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ANALYSIS OF THE 5' REGULATORY REGION OF HUMAN GLUCOCORTICOID RECEPTOR GENE

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RÉSUMÉ

Ce gène du récepteur des glucocorticoïdes est régulé négativement par son propre ligand. Cette régulation négative est reliée à l'insensibilité au traitement par des glucocorticoïdes en pratique clinique. Le mécanisme est complexe et implique plusieurs étapes telle la transcription du gène du récepteur des glucocorticoïdes et les modifications post-transcriptionnelles et post-traductionnelles du récepteur des glucocorticoïdes. J'ai isolé des fragments de plusieurs tailles de la région régulatrice 5' du gène du récepteur des glucocorticoïdes d'une banque génomique de leucocytes humains et je les ai insérés dans un plasmide rapporteur pour transfecter de façon transitoire des cellules. J'ai identifié la région à la position -2846/-2648 pb en amont du site d'initiation de la transcription du gène du récepteur des glucocorticoïdes qui est suffisante pour réguler négativement la transcription du gène du récepteur des glucocorticoïdes par des glucocorticoïdes. La co-transfection du récepteur nucléaire orphelin "steroidogenic factor-1" et du récepteur des glucocorticoïdes augmente l'activité du gène rapporteur en l'absence de glucocorticoïdes tandis que l'activité du gène rapporteur est grandement diminuée par la présence de l'hormone. La technique *Electrophoresis Mobility Shift Assay* a montré un motif d'ADN dans cette région qui est reconnue par le "steroidogenic factor-1" mais pas par le récepteur des glucocorticoïdes bien que un demi-site GRE soit situé à seulement 5 paires de bases du site de reconnaissance du "steroidogenic factor-1". De plus, des essais de *GST pull-down* ont démontré que le "steroidogenic factor-1" peut interagir avec le récepteur des glucocorticoïdes d'une façon hormono-dépendante *in vitro*. La région d'interaction avec le "steroidogenic factor-1" est située dans le domaine de liaison à l'hormone du récepteur des glucocorticoïdes. Ces observations étendent la théorie classique qui veut que l'interaction des récepteurs nucléaires orphelins avec

l'ADN se produit seulement comme un monomère, impliquant un nouveau mécanisme de la fonction des récepteurs nucléaires orphelins. Le fait que ces récepteurs nucléaires interagissent entre eux suggère aussi que le recrutement des co-activateurs et co-répresseurs de la transcription par différentes surfaces des récepteurs nucléaires peut spécifier la vitesse de la transcription du gène cible. Mes résultats apportent des évidences additionnelles dans la régulation de l'expression du gène du récepteur des glucocorticoïdes sans liaison directe à l'ADN.

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SUMMARY

That glucocorticoid receptor gene is downregulated by its own ligand is related to the insensitivity to glucocorticoid treatment in clinical practice. The mechanism is complex and involves several stages such as glucocorticoid receptor gene transcription, post-transcription and post-translation modification of the glucocorticoid receptor. Here I have isolated various lengths of the 5' regulatory region of glucocorticoid receptor gene from human leukocyte genomic library and fused them to reporter plasmid to transiently transfect various cells. I have identified the region at –2846/-2648 base pairs upstream of transcription initiation site of glucocorticoid receptor gene, which is sufficient to downregulate the glucocorticoid receptor gene transcription by glucocorticoids. Co-transfection of the orphan nuclear receptor steroidogenic factor-1 and glucocorticoid receptor increases the reporter gene activity in the absence of glucocorticoids, while reporter activity is greatly decreased in presence of the hormone. Electrophoresis Mobility Shift Assay showed a DNA motif within this region that is recognized by steroidogenic factor-1, but not by glucocorticoid receptor, although a GRE half site is only 5 base pairs away from the Steroidogenic Factor-1 recognition site. Further GST pull-down experiments demonstrated that Steroidogenic Factor-1 could interact with glucocorticoid receptor in a hormone dependent manner *in vitro*. The interaction region with steroidogenic factor-1 is located in the glucocorticoid receptor hormone-binding domain. These observations extend the classical theory that orphan nuclear receptor interaction with DNA occurs only as a monomer, implying a new mechanism of orphan nuclear receptor's function. That nuclear receptors interact with each other also suggests that recruitment of transcription co-activators or co-repressors by different nuclear receptor surfaces may specify the rate of target gene transcription. My results provide

additional evidence for glucocorticoid receptor regulated gene expression without direct DNA binding.

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LIST OF ABBREVIATIONS

GR	glucocorticoid receptor
SF-1	steroidogenic factor-1
GRE	glucocorticoid responsive element
HRE	hormone responsive element
FSH	follicle stimulating hormone
LH	luteinizing hormone
TSH	thyroid stimulation hormone
ACTH	adrenocorticotropin
PR	progesterone receptor
ER	estrogen receptor
AR	androgen receptor
NGFI-B	nerve growth factor induced gene B
DBD	DNA-binding domain
LBD	ligand-binding domain
AF-1	activation function-1
MAPK	mitogen-activated protein kinase
SRC	steroid receptor coactivator
CTE	carboxyl-terminal extension
TFIID	transcription factor II D
GRIP-1	GR interacting protein-1
CREB	cAMP responsive element binding protein
CBP	CREB-binding protein
TIF2	transcriptional intermediary protein 2
HAT	histone acetyltransferase
bHLH	basic helix-loop- helix
P/CAF	P300/CBP associate factor
AP-1	activation protein-1
NF-κB	nuclear factor-κ B
TBP	TATA binding protein

TAFs	TBP associating factors
CAT	chloramphenicol acetyltransferase
ERR-2	estrogen receptor related protein-2
PBS	phosphate-buffered saline
PMSF	phenylmethanesulfonyl fluoride
TR	thyroid hormone receptor
RAR	retinoic acid receptor
RXR	9-*cis* retinoic acid receptor
VDR	vitamin D_3 receptor
NcoR	nuclear corepressor

CHAPTER 1

INTRODUCTION

1.1. Steroid Hormone

Steroid hormones have diverse and important biological functions, including effects on metabolism, development and differentiation (1). They are produced by steroidogenic tissue of adrenal or gonadal origin. The pathway of steroid biosynthesis is complex, and many steroid precursors are found in the synthetic pathway of any particular steroid hormone (Figure 1). Steroid hormones are synthesized within the cell's smooth endoplasmic reticulum. A complex multiple enzyme system is required for the synthesis of steroids. These enzymes are present within the mitochondria as well as the cytoplasm. Steroid synthesis, therefore, could be blocked by molecules that inhibit protein synthesis.

The adrenal steroidogenic tissue produces glucocorticoids as well as mineralocorticoids. The steroidogenic tissue of the gonads produces various sex hormones: androgens (masculinizing), estrogens (feminizing) and progestins (related to pregnancy, gestation). The testes produce testosterone; in the ovary, estradiol and progesterone are the main steroids synthesized and secreted. During pregnancy, the placenta is an additional source of estrogens and progestins.

The pituitary hormones such as follicle stimulating hormone (FSH), luteinizing hormone (LH), thyroid stimulation hormone (TSH) and adrenocorticotropin (ACTH) stimulate steroid hormone secretion from

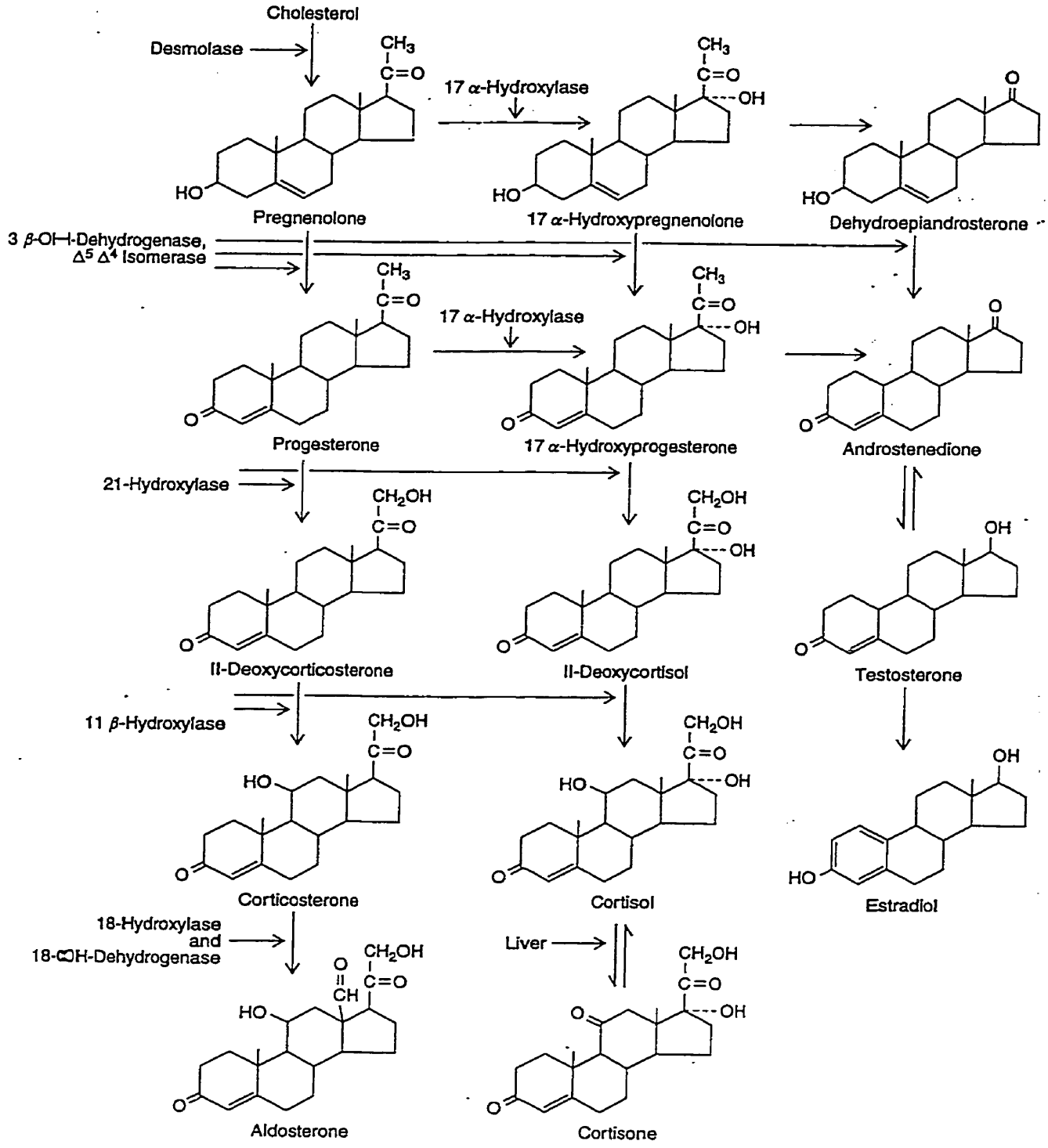


Figure 1: Pathway of adrenal steroid hormone biosynthesis

gonads, thyroids and adrenals respectively. They are controlled through negative feedback mechanism. Target organ hormones, gonad steroids and thyroxin or adrenal glucocorticoid feed back to the hypothalamus and pituitary to inhibit further secretion of pituitary hormones (Figure 2).

1.2. Steroid Hormone Receptor

The biological effects of steroid hormones are mediated by binding tightly to their specific intracellular receptor proteins, called steroid hormone receptor (2). The steroid hormones are synthesized and secreted by endocrine cells. They travel via the blood stream to their target cells, enter these cells by simple or facilitated diffusion, and then bind to specific steroid hormone receptors. Steroid hormone receptors are members of a large group of ligand-activated proteins (1, 3 and 4). They belong to the superfamily of nuclear receptors. They direct signal transduction systems in which the receptors receive the signal input by binding the hormone and are transformed into their hormone-activated form that has high affinity for DNA. The hormone-receptor complexes translocate from the cytosol to the nucleus and then bind as a monomer or dimer to specific hormone responsive elements (HREs), in the genome, where they alter the transcription rates of specific genes (5-9). The interaction between the receptors and the particular HREs is thought to allow the receptor protein to serve as enhancers or repressors of gene transcription for a wide variety of target genes (10). Specific HREs are known for estrogen, thyroid hormone, progesterone and glucocorticoids. The estrogen response element (ERE) is similar to, but distinct from the glucocorticoid response element (GRE) and thyroid response element (TRE). The progesterone and glucocorticoid responsive elements are very similar and are functionally identical (10). The interaction between the receptor and HRE permits the recruitment of the general transcription

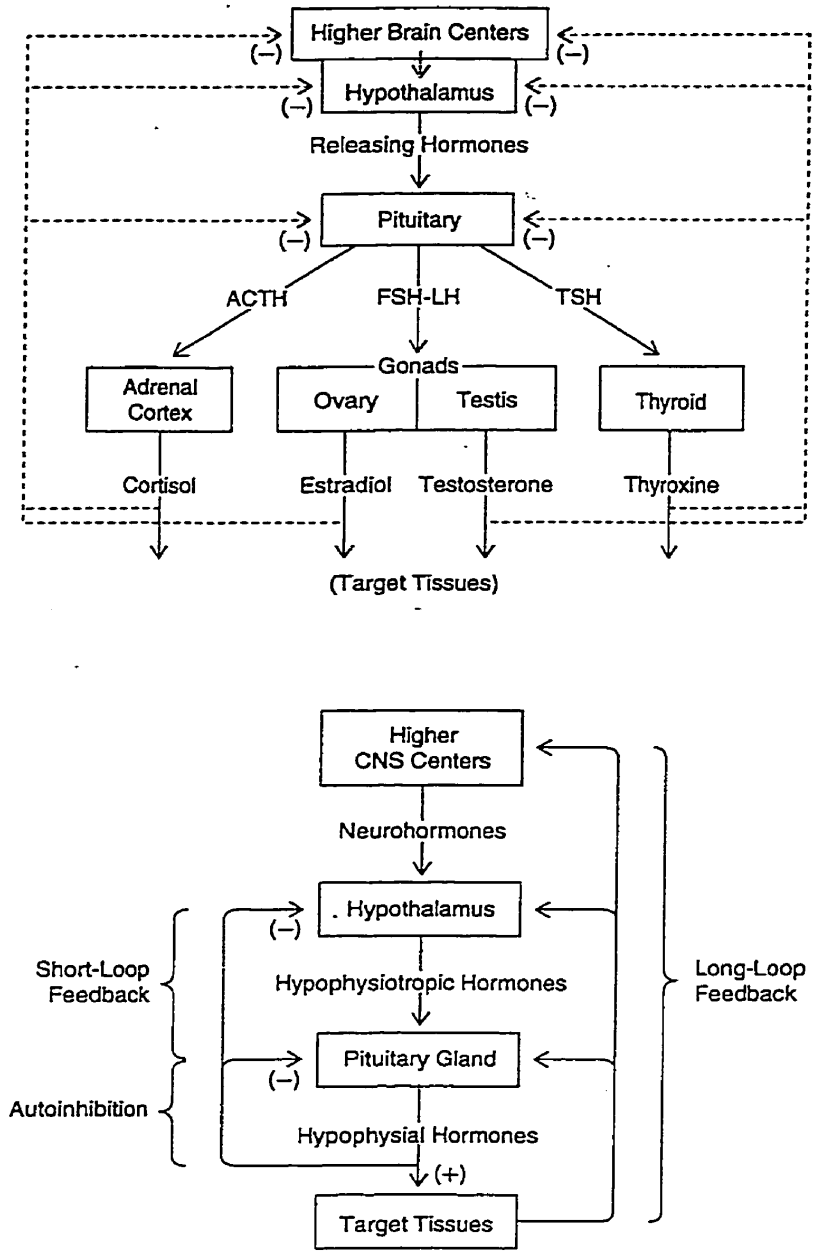


Figure 2: Model of feedback control of pituitary hormone secretion and model of long and short loop mechanism and autoregulation of pituitary hormone secretion

machinery and transcription coactivators through which a remodeling of the chromatin status occurs, in which either enhances or represses gene transcription. It is now recognized that this model is an oversimplification of a much more complex mechanism of steroid hormone action.

The nuclear receptor superfamily consists of a surprisingly large number of genes, and represents the largest known family of transcription factors in eukaryotes. It includes receptors for progestins (PR), form estrogens (ER), for androgens (AR), for glucocorticoids (GR) and mineralocorticoids (MR). In addition, it includes receptors for thyroid hormone (TR), for all-trans retinoic acid (RAR), for 9-*cis* retinoic acid (RXR) and for vitamin D_3 (VDR). Furthermore, cloning by various means has identified a large number of previously unknown genes having sequence homology to steroid hormone receptor superfamily. Since the ligands are not known, they have been termed "orphan receptors". Examples of these are steroidogenic factor 1 (SF-1)/FTZ-F1/AD4BP, DAX-1, nerve growth factor induced gene B (NGFI-B)/Nur77, RORα- β-, RXRα-β-.

The primary sequences of the steroid receptors are known. The typical steroid receptor, exemplified by GR, AR, MR, PR and ER, can be divided into four independent but interacting functional modules: the A/B domain, DNA-binding domain (DBD), the hinge region, and the ligand-binding domain (LBD). The A/B domain displays the most variability both in terms of length and the primary sequence. A large number of transcriptional units encoding nuclear receptors use alternative splicing, different promoters and distinct translation start sites to generate multiple modulator domains, leading to the expression of many receptor isoforms from a single gene (11, 12 and 13). This phenomenon is best exemplified by the family of retinoic acid receptors (14, 15), in which the gene encodes at least eight receptors with similar DNA- and ligand-binding properties but distinct biological functions. This region contains a transcription activation functional domain, referred to as AF-1.

Studies of the estrogen and progesterone receptors have clearly demonstrated that the AF-1 domain has promoter- and cell context-dependent activities, suggesting that the amino terminal region of nuclear receptors may interact with cell specific cofactors (11, 13). Although no significant amino sequence homology exists between any of the members of the superfamily within this domain, unrelated modulator domains have been shown to confer similar properties to distinct receptors. Like the estrogen receptor α- and β-, the AF-1 activity is enhanced through phosphorylation by mitogen-activated protein kinase (MAPK) (16, 17 and 18). This domain can also interact directly with steroid receptor coactivators (SRCs) to enhance the activity of the receptor complex (19, 20, 21 and 22).

The steroid receptors bind DNA as monomer, homodimer and heterodimer (23). Alternative heterodimeric interactions between nuclear receptor have been reported and may be of physiological significance (24, 25 and 26). HREs contain one or two consensus core half-site sequence. For dimeric HREs, the half-sites can be configured as inverted, everted or direct repeats. Steroid receptors bind to the half-site consensus sequence TGTTCT, while the estrogen receptors and other nuclear receptors bind to the half site consensus sequence AGGTCA. For the monomeric HREs, a single half site is preceded by a 5'-flanking A/T rich sequence. Natural HREs rarely contain two perfect consensus half sites. The DBD of the nuclear receptors is the most conserved domain. It is composed two Zinc finger modules encoding by 66-70 amino acid residues and a carboxyl-terminal extension (CTE) that spans approximately 25 residues. On the basis of mutagenesis experiments, the DBD has been further divided into sub-domains involved in direct recognition of the core half-site sequences (P-box) (27) and the dimerization determinants (D box) (28, 29). The CTE play dual roles in providing both protein-DNA and protein-protein interfaces.

The hinge region of steroid receptors is also highly variable in both length and primary sequence. As its name indicates, its main function is to serve as a hinge between the DBD and LBD. The hinge has to be very flexible to let the DBD rotate to allow some receptors to bind as dimers to both direct and inverted hormone respond element (23). This region has also been reported to serve as a docking site for corepressors (30,31).

The LBD domain is a multifunctional domain that mediates ligand binding, dimerization, interacting with heat shock proteins, nuclear localization and transactivation functions. Nuclear receptor LBDs can be defined by a signature motif overlapping with helix 4 (32). In addition, ligand dependent transactivation is dependent on the presence of a highly conserved motif, referred to as activation function-2 (AF-2), localized at the carboxyl-terminal end of the LBD. X-ray crystallographic experiments suggest that LBDs have similar structures: they consist of 300 amino acids which fold 11-13 α-helixes into three layers that bury the ligand binding site within a specific hydrophobic pocket, the core of the LBD (33, 34). Ligand dependent transactivation involves the recruitment of coactivators, a process in which the AF-2 plays an obligatory role and induces a conformational change in the LBD, allowing coactivator to bind (35). The AF-2 motif folds back against the core LBD upon ligand binding, closing the ligand binding pocket and forming a novel interface involving residues from the AF-2 itself and at least three other helices (36). While transcriptional competent interfaces are induced by receptor agonists, binding of antagonists to the LBD leads to the formation of a nonfunctional interface that prevents interaction between the nuclear receptors and coactivator protein (33). The LBD also determines the tight binding of steroid receptor to heat shock protein 90, an association that is critical for the proper folding and functioning of nuclear receptors (37).

1.3. Structure and Function of human GR

Until the last few years, structure studies of steroid receptors have been hampered by the limited availability of pure protein. By the early 1980s, by using high-affinity synthetic analogs of ligands, a number of important receptors were purified by Govindan (GR) (38,39), Green (ER), and O'Malley (PR). Subsequently, cDNA for steroid receptors were identified by screening expression libraries with antibodies that were generated against purified receptors by Miesfeld, Hollenberg, Govindan (GR) (40, 41, 42 and 43), Govindan, Chang, Waiter, Green, Maxwell (ER), Lubahn (AR), Loosfelt (PR) and Arriza (MR). Complete sequencing of cloned cDNAs in several species and in humans has revealed similar primary structures for all steroid hormone receptors. Biochemical studies of these genes or cDNAs of steroid receptors demonstrated that steroid hormone receptors, like many transcriptional regulatory proteins, are polypeptides, which are organized into relatively discrete domains on the structure according to their different functions. These discrete functional domains emerge from the sequence alignments, deletion and site directed mutagenesis and domain swapping experiments (1, 5).

The human glucocorticoid receptor is among the best-characterized eukaryotic transcription factors (44). Several major landmarks have contributed to our understanding of the structure and function of hGR. These include: 1) the synthesis of tritium-labeled steroid with high specific affinity; 2) the demonstration of a high affinity, low capacity binding species, the putative receptor protein for glucocorticoids; 3) the interaction of the receptor with the nucleus or DNA; 4) the demonstration that glucocorticoids can regulate specific genes, their messenger RNAs and their transcription; 5) the purification of the GR complex; 6) the demonstration of specific GR binding sequences within the genome; 7) the demonstration by gene transfer

of glucocorticoid responsive sequences and 8) the cloning of complementary DNA for some steroid receptors. Especially, the purification of the receptor proteins and the cloning of the gene or cDNA have led to the identification of functional domains for hormone binding, DNA binding, and transcriptional activation. Furthermore, sequence analysis revealed a remarkable structural conservation among the hormone receptors.

Our group has developed two rapid and high-yield purification methods for the glucocorticoid receptor based on differential DNA affinity and ligand affinity chromatography, which make it possible to get the antibody of GR and has finally cloned the full length cDNA of human GR gene. We have shown that several amino acids within the LBD of GR are critical for transactivation function of GR.

1.3.1. Structure of human GR

With the cloning human GR gene cDNA, the modular structure of hGR was demonstrated by *in vivo* and *in vitro* experimentation with mutants and chimeric receptors. The hGR can be subdivided into several functional domains (Figure 3). The central DBD is located between amino acids 420-486. It was subjected to detailed analyses using both genetic and biochemical approaches (45, 46). Insertion and point mutations in this domain impair the ability of the receptor to bind to DNA specific sequence (TGTTCT), referred to as the GRE that induce transcription. This domain is the most highly conserved among steroid hormone receptors. And the sequence in this domain is very rich in basic amino acids, such as cysteine, lysine, and arginine, which could facilitate binding to the negatively charged DNA. The arrangement of the basic amino acids within this domain is quite interesting. It is a globular structure that can be subdivided into two

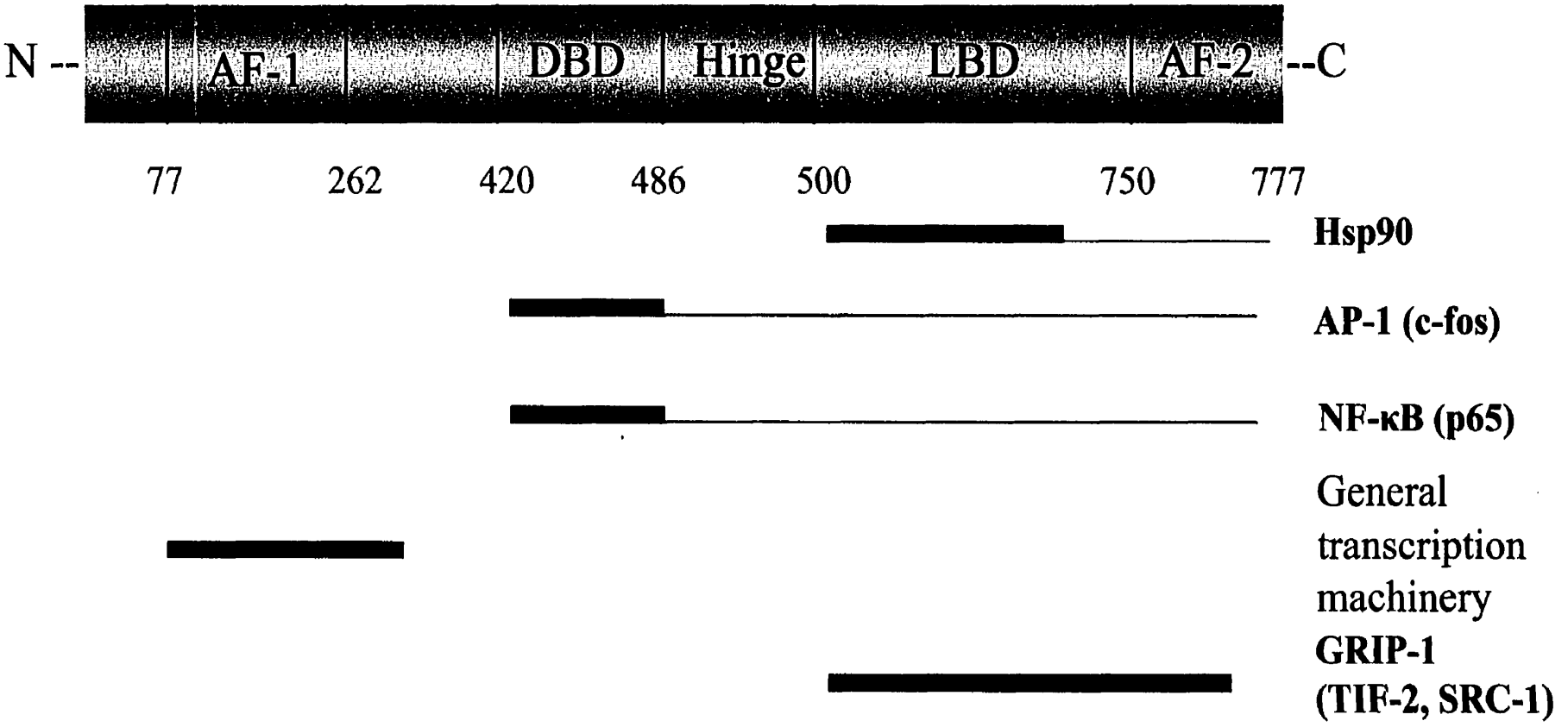


Figure 3: Domain structure of glucocorticoid receptor and GR-interacting proteins
human GR is composed of independent functional domains that including DBD and LBD, the primary function of which are to recognize specific DNA sequences and ligands, respectively. Human GR possess two transcription activation functions (AF-1and AF-2) located at the amino and carboxy termini. Human GR exert its function by interacting with other proteins.

modules. Each module consists of a Zinc-coordinated center and an amphipathic α-helix. The Zinc finger in the GR DBD is quite different from TF III A type Zn finger: in the TF III A Zn-finger, the Zn is coordinate by two cysteines and two histidines, rather than by four cysteines (47, 48 and 49). The first module contains the first Zn finger. It stars with a short segment of anti-parallel β-sheet and ends with a α-helical structure between the second pair of Zn coordinating cysteines. The β-sheet helps to orient the residues that contact the phosphate backbone of DNA. The helical structure (P-box and downstream amino acids) provides important deoxynucleotide contacts and fits into the major groove of the DNA helix. The second module is more important for phosphate contacts and for dimerization (D box between A476 and D481) of the two DBD modules. The two modules form a globular structure through the interaction of aromatic side chains of conserved amino acids in the amphipathic helices (F463, F463, Y497 and Y474).

The LBD located in the carboxyl-terminal half of hGR consists of 300 amino acids, which folds into a complex tertiary structure, creating a specific hydrophobic pocket that surrounds the ligand. The LBD also contains sequences responsible for receptor dimerization, heat shock protein interaction and one of the two transcriptional activation domains (AF-2). The sequence of this domain contains loci that are very rich in hydrophobic amino acids and moderately conserved. The LBD has three functions: 1) repression of transcriptional activating activity of receptor; 2) binding hormone and 3) determining hormone regulation stimulation of receptor function. The LBD also determines the tight binding of GR to heat shock protein 90, an association that is critical for proper folding and functioning of receptor. Site mutation studies showed that cysteines in LBD fulfill an important function in GR by facilitating the interaction of specific ligands. This process involves direct interaction with factors such as heat shock proteins by maintaining a tight hydrophobic pocket, which prevents access of

inappropriate ligands in circulation. Conservation of cysteine 665 regulates the interaction of glucocorticoids and mineralocorticoids with hGR, thus defining tissue and ligand specificity in corticosteroid action (50, 51). Cysteine 638 plays a role in limiting the agonist and antagonist occupancy as well as in determining the level of transcription activation induced by individual ligands. The glycine at position 567 is crucial for normal receptor function, since substitution mutant G567A exhibits dramatic change in function. It fails to bind cortisol and RU486 and has absolutely no affinity for ALDO in CV-1 transfected cells.

The N-terminal region of the hGR contains the transcription activation domain (AF-1), which is mapped between amino acids 77 and 262 (52, 53). This domain has the least homology with other members of steroid receptors both in length and in sequence. It activates reporter gene when fused to GR or heterologous DBD. Deletion analysis reveals that a 58-amino-acids core domain between residues 187 and 244 is almost as active as intact AF-1 domain and squelches basal transcription to comparable level (54). This inhibition is thought to result from protein-protein interaction with components of general transcription machinery, coactivator and other transactivator. Using nuclear resonance spectroscopy, the structural studies had shown that AF-1 domain is largely unstructured in aqueous solution. Recently, a number of proteins have been identified that modulate GR activity such as GRIP1, TIF-2, SRC-1 and P300/CBP. But the only proteins that have been shown to be direct targets for the AF-1 domain are TBP and the ADA2 protein. TBP is the DNA binding subunit of the TFIID coactivator complex (55). TFIID has the capability to phosphorylate other components of the transcription complex and to acetylate histones. ADA2 is a subunit of the ADA adapter complex that can enhance interaction between some activation domains and TBP. This complex also has histone acetyltransferase activity.

An additional region involved in transactivation has been mapped to the ligand-binding domain and is called the AF-2. This region is highly conserved within the family of steroid hormone receptors and forms part of an amphipathic α-helix that is thought to undergo a dramatic conformational rearrangement when the receptor binds ligand. A novel approach, in which the GR cross linked to a ^{32}P-labelled GRE DNA sequence was used as a probe, identified a protein of 170 kDa (GR interacting protein GRIP 170), which co-purified with an activity that gave a modest but reproducible stimulation of GR-induced transcription (56). In addition, the GR has been reported to interact with the CREB-binding protein (CBP) in a ligand dependent manner (57). GRIP-1 belong to a family of related proteins that have been identified as binding to the PR and ER and termed nuclear coactivator family, which includes SRC-1 (steroid receptor coactivators-1) (58) and TIF2 (transcriptional intermediary protein 2) (59)

1.3.2. Function of human GR

In the absence of the hormone, hGR is predominantly located in cytoplasm forming complex with other proteins, notably hsp 90 and 70. Upon binding glucocorticoid hormones, the complex dissociates and the receptor enters the nucleus, dimerizes and binds the GRE. To modulate its target genes, hGR interacts with various components of the general transcription machinery, coactivators, corepressors and cointegrators P300/CBP. The recruitment modulates the chromatin structure to allow or exclude the assembly of other gene regulatory protein and or general transcription machinery on the DNA (Figure 4).

To regulate target gene transcription specificity, two aspects have to be considered. One is the cell context. Different genes are turned on in

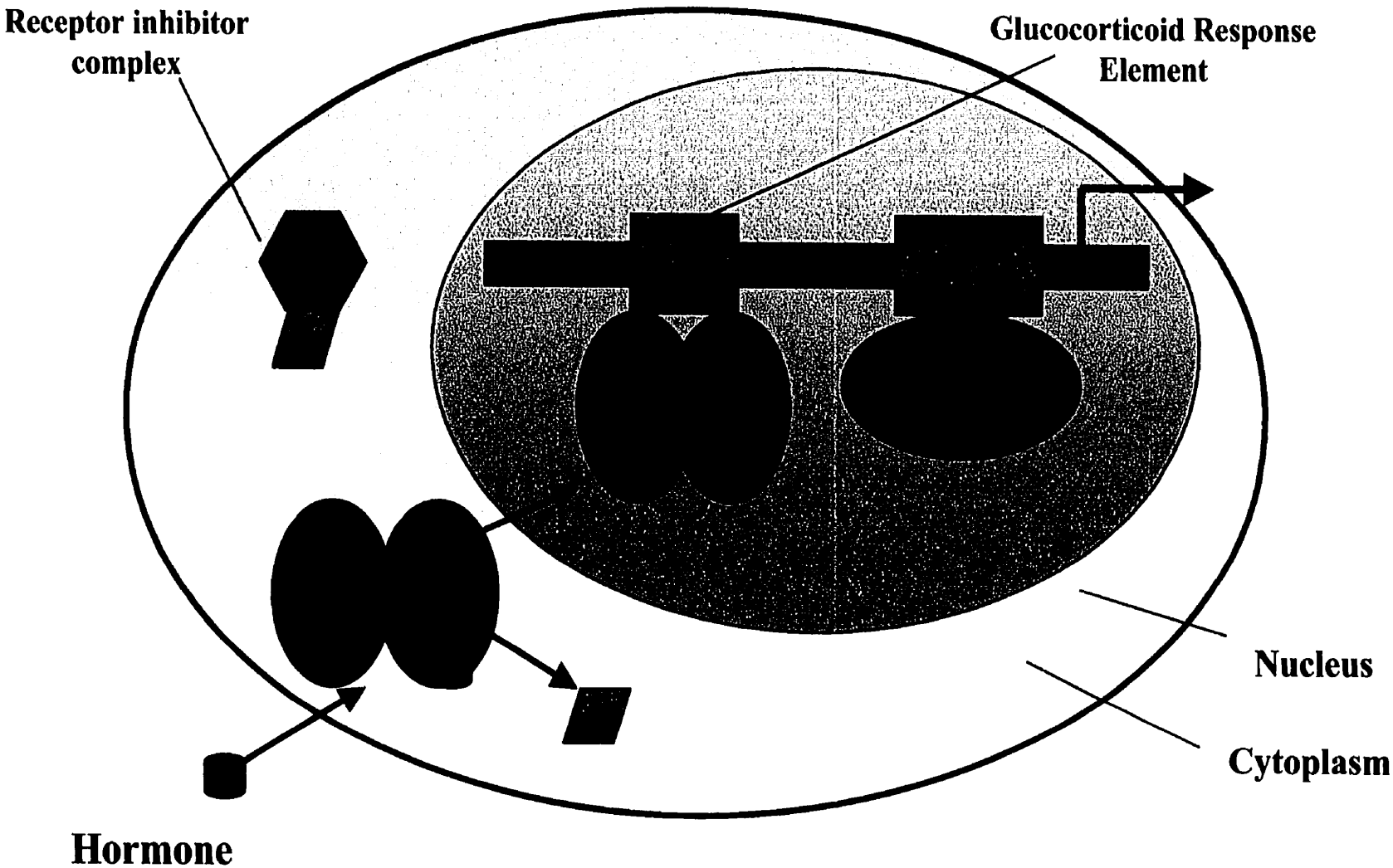


Figure 4: Model for glucocorticoid hormone action. In the absence of the hormone, hGR is located in cytoplasm forming complex with proteins such as hsp90. Upon binding glucocorticoids, GR transforms into active state, dissociates with the inhibitors, forms homodimers and translocates to nucleus, where it binds GRE. To modulate its target genes, hGR interacts with the general transcription machinery and coactivators. The recruitment modulates the chromatin structure, thus either enhance or repress target gene transcription.

different cells and different tissues. That means each phenotype of cells has its own series of transcription activators and coactivators. Second, different genes have different sequences in its transcription regulation region. The response elements can be ligands for regulators, acting as allosteric effectors that determine the regulators' conformation (60). A given regulator may activate transcription in the context of one gene, repress transcription in another and bind but exert no regulatory effects in a third. Hence DNA acts as an allosteric ligand whose binding alters the regulator's affinity for other ligands, such as coactivators or corepressors. If the regulator assumes distinct conformations on binding to different genomic sites, then the DNA sequence of the response element can specify which surfaces are available to contact target factors and thereby determine how the protein regulates transcription at that site. So when the receptor binds to GRE with the major groove in the DNA helix, the target DNA acts as an allosteric regulator of recognition by providing a scaffold to bind the subunits in the correct position for dimerization and by stabilizing substructure that support a strong dimer interface.

Stimulation of eukaryotic transcription by RNA polymerase II requires reconstruction of chromatin and the ordered assembly of a pre-initiation complex at the promoter of the regulated gene (61). The pre-initiation complex contains basal transcription factors, RNA polymerase II and additional accessory factors such as coactivators. GR, as a transcriptional factor, may function at one or more steps in the processes of transcriptional initiation and elongation. One important way in which many activators are thought to operate is by interacting with components of the transcriptional machinery to recruit them either to the promoter or to promote or stabilize the binding of the general transcription factors to DNA. Interaction may occur directly between basal transcription factors and GR and or between GR and coactivators or adapters, which form a bridge between the GR and the basal factors. It is recently reported that purified transcriptional factor TFII

D could enhance the N-terminal of GR activity by 9-fold in Hela nuclear extract (62). TF II D is a multiple protein complex containing TATA- box binding protein (TBP) and tightly associated factors termed TBP associated factors (TAFs). TF II D has the capability to phosphorylate other components of transcriptional complex and to acetylate histones in addition to its established role in specifying site of transcription complex formation. The N-terminal domain of GR has been shown to interact directly with TFIID complex and in particular interact with TBP. This interaction was markedly identified by a mutation in the AF-1 domain. The interaction between AF- 1 of GR and TF II D is specific, since no interaction was seen with TF III B, an analogous protein complex involved in RNA polymerase III.

Understanding of the mechanism of AFs function has been recently enhanced by the discovery of a family of 160-kDa proteins that bind in a hormone-dependent manner to the LBD of GR and thereby enhance transcriptional activation (63). This family consists of three genetically distinct but structurally and functionally related proteins: SRC-1/NcoA-1 (64), TIF2/GRIP1/NcoA-2 (65) and P/CIP/ACTR/RAC3 (66). Members of the P160 family share ~40% sequence identity with regions of high and low homology interspersed across their 1400 amino acid length. These transcriptional coactivators do not enhance the expression of genes by themselves because they do not bind to DNA and are not components of the basal transcription machinery. Rather, they are recruited to the promoters through their contacts with the NRs. The P160 coactivators can bind, and may exist in a complex with two other types of coactivators: CBP or its homologue P300 and P/CAF (P300/CBP-associated factor) (67). All three coactivator families help to mediate the activities of GR and all three contain histone acetyltransferase activities that may contribute to their functions as coactivators by locally affecting chromatin conformation (67). CBP can also bind to components of the basal transcription machinery (68). The ability of P160 coactivators to interact with GR LBD is essential for their coactivator function (69), which is

presumably accomplished by serving as adapters that link AFs in GR LBD with components of chromatin and the transcription machinery. Each P160 coactivator molecule contains three leucine-rich motifs (LXXLL, where *X* is any amino acid), called NR boxes, clustered in the central region of the polypeptide chain (70, 71), called the NR interaction domain (NID). Each NR box motif is capable of binding specifically and in a hormone-dependent manner to GR LBD, but the NR binding preferences of each NR box are somewhat different (71). Thus, although no single NR box motif has a universal ability to bind all nuclear receptor LBDs efficiently, the multiple motifs presenting in the P160 proteins collectively contribute to the broad NR binding specificity. P300 and its family member, CBP function as key transcriptional coactivators by interacting with the activated forms of certain transcription factors (72). Over-expression of P300/CBP potentiates ligand dependent transcriptional responses of receptors shown to interact with P300/CBP. Over-expression of CBP relieves AP-1 antagonism by glucocorticoid receptors, consistent with the hypothesis of competition between AP-1 and nuclear receptors. Microinjection of antibody of P300/CBP blocks ligand dependent transcription activation of GR, RAR and RXR. In a search for additional cellular targets of P300/CBP, a protein-protein cloning strategy identified SRC-1 as a P300/CBP interactive protein. P300 and SRC-1 interact, specifically, *in vitro* and they also form complexes *in vivo*. P/CAF was discovered on the basis of sequence homology to the yeast GCN5 protein. It contains an extended amino terminus, which enables it to interact with CBP and members of the NcoA family (73, 74). It is found that P/CAF is part of a P300/CBP/NcoA complex that is implicated in nuclear receptor actions. Although P300/CBP is required for the functions of many transcription factors, the role of P/CAF and NCoAs appear to more selective, implying that different combinations of coactivator complexes may determine the transcription factors specificity.

GR also regulates gene transcription via direct interaction with other transcription factors, a process that does not depend on DNA binding. A good example is the metalloprotease as the target gene of glucocorticoid repression. The repression is not dependent on protein synthesis, but caused by interfering with AP-1 activity by direct protein interaction (75). Point mutations in the DNA binding domain have shown that the repression of AP-1 activity and the transactivation functions of GR are separable entities. DNA binding and activation of glucocorticoid-regulated promoters require GR dimerization, while repression is a function of GR monomers. A more complex picture has emerged from GR/AP-1 interaction on proliferin gene, where the target sequence for the receptor is an overlapping GRE and AP-1 response element. The GR has a positive effect in the presence of c-jun homodimer and a repression function with c-fos/c-jun heterodimer. The sequence in the N-terminal of the receptor was reported to be important for GR dependent repression in this case. Glucocorticoid is also known to inhibit transcription of key genes involved in the immune response such as interleukin IL–6 and –8. The region shown to be critical for glucocorticoid mediated down regulation of the IL-6 promoter was mapped to a NF-κB response element. NF-κB is heterodimer of 50 kDa (p50) and 65kDa (p65/rel A) subunit (76). GR inhibits NF-κB binding to DNA in a way that the DBD of GR interacts directly with p65 subunits. It has recently been reported that some of the negative effects of glucocorticoids are dependent upon protein synthesis and glucocorticoids induce the production of the inhibitor protein IκB, which keeps NF-κB from entering the nucleus. Moreover, recent experiments with mutant mouse carrying DNA-binding deficient GR demonstrate that development and survival of these mice do not require HRE-mediate gene regulation (77).

1.4. Structure and Function of Orphan Receptor

Once investigators realized that nuclear receptors shared extensive homology at the amino acid and nucleotide sequence levels, a search for new members was undertaken using low-stringency screening of cDNA libraries with well conserved DBD fragments as probe. This cloning exercise led to two unexpected results. First, individual ligands, such as T3 were shown to regulate development and physiology through multiple receptors. This finding was first exemplified by the characterization of a second receptor for T3. Second, the search for new members of the superfamily led to isolation of multiple cDNAs encoding proteins with structural features found in nuclear receptors. However, since ligands could not be linked to these putative receptors based on structural studies alone, these new members of the superfamily were referred to as "orphan nuclear receptors". So the orphan nuclear receptors are defined as gene products that embody structural features of nuclear receptors, which were identified without any prior knowledge of their association with a putative ligand.

The vast majority of orphan nuclear receptor possesses all functional domains that characterize classic nuclear receptors. Some receptors have a very short modulator domain, and therefore lack AF-1, while Rev-Erbα and -β lack the conserved AF-2. In addition, the orphan nuclear receptor superfamily includes members possessing either a conserved DBD or LBD, but not necessarily both in the same molecule. Both DAX-1 and SHP lack a nuclear receptor-like DBD, but these proteins can bind DNA using these unrelated domains. DAX-1 has been shown to bind hairpin loop structures in DNA via its unique amino-terminal domain (78).

With the exception of the DAX-1, orphan nuclear receptors recognize specific HREs through their well-conserved DBD in a manner similar to that of

classic nuclear receptors. Functional studies of orphan nuclear receptors have considerably changed previously held dogma on how nuclear receptors can bind DNA. First, identification of RXR led to the discovery that a large subset of classic and orphan nuclear receptors binds DNA as heterodimer (79). Second, several orphan nuclear receptors can bind DNA with high affinity as monomer (80). Monomeric nuclear receptors utilize the CTE to recognize distinct A/T rich sequences located upstream of a single core half site. The CTE-DNA interaction provides additional protein-DNA contacts in monomeric sites necessary for specific and high-affinity binding (81). The distinct amino-terminal domains contained in orphan nuclear receptor RORα have been shown to interact with a common CTE to regulate the receptor's binding site specificity. The hinge and amino-terminal domain appear to orient the zinc finger modules and the CTE relative to each other and are required to achieve proper interactions with core AGGTCA half site and the specific A/T rich moiety.

All vertebrate orphan nuclear receptors possess a highly recognizable LBD. The presence of a conserved LBD is often interpreted as a strong indication that all vertebrate orphan nuclear receptors possess the intrinsic ability to bind a specific ligand. However, since the LBD mediate multiple functions (such as dimerization and coactivator interaction), its presence may only be required for those activities, which could be regulated via covalent modifications or protein-protein interaction. Moreover, widely phylogenetically divergent receptors can bind similar ligands, suggesting that the ligand-binding function of the nuclear receptors has evolved independently several times during evolution.

1.5. Structure and Function of SF-1

SF-1 was initially characterized because of its capability to bind to conserved AGGTCA consensus motif in the proximal promoter regions of the cytochrome P450 steroid hydroxylases. Because the DNA-binding site for SF-1 was strikingly similar to the binding site of other members of nuclear hormone receptor, Lala et al (82) used a probe comprising the DNA-binding domain of RXRβ to screen a mouse cDNA library. While Honda et al (83) purified the corresponding protein by oligonucleotide affinity chromatography, determined partial peptide sequence and screened the cDNA library. In each case, isolated cDNA clones were expressed in transient assays and were found to enhance promoter activity of the steroid hydroxylases.

Several naturally occurring variants of SF-1 are produced by the FTZ-F1 locus. A second FTZ-F1 homologue, termed embryonic long terminal repeat-binding protein (ELP), was isolated from marine embryonic carcinoma cells. Recently, additional isoforms of ELP-1, ELP-2 and ELP-3 were cloned from the same cell line. It is now recognized that each of the ELP-isoforms along with the SF-1, are transcribed from the single FTZ-F1 gene as a result of alternative promoter usage and differential splicing. The transcripts of ELP-3 and SF-1 differ in their 5'-untranslated regions, but encode an identical SF-1 protein. In contrast, ELP-1 and ELP-2 contain additional 77 amino-terminal amino acids relative to SF-1. ELP-2 and SF-1 are otherwise identical, whereas the carboxyl terminus of ELP-1 is 74 amino acids shorter reflecting alternate splicing (84).

SF-1 cDNA shares conserved regions that correspond to known functional domains of other members of nuclear receptor superfamily. It contains two zinc finger modules. The proximal (P) box in the first zinc finger, the distal

(D) box within the second zinc finger and the intervening linker region mediate DNA binding. Interestingly, the P box is a hybrid one combining residues characteristic of GR and ER subclasses of nuclear receptor. It interacts as monomer with CAAGGTCA recognition motif. SF-1 also has an additional 30 amino acid carboxyl terminal extension adjacent to the second zinc finger motif, referred to as the A box, which recognizes additional bases 5' to the CAAGGTCA motif. These 30 amino acids comprising an A-box are conserved absolutely in all mammalian species (81).

SF-1 contains a transactivation domain AF-2 in the C-terminal region homologous to the ligand-binding domain of ligand-activated nuclear receptor (85). In particular, the AF-2 domain is found at the carboxyl terminus of many ligand inducible nuclear receptors forming an amphipathic α-helix that apparently is essential for transcriptional activity. This region is conserved absolutely in all SF-1 proteins. This domain has been demonstrated to form complexes with SRC-1 and P300/CBP *in vivo* and *in vitro* (85). In hinge region, there is a conserved stretch of seven consecutive prolines that lie within a proline-rich domain (amino acid 124-226). This domain has been proposed to mediate transcriptional activation by SF-1. This region is also referred to as the AF-1 domain (86). Another motif near the C-terminal region of SF-1 (amino acid 427-430) is a potential consensus site for phosphorylation by cAMP-dependent protein kinase (87). This sequence may provide a mechanism for interaction between the cAMP-dependent signaling pathway and SF-1 transcriptional activation (Figure 5).

Expression of SF-1 is associated with the precursors of adrenal steroidegenic tissue and gonadal steroid-producing cells. Gene knockout experiments provide strong evidence for a direct role for SF-1 in regulating mammalian sexual development as well as differentiation of steroidegenic tissue (88). SF-1 usually constitutively activates gene expression, and its activity is regulated by phosphorylation: *in vitro*, protein kinase A-induced

Figure 5: Domain structure of steroidogenic factor 1. Human SF-1 composed of similar function domains as other members of nuclear receptor superfamily. DBD domain contains a hybrid P box, which combines residues characteristic of GR and ER subclasses of nuclear receptor. It interacts as monomer with CAAGGTCA recognition motif. While additional 30 C-terminal amino acids of the second Zinc finger recognize additional bases 5' to the AGGTCA hexamer. It contains a transactivation domain AF-2 in the C-terminal region homology to the ligand-binding domain of ligand-activated nuclear receptor. In the hinge region, seven consecutive proline stretches mediate transcriptional activation. This region is referred as the AF-1 of SF-1. Conserved motif near C-terminal region has a potential consensus site for phosphorylation by cAMP-dependent protein kinase.

phosphorylation of SF-1 reduces the receptor's binding affinity; while *in vivo*, SF-1 phosphorylation may regulate cAMP-dependent gene induction (87). In addition, phosphorylation of AF-1 in the hinge region increases SF-1 transcription activity via direct recruitment of the coactivator GRIP-1. While 25-hydroxycholesterol has been shown to potentiate SF-1 activity, no high affinity specific ligand has been isolated (89). Elimination of the putative AF-2 domain at the carboxyl terminus of SF-1 resulted in complete loss of transactivation function. Several lines of evidence demonstrated that SF-1 interacts with SRC-1. Full length SRC-1 enhances SF-1 mediated transcription activation, whereas a dominant negative form of SRC-1, consisting of its interaction domain alone, inhibited the activity of SF-1 (85). In mammalian two hybrid assays, fusion of VP16 activation domain to the interaction domain of SRC-1 confirmed the interaction between SRC-1 and SF-1 and demonstrated that the AF-2 domain is required for interaction with SRC-1. Furthermore, SRC-1 together with CBP or a closely related factor, P300, synergistically enhanced transcriptional activity of SF-1.

Another orphan nuclear receptor, DAX-1, is expressed in a pattern overlapping with SF-1, an observation that led to the suggestion that the two receptors may cooperate in the development of the HPA. DAX-1 is an atypical orphan receptor, harboring a conserved LBD like domain (90). It possesses an amino terminal domain that has repetitive regions rich in glycine and alanine residues. It has been found that DAX-1 can antagonize SF-1 transcriptional activity by interacting with a repressive domain within the carboxyl terminus of SF-1. Further, DAX-1 can recruit nuclear receptor corepressors NCoR to SF-1 (91). Recently, SF-1 has been shown to interact functionally with other transcription factors. SF-1 synergizes with the estrogen receptor to stimulate expression of the gonadotropin II β- subunit gene (92). SF-1 and Sp1 also function cooperatively in the transactivation of cholesterol side-chain cleavage (Cyp11A1) gene promoter.

SF-1 is a homologue of the *Drosophila* orphan nuclear receptor, *fushi tarazu factor* 1 (FTZ-F1), a transcription factor that regulates *fushi tarazu* homeobox gene expression during early development (93). The SF-1 gene is expressed constitutively in specific endocrine tissues: the hypothalamus, anterior pituitary, adrenal gland, gonads, and placenta. The importance of SF-1 is underscored by its ability to regulate the expression of many genes characteristic of these tissues, such as the gene of steroidogenic enzymes and the steroidogenic acute regulatory protein. In mammals, SF-1 plays a key role in the development and differentiated function of the adrenal gland and gonads. Disruption of the FTZ F-1 locus in the mice precludes the development of the adrenal gland and gonads. Genetic males appear sex reversed because of the absence of male external genitalia and preservation of *Mullerian* structures. These mice also have abnormal gonadotropin production, apparently reflecting a role for SF-1 in the development of the ventromedial hypothalamus and function of the pituitary gonadotropes. This array of physiological effects of SF-1 parallels its expression in the hypothalamus and gonadotropic cells in the pituitary.

1.6. Gene Structure and Regulation of Expression

The phenotype differences that distinguish the various kinds of cells in higher eukaryotes are largely due to differences in the expression of genes that code for proteins, that is, those transcribed by RNA polymerase II. In principle, the expression of these genes might be regulated at multiple stages. So does the transcription of these genes. It could be regulated in a gene specific way at any one of several sequential steps: (activation of gene structure---initiation of transcription---processing the transcript---transport to cytoplasm---translation of mRNA).

The existence of the first step is implied by the discovery that genes may exist in either of two structural conditions. Relative to the state of most of the genome, genes are found in an "active" state in the cells in which they are expressed. Transcription of a gene in the active state is controlled at the stage of initiation, that is, by the interaction of the RNA polymerase with its promoter. For most genes, this is a major control-point; probably it is the most common level of regulation. The primary transcript is modified by capping at the 5' end and also by polyadenylation at the 3' end. Intron must be spliced out from the transcripts of the interrupted genes. The mature mRNA must be exported from the nucleus to the cytoplasm and translated to protein.

But having acknowledged that control of gene expression can occur at multiple stages and that production of RNA cannot inevitably be equated with production of protein, it is clear that the overwhelming majority of regulatory events occur at the initiation of transcription. A regulatory transcription factor serves to provide common control of a large number of target genes.

Promoters for RNA polymerase II contain a wide variety of short *cis*-acting elements, each of which is recognized by a *trans*-acting factor. The *cis*-acting elements are located upstream of the initiation site and maybe present in either orientation and at a variety of distances in regard to the initiation site. The *trans*-acting factors, upon binding to the *cis*-elements, interact with the components of the general transcription machinery or interact with coactivators. So a gene is regulated by a sequence at the promoter or enhancer that is recognized by a specific protein. The protein functions as a transcription factor needed for RNA polymerase to initiate. Active protein is available only under conditions when gene is to be expressed. The activity of the inducible transcription factors may be regulated in several ways, such as expression in specific cell context; modification by phosphorylation or

dephosphorylation; being activated by binding ligand; release from silence states and translocation from cytoplasm to nucleus.

In a eukaryotic cell, there are three different RNA polymerases I, II and III, each dedicated to the transcription of different sets of genes with characteristic promoters. These promoters are recognized by two types of transcriptional factors: the basal or general transcription factors (GTFs), which interact with the core promoter elements and the modular or sequence specific transcriptional factors, which generally interact with sequences located further upstream. GTFs are sufficient to determine RNA polymerase specificity and to direct low levels of transcription, whereas the specific sequence transactivator acts by enhancing or reducing the basal level transcription (94). The initiation step of transcription mediated by RNA polymerase II is the binding of TFIID to the promoter at a short distance from the transcriptional site. TFIID is a multi-protein complex composed of TATA binding protein (TBP) and the highly conserved TBP associated factors (TAFs). After TFIID binding, it is that of TFIIB, a GTF with affinity for single-stranded DNA, which apposes to sequence adjacent to the TATA box in response to a critical change in DNA topology induced by TBP. Recruited by TFIIB of another GTF, TFIIF is followed by binding of RNA polymerase II (Figure 6).

1.7. GR Gene Autoregulation

The cellular glucocorticoid receptor concentration is modulated by a number of factors, including glucocorticoids, which autoregulate their own receptors. Regulation of hGR expression, especially the feedback control of hGR, is an important aspect of physiologic control since the sensitivity of target cells to a hormone signal is directly related to receptor concentration. Steroid

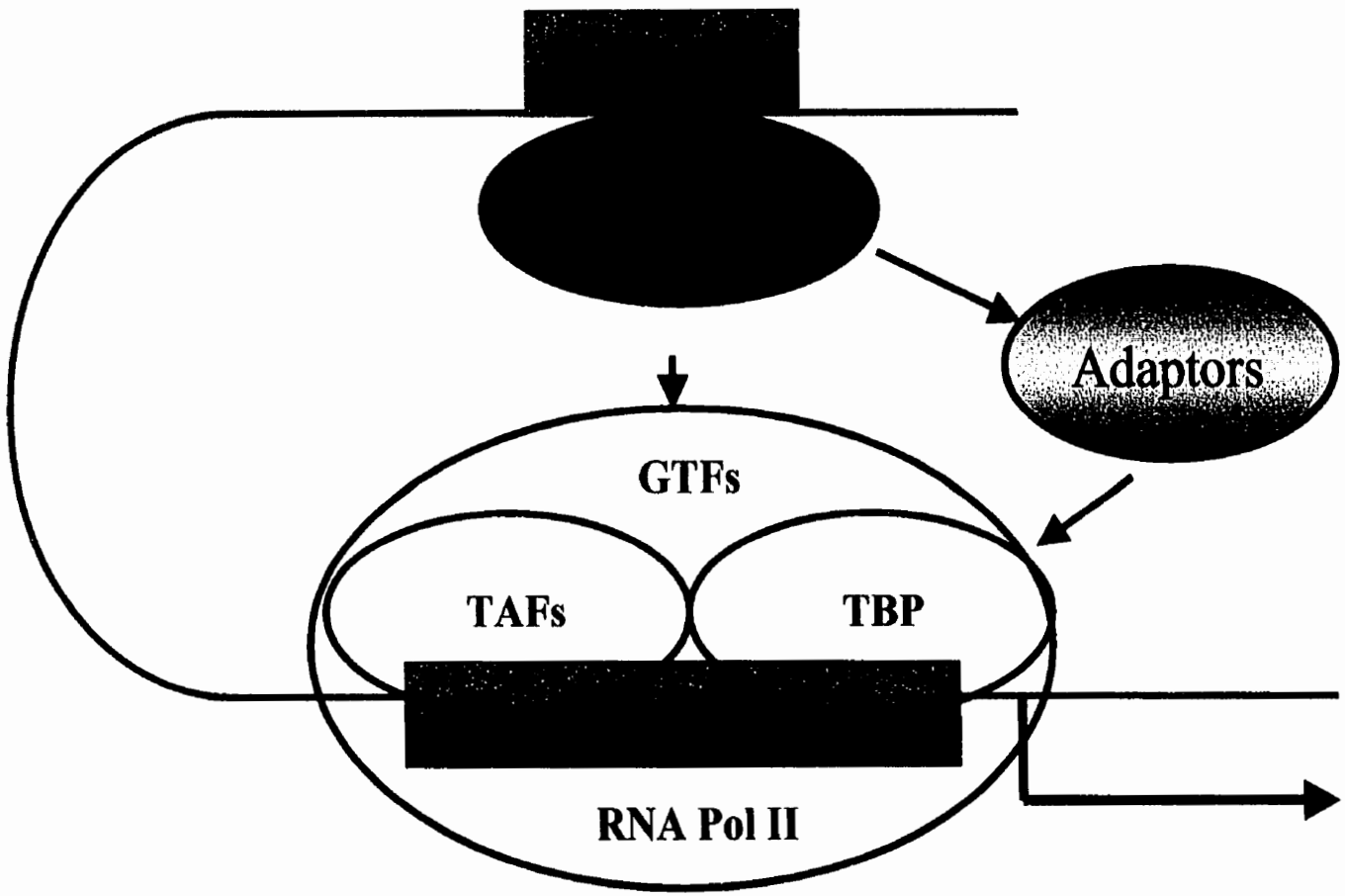


Figure 6: Gene transcription regulation Transcription factors recognize *cis*-element within the regulatory region of target genes. They could interact with general transcription factors as well as other transcription coactivators. These protein complex modulates the activation state of chromatin, thus either enhance or repress gene transcription.

receptors are known to undergo homologous downregulation after exposure to cognate ligands both in cultured cells and in animals. This reduction in cellular receptor levels leads to insensitivity upon subsequent hormone administration. The GR represses its own synthesis in a hormone dependent manner. This repression is restricted to ligands of GR, is dose- and time-dependent, is reversible upon hormone withdrawal and requires a functional receptor.

By using Northern blot hybridization and quantitative slot blot hybridization, it has been shown that within the two different cell lines (human IM-9 lymphocytes and rat pancreatic acinar AR42J) glucocorticoid treatment led to approximately 50% decrease in the steady state glucocorticoid receptor mRNA levels (95). This decrease occurred with a half time of 3h for IM-9 cells and 6h for AR42J cells. Measurement of GR mRNA levels by slot blot hybridization demonstrated that a tissue-specific difference in glucocorticoid receptor mRNA accumulation was found after adrenalectomy; dexmamethasone treatment resulted in a consistent decrease 40-60% glucocorticoid receptor mRNA in all tissues (96). Furthermore, glucocorticoid treatment of cells expressing transfected hGR cDNA resulted in downregulation of hGR mRNA in the presence of cycloheximide or actinomycin D. This suggests that a glucocorticoid inducible protein was not essential for the down regulation. Both the agonist Dex and the antagonist RU 486 can lead to decreased transcription of GR cDNA, as assessed by nuclear run-on experiments. In addition, a mutant hGR that is incapable of binding to a GRE was unable to autoregulate its expression, suggesting that DNA binding is a crucial event in this process. Positive autoregulation of GR expression was also observed in the glucocorticoid sensitive human leukemia T cell line 6TG1.1 and in the human B-cell line IM-9.

It has been shown that transfected human GR cDNA contains sufficient genetic information to recapitulate the downregulation of both hGR mRNA

and receptor protein observed *in vivo* (97). Using immuno-precipitation assay, it was demonstrated that GR could specifically interact with a cDNA insert corresponding to the far 3' non-translated end of GR cDNA (98). This observation is further substantiated by DNAase-I protection assay. This particular fragment contains three copies of the degenerate consensus sequence of AGAACA. It is suggested that down regulation of GR mRNA by glucocorticoid hormone could be caused by interaction between GR protein and its own gene in analogy to the model of positive gene regulation by glucocorticoids. It is also possible that this interaction could alter the rate of GR mRNA degradation. It was reported that the fragment from +527 to +1526 within the GR cDNA could interact with GR protein, which is partially responsible for the observed down-regulation. The phosphorylation status of the receptor was shown to have an effect on the repressive function of the GR. Webster et al (99), showed that a mouse GR, in which all of the phosphorylation sites had been abrogated was no longer able to down regulate its own synthesis.

The 5' region of hGR gene was first isolated by Govindan from a human leukocyte genomic library in λEMBL3 (100). DNA sequencing analysis showed that the promoter is extremely rich in G+C (72% GC content), which is known to be involved in the regulation of many housekeeping genes such as GR as well as a number of cellular oncogenes. There are three repeats of "CCGCCC" and eight repeats of "GGGCGG", enriching the promoter region with 11 Sp-1 binding sites. It contains a "TAATA" box and a "CAT" box and two "CACCC" motifs. Although the perfect "TATAA" box is not present in the promoter region, it occurs downstream in intron 1. It also contains a motif similar to the GRE, which included two interchanged nucleotides "TCTTGT". By using primer extension and S1 nuclease mapping, it is shown that exon I of hGR gene contains a major part of 5' non-coding sequences of hGR mRNA and exon II contains coding sequences for the first 394 amino acid residues of the modular region of hGR. The major transcription initiation site was

found to be 134 bp upstream of the ATG initiation codon. On the transcription level, deletions performed on GR promoter constructs fused to the chloramphenicol acetyltransferase (CAT) reporter gene suggest that a site important in the hormone-mediated downregulation of GR gene expression is located at position –470 to –1030. This contains a putative AP-1 site (-893 to –899). Cross talk between the GR system and the Jun/Fos system signal pathway can occur through transcriptional interference. The region between –245 and –750 located 5' to mRNA start site is characterized by the presence of three binding sites (-406, -566 and -718) for GCF-1, a transcription repressor. The GCF binding site is also recognized by Sp-1, which regulates gene expression positively. Another 95-kDa protein GRF-1, a repressor of hGR gene, interacts with a similar specific region in hGR gene promoter and is up regulated by glucocorticoid (101). Two other DNA binding factors have been isolated by DNA affinity chromatography. These two factors show immunological similarities with autoantigen Ku with apparent molecular masses of 80 and 62 kDa. They form heterodimers and increase the transcription efficiency of hGR gene by interacting with the sequence motif GAAGTGACACACTTC at –892/-878 upstream of the initiation site of hGR gene (102).

ERR-2 (estrogen receptor related protein-2) has been shown to function as a potent repressor of transcriptional activity mediated by glucocorticoid receptor. Transient transfection of different cell lines with a steroid responsive reporter plasmid and receptor expression plasmid revealed that transcriptional activity mediated by GR in response to agonists was strongly suppressed by co-expression of ERR-2. The orphan receptor displayed no promoter activity when expressed without GR. The inhibitory activity of ERR-2 is cell-specific and also receptor specific because transactivation mediated by the progesterone receptor is unaffected by ERR-2 (103).

CHAPTER 2

OBJECTIVE

As described in details in chapter I, homologous down regulation of glucocorticoid receptor is a complex procedure and it can be at transcriptional level as well as the post-transcription and post-translation level.

The objective of my present investigation is to study the transcriptional regulatory elements present in the regulation region on hGR gene at the transcriptional level and subsequently to analyze promoter activity. Although most of the studies carried out earlier were within 1 kilo base upstream of the hGR initiation site, until now no one has located functional GREs, which are responsible for the autoregulation of human GR gene. Thus I wished to find the *cis*-acting elements further upstream which homologously downregulate hGR gene and tried to locate the functional GRE. Also because several earlier observations had shown that GR could regulate transcription by protein-protein interaction, which is not dependent on DNA binding, I wished to find out the possible existence of other transcription factors that could mediate glucocorticoid effects on GR gene expression.

CHAPTER 3

MATERIAL AND METHOD

3.1. Plasmid Construction

The PCR kit, "Expand High Fidelity PCR System" (Boehringer Mannheim), was used to amplify the various lengths of the 5'-regulatory region of hGR from the human leukocyte genomic library. The advantage using this kit is that the Expand High Fidelity PCR System consists of a mixture of enzymes thermostable Taq DNA and Pwo DNA polymerases, which give the PCR product high yield, high fidelity and high specificity from the genomic DNA template. It is especially optimized to amplify most efficiently DNA fragments up to 5 kilo bases from genomic DNA.

The genomic DNA library is constructed using human leukocyte DNA, which is partially digested with MboI and inserted at the BamH I site of the λEMBL-3. This phage genomic DNA library was amplified and the DNA was extracted for use as template. The primers were designed according to the sequence of the GR promoter with restriction digestion site BamH I at both ends to facilitate further subcloning.

The sequences of the adapter-primer used are as follows:

1) GGAATTCCGGATCC -2692 GGGCTATTTAACTCAC
2) GGAATTCCGGATCC -2677 GTGAGTTAAATAGCCC
3) GGAATTCCGGATC -1786 CGTCCAAGCCTTCCCCGACGC
4) GGAATTCCGGATC -1627 CTAGGAGATGTTCCTGTTAG
5) GGAATTCCGGATC -1646 CTAACAGGAACATCTGTAG
6) GGAATTCCGGATC -1475 CCCACCCCCGAATCTTG

7) GGAATTCCGGATCC -2846 GGGGAGATGTGTTCTATTAG

8) GGAATTCCGGATCC -2486 GCCCTTGCTTTGGATGC

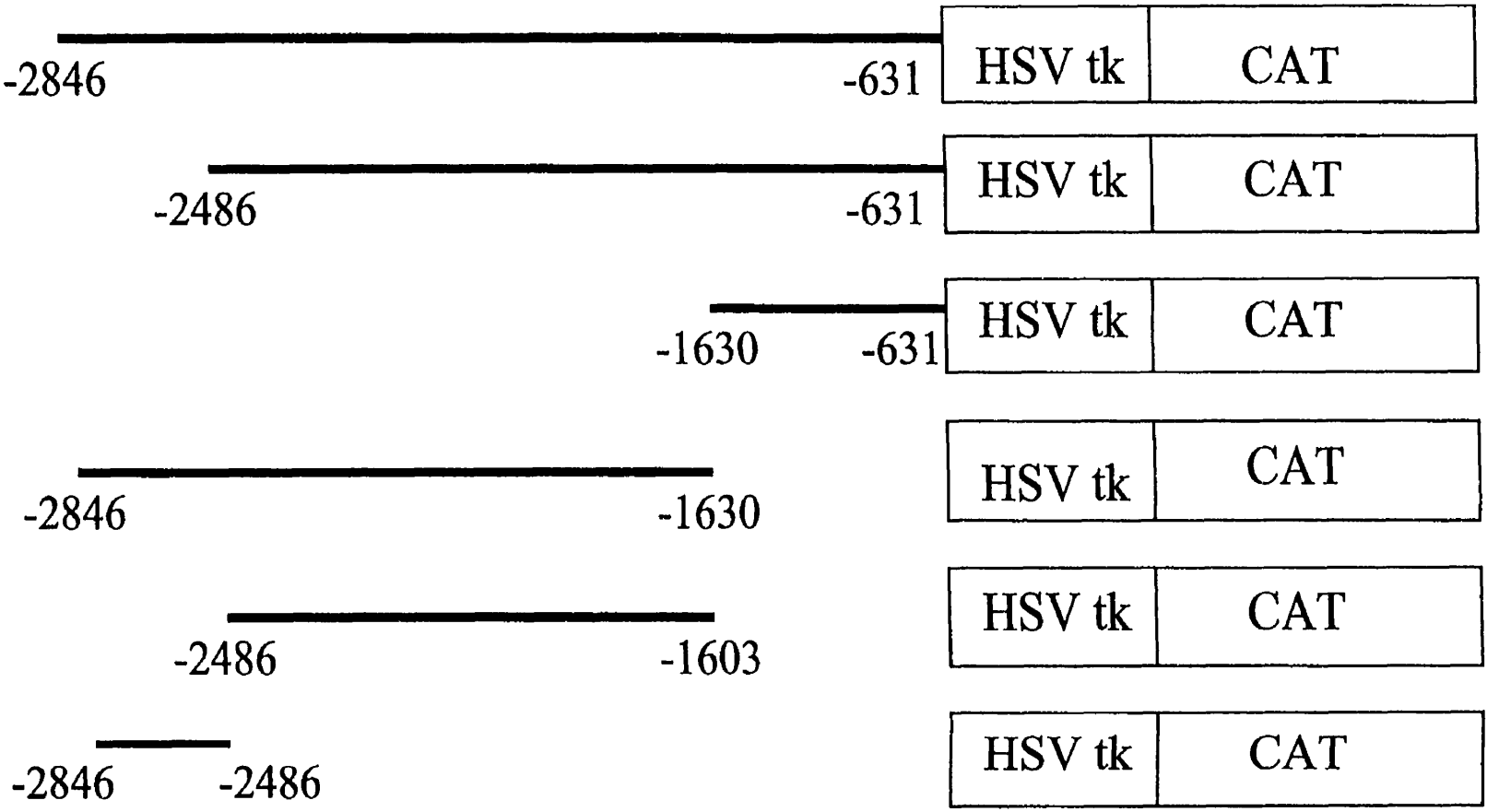
The fragments of human GR gene are from –2846 to –2486, from –2846 to -2677, from –2677 to –2486, from –1786 to –1475, from –1786 to –1630, from –1630 to –1475,from -2846 to –1475, from –2846 to –1630, from –2677 to –1475 (Figure 7).

The PCR reaction condition such as the incubation time, annealing temperature (from 42°C to 58°C), concentration of DNA polymerase enzyme, template DNA and magnesium ions (from 1 mM to 3 mM) were optimized.

PCR reactions were set up in a sterile microcentrifuge tube on ice. Half microgram of genomic DNA was used. The upstream and downstream primers were adjusted in final concentration of 300 nM. The dNTP mix was 500 uM. The Magnesium ion final concentration was 1.5 mM. One unit of PCR enzyme mix was used in each PCR reaction. The final volume was made up to 50 ul with water and mixed by pipetting. The enzyme mix was added last to the reaction system in order to circumvent the need for hot start without dNTPs. This could avoid the interaction of the enzyme mixture with primer or template through the 3'- 5' exonuclease activity of Pwo.

The PCR reactions were performed by beginning with 94°C for 5 min to denature the template, then 30 cycles with each cycles: denaturing for 15s at 94°C, annealing for 30s at 52°C, elongation for1 min at 72°C. During the last cycle, the reaction the elongation time prolonged up to 10 minutes at 72°C and was cooled down to 4°C. Five ul of the PCR product was used to check the DNA size using agarose gel along with a DNA marker.

To the rest PCR product, equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added. The tube was vortexed and centrifuged at 12,000 g for 1 minute at room temperature. The aqueous phase was transferred to a fresh



-2543 GCAACTAGGTCAAGCAGTGTTCTCATA -2516

Figure 7: Plasmid construction of 5' regulatory region of GR gene Human genomic DNA was used as the PCR template to amplify various lengths of 5' regulatory region of GR gene. PCR products were cloned into the vector pBLt kCAT. These plasmid constructions were used as the reporter gene for transient transfection.

tube. Two volumes of 100% ethanol were added to precipitate the DNA. The DNA was re-dissolved in 20 ul TE buffer and was digested with 5 units of restriction enzyme BamH1 in 37°C water bath for 30 minutes. After checking again on the DNA agarose gel, the fragments with desired sizes were purified by the sucrose density gradient centrifugation. The density gradient is arranged from 5% to 20% sucrose in Tris-Cl pH=7.6 together with ethidium bromide. The sample was added on the top of the centrifuge tube containing density gradient sucrose, with 5% sucrose on the top and the 20% at the bottom. Centrifugation was performed by using the rotor SW 60 from Beckman at room temperature 30,000 rpm for overnight. The fragment was visualized under the ultraviolet and the desired fragment was taken out by a one ml syringe. Following the extraction with butanol to get rid of ethium bromide, the fragment was precipitated with ethanol in the presence of 0.3 M sodium acetate. The purified fragment was then ligated to the BamH I site of the pBLtkCAT reporter vector with or without tk promoter. After restriction enzyme digestion, the recombinant clones were sequenced by using T_7 Sequencing Kit to confirm the right clone with proper orientation.

3.2. Sequencing

To prepare the template, mini preparation of plasmid DNA was extracted from 4 ml of overnight bacteria culture. The overnight culture was centrifuged and the supernatant was discarded. The bacteria pellet was resuspended with 100 ul of lysis buffer I (50 mM of glucose; 25 mM of Tris-Cl pH=8.0; 10 mM of EDTA pH=8.0), then was added 200 ul of lysis buffer II (0.2 M NaOH; 1% SDS) vortex briefly. Then the lysis buffer III (3M KOAC, 11.5% HOAC) was added. The tube was centrifuged to collect the supernatant. After using phenol and chloroform to extract the protein, the

upper liquid phase was precipitated with 100% ethanol. The plasmid pellet was dissolved in a final volume of 100 ul, and 20 ul was used as template.

To denature the template, 12 ul of distilled water and 8 ul of 2 M NaOH were added to 1.5 ml microcentrifuge tube, finishing with ddH_2O up to final volume 40 ul. The tube was vortexed gently and centrifuged briefly to collect the liquid at the bottom and was incubated at room temperature for 10 minutes. Seven ul of 3M sodium acetate pH4.8 and 4 ul distilled water were added and the tube was vortexed, added 120 ul 100% ethanol, mixed well and placed on dry ice for 15 minutes. The precipitate DNA was collected by centrifugation, and the pellet was washed with ice-cold 70% ethanol without disturbing. The tube was re-centrifuged for 10 minutes to remove the supernatant and the pellet was dried by air. The denatured template was dissolved in the mixture of 5 ul of distilled water, 1 ul of 2.5 uM universal primers and 1 ul of 10 time annealing buffer. The tube was vortexed gently and centrifuged briefly, and the annealing reaction was performed by incubating at 65°C waterbath for 5 minutes, transferring quickly to 37°C waterbath for 10 minutes and keeping at room temperature for at least 5 minutes. To the tube containing the annealed template and primer, 1.5 ul labeling mix, 0.5 ul of labeled α-^{35}S dATP and 1 ul of diluted T_7 DNA polymerase (1ul of T_7 DNA polymerase dilute with 8 ul of dilution buffer) were added. The components were mixed by gently pipetting and incubated at room temperature for 5 minutes. While this incubation is in progress, four set of tubes was set up by pipetting 1.25 ul of 'G' mix short, 'A' mix short, 'T' mix short and 'C' mix short respectively. Warm the four sequencing mixes by placing the microcentrifuge tubes at 37°C for at least 1 minute. After the labeling reaction has been incubated for 5 minutes, 2.2 ul of the labeling reaction was transferred to the four sets of pre-warmed tubes containing 1.25 ul of the G mix-short, A mix-short, T mix-short and C mix-short respectively. A fresh pipetting tip was used for each transfer. The reaction was incubated in 37°C waterbath for 5 minutes, and then was added 10 ul of

stop buffer to each tube. The tubes were heated in 90°C waterbath for 4 minutes and chilled in ice quickly.

The sequencing gel was pre-run at 40 W constant power for half an hour with 0.5 time TBE. The sample was carefully loaded into the well, with each well a fresh tip. The sequencing gel was run at 2000 voltages 20 mA. After the bromophenol blue in the samples loaded reach to the bottom of the gel, switch off the power. The gel was removed by attaching to a piece of dry filter paper (Whatman Number 1), covered with plastic wrap, dried at 80 °C with vacuum, exposed overnight at room temperature and autoradiographed using Fuji RXO-G film with intensifying screens.

3.3. Cell Culture and Transient Transfection Assay

Several cell lines are used including CV-1, Hela and HepG-2. The monkey kidney cells are deficient in glucocorticoid receptor. The HepG-2 has high expression of SF-1. Cells were passaged in Minimal Essential Medium (MEM, sigma) supplemented with 10% charcoal-treated fetal bovine serum (DCC-FBS), gentamucin (16ug/ml), and fungizone (1 ug/ml). The cells, grown to 70%-80% confluency in 250 ml culture flask, were collected by mild trypsinization, and 1.5 X 10^6 cells / 100 mm Petri dish were plated. Two hours prior to transfection, the medium from the plate was aspired, and 4 ml of DMEM supplemented with 10% FBS was added. Calcium phosphate-precipitated DNA (960 ul), 2.5 ug of receptor expression vectors (hGR and/or hSF-1), 2.5 ug of reporter plasmid with various length of 5' regulation region of hGR linking to chloramphenicol acetyltransferase and 5 ug of pCH110, the β-galactosidase expression vector were used for standardization of transfection (Pharmacia, Montreal, Canada) and were added dropwise to the medium (Herbommel et al., 1984). The calcium phosphate precipitate was

left in contact with the cells for 12 hours. Then, 2 ml of 15% glycerol in Hepes-buffered saline (HBS) was added to the medium and gently mixed. This step was found to be crucial for an efficient transfection and to quantitatively remove the calcium phosphate precipitate. After 3-5 minutes, the medium was removed by aspiration. The cells were washed twice with 4 ml of Tris-HCl (pH 7.4), 1 mM MgCl2, 1 mM CaCl2, and 140 mM KCl (TBS), and 4 ml of MEM supplemented with 5% dextran-coated charcoal-treated fetal bovine serum (DCC-FBS) was added to the transfectants. Hormones were added as indicated from a 1000 times concentrated stock solution in ethanol, and the incubation was continued for an additional 24 hours.

The following day, the medium from the Petri dishes was collected in 15ml plastic centrifuge tubes (Falcon), and the cells were washed twice with 4 ml of phosphate-buffered saline (PBS). Following brief trypsinization, the enzyme was blocked by the addition of the medium collected from each Petri dish. The cells were collected by centrifuge at 1500g for 10 minutes at room temperature, suspended in 500 ul of PBS, transferred into 1.5ml reaction tube, and centrifuged at 2500g for 5 minutes to collect the cells. The PBS was removed by aspiration, and the cells were resuspended in 100 ul of 0.25 M Tris-HCl (pH 7.8) containing 0.05mM phenylmethanesulfonyl fluoride (PMSF). The cells were lysed by three cycles of freeze-thaw, and the cell extracts were collected by centrifugation at 15,000 g for 5 minutes at room temperature. The transfection efficiency was measured by determining the β-galactosidase activity in 10 ul of the cell extract. Extracts containing 10 units of the β-galactosidase activity were used in assaying the CAT. The results of three independent experiments varying by less than 5% were considered for evaluation.

3.4. GST Fusion Protein Expression and Purification

The full-length of hGR cDNA encoding 777 amino acids was constructed from overlapping cDNA clones isolated from MCF7-λgt11 and -λgt10 cDNA libraries. The human GR cDNA clones (hGR AF-1 amino acid from 76 to 262, hGR LBD from amino acid 500 to 777) were subcloned into the pGEX vector in proper reading frame and orientation. The full-length of hGR cDNA was also cloned to pcDNA1 vector down stream of the T7 promoter, which is used to express radioactivity labeled hGR *in vitro*. The cDNA encoding human SF-1 was cloned into the pGEX vector in right reading frame and orientation and pcDNA 1 vector to express protein. The plasmid constructions were analyzed by restriction enzyme and sequenced to confirm the reading frame.

The plasmid were transformed into the frozen competent cells pBL21 (DE) plysS, an E.coli strain which contains a lambda lysogen expressing the T7 polymerase under the control of the lacUV5 promoter and a plasmid which constitutively expresses T7 lysozyme. The expression of T7 polymerase is necessary for transcription of recombinant protein cloned into pGEX vector. In addition, the presence of the T7 lysozyme reduces basal expression of genes cloned into pGEX vector by inhibiting the T7 polymerase. In the presence of isopropyl-β-D-thiogalactopyranoside (IPTG), the cells are induced to express high level of T7 RNA polymerase and high level GST fusion protein

To determine the optimal introduction time for maximum expression for a particular protein, 3 ml of LB containing Ampicillin (50ug/ml) were inoculated from a single recombinant E.coli colony and were amplified for overnight at 37 °C by shaking vigorously 250 rpm. The next day, 25ml of 2YT were inoculated to OD_{600} of 0.1. Grow the culture at 37 °C by vigorously shaking to OD_{600} = 0.4-0.6. A 1ml aliquot of cells was removed prior to IPTG induction, centrifuged in a microcentrifuge and the supernatant was

aspirated. The pellet was frozen at –20 °C. This will be the time zero sample. IPTG was added to a final concentration of 0.1 mM and the flask was continued to shake. After 1 hour of incubation, 1ml sample was removed again, centrifuged as described above and the cell pellet was frozen at –20 °C. Continue to take samples at 1hour intervals and repeat the procedure as described above. The next day, thaw the frozen pellet on ice to break the membrane of the cells. One ml of NETN buffer was added to suspend the viscous pellet. Sonicate briefly till the lysate looks transparent and no longer viscous. The tube was centrifuged full speed to discard the protein debris. Analyze 20ul of each of the supernatant from pellet samples on SDS-PAGE. Stain the gel with Coomassie blue and look for a band with increasing intensity in the expected size range for protein. From this expression experiment, the optimal time to harvest the cells was determined after IPTG induction.

After determining optimal induction time for maximum expression for a particular protein, 2 ml of LB Ampicillin (100 ug/ml) was inoculated from a single recombinant colony. The bacteria were cultured at 37 °C by vigorous shaking at 250 rpm. The next day, 20 ml LB Ampicillin medium was added, shaken at 37 °C until OD_{600}=0.5. IPTG was added to the final concentration of 0.1 mM. Continue shake the culture for the optimal time. The culture was cooled down on ice and centrifuged to collect the bacteria pellet. The pellets were frozen in –20 °C for overnight. The next day, the pellet was thawed on ice, suspended with 1ml NETN buffer, briefly sonicated until the lysate looks transparent and no longer viscous. The tube was centrifuged full speed to discard the protein debris and the supernatant was collected for following purification. Apply 20 ul supernatant on SDS-PAGE gel to check the intensified protein bands.

To purify the GST fusion protein, Glutathion Sepharose 4B was prepared according to the manufacturer and suspended finally in NETN buffer

containing 0.5% power milk. 200 ul of crude extract after sonication was incubated with 20 ul packed sepharose beads at room temperature and shaken for 30 minutes. Put the tube back in ice, wash the beads three times with NETN buffer and elute it with 20 mM reduced glutathion buffer in ice for 15 minutes. It is important to keep the protein in low temperature to protect it from degradation.

3.5. DNA Mobility Shift Assay

In order to make DNA probe labeled with ^{32}P-γATP, single oligos (purchased from Life Technology) –2543 (GCAACTAGGTCAAGCAGTGTTCTCATA) –2516 and its complementary were dissolved with TE buffer to final concentration of 1 ug/ul. Half microgram of single strand oligos was mixed with its complemented oligos together with one time annealing buffer (100 mM Tris-Cl pH 7.6, 10 mM $MgCl_2$ and 16 mM DTT) to final volume up to 20 ul. This mixture was put into 65°C water bath for 5 minutes, quickly transferred to 37°C water bath for 10 minutes and kept in room temperature for at least 5 minutes. Add 3 ul of ^{32}P-dATP and 4 ul of 1mM mixture of dTTP, dCTP and dGTP. Add 2 unit of T_4 DNA polymerase. Use the pipette to mix gently. Keep it at room temperature for 30 minutes to fill in the sticky end of the double strand of oligos.

In order to purify the labeled probe, the ^{32}Pγ ATP-labeled oligos was then applied on 5% of non-denatured polyacrylamide gel. The gel was run at 200 voltages for 30 minutes at room temperature, covered the wet gel with Sara film; mark each corner with the radioactive ink and expose to the X-film for 30 seconds. After develop the film, the band was cut out at the position corresponding to the band on the X-film. Then the gel was eluted at 37°C for overnight with 300ul elution buffer containing 0.5 M sodium chloride, 0.02 M

Hepes pH 8.0, 2mM EDTA. The next day, centrifuge the incubation tube and the supernatant was extracted with equal volume of phenol: chloroform: isoamyl alcohol (25: 24: 1), then precipitate with 1 ml of ethanol at –70°C for half hour. The radioactivity of the probe was measured and the probe was resuspend in 5000 cm/ul TE buffer.

To perform EMSA, 2.5 ug of crude extract from expression vector transformed *E. coli* bacteria was used. The crude extract was incubated with 2000 cpm ^{32}PγATP labeled probe –2543 (GCAACTAGGTCAAGCAGTGTTCTC ATA) –2516 in the presence of 0.5 ug Poly dI • dC and 0.5 ug BSA at room temperature for 20 minutes up to the final volume of 20 ul DNA binding buffer (12 mM Hepes pH 7.9, 60 mM KCl, 10% glycerol, 1 mM PMSF, 0.5 mM EDTA, 1 mM DTT). When indicated, a 100 fold molar excess of non-labeled probe as the competitor DNA was included in the incubation. In order to determine the specific bases, which are important for fusion protein SF-1 recognition, synthesized oligos with one or two mutant bases from the ^{32}P-labeled probe were used as the non-labeled competitor. The mutated oligos are:

(Mutant 1) GCCCCTAGGTCAAGCAGTGTTCTCATA;
(Mutant 2) GCCCCAAGGTCAAGCAGTGTTCTCATA;
(Mutant 3) GCCCCTATTTCAAGCAGTGTTCTCATA;
(Mutant 4) GCCCCTAGGGAAAGCAGTGTTCTCATA;
(Mutant 5) GCCCCTAGGTCTTGCAGTGTTCTCATA;
(Mutant 6) GCCCCTAGGTCAAGCAGGGTTCTCATA;
(Mutant 7) GCCCCTAGGTCAAGCAGTTTTCTCATA;
(Mutant 8) GCCCCTAGGTCAAGCAGTGTCCTCATA;

The complexes were resolved on 5% (80: 1 cross-linked) acrylamide gels in buffer 0.5 X TBE. The electrophoresis was conducted at 200 voltages on 20 X 20cm gel (100 minutes-run time). The gel was dried at 80°C with vacuum, exposed overnight at –20 °C and autoradiographed using Fuji RXO-G film with intensifying screens.

3.6. Supershift Assay

The polyclonal antibody was used against the GST-SF1 fusion protein to perform supershift assay. Immunization procedure was as described (101) and antibody was assayed by enzyme linked immunosorbent assay. One ul of 1 to 1000 diluted GST SF-1 antibody was incubated with 2.5 ug of crude extract of GST SF-1 at room temperature for 15 minutes. Then 2000 cpm probe was added in the presence of 0.5ug Poly dI • dC and 0.5 ug BSA at room temperature for 20 minutes in the final volume of 25ul DNA binding buffer. The complexes were resolved on 4% (80: 1 cross-linked) acrylamide gels in buffer 0.5 X TBE. The electrophoresis was conducted at 200 voltages 20X20cm gel (100 minutes-run time). The gel was dried at 80 °C with vacuum, exposed overnight at –20 °C and autoradiographed using Fuji RXO-G film with intensifying screens.

3.7. GST Pull-down Assay

Human GR_{1-777} and hSF-1 are cloned into the pcDNA vector with T7 promoter upstream of the initiation site. GR and SF-1 are translated *in vitro* by using TNT Rabbit Reticulocyte Lysate System (from Promega). The circular DNA template was purified from cesium chloride centrifugation. The ^{35}S labeled protein was produced by following the procedure as described in the protocol from manufacturer. The reaction components were assembled into a 1.5 ml polypropylene microcentrifuge tube. The components are 25 ul TNT Rabbit Reticulocyte Lysate, 2 ul of TNT reaction buffer, 1 ul T7 RNA polymerase, 1 ul of amino acid mixture minus methionine 1mM, 4 ul of ^{35}S methionine (100 Ci/mmol at 10 mCi/ml), 1 ul of Rnasin Ribonuclease

inhibitor (40 u/ul) and 2 ul DNA template (0.5 ug/ul). The reaction was finished up to a volume of 50 ul. All the components are added and gently mixed by pipetting. After the translation reaction at 30 °C for 2 hours, 2 ul was applied on the SDS acrylamide gel to check the protein production.

Protein-protein interaction *in vitro* was performed in the following reaction system. ^{35}S-Methionine labeled protein from above was diluted 8 folds and aliquot of 5ul was mixed with 5 ul of crude extract of GST fusion protein in a final volume of 25 ul of incubation buffer (10% glycerol, 100 mM potassium chloride, 20mM Tris-HCl pH 7.9, 0.05% NP-40, 0.05 mM EDTA, 1 mM DTT, 1 mM PMSF and 0.5 % powder milk). The incubation was continued for 10 minutes at room temperature. The bound and free proteins were separated by adding 10 ul packed Glutathion Sepharose 4B beads. The reaction mixture was incubated at 4°C for 30 minutes with occasional agitation. After the beads were washed three times with incubation buffer, proteins were eluted with 20 mM reduced glutathion buffer in ice for 15 minutes. The elution was then 1:1 diluted with 2 folds SDS sample buffer and resolved on 12% SDS acrylamide gels. Gels were treated with Enhancer (NEN), dried and analyzed by autoradiography.

CHAPTER 4

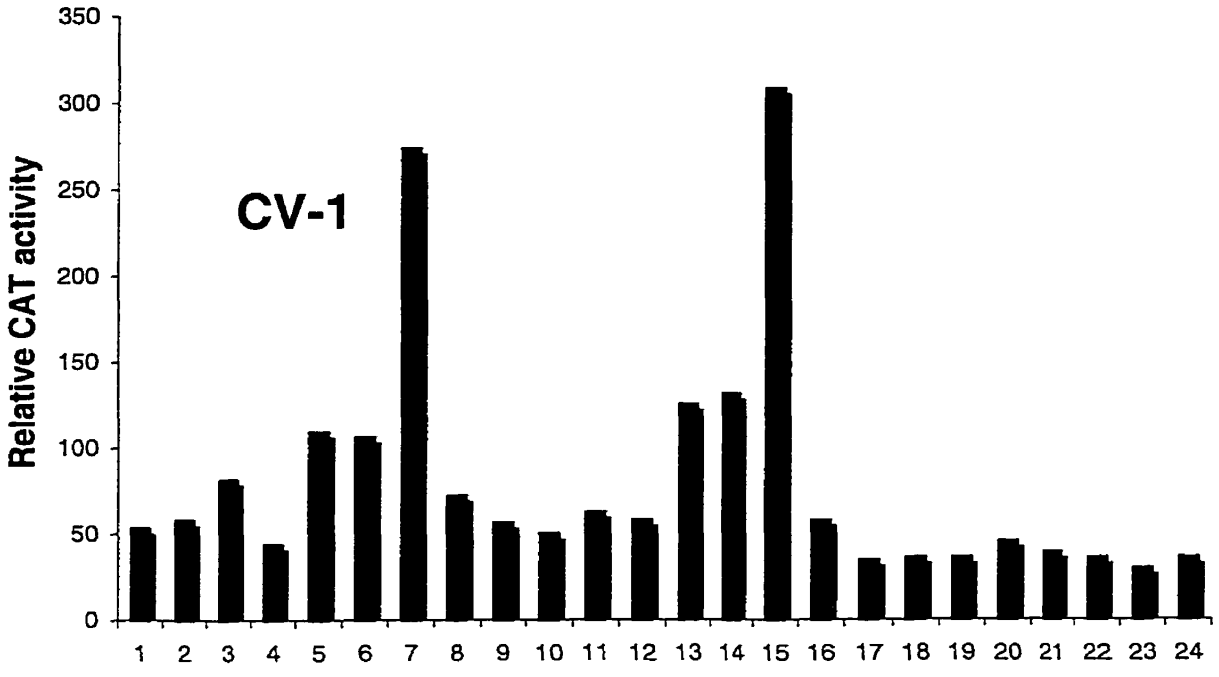
RESULT

4.1. Sequence between –2846/-2486 of human GR gene contains element, which is responsible for the human GR gene downregulation.

My primary aim was to study the homologous down regulation of human GR gene. So I performed a series of transient transfection experiments with the human GR promoter in different cell lines. I selected the CV-1 cells, Hela cells, and HepG2 cells. In CV-1 cells, there is no endogenous GR expression. But there is expression of GR in Hela cells. HepG2 cells have a very high level of SF-1 expression. The phenotype differences that distinguish between the various kinds of cells are largely due to differences in the expression of genes that encode for proteins. So choosing different kinds of cell types with different expression of transcriptional activators as well as coactivators could give more information from multiple points of views. CV-1 cells were transfected with pHGR-2486/-631 tk CAT, basal CAT expression was observed in the cell extract irrespective of whether the cells were transfected with human GR cDNA alone, human SF-1 cDNA alone or together in the presence or the absence of 1 ul Dex. But when pHGR-2846/-631 tk CAT was transfected in the presence of human GR cDNA, CAT activity increased 0.5 folds in the absence of Dex, but decreased in the presence of Dex. Cotransfections with human SF-1 cDNA alone are a 1 fold higher of expression of CAT activity than the control and no change in response to the

Dex. When transfected with human GR and SF-1 cDNA, it gave a very high expression of CAT activity more than 5 folds higher than the control and the same level of CAT activity in the presence of Dex. This indicated that the sequence between –2846/-2486 contains elements with down regulation function. To further confirm this, CV-1 cells were transfected with p-2846/-2486 tk CAT, it gave almost the same results as the transfection of pHGR-2846/-631 tk CAT when cotransfected with human GR cDNA alone, human SF-1 cDNA alone or together in the presence or absence of Dex (Figure 8).

Next Hela cells, which contain endogenous expression of GR, were first transfected with p-2846/-631 tk CAT. Cotransfection of human GR cDNA in the absence of Dex gave three folds higher CAT activity than that in the presence of Dex, but approximately the same as the control. When Hela cells were cotransfected with human SF-1 cDNA, CAT activity is three folds higher than the control in the absence of Dex. In the present of Dex, CAT expression is the same as the control and 5 folds less than that in the absence of Dex. When cotransfected with human GR and SF-1 cDNA together, CAT activity is even higher 4 folds in absence of hormone than the control and 8 folds higher in the presence of the hormone. When the hGR –2486/-631 tk CAT was transfected to Hela cells, basal CAT expression was observed again either cotransfected with human GR cDNA alone or human SF-1 cDNA alone or together in the presence or absence of Dex. This was similar to the observation with CV-1 cells. When pHGR-2846/-2486 tk CAT was transfected in Hela cells, cotransfection with human GR cDNA alone in the absence of hormone gives 5 folds CAT activity than that in the presence of Dex. When pHGR-2846/-2486 tk CAT was transfected together with human SF-1 cDNA alone in the absence of hormone, it gave CAT activity 9-fold of that in the presence of Dex and 3 folds of the control. When pHGR-2846/-2486 tk CAT together with human GR and SF-1 cDNA were transfected in the absence of hormone, CAT activity was 8 folds higher than that in the presence of Dex and 2.5 fold of the control. These results were

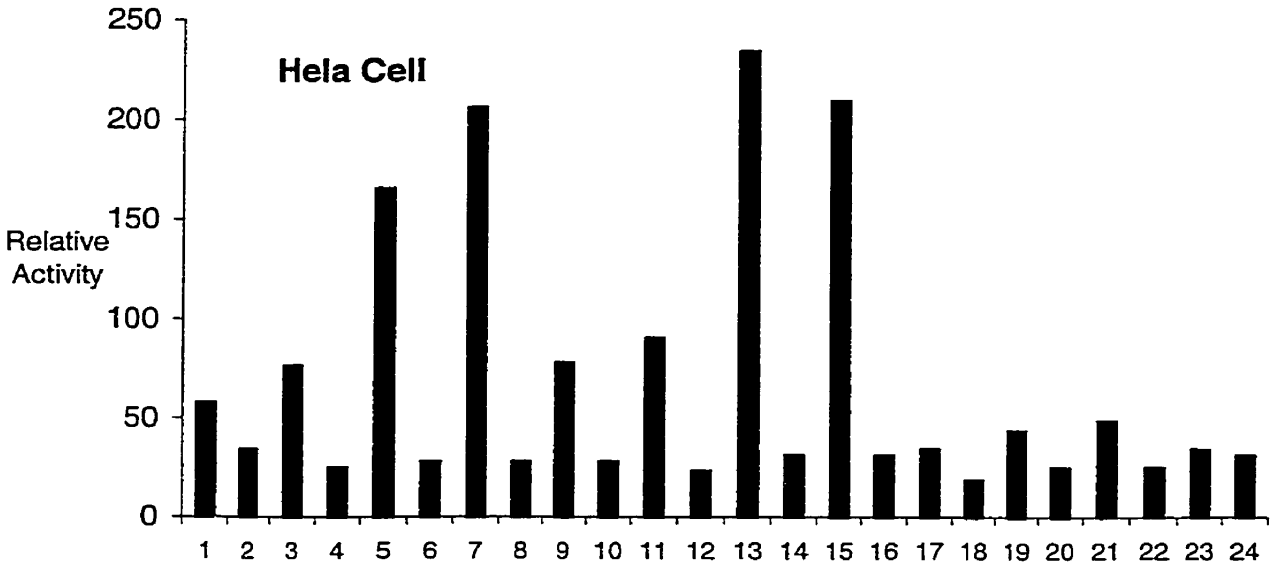


1 uM Dex	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
pcDNA GR1-777			+	+			+	+			+	+			+	+			+	+			+	+
pcDNA SF-1					+	+	+	+					+	+	+	+					+	+	+	+
pHGR -2846/-631 tk CAT	+	+	+	+	+	+	+	+																
pHGR -2846/-2486 tk CAT									+	+	+	+	+	+	+	+								
pHGR -2486/-631 tk CAT																	+	+	+	+	+	+	+	+
pCH110	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Figure 8. Transient transfection of human GR-tk CAT chimeras in CV-1 cells.
Semiconfluent CV-1 cells were transfected with different lengths of human GR gene promoter linked to tk CAT in presence of 5 ug of pCH110 plasmid as the control. Following the determination of β-galactosidase in an aliquot of total cell extract, CAT activity was measured in extracts containing 10 units of β-galactosidase activity. The acetylated chloramphenicol derivates were visualized by autoradiography and determined by scintillation counting. The values are the average of triplicate experiments.

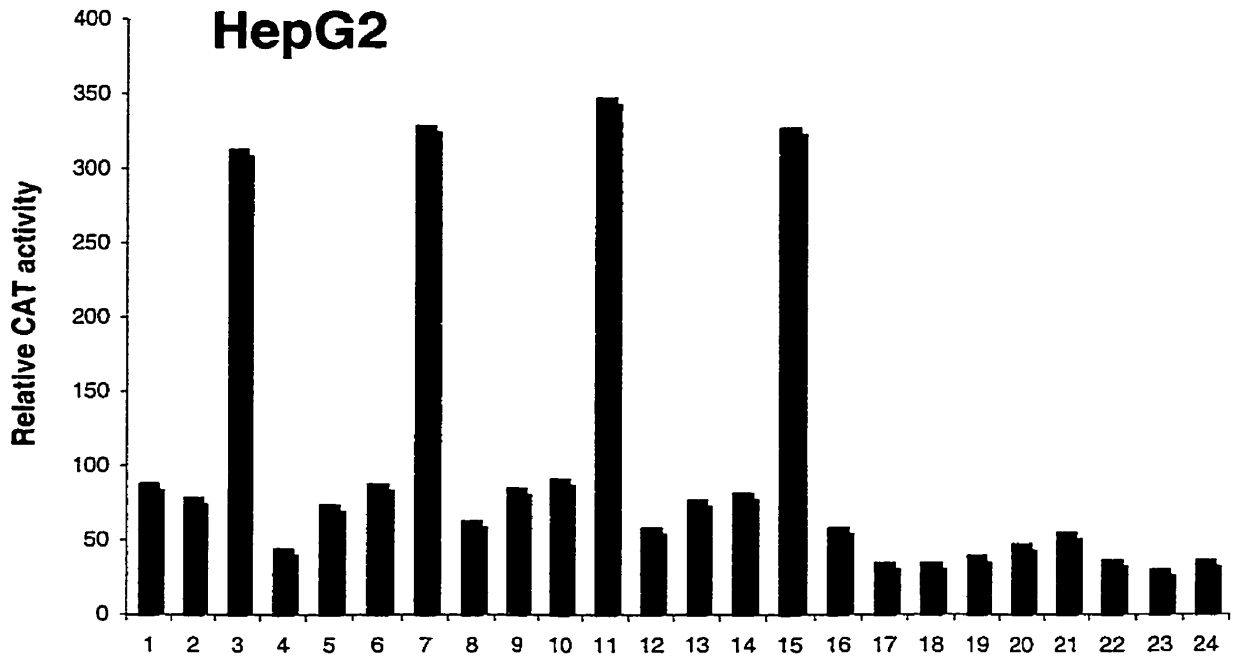
similar with the transfection experiments in CV-1 cells, suggesting the sequence between –2846/-2486 of human GR gene could downregulate human GR gene expression (Figure 9).

The same transient transfection experiments were performed in HepG2 cells, because these cells contain high expression of human SF-1. When HGR-2846/-631 tk CAT was transfected with human GR cDNA, CAT activity was six folds higher in the absence of Dex than that in the presence of Dex and approximately four times as much as the control. When cotransfected with human SF-1 cDNA, CAT activity was approximately the same as the control in the absence or presence of Dex. When cotransfected with human GR and SF-1 cDNA together in the absence of hormone, CAT activity was even higher (4 folds of the control) and 7 folds higher in the presence of the hormone. When I transfected the hGR –2486/-631 tk CAT to HepG2 cells, again it only showed basal CAT expression in the cell extract whether it was cotransfected with human GR cDNA alone or human SF-1 cDNA alone or together in presence or absence of 1 ul Dex. This is similar to the results from CV-1 cells. When pHGR-2846/-2486 tk CAT was transfected in Hela cells, cotransfection of human GR cDNA alone in the absence of hormone showed seven folds CAT activity than that in the presence of Dex and 4 folds as much as the control. When pHGR-2846/-2486 tk CAT was transfected together with human SF-1 cDNA alone in the absence of Dex, CAT activity showed almost the same as that in the presence of Dex and the control. Cotransfection of pHGR-2846/-2486 tk CAT together with human GR and SF-1 cDNA in the absence of hormone showed 7-fold CAT activity of that in the presence of Dex and 4 fold of the control. The conclusion from these results is that the sequence between –2846/-2486 of human GR gene contains element, which is responsible for the human GR gene downregulation (Figure 10).



1 uM Dex	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
pcDNA GR1-777			+	+			+	+			+	+			+	+			+	+			+	+
pcDNA SF-1					+	+	+	+					+	+	+	+					+	+	+	+
pHGR -2846/-631 tk CAT	+	+	+	+	+	+	+	+																
pHGR -2846/-2486 tk CAT									+	+	+	+	+	+	+	+								
pHGR -2486/-631 tk CAT																	+	+	+	+	+	+	+	+
pCH110	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Figure 9. Transient transfection of human GR-tk CAT chimeras in Hela cells. Semiconfluent Hela cells were transfected with different lengths of human GR gene promoter linked to tk CAT in presence of 5 ug of pCH110 plasmid as the control. Following the determination of β-galactosidase in an aliquot of total cell extract, CAT activity was measured in extracts containing 10 units of β-galactosidase activity. The acetylated chloramphenicol derivates were visualized by autoradiography and determined by scintillation counting. The values are the average of triplicate experiments.



1 uM Dex	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
pcDNA GR1-777			+	+			+	+			+	+			+	+			+	+			+	+
pcDNA SF-1					+	+	+	+					+	+	+	+					+	+	+	+
pHGR -2846/-631 tk CAT	+	+	+	+	+	+	+	+																
pHGR -2846/-2486 tk CAT									+	+	+	+	+	+	+	+								
pHGR -2486/-631 tk CAT																	+	+	+	+	+	+	+	+
pCH110	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

Figure 10. Transient transfection of human GR-tk CAT chimeras in HepG2 cells. Semiconfluent HepG2 cells were transfected with different lengths of human GR gene promoter linked to tk CAT in presence of 5 ug of pCH110 plasmid as the control. Following the determination of β-galactosidase in an aliquot of total cell extract, CAT activity was measured in extracts containing 10 units of β-galactosidase activity. The acetylated chloramphenicol derivates were visualized by autoradiography and determined by scintillation counting. The values are the average of triplicate experiments.

4.2. SF-1 could recognize the sequence between –2543 to –2516 of GR gene, but GR could not.

Sequencing of the region between –2846/-2486 of human GR gene showed an interesting motif, in which one half of GRE and one SF-1 response element are separated by 5 bases. This lead to the question whether GR and SF-1 or SF-1 like proteins can form a heterodimer, thus changing the transcription rate of the GR gene. If this is true, it is possible that due to heterodimer of GR and SF-1, GR may interfere in SF-1 transcriptional activity and the transcription factor complex can provide an alternative surface to recruit different transcription coactivators or corepressors. It is also possible that heterodimer formation can facilitate the association or dissociation of transcription coactivators, thus giving different transcription rate from the GR or SF-1 on the human GR gene. In order to test these hypotheses, first I wanted to perform EMSA to clarify whether GR or SF-1 can recognize this motif. As described in the material and method, GST fusion protein of hGR $AF\text{-}1_{76\text{-}262}$, hGR $LBD_{500\text{-}777}$ and full-length hSF-1 was incubated with ^{32}P labeled probe, and the complex was separated on polyacrylamide gel. Binding proteins were certified by adding antibody of hGR and SF-1 to perform the supershift assay. In order to determine the exact binding site, which is important for fusion protein of GR and SF-1 recognition, synthesized oligos with one or two mutant bases from the ^{32}P labeled probe were used as the non-labeled competitor. So that if the mutated non-labeled oligos can compete with the binding of the fusion protein it could means the mutant base pair is the protein-binding site. On the contrary, if the mutated non-labeled oligos cannot compete with the binding of the fusion protein, it indicated that the mutant base pair is not the protein-binding site. It is found that GST fusion protein of human GR cannot bind anywhere of the DNA motif. But GST SF-1 fusion protein can recognize the ^{32}P labeled probe –2543

(GCAACTAGGTCAAGCAGTGTTCTCATA) –2516. This binding is dose-dependent manner in that the more the protein was added in the reaction system, the more the intensive shifted band. This binding could also be competed by adding 10 folds and 100 folds cold probe. One hundred folds molar excess non-labeled probe was able to compete almost all the binding of SF-1 to the probe –2543 (GCAACTAGGTCAAGCAGTGTTCTCATA) –2516. When 100 folds molar excessive palindromic GRE oligos TGTTCTGGATGTTCT was added, there was no change in the intensity of the shift bands. This signifies that the SF-1 cannot bind to the palindromic GRE. But 100 folds molar excess of mutant oligos (M1) GCCCCTAGGTCAAGCAGTGTTCTCATA and (M2) GCCCCAAGGTCAAGCAGTG TTCTCATA could compete with SF-1 binding to wild type sequence of the GR gene. On the other hand, the mutant M3--M8 cannot compete SF-1 binding to wild type sequence of GR gene. This means that the base pairs at approximately –2543 to –2516 (GCAACTAGGTCAAGCAGTGTTCTCATA) is important for the binding of GST fusion protein of SF-1. The result of the super shift certified the binding protein is SF-1, because 1 ul of 1000 diluted antibody against SF-1 could not shift the labeled probe, but can supershift half of the shifted labeled probe. Two ul of 1000 time diluted antibody against SF-1 could supershift the entire labeled probe. I concluded from the above results that SF-1 could bind the sequence motif probe –2543 (GCAACT AGGTCAAGCAGTGTTCTCATA) –2516 of human GR gene, but human GR cannot. The binding site for the SF-1 is at –2543 to –2516 (GCAACTAGGTCAAGCAGTGTTCTCATA) (Figure 11, 12).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14
GST SF-1 (ug)	-	1	2	2	2	2	2	2	2	2	2	2	2	2
Competitor fold	-	-	-	10	100	100	100	100	100	100	100	100	100	100
synthetic motif	-	-	-	wt	wt	GRE	M1	M2	M3	M4	M5	M6	M7	M8

Figure 11. Electrophoretic mobility shift assay of DNA-protein interaction with hGR gene fragment and GST SF-1 expressed in bacteria. Competition with synthetic palindromic GRE and Mutant M1-M8. 2.5 ug of crude extract from expression vector transformed *E. coli* bacteria was used. The crude extract incubate with 2000 cpm $^{32}P\gamma$ ATP labeled probe –2543 GCA ACT AGG TCA AGC AGT GTT CTC ATA –2516 in the presence of 0.5 ug Poly dI • dC and 0.5 ug BSA at room temperature for 20 minutes in the final volume of 20 ul DNA binding buffer. When indicated, a 100 fold molar excess of non-labeled probe as the competitor DNA was included in the incubation. Synthesized oligoes with one or two bases muted from the ^{32}P labeled probe were used as the non-labeled competitor. The mutated oligo are:

(Mutant 1) GC***CC***CTAGGTCAAGCAGTGTTCTCATA;

(Mutant 2) GCCCC***A***AGGTCAAGCAGTGTTCTCATA;

(Mutant 3) GCCCCTA***TT***TCAAGCAGTGTTCTCATA;

(Mutant 4) GCCCCTAGG***GA***AAGCAGTGTTCTCATA;

(Mutant 5) GCCCCTAGGTC***TT***GCAGTGTTCTCATA;

(Mutant 6) GCCCCTAGGTCAAGCAG***G***GTTCTCATA;

(Mutant 7) GCCCCTAGGTCAAGCAGT***T***TTCTCATA;

(Mutant 8) GCCCCTAGGTCAAGCAGTGT***C***CTCATA;

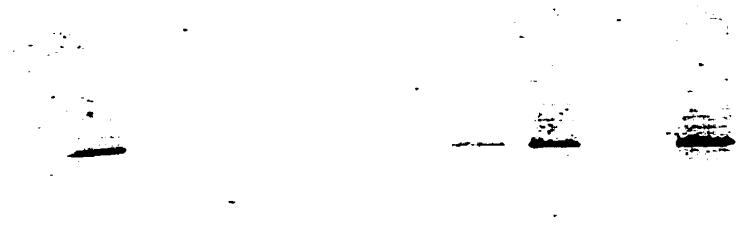
The complexes were resolved on 5% (80: 1 cross-linked) acrylamide gels in buffer 0.5 X TBE. The electrophoresis was conducted at 200 voltages 20 X 20cm gel (100 minutes-run time). The gel was dried at 80 °C with vacuum, exposed overnight at –20 °C and autoradiographed using Fuji RXO-G film with intensifying screens.

	1	2	3	4	5	6
GST SF-1 (ug)	-	-	2	2	2	2
GST SF-1 Antibody (ul)	-	1	-	1	2	3
Synthetic probe 2000cpm	+	+	+	+	+	+

Figure 12. Electrophoretic mobility shift assay of DNA-protein interaction with hGR gene fragment and GST-SF-1 expressed in bacteria. Supershift GST-SF-1 in the DNA-protein complex two ug of crude extract from expression vector transformed *E. coli* bacteria was used. The crude extract incubate with 2000 cpm $^{32}P\gamma$ ATP labeled probe –2543 GCA ACT AGG TCA AGC AGT GTT CTC ATA –2516 in the presence of 0.5 ug Poly dI • dC and 0.5 ug BSA at room temperature for 20 minutes in the final volume of 20 ul DNA binding buffer. Supershift were performed by adding 1 ul of 1 to 1000 diluted GSTSF-1 antibody to incubate with 2.0 ug of crude extract of GST SF-1 at room temperature for 15 minutes before adding $^{32}P\gamma$ ATP labeled probe.

4.3. GR and SF-1 could form complex *in vitro* in a hormone dependent manner.

Since the SF-1, but not human GR, can bind the DNA motif in human GR gene and GR can down regulate GR gene expression by exerting effect on this motif, it is reasonable to speculate that GR may not interact with DNA but with SF-1. So next I wanted to clarify whether GR and SF-1 can interact with each other by forming heterodimers *in vitro*. Human GR_{1-777} and hSF-1 cDNA were cloned into the pcDNA vector with the T_7 promoter upstream of the initiation site and the circular plasmid were used as the template to be translated *in vitro* and labeled with ^{35}S by using the TNT Rabbit Reticulocyte Lysate System (Promega). The bacteria expressed proteins GST, GST hGR AF-1 $_{76-262}$, GST hGR $LBD_{500-777}$ were used as baits by incubating with the radioactively labeled SF-1 in the presence or the absence of Dex. In the GST control lane one cannot see any radioactivity labeled protein. GST hGR AF-1_{76-262} also cannot bind any labeled protein, even when high level (5 ul) of ^{35}S-SF-1 was used. But when GST hGR $LBD_{500-777}$ was used to fish out the SF-1, band of radioactively labeled SF-1 was observed in the presence of Dex only. Input of 5 ul of ^{35}S SF-1 gave a more intensive band than input of 1 ul of ^{35}S SF-1. These results suggest that GST-hGR can interact with SF-1 *in vitro* and the interaction domain of hGR is in the hormone-binding domain. The interaction between GR and SF-1 is hormone dependent. To double confirm the conclusion from above, hGR cDNA was used as the template to make labeled peptide of hGR $LBD_{500-777}$. GST SF-1 of different quantity ranging from 1 ul to 10 ul was incubated with hGR $LBD_{500-777}$ in the presence or the absence of Dex. GST control cannot recruit the ^{35}S labeled hGR. GST SF-1 did recruit the ^{35}S labeled hGR only in the presence of Dex. This result further confirmed that hGR LBD can interact with SF-1 in a hormone dependent manner (Figure 13).



	1	2	3	4	5	6	7	8	9
^{35}S SF-1	10%	1ul	1ul	2.5 ul	5ul	1ul	1ul	5ul	5ul
GST control		+							
GST GR-AF-1			+	+	+				
GST GR-HBD						+	+	+	+
1 uM DEX	-	-	-	-	-	-	+	-	+

	1	2	3	4	5	6	7	8	9	10
^{35}S hGR$_{1-777}$	10%		1ul	1ul	2.5ul	2.5ul	5ul	5ul	10ul	10ul
GST control		+								
GST SF-1			+	+	+	+	+	+	+	+
1 uM DEX			-	+	-	+	-	+	-	+

Figure 13. GST pull down experiment with sub-function domains and SF-1. hGR $_{1-777}$ and full length hSF-1 are translated in vitro by using TNT Rabbit Reticulocyte Lysate System (from promega). Protein-protein interaction in vitro was performed with ^{35}S-methionine labeled protein from 50 ul reaction diluted 8 fold and 5ul aliquot was mixed with 5 ul of crude extract of GST fusion protein in a final volume of 25 ul of incubation buffer. The incubation was continued for 10 minutes at room temperature. The bound and free proteins separated by adding 10 ul packed Glutathion Sepharose 4B beads. The reaction mixture was incubated at 4°C for 30 minutes with occasional agitation. After the beads were washed three times with incubation buffer, proteins were eluted with 20mM reduced glutathion buffer in ice for 15 minutes. The elution was then 1:1 diluted with 2X SDS sample buffer, and resolved on 12% SDS acrylamide gels. Gels were treated with Enhancer (NEN) dried and analyzed by autoradiography.

CHAPTER 5

DISCUSSION

The regulatory region of DNA responsible for the control of gene expression requires specific interactions of multiple recognition elements with sequence specific DNA binding proteins. These factors influence the transcription of responsive genes in both a negative and a positive direction under various conditions of differentiation and development. Some of the *trans*-acting factors that interact with specific DNA sequences surrounding the start of transcription have been identified (104, 105). The human glucocorticoid receptor, like other members of the superfamily of nuclear receptors, is a ligand dependent *trans*-acting regulatory protein. It has long been known that GR itself was down regulated by glucocorticoids. GR expression level undergoes homologous downregulation after exposure to glucocorticoids both in cultured cells and in animals. This reduction in cellular receptor levels is desensitized upon subsequent hormone administration in clinic practice. The GR represses its own synthesis in a hormone dependent manner. This repression is restricted to ligands of the GR, is dose- and time- dependent, is reversible upon hormone withdrawal and requires a functional receptor.

It had been shown that many the cell lines human IM-9 lymphocytes and rat pancreatic acinar AR42J glucocorticoid treatment led to approximately 50% decrease in the steady state glucocorticoid receptor mRNA levels (95). Measurement of GR mRNA level by slot blot hybridization showed that a tissue-specific difference in glucocorticoid receptor mRNA accumulation occurred after adrenalectomy; Dex treatment resulted in a consistent decrease (40%-60%) glucocorticoid receptor mRNA in all tissues (96). Positive autoregulation of GR expression was also observed in the glucocorticoid

sensitive human leukemia T cell line 6TG1.1 and in the human B-cell line IM-9. It has been shown that the transfected human GR cDNA contains sufficient genetic information to recapitulate the downregulation of both hGR mRNA and receptor protein observed *in vivo*. Furthermore, glucocorticoid treatment of cells expressing transfected hGR cDNA resulted in downregulation of hGR mRNA in the presence of cycloheximide or actinomycin D. This suggests that a glucocorticoid inducible protein was not essential for down regulation. Both the agonist Dex and the antagonist RU 486 can lead to decreased transcription of GR cDNA as assessed by nuclear run-on experiments. In addition, a mutant hGR that is incapable of binding to a GR response element was unable to autoregulate its expression, suggesting that DNA binding is a crucial event in this process. Using immonoprecipitation assay had demonstrated that GR could specifically interact with a GR cDNA clone. Further nuclease protection experiments indicated the presence of several internal GR binding regions in GR cDNA (97, 98). Recently, the phosphorylation status of the receptor was shown to have an effect on the repressive function of the GR. Webster *et al* (99), showed that a mouse GR in which all of the phosphorylation sites had been abrogated was no longer able to down regulate its own synthesis.

Deletions performed on GR promoter constructs fused to the chloramphenicol acetyltransferase (CAT) reporter gene identified a site important in the hormone-mediated downregulation of GR gene expression that is located at the positions –470 to –1030. This contains a putative AP-1 site (-893 to –899). Cross talk between the GR system and the Jun/Fos system signal pathway can occur through transcriptional interference (100). The region between –245 and –750 located 5' to mRNA start site is characterized by the presence of three binding sites (-406, -566 and -718) for GCF-1, a transcription repressor. The GCF binding site is also recognized by Sp-1, which regulates gene expression positively. A 95-kDa protein GRF-1 (101), a repressor of hGR gene, interacts specifically in the same region in

hGR gene promoter and is up regulated by glucocorticoid. Two other DNA binding factors have been isolated by DNA affinity chromatography. These two factors show immunological similarities with autoantigen Ku with apparent molecular masses of 80 and 62 kDa (86). They form heterodimers and increase the transcription efficiency of hGR gene by interaction with the sequence motif GAAGTGACACACTTC at –892/-878 upstream of initiation site. Another nuclear receptor ERR-2 (estrogen receptor related protein-2) has been shown to function as a potent repressor of transcriptional activity mediated by glucocorticoid receptor when transiently cotransfected with reporter plasmid and GR cDNA in different cell lines (102). The inhibitory activity of ERR-2 is cell-specific and also receptor specific because transactivation mediated by the progesterone receptor is unaffected by ERR-2.

In the present of study, I have investigated the homologous down transcriptional regulatory elements present in the regulation region of the hGR gene at the transcription level and subsequent analysis of promoter activity. Since most of the earlier studies were performed were within 1kilo base upstream of hGR initiation site, I wished to verify the possible *cis*-acting elements that homologous down regulate hGR gene further upstream. A series of transfection experiments were performed with various length of human GR promoter in different cell lines. CV-1 cells, Hela cells and HepG2 cells were selected for these experiments. In CV-1 cells, there is no endogenous GR expression. But there is GR expression in Hela cells. And HepG2 cells have a very high level of SF-1 expression. It has not been reported whether there is expression of SF-1 or SF-1 like protein in CV-1 and Hela cells and whether there is endogenous GR in HepG2 cells. The priority of choosing different cell lines is that not only they have different expressions of GR and SF-1 or SF-1 like protein, but also have different expressions of other transcription factors or coactivators. The phenotype differences that distinguish the various kinds of cells are largely due to differences in the

expression of genes that code for proteins. So careful choice of different kinds of cell types with different expressions of transcriptional activators as well as coactivators could give more information from multiple view points.

Transient transfection of various lengths of GR promoter region fused to CAT into different cells imply that DNA sequence between –2846 and –2486 in the regulation region of human GR gene determine the homologous down expression. EMSA indicates that SF-1 can recognize a motif between –2846/-2648 upstream of GR gene transcription initiation site. In this region, a GRE half site is 5 base pairs away from a SFRE. The DNA binding domain of SF-1 is a hybrid one, in which the P box is combining residues characteristic of GR and ER subclasses of nuclear receptor. SF-1 interacts as monomer with CAAGGTCA recognition motif. All these lead me to hypothesize that SF-1 and GR may interact with each other and both bind to the DNA to form a stable complex, which suggests that this interaction may be mediate through the DNA binding domain. But electrophoretic mobility shift assay showed different results that GR can not bound to the half GRE, neither could be synergized by the SF-1 bound to SFRE, which suggests that GR may alternate GR gene transcription involving non-DNA dependent interaction. In order to clarify this, GST pull down assay was carried out to test whether GR regulate GR gene by interacting with SF-1. The results showed that GR could interact with SF-1 *in vitro* in a hormone dependent manner. The interaction domain of GR is located in the hormone-binding domain.

The interaction between the nuclear receptors is not a new observation. Steroid receptors have been known to form heterodimers when expressed in the same cells. This interaction plays an important role in transcription initiation (106, 107). It had been shown that GR and AR form heterodimers *in vitro*, by using electrophoretic mobility shift assay. GR and AR inhibit each other's transcriptional activity in cultured cells. Cotransfection of

complementary salt bridge mutants can re-establish heterodimerization and restore AR inhibition of GR transcription activity. MR and GR have also been shown to form heterodimers through putative salt bridges in the zinc finger region. Mutant GR D481R and MR D645R can disrupt the salt bridge, thus markedly decreasing the transcription activity, while cotransfection of mutant MR R643D and GR D481R can restore the transcription activity. In contrast to this observation, it was reported that MR and GR activate transcription synergistically through heterodimer formation. These disparate results may be a context dependent regulatory switch, such as different transcriptional coactivators (106, 107), different target DNA sequences or flanking sequence. It could also be due to the altered post-translation modifications such as phosphorylation and acetylation. Our results demonstrated that GR interact with orphan nuclear receptor SF-1 *in vitro.* This interaction is mediated in a hormone dependent manner through GR DBD and LBD domains. Although the DBD of SF-1 is similar to other steroid receptors, it has a hybrid P box, half like GR and half like ER. So it is appealing to speculate that GR and SF-1 interact through the zinc finger region, and the hormone-binding domain can synergize the interaction. The difference from the heterodimerization of GR with AR or GR with MR is that only SF-1 binds to the target DNA sequence while GR does not. Since GR can recruit many transcription coactivators, including members of NcoA family, the P300/CBP and P/CAF. These proteins possess acetyltransferase activity that acetylates the histones as well as non-histones. This modification of the target proteins may result in chromatin remodeling, changing the stability of the transcription factors and the transcription machinery, subsequent changing the transcription initiation rate.

It has long been known that acetylation of histones and non-histones play an important role in gene transcription. Chromatin structure is generally repressive to the interaction of sequence-specific binding protein with DNA. Hence, nucleosomes must be remodeled in the promoter region during gene

activation. Covalent modification of core histones has long been correlated with changes in chromatin that occur during gene activation and replication (108, 109, 110 and 111). The amino- and carboxyl- terminal ends of histones are subjected to phosphorylation, methylation, ubiquitination and ADP-ribosylation. The possibility is that these various modifications are used in combination to create a 'histone code' that affects chromatin function in different ways (112). The accumulated literature on histone acetylation supports a general model in which histone acetylation contributes to the formation of a transcription competent environment by 'opening' the chromatin and allowing general transcription factors to gain access to the DNA template. Conversely, histone deacetylation mainly contributes to a 'closed' chromatin state and transcriptional repression (113, 114). In particular, amino terminal extensions of the core histones that comprise nucleosomal histone octamers can be acetylated on certain lysine residues, whose positions are nearly invariant through eukaryotic evolution. Acetylation of these lysines tends to correlate with active genetic loci. In contrast, these lysines show reduced acetylation at silenced or heterochromatic chromosomal regions. There are two general models to explain the effect of acetylation. In the first, neutralization of the positive charge of lysine residues by acetylation lowers the affinity of histone octamers for the negatively charged DNA. In the second, the acetyl group functions as signals for interaction of histones with other regulatory proteins.

It has become more and more clear that transcription coactivators play an important role in regulating gene expression. It has shown that most of the transcription coactivators possess HAT activity. Up to now many of these co-factors in the literature have been demonstrated to form complexes with GR. It seems that different combinations of co-factors specify target gene transcription.

The observation that estrogen, progesterone and glucocorticoid receptors could interfere with each other's function and the identification of a conserved region required for ligand-dependent transcriptional activity provided initial evidences for the existence of coactivator proteins that mediate AF-2 function. Biochemical approaches indicated that P300/CBP could interact with nuclear receptor in hormone and AF-2 dependent manner (115). P300 shows considerable homology with CBP. P300/CBP has been implicated in the functions of a large number of regulated transcription factors by direct physical interaction. Specific antibodies against CBP effectively inhibit ligand induced gene expression in microinjection assays. *In vivo* studies, using fibroblasts cells isolated from a P300 knockout -/- mouse, loss of P300 severely affected RNA polymerase II dependent transcription (116). One of the general functional properties that P300/CBP harbors HAT (histone acetyltransferase) activity. Compact chromatin structure imposes a repression effect on transcription *in vivo*. Histone acetylation results in decreased affinity between core histone subunits and DNA, and is also correlated with transcription activation. It has also been shown that TBP and CBP can interact with both the GR AF-1 domain and the ligand-binding domain, offering a possible mechanism for synergistic interaction between the AF-1 domain and other receptor activation domain (117). In addition to interaction with the nuclear receptor, P300/CBP interacts with members of the SRC family, indicating it may form a ternary complex with the SRC family and nuclear receptor (118). Functional evidence is that CBP synergizes with human SRC-1 in the transactivation of ER and PR.

P300 associated protein (P/CAF) was the first mammalian HAT discovered on the basis of sequence homology to yeast Gcn5 protein (119). P/CAF contains an extended amino terminus, which enables it to interact with CBP and members of the SRC family. The interface for P/CAF to interact with nuclear receptor is separate from that interacting with P300/CBP or SRCs. Although the P300/CBP is required for the functions of many transcription factors, the

role of P/CAF and SRC however appear to be more selective (120). This raises the possibility that assembly of P300, P/CAF and SRCs is determined by factors such as the DNA-bound transcription factors. Signal transduction pathways are also conceivably involved in regulating such complex assembly. HAT activity is not the only functional property of P300 and P/CAF. HAT selection is probably determined by a particular transcription factor and/or other components of the complexes assembled on it.

Biochemical analysis also revealed two other proteins P160 (63) and P140, the two major species of proteins interacting with ligand GR. P160 factors are also capable of interacting with P300/CBP via a separate domain. P160 also has HAT activity and belongs to the SRC family, in which multiple variations have been isolated. To date, three distinct members of the SRC family exist as multiple splice variants including SRC-1/NcoA-1, TIF2/GRIP1/NcoA-2 and P/CIP/ACTR/RAC3. GRIP-1 was purified by cross-linking GR to a ^{32}P-labelled GRE DNA sequence and used as a probe. All these factors potentiate transcription activation by several nuclear receptors and have an amino-terminal region that contains a PAS-A-basic helix-loop-helix (bHLH) domain, which functions as dimerization motifs (121). The central regions of SRC-1 and GRIP-1 are characterized by a high percentage of serine and threonine residues and contain ER interacting domains, but not the SRC-1 region interacts with PR. A third region of increased homology is observed in the carboxyl terminus of SRC-1 and TIF-2 /GRIP-1. This region, which was found to interact with the progesterone receptor in the case of SRC-1, also functioned to inhibit progesterone dependent function when over-expressed in cells. This implies that SRC-1 and GRIP-1 may have two independent nuclear receptor interacting domains. Analysis of nuclear receptor coactivators has revealed the existence of multiple highly conserved amphipathic 'LXXLL' (where L is leucine and X is any amino acid) helical motifs (70). These motifs play an important role by associating with critical residues in coactivator interface region to the LBD of nuclear receptor.

Mutation of these residues abrogates both coactivator recruitment and transcription. Secondary structure analysis of these motifs indicates that they form amphipathic α-helices and that the conserved leucines form a hydrophobic surface on one face of the helix. Extensive mutagenesis analysis revealed that different LXXLL motifs within SRC-1/NcoA-1 are selectively required to support functions of different nuclear receptors (71). This functional specificity co-related with the difference in affinity between each LXXLL motif and nuclear receptors. Such selectivity may arise from the amino acid residues located adjacent to the LXXLL motifs.

The HATs are also able to acetylate non-histone substrates as well. These include the tumor suppressor and DNA binding activator p53 (122), two basal transcription factors, TF II E and TF II F (123) and the transcription factors GATA-1 (GATA binding protein 1) that regulates the hematopoietic cell lineage (124). Most recently, androgen receptor has been demonstrated to be modified by acetylation *in vivo* as well *in vitro* (125). P300/CBP acetylated the AR at a highly conserved lysine-rich motif carboxyl terminal to the zinc finger DNA binding domain. ^{14}C acetate-labeling experiments demonstrated that AR acetylation by P300 in cultured cells requires the same residues identified *in vitro*. Point mutation of the AR acetylation site (K632A/K633A) abrogated dihydrotestosterone dependent transactivation of the AR in cultured cells. Mutation of the CH3 region of the P300 or of the histone acetylase domain reduced ligand dependent AR function. The identification of AR as a direct target of histone acetyltransferase coactivators has important implications for targeting inhibitors of AR function.

The p53 tumor suppressor protein exerts antiproliferation effects in response to various types of stress. The biochemical activity of p53, that is required for tumor suppression, involves the ability of the protein to bind to specific DNA sequences and to function as a transcription factor. P53 is a short-lived protein that is maintained at low and often undetectable levels in normal

cells. Tight regulation of p53 is critical for normal cell growth and development. The mechanism by which p53 is activated is generally thought to involve mainly post-translational modification. Post-translational modification of the carboxyl terminus of p53 has been shown to play an important role in controlling p53- specific DNA binding. Thus modification of a highly basic region within the carboxyl-terminal 30 amino acids of the p53 by phosphorylation, antibody binding or deletion of this region can convert p53 from inert to an active form. Recently, Evidence was provided that p53 can be modified by acetylation both *in vivo* and *in vitro*. Remarkably, the site of p53 that is acetylated by its coactivator, P300, resides in a C-terminal domain known to be critical for the regulation of p53 DNA binding. The acetylation of p53 can dramatically stimulate its sequence-specific DNA-binding activity, possibly as a result of an acetylation-induced conformational change. P300/CBP mediates acetylation of specific lysine residues in the C terminus of p53 with a consequent activation of DNA binding. The acetylation mediate neutralization of positive charge could disrupt interactions between the C-terminus and the core domain, thus permitting the DNA binding domain to adopt active conformation. In this case, P300/CBP is the transcription factor acetyltransferase (FAT) in addition to HAT activity. This extends the molecular mechanism of acetyltransferase/coactivator functions in transcriptional activation by implicating a FAT function that may operate in place of, or in addition to, HAT function. Thus, coactivators possessing acetyltransferase activity could be recruited by sequence specific activators to acetylate either the activators themselves or the general transcription machinery or both with consequent activation.

In absence of hormone, nuclear receptors repress transcription of target gene via their association with corepressor complexes that contain histone deacetyltransferase activity. Hormone binding triggers the release of corepressors and subsequent association of an array of coactivators. Among

the nuclear receptor coactivators identified, the protein P160 family has been characterized to possess the central receptor interaction domain and the carboxyl terminal domains responsible for HAT activity and for interaction with P300/CBP and P/CAF. Multiple nuclear receptor boxes, LXXLL, are located in the central region of the coactivators, which function as protein-protein interaction modules by directly contacting a hydrophobic groove formed on the surface of receptor ligand binding domain upon binding of agonist hormone. In contrast, antagonist ligands induce the receptor to assume a conformation where intermolecular interaction between the AF-2 and the LBD core occlude the coactivator association. Amino acids flanking the LXXLL motif may serve to modulate the specificity and affinity of the coactivator-receptor interaction as evidenced by the fact that different receptors display preferences for different LXXLL containing α helix. Evans (126) reported that, upon hormone binding, the nuclear receptor recruits the P160 coactivator and P300/CBP to the target gene. The acetyltransferase activity of P300/CBPacetylates histones to remodel chromatin and also begins to modify other target proteins such as the P160 coactivators. The acetylation sites in the ACTR are located at K629 and K630, which are next to 631LLQLLT636. This acetylation neutralizes the positive charges of these two lysine residues adjacent to the core LXXLL motif and disrupts the association of HAT coactivator complex with promoter bound estrogen receptors. These results provide strong *in vivo* evidence that histone acetylation play a key role in hormone induced gene activation and demonstrates a novel regulatory mechanism of cofactor acetylation in hormone signaling.

Mechanism of GR mediated repression of gene expression is much more complex than its transcriptional activation of target gene. Work has been done to demonstrate that GR represses transcription of target genes either through direct GR interaction with DNA (such as negative GRE) or through protein-protein interaction with other classes of transcription factors, again

emphasizing the role of transcription coactivators. In addition, recent data suggest that GR mediated repression may also result from antagonism with other members of the nuclear receptor family. Corticotrophin-releasing hormone (CRH) plays a primary role in mediating activation of the hypothalamic-pituitary-adrenal axis and is an important target of negative regulation by glucocorticoids. Malkoski (127) defined those *cis*-acting regions of the CRH largely through a consensus cAMP-response element at –224 bp relative to the transcription start site. In the absence of Dex, cAMP stimulated CRH promoter. Dex dependent repression of cAMP stimulated transcriptional activation was localized to promoter sequences between –278 to –249 bp. Specific, high affinity binding of a GR DNA binding domain to this promoter region was shown by EMSA. These studies concluded that a highly conserved region between –278 to –249 bp is crucial for glucocorticoid dependent repression, and that GR is capable of direct interaction with this functionally defined negative GRE of human CRH promoter.

The osteocalcin gene encodes a protein that is synthesized in bone and is positively regulated by the hormonally active form of vitamin D_3 (1, 25 dihydroxyvitamin D_3). It has been shown previously that glucocorticoid can repress both 1,25 OH_2D_3 induction and the basal activity of the osteocalcin promoter. More recently, it has been shown that the location of the GR binding site overlaps with the TATA box element for this promoter. Furthermore, this TATA box element has been characterized as a weak element, because when the hormone activated GR binds to its site on this promoter, it displaces the TBP, and transcription from the promoter is ablated (128).

A number of genes were found to be repressed by glucocorticoids and yet contained no GR binding sites. However, many of these genes did possess AP-1 response element. These elements are bound by jun-jun homodimer or jun-fos heterodimer, which act as crucial transcriptional factors. One

example is collagenase gene, which is repressed by GR but is activated through the transcription activator AP-1 (129). GR and Jun can reciprocally repress each other's transcription by a mechanism independent of DNA binding. Overexpression of Jun can prevent the glucocorticoid-induced activation of genes carrying a functional GRE. Conversely, GR can repress AP-1 mediated transcriptional activation. There is now evidence of GR binding to the c-fos and c-jun components of AP-1. The integrity of the GR DBD appears to be critical for the repression activity, although additional sequences outside DBD may also play a role. AP-1 can also antagonize the activity of GR, and the basic zipper regions of c-fos and c-jun are necessary for this interaction. The N-terminus of c-fos containing the transactivation domain has also been implicated.

Recently it has also been suggested that GR mediate repression of the gene encoding proopiomelanocortin (POMC) by antagonizing other members of the nuclear receptor family, the Nur77. Nur77 forms homodimers and effectively activates transcription upon interaction with a novel DNA-response element NurRE, which has been found within the 5' flanking sequences of the POMC gene. Previously, it was thought that the orphan nuclear receptor Nur77 acted on transcription solely as a monomer or as a heterodimer with the retinoic acid receptor RXR. However, reporter plasmid containing NurRE responded to physiological stimuli, whereas a reporter that contained the binding site for Nur77 monomer (NBRE/nGRE) did not. The possible mechanism involved may be the mutually exclusive binding of GR and Nur77 at the nGRE in regulation region of POMC, or the protein-protein interaction at the site of NurRE of the same nature as the GR interference with AP-1 activity. Besides interaction between GR and Nur77, they may also interact with other common target proteins, such as coactivators or corepressors, since P300/CBP has several binding sites with nuclear receptor (130).

In general, the mechanism of GR mediated repression of target gene expression is complex. On one law, DNA response elements act as allosteric effectors that determine GR conformation to specify which surfaces are available for contact with target factors, and thereby determine how the protein regulates transcription at that site. On the other law, once GR binds to GRE, it can either recruit transcription coactivators or corepressors or directly interact with the transcriptional machinery. The transcription specificity is thus determined by cell context. GR can also regulate target gene transcription by DNA-nondependent manner. This mechanism involves physical interaction with other DNA binding transcription factors, thus changing their affinity with transcription coactivators.

According to my results, in the absence of hormone, GR will associate with hsp90 or hsp70 or transcription corepressors in the cytosol, and is kept in an inactive form. It cannot form a homodimer and translocate to the nucleus. Using GST fusion protein of GR AF- 1 domain to screen expression cDNA library, our group has cloned several transcriptional regulatory proteins including one transcription corepressor (Govindan *et al.* Unpublished). These could help to explain why the CAT activity in absence of Dex is high because SF-1 recognizes the DNA sequence motif CAACTAGGTCA and binds DNA as a monomer; SF-1 could also recruit SF-1 associated coactivators, while at the same time GR could confine the transcriptional corepressors outside of nucleus. In this way, SF-1 enhances the transcription activity of human GR gene. But in the presence of dexmethasone, human GR binds the hormone with high affinity. This transforms the GR into an active conformation. GR dissociates from other inhibitors, forms a homodimer and enters the nuclear. In the nucleus, GR can interact with common coactivators, which are important for SF-1 to upregulate target gene transcription. Also, GR may form a heterodimer with SF-1. This could in one way serve to expel the transcription coactivators associated from SF-1, or alternatively serve to produce a conformational

change, which may provide a different surface capable of recruiting nuclear corepressors. All of these may result in down regulation of the GR gene transcription.

My work provides a clue that GR may downregulate itself by interacting with other nuclear receptors in a DNA nondependent manner. Although my experiments are performed on SF-1, the conclusions are not necessarily limited to SF-1; it is also possible that SF-1 like proteins have an important and real role in this mechanism. Future work must therefore use DNA affinity chromatography to purify other SF-1 like proteins in tissues, which exhibit homologous down regulation of GR gene expression. On the other hand, defining the interaction between coactivators and corepressors with GR or SF-1 like proteins or complexes of both proteins is also necessary. There are reports that P300/CBP and P/CAF acetylate androgen receptor on conserved lysine residues carboxyl terminal to the zinc finger DNA binding domain, which governs hormone dependent transactivation. It would be interesting to verify whether this could also happen to the glucocorticoid receptor.

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