclear receptors. Direct demonstration of retinoid binding to nuclear receptors correlating to biological activity was complemented only recently by studies showing transcriptional activation by retinoids and the ability of retinoids to modulate RAR mRNA expression in melanoma cells. Here we describe a new class of 13-*cis* RA analogs, derivatives of the 6'substituted naphthalene-2-carboxylic acid, and present evidence that several of these analogs can differentially induce hybrid receptors bearing the ligand domain of individual RAR subtypes. Our data indicate that retinoids can be synthesized that may govern specific biological functions by virtue of their RAR subtype specificity.

**L 409** RETINOIC ACID MODULATION OF BONE MORPHOGENETIC PROTEIN (BMP) 2 AND BMP 4 IN OSTEOBLAST: ROLE OF THE TRANSCRIPTION FACTOR EGR-1 AND THE ORPHAN RECEPTOR NUR 77.

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The bone morphogenetic proteins (BMP 2-7) are a set of morphogenetic growth factors, belonging to the TGFβ-superfamily. They most likely play key roles in early differentiation, and in a variety of differentiating tissues, such as bone, cartilage, heart, skin, nervous, and reproductive tissues, as exemplified by their expression patterns. We have been analyzing BMP 2 and BMP 4 expression, as well as expression of the transcription factors EGR-1 and Nur 77 in MG-63 osteosarcoma cells and in rat fetal calvarial (FRC) primary osteoblast cultures using retinoic acid (RA). In both culture systems, RA induces a rapid increase (1/2 hr) and subsequent disappearance (3-6 hrs) of BMP 2 and BMP 4 mRNA, and Egr-1 and Nur 77 mRNA. Treatment of MG-63 cells with cycloheximide (10ug/ml) does not prevent the increase in BMP 4 mRNA after RA treatment, but does prevent the appearance of BMP 2 mRNA. The rapid disappearance of BMP 4 mRNA by 3 hr is also prevented by cycloheximide. Egr-1 and Nur 77 mRNA are also inducible at 1/2 hr by RA in presence or absence of cycloheximide and exhibit prolonged higher steady-state mRNA levels at 3 hrs in the presence of cycloheximide. These results suggest that the ligand activated retinoic acid receptor (RARα) in collaboration with Egr-1 and Nur 77 are directly involved in transcriptional regulation of BMP 2 and BMP 4 genes. Preliminary support for this conclusion comes from the treatment of MG-63 cells with anti-sense Nur 77 oligonucleotides in which BMP 4 expression is inhibited and BMP 2 expression is enhanced at 1/2 hr after RA treatment. The 5' flanking region of the mouse BMP 2 gene has been isolated and will be used to test our hypothesis by co-transfection of Egr-1 and Nur 77 expression vectors with 5'-flanking BMP 2-CAT constructions into MG-63 cells and co-transfection of RARα expression vectors with BMP 2-CAT constructions into COS cells.

**L 410 ACTIVATION OF THE RAT PROLACTIN DISTAL ENHANCER INVOLVES SYNERGISM BETWEEN PIT-1 AND ESTROGEN RECEPTOR. Holloway, J.M., Yu, V., Scully, K., Ingraham, H.A., & Rosenfeld, M.G.**

University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093-0648. To investigate the transcriptional regulation of the rat prolactin enhancer/promoter gene, transfections were performed in CV-1(Green Monkey Kidney Cells) to determine the individual transcriptional activities of Pit-1 and estrogen receptors. Full activation of the rat prolactin enhancer/promoter involves synergism between Pit-1 and estrogen receptors binding in the distal enhancer region between bp -1600 and -1540 downstream of the transcriptional start site. Gel shift analysis and ABCD binding assays suggest that synergism does not involve binding affinity increases due to interactions between Pit-1 and estrogen receptor. Synergism between these receptors requires DNA binding of both receptors and a minimum of one transcriptional "tau" domain be present in both receptors. Results indicate that while Pit-1 may bind to as many eight sites in the rat prolactin enhancer/promoter, binding to the 1D (distal) Pit-1 binding element in the enhancer is obligatory for overall expression, synergism, and hormone activation by estrogen receptor. Further studies will hopefully suggest a mechanism for the synergism between Pit-1 and estrogen receptor.

**L 411**ISOLATION OF NOVEL ECDYSONE-INDUCIBLE GENES FROM *DROSOPHILA*,

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Ecdysone is a steroid hormone that triggers metamorphosis in *Drosophila*. The genetic response to ecdysone can be visualized directly in the transcription puffing patterns of late larval salivary gland polytene chromosomes. Detailed analysis of these puffing patterns by Michael Ashburner and colleagues led to the formulation of a model wherein ecdysone binds to a receptor protein; the hormone-receptor complex directly induces a set of "early" puffs (genes); the products of these early genes then induce the expression of a set of late puffs (genes) and repress their own expression. Thus, genes that respond directly to ecdysone are of particular interest as they will include key regulatory products which coordinate the genetic hierarchy that initiates metamorphosis. Two ecdysone-inducible genes, E74 and E75, have been cloned by chromosomal walking through early puff loci. Each encodes a set of DNA binding proteins with features characteristic of transcription factors and are thus good candidate regulatory genes.

We have extended the analysis of the ecdysone regulatory hierarchy by examining transcription directly in all larval tissues. I have constructed a subtracted cDNA library enriched for ecdysone-inducible genes as an initial step towards assessing the global genetic response to ecdysone. Individual clones from this library are tested by slot blot hybridization to RNA isolated from tissues either treated or not with ecdysone to determine if their cognate genes respond directly to the hormone. Clones selected in this manner are then tested by Northern blot hybridization; of seven clones thus identified, six represent novel ecdysone-inducible genes, one is identical to *E75*. I have thus far screened less than one percent of the complexity of the subtracted library and anticipate identifying additional clones. Definition of the temporal and tissue-specific profiles of expression of these genes is in progress. Their patterns of expression are consistent with a role in metamorphosis. Hybridization to polytene chromosomes will reveal which clones correspond to puff loci. Using these and other criteria, one or more of these genes will be selected for detailed molecular analysis in an effort to assess how they might be integrated into the ecdysone regulatory hierarchy that is activated at the onset of metamorphosis.

## L 412 CLONING OF AN INSECT PROTEIN BELONGING TO THE STEROID AND THYROID HORMONE RECEPTOR SUPERFAMILY

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We have cloned the cDNA sequence coding for a Chironomus tentans protein (536 amino acids) which exhibits a good amino acid sequence homology to various members of the steroid and thyroid hormone receptor superfamily. This homology holds true not only for the zinc finger-containing DNA-binding domain but also the ligand binding domain, and particularly for the highly conserved regions within that domain. The Chironomus protein shows a far better sequence homology to the Drosophila ecdysone receptor (M. Koelle and D. S. Hogness, personal communication) than to E75A, svp, 2C and tailless, other members of the Drosophila nuclear receptor superfamily. Therefore, we speculate that the cloned sequence codes for a Chironomus ecdysone receptor.
On Northern blots cDNA probes of the cloned gene hybridize to an approximately 4.2 kb polyadenylated RNA. Its expression seems to be developmentally regulated and correlates to the changes of the ecdysone titer.
The gene is located on chromosome 2, region 17C, as determined by in situ hybridization to salivary gland polytene chromosomes.

## L 413 THE CHICKEN AVIDIN GENE FAMILY; STRUCTURAL COMPARISON OF THE GENES AND THEIR PROTEIN PRODUCTS

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The chicken avidin gene family provides a versatile system to study regulation of gene expression. Avidin is induced in the oviduct by progesterone and in most of the tissues due to inflammation. A family of avidin related genes (avr1-avr5) was found from two genomic clones. The amino acid sequences derived from the four putative exons of the avrs have an identity of 70-80% with the egg-white avidin. Since traditional cloning of the avidin gene (i.e. the gene encoding the egg-white avidin) was unsuccessful, it was amplified from the chromosomal DNA using PCR. The exon-intron structure of the amplified region was similar to that of the avrs. Isolation of the rest of the avidin gene and its 5'-flanking region as well as that of the avr3 is carried out by PCR methods. The amino acids important for biotin-binding in avidin are also conserved in the predicted AVR prcteins. Our recent results indicated that at least the avr3 gene is transcribed at a low level (see Kunnas et al.). The 3D-coordinates of the egg-white avidin were kindly provided us by Prof. W. Hendricksson (Univ. Colombia, NY). A 3D-model of the predicted AVR3 protein is used to study whether it had a biotin-binding activity similar to that of avidin. Interesting sequence homologies to known gene regulatory elements (e.g. GRE/PRE, HSE) have been found at the 5'-flanking region of the avr4 including adjacent AP-1 and APRE (acute phase response element). Transfection studies have not shown a significant induction of the marker gene by progesterone or heat-shock.

## L 414 ESTROGEN RECEPTOR MEDIATED REGULATION OF GONADOTROPIN RELEASING HORMONE (GnRH) PROMOTER ACTIVITY IN HYPOTHALAMIC CELLS

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Gonadal sex steroids may act directly or indirectly to regulate GnRH gene expression. To determine whether the rat gonadotropin releasing (rGnRH) gene contains DNA sequences that directly mediate estrogen (E) responsiveness, a series of luciferase (LUC) expression vectors containing 5'-flanking DNA and including 116 base pairs of the first exon were transiently transfected into an immortalized mouse hypothalamic neuronal cell line, GT1-7 (provided by P. Mellon). Initial experiments determined there were no effects of E on the endogenous mouse GnRH mRNA levels or the 5'-deletional constructs of the rGnRH promoter, supporting that the GT1-7 cells are deficient in estrogen receptor (ER). However, cotransfection of an expression vector containing the complete coding region of the human ER created an estrogen responsive cell line in which to examine E regulation. Luciferase activity was measured after 16 hrs of E ($5 \times 10^{-8}$M estradiol benzoate) treatment. Data were normalized to the activity of an internal SV40 ß-galactosidase control. Basal luciferase activity directed by 424 bp of rGnRH upstream flanking DNA was inhibited by 40-60%, whereas a RSV promoter construct was unaffected. The extent of the inhibition was dependent on the amount of ER plasmid cotransfected (0.5-5.0 $\mu$g). In addition, cotransfection of the control vector lacking ER sequences failed to regulate GnRH promoter activity. Deletion of sequences from -424 to -171 resulted in a loss of this inhibitory effect of estrogen. These studies suggest that the region -424 to -171 upstream of the transcriptional initiation site is involved in the negative regulation of the rGnRH gene by estrogen in hypothalamic cells. Mapping of this region and functional studies are underway to determine the mechanism of these observations.

## L 415 RETINOID X RECEPTOR/COUP-TF INTERACTIONS MODULATE THE CELLULAR RETINOID RESPONSE.

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Our laboratory has recently shown that minimal hormone response elements for the vitamin D3 (VDR), thyroid hormone (TR), and retinoic acid receptors (RAR) are composed of two direct AGGTCA repeats (DRs) with specificity determined through the number of nucleotides separating the two half-sites. Accordingly, spacers of 3, 4, and 5 nucleotides were shown to confer responsiveness to the VDR, TR, and RAR, respectively. A prediction of this "3-4-5 rule" is that the remaining spacing options might serve as targets for other nuclear receptors. In investigating this possibility, we found that both the retinoid X receptor (RXR) and COUP-TF, a member of a group of "orphan" receptors which lack identified ligands, bind preferentially to a DR-1 element in vitro. In vivo studies reveal that RXR can activate through a COUP-TF response element and that COUP-TF is a specific and potent repressor of RXR-mediated transactivation through DR-1. Recombinant RXR and COUP-TF were shown to form a heterodimer on DR-1. Thus, our results support a general proposal in which half-site spacing preferences can be exploited to elucidate the complex and interactive regulatory networks involving both the known and orphan receptors.

**L 416** REGULATION OF THE RARβ-PROMOTER: A ROLE FOR E1A IN RA-INDUCED RARβ EXPRESSION, Frank A. E. Kruyt, Alberta J. M. Walhout, Gert Folkers and Paul T. van der Saag
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Retinoic Acid (RA) influences many biological processes such as cell growth, differentiation and development. It exerts its effects via specific sets of nuclear receptors (RA-receptors; RARs) and retinoid X receptors (RXRs), which belong to the multigene family of steroid/thyroid hormone receptors. Three types of RARs have been identified (α, β and γ) which can modulate gene expression via RA response elements (RAREs). Such an element has been identified in the RARβ promoter, whose expression is strongly induced by RA.

We have studied the RA-induced activation of the RARβ promoter in P19 EC cells by CAT assays and identified more RA-confering sequences between nucleotides -180 to -63. This promoter fragment contains two elements closely resembling the consenses sequences of the cyclic AMP- (CRE) and TPA response element (TRE). To test which element(s) mediate(s) activation by RA, we deleted either one of the elements or both. Mutation of the CRE was found to abolish the stimulatory effect of RA. In gel-retardation experiments using nuclear extracts of untreated and RA-treated P19 EC cells, only the CRE was able to specifically bind nuclear factors. Furthermore, we studied RA-induced RARβ expression in a wide variety of non EC cell lines. In the majority of the cells tested, no RA-induced RARβ expression could be detected, with the exception of 293 cells, which express the oncogenic E1A proteins. Considering the postulated existence of an E1A-like activity in EC cells we investigated whether E1A is able to influence RA-induced transcription of the RARβ gene. A comparative analysis between END-2 cells (a differentiated clone of P19 EC cells) and with E1A expression vectors stably transfected derivatives revealed that E1A is able to facilitate RA-induced RARβ activation. Transient (co)transfection assays in END-2 cells showed that E1A exerts its effect in a more direct fashion. These data suggest a role for E1A in RA-induced RARβ expression.

**L 417 RETINOIC ACID RECEPTORS FUNCTION AS MASTER REGULATORS**

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During recent years it became well accepted that retinoid actions on gene regulation and the resulting pleiotropic effects on fetal development, cellular differentiation, and growth are mediated through the activation of nuclear receptors. From each of the three receptor subtypes identified (RARα, β, and γ) multiple isoforms exist which differ in their N-terminal region while otherwise (i.e. DNA and ligand binding domain) identical. Here, we report that expression of RARγ2, one of the two major RARγ isoforms, is regulated by its own promoter which contains a retinoic acid response element (γ2RARE). The magnitude of the γ2RARE RA response is lower than that obtained with the β2RARE and has to be settled closer to that described for the RAREs in the ADH3, CRBPI, and laminin B1 promoters. A change of a G into C in the second half-site transforms the γ2RARE into a strong RARE. Multiple Sp1 binding sites (GC-boxes) adjacent to the γ2RARE contribute significantly to the total promoter activity. Although activating transcription from the palindromic thyroid hormone response element $TRE_{pal}$, RARγ1 is repressing induction of the RARγ2 promoter. Recently, we have described a similar repression by RARγ1 for the autoregulated RARβ2 promoter. This repression is most likely due to strong binding but poor activation from these response elements by RARγ1 and might in parts be responsible for the mutually exclusive expression pattern of RARγ1, RARβ2 and RARγ2.

**L 418** THREE RETINOID X RECEPTORS GENES ARE DIFFERENTIALLY EXPRESSED DURING EMBRYOGENESIS AND IN THE ADULT ANIMAL, David J. Mangelsdorf, Uwe Borgmeyer, Estelita S. Ong, Joel Zhou, Richard Heyman, and Ronald M. Evans, Gene Expression Laboratory, The Salk Institute, San Diego, CA 92186-5800.

The retinoid X receptors (RXRs) are a subfamily of the nuclear hormone receptors which respond specifically to retinoic acid, but which differ substantially from the previously described retinoic acid receptors (RARs) in both structure and ligand specificity. Three RXR genes have now been identified and are designated RXRα, β, and γ. All three RXRs are located at distinct chromosomal loci. Northern blot and in situ hybridization techniques indicate that all three RXRs have patterns of expression which are unique between themselves and the RARs. Detailed analyses reveal RXRβ to be ubiquitously expressed during development and in the adult. RXRα displays high level expression during organogenesis and in the adult skin and metabolic organs. RXRγ shows the most restricted expression, being limited to the embryonic CNS and the adult heart, brain, liver, and muscle. The distribution of RXR mRNA along with the known RXR target genes suggest these receptors play a role in metabolism and in the developing nervous system.

**L 419** Cellular Distribution Of Alpha and Beta Thyroid Hormone Receptor mRNA During Metamorphosis of *Xenopus laevis*, Jeffrey N. Masters*¶ and Michael S. Beattie¶§, * Ohio State Biotechnology Center, ¶ Department of Cell Biology, Neurobiology and Anatomy, § Division of Neurosurgery, The Ohio State University, Columbus, OH 43210

The metamorphosis from larval to juvenile form in anuran amphibians encompasses a radical transformation from swimming to quadrupedal locomotion and includes the addition and innervation of limb musculature, additions and deletions of central neuronal elements and connections, and tail resorption. This process is dependent on thyroid hormones (TH) which rise dramatically during metamorphic climax and fall to very low levels immediately after metamorphosis. The alpha and beta forms of the *Xenopus* thyroid hormone receptor (THR) mRNAs have been recently shown to be independently regulated during development. The αTHR is present relatively early in development while βTHR expression correlates with metamorphosis. Thus we hypothesize that the αTHR may mediate generative processes while the βTHR mediates the degenerative components during Xenopus development. Preliminary *in situ* hybridization with probes specific for the αTHR and βTHR mRNA (provided by D. Brown) at stage 52 showed no detectable expression of the β receptor while the α receptor was concentrated in areas undergoing rapid cell growth; these areas include the hindlimb buds, ventricular zone, the spinal cord and dorsal root ganglia. The absence of the βTHR at this developmental stage is consistent with published reports using total RNA blots. We are currently analyzing frogs which span metamorphic climax to determine the precise localization of the two forms of the THR.

**L 420** EVIDENCE THAT THE MURINE PPAR MEDIATES THE INDUCTION OF A CYTOCHROME P450 BY PEROXISOME PROLIFERATORS, A. Scott Muerhoff, Keith J. Griffin, and Eric F. Johnson, Division of Biochemistry, Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA 92037

Clofibric acid, a peroxisome proliferator, induces the expression of mRNAs encoding the rabbit cytochrome P450, CYP4A6, ≈10-fold in liver and ≈5-fold in kidney *in vivo*. The role of the peroxisome proliferator activated receptor (PPAR) in the induction of CYP4A6 was investigated using luciferase reporter gene constructs driven by the promoter and 5' flanking sequences derived from the CYP4A6 gene. Expression plasmids harboring PPAR cDNAs that encoded either the wild type (PPAR-wt) or a Glu->Gly mutant (PPAR-G) and that were obtained from mouse liver mRNAs using specific primers and PCR amplification were used in cotransfection experiments. Clofibrate does not significantly alter the expression of luciferase in COS-1, HepG2 or the RK13 rabbit kidney epithelial cell lines transfected with a luciferase reporter construct containing 2.5 kb of CYP4A6 5' flanking sequence. Cotransfection of the PPAR-wt with the CYP4A6/luciferase construct leads to a peroxisome proliferator dependent, ≈10-fold activation of luciferase expression in COS-1 cells. This *trans*-activation is seen in the absence of peroxisome proliferators in either RK13 or HepG2 cells. In contrast, *trans*-activation by PPAR-G is dependent on peroxisome proliferators in all three cell lines. PPAR-G is activated by the peroxisome proliferators: clofibric acid, di(2-ethylhexyl)phthalate, 2,4,5-T and Wy14643, and the $ED_{50}$ values determined in RK13 cells are very similar to those that were reported (Issemann & Green (1990) Nature 347:645) for chimeric receptors constructed from the ligand binding domain of the PPAR and DNA binding domains derived from either the glucocorticoid or estrogen receptors. Deletion analyses indicate that *trans*-activation by the PPARs of luciferase transcription driven by the CYP4A6 promoter is dependent on a short segment of the 5' flanking sequence of the CYP4A6 gene localized to -725 to -663 relative to the cap site. This is the first report to demonstrate a role for members of the steroid/thyroid receptor superfamily in the induction of cytochrome P450 monooxygenases by foreign compounds. Work is in progress to characterize the responsive element for the PPAR in greater detail. (This work is supported by NIH grant HD04445 (EFJ) and a postdoctoral fellowship to ASM from the American Heart Association California Affiliate.)

**L 421** Identification of the thyroid hormone response element in the human skeletal α-actin gene

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The skeletal isoform of α-actin mRNA (normally expressed at low levels in the adult myocardium) has been shown to increase in the adult heart after the imposition of hemodynamic overload/aortic restriction. Furthermore, T3 was recently shown to cause a rapid increase in the amount of skeletal α-actin mRNA in hearts from normal and hypophysectomised animals. This data indicates skeletal α-actin is an ideal marker for the pathophysiological state of the adult heart. We used transient transfection analysis to show that T3 induces the expression of the native skeletal α-actin promoter between nucleotide positions -2000 and +239 linked to the Chloramphenicol Acetyl Transferase(CAT) reporter gene in COS-1 fibroblasts and myogenic C2C12 cells. This T3 [10-100nM] induced transcriptional activation is dependent on the expression of the thyroid hormone receptors (TRs) from transfected α1- and β1- c-*erbA* cDNA expression vectors. Electrophoretic mobility shift assays [EMSA] were used to identify a thyroid hormone response element (TRE) which is located between nucleotide positions -173 and -149 with respect to the start of transcription at +1. This sequence, fits the core sequence binding motif for the thyroid hormone receptor (TR), $G/_A\,GG\,T/_A C\,A/_G$. The skeletal α-actin TRE and defined rodent growth hormone TREs interacted with an identical nuclear factor *in vitro* in muscle cells, that was developmentally regulated during myogenesis. The α-actin TRE was cloned into an enhancerless SV40 promoter, linked to the CAT gene and found to function in a T3-dependent manner[10-100nM). The skeletal α-actin TRE is juxtaposed next to SRF and SpI binding sites, at its 5' and 3' flanks, respectively. In conclusion, these results indicate that T3 induced increases in α-actin mRNA in animals are mediated by a direct transcriptional mechanism which may involve interactions with ubiquitous proteins.

**L 422** EXPRESSION OF v-erbA AND c-erbA IN XENOPUS LAEVIS OOCYTES, Sylvia C.B. Nagl and Lizabeth A. Allison, Department of Zoology, University of Canterbury, Christchurch, New Zealand

v-erbA, one of the two oncogenes of the avian erythroblastosis virus (AEV), constitutes a highly mutated version of its cellular homologue, c-erbA, which encodes the thyroid hormone receptor. v-erbA cooperates with v-erbB, the second oncogene of AEV, to induce transformation of erythroblasts in tissue culture and to induce acute erythroleukemia in chicks. The v-erbA protein acts as a dominant repressor in animal cells, blocking activation of gene expression by thyroid hormone, whereas, in yeast the v-erbA gene product acts as a hormone-regulated activator. Since these findings indicate that the dominant repressor functions of v-erbA are not universal and depend on cellular context, studies in other cell types acquire additional importance.

We have shown that Xenopus laevis oocytes are a suitable expression system for the study of regulatory interactions between the v-erbA gene product and the thyroid hormone receptor. Transcriptional activity was assessed by the ability of these proteins to regulate expression of a thyroid hormone-responsive CAT reporter gene (pBL-CAT2-TREp). CAT protein expression was increased up to 20-fold in oocytes that had been injected with 5 ng of a thyroid hormone receptor expression plasmid, RS-rTRα, and incubated in the presence of 100 nM thyroid hormone ($T_3$). In contrast, injection of 5 ng of a v-erbA expression plasmid, RS-v-erbA, resulted in CAT protein expression below control levels in the presence or absence of $T_3$. The presence of v-erbA protein in microinjected oocytes will be confirmed by Western blotting. Results of coinjection experiments (RS-rTRα plus RS-v-erbA) will be presented.

**L 423** DELIMITING THE TRANSCRIPTIONAL ACTIVATION DOMAIN OF NGFI-B, Ragnhild E. Paulsen, Christine A. Weaver and Jeffrey D. Milbrandt, Department of Pathology, Washington University, St. Louis, MO 63110.

Steroid hormone receptors are structurally organized in different functional domains, of which the amino-terminal domain is the least well characterized. It has been ascribed a general role in transactivation, but the molecular mechanisms of this function are still unknown. We have therefore undertaken a study of NGFI-B, the only inducible member of this superfamily, to define the functional subdomain in the amino-terminus that is responsible for transactivation.

A large deletion (170 amino acids) of the amino-terminus of NGFI-B, which removed 64 % of the residues upstream of the zinc fingers gave a transcriptionally inactive protein (less than 2 % of wildtype activity) when the construct was inserted into a mammalian expression vector (cmv-neo) and assayed in a cotransfection assay in CV1-cells using 8 copies of a NGFI-B response element upstream of a luciferase reporter gene. This protein localized to the nucleus as shown by immunohistochemistry and bound DNA with high affinity as demonstrated by gel retardation assay, arguing that the loss of activity was due to the loss of an activation domain in the deleted region. A series of deletion mutants were made to further delimit the residues responsible for transcriptional activation, and revealed a 45-amino acid sequence rich in serine and threonine residues as the subdomain most likely to be responsible for transactivation, raising the possibility that phosphorylation may be involved in the transactivation prosess. This is especially interesting because NGFI-B is differentially phosphorylated depending on which extracellular stimulus has induced its synthesis.

**L 424** INHIBITION OF AP-1 ACTIVITY BY c-erbA AND RETINOIC ACID RECEPTOR, BUT NOT v-erbA: IDENTIFICATION OF CRITICAL REGIONS, Fahri Saatcioglu, Tiliang Deng, Martin Zenke*, and Michael Karin, Department of Pharmacology, School of Medicine, University of California, San Diego, La Jolla, CA 92093; and *Institut of Molecular Pathology, Dr. Bohr Gasse 7, A-1030 Vienna, Austria.

It has recently been shown that glucocorticoid and retinoic acid receptors can inhibit the expression of the collagenase and other AP-1 dependent genes in a ligand-dependent manner through an interaction which renders both the receptor and AP-1 unable to bind to DNA. It is thought that this event may involve other members of the steroid/thyroid hormone receptor superfamily. We have begun to investigate the details of this phenomenon and chose the thyroid hormone receptor (c-erbA) as our model system. We found that c-erbA can also interfere with AP-1 activity in vivo. While c-erbA efficiently blocked AP-1 activity in HeLa and CV1 cells, the viral homologue v-erbA had no effect. Chimeras between c-erbA and v-erbA were used to locate the region most important in the interaction of c-erbA and AP-1 to the carboxy terminus of the receptor. When a similar region at the analogous position in the retinoic acid receptor alpha (RARα) was deleted, it also lost its ability to interfere with AP-1 activity. This region was also found to be important for the ability of both c-erbA and RARα to transactivate from their response elements. We will present mutagenesis as well as transformation data documenting the importance of this region.

**L 425** A CYCLOHEXIMIDE-SENSITIVE PROTEIN COMPLEX MEDIATES STEROIDAL INDUCTION OF THE OVALBUMIN GENE, Lora A. Schweers and Michel M. Sanders, Department of Biochemistry, University of Minnesota, Minneapolis, MN 55455

A steroid-dependent regulatory element (SDRE) between -900 and -759 in the ovalbumin gene is required for regulation by four classes of steroid hormones. To further define sequences required for induction by steroids, 5' deletion mutants were made in the SDRE by exonuclease III digestion of a plasmid containing 900 bp of ovalbumin 5' flanking sequence fused to the chloramphenicol acetyltransferase (CAT) gene. These mutants were tested for hormone responsiveness by transfection into steroid-responsive primary oviduct cell cultures and expression levels measured by CAT assay. Deletion of 8 bp to -892 totally abolished induction by each of the four steroids. DNase I footprinting with oviduct nuclear extracts defined two footprints from -900 to -860 and from -848 to -801. Gel shift assays of the footprinted sequences and oviduct nuclear proteins produced four DNA/protein complexes, all of which were eliminated by competition with a fragment containing sequences from -848 to -801. Treatment of these complexes with deoxycholate abolished binding of the highest molecular weight complex (complex I), suggesting that this complex contains more that one associated protein. These and other results imply that protein binding to the two sites is cooperative. To determine whether these complexes are steroid-dependent, footprinting was performed with nuclear extracts from estrogen-treated and -withdrawn chicks. Preliminary results indicate that both footprints are steroid-dependent. Because concomitant protein synthesis is required for transcription of the ovalbumin gene, steroids may induce an essential transcription factor. Gel shift assays with cycloheximide treated oviduct nuclear extracts indicated that complex I was abolished by cessation of protein synthesis. These data suggest that a cycloheximide-sensitive complex of steroid-dependent proteins binds to the 5' portion of the SDRE to mediate steroidal induction.

**L 426** THE ESTROGENIC AND ANTIESTROGENIC PROPERTIES OF TAMOXIFEN IN $GH_4C_1$ PITUITARY TUMOR CELLS ARE GENE SPECIFIC, James D. Shull, Faith Beams, Tonia Baldwin, Cindy A. Gilchrist, and Marjorie J. Hrbek, Eppley Institute for Research in Cancer, University of Nebraska Medical Center, Omaha, NE, 68198-6805

We have examined the effects of the antiestrogen tamoxifen (TAM) and the estrogen 17β-estradiol (E2) on several estrogen regulated responses in $GH_4C_1$ pituitary tumor cells. Following five days of treatment with either TAM (1.0 μM) or E2 (1.0 nM), the level of prolactin (PRL) mRNA was markedly increased. In contrast, only E2 was able to stimulate cell proliferation as evidenced by the effects of TAM and E2 on thymidine incorporation by whole cells, cellular DNA, or deoxythymidine triphosphate (dTTP) incorporation by nuclei isolated from treated cells. The abilities of TAM and E2 to induce progesterone receptor (PR) and PR mRNA were also examined. TAM alone was unable to induce PR, whereas E2 was very effective in inducing PR. When added in combination with E2, TAM acted as a classical antiestrogen, partially blocking the induction of PR by E2. To determine whether the inabilities of TAM to stimulate cell proliferation and induce PR synthesis were a function of TAM concentration, dose response experiments were performed. TAM at concentrations ranging from $10^{-8}$ to $10^{-6}$ M was effective in inducing PRL mRNA, but at none of the tested concentrations was TAM effective in stimulating cell proliferation or inducing PR synthesis. These data illustrate that the estrogenic properties of TAM in $GH_4C_1$ cells are gene specific and suggest that the structural features of TAM are not the sole determinants of whether this ligand functions as an estrogen agonist or antagonist. Two models are presented in an attempt to explain these data at the molecular level. Supported by grants from the NIH, ACS, and Nebraska Department of Health.

**L 427** TRANS-ACTIVATION STUDIES OF THE CHICKEN THYROID HORMONE RECEPTORS; TRα-p46 and p40 and TRβ0 and β2

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Vertebrates produce various forms of T3 receptors (TRs) from two related genes, α and β, which are differentially expressed in a tissue-specific manner. In the chicken, the variant TRs differ primarily in the N-termini. The cTRα mRNA contains two different translational start codons which give rise to 2 proteins that differ by 36 amino acids in the N-termini. The larger protein, p46, can be phosphorylated on two serine residues, whereas the shorter form, p40, lacks the domain encoding these sites. The cTRβ gene produces 2 mRNAs that encode receptor with distinct N-termini due to differential splicing. One form, cTRβ0, has a primary structure virtually identical to that of the cTRα-p40 receptor, whereas the N-terminal domain of cTRβ2 shows homology to rTRβ2.

Expression of cTRα mRNA is ubiquitous whereas those of TRβ is restricted and is developmentallly regulated. cTRβ2 mRNA is only expressed in the early developing chick retina. The differences in localisation of TRα and β gene expression suggest cell- and stage-specific functions for TRs in chick development, and the existence of alternative forms of both TRα and β suggests that they have different trans-activating and tissue-specific properties. Our present data show that cTRβ2 is a stronger trans-activator than cTRβ0 when acting on $T_3$RE palindrome. Data will be presented describing the trans-activating properties of the four different cTRs, and correlated to the orientation and spacing of the half-sites (AGGTCA) in the thyroid hormone response element.

**L 428** A *CAENORHABDITIS ELEGANS* MEMBER OF THE NUCLEAR HORMONE RECEPTOR GENE FAMILY IS EXPRESSED DURING EARLY EMBRYOGENESIS, Ann E. Sluder and Gary Ruvkun, Dept. of Molecular Biology, Massachusetts General Hospital, Boston, MA 02114

As a means of identifying candidate nuclear hormone receptors in the nematode *C. elegans* we probed genomic libraries with degenerate oligonucleotides encoding a highly conserved region within the "zinc finger" motif of known receptors. The predicted gene product of one clone isolated from this screen, *crf-2*, is 50% identical to the mouse thyroid hormone receptor within the zinc fingers, but exhibits no significant similarity to known receptors outside of the DNA binding domain.

To obtain clues to the function of *crf-2* we have examined its expression pattern. *In situ* hybridization experiments (D. Greenstein, pers. comm.) and developmental Northern blot analysis detected *crf-2* transcript in early to midstage embryos and in gravid hermaphrodites. Analysis of mutants defective in gonad or germline development has shown that *crf-2* is transcribed in the maternal germline. Whether or not *crf-2* is also transcribed zygotically remains to be determined.

To define the *crf-2* expression pattern further, a *crf-2/lacZ* fusion gene has been constructed and used to transform worms. Immunostaining of transgenic animals detects fusion protein in particular embryonic cells from just before gastrulation through mid-embryogenesis. Efforts to generate antibodies against the *crf-2* protein are in progress in order to compare the expression pattern of the native protein with that of the fusion.

The expression studies suggest that a mutation in *crf-2* might have a zygotic or maternal-effect lethal phenotype. The physical map location of *crf-2* places it in a genetically well-defined region of Chromosome I. The *crf-2* gene does not appear to correspond to any of the existing mutations in the region, and we are currently screening for additional maternal-effect and zygotic lethal mutations.

**L 429** STRUCTURAL AND FUNCTIONAL ANALYSIS OF A RETINOIC ACID REGULATED PROMOTER. Joseph P. Stein, Terrie A. Burrell, Janice S. Gilsdorf and Mark J. Hozza, Department of Pharmacology, SUNY Health Science Center, Syracuse, NY 13210

Retinoic acid increases the relative transcription rate of the tissue transglutaminase (TGase) gene about 10-fold in mouse peritoneal macrophages, suggesting the probable presence of upstream regulatory sequences that mediate retinoic acid regulation of the transglutaminase gene. However, our attempts to identify a retinoic acid response element have been hampered by the absence of a full-length TGase cDNA. Our longest TGase cDNA clone, 3,468 bp, contained the complete amino acid coding sequence but only 29 bp of 5' untranslated sequence. In order to obtain the sequence of the entire 5' untranslated region, and thus identify the transcription start (CAP) site, we adapted a procedure known as Rapid Amplification of cDNA Ends. A TGase-specific oligonucleotide primer and macrophage mRNA were used to generate a tailed cDNA copy of the 5' end of the TGase mRNA. Using synthetic oligonucleotides and PCR, these cDNA copies were amplified and cloned into a plasmid vector. Sequence analysis of 3 such clones identified the precise 5' end of the TGase mRNA. This sequence was used to identify the CAP site of the TGase gene in a clone isolated from an adult Balb/c mouse genomic DNA library. Four fragments that together encode the 5' portion of the TGase gene, including exons 1 and 2 and 3 Kb of upstream flanking sequence, have been subcloned into plasmid vectors and partially sequenced. Proximal to the TGase CAP site are several sequence elements typical of eucaryotic promoters: a CAAT box, a TATA box, and an extensive "GC-rich" region. We have cloned several transglutaminase promoter constructs in a promoterless plasmid vector containing a "G-free" cassette, which permits a functional analysis of putative promoter sequences using the technique of *in vitro* transcription. In addition, overlapping 200 bp fragments that span 2 Kb of the proximal 5' flanking sequence have been synthesized for use in band shift assays with retinoic acid receptor extracts. These experiments should identify a true retinoic acid response element, and further our understanding of the mechanism by which retinoic acid regulates specific gene expression.

**L 430** EXPRESSION DOMAINS OF RETINOIC ACID RESPONSIVE GENES IN VIVO

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We have created transgenic mice carrying retinoic acid responsive gene promoters driving b-galactosidase expression. The first contains the 8 kb promoter of the mouse RARβ2 gene, a known primary target for retinoic acid transactivation which contains a well characterized RA response element (RARE). The second contains this same response element in the heterologous context of a truncated basal promoter. Comparison of the embryonic expression pattern of these transgenes defines domains (and tissues) of the embryo which are likely to contain retinoic acid, domains in which the RARβ2 promoter is not expressed despite the presence of RA, and domains permissible for RARβ2 expression. This implicates cis-acting elements in the RARβ2 promoter (in addition to the RARE) which regulate the spatial expression of this gene. Tissue culture transfection assays have been established which exhibit some of these regulatory processes, and which may facilitate the molecular characterization of these sequences.

**L 431** Sex-Dependent Expression and Clofibrate Inducibility of Cytochrome P450 4A Fatty Acid Omega-Hydroxylases. Male Specificity of Liver and Kidney CYP4A2 mRNA and Tissue-specific Regulation by Growth Hormone and Testosterone. Scott S. Sundseth and David J. Waxman*, Wellcome Research Labs, Burroughs-Wellcome Co., Research Triangle Park, NC 27709 and *Department of Biological Chemistry & Molecular Pharmacology and Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115

The induction of liver cytochrome P450 4A-catalyzed fatty acid omega-hydroxylase activity by clofibrate and other peroxisomal proliferators has been proposed to be causally linked to the ensuing proliferation of peroxisomes in rat liver. The clofibrate induction of both P450 4A and peroxisomal enzymes proceeds via transcriptional mechanisms, and is putatively mediated by the peroxisome proliferator-activated receptor, a novel steroid receptor gene superfamily member. Since female rats are less responsive than males to peroxisome proliferation induced by clofibrate, the influence of gender and hormonal status on the basal and clofibrate-inducible expression of the 4A P450s was examined. Northern blot analysis using gene-specific oligonucleotide probes revealed that in the liver, P450 4A1 and 4A3 mRNAs are induced to a much greater extent in male as compared to female rats following clofibrate treatment, while P450 4A2 mRNA is altogether absent from female rat liver. Male-specific expression of P450 4A2 mRNA was also observed in the kidney. Western Blot analysis indicated that a similar sex-dependence characterizes both the basal expression and the clofibrate inducibility of the corresponding P450 4A proteins. This suggests that the lower responsiveness of female rats to clofibrate-induced peroxisome proliferation may refelect the lower inducibility of the P450 fatty acid hydroxylase enzymes in this sex. Investigation of the contribution of pituitary-dependent hormones to the male-specific expression of 4A2 revealed that this P450 mRNA is fully suppressed in liver following exposure to the continuous plasma growth hormone profile that characterizes female rats. In contrast, kidney 4A2 expression, although male-specific, was not suppressed by continuous growth hormone treatment, but was positively regulated by testosterone. The male-specific expression of liver and kidney P450 4A2 is thus under the control of distinct pituitary-dependent hormones acting in a tissue-specific manner.

L 432 ESTROGEN OPPOSES 1,25(OH)$_2$D$_3$-INDUCED UPREGULATION OF $\alpha_v$ INTEGRIN SUBUNIT mRNA BY OSTEOCLAST PRECURSORS, Steven L. Teitelbaum, Jose I. Alvarez, Jean C. Chappel, F. Patrick Ross, Keith A. Hruska, and Meetha M. Medora, *Departments of Medicine and Pathology*, Jewish Hospital at Washington University Medical Center, St. Louis, MO 63110.

We have shown previously that the integrin, $\alpha_v\beta_3$ is responsible for osteoclast adhesion to the bone surface through its recognition of osteopontin/bone sialoprotein. Thus, modulation of this attachment integrin is potentially a pivotal event in controlling the resorptive process. In the present experiments, we explored expression of the $\alpha_v$ subunit, whose rate of synthesis regulates $\alpha_v\beta_3$ expression. We first examined the effect of 1,25(OH)$_2$D$_3$, an agent promoting osteoclast differentiation, on $\alpha_v$ mRNA levels in chicken osteoclast precursors. A labeled cDNA containing the 3' end of the chicken $\alpha_v$ message was used to probe Northern blots of osteoclast precursor RNA. Up to a 30-fold increase in $\alpha_v$ mRNA expression was observed with 1,25(OH)$_2$D$_3$ ($10^{-8}$M) in cells cultured for 5 days. The effect of 1,25(OH)$_2$D$_3$ was visible by 24 hours of treatment and maximal at 4-5 day. The induction was dose-dependent with a threshold at $10^{-11}$M and maximum at $10^{-8}$M. Upregulation was also seen with 25(OH)$_2$D$_3$ ($10^{-7}$M, $10^{-6}$M) and 22-oxa-1,25 dihydroxyvitamin D$_3$ ($10^{-10}$M - $10^{-8}$M). The 1,25(OH)$_2$D$_3$-induced increase in $\alpha_v$ mRNA levels is paralleled by enhanced synthesis and surface expression of $\alpha_v\beta_3$. Most importantly, stimulation of $\alpha_v$ mRNA by 1,25(OH)$_2$D$_3$ was inhibited by $\beta$-estradiol in a dose-dependent manner. These results indicate that upregulation of $\alpha_v$ mRNA is a component of vitamin D-induced osteoclast differentiation. Furthermore, our findings suggest that the inhibitory effects of estrogen on bone resorption may be mediated by decreasing $\alpha_v$-bearing integrin expression by osteoclasts, which in turn would impact on the ability of the cells to bind to and ultimately resorb bone.

L 433 CHARACTERIZATION OF THE PROSTATE-SPECIFIC ANTIGEN PROMOTER AND THE HUMAN GLANDULAR KALLIKREIN-1 PROMOTER, Jan Trapman, Peter H.J. Riegman, Remko J. Vlietstra, Lex Suurmeijer, Hetty A.G.M. van der Korput and Kitty B.J.M. Cleutjens, *Department of Pathology*, Erasmus University, Rotterdam, The Netherlands.

Prostate-specific Antigen (PSA) and human Glandular Kallikrein-1 (hGK-1) are kallikrein-like serine proteases, which are exclusively synthesized in epithelial cells of the human prostate. The PSA(APS) and hGK-1(KLK-2) gene form together with the KLK-1 (tissue kallikrein) gene, which is not expressed in prostate, a 60 kb locus on human chromosome 19. Transcription from the PSA and hGK-1 gene is androgen-regulated in LNCaP cells. Structural analysis of the promoter region of the PSA gene revealed the presence of at least one motif (AGAACAgcaAGTGCT; at -170 to -156) resembling the HRE (hormone responsive element) consensus sequence, recognized by the glucocorticoid and the progesteron receptor. A closely related sequence (GGAACAgcaAGTGCT) was found in the hGK-1 promoter at a similar position. To find out whether these motifs could serve as functional AREs (androgen responsive element), PA-CAT and hGK-1-CAT reporter gene constructs and the androgen receptor expression plasmid pARo were co-transfected in COS cells. Results obtained from deletion mapping and point mutational analysis of the promoters showed that the above mentioned sequences are indispensable for androgen-regulation. Importantly, androgen-stimulated transcription could be enhanced by upstream sequences, indicating that the androgen receptor acts in concert with other transcription factors for maximal activity. Similar data were obtained when a glucocorticoid receptor expression plasmid was used in these experiments.

L 434 DIFFERENTIATION "IN VIVO" OF BROWN ADIPOSE TISSUE: ONTOGENY OF NUCLEAR THYROID HORMONE RECEPTORS AND EXPRESSION OF c-ERB-A PROTO-ONCOGENES IN RAT FETAL BROWN FAT. F.Villarroya, A.Tuca, M.Giralt, T.Mampel, O.Viñas, R.Iglesias. Dept. of Biochemistry and Molecular Biology. University of Barcelona.Spain.

Differentiation "in vivo" of rat brown adipose tissue occurs in the fetal life. Between days 18 and 20 of rat fetal development there is a specific induction of the uncoupling protein gene expression, the marker of differentiated adipocytes. Peak values of thyroxine 5'-deiodinase activity, similar to those found in maximally thermogenic activated adult brown fat, are detected in this moment of fetal brown adipose tissue development. Nuclear L-$T_3$ levels in fetal brown fat increase four fold between the day 18 and 19 of the fetal life. Thus, nuclear L-$T_3$ content in 20-day old fetal brown fat is higher than in any other fetal tissue. Nuclear thyroid hormone receptors, assessed as nuclear L-$T_3$ binding sites determined by Scatchard analysis, rise two fold between the days 18 and 20 of fetal life. The number of nuclear L-$T_3$ binding sites in 20-day old fetal brown fat is higher than in any other fetal tissue studied and similar to that in adult brown fat. The expression of $\alpha$ and $\beta_1$ c-erbA genes in developing brown adipose tissue indicates that, as in adult brown fat, 6.5 kb $\beta_1$ c-erbA, 2.6 kb $\alpha_2$ c-erbA and 5.5 kb $\alpha_1$ c-erbA transcripts are present in the fetal tissue. They are detected as early as in the day 18 of fetal development and their relative abundance parallel the mentioned changes in nuclear $T_3$ binding sites. The pattern of development of thyroid hormone status and receptor equipment in brown adipose tissue is clearly different from the other rat tissues (liver, brain) as it shows a neatly earlier maturation. It may be related to the need of a fully differentiated brown fat function just after birth when a high thermogenic activity is required and supports the hypothesis that thyroid hormones may play an important role in brown fat differentiation (uncoupling protein gene induction) in the fetal life.

L 435 HORMONAL REGULATION OF ENZYMES INVOLVED IN PEROXISOME PROLIFERATION. Stephanie J. Webb & Russell A. Prough, Department of Biochemistry, University of Louisville School of Medicine, Louisville, KY 40292

Dehydroepiandrosterone (DHEA) is a C19-steroid with no known direct endocrine function. Administration of DHEA has been shown to prevent a number of pathophysiological conditions, including cancer, obesity, diabetes, and atherosclerosis in rodents. DHEA administration to male Sprague-Dawley rats resulted in time- (4 days) and dosage- (100 mg/kg body wt. i.p.) dependent increases of rat hepatic NADPH:cytochrome P450 oxidoreductase and cytochrome P450IVA activity concomitant with peroxisome proliferation. Peroxisome proliferation is characterized by an induction in the peroxisomal $\beta$-oxidation enzymes and the microsomal cytochrome P450IVA isozymes. P450IVA family isozymes hydroxylate medium-chain saturated fatty acids at the $\omega$- and $\omega$-1 positions, and unsaturated fatty acids at multiple positions, forming either hydroxy fatty acids or epoxy fatty acids. The $\omega$-hydroxylated fatty acids probably serve as substrates for the peroxisomal $\beta$-oxidation pathway. Administration of DHEA (either *i.p.* or *p.o.*) induced NADPH:cytochrome P450 oxidoreductase activity, palmitoyl CoA oxidase activity (a marker for peroxisome proliferation), and at least two proteins immunochemically related to P450IVA. Induction of the specific content of hepatic P450IVA and its flavoprotein oxidoreductase was correlated with increased levels of total and poly A$^+$ enriched mRNA specific for these enzymes. Thyroid hormone, 3,5,3'-triiodothyronine ($T_3$), regulates the expression of a number of drug metabolizing enzymes, including NADPH:cytochrome P450 oxidoreductase, as well as enzymes induced by peroxisome proliferators. Administration of $T_3$ (50 $\mu$g/100 g body wt. *i.p.*) induced the activity, protein content, and mRNA level of NADPH:cytochrome P450 oxidoreductase. Coadministration of $T_3$ and DHEA additively induced the expression of the oxidoreductase. In contrast, $T_3$ administration decreased the expression of P450IVA protein and mRNA levels in the absence or presence of DHEA. These data suggest that endogenous $T_3$ may regulate expression of the P450IVA family and possibly peroxisomal $\beta$-oxidation. Supported by U.S.P.H.S. Grants ES04244 and CA43839.

**L 436** CHARACTERIZATION OF THE PITUITARY-SPECIFIC THYROID RECEPTOR ß2 PROMOTER REGION AND ITS EXPRESSION IN THYROTROPES, William M. Wood, Janet M. Dowding, Angela R. Nelson, Virginia D. Sarapura, David F. Gordon and E. Chester Ridgway, Department of Medicine, U.C.H.S.C., Denver, CO 80262.

In rodents there are at least two isoforms of the thyroid hormone ß receptor (TRß1 and TRß2) which differ at their amino termini. Thus both isoforms contain identical DNA and ligand binding domains but diverge in sequence just upstream of the DNA binding region as a result of alternative splicing of amino terminal exons. We have previously shown that the mouse TRß2 protein expressed in thyrotrope cells initiates translation from an AUG codon 39 residues downstream from the AUG utilized in rat GH3 cells. Since the expression of TRß2 is restricted to the pituitary gland, it is believed that transcription of the TRß1 and TRß2 mRNAs arises from two different promoters. In order to isolate the DNA locus corresponding to the putative pituitary-specific promoter, we screened a mouse genomic library with a cDNA probe unique to the amino terminal region of TRß2. Two independent positive recombinants were isolated. One of these which included an insert 16 kb in size contained the TRß2 amino terminal cDNA sequence entirely within one exon. Complementary DNA probes corresponding to the common C-terminal region or to the divergent amino terminal TRß1 exon(s) did not hybridize with either of the TRß2 positive clones suggesting that the TRß2 genomic locus must be relatively distant from the other exons of the TRß gene. Approximately 3 kb of DNA upstream of the AUG codon, the entire coding TRß2 exon and 100 bp of the subsequent intron were sequenced. Consensus TATA sequences are located 400 and 1000 bp upstream of the AUG codon (designated as +1). Three CAAT box motifs occur at -300, -600 and -1050. The most upstream TATA and CAAT elements are arranged in a manner reminiscent of a classical core promoter. Transcription initiating at this point would result in an extremely large 5' untranslated region unless an intron which interrupts the 5' untranslated region is present. No discernible classical thyroid hormone response elements are present although there are several consensus half sites (GGTCA) scattered throughout the upstream flanking region. A motif resembling the pituitary-specific Pit I binding site occurs at -130. A construct containing 3 kb of the DNA immediately 5' flanking of the AUG codon fused to a luciferase reporter gene expressed at a level >1000 fold higher in TtT-97 thyrotropic tumor cells than a promoterless luciferase plasmid suggesting that this region contains a promoter which is highly active in thyrotrope cells. In summary we have isolated the genomic locus corresponding to the unique amino terminal coding exon of the pituitary-specific thyroid hormone receptor (TRß2) and have demonstrated that 3 kb of the upstream flanking DNA functions as an active promoter in thyrotrope cells.

**L 437** REGULATION OF THE HIV-1-LTR BY THYROID HORMONE RECEPTOR, Vandana Yajnik* and Herbert H. Samuels, Departments of Pathology*, Medicine, and Pharmacology, New York University Medical Center, 550 First Avenue, New York, N.Y. 10016

Members of the steroid/thyroid hormone receptor gene family have been reported to regulate transcription from the LTRs of a number of viruses. We have observed that thyroid hormone (T3) stimulates expression from human immunodeficiency virus type I (HIV-1)-LTR-CAT constructs. HeLa cells, which lack thyroid hormone receptor (T3R), were co-transfected with an HIV-1-LTR-CAT vector (pU3R-III-CAT(-453/+80)) and a vector expressing T3R. T3 stimulated CAT expression approximately 8-10 fold (T3/basal). Similar experiments were performed using glucocorticoid, retinoic acid, and vitamin D receptors. These receptors and their ligands did not stimulate the HIV-1-LTR. The sequences important for T3 stimultion of the HIV-1-LTR were mapped by coupling detailed functional analyses with receptor binding studies. Gel mobility shift assays were conducted to examine the binding of purified chick T3R-alpha to the -167/+80 fragment of the HIV-1-LTR. Without T3, the receptor formed gel shift complexes of low and high mobility. When T3 was added to the incubation there was a marked reduction in the abundance of the low mobility complex and an increase in the high mobility complex. We have noted two regions in the -167/+80 sequence of the LTR which bind T3R. The more distal element contain sequences identical to known thyroid hormone response elements (TREs). Introduction of mutations into these motifs completely abolish stimulation of the HIV-1-LTR by T3R. When these sequences were cloned upstream of a heterologous promoter, they mediated trans-activation by T3R in HeLa cells. Deletion of an essential cytosine residue in these TREs markedly reduced T3 stimulation. The more proximal T3R binding site is localized closer to the TATA box. Although this element binds to purified chick T3R-alpha, it does not mediate trans-activation of the HIV-1-LTR when the more distal TREs are deleted. However, expression of the HIV-1 tat trans-activator converts the inactive proximal response element to one which now mediates strong T3 stimulation by T3R. These studies indicate that the HIV-1-LTR contains both tat dependent and independent TREs and provides a model for analysis of activation of T3R by other transcription factors.

*Late Abstracts*

RECOMBINANT MINERALOCORTICOID RECEPTOR EXPRESSED IN BACTERIA ADOPTS A HIGH STEROID BINDING AFFINITY STATE, Claudio A. Caamaño, M. Inés Morano and Huda Akil, Mental Health Research Institute, University of Michigan, Ann Arbor, MI 48109-0720.

One of the limiting steps for establishing structure-function relationships in steroid receptors is the production and isolation of its constitutive units in a way that facilitates its assembly into biologically active complexes. The DNA- and steroid-binding domains of the rat mineralocorticoid receptor were expressed in *Escherichia coli* as a fusion protein with glutathione-S-transferase. The expressed recombinant protein, that comprised between 3 and 5 % of the total cytosolic fraction, could be isolated by a single affinity chromatography step under mild conditions (5 mM glutathione) in the absence of detergents. The identity of the induced protein was confirmed by Western blot analysis using antibodies to both mineralocorticoid receptor and carrier protein. Although the [$^3$H] aldosterone binding activity of the purified preparation was negligible, further incubation with rabbit reticulocyte lysate rendered the preparation active in a time and temperature dependant process. The apparent Kd value for aldosterone (0.7 ± 0.2 nM) and the steroid binding specificity were in accordance with those reported for the native mineralocorticoid receptor and the binding capacity of different preparations varied between 17 and 32 pmoles/ mg of protein. Since this receptor generated in bacteria exhibits a correct steroid binding activity after treatment with reticulocyte lysate, this system is potentially useful for structural and functional studies of the receptor.

GENETIC SEQUENCES OF HORMONE RESPONSE ELEMENTS SHARE SIMILARITY WITH PREDICTED ALPHA HELICES WITHIN DNA BINDING DOMAINS OF STEROID RECEPTOR PROTEINS: A BASIS FOR SITE-SPECIFIC RECOGNITION, Lester F. Harris, Michael R. Sullivan and David F. Hickok, Cancer Research Laboratory, Abbott-Northwestern Hospital, Minneapolis, MN 55407

**We report conservation of genetic information between regulatory proteins' DNA recognition helices and the DNA sequences to which they bind. We also report findings of amino acid/nucleotide hydrogen bonding calculations using computer models derived from atomic coordinates of protein/DNA complexes. We present a hypothesis for a DNA site-specific recognition code where functional sites on amino acid side chains recognize genetically conserved sites of stereochemical complimentarity on their cognate codon or anticodon nucleotides within hormone response element major groove half-sites. Our observations support a deterministic origin of the genetic code and suggest that hormone response elements, operators and regulatory proteins' DNA binding alpha helices may be conserved remnants of a successful prebiotic process of molecular recognition and synthesis. Prior to the structural determination of steroid receptor proteins' DNA binding domains, we predicted the location, sequence and the alpha helical secondary structure of the DNA reading head, specifcally for the glucocorticoid receptor and by inference several other members of the steroid hormone receptor superfamily. These predictions were recently confirmed by NMR and X-ray crystallographic structural determinations of the DNA binding domains of the glucocorticoid and estrogen receptors. Our observation that the cDNA coding for the glucocorticoid receptor's DNA recognition helix contains a region of sequence similarity to the mouse mammary tumor virus 5' long terminal repeat glucocorticoid response element, suggests that this cDNA sequence may be a target site for glucocorticoid receptor binding and autoregulation.**

:DICTING HORMONE STRUCTURE AND FUNCTION BY
REOCHEMICAL FIT OF CANDIDATE LIGANDS INTO DNA,
. Hendry, Thomas G. Muldoon, Darrell Brann and
Mahesh, Drug Design Lab, Department of Physiology and
gy, Medical College of Georgia, Augusta, GA 30912
iety of molecular modeling techniques including
aphics and energy calculations, we have shown that
tant naturally occurring small molecules including
l, insect and mammalian hormones fit in a "lock and
r into cavities between base pairs in partially unwound
ded DNA. Certain mammalian hormones (steroid and
nones), fit particularly well in the sequence 5'-TpG-
; however, each hormone possessed a unique pattern of
ınd donor/acceptor linkages with DNA (J. Steroid
lol. Biol. 39:133, 1991; 31:493, 1988; 24:843,
ımmon feature of each of these structures was the
functional groups which could simultaneously and
cally link both DNA strands. While we have been
/oid proffering a direct role for these findings in
ion, several basic postulates have evolved: 1) the
stereochemical complementarity of a naturally
ıall organic molecule with DNA, the more essential is
function; 2) the specific biological function of a given
ule is determined by the particular site in DNA which it
the exact pattern (number, type and location) of
tor linkages formed at that site; 3) within any group of
tested for a given biological activity, those structures
he greatest complementarity with DNA have the greatest
e of the puzzling aspects of these studies has been the
any structures which link certain donor/acceptor
DNA. If our postulates are correct, structures which
ed to possess such missing linkages should have unique
ıctions. It follows that this information should be
g design as well as in the prediction of as yet unknown
ewly discovered orphan receptors. We recently found
etrahydroprogesterone fulfills these criteria.