THE STRUCTURE AND FUNCTION OF STEROID RECEPTOR PROTEINS

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I. INTRODUCTION

The observed physiological response to hormone treatment is the coordinate regulation of tissue-specific gene networks. The effects of steroid hormones are mediated by intracellular receptor proteins. These receptors have been a favorite subject of study since they can be conveniently labeled by radioisotopic steroid ligands (covalently and noncovalently) and their mechanism of action is at the level of gene regulation: steroid receptors (SRs) are transcriptional regulatory factors. There has been much progress in recent years toward the elucidation of various components of the steroid response and the molecular mechanisms which transmit this endocrine signal. Most notably, the sites of action on the DNA have been identified at the sequence level, and investigations into the structure and function of steroid receptors have been extremely informative due to the recent cloning of SR cDNA sequences. The purpose of this present review is to give some general background on the studies of steroid hormone action and to discuss in some detail current topics which have become important as a result of isolating and characterizing SR-coding sequences from a number of sources. No attempt is made to summarize the entire field of steroid hormone action but, rather, this review focuses on a few model systems. The primary example used is the glucocorticoid receptor (GR) since it has been the most intensely studied.[1]

The development of radioisotopic ligands allowed researchers to characterize SRs using standard biochemical techniques. For example, Wrange et al.[2] worked out a column chromatography strategy which utilizes DNA cellulose as one of the steps. Once sufficiently pure SR protein became available, polyclonal and monoclonal antibodies were produced and used as immunological agents to further characterize these proteins.[3-7] As a result of these biochemical and immunological studies, it was proposed that the GR is comprised of three distinct protein domains which can be characterized by steriod binding, DNA binding, and a major epitope for immunoreactivity.[8] Other properties of SRs have also been reported, including the role of phosphorylation in SR activity,[9,10] conformational changes as measured by two-phase partitioning, [11] and interactions with other cellular proteins.[12]

In parallel to the biochemical studies, a number of laboratories have been addressing the question of steroid hormone action *in vivo* using molecular biological approaches in an attempt to understand how ligand-activated SRs are able to "modulate" the expression of specific genes. The most thoroughly studied example of SR-regulated gene expression has been the induction of mouse mammary tumor virus (MMTV) transcription by the GR. These studies have shown that upon binding of dexamethasone (dex; a synthetic glucocorticoid) the cytosolic GR is actively targeted to the nucleus,[13] where it binds to specific DNA sequences referred to as glucocorticoid response elements (GREs) found within and near

hormone-responsive genes.[14] This binding results in altered chromatin structures at the GRE and simultaneous (or subsequent) changes in the frequency of transcriptional initiation at the nearby promoter.[15] For instance, the induced transcriptional initiation rate at the MMTV promoter is reached with a half-time of 7 to 9 min after hormone addition.[16] The regulation is strictly at the level of transcriptional initiation in that there are no observable changes in transcriptional elongation rates, RNA stability, mRNA processing, or polyadenylation.[16]

MMTV GREs have been shown to be GR-dependent transcriptional enhancers, able to regulate the expression of heterologous promoters in a distance- and orientation-independent manner.[17] Similar GREs have now been characterized in a variety of hormone-responsive genes including the human metallothionine IIA gene,[18] the chicken lysozyme gene,[19] the human growth hormone gene,[20] and the rat tyrosine aminotransferase gene.[21] More recently, other hormone-responsive elements have been described for estrogen regulation of vitellogenin genes,[22] progesterone regulation of uteroglobin,[23] and androgen regulation of both MMTV[24] and the prostate-specific gene, C3.[25]

The manipulation of *cis*-acting DNA sequences via transient expression assays allowed for the identification and characterization of the SR-binding site; however, a more difficult task was the recovery of SR-coding sequences to facilitate a molecular genetic analyses of these *trans* regulators. The biochemical studies have been plagued by the low abundance and instability of SR proteins.[1] Genetic approaches to the study of steroid hormone action have been limited; their major contribution was in demonstrating the requirement of SRs for the *in vivo* response.[26,27] Therefore, many labs set out to isolate SR cDNA sequences using antibodies as reagents to selectively enrich polysomes or as probes to screen bacteriophage expression libraries. This was first accomplished by our group with the cloning of rat GR cDNA,[28,29] and, more recently, others have completed the isolation of the human[30,31] and mouse[32] GR; the human,[33,34] chicken,[35,36] rat,[37] and frog[38] estrogen receptor (ER); the rabbit[39] and chicken[40,41] PR; the human mineralocorticoid receptor (MR);[42] the chicken[43] and human[44] thyroid hormone receptors (TR); the chicken[45] and human[46] vitamin D receptor (VDR); and the human retinoic acid receptor (RAR).[47,48] Analysis of these SRs has revealed that they all share a common sequence motif functionally corresponding to the DNA-binding domain. Various components of these SR protein domains are discussed in the following sections.

Four topics for this review have been chosen which are the direct result of structure-function analyses of cloned SR sequences. First, experiments which are directed at deciphering the effects of ligand binding on SR "transformation", cellular localization, DNA binding, and gene regulation are presented. Second, the surprising finding that the small (~80 amino acids) conserved DNA-binding domain of the SRs encodes multiple functions is reviewed. Third, several models of SR-mediated transcriptional regulation, particularly in light of recent data concerning both positive and negative regulation by the GR, are presented and reviewed. Fourth, what is known about cell-specific hormone responses is reviewed, and a general molecular biological approach which is being used in our laboratory to dissect these complex systems is presented.

One of the most exciting new directions in the study of steroid hormone action is the application of molecular genetics to the investigation of well-characterized cellular and physiological responses which are clinically relevant. The hope here is to demonstrate not only what has been learned using molecular genetics to examine SRs, but also to point out potential future directions that are now possible.

II. THE EFFECT OF LIGAND BINDING ON SR ACTIVITY

A number of results have challenged the hypothesis that steroid binding acts as a single trigger in activating SRs through a conformational change leading to an increase in specific

DNA binding. First, it has been shown that ligand-free GR can bind specifically to GREs *in vitro* using either purified GR[49] or *de novo* synthesized GR in the absence of steroids.[50] Second, antagonists are able to stimulate nuclear translocation of the GR, and GR complexed with a variety of these antagonists is capable of specifically binding the GRE *in vitro*. Yet these complexes cannot cause GR-dependent transcription *in vivo*.[13,51] Third, multiple signals in the GR encode nuclear targeting information; one is hormone dependent and the other is not.[13] Fourth, GR deletion mutants which cannot bind steroid are localized in the cytoplasm;[52] other mutant GRs are constitutively in the nucleus and are fully active.[13,52]

Several studies have measured DNaseI hypersensitivity *in vivo* and found that there is a direct correlation between the induction of chromatin alterations and SR-dependent transcriptional activity.[15,53,54] However, GRE binding *in vitro* can be observed with mutant GRs that are unable to induce DNaseI hypersensitivity or transcription *in vivo*.[50,55] Taken together, these results suggest that steroid binding to the receptor may be effecting more than one SR function, independent of DNA binding, such as cellular (or nuclear) localization and transcriptional activity. Moreover, they suggest that the *in vivo* requirement of ligand is to alter SR conformation such that it is able to "functionally interact" with steroid-responsive promoters. SR sequences which encode the ligand-binding domain may also be able to inhibit SR activity in the absence of ligand.[52] These data strongly imply that sequence-specific DNA binding *in vitro* is not an accurate measurement of potential SR transcriptional regulatory activity *in vivo*.

Functional mapping of the steroid-binding domain of the GR has shown that this domain is relatively large, consisting of at least the carboxy-terminal 300 amino acids.[50,52] Multiple GR-derivative proteins were tested *in vitro* by labeling with both noncovalent and covalent radioisotopic ligands to localize the sequences required for this binding.[50] Several internal deletions in this region were able to diminish ligand binding without totally eliminating it. Secondary structure predictions for this region suggest a potential hydrophobic pocket.[29,50] Recently, two groups have identified amino acids involved in glucocorticoid binding to the GR using affinity labeling. Simons et al.[56] have shown that dex-mesylate (DM) covalently labels cysteine 656 of the rat GR. Carlstedt-Duke et al.[57] have shown by photoaffinity labeling that triamcinolone acetonide (TA) interacts with amino acids methionine 622 and cysteine 754. These positions are all within hydrophobic regions and are relatively homologous to sequences in PR and MR, but not other SRs.[39-42]

Since the methods utilized for labeling with DM and TA are different, it is not possible to determine if the receptor interaction with these two ligands is identical. Presumably, these ligands cause distinct structural changes in the GR which affect GR activities since DM is an antagonist and TA is an agonist *in vivo*. More definitive explanations will require crystallographic data from other SR complexes using a variety of agonists and antagonists. The ability to overexpress GR from cloned cDNA may facilitate these approaches by providing large quantities of "virgin" GR synthesized in *Escherichia coli* or yeast.

Another approach has been to construct GR mutant proteins which have steroid-independent transcriptional regulatory activity *in vivo*. Godowski et al.[52] have shown that carboxy terminal deletions of more than 195 amino acids, but less than 270 amino acids, lead to GR proteins that constitutively target to the nucleus and regulate transcription. Carboxy-terminal mutations which are less than 195 amino acids do not bind hormone and are localized to the cytoplasm; there is a direct correlation between nuclear localization and transcriptional regulation. However, ligand-induced nuclear translocation cannot be the critical step which controls SR activity because a number of studies have shown that both DM- and RU486-bound GR is translocated to the nucleus in the absence of transcriptional regulatory activity.[13,58] Moreover, immunological and biochemical studies indicate that both ER[59,60] and TR[61] are normally found in the nucleus under hormone-free conditions. Therefore, *in vivo* SR activity seems to require more than simply nuclear localization. The initial observation

that ligand binding is required for SR-transcriptional regulatory activity *in vivo* is still correct. However, since it appears that specific binding *in vitro* may not require ligand, there must be more to the steroid response than simply the ability of SRs to recognize their binding site on naked DNA. At present, there are no *in vitro* transcription systems which convincingly demonstrate SR-dependent regulation. When there are, then it should be possible to investigate the mechanism of ligand-dependent SR transformation under controlled conditions.

SR transformation could be due to derepression,[52] involving both intra- and intermolecular interactions. These interactions are not realized with GR bound to the antagonists RU486 and DM,[1] indicating that there are, in fact, major differences between specific DNA binding *in vitro* and *in vivo*. The only conclusion at this point is that binding of these antagonists does not inhibit specific GRE binding *in vitro*.[50] What other step(s) may be inhibited is unclear. It is interesting to note that a GR variant (nti) has similar properties to antagonist-bound GR. The nti GR binds steroid and DNA and is translocated to the nucleus, but it seems deficient in SR-transcriptional regulatory activity.[62] The precise nature of the nti mutant phenotype is a subject of investigation and is described later; however, at face value, it suggests that transcriptional regulation requires SR activity in addition to specific DNA binding.

III. A HIGHLY CONSERVED REGION ENCODES THE DNA-BINDING DOMAIN

There have been two surprising results to come out of the isolation and characterization of SR cDNA sequences that may be clues to understanding the mechanism of steroid hormone action. First, functional mapping studies indicate that specific DNA binding *in vivo* of the GR to the GRE is encoded within a small 86 amino acid region of the GR.[55] This domain can be separated from the rest of the protein by limited protease digestion,[8] yielding a peptic fragment coincident with the same region identified by deletion mapping studies to contain the DNA-binding domain.[50] It has been shown that a 150 amino acid peptide encoding this domain can be synthesized in *E. coli*, purified by column chromatography, and used in DNA-binding experiments to yield specific footprints on the GRE.[63] Second, this same region is highly conserved not only across species barriers for the same SRs (such as *Xenopus* and human ER), but also between classes of receptor proteins, whereas very little conservation is seen in other regions of these molecules. Figure 1 is a schematic drawing summarizing the homologies between the variety of receptors that have now been cloned and analyzed.

Deletion experiments were performed to better map the functional activity of *in vitro* DNA binding.[50,64,65] The most extensive of these analyses were performed on the rat GR by Rusconi and Yamamoto[50] and can be summarized as follows. Using both deletion analyses and retention of function analyses, a small region was shown to contain all of the activities required for specific DNA binding by the GR to the GRE. These experiments included looking both for specificity of binding and, to a lesser extent, affinity of binding. It was concluded that the amino terminus and the carboxy-terminal half of the GR are not required for specific DNA binding *in vitro*. These experiments were accomplished by synthesizing various deletion mutants of the GR *in vitro* using reticulocyte lysate extracts and then immunoprecipitating protein-DNA complexes utilizing end-labeled DNA fragments.

With the addition of TR and RAR, this family of cytosolic and nuclear receptors has grown. Since they are all transcriptional regulatory factors whose activity is controlled by ligand binding, they are referred to here as "ligand-regulated factors" (LRFs). The ancestral LRF protein using my definition consisted of two structurally independent domains (ligand binding domain and the regulatory DNA binding domain). LRFs mediate their action through binding to appropriate ligand-response elements (LREs) located within and near LRF-regulated genes. Figure 1 illustrates that between the different LRFs these two domains are

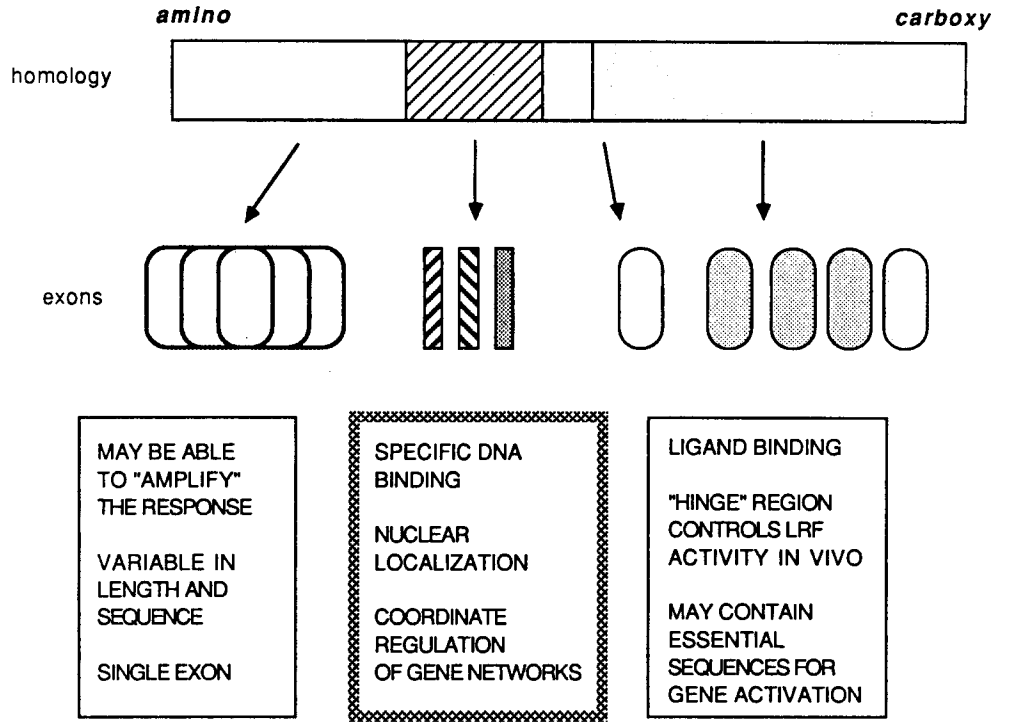

FIGURE 1. Sequence homology among members of the LRF family. The three major domains are divided into regions of high (striped), low (stippled), and no (open) homology. The approximate intron-exon borders of the rat GR gene are shown.[75]

easily identified and are, in fact, separated by a small, nonconserved region which may act as a "hinge". The high conservation within the DNA-binding domain, as seen by the sequence homology and extrapolated from the DNA binding experiments of Rusconi and Yamamoto,[50] suggests that this domain is the primary basis for the coordinate regulation of distinct LRF gene networks. The ligand domain is less well conserved and reflects the variety of chemical structures present among the different ligands. Therefore, the complexity, activity, and specificity of the endocrine messenger molecules (LRFs) result from the functional interaction of these two distinct domains.

The ability to interchange the ligand domain and the DNA-binding domain of two separate receptors has demonstrated that these domains are, indeed, independent entities. Green and Chambon[66] were the first to show that by combining the GR DNA-binding domain with the ER steroid-binding domain (GR-ER chimera) they could construct a molecule which regulates MMTV expression through estradiol binding. In addition, Petkovich et al.[47] have recently shown that a human ER-RAR chimera is able to control the expression of vitellogenin-tk cat-vectors (ERE-regulated) via retinoic acid. In both of these cases, it has been demonstrated that the ligand domain is acting upon the DNA-binding domain to regulate receptor activity. This type of gene swapping experiment is very exciting. One can imagine that by constructing molecules which combine one LRF ligand-binding domain with a heterologous LRF DNA-binding domain it will be possible to design novel molecules that may be useful "probes" in transgenic mice. For example, one could examine the diversity of cell types effected by retinoic acid by monitoring the activity of ER-RAR chimeras using ERE reporter genes.

The primary amino acid sequence of the DNA-binding domain of LRF family members is shown in Figure 2. Pairs of nearby cysteines are thought to be coupled together to form what has been referred to as a finger.[67] The cysteines are most likely coordinated together

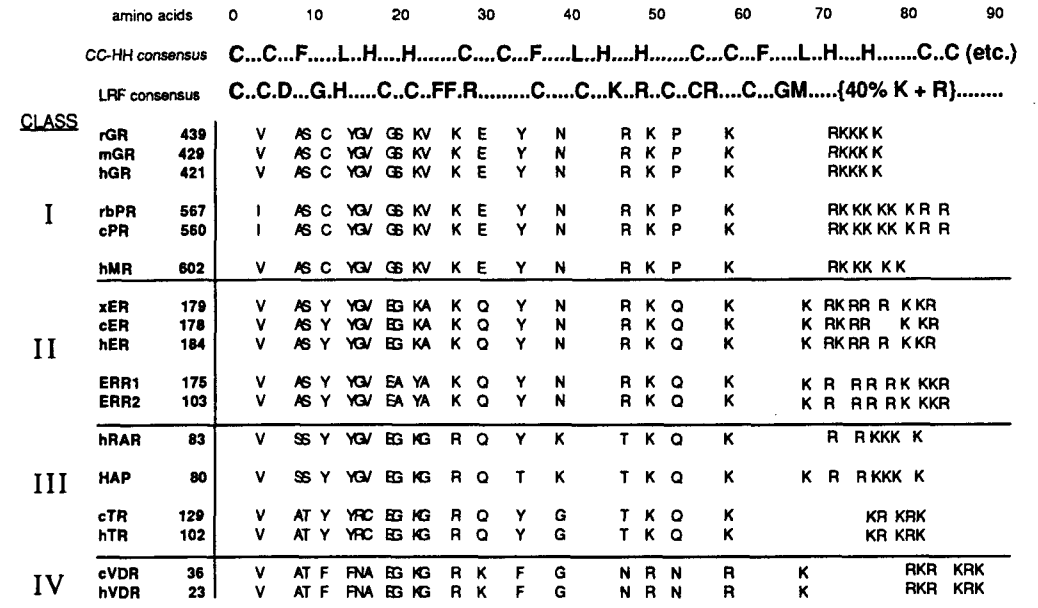

FIGURE 2. Amino acid sequences for the 90 residues encoding the DNA-binding domain of the LRFs sequenced to date. Residues of 100% conservation constitute the LRF consensus sequence and are shown at the top in bold letters. Positions that are highly conserved between various LRFs are also given. All other positions are only moderately or minimally conserved. The number of amino-terminal residues preceding the DNA-binding domain is given for each LRF. The sequences have been compiled from the data reported for the rGR,[29] mGR,[32] hGR,[30] rbPR,[39] cPR,[40] hMR,[42] xER,[38] cER,[35] hER,[33] ERR1 and ERR2,[97] hRAR,[47] HAP,[98] cTR,[43] hTR,[44] cVDR,[43] and hVDR.[46] The rER[37] sequence is 100% identical to the hER[30] across this region and was therefore omitted from the figure.

by a zinc ion to form the finger structure.[68] There are several characteristics which distinguish LRF family members from other proteins which have been shown to contain C_2H_2 fingers (such as TFIIIA and SP1).[69,70] First, the LRF proteins contain only two DNA-binding fingers; there are many conserved amino acids within this region that are unique to LRFs (compare the LRF consensus to the CC-HH consensus). Notably, there are no conserved histidines, and the sequence of finger 1 differs significantly from finger 2. Second, these two fingers are always located at the amino end of the protein relative to the ligand-binding domain. The length of the amino terminus is highly variable, as indicated in Figure 2. Third, the 18 LRFs sequenced to date can be divided into four related classes depending on other conserved amino acids. Classes I and IV are the least similar; classes II and III are very similar. Last and most important, ligand binding is required to regulate the specific DNA binding and transcriptional regulatory activity of LRF proteins *in vivo*. The interchangeability of these two domains between LRF family members[47,48,66,71] indicates that this modulatory mechanism is, indeed, an important conserved function.

Obviously, the relatively few amino acid substitutions between LRFs within the DNA-binding domains (see Figure 2) reflect the subtle differences in specific protein-DNA interactions distinguishing the LRF-responsive gene network (see Section V). One prediction would be that the LREs will also reflect four classes of DNA-binding sites having coevolved with the subsets of LRFs shown in Figure 2. A similar type of *cis-trans* coevolution has been proposed to explain the species specificity of mouse and human ribosomal RNA gene expression.[72] Experiments are in progress to identify which amino acids are required for GR-GRE DNA interactions. This is being done using both oligonucleotide-directed mutagenesis and genetic selections for *cis* and *trans* mutants in yeast.[73] The appropriate selectable markers allow one to isolate mutants in the GRE which can then be used to select for

revertants in the *trans*-acting GR protein. Conceivably, these types of experiments could be done for a variety of LRF proteins and, thereby, delineate the protein-DNA interactions which specify the LRF gene regulatory networks.

Interestingly, the genomic organization of this DNA-binding domain suggests that it is made up of smaller components. Figure 1 shows the organization of the rat GR gene. Note that each finger is encoded in a separate exon, and a third exon encodes the arginine/lysine-rich region which has been shown to be required for DNA binding and nuclear translocation.[13,50] This genomic organization suggests that various components came together in the ancestral protein, most likely through exon shuffling,[74] giving rise to a DNA-binding domain which subsequently diverged.

The third distinct region of LRFs is the amino-terminal "domain". Figures 1 and 2 reveal that this region is the least conserved; it is highly variable in both sequence and size. Functional studies of the GR[55] and the ER[71] demonstrate that it is dispensable for the regulation of MMTV and vitellogenin transcription, respectively. Since there is little similarity in the amino terminal domain between these two closely related SR proteins, it is thought that the function may be either degenerative or so subtle that it is difficult to test directly. One of the more striking comparisons is between the hVDR and the hMR. The amino terminus of the hVDR is only 23 amino acids[45] compared with 602 amino acids in the hMR amino terminus,[42] even though there is considerable conservation in the DNA- and ligand-binding domains (see Figure 2). By first approximations, therefore, both functional studies and evolution of the LRF family suggest that the contribution of the amino-terminal domain to LRF activity may not be critical. One possibility, however, is that this region does encode an important function, but the requirements for this activity are not sequence specific and/or required by all LRFs for *in vivo* activity. Section IV discusses the idea that "acidic" amino acids may be important for positive regulatory activity. The only recognizable sequence in this motif is the presence of aspartate and glutamate residues.

Figure 3 shows that the nti GR from mouse lymphocytes is mutated by virtue of a splicing error, which eliminates the amino-terminal-encoding exon.[75] The phenotype of the nti GR is such that it is defective in mediating the hormone response and may be revealing a function for this upstream exon in lymphocytolysis.[76] The analysis of nti cDNA sequences indicates, however, that the mutant phenotype (failure to lyse cells) may be due to both the loss of the amino terminus and the addition of exon 1 sequences, joining a normally terminated open reading frame (ORF) to exon 3 (Figure 3). The alternate splicing of at least three different 5′ exons adds diversity to this complex mutant.

We are currently carrying out experiments in an attempt to understand how the evolutionary divergence of the amino terminus and the ostensive dispensability of this domain concur with the nti GR phenotype. We are examining this apparent paradox by expressing nti cDNA sequences in functional cotransfection assays to determine if we can separate the lysis phenotype from transcriptional regulatory activity. It could be that the lysis phenotype has a high threshold for GR responsiveness and, therefore, requires an intact GR, even though these same amino-terminal sequences are not required for activation of MMTV gene expression.[55] If this is true, then it would suggest that the amino-terminal sequences of the GR are only required under specific circumstances in which the target gene would otherwise be suboptimally induced. It should be pointed out that the receptor swapping experiments described here have not revealed any requirement for the amino-terminal domain to differentiate between ER, GR, and RAR. This author favors the notion that the GR amino terminus "augments" basal GR activity. This additional activity is required for the induction of some, but not all, GR-responsive genes. Perhaps there are different thresholds of GR responsiveness which correlate to the quantity and quality of the GREs in and around regulated genes.

A recent report by Kumar et al.[71] has shown that extensive analysis of the human ER reveals the familiar three functional domains.[64] Using transient transfection assays, they

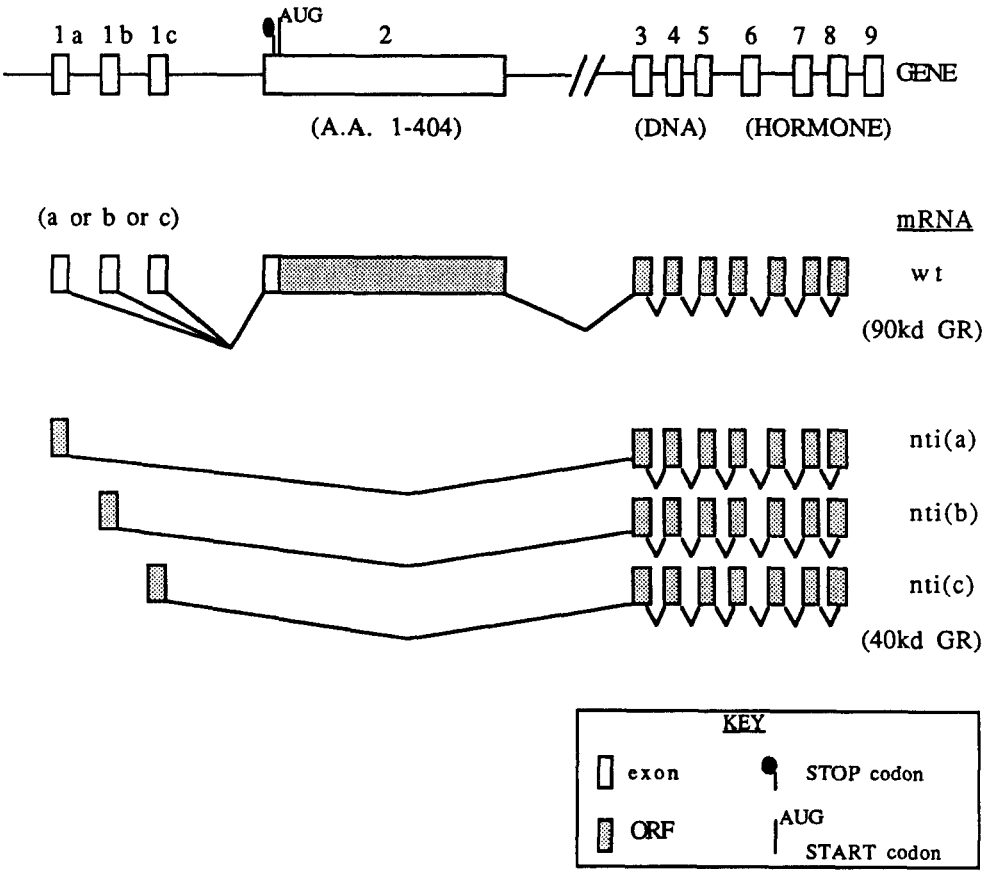

FIGURE 3. The genomic organization of the GR gene. Schematic drawing shows the splicing defect in the nti mutant which eliminates exon 2 from the mature transcript. The splicing of exons 1a, 1b, or 1c to 3 results in the potential addition of novel amino acids to the amino terminus of the nti GRs.

have reported that the finger region encodes target gene specificity and the ligand domain is required for activation. Competition assays suggest that deletion of the ER ligand domain does not result in constitutive activation, in contrast to what has been shown for the rat[52] and human[65] GR. In addition, Kumar et al.[71] report that the amino-terminal domain of the human ER is not absolutely required for vitellogenin ERE activation, but it does seem to be necessary for activation of the human PS2 gene promoter. Perhaps there is an analogous situation with the nti GR mutant in that this truncated GR may be able to activate the MMTV GRE but not the putative lysis gene GRE.[27]

One can imagine that various contextual factors may be involved in differentiating between target genes. For example, surrounding sequences may influence the binding affinity of the LRF for the LRE and thereby compromise the ability of the LRF to mediate transcriptional regulation. Variations in DNA-binding affinities may affect the abilities of these SRs to mediate transcription, i.e., the PS2 ERE may be bound less tightly by some ER mutants which are capable of binding normally to the vitellogenin ERE. The sequences of the PS2 ERE and vitellogenin ERE are similar, but not identical. Therefore, ER DNA-binding affinities for the ERE may be a sensitive way to physiologically distinguish these two genes. Unfortunately, absolute DNA-binding affinities have not yet been reported for various LRF-LRE interactions. However, using the genetic approaches described earlier to examine GR-GRE interactions, it should soon be possible to correlate *in vitro* DNA binding to *in vivo* transcriptional activity.

IV. MECHANISMS OF SR-MEDIATED TRANSCRIPTIONAL REGULATION

The best understood system for the study of transcriptional regulation has been GR-modulated MMTV expression. Yamamoto proposed[17] that GR recruits the rate-limiting transcription factors into a stable promoter complex, resulting in increased transcriptional initiation rates. This model suggests that both protein-DNA interactions and protein-protein interactions are required for the observed transcriptional induction. These precise interactions have not been identified; however, preliminary data[73] suggest that there are sequences distinct from DNA binding which are required for transcriptional regulation. These sequences are primarily composed of acidic amino acids (glutamate and aspartate) and have been identified in several yeast regulatory proteins.[77,78] One model is that the acidic residues of GCN4 and Gal4 (and perhaps the LRFs) interact with the pentapeptide repeated "tail" of RNA polymerase II molecules and thereby increase initiation frequencies.[79] Exactly how this interaction is mediated, or if the acidic residues (or even the pentapeptide repeat) are, in fact, the primary activation sequences, remains to be investigated directly.

A second model which has been proposed suggests that in some cases binding of the LRF to the LRE relieves negative regulation through derepression.[80] Recently, Hager and colleagues[81] presented evidence that binding of the GR to the MMTV GRE displaces a nucleosome. They propose that nuclear factor 1 (NF1) is then able to bind to the MMTV promoter and stimulate transcription. This model does not require a protein-protein interaction between the GR and NF1. A third model, which would be suggested from the above two, is that DNA binding brings about a torsional or structural change in the DNA double helix, resulting in increased initiation frequencies. One can imagine, for example, that helix destabilization may allow other proteins to bind. This model which has been proposed for other types of enhancer systems often includes the possibility that DNA-modifying enzymes, such as topoisomerases, will be an integral part of this mechanism.[82,83] It may be that some combination of these three mechanisms will be required for LRF action.

Transcriptional repression by SR proteins has also been studied in a number of cases. Genes such as propiomelanocortin,[84] prolactin,[85] and α-fetoprotein[86] have been shown to be transcriptionally repressed by glucocorticoids. Results emerging from the study of prolactin transcriptional repression by GR have suggested that GR binding to the prolactin $^{-}$GRE (to distinguish it from $^{+}$GRE) interferes with promoter utilization. Similar to POMC repression,[84] GR binding to a prolactin promoter-embedded $^{-}$GRE most likely leads to interference of promoter-binding factors in the prolactin gene regulatory region.[87] This type of interaction would displace or prevent the binding of promoter factors such as the CTF or SP1 to this overlapping sequence.

It has also been shown that a further upstream $^{-}$GRE-binding element in the prolactin promoter is involved in repression. Sakai et al.[87] have suggested that this $^{-}$GRE sequence encodes a binding site for a putative enhancer-activating protein. In cells containing the enhancer-binding protein, the prolactin promoter is maximally transcribed. However, when activated GR binds to this $^{-}$GRE, the enhancer-activating protein is no longer active (or bound), resulting in the apparent repression of the prolactin promoter. They have shown that the upstream prolactin $^{-}$GRE can still be negatively regulated by glucocorticoids when moved in a distance- and orientation-independent matter relative to a heterologous promoter. One of the observations in these experiments has been that some cells are functionally deficient in this putative enhancer-binding protein (such as the AR4-2J pancreatic cell line). The prolactin promoter is transcribed at a low rate in these cells, even in the presence of dex. Moreover, the $^{-}$GRE is not activated by GR binding. For example, it was shown by transiently cotransfecting two plasmids into AR4-2J cells that GR does bind and activate an MMTV $^{+}$GRE; however, the prolactin $^{-}$GRE reporter is not enhanced in the same transfection.[87]

Therefore, it seems as though the GR-$^{-}$GRE interactions on the prolactin fragment are distinct from those on the MMTV $^{+}$GRE. It could be that the GR binds to the $^{-}$GRE in a structurally distinct way. This idea comes from experiments in which the prolactin $^{-}$GRE was mutagenized and functionally converted to a $^{+}$GRE.[88] The sequence of this "mutated" prolactin $^{-}$GRE is nearly identical to the sequence of a $^{+}$GRE. The question of how the same *trans*-acting GR can bind to one $^{+}$GRE sequence and activate, whereas when it binds to a similar $^{-}$GRE it represses (or fails to activate), remains to be answered. It seems likely that elucidation of the mechanisms required for upstream GR-dependent prolactin repression will be enlightening in regard to our understanding of GR-$^{+}$GRE interactions.

Although the majority of investigations into the mechanisms of steroid hormone action has focused on transcriptional activation, it is clear that repression may be as prevalent a mode of regulation as induction.[90] If repression requires only sequences from the LRF DNA-binding domain, as results seem to indicate,[87,88] then this may partially explain the incredible conservation in this region (Figure 2). Transcriptional repression (mediated exclusively by DNA binding) may be the important function that "drives" evolutionary conservation of these sequences between LRFs.

V. CELL SPECIFICITY OF HORMONE RESPONSES

It is clear from the data presented in Section IV that the molecular basis of steroid hormone action is the regulation of transcription. The primary response to activated hormone receptors is the induction or repression of specific target genes.[17] In turn, these primary gene products most likely carry out other cellular functions such as phosphorylation, proteolysis, or even transcriptional regulation of secondary target genes, leading to the observed physiological phenotype.[89] Figure 4 is a schematic drawing which maps out two hypothetical LRF-responsive gene networks. The two-fingered LRF protein represents any of the receptors which have been discussed in this review. In Figure 4A, the induction of genes a, b, c, and d by the activated LRF results in a multitude of responses; alternatively, gene e is repressed by LRF binding. In the simplest case, gene product A biochemically contributes to the phenotype. At the next level, gene product B is itself a gene regulator which induces gene j and represses gene k. Gene product J contributes to the phenotype, whereas the intracellular reduction of gene product K becomes critical. Gene product C could be a kinase which phosphorylates the preexisting proteins F and H. In this hypothetical scheme, the F protein is activated by phosphorylation, and the H protein is inactivated by phosphorylation. Finally, gene product C phosphorylates gene product D, which is also ligand regulated. Taken together, the cell-specific biochemical response is the result of the addition of four protein products (A, J, F, and D) and the loss of proteins K and E along with the inactivation of protein H.

Figure 4 also suggests that this "gene network" is cell specific. One can imagine that the hormone responsiveness of the primary genes a through e is developmentally determined in each cell type. For example, if Figure 4A represents the GR-regulated gene network in hepatocytes and Figure 4B is that of a lymphocyte, it is apparent that some genes are regulated in both cell types (a and e), while others are cell specific (b, c, d and x, y, z). This scheme implies that the same LRF can turn on a variety of primary target genes in different cells, thereby leading to distinct phenotypes in hepatocytes and lymphocytes. Figure 4B illustrates that a single protein product may be the determining factor which is critical to the phenotype. If Z′ is the definitive "lysis gene product" required for dex-induced killing of Wehi7 cells, then to understand lymphocytolysis one should isolate and characterize the products of the y and z genes. Moreover, this hypothetical gene network could be used to explain the major difference between dex-sensitive (immature T-cells?) and dex-resistant (mature T-cells?) thymocytes.[27] For example, genes a, x, y, and e could be hormonally

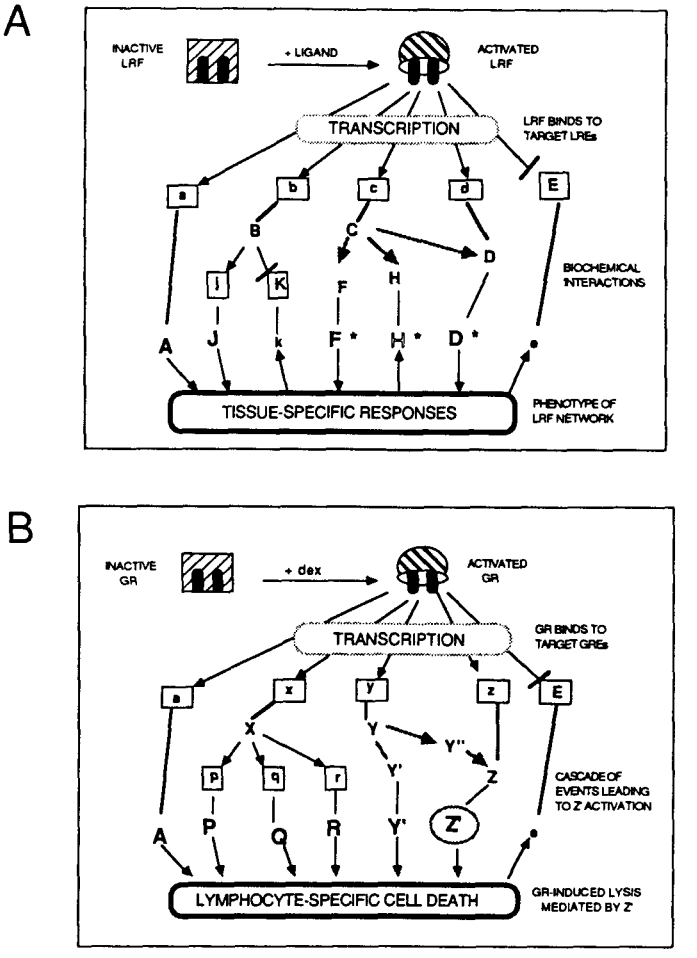

FIGURE 4. Hypothetical LRF-modulated expression of two gene networks in hepatocytes (A) and lymphocytes (B). Lower case letters are genes and upper case letters are gene products.

regulated in both thymocyte cell types. However, in this scenario, the GR responsiveness of gene z would be developmentally determined such that it is not expressed in the dex-resistant cells. This would result in a modified (but very similar) GR-modulated thymocyte-specific gene network in each of the two cell types.

A second mode of cell-specific LRF responsiveness would be at the level of receptor expression. The ER, PR, and TR appear to be tissue specific in their expression.[90] These receptors will lead to regulation of their respective gene networks only in those tissues in which they are expressed. In contrast, the GR is expressed in essentially all cell types,[1,91] and, therefore, one needs to explain the cell specificity of the glucocorticoid response, i.e., it seems not to be at the level of GR expression. As has been proposed by others,[17,92] the various genes in the gene network could be "accessible" to regulatory factors by at least two different mechanisms. One would be at the level of chromatin organization and CpG methylation (epigenetic effects); certain genes will be developmentally turned on or turned off depending on the "state" of the DNA. A second level of control is the presence or absence of additional cell-specific transcription factors which work together with the LRFs to regulate transcription. For example, in the hepatocyte (or dex-resistant lymphocyte), it

could be that lysis gene z is not activated because chromatin or methylation has shut it off, or that other lymphocyte-specific factors are required.

The cell-specific hormone responses of hepatocytes and lymphocytes have been studied for many years at the biochemical level.[90] Recent work involving regulation of transcription by LRFs (augmented by the recent cloning of many LRF proteins) allows one to begin a more systematic study of the target genes in these various networks. For example, by isolating the primary sequences of the LRF-responsive genes a, b, c, and d from liver cells (and identifying their biological function), it should be possible to determine some of the complex interactions between gene products A, J, K, F, H, and D which dictate to the physiological phenotype.

In our laboratory, we are currently developing modified strategies of subtractive cDNA cloning[93,94] to recover the primary gene sequences of GR-regulated genes in hepatocytes and lymphocytes. This approach assumes that the basis of the cell-specific glucocorticoid response is the transcriptional activation and repression of primary target genes through the binding of the GR to the GRE. It is interesting to note that over 10 years ago, Ivarie and O'Farrell[95] used 2-D protein gels to show that relatively few protein products are regulated by glucocorticoids in liver cells. They found that only 5 to 10 proteins out of ~1000 are affected by short-term dex treatment. Based on these early results, we predict that the primary regulated genes in the glucocorticoid gene network in both hepatocytes and lymphocytes will consist of no more than a handful of genes.

Beyond isolating these gene sequences, there is the more difficult task of identifying the functions of the gene products. This will constitute a formidable endeavor and is a problem that plagues every approach in which genetic schemes are used to isolate genes for which no biochemical activity is known *a priori*. In addition to the usual approach of looking for comparative homologies, we will also attempt to identify functions on the basis of expression in transfected cells, perhaps looking for a dominant negative phenotype.[96] We are particularly interested in isolating and characterizing the lysis gene(s) from Wehi7 cells and expect that this gene product will have a testable phenotype in both lymphocyte and nonlymphocyte cells.

VI. SUMMARY

This review has highlighted several topics in the study of steroid hormone action. The unanswered questions regarding the mechanism of ligand-controlled LRF activity, the extent of evolutionary conservation and specificity of DNA binding, and the validity of various models of transcriptional regulation mediated through gene networks point to the future direction of research in this field. Steroid hormones are used extensively in clinical treatments, especially glucocorticoids.[90] Our laboratory is attempting to determine which gene networks are responsible for some of these clinical phenotypes. Figure 5 points out that the study of glucocorticoid action holds a unique position because it spans both the basic sciences and the field of applied molecular biology. Now that we have a fundamental knowledge of the necessary elements required for steroid-dependent regulation of gene expression, we can better investigate the clinical responses to steroid therapy (which include devastating side effects) by isolating and characterizing the important target gene(s).

In this author's opinion, future directions in the study of steroid responsiveness will have to include a systematic approach toward deciphering a variety of these LRF-regulated gene networks in experimentally feasible systems. Hopefully, work in this area may be revealing and perhaps beneficial to ongoing clinical studies. In addition, the study of mechanisms of transcriptional induction and repression, using the model system of LRFs, could be applicable to many gene regulatory systems which are controlled by such processes as development and differentiation.

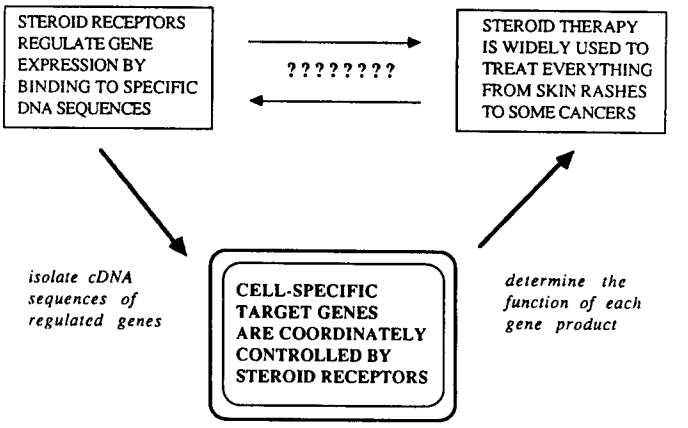

FIGURE 5. Schematic representation of the interrelationships between the use of steroids in clinical applications, our current understanding of the mechanisms of steroid action at the level of transcriptional regulation, and the direction of research that will identify clinically important steroid-regulated target genes.

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