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FUNCTIONAL INTERACTION BETWEEN THE GLUCOCORTICOID RECEPTOR AND C/EBPβ

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Thesis submitted to the Department of Biochemistry in partial fulfillment
Of the requirements for the degree of Master of Science.

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ABSTRACT

Glucocorticoid receptor (GR) is a ligand activated sequence specific transcription factor that belongs to the nuclear receptor superfamily. Most often GR regulates transcription through binding to glucocorticoid DNA response elements (GREs) found in the regulatory regions of target genes. Regulation of transcription by GR usually occurs in a combination with other heterologous transcription factors whose DNA response elements are found in proximity of GREs in the gene regulatory regions.

The Herpes Simplex Virus thymidine kinase (HSV tk) proximal promoter contains two DNA binding sites for Sp1, and one "CCAAT" DNA motif. Surprisingly, when GR was transiently co-transfected with a CAT reporter gene driven by the HSV tk promoter, it induced HSV tk transcription 5 fold in response to dexamethasone in the absence of any apparent GREs. Activation of HSV tk transcription was dexamethasone dependent, as RU 486, a GR antagonist was unable to activate transcription. Activation of HSV tk was also GR specific as androgen receptor was unable to activate HSV tk transcription following DHT treatment.

To map the region of GR responsible for GRE independent activation of HSV tk transcription, several different GR expression constructs were used. A GR DNA binding mutant was able activate transcription at least as efficiently as wild type GR. This clearly demonstrated that regulation occurred in the absence of DNA binding by GR. Significantly, GR's ligand binding domain alone was

found to be sufficient for full transcriptional activity. These results, together, demonstrated that GR's GRE independent activation of HSV tk transcription following dexamethasone treatment probably occurred by a ligand dependent coactivation function and required the Af-2 activity of the receptor alone.

Using synthetic promoters containing individual multimerized HSV tk DNA response elements, it was determined that GR activated transcription specifically through the tk "CCAAT" enhancer binding sequence. When individual CCAAT enhancer binding protein (C/EBP) isoforms were coexpressed together with a GR DNA binding mutant, GR specifically enhanced C/EBPβ mediated transcription following dexamethasone treatment. This result was confirmed using a mammalian two hybrid assay where the C/EBPβ isoform (but not C/EBPα or C/EBPδ) was able to form a protein complex *in vivo* with GR's ligand binding domain. Finally, an immunoprecipitation biding assay revealed that full GR was able to directly interact with C/EBPs. However in this instance selectivity in binding was not apparent.

This thesis provides evidence that GR can positively regulate transcription, in a non conventional, GRE independent manner as a ligand dependent coactivator for C/EBPβ.

DEDICATION

This thesis is dedicated to my family, and especially to my mother, for her encouragement and support in times when I really needed it. I would like to thank my family for their inspiration, patience and teaching me that I can accomplish my goals with hard work. I am also grateful to them for making me proud and never letting me forget my Polish heritage.

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ABBREVIATIONS

aa	amino acid
AF	trans activation function
AP-1	activating protein 1
AR	androgen receptor
Bob-1	B cell specific coactivator
bZIP	basic leucine zipper
CAT	chloramphenicol acetyl transferase
CBP/p300	CREB binding protein
C/EBP	CCAAT enhancer binding protein
DBD	DNA binding domain
CTD	carboxyl terminal domain
DEX	dexamethasone
DHT	dihydroxytestosterone
DNA	deoxyribonucleic acid
DTT	dithithreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetraacetic acid
ER	estrogen receptor
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GTF	general transcription factor
HEPES	N-2-hydroxyethylpiperazine-N'-2ethane sulfonic acid
HSP	heat shock protein
HSV tk	Herpes Simplex Virus thymidine kinase
LB	liquid broth

LTR	long terminal repeat
MSV	Moloney sarcoma virus
NLS	nuclear localization signal
OCT	octamer transcription factor
OD	optical density
PMSF	phenylmethyl sulfonyl flouride
PPAR	peroxisome proliferator activated receptor
PR	progesterone receptor
RAR	retinoic acid receptor
RNA POL II	RNA polymerase II
RSV	Rous sarcoma virus
RXR	retinoic x receptor
RU 486	RU 38486
SDS PAGE electrophoresis	sodiumdodecyl sulfate polyacrylamide gel
SRC-1	steroid receptor coactivator 1
SP1	specific protein 1
STAT	singal transducer and activator of transcription
SV40	simian virus 40
TBP	TATA binding protein
TR	thyroid hormone receptor
TF	upstream transcription factor

I. INTRODUCTION

Transcription of most eukaryotic genes is regulated by an assortment of sequence-specific DNA binding proteins, called transcription factors, that bind in *cis* to regulatory sites flanking the target genes [1]. To promote high level, and tissue specificity of gene expression combinations of several transcription factors are usually required. Glucocorticoid receptor (GR) mediates transcription in response to hormone by directly binding to the glucocorticoid response element (GRE) [2]. However, in this study I have observed that ligand activated GR also can mediate transcription in a non conventional way that is independent of DNA binding. This GRE independent activation of transcription is mediated through a second heterologous transcription factor C/EBPβ [3] binding to its "CCAAT" DNA response element [4]. This thesis describes my initial identification of GRE independent transcriptional regulation by GR and mapping the effect to a DNA independent interaction between GR and C/EBPβ.

1. Regulation of Transcription - Recruitment of The Basal Transcription Machinery Complex

Expression (or transcription) of structural genes in eukaryotes is a highly regulated process in which the recruitment of RNA polymerase II (RNA pol II) to specific regions of DNA (or promoters) of genes is a major regulatory step [5, 6].

The molecular machinery responsible for the recruitment of RNA pol II and the initiation of mRNA synthesis are collectively called transcription factors.

Upstream transcription factors (TFs) are proteins that act in *trans* by binding to *cis* DNA regulatory elements, in promoters or transcriptional enhancers, to influence the expression of genes at a specific time and place (for review see [1]). Through binding to their cognate DNA response elements, upstream TFs are able to recruit general transcription factors and influence the assembly of the basal transcriptional machinery complex, which in turn recruits RNA pol II complex.

a) Assembly of General Transcription Factors is the Major Step in the Formation of the Basal Transcriptional Machinery

Transcription of all genes requires the sequential assembly of several different proteins, general transcription factors, at a specific site in the promoter region of structural genes to form the basal transcription machinery complex [6, 7]. The most commonly occurring as well as the most widely studied core promoter site is Thymidine/Adenine rich TATA box that is typically located 20-40 base pairs upstream from the transcription initiator site [8]. Assembly of a host of proteins called general transcription factors (GTFs) on the TATA box has to occur in a sequential order for efficient synthesis of mRNA [9]. The sequential assembly is initiated by a general transcription factor complex, TFIID, binding to the TATA box [10]. Binding to the TATA box by TFIID occurs via TBP, which is

the core subunit responsible for recognizing this DNA template [11]. At least two GTFs, TFIIB and TFIIF, along with other factors are then able with TFIID to directly recruit RNA pol II to the TATA promoter and form the preinitiation complex [12]. RNA pol II holoenzyme (SRB/holoenzyme complex) then aids the recruitment of TFIIE and TFIIH which complete the formation of preinitiation complex. Phosphorylation of the carboxyl terminus domain of the large subunit of RNA pol II follows, causing the RNA pol II to be released from the TATA box region and begin transcribing mRNA. Assembly of GTFs is essential for efficient transcription by RNA pol II of structural genes in the cell ([7] and references within).

b) Upstream Transcription Factors Regulate General Transcription Factor Assembly

Assembly of the preinitiation complex by the basal transcriptional machinery in the cell is tightly controlled by a combination of positive and negative regulatory factors which include DNA sequence specific activators, or upstream transcription factors [9]. TFs are proteins that regulate transcription by binding to specific DNA sequences in promoters and enhancers . TFs activate RNA pol II transcription by direct protein-protein contacts ultimately leading to the recruitment of the basal transcription machinery complex [1, 13].

Several TFs have been shown to directly contact the basal transcriptional machinery and thus activate transcription by recruitment of GTFs and enhanced assembly of the basal transcription machinery complex [1, 2]. For example,

POU factors, nuclear receptors, and several other transcription factors can directly interact with TBP, TFIIB, TFIID and therefore have the potential to activate transcription by direct recruitment of GTFs [12, 14-18].

TFs can also interact with coactivators. Coactivators are nuclear adopter molecules that contact transcription factors and recruit the basal transcriptional machinery by direct protein-protein interaction. Contact with both the basal transcription machinery and TFs by coactivators does not require DNA binding. In other words, coactivators act as a "bridge" between TFs and transcription initiation complex that is independent of DNA binding. Observations that the transcriptional activation of a factor could be titrated or "squelched" upon overexpression of related activation domains led to speculation that the upstream transcription factors required intermediary factors or "coactivators" to fully activate transcription [19, 20] To date coactivators identified include B-cell specific coactivator for Oct-1 and Oct-2 termed Bob1 [21-24], CREB Binding Protein (CBP)/p300 for CREB [25, 26], and a variety of coactivators for nuclear receptors termed GRIP-1[27, 28],TRIP-1 [29], RIP 140 [30], RIP 160 [31, 32], TIF1 [33], TIF2 [34], SRC-1 [35], and CBP/p300 [25, 26].

Recently, CBP/p300 has been shown to also contain histone acetyl transferase activity [36]. *In vivo,* the DNA template is wrapped around an octamer histone complex, the nucleosome, to form transcriptionally repressed chromatin fiber. Reshaping of the chromatin structure is believed to be necessary to relieve the chromatin inactive state, and thus allow access for TFs to DNA template to potentiate transcription. Post-translational modification of

nucleosomes, particularly acetylation of histones, can destabilize the nucleosome structure and therefore relieve transcriptional repression by allowing access for TFs to their DNA response elements [37]. It is now becoming apparent that many TFs can target histone acetylases through CBP/p300 in response to their signals to activate transcription [38].

In addition to coactivators, a new group of cofactors, termed corepressors, that interact with certain nuclear receptors have been identified [39, 40]. Two related proteins SMRT and N-CoR interact with unliganded thyroid receptor (TR) and retinoic acid receptor (RAR) and are released following ligand treatment. Hormone treatment dissociates corepressors from nuclear receptors and relieves suppression by allowing nuclear receptors to interact with coactivators and thus activate transcription. Corepressors have recently been shown to interact with histone deacetylases [36, 41, 42]. In contrast to histone acetylation, deacetylation stabilizes the repressed chromatin state and promotes transcriptional repression. A recent series of articles demonstrates that the corepressor deacetylase activity is essential for transcriptional repression that is directed by many unliganded nuclear receptors [43-48]. This new function for corepressors, along with the new function for coactivators, provides an elegant and sophisticated picture of how some nuclear receptors modulate transcription by influencing chromatin structure in the cell.

In addition to acetylase/deacetylase activity chromatin structure can also be regulated by general transcriptional activators, the SWI/SNF complexes ([2, 9, 12, 37, 43] and references within). SWI/SNF complexes relieve the inactive

chromatin state by actively displacing nucleosomes from the DNA template. Activation of transcription by TFs is often dependent on SWI/SNF complexes in yeast.

All sequence specific TFs contain at least three functional domains; a DNA binding domain (DBD), transactivation domain (AF), and nuclear localization sequence (NLS). The DNA binding domains include any one of many different kinds of structural motifs necessary for recognition of specific DNA response elements found in the regulatory regions of genes. Several well characterized DBD motifs for TFs include the helix-loop-helix motif, the basic leucine zipper (bZIP), and the zinc finger [49] (for review of DBDs see [50] and references within). DNA binding domains are responsible for the TF's specificity and interaction with DNA response elements. Activation of transcription by TFs generally requires binding to their cognate DNA binding sites in regulatory regions of genes. The second common domain to all TFs comprises a basic cluster of amino acids known as the nuclear localization sequence (reviewed in [51]) . NLS are needed for nuclear import and thus localization to the nucleus.

The third common and important domain to all transcription factors is the transcriptional regulatory domain (AF) [1], that is able either to activate or repress transcription. AFs activation or repression function is result of direct contact with the basal transcriptional machinery and/or indirectly through coactivators [10].

2. *Glucocorticoid Receptor Structure and Function*

Glucocorticoid receptor (GR) is a member of the nuclear receptor superfamily that is marked by its high homology in DNA binding and ligand binding domains [52]. Over 150 members of the nuclear receptor family have been identified and have been shown to play a crucial role in development, cell differentiation, and organ homeostasis [2, 53-55].

The nuclear receptor superfamily is divided into four different classes based on evolutionary homology and DNA binding/dimerization characteristics [52]. The first class includes the steroid receptors which bind to DNA as homodimers. Each partner of the homodimer recognizes the hexameric half-site, which are inverted repeats separated by 3 base pairs. For GR and the other steroid receptors the hexamer half-site is 5'-AGAACA-3', while for ER the hexamer half-site is 5'-AGGTCA-3'. The homodimer is positioned on the DNA in a way that allows the partners to contact and dimerize in a DNA-dependent manner in a head to head arrangement through the DNA binding domain.

The second class receptors comprises receptors that can heterodimerize with retinoid-X-receptor (RXR) [54]. These receptors heterodimerize to direct repeats (DRs) that contain the 5'-AGGTCA-3' hexameric half-site and variable spacing between 1 and 5 nucleotides. The spacing between the half-sites in DRs determines the heterodimer partner for RXR as well as the 5' or 3' position on DNA (polarity) for each receptor in the heterodimer. This class of nuclear receptors is the only one which has been shown to interact with and be regulated

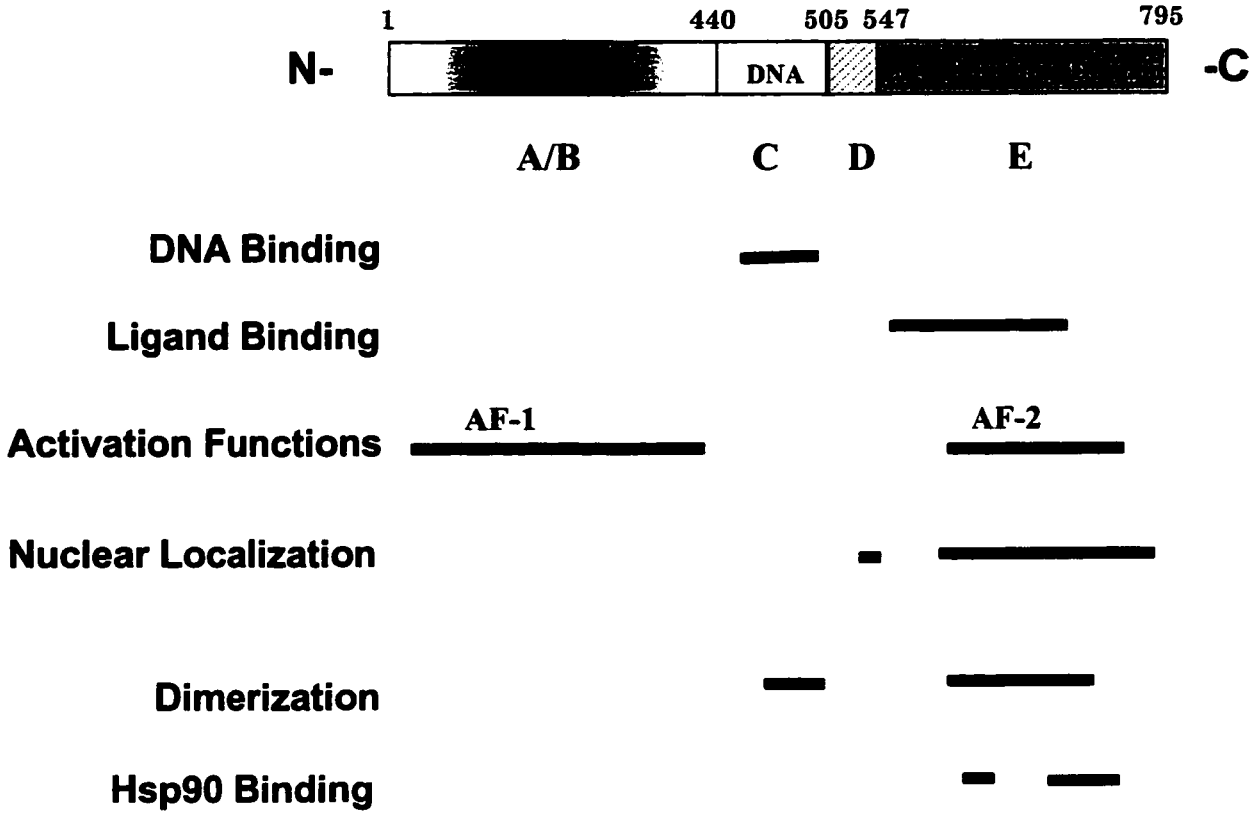
by nuclear co-repressors. Finally, the third and fourth class comprises orphan receptors that bind to DNA as homodimers, or as monomers, respectively.

Nuclear receptors have a modular structure that can be divided into five different domains labeled A to E (summarized in figure 1 for GR) [56].

The N-terminal, A/B transactivation domain, is a highly variable domain between receptors. This domain ranges from 100 amino acids in thyroid receptor to 600 amino acids in mineralocorticoid receptor [57]. By contrast, the central C DNA binding domain is highly conserved in nuclear receptors (> 90%). This approximately 70 amino acid long domain contains two cys 4 zinc finger motifs, that mediate DNA binding [49, 58]. The first zinc finger motif coordinates the DNA specificity by making contacts with bases found in the major groove of DNA, while the second zinc finger makes extensive contacts with the DNA phosphate backbone [58-60]. This domain also contains exposed surfaces that make additional protein-protein contacts [61] and is also responsible for DNA dependent dimerization. The DNA binding domain directs the receptor to specific promoter sequences. The D region separates the DNA binding domain and the ligand binding domain. This domain contains an SV40 T-antigen like NLS [62] that is primarily responsible for nuclear import of GR [63]. In addition, this region appears to function as flexible hinge which appears to be crucial for the spatial arrangement of the receptor. The carboxy terminal E region is a moderately conserved region between receptors that contains several overlapping function. Besides hormone binding,

Figure 1: Schematic Illustration of rat GR structure and its functional characteristics

Glucocorticoid receptor schematic diagram representing its modular domains and functions was adapted from Tsai, MJ and B. O'Malley, 1994 [57]. The amino acid positions that span each domain are indicated above the GR structure. Domains A-E indicated below the main structure represent the functional domains conserved in all members of the nuclear receptor superfamily [52]. Functional characteristics found within each of the domains are summarized below the receptor structure. Two transactivation functions (AFs) are localized in the A/B and the E domains [64, 65]. AF-1 is constitutively active, while AF-2 activity is induced by the ligand. GR also contains two dimerization domains found in the DNA (C domain) and the ligand binding domain. Dimerization function located in the C domain is dependent on DNA binding [66]. A second dimerization domain is located in the E domain and is proposed to be located between amino acids 730-764 based on homology with ER [67]. Multiple sites within the ligand binding domain have been shown to associate with hsp 90. Finally, GR structure comprises two nuclear localization signals (NLS's) [63]. The first NLS is found in the hinge region (D domain) and is homologous to the SV40 T-antigen bipartite NLS [68]. NLS-2 has not been well characterized and spans the whole ligand binding domain.
The ligand binding core encompasses amino acids 537-673 which was identified by the product of trypsin digest that retained significant affinity and specificity that is seen with the whole receptor [69].


1
440
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795
N-
DNA
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A/B
C
D
E
DNA Binding
Ligand Binding
Activation Functions
AF-1
AF-2
Nuclear Localization
Dimerization
Hsp90 Binding

for GR this domain also contains the ligand dependent transactivation function, a dimerization function, as well as a second NLS [67]. Steroid receptors also associate with hsp90 complex in the absence ligand through determinants in the ligand binding domain [70]. The ligand binding domain also determines the ligand specific regulation of transcription. Both the agonist dex, and antagonist RU 486 bind to the LBD [71]. However, binding by agonist or antagonist as determined by proteolytic mapping proceeds by distinct amino acid residues resulting in a different conformational state of the LBD. These different conformational changes by agonist/antagonist on GR's LBD mediate different transcriptional effects through AF-2. In addition, withdrawal of RU 486 following its treatment has also been found to block the redistribution of GR back into the cytoplasm, showing that RU 486 treatment can also affect GR's nuclear trafficking [72].

3. Activation of Transcription by Glucocorticoid Receptor

GR has been a classic nuclear receptor used to study steroid hormone action [73]. GR in its "simplest" pathway binds to *cis* DNA elements in the regulatory regions of genes to promote the assembly of the basal machinery complex. However, GR can also mediate transcription of other heterologous transcription factors by modifying their or its own activity through direct protein-protein interaction.

a) Glucocorticoid Receptor Activates Transcription by Direct and Indirect Recruitment of GTFs

In vivo, the unliganded GR is localized in the cytoplasm where it is associated with a complex of heat shock proteins (hsp) [74]. The heat shock protein complex includes 2 molecules of hsp90, hsp70, p60, and hsp59 along with other factors which have so far not been well characterized [74]. GR is maintained in the hsp-GR complex in order to keep an open conformation that allows the receptor to bind to its ligand with high affinity [75, 76]. The natural ligand for GR is the lipophilic steroid hormone, cortisol. Because of its lipophilic nature, the ligand can enter the cell through passive diffusion and bind to the open conformation receptor in the receptor-hsp complex . Ligand binding promotes the dissociation of the hsp complex from GR, which produces a 'transformed', transcriptionally active, ligand bound receptor. The ligand activated receptor is then rapidly transferred to the nucleus where it regulates transcription by binding to the regulatory regions of target genes. GR binds to its *cis* DNA element as a homodimer [2]. The *cis* DNA element that GR recognizes is the TGTTCTnnnAGAACA palindromic sequence (termed Glucocorticoid Response Element (GRE)) in which each hexamer half site is recognized by the one of the receptors from the homodimer. DNA bound liganded GR homodimer has the potential to activate transcription by directly recruiting limiting components of the basal transcription machinery, or indirectly through recruitment of coactivators and chromatin destabilizing factors.

There is evidence showing that nuclear receptors can interact with the components of basal transcription machinery, although no direct evidence for GR is available yet. Several nuclear receptors have been shown to physically interact with TBP [77], TFIIB [15], and TFIID [16] (more specifically $TAF_{II}30$). Retinoic x receptor (RXR), a member of the nuclear receptor family, has been shown to interact with TBP [77]. Thyroid receptor (TR), another member of the nuclear receptor family, has been shown to contact TFIIB with its amino terminus[15]. And finally, estrogen receptor (ER) has been shown to require $TAF_{II}30$, a subunit of TFIID, for activation of transcription [78]. While the exact physiological relevance of these interactions is unclear, it is believed that contacts with GTFs contribute to transcriptional activation via nuclear receptors.

Nuclear receptors have also been shown to interact indirectly with the basal transcriptional machinery via coactivators. The first well characterized coactivator for nuclear receptors was steroid receptor coactivator-1 (SRC-1) [35]. SRC-1 has been shown to interact with the transactivation function-2 (AF-2) of the ligand binding domain of steroid receptors and enhance receptor mediated transcription in a ligand dependent manner. Enhancement of steroid receptor mediated transcription occurred only in the presence of agonist and not antagonist. Furthermore, over expression of SRC-1 repressed steroid receptor mediated squelching, showing that SRC-1 is the limiting factor. Recent data shows that SRC-1 interacts with AF-2 and is needed for ligand dependent activation; a lysine residue in the AF-2 conserved through out the nuclear receptors is necessary for recruitment of SRC-1 and the activity of AF-2 [79].

While it is clear that SRC-1 interacts with steroid receptors and enhances their activation, it is still unclear how it recruits the basal transcription machinery. Interestingly, new data demonstrates that coactivators might be targeted by nuclear receptors in response to their ligands and activate transcription by a histone acetylation function that relieves transcriptional repression of chromatin structure (see above, [36]).

Finally, *in vivo* GR may also potentiate transcription by interacting with SWI/SNF complexes [80]. SWI/SNF comprise protein complexes that reverse the repressive effects of chromatin structure and are general activators of transcription *in vivo* [81]. GR's transcriptional activation in yeast has been shown to be dependent on SWI1, SWI2, and SWI3 function [82].

b) Glucocorticoid Receptor Regulates Transcription by Interaction with Other Transcription Factors

Although GR can activate transcription by binding to complex promoter or enhancer regions and directly or indirectly promote the recruitment of GTFs, it can also modify transcriptional activity of other upstream factors through direct protein-protein interaction. For example, protein-protein interactions between GR and AP-1 [61], as well as, GR and STAT5 [89] have been reported to effect each other transcriptional activity. Each physical interaction reflects a unique mechanism by which GR regulates transcription through heterologous TFs.

Several studies have shown both positive and negative gene regulatory interactions between GR and AP-1 [61]. AP-1 proteins comprise a family of transcription factors characterized by their basic leucine zipper DNA binding motif [83]. AP-1 is either a homo or heterodimer arising from the pairing of either jun-jun or jun-fos related proteins [84]. These two components form a dimer before binding to their DNA response element.

Regulatory interactions between GR and AP-1 jun-fos heterodimer or jun-jun homodimer have been shown to have different transcriptional effects. In many cell lines the proliferin gene is activated by AP-1, but repressed by glucocorticoids [85]. By contrast, in HeLa and S2 cells glucocorticoids enhance the AP-1 activated transcription of the proliferin gene [84, 86]. Different transcriptional response by GR and AP-1 has been localized to a 25 base pair composite response, termed plfG. Even though the 25 base pair response element does not resemble a GRE or an AP-1 response element, both GR and AP-1 have been shown bind to it simultaneously [84]. Glucocorticoid repression was reported to occur by direct protein-protein contact between GR and AP-1 jun-fos heterodimer, while activation resulted from direct protein-protein contact between GR and AP-1 jun-jun homodimer [84, 87, 88].

Another intriguing property of GR is its interaction with the transcription factor, STAT5 [89]. STAT5 is a member of the Signal Transducer and Activator of Transcription family (STAT) (for review of STATs see [90, 91]). STATs were first identified in 1992 as TFs with a unique mechanism of action in response to cytokines, hormones, and growth factors. STAT proteins share a conserved

DNA binding domain, and a phosphotyrosine binding domain, the Src Homology 2 domain (SH2). The SH2 domain allows STATs to bind to Janus protein - tyrosine kinases (JAKs) found near the plasma membrane. Following signal induction, JAKs phosphorylate STATs on a conserved tyrosine residue to promote STAT dissociation from JAKs, dimerization, and translocation to the nucleus. Consequently, STAT dimers bind to their DNA response elements and activate transcription.

STAT5 transduction pathway demonstrated a new transcriptional mechanism for glucocorticoid hormone action as GR was found to act as a transcriptional coactivator for STAT5 and influence STAT5 dependent transcription [89]. STAT5 activates transcription in response to prolactin on the β-casein gene promoter. The β-casein gene promoter contains DNA binding sites for several nuclear factors [92], but no discernible GRE consensus sequence [93]. However, simultaneous prolactin and glucocorticoid hormone stimulation results in a synergistic enhancement on STAT5 activated transcription. This synergistic action is retained with GR variants that lack the DNA binding domain, demonstrating that GR enhancement is GRE independent. Co-immunoprecipitation assays and mobility shift assays show that GR and STAT5 can form a stable complex in cells in response to prolactin and glucocorticoids. This functional interaction between STAT5 and GR establishes a transcriptional coactivation function for GR. Potentiation of STAT5 activation of transcription by GR in response to glucocorticoids, and independent of GREs, is the first convincing demonstration to show that activation of transcription by a TF

is independent of its DNA response elements. Ligand dependent coactivator function for STAT5 demonstrates a new, non conventional, mechanism of transcriptional activation for GR.

4. CCAAT/ Enhancer Binding Proteins

CCAAT/Enhancer Binding Proteins (C/EBPs) are sequence specific upstream transcription factors that were first isolated and characterized 12 years ago [3, 94]. To date the C/EBP transcription factor family contains members from several different species and includes C/EBP α, C/EBP β (two isoforms LAP and LIP), C/EBP δ, C/EBP ε, Chop-10, NF-M, and NF-IL6 [3, 94-98]. All the family members recognize "CCAAT" DNA response elements, and contain the bZIP DNA binding motif [83]. This motif contains a cluster of basic amino acids necessary for specificity and binding to DNA, and a heptad repeat of leucines (the leucine zipper) that allows C/EBPs to bind to DNA as either homodimers or heterodimers [99].

C/EBP α, C/EBP β (LAP and LIP), and C/EBP δ isoforms contain overall homology which includes high homology in the bZIP motif and moderate homology activation domains. All three isoforms contain three distinct activation domains [3], out of which two of the activation domains (from C/EBPα) have been shown to directly interact with TBP [100]. Despite over 90 % homology that

is observed in the bZIP domain between these three family members, the differences in the bZIP account for distinct transcriptional regulatory properties of these three members. For example, when the bZIP domain of C/EBPβ was swapped with that of C/EBPα, synergistic activation of the CYP2D5 gene with another heterologous transcription factor Sp1, was abolished [101].

Several biological roles have been assigned to C/EBP family members. For example, *in vivo* C/EBP α, C/EBP β, and C/EBP δ along with Peroxisome Proliferator Activated Receptor γ (PPARγ) are positive mediators of adipocyte differentiation [3, 102, 103]. Adipocyte differentiation is a cascade of molecular events that is triggered by adipogenic signals. 3T3-L1 cells, tissue culture preadipoblasts that recapitulate adipose differentiation *in vivo*, can be triggered to synchronously differentiate to a mature adipocyte phenotype in response to insulin, dexamethasone, and inducers of intracellular cAMP [103]. This cocktail of hormones triggers a sequential expression of regulatory proteins in which 3 out of 4 are C/EBP α, C/EBP β, and C/EBP δ. During the first 2 to 4 days following adipocyte stimulation 3T3-L1 cells undergo a prolific phase of clonal expansion. During this phase of adipocyte differentiation, the C/EBP β and δ increase in expression is the result of cAMP inducer, and glucocorticoid treatment, respectively [3]. C/EBP β and δ then activate the expression of C/EBP α and PPARγ which occurs ~2 days after stimulation [3, 102]. Both C/EBP α and PPARγ contain CCAAT response elements in their promoters which are initially regulated by C/EBP β and δ. Expression of C/EBP α and PPARγ are sufficient for the final stages of terminal differentiation, which includes the activation of a

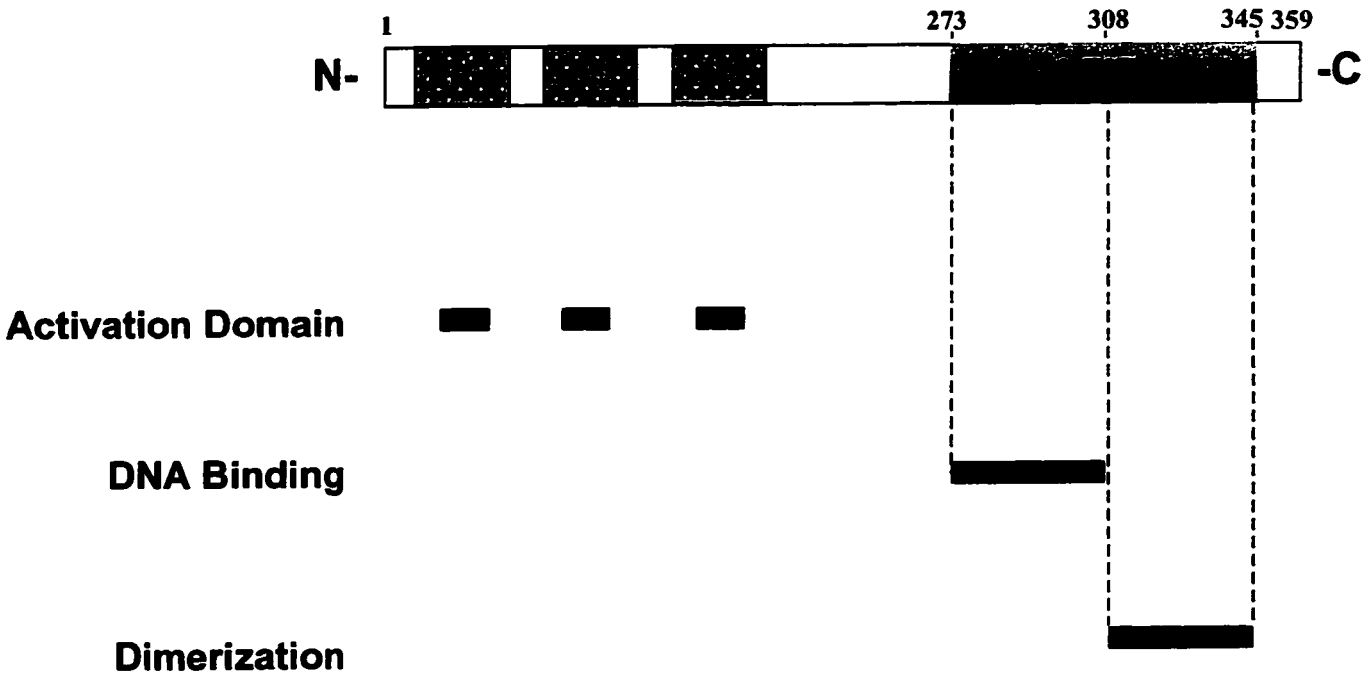
variety of fat- specific genes [104, 105]. In addition, C/EBP α is the key regulator responsible for arrest in mitotic growth in differentiated adipoblasts [106]. PPARγ is a member of the nuclear receptor superfamily that is selectively expressed in adipose tissues [107]. The expression profile of PPARγ mimics that of C/EBPα suggesting that it is also a key regulator in the final stages of adipocyte differentiation. Similarly to C/EBPα, PPARγ expression and activation in fibroblasts can convert fibroblasts into uniform adipocytes [108].

Significantly, C/EBPs, and distinctly C/EBPβ,have been demonstrated to mediate transcriptional activity in the cell by forming protein-protein interactions with other heterologous transcription factors. These protein-protein interactions with distinct regulatory proteins ranging from nuclear receptors to the retinoblastoma gene have been postulated to transcripitonally regulate a large range of biological effects [109-116].

Of particular interest to this research project, the mediation of transcriptional activity between GR and C/EBPβ has been reported. Synergistic action of GR with a factor recognizing "CCAAT" DNA motif was first reported in 1988 [117]. In that series of experiments, it was observed that hormone mediated inducibility of the tyrosine aminotransferase gene could be attained by GRE site in combination with a "CCAAT" DNA motif. However, hormone inducibility was abolished by cluster mutations of the "CCAAT" DNA motif. This result strongly suggested a synergistic action between GR and the factor recognizing the "CCAAT" DNA motif.

Figure 2: Schematic representation of CCAAT/Enhancer Binding Proteins α, β, δ and their functional characteristics

CCAAT/Enhancer Binding Protein α shown as an example for C/EBPα,β, and δ isoforms [3]. The amino acid positions for C/EBP α are shown above the main schema. C/EBPα,β, and δ contain three distinct transactivation domains located near the amino terminus of the protein. Two of these domains have been shown to directly interact with TBP for C/EBPα [100]. The bZIP motif located between amino acids 273 to 345 is highly conserved through out the C/EBP family. The basic amino acid portion of bZIP, located between amino acid 273 to 308 of bZIP, directly contacts and binds to DNA. The remaining zipper motif of bZIP (amino acids 308 to 345) contains a heptad repeat of leucines that is responsible for homo and heterodimerization between C/EBP family members.


1
273
308
345
359
N-
-C
Activation Domain
DNA Binding
Dimerization

A more convincing report of the synergistic interaction between ligand activated GR and NF-IL6 [96] (C/EBPβ homologue) was shown in 1993 [113]. Synergistic activation of the rat α1-acid glycoprotein gene transcription by ligand activated GR and NF-IL6 was shown to occur via a direct protein-protein interaction. The interaction between NF-IL6 and GR was mapped using an *in vitro* binding assay to the bZIP domain of NF-IL6. For GR, the LBD, as well as the DNA binding domain was found to be required for synergistic activation of rat α1-acid glycoprotein gene transcription. Interestingly, synergistic activation of transcription was able to occur in the absence of GR's AF-1 function. These results show clear evidence that direct protein-protein interaction between these two distinct proteins is responsible for synergistic activation of the rat α1-acid glycoprotein gene.

5. OBJECTIVES

Ligand activated GR regulates transcription primarily through GREs found in the regulatory regions of genes. Herpes Simplex Virus thymidine kinase (HSV tk) proximal promoter [118] contains two SP1 DNA binding sites, one C/EBP DNA binding site, but no discernible GREs. Initially my intent was to use this supposedly glucocorticoid unresponsive promoter as a negative control for another set of experiments. Surprisingly, transiently expressed GR activated HSV tk transcription in response to synthetic glucocorticoids. This intriguing result initiated this project and has led me to examine the mechanism by which

GR activates HSV tk transcription. The first objective of this project was to examine and map the GR domain that is responsible for activation of HSV tk transcription in the absence of GREs. The second objective was to examine and identify the DNA response elements in HSV tk proximal promoter, and ultimately the transcription factor through which GR was activating HSV tk transcription.

II. MATERIALS AND METHODS

1. *Receptor Ligands*

GR ligand, 1,4-Pregnadien-9α-fluoro-16αmethyl-11β,17,21-triol-3,20-dione (dexamethasone) and androgen receptor ligand, 5α-androstan-17β-ol-3-one (DHT) were both purchased from Steraloids. The GR antagonist RU 38486 (RU 486) was obtained from Russel-Uclaf.

2. *Expression constructs*

Wild type rat GR eukaryotic expression constructs [119] were a kind gift from Dr.K. Yamamoto (UCSF, California). Other constructs were derived from this vector as described below. Full length wild type GR, DNA binding mutant L501P, as well as the N terminus and C terminus constructs are driven by the Rous Sarcoma Virus (RSV) Long Terminal Repeat (LTR) followed by an SV40 poly adenylation signal. Ligand binding domain of rat GR fused to the Gal4 DNA binding domain was provided as a gift form Dr.Y. Lefebvre (University of Ottawa). C/EBP expression vectors were graciously provided by Dr. S. McKnight (Tularik, San Francisco, California). C/EBP α, β, δ eukaryotic expression vectors are driven by the Moloney Sarcoma Virus (MSV) LTR followed by a thymidine kinase

(tk) poly Adenylation signal. The initial CAT reporter construct used was Herpes Simplex Virus thymidine kinase promoter (HSVtk) CAT EREOct (wt/mut) [120]. Additional CAT reporter constructs were created by subcloning different DNA binding elements from HSV tk minimal promoter into the G5E1bCAT reporter construct. G5E1bCAT reporter plasmid contains 5 Gal4 DNA binding elements followed by E1b TATA box driving the expression of the CAT gene [121]. To create the 4xSp1G5E1bCAT, 4xC/EBPG5E1bCAT, and 4xOctG5E1bCAT reporter constructs Beckman Oligo 1000 DNA synthesizer was used to make Sp1, C/EBP, and Octamer DNA binding elements; 5'-CTA GCG ACC CCG CCC AGC GTG-3', 5'-CTA GGA GTG TCA TTG GCG AGG-3', and 5'-AGC TTG CTT ATG CAA ATA AGG TG-3', respectively. After self-ligation , DNA binding elements were inserted into the compatible Xba1 site in G5E1bCAT. Positive clones were screened and selected for 4 DNA binding elements using restriction digest and then verified by DNA sequencing [122] with Sequenase® Kit purchased from United States Biochemical Corporation. In addition the HSVtk-E1bCAT reporter construct was also created . GeneAmp©PCR Kit (Perkin Elmer) was used to amplify HSV tk promoter from -109 to –40 bp (- HSV tk TATA box). PCR reaction was set for 30 cycles of 1 minute at 95°C , 2 minutes at 54°C , 2 minutes at 72°C , and final cycle 8 minutes at 60°C . Two 24mer primers used to amplify HSVtk were, 5'-CGC TAC TGC AGG TCG ACG GAT CCG-3' and 5'-CGC TCT AGA CGA AGT GGA CCT CGG-3', containing Pst 1 or Xba 1 site, respectively, were synthesized using Beckman Oligo1000 DNA synthesizer. The 90 bp PCR product was gel purified

using the GeneClean kit (Bio Can) restriction enzyme digested, and subcloned into Pst 1 and Xba 1 sites of G5E1bCAT reporter plasmid. Positive clones were screened by restriction digest and verified by sequencing with Sequenase® kit purchased from United States Biochemical Corporation. The internal control reporter plasmid used to check for transfection efficiency was the RSV β-gal construct. The *in vitro* translation constructs of C/EBPβ and C/EBPδ were created by gel purifying EcoR1 - BamH1 β and δ cDNAs inserts (Qiagen Quick Kit). Each insert was then ligated into the EcoRI and BamH1 sites in the pGEM-7Z vector (Pharmacia Biotech), positive clones were then selected and checked by restriction digests and DNA sequencing.

3. *Bacteria culture and plasmid preparation*

Plasmid DNAs were transformed by the $CaCl_2$ heat shock method [123] into the competent *Escherichia coli* - DH5α strain and plated on agar plates containing 100 μg/ml ampicillin (Bristoll Laboratories). Reporter plasmids were transformed into *Escherichia coli* Rb404 (*dam*$^-$ *dcm*$^-$ strain) to prevent the formation of cryptic GRE elements by DNA methylation of BamHI sites [124]. Colonies were allowed to grow overnight, single colonies were picked and used to inoculate 5 ml overnight cultures. These in turn were used as inoculation for the larger 500 ml liquid broth (LB) scales and grown overnight. The standard alkaline-lysis protocol was used to isolate the plasmid DNA [125, 126]. This was

followed by 2 consecutive cesium chloride gradients [127], and dialysis. This procedure isolated DNA plasmids which were 85 - 95 % supercoiled as estimated by agarose gel electrophoresis and ethidium bromide staining.

4. Transient Transfection Analysis of reporter gene expression

(i) Cell Maintenance

Cos 7 cells, african green monkey kidney fibroblast cell line (ATCC, Maryland, USA), were used for all transient transfection experiments. Cos 7 cells were maintained in Dubelco's Modified Eagle Medium (DMEM) at 37°C in humidified atmosphere (5% CO_2, 95% O_2). DMEM was made with the following ingredients: 900 ml of pyrogen free water, DMEM powder (GIBCO BRL), 20 μM HEPES (N-2-hydroxyethylpipeerazine-N'-2-Ethane Sulfonic Acid), 10 ml of Non-Essential Amino Acids (GIBCO BRL), 0.3 % L-Methionine (GIBCO BRL), 100 U Streptomycin (GIBCO BRL), 100 U Penicillin (Sigma), 0.05 M $NaHCO_3$ (GIBCO BRL), supplemented with 10 % heat inactivated Fetal Calf Serum (FCS), (GIBCO BRL). To passage the cells, 90-100% confluent cells were first washed with 1xPhosphate Buffered Saline (1xPBS) (0.1 M KH_2PO_4 (pH 7.2), 0.15 M NaCl(all purchased from VWR)), and then incubated with 2 ml of 0.25x trypsin (GIBCO BRL) at 37°C for 2 min. Trypsin treatment was stopped by adding 5 ml of DMEM supplemented with 10 % FCS. Cells were then counted using a

SPotlite® hematocymeter (Hausser Scientific) and seeded at $6x10^5$ per 75 cm flask (Falcon).

(ii) Transient Transfections

For transient transfections, cells were treated with 0.25x trypsin and seeded at a density of $2x10^5$ cells per 35 mm plate (Falcon) the night before the transfection. The cationic detergent Lipofectamine® (GIBCO BRL) was used to transfect the cells with specific DNA the following day. The transfection procedure was the same as described in the GIBCO BRL protocol [128]. Up to 2 μg of DNA mix along with 5 μl of Lipofectamine were placed in 200 μl of Optimem media (GIBCO BRL, Montreal, Quebec) and incubated at room temperature for 60 min to allow the cationic detergent and DNA to form a stable complex. After 60 min cells were washed with 1 ml of Optimem. Additional 800 μl of Optimem was added to DNA/Lipofectamine mixture which was then slowly added on top of the cells. Cells were incubated for 6 h at 37°C , after which 1 ml of DMEM supplemented with 20 % FCS was added. In the first experiments without C/EBP isoforms the ratio of DNA added was; 0.3 μg of CAT reporters, 0.6 μg of GR expression vectors, and 0.3 μg of the β-galactosidase reporter. In the experiments where C/EBP isoforms were cotransfected, DNA was added with the following ratio: 0.3 μg of CAT reporters, 0.6 μg of glucocorticoid receptor constructs, 0.3 μg of C/EBP constructs, and 0.3 μg of β-galoctosidase reporter.

Sixteen hours post transfection, 2 ml of fresh DMEM media + 10 % FCS supplemented with appropriate ligand or the ethanol carrier was added to the cells. The concentrations of ligands used were: 0.2 μM dexamethasone, 1.0 μM RU 486, and 0.05 μM DHT for plates transfected with androgen receptor. Cells were then allowed to grow for additional 48 hours after which they were harvested and cellular extracts were analyzed for Chloroamphenicol Acetyl Transferase (CAT) and β-galoctosidase activity.

5. Determination of Protein Concentrations by Bradford Assay

The Bio-Rad Microassay procedure was used to measure the concentration of protein present in the cytosolic extracts. Approximately10 μl of cytosolic extract was placed in 0.8 ml of H_2O, to which 0.2 ml of dye reagent concentrate was added. Samples were vortexed (avoiding excess foaming), and allowed to stand at room temperature for 15 minutes. Absorbance of each sample was measured at λ=595 nm, after which the protein concentration could be extrapolated using the standard curve. To obtain a standard curve, diluted concentrations of bovine serum albumin (New England Biolabs) were placed in 0.8 ml of H_2O. 0.2 ml of dye reagent concentrate (Bio-Rad) were added to each sample, vortexed avoiding excess foaming, and allowed to stand at room temperature. After 15 minutes samples absorbance was measured (λ=595 nm)

to obtain a standard curve. This curve was then used to estimate the concentrations of proteins present in the cytosolic extracts. Measured protein concentrations was used in the CAT and β-galactosidase assays.

6. β-*galactosidase Assays*

β-galactosidase assays were performed according to standard procedures [129]. To measure β-galoctosidase activity, 50 μl of the supernatant was added to 550 μl of Z buffer (60 mM Na_2PO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM Mg_2SO_4 (all purchased from VWR), 50 mM β-mercaptoethanol (Sigma), pH 7.0). Reactions were started by adding 120 μl of ONPG (4mg/ml O-Nirophenyl β-D-galctopyranoside (Sigma) in 100 mM phosphate buffer, pH 7.0) and incubated at 30°C. The reaction time was carefully monitored with a stop watch. Following the appearance of yellow colour 300 μl of 1 M Na_2CO_3 (VWR) was added to stop the reaction. Activity of samples were measured at OD(λ=420 nm) and used in the following formula for β-galoctosidase units:

β-galactosidase units = $OD_{(420)} \div$(0.0045x reaction time (min) x extract volume (50 μl) x protein concentration (μg/μl))

7. *Chloroamphenicol Acetyl Transferase Assays*

CAT assays were performed as previously described by Giffin et al. [130]. Cos 7 cells were harvested three days post transfection. Cells were first washed with 1xPBS, scrapped using a rubber policeman, and then collected into 1.5 ml Eppendorf tubes at 4°C. Cells were centrifuged for 1 min at 2000 r.p.m. (4°C) and resuspended in 200 μl of Freeze-Thaw buffer (FT buffer) (0.25 M sucrose, 10 mM Tris-HCl pH 7.4 (VWR), 10 mM EDTA (ethylenediamine tetraacetic acid) (Sigma). Cells were lysed by three freeze-thaw cycles in dry ice-methanol and followed by incubation at 37°C. Following freeze/thaw, samples were centrifuged at 4°C for 5 min and supernatnat was collected. Isolated supernatants were used in CAT, β- galactosidase, and Bradford assays. For CAT assays, 100 μg of protein was mixed with 1 μl of 0.2 μCi of ^{14}C Chloroamphenicol (Amersham) and 20 μl of 0.4 mg/ml Acetyl CoA (Pharmacia). The final volume was brought up to 200 μl in all samples with 0.25 Tris-HCl buffer (pH 7.4). Samples were incubated at 37°C for 2.5 hours (EREHSVtkCAT) or 4 hours (G5E1bCAT constructs). Reactions were stopped with 800 μl of ethyl acetate (Fisher Scientific). Samples were vortexed and spun for 5 min. Chloroamphenicol was then extracted from the aqueous phase and concentrated in a speed vacuum. Samples were resuspended in 10 μl of ethyl acetate, ran and separated on a thin layer chromatography plates (TLC plate) (VWR) with freshly prepared 95:5 chloroform:methanol solution (VWR). Conversion of acetylated chloramphenicol

was quantified using phosphorimager analysis (Bio-Rad), where conversion percentages were calculated using the following formula:

Conversion % = (Acetylated Chloramphenicol) ÷ (Total Chloroamphenicol)

Each converted CAT activity was corrected for β-galactosidase, where % conversion was divided by β-galactosidase activity. The resulting % conversion per β-galactosidase unit was displayed as a histogram in relative CAT units/β-gal units.

8. Co-immunoprecipitation of C/EBPS with full length myc-Tagged GR Affinity Purified from Sf-7 Cells

(i) SDS-Polyacrylamide Gel Electrophoresis

SDS-PAGE was carried out using a standard previously described protocol [131]. Protein samples were diluted in Laemmli-sample buffer and denatured by boiling for 3 min. Samples were loaded on 10 % gel [131] with a 4 %, 1 cm stacking gel. Gels run on mini-protein gel apparatus (Bio-Rad) and ran at 170 volts for 45 min. Gels used for resolving radiolabeled proteins were dried in a vacuum for 45 min at 80 °C, and then exposed for on a phosphorscreen for

phosphorimager analysis. Gels used for western blots were transferred to membranes using standard procedure [132].

(ii) Western Blotting

Western blotting for GR was done using standard previously described protocol [132]. Protein samples were loaded and run on SDS-PAGE gels. Following SDS-PAGE, protein samples were electroblotted from the SDS-PAGE gel to a PVDF membrane (Immobilon-P, Millipore) in standard electrode buffer supplemented with 20% methanol (v/v). PVDF membranes were charged with 100% methanol before the transfer. Following the transfer, membranes were first rinsed with 1xTBS+0.5% Tween, and then blocked for 1 h at room temperature with 1xTBS+0.5%Tween/5% skim milk. The primary antibody used was anti-GR antibody, BUGR 2 (1:2000 dilution) [133]. Detection of the BUGR 2 signal was done by enhanced chemiluminescence (ECL, Amershan) using horse radish peroxidase conjugated sheep anti-mouse antibody (1: 50 000 dilution) (Amersham) as the secondary antibody.

(iii) Coupled in vitro Transcription-Translation of C/EBP β and δ Isoforms

To *in vitro* transcribe and translate C/EBP β and δ isoforms, 1 μg of the indicated pGEM-7 plasmid DNA was incubated with T7 RNA polymerases using the Promega TNT coupled rabbit reticulocyte lysate system [134] including 10 mCi/ml of translation grade ^{35}Sulfur methionine (1000 Ci/mmol, Amersham).

Translations were performed as suggested by the manufacturer; each translation was incubated at 30 °C for 90-120 minutes in a final volume of 50 µl with 0.1 mM DTT added. Translations were stopped by adding an excess of unlabelled L-methionine (5mM). Each reaction was then quantified by using a portion of the sample on SDS-PAGE. Polyacrylamide gels were then dried in a vacuum at 80 °C. Translation quality and quantity were checked using phosphorimager analysis.

(iv) Preparation of Affinity Purified myc-Tagged GR From Sf-7 Cells

To affinity purify myc-tagged GR, $1x10^6$ of parental Sf-7 cells (ATCC) or Sf-7 cells stably integrated wild type myc-tagged GR (generated by David Rodda) were allowed to grow for 48 hours (or until 95-100 % confluent). Plates were treated with either 10^{-6} M dex, or 10^{-6} M RU 486 for 1 h before harvesting cells to transform the receptor.

Nuclear extracts were prepared using a standard procedure. To prepare nuclear extracts, cells first were resuspended in 400 µl low salt buffer (10 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.2 mM PMSF (phenylmethyl sulfonyl flouride) (Sigma), 0.5 mM DTT (Dithithreitol) (VWR)) and incubated on ice. After a 10 min incubation, samples were centrifuged to pellet the nuclei. Pellets were then resuspended in 150 µl of high salt buffer (20 mM HEPES-KOH pH 7.9, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 mM DTT, 25 % glycerol (GIBCO BRL)) and incubated on ice for 20 minutes.

Following the incubation, supernatants were then collected and used as nuclear extracts. The amount of protein present in each extract was quantified by Bradford assay. Nuclear extracts were then used to affinity purify myc-tagged GR.

Stably intergrated myc-tagged GR in Sf-7 cells was also transformed by adding 0.4 M NaCl to whole cell extracts [135]. Cell extracts were incubated with 0.4 M NaCl on ice for 60 min, and then at 25 °C for 25 min. This yielded an unliganded, hsp 90 free receptor [136].

To immunoprecipitate wild type myc-tagged GR, 150 μg of myc-tagged GR nuclear extract and control Sf-7 nuclear extract was used. Correction for salt concetrations was done with 0.6x Binding Buffer (minus salt) (Binding Buffer: 25 mM HEPES pH 7.9, 0.5 mM EDTA, 0.2 mM DTT(Dithithreitol) (VWR), 0.2 mM PMSF (phenylmethyl sulfonyl flouride) (Sigma), 0.1 % Nonident NP-40 (Sigma), 12 % Glycerol (GIBCO BRL)).

To immunoprecipitate myc-tagged GR, monoclonal anti-myc antibody, 9E10 was used(kindly provided by Dr. John Bell, University of Ottawa). Incubation with 9E10 was for 1 to 2 hours at 4 °C. To precipitate the antibody-GR complex protein A sepharose (Sigma) that had been pre-blocked with 50mg/ml BSA (New England Biolabs) was added to the samples and incubated for 30 minutes at 4 °C. Samples were washed three times to get rid of non-specific protein binding. The specificity of immunoprecipitation for GR were confirmed by anti-GR antibody, BUGR 2 [133], using a standard western blot procedure [132].

(v) Binding Assay

Myc-tagged GR-9E10-protein A sepharose beads complex was incubated in 150 μl of 0.6x binding buffer and 5 μl of rabbit reticulocyte lysate at 4 °C for 2 minutes. This was done to preblock protein A sepharose beads. Samples were then centrifuged at 4000 rpm (4°C), following which the precipitate was resuspended in another 150 μl of 0.6x Binding buffer. Binidng of 5 μl of the desired *in vitro* translated C/EBP isoform was incubated with affinity purified GR for 2 h at 4 °C.

After the 2 h incubation samples were washed 3 times with 500 μl of 0.6x binding buffer. Following the washes samples were resuspended in 20 μl of SDS running buffer, boiled for 5 minutes, and ran on SDS-PAGE The gel was then dried and bands were quantified by phosphorimager analysis.

III. RESULTS:

1. Activation of Herpes Simplex Virus Thymidine Kinase Transcription by Ligand Bound Glucocorticoid Receptor Occurs in the Absence of GREs

Most of the experiments presented in this project used the transient transfection assay to study the regulation of reporter genes. Transient co-transfection of GR expression constructs along with chloroamphenicol acetyl transferase (CAT) reporter genes were performed in Cos 7 cells. The CAT gene expression was driven either by several different synthetic promoters or the HSV tk proximal promoter (-/+ octamer motifs).

I started my project using the HSV tk CAT reporter as a constitutive promoter into which octamer transcription factor wild type and mutant DNA response elements were subcloned (Figure 3.a). Our expectation was that glucocorticoids would act to repress octamer motif dependent transcription as it had been reported previously that GR could decrease the binding of octamer factors 1 and 2 to octamer DNA motifs [137, 138]. No effects were expected on the HSV tk promoter alone. The HSV tk proximal promoter [118] contains two Sp1 DNA binding sites, one C/EBP DNA binding, and no discernible GRE (see figure 4.a).

When a CAT reporter construct driven by the HSV tk proximal promoter (containing either the octamer wild type or mutant DNA response elements) was co-transfected with wild type rat full length GR, a 4 fold increase in transcription from the basal level was observed in response to treatment with synthetic steroid

dexamethasone (dex) (see figure 3.b, lane 1 and 2). This result was surprising for two reasons: (1) Repression rather than activation of transcription was expected in this experiment, (2) HSV tk proximal promoter does not contain discernible GREs, and therefore should not have been positively affected by GR.

One possibility that the reporter plasmid might contain cryptic GREs had escaped our detection. It has been previously reported that *dam* methylation of the N6 position of adenine in the BamHI restriction site creates a cryptic GRE when plasmids are grown in a *dam*$^+$ DH5α *E.coli* strain [124]. To prevent the formation of cryptic GREs in the CAT reporter constructs, the reporter plasmids were re-grown in *dam*$^-$,*dcm*$^-$ bacteria strain (*E.coli,* Rb404). However, HSV tk CAT reporter plasmids that were re-grown in *E.coli* Rb404 strain showed the same effect, authenticating GRE independent activation of HSV tk transcription by ligand bound full length GR (figure 4.b, lane 1).

Further, dexamethasone treatment in the absence of co-transfected GR did not display an effect on the transcriptional level on HSV tk CAT construct in Cos 7 cells, illustrating that activation of transcription was not a fortuitous effect of the hormone treatment in this system (figure 4.b, lane 4).

Consequently, in all the following experiments all reporter plasmids (including the internal control β-galactosidase reporter construct) were grown in the *E.coli* Rb404 bacterial strain as a preventive measure to exclude the presence of cryptic GRE formation.

Figure 3: GR Activates HSV tk Transcription In the Absence or Presence of Octamer DNA Motifs

A. Schematic representation of the Herpes Simplex Virus thymidine kinase proximal promoter with wild type sequence or mutated octamer sequence response element linked to the CAT reporter gene. **B.** Cos 7 cells were co-transfected with HSV tk oct wild type or mutant CAT reporter construct and GR. Ligand treatment was performed wit 0.2 μM Dex in ethanol as indicated . CAT activities were measured as described in detail in the materials and methods section. All CAT activities were corrected for β-galactosidase activity used as an internal control.

A.


Oct wt or mt
SP1
C/EBP
SP1
TFIID
CAT
-109
-26

B.

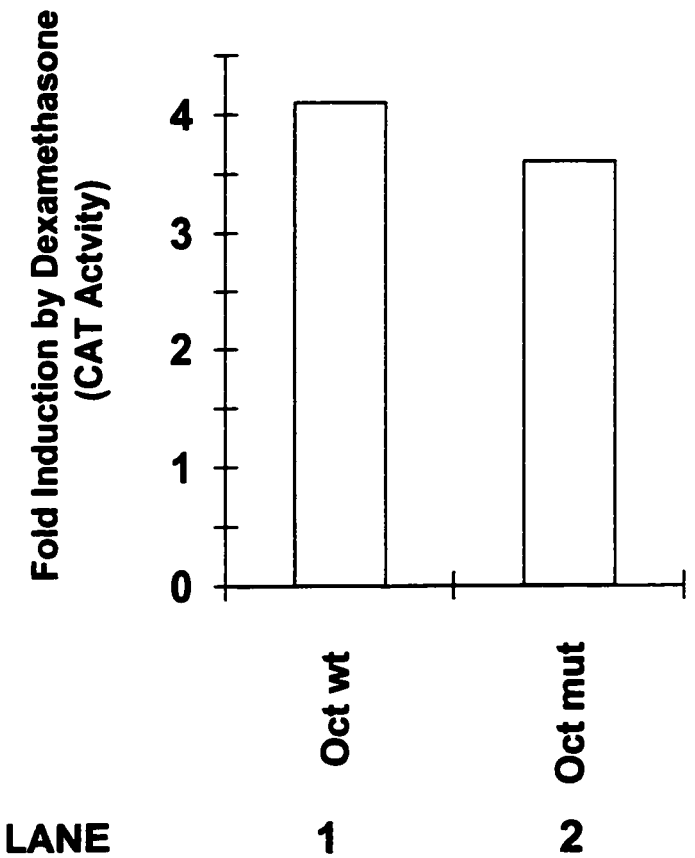
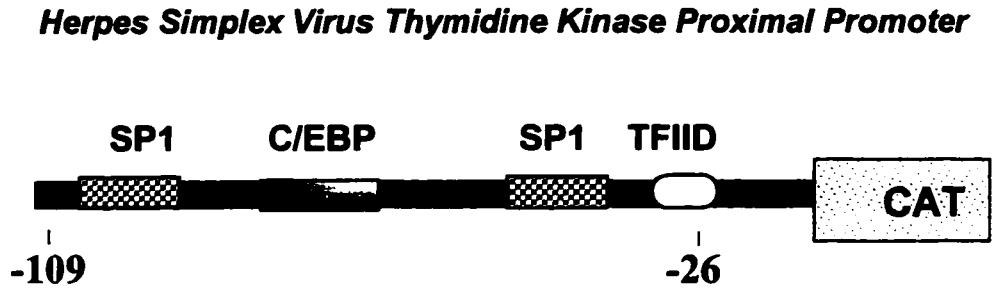

Fold Induction by Dexamethasone
(CAT Actvity)
0
1
2
3
4
Oct wt
Oct mut
LANE
1
2

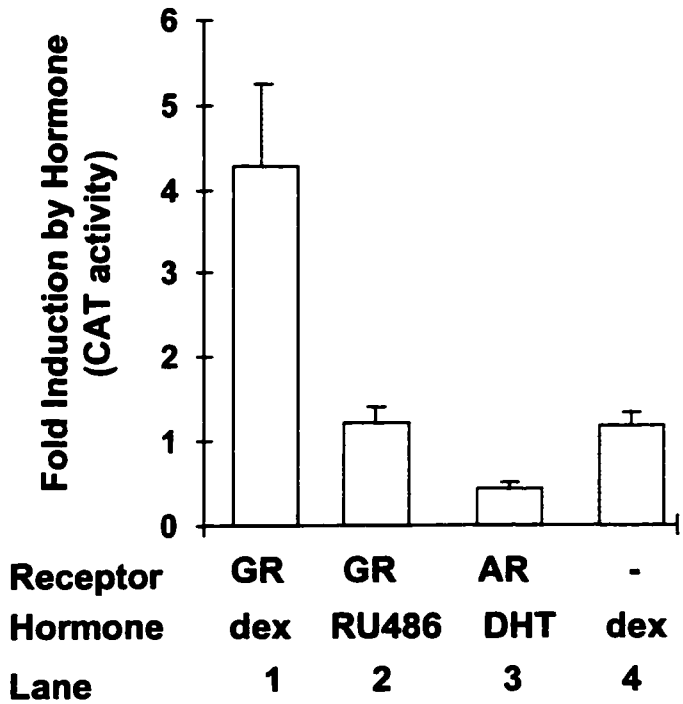
Figure 4: GR Activates HSV tk Transcription In the Absence of GREs Specifically In Response To Dexamethasone Treatment

A. Schematic representation of the Herpes Simplex Virus thymidine kinase proximal promoter (-109 to +51 bp) linked to a CAT reporter gene. HSV tk contains two GC rich Sp1 DNA binding sites found at -100 and -40 bp positions, and one "CCAAT" DNA response element at position - 80. HSV tk does not contain any discernible GREs. **B.** Cos 7 cells were co-transfected with the HSV tk CAT reporter construct and GR or AR expression plasmids prepared from *E.coli* Rb404 strain as indicated. Ligand treatment was performed wit 0.2 μM Dex, 1.0 μM RU 38486, or 0.05 μM DHT in ethanol as indicated . CAT activities were measured as described in detail in the methods and materials section. All CAT activities were corrected for β-galactosidase controls. Error bars represent the standard deviation obtained from three independent experiments performed in duplicates.

A.


Herpes Simplex Virus Thymidine Kinase Proximal Promoter
SP1
C/EBP
SP1
TFIID
CAT
-109
-26

B.


Fold Induction by Hormone (CAT activity)
6
5
4
3
2
1
0
Receptor GR GR AR -
Hormone dex RU486 DHT dex
Lane 1 2 3 4

2. Activation of HSV tk Transcription Is Dexamethasone Dependent and Glucocorticoid Receptor Specific

In my first result I observed that induction of HSV tk transcription by GR in the absence of GREs occurred specifically in response to dexamethasone treatment. As a result, the next two questions I sought to address were; (1) was the GR effect dependent on hormone agonist in the absence of GREs, and (2) could other members of the steroid receptor family similarly activate HSV tk transcription in response to its hormone treatment.

To address the first question, Cos 7 cells that were co-transfected with GR and HSV tk CAT reporter and treated with GR antagonist, RU 486 (figure 4.b, lane 2). By contrast to dexamethasone treatment, an induction of HSV tk transcription was not observed with RU 486 treatment.

To address the second question, Cos 7 cells were co-transfected with androgen receptor (AR), instead of GR. Following dihydroxytestosterone (DHT) treatment (the active form of andorgen), a consistent 2 fold decrease from the basal level was observed (figure 4.b, lane 3). This apparent repression of HSV tk transcription by AR was not pursued further. However, this result clearly indicated that activation of HSV tk transcription was specific for GR.

Together, these results have shown that induction of HSV tk transcripiton occurred specifically with GR and was dexamethasone dependent.

3. Ligand Binding Domain of GR is Sufficient for Full Activation of HSV tk Transcription

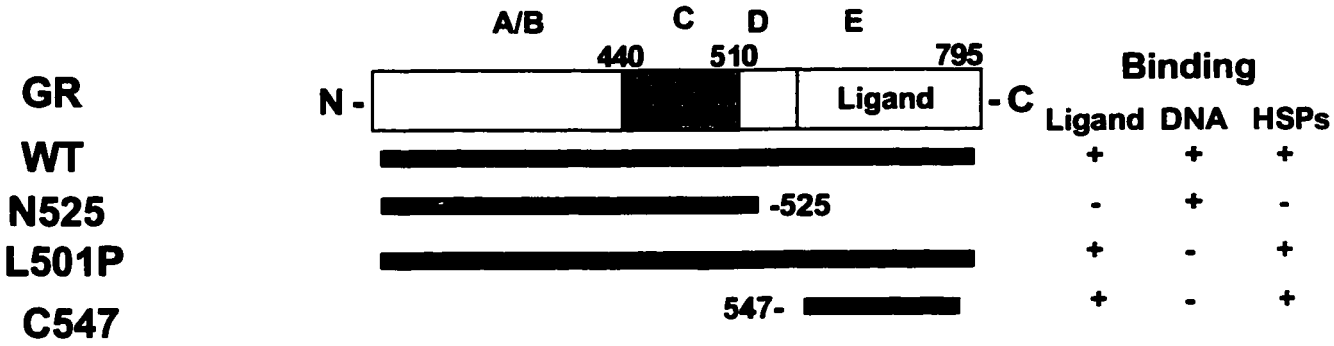
In the next series of experiments, my objective was to investigate and map the region of rat GR that was responsible for HSV tk activation of transcription in the absence of GREs. To map this region, I used four different GR expression constructs (summarized in figure 5.a), and transiently expressed them with the HSV tk CAT reporter construct in Cos 7 cells. Besides wild type full length rat GR, three additional GR constructs used were: (1) GR L501P, a site-directed mutant constructs that lacks the ability to bind DNA [119], (2) GR N525, a receptor lacking the LBD, and (3) GR 547C, construct containing the full length LBD alone. As shown in the earlier experiment, wild type full length GR showed over 4 fold induction following dex treatment (figure 5.b, lane 1). GR L501P induced HSV tk transcription actually slightly better (~ 5 fold), than the wild type GR, following dex treatment (figure 5.b, lane 2). This form of receptor possibly activates better than the wild type, since it is not being directed to GREs elsewhere in the nucleus. Again, this result was consistent with the previous observation that HSV tk activation of occurred in the absence of a GRE and confirmed that GR was activating transcription without binding directly to DNA. By contrast, the GR N525 construct lacking the LBD did not show an increase in transcription above the basal level of HSV tk CAT alone (figure 5.b, lane 3). However, the GR 547C construct containing the LBD alone retained full transcriptional induction activity of wild type GR following dex treatment (figure 5.b, lane 4). This set of experiments is consistent with the initial observation that HSV tk activation of transcription occurs independent of GREs, and shows that

GR LBD alone is sufficient and able to retain the full transcriptional activity of wild type GR in activation of HSV tk transcription.

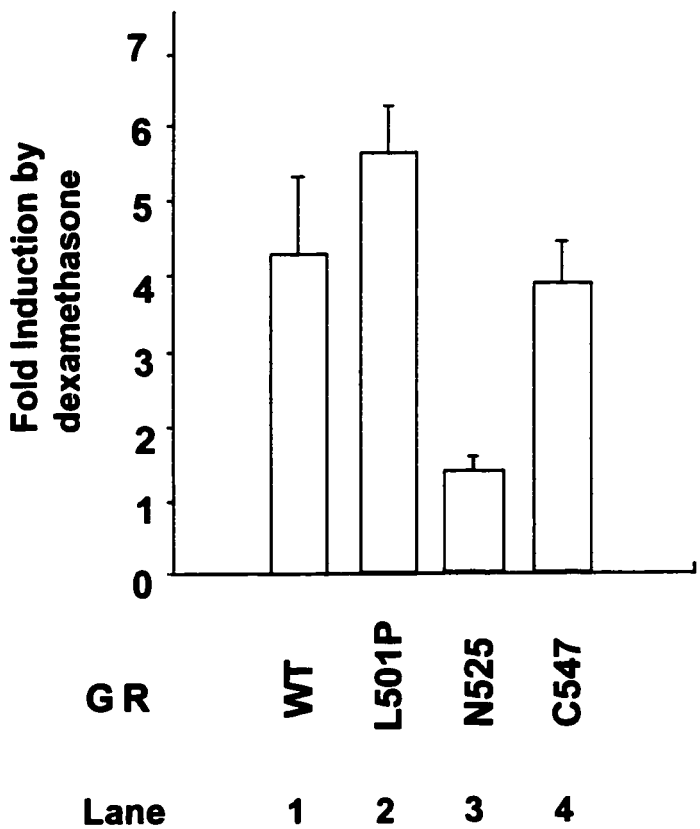
Figure 5: The Ligand Binding Domain of GR is Sufficient To Activate HSV tk Transcription

A. Schematic representation of rat GR expression constructs used in this series of experiments. GR N525 expression construct spans amino acid 1 to 525 and lacks the ligand binding domain. GR L501P constructs contains a mutation in its DNA binding domain in which the leucine at 501 position is changed for a proline, this mutation prevents this construct from binding to DNA. GR 547C construct contains just the ligand binding domain of GR spanning amino acids 547 to 795. **B.** Cos 7 cells were co-transfected with HSV tk CAT and GR expression constructs as indicated. Ligand treatment was performed with 0.2 μM dex in ethanol. Results are represented as fold induction of CAT activity by dexamethasone. All CAT activities were corrected for β-galactosidase activity as indicated in methods and materials. Error bars represent the standard deviation obtained from three independent experiments performed in duplicate.

A.


A/B
C
D
E
440
510
795
GR
N -
Ligand
- C
Binding
Ligand DNA HSPs
WT
+ + +
N525
-525
- + -
L501P
+ - +
C547
547-
+ - +

B.


Fold Induction by dexamethasone
0
1
2
3
4
5
6
7
GR
WT
L501P
N525
C547
Lane
1
2
3
4

4. Activation by Dex Activated GR That Lacks the Ability To Bind DNA Occurs Through C/EBP DNA Response Elements

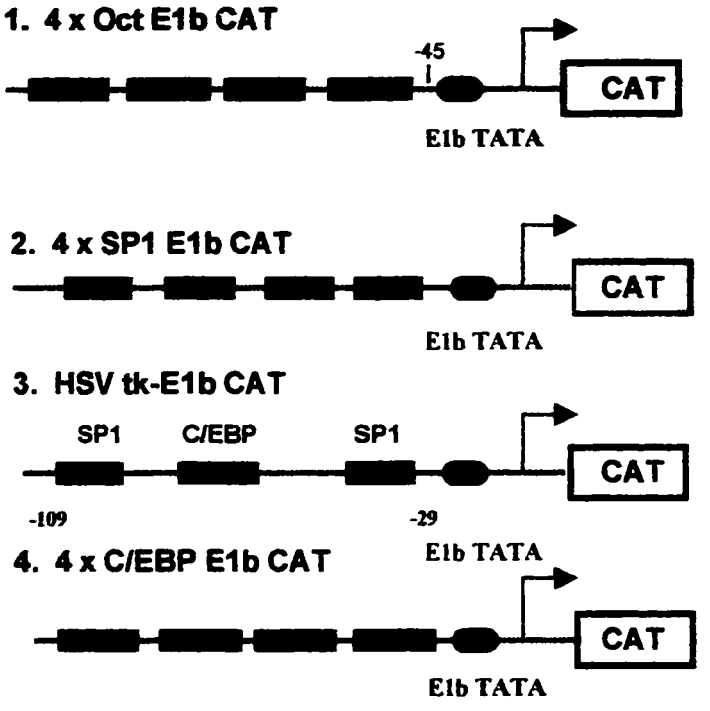
The results of my experiments to date suggested that GR might be activating transcription in the absence of DNA binding by affecting the activity of one of the transcription factors for which DNA response elements occurred in the HSV tk proximal promoter. To address the question of where the effect was occurring on HSV tk promoter, and whether it was occurring through one or a combination of DNA response elements, I constructed four additional synthetic promoter CAT reporter constructs isolating the various tk regulatory elements (summarized in figure 6.a). Each of the first three synthetic promoter reporter constructs contained a series of four DNA response elements for Sp1, oct, or C/EBP. The fourth CAT reporter construct contained HSV tk proximal promoter (-109 to -40) with the E1b TATA instead of HSV tk TATA box. This reporter was designed to test whether both Sp1 and "CCAAT" DNA motifs were needed for glucocorticoid responsiveness ,and to see whether activation was promoter specific.

These four CAT reporter constructs were co-transfected with the GR L501P expression construct into Cos 7 cells, treated with dex, and cellular extracts were analyzed for CAT activity. A synthetic CAT reporter construct containing 4 octamer DNA motifs adjacent to E1b TATA box was used as a control to demonstrate that this minimal E1b promoter was not dexamethasone responsive. As predicted, the addition of four octamer motifs

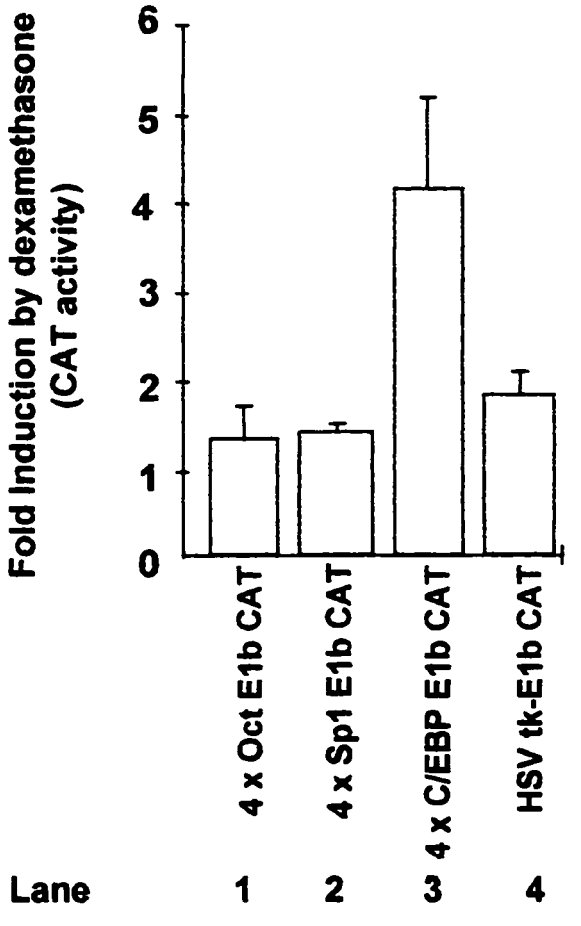
Figure 6: GR Specifically Enhances Transcription Through "CCAAT" DNA Response Element In The Absence of GREs

A. Schematic diagrams representing the different synthetic promoters tested for glucocorticoid responsiveness of the CAT gene. 4xOct E1b CAT reporter construct contains four octamer motifs and an E1b TATA box driving the expression of the CAT gene the 4xSp1 E1b CAT and 4xC/EBP E1b CAT constructs contain four Sp1 DNA response elements and four "CCAAT" DNA response elements, respectively, in front of the E1b TATA box driving the expression of the CAT gene. HSV tk CAT contains HSV tk promoter from -109 to -40 in front of the E1b TATA box driving the expression of the CAT gene. **B.** Cos 7 cells were co-transfected with GR L501P and CAT reporter constructs as indicated. Ligand treatment was performed with 0.2 μM dex. CAT activity is represented as fold induction by dexamethasone, that is corrected for β-galactosidase activity. Error bars represent the standard deviation obtained from three independent experiments performed in duplicate.

A.


1. 4 x Oct E1b CAT
-45
CAT
E1b TATA
2. 4 x SP1 E1b CAT
CAT
E1b TATA
3. HSV tk-E1b CAT
SP1
C/EBP
SP1
CAT
-109
-29
E1b TATA
4. 4 x C/EBP E1b CAT
CAT
E1b TATA

B.


Fold Induction by dexamethasone
(CAT activity)
6
5
4
3
2
1
0
4 x Oct E1b CAT
4 x Sp1 E1b CAT
4 x C/EBP E1b CAT
HSV tk-E1b CAT
Lane
1
2
3
4

failed to elicit a response to GR and dex treatment. Following dex treatment the only CAT reporter construct that showed a strong increase in transcription following dex treatment contained C/EBP DNA response elements (figure 6.b, lane 3). Activation of transcription following dex treatment was 4 fold with 4xC/EBP E1b CAT reporter. Interestingly, the HSV tk CAT reporter in which the HSV tk TATA was substituted with the E1b TATA box showed only a 2 fold increase in transcription following dex treatment. Thus one "CCAAT" DNA response element was able to function in the context of HSV tk minimal promoter, while four copies were needed with E1b minimal promoter to see the same effect. This result suggested that the different distance (16 bp) between the "CCAAT" element and the TATA box in these two different reporter constructs determined the strength of the response of the C/EBP elements to liganded GR. Thus the element that mediated the GR effect following dex treatment was the C/EBP binding element.

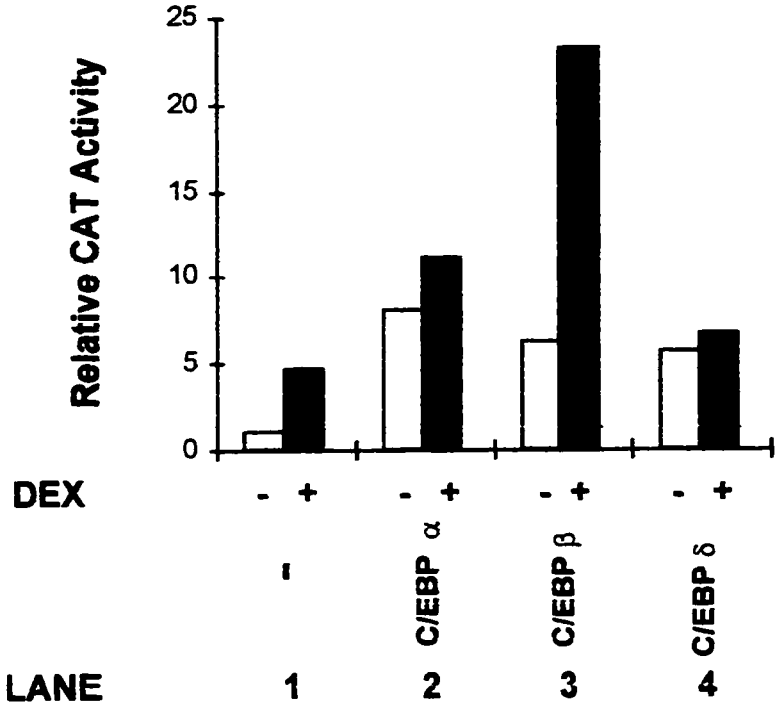
5. Dex Activated Glucocorticoid Receptor Specifically Enhances C/EBPβ Mediated Transcription on a "CCAAT" DNA Response Element

Members of the C/EBP transcription factor family are known to bind and activate transcription through the "CCAAT" DNA response elements. Because activation of transcription by GR in the absence of GREs occurred through "CCAAT" DNA response elements, I sought to determine (1) whether GR that

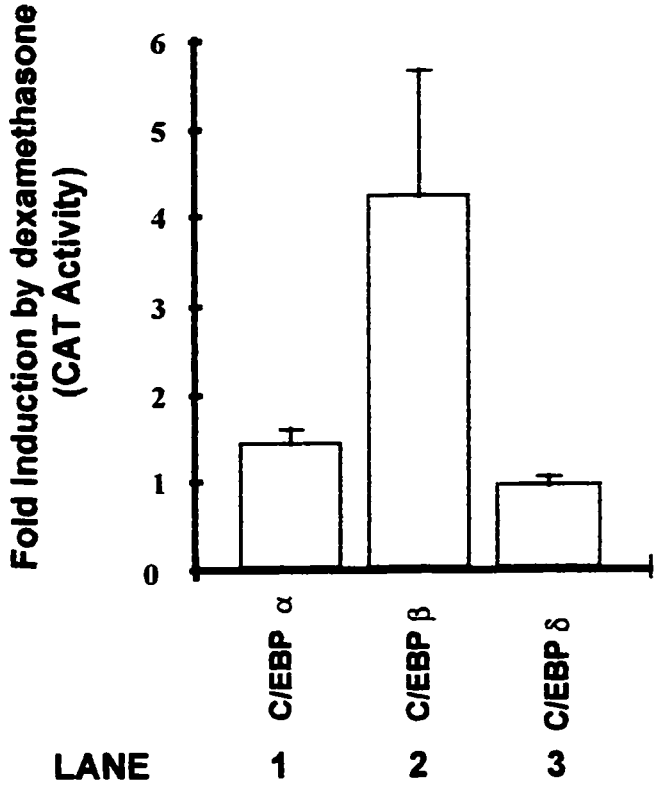
Figure 7: Enhancement of Transcription by GR Through a "CCAAT" DNA Response Element Occurs Specifically Through C/EBPβ

A. Cos 7 cells were co-transfected with GR L501P, 4xC/EBP E1b CAT and C/EBP α, β, and δ isoforms as indicated below the histogram. Cells were treated with 0.2 μM dex in ethanol. CAT activities were corrected for β-galactosidase. **B.** CAT activities from A represented as fold induction by dex for the different C/EBP isoforms as indicated below the diagram. Error bars represent the standard deviation obtained from three independent experiments performed in duplicate.

A.


Relative CAT Activity
25
20
15
10
5
0
DEX
- + - + - + - +
I
C/EBP α
C/EBP β
C/EBP δ
LANE
1 2 3 4

B.


Fold Induction by dexamethasone
(CAT Activity)
6
5
4
3
2
1
0
C/EBP α
C/EBP β
C/EBP δ
LANE
1 2 3

lacked the ability to bind DNA was able to compliment transcriptional activity of overexpressed C/EBP transcription factors, (2) whether this effect was general or specific to one of the C/EBP family members. For this reason I obtained three C/EBP isoform eukaryotic expression constructs of C/EBPα, C/EBPβ, and C/EBPδ (SL. McKnight, Tularik, San Francisco). To determine whether I could recapitulate dex activated GR effect with overexpressed C/EBP isoforms, I co-transfected each isoform with GR L501P construct and 4xC/EBPE1bCAT reporter construct into Cos 7 cells (figure 7). Each of the C/EBP isoforms activated transcription between 5 and 8 fold from the 4xC/EBPE1bCAT reporter construct. However, dexamethasone treatment only significantly enhanced C/EBPβ activation of transcription (figure 7.a,b). The specific effect of dexamethasone potentiating activation by each C/EBP isoform is summarized as fold induction by dex in figure 7.b. This experiment clearly showed that GR effected transcription specifically enhanced C/EBPβ transcription four fold following dex treatment. These results also demonstrated that activation by dex activated GR was not general for the three C/EBP family members tested, but rather specific for C/EBPβ.

In addition, this experiment also addressed another important question. In the previous section the possibility that GR acted indirectly to induce endogenous C/EBP expression (production) which in turn led to increase transcription through the "CCAAT" DNA response element, remained uncertain. However, as GR activated transcription through an overexpressed C/EBPβ isoform demonstrated

that activation of transcription occurs through complementation rather than by inducing the synthesis of endogenous C/EBP.

6. Mammalian Two Hybrid Assay Reflects That GR LBD Binds Specifically to C/EBPβ in vivo

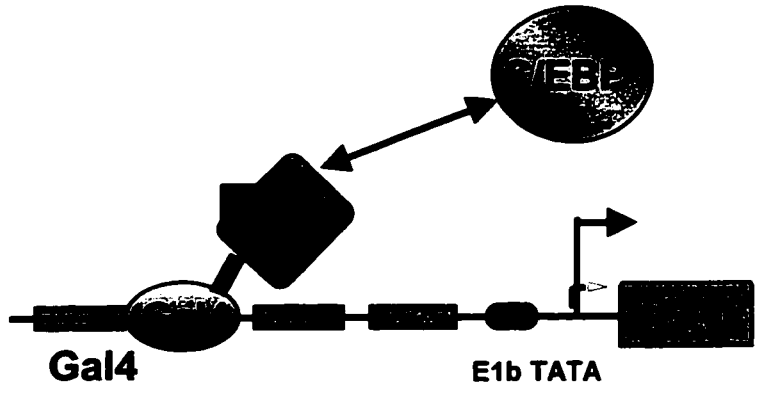
To investigate whether complementation of C/EBPβ by dex activated GR occurs through formation of a protein complex *in vivo*, I next used the mammalian two hybrid assay. In this experiment, the GR ligand binding domain fused to the Gal4 DNA binding domain (Gal-LBD) was used as the bait for a two hybrid assay in Cos7 cells. The reporter gene in this is assay was driven by 5 copies of Gal4 DNA response elements. To compliment Gal-LBD, different C/EBP expression constructs were co-transfected separately as illustrated in Figure 8.a . This assay was modified from the typical two hybrid assay in that the endogenous C/EBP transcriptional activation domains were used rather than Gal4 or VP16 domains typically employed. Cells co-transfected with the appropriate vector combination were treated with 0.2 μM dexamethasone, and cellular extracts were analyzed for CAT activity.

Expression of Gal-LBD activated transcription 4 fold following dex treatment (figure 8.b lane 1). This result was expected, since GR LBD has the ability to activate transcription through its own activation domain (AF-2). However, when the three C/EBP expression vectors were co-transfected with

Figure 8: Dex Bound Ligand Binding Domain of GR Specifically Interacts With C/EBPβ In a Mammalian Two Hybrid Assay

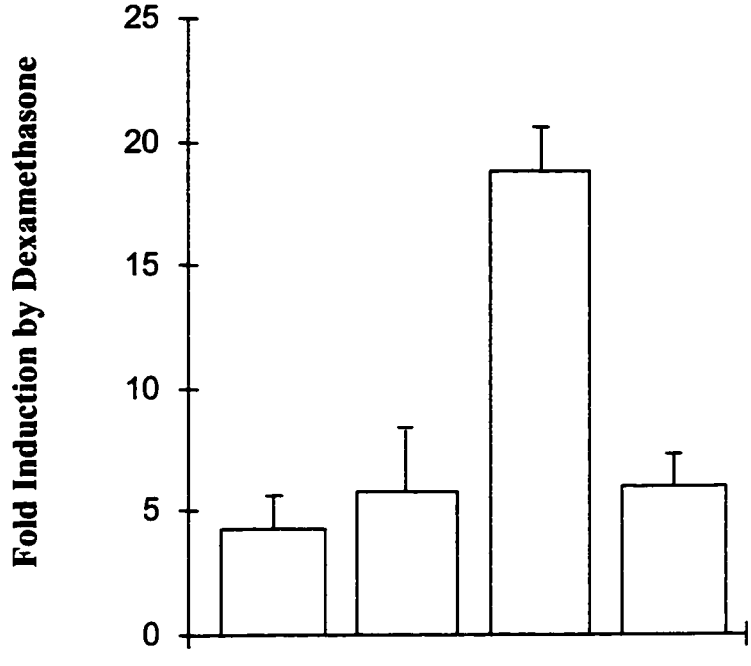
A. Schematic illustration of the modified mammalian two hybrid assay used to investigate protein protein interaction between the GR-LBD and C/EBPs. **B.** Cos 7 cells were cotransfected with G5E1bCAT reporter, containing 5 copies of Gal4 DNA response elements, Gal-LBD fusion construct, and C/EBP expression constructs as indicated in the chart below the histogram. Cells were treated with 0.2 μM Dex. CAT activity was measured and corrected for β-galactosidase activity as indicated in methods and materials. Error bars represent the standard deviation obtained from three independent experiments performed in duplicates. **C.** Cos 7 cells were cotransfected with G5E1b CAT reporter, Gal0 expression construct, and the different C/EBP isoform constructs as indicated. CAT activity was measured as described in materials and methods and corrected for β-galactosidase activity used an internal control control.

A.



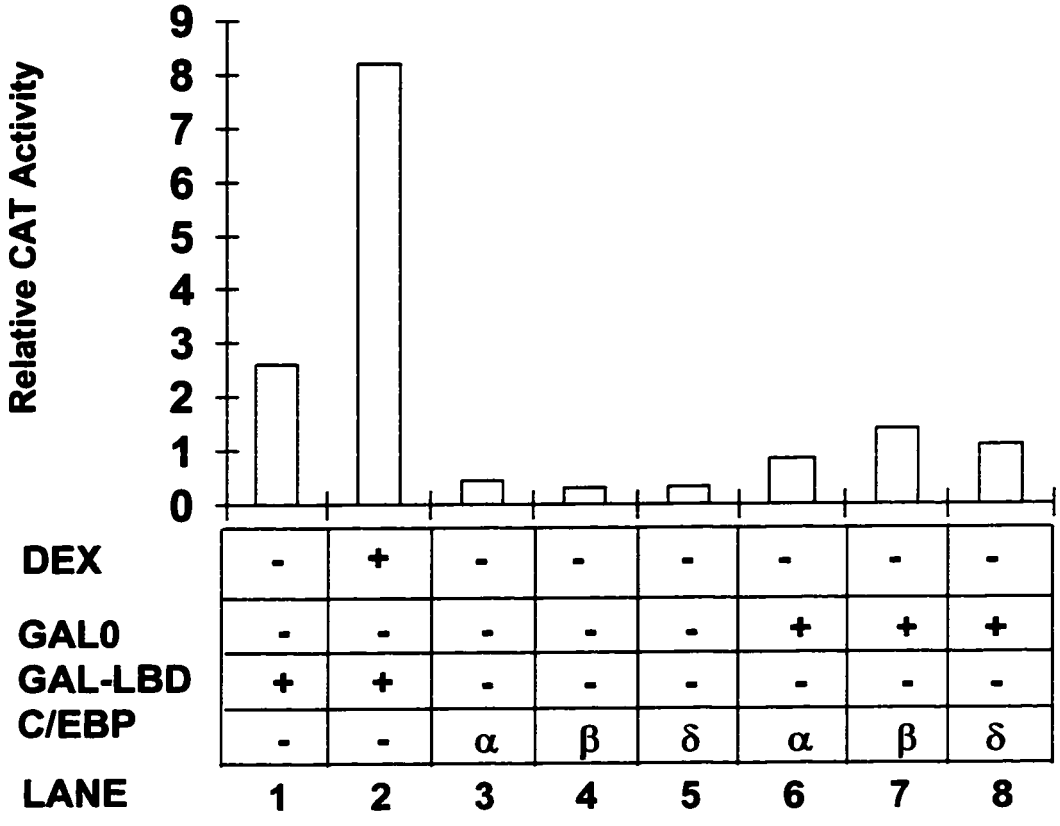
G5 E1b CAT reporter

B.



Gal-LBD	+	+	+	+
C/EBPα	-	+	-	-
C/EBPβ	-	-	+	-
C/EBPδ	-	-	-	+

C.


Relative CAT Activity
9
8
7
6
5
4
3
2
1
0
DEX
GAL0
GAL-LBD
C/EBP
LANE
- + - - - - - -
- - - - - + + +
+ + - - - - - -
- - α β δ α β δ
1 2 3 4 5 6 7 8

Gal-LBD and treated with dex, an additional increase to a total induction of 18 fold specific for C/EBPβ was observed (figure 8.b). No significant increase was seen with when C/EBPα or δ were co-transfected with dex bound Gal-LBD over GAL-LBD alone (figure 8.b). This result indicates that dex activated GR LBD and C/EBPβ, in contrast with C/EBPα or δ, are able to form a complex *in vivo* that activated transcription from Gal4 binding sites. To confirm that the effect was specific for the LBD, I also used the Gal4 DNA binding domain alone (GAL0) together with the different C/EBPs. This co-transfection did not yield any increase in activation above the basal level of GAL0 (figure 8.c). Finally, I examined whether the different isoforms of C/EBP could activate the G5E1bCAT reporter construct in the absence of the Gal-LBD. Again I did not see any activation of transcription by C/EBPs through the G5E1bCAT reporter plasmid (figure 8.c).

7. Co-immunoprecipitation of C/EBPS with full length myc-Tagged GR Affinity Purified from Sf-7 Cells

As a final experiment , immunoprecipitation binding assays between full length GR and C/EBPβ and C/EBPδ were done to confirm that GR compliments C/EBPβ activity by direct protein protein interaction. In this experiment, myc-tagged wild type GR was affinity purified by immunoprecipitation with a monoclonal myc-tag antibody, 9E10 and used as the binding matrix for *in vitro* translated C/EBPs. Immunoprecipitations were performed on two cell lines, Sf-7 parental cell line (- GR), and Sf-7 cell line which had stably integrated myc-

tagged GR (+GR). Western analysis performed on -GR, and +GR extracts using a GR polyclonal antibody, BuGR, confirmed the efficiency and specificity of the immunoprecipitation (figure 8.b).

C/EBPβ and C/EBPδ were subcloned into *in vitro* translatable vectors and translated with radiolabeled ^{35}S methionine. Sf-7 -GR and +GR cell lines were treated with different ligands or salt (to transform GR) prior to immunoprecipitation as indicated above each gel in figure 9.a. The actual results of the immunoprecipitation binding assays between GR and C/EBPβ or C/EBPδ are presented in this figure. The summary of these experiments is presented as a histogram in figure 8.c in which binding assays were quantified using phosphorimager analysis, following which +GR binding assay samples were divided by -GR samples for each treatment to determine the fold binding to GR.

Extracts which contained the myc-GR immunoprecipitated C/EBPβ more efficiently than protein A alone (figure 8.c, lane 1). Interestingly, a similar result was observed with RU 486 and salt treatment for GR and C/EBPβ (figure 8.c, lanes 2,3). Salt treatment was used to transform the myc tagged glucocorticoid receptor. More surprisingly, the same result was also observed for the binding assay between GR and C/EBPδ following dex, RU 486, and salt treatment (figure 8.c, lanes 4-6). These results indicate that full length GR and C/EBPβ and C/EBPδ are able to interact by direct protein-protein contacts. However, only agonist bound GR/C/EBPβ complex appears to be competent to potentiate transcription. In the future it would be desirable to continue probing these

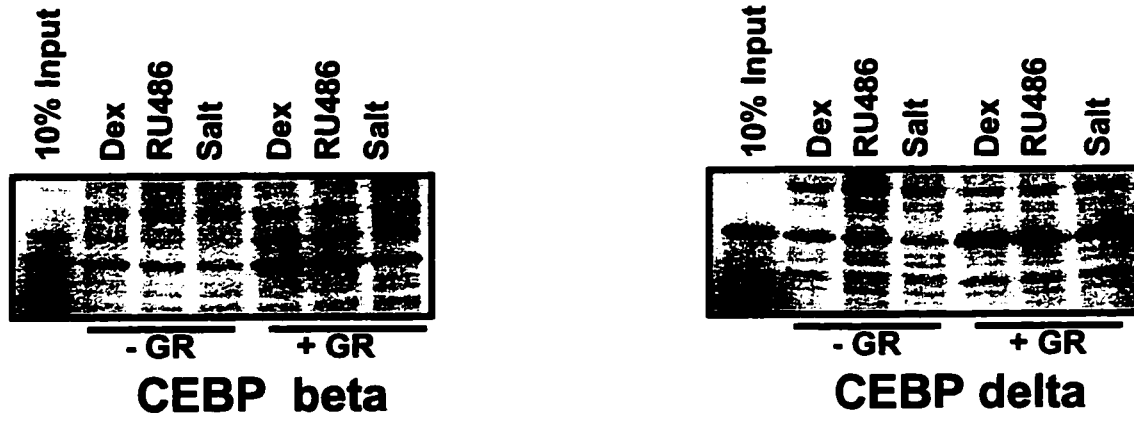
interactions using GST pull down assays with the C/EBPs used as the matrix to pull down various *in vitro* translated segments of GR. The result of these future experiments will help determine and clarify the specificity of GR and C/EBPβ direct protein-protein interaction and its relevance to transcriptional regulation.

Figure 9: Co-immunoprecipitation of C/EBPS with full length myc-Tagged GR Affinity Purified from Sf-7 Cells

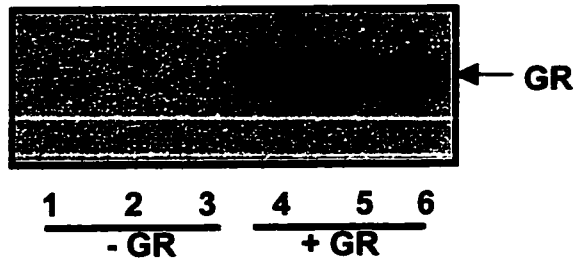
A. Binding assays between affinity purified myc-tagged full length GR and in vitro translated C/EBPβ or C/EBPδ. 35S-met labeled C/EBPβ and C/EBPδ were incubated with myc-tagged purified GR immunoprecipitated from Sf-7 cell line (+GR), or the parental Sf-7 cell line as negative control (-GR) as described in detail in methods and materials. Cell treatments as indicated above the gels were performed 1 h prior to immunoprecipitation. The bound proteins were analyzed by 12 % SDS-PAGE and visualized using phosphorimager analysis. Ten percent of the input protein are shown in the first lane for each gel. **B.** Western blot with anti-GR antibody, BuGR, done for both the parental cell line lacking myc-tagged GR (lanes 1 to 3) and with myc-tagged GR (lanes 4 to 6). Western analysis was performed as described in methods and materials section.

C. Histogram summarizing the binding results for C/EBPβ and δ. Gels in diagram A. were quantified using a phosphorimager, and are represented as fold binding between +GR/-GR samples for each treatment as indicated below histogram.

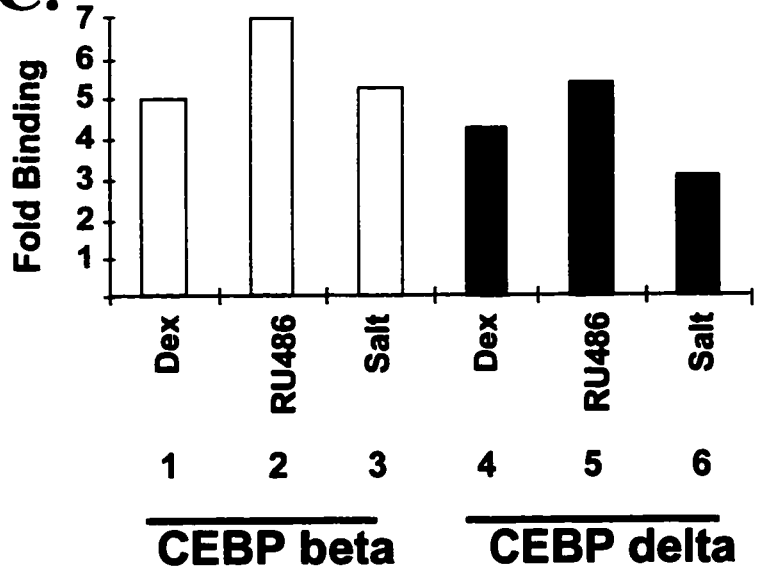
A.


10% Input
Dex
RU486
Salt
Dex
RU486
Salt
- GR
+ GR
CEBP beta
10% Input
Dex
RU486
Salt
Dex
RU486
Salt
- GR
+ GR
CEBP delta

B.


GR
1 2 3 4 5 6
- GR
+ GR

C.


Fold Binding
7
6
5
4
3
2
1
Dex
RU486
Salt
Dex
RU486
Salt
1 2 3 4 5 6
CEBP beta
CEBP delta

IV. Discussion:

1. Glucocorticoid Receptor Activates HSV thymidine kinase Transcription Following Dexamethasone Treatment in the Absence of GREs

In order to study transcriptional regulation by GR, I employed the transient transfection approach in which GR expression construct were co-transfected along with a HSV tk CAT reporter constructs into Cos 7 cells. The HSV tk proximal promoter spanning -109 to -5 base pairs employed (figure 4.a), contains two DNA response elements for Sp1 and one "CCAAT" DNA response element. However, the HSV tk sequence does not contain discernible GREs and therefore should not have been responsive to glucocorticoids. Initially, when I was setting up a set of transient assays to study GR's transcriptional regulation of Oct 1 and 2, it became apparent that the control sequence, the Herpes Simplex Virus thymidine kinase promoter, activated transcription 4 fold following dexamethasone treatment (figure 3, and figure 4.b lane 1). This result was surprising because it showed that GR was regulating transcription in a non conventional manner without GREs.

The possibility of cryptic GRE formation through *dam* methylation was excluded in the reporter plasmid constructs by re-growing in a *dam*$^-$, *dcm*$^-$ *E.coli* strain. As mentioned earlier in the results section, *dam* methylation by *E.coli* can create cryptic GREs in BamHI restriction sites [124]. The CAT reporter plasmids that were re-grown in this fashion, activated transcription to the same extent as initially observed. Therefore, together, these results were the initial evidence that

strongly suggested to a GRE independent activation of HSV tk transcription by GR following dexamethasone treatment. Transcriptional regulation in the absence of direct DNA binding is beginning to be reported for GR and other nuclear receptors [89, 113, 114, 139]. Of particular relevance to this project, GR has been found to enhance STAT5 transcription in the absence of a GRE following hormone treatment. In this case, GR mediated transcription as a ligand dependent coactivator for STAT5 [89].

2. Activation of HSV tk Transcription Is Ligand Dependent and Glucocorticoid Receptor Specific

In the next set of experiments, it was investigated whether activation of HSV tk transcription by GR can occur with the GR antagonist, RU 486. RU 486, like dexamethasone, binds to the ligand binding region of GR. However, the receptor's LBD folding is different than with dexamethasone, producing an unconventional conformation of the LBD which allows the receptor to bind DNA, but is unable to activate transcription [140]. In my experiments, RU 486 treatment did not activate GRE independent transcription above the basal level (figure 4.b, lane 2). This result showed that activation of HSV tk by GR was dexamethasone dependent, and pointed to a requirement for the AF-2 in LBD.

To investigate whether other steroid receptors could activate HSV tk transcription, I co-transfected androgen receptor (AR) with HSV tk CAT reporter gene into Cos 7 cells. Following DHT treatment, a consistent 2 fold repression of HSV transcription was observed. This result contrasted the earlier observation in

which GR activated transcription from this reporter plasmid, and thus was strong evidence that activation of HSV tk transcription was not a general property to nuclear receptors. The statistically significant 2 fold repression of HSV tk transcription by DHT treated AR could have possibly occurred by competition for common coactivators of AR and the upstream transcription factors whose DNA motifs are found in the HSV tk promoter. However, investigating the mechanism of repression by AR was disregarded in the scope of this study.

This experiment also confirmed that the HSV tk reporter gene did not contain GREs. All steroid receptors (with the exception of ER) are able to activate transcription from the same palindromic DNA response element (GRE = hormone response element = androgen response element) following appropriate hormone treatment ([141] and references within). AR's absence of transcriptional activation from the HSV tk CAT reporter gene following DHT treatment indicated the lack of discernible GREs in the HSV tk CAT reporter plasmid.

Together these results show that GRE independent activation of HSV tk transcription was dexamethasone dependent and GR specific.

3. The Ligand Binding Domain of GR Is Sufficient For Full Transcriptional Activation of HSV tk Promoter

Four different rat GR constructs were used to map the region necessary for activation of HSV tk transcription, the wild type, L501P, N525, and 547C.

GR L501P expression construct contains a site directed mutation in the DNA binding in which the leucine at the 501 amino acid position was replaced with a proline [119]. This mutation prevents GR's ability to bind specifically to DNA [142]. When GR L501P was expressed with HSV tk CAT reporter gene, it activated transcription 5 fold following dexamethasone treatment. This result clearly indicated that GR's ability to bind DNA was not required for HSV tk activation. GR L501P activation of HSV tk transcription also supports the previous observation that activation of HSV tk transcription by GR occurred independently of DNA binding sites in the reporter plasmid. It was also observed that GR L501P activation was more effective than that of wild type GR. This possibly occurred because more GR L501P was available for GRE independent activation since GR L501P did not have the ability to bind to endogenous GRE sites in the cell. Another student in the lab, G. Préfontaine, has determined that the wild type GR and GR L501P expression vectors are expressed at the same levels in the cell [142].

GR N525 expression construct that lacked the ligand binding domain did not increase HSV transcription above the basal level when cotransfected with the reporter construct. This result indicated that the region necessary for HSV tk activation of transcription was not found either in the N terminus or the DNA binding domain. Therefore, the transcriptional effect that was observed earlier was not dependent on the AF-1 function or the DBD, showing that AF-1 domain was not responsible for induction of HSV tk transcription.

Studies comparing AF-1 and AF-2 functions have shown that individual domains show low levels of activity when expressed independently [143]. But when the two AFs are expressed together, they synergize to maximally activate transcription, showing that both AFs are required for full receptor function as an upstream transcription factor. This effect was not apparent in these experiments.

The last construct tested in this series of experiments was GR's ligand binding domain alone spanning amino acids 547 to 795. When this expression construct was cotransfected with HSV tk CAT reporter into Cos 7 cells, the same four fold induction of transcription was observed following dexamethasone treatment as with the full length wild type GR expression construct. This result, consistent with the previous series of experiments, showed that the AF-2 function was required and was indeed sufficient for activation of transcription. The lack of the DNA binding domain in this expression construct was also consistent with the activation of HSV tk transcription occurring independently of direct DNA binding. The fact that the N terminal domain of GR, and more specifically AF-1 function, was irrelevant for activation of transcription was surprising. By comparison, the AF-2 domain alone had the same effect as the two AFs expressed together in the wild type receptor. Interestingly, similar functional results were also observed with GR and NF-IL6 synergistic activities in which the AF-1 function was also not required for full transcriptional activation by GR [113].

Significantly, the four fold activation following dexamethasone treatment observed in this and the previous result, was similar to transcriptional enhancement observed with transiently expressed coactivators such as SRC-1,

Indeed, these coactivators typically yield an approximate three fold increase in activation of transcription by their sequence specific transcription factors [35].

Together; GRE independent activation, AF-2 sufficiency for full transcriptional activity, and the lack of requirement for GR's DBD for transcriptional activity, and four fold induction, provide evidence that GR was not functioning as a sequence specific transcription factor but rather as a ligand dependent coactivator for another heterologous transcription factor whose DNA response element is found in the HSV tk promoter.

4. Glucocorticoid Receptor Activates Transcription Through The "CCAAT" Response Element Following Dexamethasone Treatment

To investigate whether dexamethasone bound GR activated transcription as a ligand dependent coactivator for an upstream factor (or factors) whose element or a combination of elements was present in the HSV tk promoter, I created the four different CAT reporter constructs summarized in figure 6.a. The first two reporter contain four copies of either Sp1, or "CCAAT" DNA response elements in front of the E1b TATA box, respectively. The third CAT reporter construct contains the HSV tk sequence (-109 to -40 bp) in front of the E1b TATA box. Finally, 4xOct E1b CAT reporter was used as a negative control for E1b minimal promoter. When each CAT reporter was co-transfected with GR L501P expression construct into Cos7 cells and treated with dexamethasone, four fold activation was observed specifically with 4xC/EBP E1b CAT reporter. No significant increase in activation was observed with either the 4xSp1 E1b CAT or

4xOct E1b CAT reporter constructs following dexamethasone treatment. These results indicated that GR activation of transcription in a GRE independent manner occurred through the "CCAAT" DNA response element and probably through its upstream transcription factor (C/EBP).

Somewhat surprisingly, only two fold activation was observed with the HSV tk E1b CAT reporter construct. The HSV tk CAT and the HSV tk E1b CAT reporter constructs have a 16 bp difference between the TATA box and the "CCAAT" motif. This suggest the possibility that the proper distance between the TATA box and the "CCAAT" motif sequence is important for GR mediated activation in the context of the HSV tk promoter. Two fold induction in the context of the E1b minimal promoter was able to be overcome by increasing to four copies of the "CCAAT" DNA response element placed in front of the E1b TATA box in the same reporter plasmid. The difference between 4xC/EBP E1b CAT and HSV tk E1b CAT promoters' activities was not further analyzed.

Finally, there is no data available in the literature on which C/EBP transcription factors isoform is expressed in Cos 7 cells which are derived from kidney fibroblasts. However, when C/EBPβ was initially cloned mRNA expression was observed in mouse kidney tissues using Northern analysis [3].

*5. Activation of Transcription by Dexamethasone Activated GR Through the "CCAAT" DNA Response Element Occurs Specifically Through C/EBP*β

From the previous series of experiments, it was determined that GR activated transcription through the "CCAAT" DNA response element,

independently of a GRE, following dexamethasone treatment. CCAAT enhancer binding proteins (C/EBPs) are upstream transcription factors that activate transcription through the "CCAAT" DNA response element [3]. To determine whether the GR ligand dependent coactivation function through the "CCAAT" DNA response element was general to all C/EBPs or specific to one of the C/EBP isoforms, three different C/EBP expression constructs were used. Mammalian C/EBP α, β, and δ expression constructs were cotransfected with GR L501P, and 4xC/EBP E1b CAT reporter construct into Cos 7 cells. All three isoforms were able to activate transcription 5-8 fold from the synthetic 4xC/EBP E1b promoter. However, when the cells were treated with dexamethasone an additional 4 fold enhancement of transcription was only seen with the C/EBPβ isoform. These results together indicate that GR's GRE independent enhancement of transcription through "CCAAT" DNA response elements occurs specifically through C/EBPβ.

There are several biological processes in which the ligand dependent GR coactivation function might enhance C/EBPβ transcriptional activity. One obvious canditate is adipogenesis. The C/EBP transcription factor family along with PPARγ have been shown to play central roles in adipogenesis [102, 103]. As mentioned in the introduction, C/EBPβ and C/EBPδ expression levels increase in the early stages of adipose differentiation in 3T3-L1 cells , and C/EBPα expression levels in the final stages. Dexamethasone treatment is needed to promote 3T3-L1 differentiation during the same initial 48 hour time frame in which C/EBPβ and C/EBPδ are expressed. It was initially observed that

dexamethasone was required for the induction of C/EBPδ expression in the 3T3-L1 cells ([102, 103] and references within). However recent data indicate that dexamethasone is still needed in 3T3-L1 adipogenesis conversion when either C/EBPβ or C/EBPδ are expressed ectopically, and does not induce C/EBPδ expression as seen in the 3T3-L1 cells [144]. Both C/EBPβ and C/EBPδ were reported to induce PPARγ mRNA expression in 3T3 fibroblasts [144, 145]. However, full induction of PPARγ mRNA by C/EBPβ occurred after exposure to dexamethasone in the absence of ongoing protein synthesis, demonstrating a direct role for GR along and C/EBPβ in PPARγ induction. In addition, induction by dexamethasone was dose dependent with a K_D of 10 nm, which is in the approximate range for dexamethasone association with GR. Interestingly, the PPARγ promoter contains two C/EBP consensus DNA binding sites, but so far it has not been shown to contain GREs [146]. And finally, dexamethasone treatment alone did not display enhancement of PPARγ mRNA synthesis in the absence of C/EBPβ.

Together these data strongly support the possibility that GR can enhance C/EBPβ transcriptional activity as a ligand dependent coactivator in the absence of GREs. This biological process will be evaluated in the context of ligand dependent coactivation function by GR in the near future by those continuing the project.

Both C/EBPβ and GR have also been demonstrated to play a role in the inflammatory response through NF-κB [112, 147]. NF-κB is an upstream transcription factor whose transcripitonally active p65 subunit has been shown to

positively regulate the inflammatory response. NF-κBs activity is regulated by another heterologous protein IκB which traps NF-κB in the cytoplasm, preventing NF-κB from positively regulating transcription.

GR has been shown to interfere with NFκBs activity by at least two different mechanisms. First, GR was reported to repress NF-κB transcriptional activity by an interference mechanism, where GR blocked NF-κB activity by direct protein protein binding and prevention of NF-κB interaction with its DNA response element [147]. Second, GR's interference response for NF-κB has also been shown to occur by rapid induction of IκB synthesis and thus sequestration of NF-κB in the cytoplasm [148].

On the other hand, C/EBPβ has been shown to physically interact with NF-κB and positively influence NF-κB transcriptional activity [112]. Perhaps, the GR-C/EBPβ interaction observed in this study may reflect an alternative interference mechanism of NF-κB transcriptional activity in which C/EBPβ may be sequestered from NF-κB by GR. Competition for C/EBPβ by GR and NF-κB may cause physical interference in the NF-κB-C/EBPβ synergistic activation of transcription. While this interference mechanism remains a possibility, it will not be further pursued.

Finally, GR and C/EBPβ are both highly expressed in liver tissue. Compartmentalization of GR and C/EBPβ in the liver suggests the possibility for other physical and functional interactions.

Several earlier reports have provided evidence that C/EBPβ transcriptional activity can be affected by nuclear receptors. For example, estrogen receptor has been shown to repress C/EBPβ transcription following its hormone treatment in the absence of an ERE [114]. Retinoic acid, the ligand for RAR, has also been shown to reverse adipogenesis specifically through C/EBPβ [111]. And finally, GR has been shown to physically associate and synergistically activate with NF-IL6 (C/EBPβ human homologue) [113]. However, the data presented in that paper deviated significantly from the results presented in this thesis. While Nishio et al. have observed that mutation of a GRE in the AGP promoter did not effect GR's synergism with NF-IL6 , they have observed (in contrast to my results) that GR's DBD was required for GR's transcriptional activity. Subsequently, GR LBD alone was not tested for enhancement on NF-IL6 activity. As a result, Nishio et al. concentrated on the synergism mechanism and understandably did not observe GR's coactivation role in the context of C/EBPβ transcriptional activity. More interestingly, Nishio et al. have localized the physical interaction between GR and NF-IL6 to the bZIP domain using an *in vitro* binding assay. C/EBP α,β, and δ isoforms contain moderate homology in the three activation domains, and high homology in the bZIP domain [3]. It is therefore perhaps surprising that GR coactivation function observed in this thesis did not occur for all three C/EBP isoforms. On the other hand, recent data presenting synergistic interaction between Sp1 and C/EBPβ, showed that by swapping the bZIP domains of C/EBPα and C/EBPβ resulted in different transcriptional effects [101]. This information possibly explains why GR's ligand

dependent coactivation that was observed in this project occurred specifically with C/EBPβ and not C/EBPα or C/EBPδ.

6. Glucocorticoid Receptor's Ligand Binding Domain Forms a Complex With C/EBPβ In Vivo

From the last set of results it appeared that GR ligand dependent coactivation function occurred specifically through C/EBPβ through a "CCAAT" DNA response element in Cos 7 cells. To further investigate whether GR and C/EBPβ as well as the other two C/EBP isoforms can form a protein complex *in vivo,* the mammalian two hybrid assay was employed. The bait for the two hybrid assay was GR's ligand binding domain fused to the Gal4 DNA binding domain, while the activation function was performed by the different C/EBP isoforms in this modified two hybrid system. Since C/EBPs contain their activation domains, fusion two GAl4 or VP16 transactivation domains was not required. The CAT reporter construct used in this assay contained 5 copies of Gal4 DNA response element. Therefore this experiment varied and was reversed from the previous experiments in that the C/EBPs were acting as the coactivators and the ligand binding domain fused to Gal4 DNA binding domain (Gal4-LBD) was the sequence specific transcription factor (see figure 8.a). The Gal4-LBD fusion construct was cotransfected with the different C/EBP expression constructs, and 5xGal4 E1b CAT reporter into Cos7 cells. Following dexamethasone treatment, only C/EBPβ expression construct was able to coactivate LBD dependent

transcription. C/EBPα and δ displayed ~ 5 fold induction following dexamethasone treatment which was similar to the activity of the Gal4-LBD fusion construct. This result showed that GR's LBD was able to form a protein complex *in vivo* with C/EBPβ and not C/EBPα or C/EBPδ. In addition, the Gal4-LBD fusion construct limited the interaction of C/EBPs to just the GR LBD, confirming the previous results in which the GR LBD alone was sufficient for full transcriptional activation following dexamethasone treatment. Finally, C/EBPs that were cotransfected either alone with the 5xGal4 E1b CAT reporter construct or with Gal4 DNA binding domain construct did not show significant CAT activity indicating that the C/EBPs activation of transcription was specific to GR's LBD. Taken together these results show that the GR LBD and C/EBPβ can form a protein complex in Cos7 cells.

7. Immunoprecipitated Glucocorticoid Receptor and C/EBPs Can Form Direct Protein-Protein Interaction In Vitro

As a final experiment, I investigated whether GR's GRE independent activation of transcription through C/EBPβ occurs through direct or indirect interaction. For this reason, I employed an immunoprecipitation binding assay. Myc-tagged GR immunopurified from Sf-7 cells that was treated with either dexamethasone, RU 486, or NaCl prior to purification. The efficiency and specificity of the immunoprecipitation was analyzed by Western analysis using the GR specific antibody, BuGR (figure 9.b). As a negative control for GR, same treatment and immunoprecipitation was performed on the parental Sf-7 cells. C/EBPβ and C/EBPδ that were subcloned into *in vitro* translatable vectors were

expressed using ^{35}S labeled methionine were employed in the binding assay. C/EBPδ was intended to be used as a negative control for C/EBPβ since it did not display any activity with GR in the previous experiments. A summary of the results for the binding assays between GR treated with either dex, RU486, or NaCl and C/EBPβ or C/EBPδ are represented as histogram in figure 9.c. Dexamethasone treated GR interacted with C/EBPβ 5 fold over the background. Thus, dexamethasone treated GR and C/EBPβ can bind directly. Surprisingly, RU486 or NaCl treatment displayed similar binding result between GR and C/EBPβ. Both salt treatment and RU486 are able to transform GR, therefore it appears that transformation of GR is sufficient for direct protein protein interaction. However, as seen with in the previous experiment RU 486 treated GR was unable to activate transcription in a GRE independent manner. Therefore, RU 486 treatment is sufficient for direct protein protein interaction but not for GRE independent transcriptional activity.

Surprisingly, binding assay results between GR and C/EBPδ displayed similar results with the dexamethasone, RU 486, or NaCl treatment. Therefore it appears that C/EBPδ, just like C/EBPβ, is also able to form direct protein protein interaction with GR. However, GR did not display GRE independent transcriptional activity with C/EBPδ in the previous experiments. Therefore the full length receptor directly interacts with both C/EBPβ and C/EBPδ. Because GR activation of transcription in the absence of GREs occurred just with C/EBPβ and was localized to the LBD, it is possible that full length GR contains two sites that interact with C/EBPs; one site in the DBD that is common to the two C/EBPs

tested, and second site in the LBD that is specific to C/EBPβ alone. Previous evidence points to the possibility that GR interacts with NF-IL6 (C/EBPβ homologue) through its DNA binding domain

To clarify this issue, more binding assays are required. In these experiments, I plan to express the three C/EBP isoforms as GST fusion proteins and use them as the binding matrix. To analyze the possible different GR interaction sites, different *in vitro* translated GR domains will be used including the DBD alone, as well as the transformed LBD alone. This binding experiments should provide answers that will clarify the immunoprecipitation binding assay results.

V. Conclusions:

This thesis provides evidence for the identification of functional interaction between the glucocorticoid receptor and C/EBPβ transcription factor, in which GR was able enhance C/EBPβ transcriptional activity independently of GREs. In particular, I have demonstrated that:

1) Dexamethasone bound glucocorticoid receptor activated Herpes Simplex Virus thymidine kinase proximal promoter which did not contain any discernible GREs. GRE independent activation of transcription through the HSV tk promoter by GR was dexamethasone dependent and GR specific.

2) The dexamethasone bound ligand binding domain of GR was sufficient for full transcriptional activity through HSV tk proximal promoter. The AF-1 function, as well as a functional DBD of GR were irrelevant for full transcriptional activity. This information, together, revealed that GRE independent activation of HSV tk transcription occurred through a ligand dependent coactivation function of GR, rather than its role as an upstream transcription factor.

3) GR expression construct that lacked the ability to bind DNA was able to activate transcription following dexamethasone treatment through the "CCAAT" DNA response that is located in the HSV tk proximal promoter. Therefore it

appeared that GR was acting as ligand dependent coactivator for the transcription factor which binds to the "CCAAT" DNA response element.

4) In a transient transfection assay GR expression construct that lacked the ability to bind DNA specifically enhanced C/EBPβ transcriptional activity, but not C/EBPα or C/EBPδ, following dexamethasone treatment. This result demonstrated that GR was functioning as a ligand dependent - C/EBPβ specific coactivator.

5) Using a mammalian two hybrid assay, it was demonstrated that dexamethasone bound GR ligand binding domain was able to form a protein complex with C/EBPβ and not C/EBPα or C/EBPδ *in vivo.*

6) And finally, an immunoprecipitation binding assay between full length wild type GR and C/EBPs revealed that these two heterologous transcription factors were able to form direct protein protein contacts *in vitro.*

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2. Marcin Boruk, Joanne Savory, Robert J.G. Haché, **Functional Interactions between C/EBPβ and glucocorticoid receptor**, (manuscript in preparation).

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