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CHARACTERIZATION OF THE DNA-INDEPENDENT DIMERIZATION INTERFACE OF THE RAT GLUCOCORTICOID RECEPTOR

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Thesis submitted to the Department of Biochemistry, Microbiology and Immunology in partial fulfillment of the requirements for the degree of Doctor of Philosophy

University of Ottawa
Ottawa, Ontario, Canada
January, 1999

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0-612-45191-7
ABSTRACT

The glucocorticoid receptor (GR), a member of the nuclear receptor superfamily of transcription factors, binds DNA as a ligand induced homodimer. Within the DNA binding domain, a short sequence of amino acids called the D-box, has been shown by mutagenesis and structural studies to function as a DNA-dependent dimerization interface. The importance of the D-box for DNA-dependent dimerization by GR is well known. A second dimerization activity necessary for dimerization in solution in the absence of DNA has been proposed for GR, based on homology with other steroid receptors. While the DNA-dependent dimerization activity has been extensively studied, very little is known about the DNA-independent dimerization activity in GR. The subject of this thesis is the characterization of the solution dimerization activity in GR.

Three independent assays demonstrated solution dimerization of GR: co-immunoprecipitation, GST pull-down and two-hybrid assay in yeast. The GR, in its steroid free state, is packaged into a heat shock protein containing multiprotein complex. My results showed that solution dimerization of GR required dissociation of the heat shock protein complex. However, dimerization did not appear to have a strict ligand requirement as efficient dimerization occurred with both salt- and antagonist-treated receptors. Further, by all three assays, I show that amino acids 505 to 547 are required for GR solution dimerization. Interestingly, these amino acids overlap with the nuclear localization signal-1 in GR, the binding site for the NLS receptor importin α. Additionally, my data also provided evidence for protein interaction surfaces in the
amino and carboxy terminal domains of GR, results that are consistent with the idea that multiple regions of GR contribute, either directly or indirectly, to homodimerization.

Co-transfection experiments with wild type GR and a GR mutant, which had a mutation in one of its nuclear localization signals such that it was unable to efficiently transfer to the nucleus in response to hormone, demonstrated an interaction between full-length GR monomers \textit{in vivo}. Data from the co-transfection assay also suggested that GR homodimerizes in the cytoplasm prior to nuclear import.

Finally, I show for the first time that GR has the potential to heterodimerize with several members of the nuclear receptor superfamily in solution.

These results provide the first detailed characterization of the GR solution dimerization activity.
DEDICATION

To my parents, Julia and Robert, for their unconditional love and support and for their unwavering belief in my ability to see this through. To my sisters and brothers for their many phone calls and constant encouragement over the years.
ACKNOWLEDGMENTS

I once read the acknowledgments in a book that began with the sentence; “This book comes into existence trailing the kindness of others”. This statement is particularly relevant, as the completion of this thesis depended on the kindness and generosity of many people.

I wish to acknowledge my co-supervisor Drs. Yvonne Lefebvre and Robert Haché. I am deeply grateful to Dr. Lefebvre for her role in guiding my formation as a scientist. Her support, teaching, encouragement and mentoring over the years made a mentally challenging and often difficult journey rewarding and enjoyable. I would also like to acknowledge and thank Dr. Haché for his many, many excellent suggestions and for his insight into and interpretation of experimental data.

I would like acknowledge the members of my thesis advisory committee Drs. N. Tanphaichitr and L. Kliene for giving freely of their time and advice over the years. Contributors of materials (plasmids, cell lines) are listed in the thesis, however, I would especially like to thank Dave Rodda for providing the Sf7 cell line stably transfected with myc GR used in the co-immunoprecipitation assays and Brian Hsu for providing the GR_{NL1} - mutant.

I am also grateful to the following past and present members of the Hormones, Growth and Development group at the Loeb Institute for Medical Research who have assisted and encouraged me over the years: B. Hsu and M. Boruk for their friendship, encouragement and shared moments of laughter, Dr. E. LaCasse for technical advise and for keeping me supplied with papers relevant to my work, Dr. D Begin for the countless
hours spent teaching me proper tissue culture techniques, L. Pope for spending, what at
the time seemed like an eternity in the cold room isolating nuclear envelopes and for
getting me up to speed when I first started working in the lab, T. Reich for all his help
and sound advice both in and out of the lab and G. Préfontaine for performing the direct
binding assay, reading part of the thesis and for assisting with the preparation of some
figures.
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ABBREVIATIONS

ACTR .......................... activator of the TR and RAR
AF .............................. activation function
AIB1 ........................... amplified in breast cancer 1
AR ............................... androgen receptor
ARP-1 .......................... apolipoprotein regulatory protein 1
Arnt ............................. aryl hydrocarbon nuclear translocator

β-gal ............................ β-galactosidase
bHLH ............................. basic Helix-Loop-Helix
BSA .............................. bovine serum albumin

CBP ............................. CREB Binding Protein
COUP-TF ........................ chicken ovalbumin upstream promoter

DAC ............................. deacylcortivazol
DBD .............................. DNA binding domain
D-box ............................ distal box
Dex ............................... dexamethasone
DMEM ............................ Dulbecco’s Modified Eagle Medium
DMSO ............................ dimethly sulfoxide
DNA ............................... deoxyribonucleic acid
DNase I .......................... Deoxyribonuclease I
DTT ................................. dithiothreitol

ECL .................................. enhanced chemiluminescence
EDTA ................................. ethylenediaminetetraacetic acid
EMSA .................................. electrophoretic mobility shift assay
ER ..................................... estrogen receptor
ERE .................................... estrogen receptor response element

FBS ..................................... fetal bovine serum
Ftz-F1β ................................. fushi tarazu F1β

GALDBD .............................. GAL4 DNA binding domain
GALTA ................................. GAL4 transactivation domain
GC ..................................... glucocorticoid hormone
GR ..................................... glucocorticoid receptor
GREs .................................. glucocorticoid response elements
GRIPI .................................. glucocorticoid receptor interacting protein I
GR_{NL1}^- ............................. GR with a mutation in NL1
GR_{WT} ................................ wild type GR
GST ..................................... Glutathione-S-Transferase

h ........................................ hour
HAT ..................................... histone acetyl transferase
HNF4 ........................................ hepatic nuclear factor 4
HREs ..................................... hormone response elements
HRP ........................................ horse radish peroxidase
hsp ......................................... heat shock protein

IIF ........................................ indirect immunofluorescence
IP buffer ................................... ImmunoPrecipitation buffer
IPTG ....................................... isopropyl β-D-thiogalactopyranoside

JAKs ....................................... Janus kinases

K ............................................ lysine
kDa .......................................... kilodalton

LBD ......................................... ligand binding domain

min ........................................ minutes
mM .......................................... millimolar
MR .......................................... mineralocorticoid receptor

N ............................................. asparagine or nuclear
NCoA-1 ..................................... nuclear receptor co-activator-1
NCoR ....................................... nuclear receptor co-repressor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>NE</td>
<td>nuclear envelope</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor κB</td>
</tr>
<tr>
<td>NGFI-B</td>
<td>nerve growth factor inducible factor B</td>
</tr>
<tr>
<td>NGS</td>
<td>normal goat serum</td>
</tr>
<tr>
<td>NL1</td>
<td>nuclear localization signal-1</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localization signal</td>
</tr>
<tr>
<td>NPC</td>
<td>nuclear pore complex</td>
</tr>
<tr>
<td>ONPG</td>
<td>o-nitrophenyl β-D galactopyranosidase</td>
</tr>
<tr>
<td>p/CIP</td>
<td>co-integrator associated protein</td>
</tr>
<tr>
<td>P-box</td>
<td>proximal box</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PKA</td>
<td>protein kinase A</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator activated receptor</td>
</tr>
<tr>
<td>PPO</td>
<td>2,5-diphenlyoxazole</td>
</tr>
<tr>
<td>PR</td>
<td>progesterone receptor</td>
</tr>
<tr>
<td>RAC3</td>
<td>receptor associated co-activators 3</td>
</tr>
<tr>
<td>Ran</td>
<td>Ras related nuclear protein</td>
</tr>
<tr>
<td>RAR</td>
<td>retinoic acid receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>RPM</td>
<td>rotations per minute</td>
</tr>
<tr>
<td>RU486</td>
<td>RU38486</td>
</tr>
<tr>
<td>RXR</td>
<td>retinoid X receptor</td>
</tr>
<tr>
<td>SD</td>
<td>synthetic dropout</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SF-1</td>
<td>steroidogenic factor 1</td>
</tr>
<tr>
<td>SHP</td>
<td>short heterodimeric partner</td>
</tr>
<tr>
<td>SMRT</td>
<td>silencing mediator for RXR and TR</td>
</tr>
<tr>
<td>SRC-1</td>
<td>steroid receptor coactivator-1</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducers and Activators of Transcription</td>
</tr>
<tr>
<td>SV 40</td>
<td>simian virus 40</td>
</tr>
<tr>
<td>TAF</td>
<td>transactivation function</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris Buffered Saline-Tween</td>
</tr>
<tr>
<td>TIF2</td>
<td>transcriptional intermediate factor 2</td>
</tr>
<tr>
<td>TF</td>
<td>transcription factor</td>
</tr>
<tr>
<td>TR</td>
<td>thyroid hormone receptor</td>
</tr>
<tr>
<td>UAS</td>
<td>upstream activator sites</td>
</tr>
<tr>
<td>VDR</td>
<td>vitamin D receptor</td>
</tr>
</tbody>
</table>
vit D3  ........................................... 1,25-dihydroxyvitamin D₃,

w/v.............................................. weight per volume

w/w.............................................. weight per weight
I. INTRODUCTION

1. Nuclear Receptor Superfamily—A Historical Overview

The classical steroid hormone molecules which include the adrenal steroids (glucocorticoids, mineralocorticoids), the sex steroids (progestins, androgens and estrogens) and the secosteroid (1,25-dihydroxyvitamin D₃, (vit D3)) were chemically characterized during the period that started with the discovery of testosterone in 1889 and ended with the purification vit of D3 in 1971 (1). They were isolated based on their abilities to affect development, differentiation, metamorphosis and physiology. Many of these hormones are associated with known human diseases. By the middle of the century, glucocorticoids were widely accepted as therapeutic agents. For example, since 1949, adrenal cortical hormones have been used clinically for their anti-inflammatory effect (2). The importance of this effect in the management of diseases such as rheumatoid arthritis and disseminated lupus erythematosus was well recognized though not well understood as the mediators of the actions of these hormones and their mechanism of action were not known (2). The first clue about their mode of action came with the observation that ecdysteroid, the insect molting steroid hormone, induced giant chromosome puffs at specific sites in the Drosophila polythene chromosome (3-5). This suggested that steroid hormones had an effect on gene transcription in the nucleus. The development of radiolabeled hormones facilitated the identification of binding proteins for the steroid hormones. In 1962, radiolabeled estradiol was used to identify an intracellular protein, which was believed to be mediating the effects of estradiol in the cell (6). The following
year [\textsuperscript{14}C]-cortisol, a labeled glucocorticoid hormone, was shown to translocate from the cytoplasm to the nucleus (7). This led to a proposed model to account for the action of steroid hormones. In this model the hormone bound to a cytoplasmic receptor. The hormone receptor complex then translocated to the nucleus where it influenced gene expression. The subsequent cloning of steroid hormone receptors was therefore essential for proving and understanding the molecular basis of this model.

The development of the covalent affinity ligand, dexamethasone-21-mesylate (8), facilitated the purification of the glucocorticoid receptor (GR). Subsequently, the generation of specific antibodies against this receptor (9) led to the cloning of the first steroid hormone receptor, GR, in 1984 (10) and soon thereafter the estrogen receptor (ER) (11). By 1990, receptors for all the known lipophilic hormones had been identified either by low stringency hybridization or with the use of receptor specific antibodies. The cloned receptors confirmed earlier biochemical studies identifying the modular structure of the receptors. These studies had identified an N-terminal domain to which the antibodies bound, a highly conserved, central DNA binding domain (DBD) and a C-terminal ligand binding domain (LBD).

Subsequent to the observation that certain receptor regions, particularly the DBD, were highly conserved, numerous groups cloned new nuclear receptor encoding cDNAs by low stringency screening of cDNA libraries using cDNA or oligonucleotides probes encompassing the conserved regions. Using this technique, two groups identified the cellular counterpart of the viral oncogene, \(\text{v-erbA} \) (12, 13). The homology of steroid receptors to \(\text{v-erbA} \) led to the discovery of the \(\text{c-erbA} \) locus as the thyroid hormone receptor (TR). This discovery which first linked a non-steroidal ligand receptor with the
family of classical steroid hormone receptors was an important discovery as it showed that chemically distinct ligands interact with structurally related receptors. The expansion of the family continued as the receptor for the teratogenic morphogen, retinoic acid, called the retinoic acid receptor (RAR) was also found to belong to this family (14-17). The discovery of the retinoid X receptor (RXR) was also another important advance (18, 19). Though structurally similar to RAR, RXR failed to bind all-trans-retinoic acid. Thus RXR was initially termed an orphan receptor, a term that defines a protein which is structurally related to hormone receptors but for which the ligand (to which the X refers) is unknown. In 1992, 9-cis-retinoic acid was found to be the ligand for RXR (20-22). Presently, in addition to the 15 members identified as the receptors for all the lipophilic ligands known, more than 150 related members have been discovered in a diversity of animal species from worm to insect to human (23-27). Most of these are orphan receptors and they are found in every metazoan species. It is expected that some of these proteins may interact with novel ligands while others may not even be ligand dependent. The expansion of the types of ligands working through such receptors and the identification of orphan receptors meant that the general terms steroid receptor or hormone receptors no longer applied. Thus, the term nuclear receptor superfamily was invoked to indicate that the primary actions of these transcription factors were via nuclear events.

As the DNA sequence of individual receptors became available, computer analysis of the deduced amino acids sequence revealed some regions of close homology and other regions with notable differences between the different receptors. Nuclear receptors are single polypeptide chain proteins with three major modular domains (24-
28). The N-terminal domain is highly variable and ranges in size from ~ 23 amino acids for the vitamin D receptor (VDR) (29, 30) to 600 amino acids for the mineralocorticoid receptor (MR) (31, 32). It is less well characterized than other receptor domains and therefore its function is not as well defined. However, a transcriptional activation domain which functions as a constitutive activator in receptor derivatives lacking the carboxy terminus or when tethered to a heterologous DNA binding domain (DBD) has been mapped to this domain. Common to all members of the nuclear receptor family is a centrally located DNA binding domain composed of about 70 amino acids with several conserved cysteines. The DBD, which targets receptors to DNA, is characterized by two conserved zinc finger motifs each formed by the tetrahedral coordination of four cysteine residues to a zinc ion. In addition to their characteristic DBDs, nuclear receptors exhibit a large carboxy terminal domain responsible for the binding of hormone referred to as the ligand binding domain (LDB).

2. Mechanism of Action of Steroid Receptors

Classical steroid hormone receptors are found in both the nucleus and the cytoplasm of target cells in the absence of hormone. At equilibrium, the estrogen (ER) (33-39), progesterone (PR) (39-45) and androgen receptors (AR) (46-50) are predominantly nuclear. In contrast, the glucocorticoid (GR) (51-62) and mineralocorticoid (MR) (63-65) receptors are cytoplasmic. Steroid hormone receptors exist in at least two different forms as identified by their sedimentation positions on sucrose gradients. In the absence of hormone steroid receptors are found in large heterocomplexes that sediment as 8-10S on sucrose gradients (summarized for GR in Fig.
1) (66-74). These multiprotein complexes contain heat shock proteins (hsp 90,70 and 60), high molecular weight immunophilins (p56/59 and CyP-40) and other non receptor proteins (p23 and p50) (75-78). In the presence of ligand, the receptor dissociates from the heterocomplex generating a liganded, hsp free form of the receptor that sediments at 4S on sucrose gradients.

One of the major components of the heterocomplex is heat shock proteins. Hsp 90 and 70 are known molecular chaperones (79-82). Hsp 90 for example acts as a chaperone in vitro by preventing protein aggregation and thereby preserving the activity of those proteins (79). For GR and MR, hsp 90 association with the LBDs is essential for maintaining the receptors in a high affinity steroid binding conformation (83-88). However, this does not appear to be a universal characteristic of steroid hormone receptors since PR (89, 90), ER (91) and AR (92, 93) do not require hsp 90 for ligand binding. It has also been proposed that association of hsp 90 represses protein functions such as DNA binding, dimerization and transcriptional activation (75-77, 94, 95). This repression is relieved by ligand binding.

Alternatively, hsp 90 may be involved in receptor trafficking. This idea is supported somewhat by experiments in which the chicken hsp 90 was targeted to the nucleus by fusion with the nucleoplasmin NLS (hsp 90-NLS). When expressed by itself, the hsp 90-NLS was localized to the nucleus while mutants of GR and PR, which lacked NLSs, were cytoplasmic. However, when the receptor mutants, which lacked NLSs, were co-expressed with hsp 90-NLS, the receptors completely localized to the nucleus (96). Another observation that suggests a possible role for hsp 90 in receptor
Figure 1. A simplified model of glucocorticoid receptor action.

Prior to hormone binding, the glucocorticoid receptor, GR, is maintained in an inactive state in a multiprotein complex. Glucocorticoid hormone (GC) enters the cell by simple or facilitated diffusion and bind to the GR in the GR-hsp complex. Upon binding hormone, the GR dissociates from the complex and translocates to the nucleus where it binds as a homodimer to specific DNA sequences, called glucocorticoid response elements (GREs) found in the promoter regions of target genes. Steroid binding is transient. Following dissociation of hormone, GR is repackaged into the hsp complex.
trafficking is that treatment of cells containing hormone bound GRs with the antibiotic geldanamycin, inhibits steroid dependent transfer of GR to the nucleus. Geldanamycin blocks GR:hsp 90 complex assembly (97).

Finally, it is not clear why some steroid receptors such as GR and MR are predominantly cytoplasmic in hormone free conditions while others such as ER and PR are nuclear when they are all known to be packaged into similar hsp complexes. One assumption that has long been made is that the association of hsp with GR and MR blocks their NLSs. However, this is difficult to reconcile given the similarity of the composition of the GR, PR and MR complexes (77, 95, 98, 99).

Like hsp 90, the role of hsp 70 and the immunophilins in the 8S complex is thought to be related to their chaperoning activity. The hsp 70 family of proteins appears to have several functions in the cell related to protein folding, unfolding, complex disassembly and as part of the machinery involved in the passage of proteins through membranes (80-82). Whether hsp 70 performs the same functions in the 9S complex is not yet known.

Immunophilins are ubiquitous proteins which bind immunosuppressant drugs (100) and have peptidyl isomerase activity; i.e. they catalyze cis-trans isomerization of peptidylprolyl bonds (101-103). This activity suggests that they act as protein chaperones in the cell (102, 103). The fact that receptor heterocomplexes can be formed in the absence of immunophilins implies that they are not critical components of the 9S complex (104). However, it has been proposed that they too may play a role in targeting the receptor to the nucleus (98, 105).
The hsp-associated steroid receptor is said to be inactive or untransformed as this complex does not bind DNA and is unable to activate transcription (reviewed in (75)). Concomitant with or immediately following hormone binding, the receptor dissociates from the heterocomplex. This process is termed either receptor transformation to indicate the transition from an hsp associated receptor that sediments as 9S on sucrose gradients to an hsp free entity that sediments as 4S on sucrose gradients or receptor activation to signify the acquisition of DNA binding activity and the activation of other receptor functions such as dimerization and transcriptional activation and, for GR and MR, the activation of the nuclear localization functions. The two terms are used interchangeably. The activated receptors transfer to the nucleus (GR and MR) where they bind as homodimers to specific DNA sequences called hormone response elements (HREs) found in the promoter region of target genes to alter gene expression.

How do DNA-bound GR and other steroid receptors activate target genes? This question remains unclear. Steroids receptors has been shown to interact directly with components of the basal transcriptional machinery such as TF-IIB, TBP and TAFII 30 (106-109). It has been proposed that these interactions play a role in the activation of transcription by steroids receptors. Our understanding of the molecular mechanism underlying gene activation by nuclear receptors was greatly enhanced with the identification of a number of intermediary proteins called co-activators, that interact with steroid/nuclear receptors and play essential roles in mediating transcriptional effects.

Co-activators were first identified as proteins that bound the transactivation domains of nuclear receptors. This group of proteins has since grown to include steroid (SRC-1) (110), nuclear receptor co-activator-1 (NCoA-1) (111); the transcriptional
intermediate factor 2 (TIF2) (112), glucocorticoid receptor interacting protein 1 (GRIP1) (113), NCoA-2; the receptor associated co-activators 3 (RAC3) (114), CREB Binding Protein (p300/CBP), co-integrator associated protein (p/CIP) (115), amplified in breast cancer 1 (AIB1) (116) and activator of the TR and RAR (ACTR) (117). All the co-activators contain histone acetyltransferase (HAT) activity (117-122), and recent experiments have shown that co-activators exist in large protein complexes that contains several different co-activators (123, 124). Recent evidence also suggest that, in addition to histones, co-activators can also acetylate activator proteins (125-127) and general transcription factors (128). Thus, a possible mechanism for the activation of genes by nuclear receptors may involve the recruitment of large co-activator complexes which may function in part as chromatin remodeling factors and as proteins which bridge the basal transcriptional machinery to the DNA bound nuclear receptor. While the complete mechanism of transcriptional activation remains to be elucidated, it is apparent that gene activation by these transcription factors may involve multiple targets and pathways. Steroid binding is transient (t_{1/2} = 5-10 min) (129), and the loss of hormone from the receptor results in repackaging of the receptor into the hsp containing complex (58, 62) (Fig 1).

3. Structure/Function Relationship of Rat Glucocorticoid Receptor Domains

The glucocorticoid receptor was the first nuclear receptor to be cloned (10). Like other steroid hormone receptors, GR is organized into six homologous domains, A-F (summarized in Fig. 2). Many of the domains contain several overlapping functions.
Figure 2. Schematic representation of rat GR and its functional domains.

Schema of rat GR with boxes that highlight the DNA and ligand binding domains. Above the diagram, the A-F labels represent the functional domains common to all nuclear receptors (130). Numbers below the diagram indicates selected amino acid positions, primarily those for boundaries of the major domains. Some of the functions and properties of each domain are summarized. Transactivation functions, TAF-1 and TAF-2, map to the N- and C-terminal portions of the receptor respectively (131). The centrally located, DNA-dependent dimerization domain (amino acids 477 to 481) is responsible for cooperative binding of receptor DBD monomers to DNA (132, 133). A second dimerization domain located in the E region has been proposed based on homology with the estrogen receptor (134). The nuclear localization signal-1 (NL1) is a tripartite motif found between amino acids 486 and 524 (60, 135). A second NLS, NL2, maps to amino acids 540 to 795 (60). Hsp 90 binds to multiple sites within the ligand binding domain (136-139).
N-  A/B  C  D  E/F  - C  DNA  tandem binding

1  440  505  547  795

TAF-1

Dimerization

Nuclear Localization

Hsp90 Binding

TAF-2

NL1

NL2
(i) The N-terminal or A/B Domain (amino acids 1-439)

The N-terminal or A/B region of rat GR has as its most important function identified to date a transcriptional activation domain. This activity referred to as TAF-1 (transactivation function-1) or activation function (AF-1) (131, 140, 141) is hormone independent and has been mapped to amino acids 98-292 of the rat GR by deletion experiments (141). The A/B domain is often referred to as the modulatory domain since it is not directly involved in hormone binding, receptor activation, DNA binding or DNA-dependent dimerization. N-terminally truncated receptors however, exhibit increased non-specific DNA binding, postulated to be a result of the increased positive charge of the receptor (142). Alternatively, it has been suggested that the reduced ability of N-terminally truncated receptors to distinguish between specific and non-specific DNA may the result of altered protein-protein contacts in the GR dimer which result in altered protein-DNA interactions (143). Evidence to support this theory is still lacking. The binding sites for most anti-GR antibodies map to this region of the receptor.

(ii) The DNA Binding or C Domain (amino acids 440-504)

The DNA binding domain is highly conserved among nuclear receptors and is the most extensively studied region of the receptor. The DBD, required for the receptor to bind both specific and non specific DNA, is characterized by two zinc fingers each formed by the tetrahedral coordination of four cysteine residues to a zinc atom (144-146). The GR and other nuclear receptors bind to bipartite response elements. The DNA sequences responsive to glucocorticoids are called glucocorticoid response elements (GREs) and they contain two hexameric half sites spaced by three nucleotides (147-151).
In other transcription factors containing multiple zinc fingers, two or more zinc fingers can often be aligned to give a consensus sequence. However, the sequence of the two GR zinc fingers are quite distinct from each other (152), providing the first hint that the functions of the two fingers may be different. The monomers of the GR DBD bind cooperatively to DNA as a homodimer. The two zinc fingers play two major roles in GRE recognition: the N-terminal finger makes base specific contacts with DNA (152). Cooperativity in DNA binding results from favorable protein:protein contacts made through an interface in the C-terminal finger (the D-box, discussed in detail below) which is aligned by DNA binding (152-155).

Additional functions that have been mapped to the DBD include:

- **Dimerization**: The five amino acids that form the base of the C-terminal zinc finger called the distal or D-box is a DNA-dependent dimerization interface (152). Dimerization through this interface does not occur in solution even at high protein concentration (146).

- **Protein-protein interactions**: In addition to homodimerization and targeting the receptor to DNA, many protein-protein interactions have been mapped to the GR DBD. These interactions, usually with other sequence specific transcription factors, include Oct 1 and 2 (156), Stat 5 (157), Nurr 77 (158), NFκB, NF-IL6 (159) and AP-1 (160).

- **Transcriptional activation**: A transactivation domain termed enhancer 1 (enh 1) has been mapped to the second zinc finger of the GR DBD (161-163). Mutations in this domain affect transcriptional activation but not DNA binding.
• **Nuclear localization:** The GR has two NLSs one of which (NL1) maps to the hinge region and overlaps with the DBD (60, 135). NL1 is a tri-partite nuclear localization composed of three proto-signals. One proto-signal is located at amino acids 486-490 in the second zinc finger of the DBD (135).

**iii) The Hinge Region or D Domain (amino acids 505-546)**

The sequence, the D domain, which separates the DBD from the C-terminal ligand binding domain is called the hinge region as it has generally been thought of as a flexible link between the two domains. The first identified and best characterized function of the hinge region is a nuclear localization sequence, NL1, similar in composition to that seen in the SV40 T antigen (60) and which is part of a tri-partite motif that constitutes NL1.

**iv) The Ligand Binding or E/F Domain (amino acids 547-795)**

The GR (LBD), composed of the carboxy terminal one third of the protein, is a multifunctional domain that in addition to ligand binding is required for nuclear localization, dimerization and harbors a ligand dependent transactivation function, AF-2. The initial function assigned to the LBD was obviously the ability to bind ligand and traditionally, this domain is thought of as a molecular switch that regulates the activity of the receptor. It is now clear that this region of the receptor participates in several other functions regulated by ligand binding. Some of these functions include:

• **Hsp 90 Binding:** hsp 90 of the 8S receptor heterocomplex binds directly to the LBD of GR and other steroid receptors (136-139). Much work has established that hsp 90
association is required for steroid binding (83-88) and hsp 90 dissociation precedes DNA binding by the receptor (77, 95, 164, 165). A comparison of the amino acid sequence of the GR LBD and the catalytic domains of p60src-another protein with which hsp 90 is associated showed no obvious similarity (166, 167). In contrast, TR which does not bind hsp 90 (168) has some homology with the region of GR thought to be needed for hsp 90 binding (169). These results suggest the presence of multiple hsp 90 binding sites and that the tertiary structure (GR vs. p60src) or the non conserved amino acids (GR vs. TR) may be important for the specificity of hsp 90 interaction. While it has proved difficult to map a specific binding site for hsp 90, it is generally accepted that hsp 90 make contacts with multiple sites in the LBD with amino acids 537-673 being sufficient to form a stable complex.

- **Nuclear localization:** In addition to NL1, Picard and Yamamoto (60) showed that the LBD of GR had the ability to target proteins to the nucleus in a hormone dependent manner. The complexity of the LBD has so far prevented the identification of amino acids responsible for this activity called NL2.

- **Transcriptional activation:** A transactivation function (TAF 2 or AF-2) has been identified in the LBD of GR (131, 140, 141, 155, 170). Within AF-2, which was initially mapped to the entire LBD, an autonomous activating domain, termed AF-2 AD has been identified. AF-2 AD maps to the extreme C-terminus of the GR LBD and is conserved among all known transcriptionally active members of the nuclear receptor superfamily (155, 171-173). AF-2 AD is required for transactivation and for the ligand dependent
interaction between the LBD and co-activator molecules such as GRIP-1, SRC-1 and CBP/p300 (110, 113).

4. DIMERIZATION

Dimerization, which can be defined as physical interactions between related proteins, is a common theme in the regulation of signal transduction and the control of transcriptional activation by several families of transcription factors. It is a regulatory mechanism involved in signal transduction from receptors at the cell membrane as well as from nuclear receptors. Ligand induced dimerization is essential for stimulation of the intrinsic catalytic activity of growth factor receptors and it is accepted as the universal mechanism for the activation of T- and B-cell receptors, lymphokine receptors and other families of cell surface receptors. Similarly, families of transcription factors such as the nuclear receptor superfamily use homo- and heterodimerization as means of regulating transcription factor activity (reviewed in (174)). Some of the potential benefits of dimerization and how it is utilized as a regulatory tool in signal transduction and the control of transcription are briefly summarized.

PROXIMITY AND ORIENTATION Proteins are brought into close proximity with one another when they dimerize. This allows them to act in trans on one another. An example of this is the ligand induced dimerization of the cell surface receptors such as the TGF-β receptor and the EGF receptor. Both receptors activate intracellular signaling pathways. Ligand induced dimerization is the trigger that activates the intracellular kinase domains of these receptors, which then phosphorylate the partner brought into close proximity (175). Similarly, cytokine receptors are also activated by
phosphorylation. However, unlike growth factor receptors, cytokine receptors do not possess kinase domains, rather, they have a kinase, called Janus kinase (JAK), associated with their cytoplasmic domains. Like growth factor receptors, cytokine receptors rely on phosphorylation for activation and signal transduction. The JAKs are themselves activated by phosphorylation. Ligand induced dimerization of cytokine receptors bring the JAKs into close proximity and thereby allows them to cross phosphorylate and activate each other as well as their associated receptors (176).

**DIFFERENTIAL REGULATION BY HETERODIMERIZATION** Proteins that are able to dimerize with many partners may generate dimeric species with distinct functions. The relative concentration of the proteins in the cell and the relative strengths of the interaction may influence the major dimeric species formed and thus the biological outcome. An interesting example of differential regulation by dimerization is heterodimerization with dominant negative partners. A dominant negative partner is a family member that retains the ability to dimerize but lacks an important functional domain such as a DBD or a transactivation domain. The Id protein from the MyoD family of transcription factors is such a partner. Id contains the dimerization domain but lacks a DBD (177, 178). It is functionally neutral as a monomer but forms a non-productive heterodimer with MyoD and thus inhibits MyoD activity. (177, 178).

In like manner, the function of Myc, Max and Mad, bHLH (basic Helix-Loop-Helix) proteins are also regulated by heterodimerization. bHLH proteins contain a highly conserved basic region required for DNA binding adjacent to the HLH motif that mediates dimerization. Max binds to DNA as a heterodimer with Myc to activate transcription. The Myc protein does not homodimerize or bind DNA on its own. The
Max protein forms homodimers that are also able to bind DNA, however, this results in repression of transcription. Max also heterodimerizes with other bHLH proteins such as Mad and Mxi1, which like Myc, do not homodimerize or bind DNA. The activation of transcription by Myc-Max heterodimers can be inhibited by overexpression of Mad (179). Experimental data show that Myc-Max but not Mad-Max heterodimers predominate in undifferentiated macrophages. However, 48 h following the induction of differentiation, only Max-Mad complexes are detected (180). These data provide a functional correlate which demonstrates that differentiation is accompanied by a change in the composition of the Max heterodimers and therefore most likely a change in gene expression.

**ENHANCED AFFINITY** A dimeric protein generally has a larger interaction surface than a monomer. The larger interaction surface offers increased potential for protein-protein interactions or protein-DNA interactions. For transcription factors, a higher DNA binding affinity may be achieved relative to the monomer as twice as many base pairs may be recognized and contacted. Nuclear receptor monomers invariably bind DNA with a lower affinity than the corresponding dimer (181). The bHLH protein MyoD, for example, is able to bind DNA as a homodimer, however, it has a ten fold higher affinity for DNA when it heterodimerizes with E47, another family member (182, 183). Similarly, TR, VDR and RAR of the nuclear receptor superfamily bind to their cognate responsive elements with higher affinity as a heterodimer with RXR than as a homodimer (181).

**DIVERSITY** In addition to enhanced affinity, the diversity that can be achieved by dimerization of different members of a family of proteins offers enormous scope for
regulation since different dimeric combinations can potentially have distinct regulatory and functional properties (184). A specific example of how the diversity that results from heterodimerization can be used to regulate activity is the VDR/RXR and VDR/RAR heterodimer. The VDR/RXR heterodimer bind to HREs in which the two half sites are separated by three base pairs. In contrast, the VDR/RAR heterodimer utilizes HREs with a six base pair spacer (181).

**REGULATED MONOMER-TO-DIMER TRANSITION** Proteins such as the cell matrix protein, E-cadhedrin (185), and the vesicular protein, synaptotagmin (186), undergo monomer-to-dimer transition in response to changes in calcium levels. These proteins are only active as dimers, thus, the regulation of the transition from monomer to dimer may function as the rate limiting step for activation.

Another example of proteins whose functions are regulated by monomer-to dimer transition is the STAT (Signal Transducers and Activators of Transcription) proteins. In the absence of stimuli, STATS are monomeric cytoplasmic proteins. Cytokine stimulation ultimately leads to phosphorylation of STAT tyrosines by JAKs. Tyrosine phosphorylation results in intermolecular dimerization of STATS through their SH2 domains and the phosphotyrosine residues (187). Dimerization appears to control nuclear localization (188) of STATS and is essential for DNA binding. The experimental evidence suggests that while STATs dimerize in solution, multimerization of STAT dimers on DNA through a conserved amino tail domain enables STAT proteins to recognize and bind cooperatively to variations of the consensus site (189). Thus for this family of transcription factors, phosphorylation controls their oligomeric state, however,
dimerization is needed for nuclear localization, critical for DNA binding and is required to enhance binding site recognition.

5. Dimerization and Nuclear Receptors

Hormone response elements (HREs) are sequence specific DNA binding sites for nuclear receptors and thus mediate the actions of these transcription factors. The core recognition motif of HREs is composed of six base pairs (28, 181). A detailed analysis revealed two types of recognition motifs that confer transcriptional responses. The AGAACA motif is recognized by GR, MR, PR and AR (27, 28, 149, 151, 181). In contrast, the AGGTCA or AGTTCA motifs are recognized by ER, TR, RAR, VDR and peroxisome proliferator-activated receptor (PPAR) as well as all other known members of the nuclear receptor superfamily (Table 1) (27, 28, 181, 190-193). One of the more elegant features of nuclear receptor action is that such a diverse group of proteins recognize and bind to such similar DNA sequences. The question of how specificity is achieved becomes even more important when one considers that the DBD is the most closely related domain among family members (28). The observation that HREs are generally composed of two copies of the core recognition motifs in various configurations provided the first evidence that (i) nuclear receptors bound DNA as dimers and (ii) as with other transcription factor families, dimerization may be a key regulatory mechanism. Subsequent experimental data has led to the further sub-classification of nuclear receptors into four classes based on their DNA binding and dimerization properties (27).
Table 1. Binding site preferences for various nuclear receptors that bind DNA as monomers or homo- or hetero-dimers

Arrows represent core recognition motifs. $\Rightarrow n \Rightarrow$ represents a palindrome of recognition motif spaced by $n$ base pairs. $\Rightarrow n \Rightarrow$ represents a direct repeat spaced by $n$ base pairs. $\Leftrightarrow \Leftrightarrow$ represents an unspaced palindrome. $\Leftarrow n \Rightarrow$ represents an inverted palindrome spaced by $n$ base pairs. Note that for receptor pairs with multiple binding sites, binding sites are listed from highest (top) to lowest affinity. Adapted from Glass, C.K (1994) (181).
<table>
<thead>
<tr>
<th>Homo/Heterodimer</th>
<th>Binding Site</th>
<th>Core Recognition Motif</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CLASS I</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GR-GR</td>
<td>⇐3⇨</td>
<td></td>
<td>(194)</td>
</tr>
<tr>
<td>MR-MR</td>
<td>⇐3⇨</td>
<td></td>
<td>(311)</td>
</tr>
<tr>
<td>PR-PR</td>
<td>⇐3⇨</td>
<td></td>
<td>(195)</td>
</tr>
<tr>
<td>AR-AR</td>
<td>⇐3⇨</td>
<td></td>
<td>(196)</td>
</tr>
<tr>
<td>ER-ER</td>
<td>⇐3⇨</td>
<td>AGGTCA/AGTTCA</td>
<td>(147)</td>
</tr>
<tr>
<td><strong>CLASS II</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RXR-RAR</td>
<td>⇐5⇨</td>
<td></td>
<td>(197)</td>
</tr>
<tr>
<td></td>
<td>⇐2⇨</td>
<td></td>
<td>(198)</td>
</tr>
<tr>
<td></td>
<td>⇐1⇨</td>
<td></td>
<td>(199)</td>
</tr>
<tr>
<td></td>
<td>⇐1⇨</td>
<td></td>
<td>(200)</td>
</tr>
<tr>
<td>RXR-TR</td>
<td>⇐4⇨</td>
<td></td>
<td>(197)</td>
</tr>
<tr>
<td>RXR-VDR</td>
<td>⇐3⇨</td>
<td></td>
<td>(197)</td>
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<tr>
<td>RXR-PPAR</td>
<td>⇐1⇨</td>
<td></td>
<td>(202)</td>
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<tr>
<td>RXR-RXR</td>
<td>⇐1⇨</td>
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<td>⇐4⇨</td>
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<tr>
<td>TR-RAR</td>
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<td>VDR-RAR</td>
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</tr>
<tr>
<td><strong>CLASS III</strong></td>
<td></td>
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<td></td>
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<tr>
<td>RXR-COUP</td>
<td>⇐1-5⇨</td>
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<tr>
<td>RXR-ARP</td>
<td>⇐10</td>
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<td>(209)</td>
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<tr>
<td>RXR-HNF</td>
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<tr>
<td>SF1</td>
<td>⇐10</td>
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<td>(213)</td>
</tr>
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</table>
Class I receptors or the classical steroid subgroup of which GR is a member, bind to response elements containing two hexameric half sites, AGAACA. These receptors bind exclusively as ligand induced homodimers to palindromes separated by an invariant, non-conserved three base pair spacer (214). Included in this group are MR, PR and AR. The one exception is ER $\alpha$ and $\beta$ which, although they are classical steroid receptors, bind as ligand induced homodimers and heterodimers to a different half site sequence, AGGTCA, arranged as a palindrome with a three base pair spacer.

In contrast, the class II receptors for non-steroid hormone such as VDR, TR, PPAR and RAR bind preferentially as heterodimers with RXR to direct repeats of the AGGTCA sequence separated by a variable length nucleotide spacer (215). TR, RAR and VDR can also bind as homodimers to other configurations of the half site such as palindromes and inverted palindromes. However, heterodimerization with RXR increases their DNA binding affinity and the heterodimer is believed to be the functionally active form in vivo. As class II receptors all recognize and bind to the same DNA sequence, the question of specificity has been raised. This was resolved when it became clear that each heterodimer displays distinct HRE specificity with dimerization playing a central role in ensuring specificity. For example, the RAR/RXR heterodimer binds to and activates transcription preferentially through direct repeats separated by 2 or 5 base pairs whereas VDR/RXR and TR/RXR heterodimers bind to direct repeats separated by 3 and 4 nucleotides, respectively (28). Class III are the orphan receptors which bind to direct repeats primarily as heterodimers with RXR (215) while class IV are orphan receptors that bind as monomers to the extended core sequence AAAGGTCA (212, 213, 216, 217).
While the benefits conferred by dimerization on class II receptors are obvious, the advantages of dimerization by classical steroid receptors such as GR, PR, MR and AR are not as clear. One benefit attributed to dimerization is enhanced DNA binding affinity. With ER for example, a direct correlation between the ability to dimerize in solution and the ability to bind DNA with high affinity is well established (134). A similar link has been established between solution dimerization and high affinity DNA binding by GR (218). In contrast, evidence suggests that solution dimerization may not be a prerequisite for high affinity DNA binding by PR (219) indicating a possible difference between the receptors in this respect. It is well known that dimerization is necessary for transcriptional activation, however, whether or not solution dimerization is necessary for other receptor functions remains unknown. Thus, the biological relevance of solution dimerization for steroid hormone action will only be understood when comprehensive studies of the solution dimerization activity become available.

6. Regulation of nuclear receptor function by hetero-dimerization

(i) Classical steroid hormone receptors

Traditionally dimerization, particularly heterodimerization, was thought to be a key component of transcriptional regulation by all nuclear receptors except for GR, MR and AR. However, recent reports of heterodimerization among these receptors would seem to challenge this notion. The GR and MR are, under certain conditions, able to form a GRE-binding heterodimeric complex (220, 221). This is not surprising considering that GR and MR share complete sequence identity within the DBD region responsible for DNA binding (P-box) (28). However, whether heterodimerization occurs
in vivo and what role if any it plays in the functioning of GR and MR is not yet clear. On the one hand Trapp and co-workers reported that co-expression of GR and MR in neuronal cells resulted in synergistic activation of transcription (220), an effect they ascribed to the formation of GR:MR heterodimers. In contrast, Lui et al demonstrated an inhibition of GR mediated transcription when MR was co-expressed (221). They also postulate the formation of heterodimers. Both studies concur in showing heterodimers on DNA. The explanation for the opposite transcription results is still unclear. However, since GR and MR are co-expressed in some tissues such as in central neurons and glial cells (222), and considering that both receptors respond to physiological glucocorticoids and bind the same response element (223) it is reasonable to hypothesize that heterodimerization may extend the potential of corticosteroids in regulating responsive genes.

The GR and AR mutually inhibit each others activity when the receptors were co-expressed (224). Titration experiments suggested that inhibition was not due to squelching or occlusion (the blocking of GRE by AR homodimers without direct interaction between the receptors or vice versa) and was consistent with the formation of an inhibitory heterodimer (224). Consistent with this theory, the GR and AR DBDs form heterodimers on DNA (224). Androgens are known to cause an increase in smooth muscle and prostate proliferation (225-227) while glucocorticoids have the opposite effect (226-229). The observed interaction between the GR and AR suggested that heterodimer formation could be responsible for, at least in part, their inhibitory interaction at least at the level of transcriptional.
(ii) Non-Steroidal Ligand Activated Nuclear Receptors

In addition to being an important mechanism for the regulation of DNA binding, dimerization also regulates other functions of class II receptors. One consequence of homo and heterodimerization by this subset of nuclear receptors is altered ligand binding specificity. For example, both RAR and PPAR, in the absence of ligand, exist as heterodimers with RXR (27, 181, 215). The PPAR-RXR heterodimer can be activated by both RXR and PPAR specific ligands (202). In contrast, the RAR-RXR heterodimer is selectively activated by RAR specific ligands (230, 231). It is possible that the selective recognition of ligands may function, at least in part, as a mechanism which ensures that receptor-specific response is elicited from the RXR heterodimers.

Another result of dimerization by this group of nuclear receptors is the generation of heterodimeric species with novel receptor co-repressor interactions not seen with receptor homodimers. In contrast to steroid receptors which are found in heat shock protein complexes in the absence of hormone, the TR, RAR, VDR, PPAR and RXR are bound to cognate DNA and repress transcription of target genes in the absence of hormone (130, 232, 233). Ligand independent transcriptional repression was initially mapped to a repression function in the LBD of the subset of receptors (234, 235). Insight into the molecular mechanism of transcriptional silencing came with the identification of two related proteins known as nuclear receptor co-repressor (NCoR) (236, 237) and silencing mediator for RXR and TR (SMRT) (238) that mediate transcriptional repression by several nuclear receptors. It has been shown that co-repressors like co-activators are found in multiprotein complexes which, when associated with nuclear receptors, functions as transcriptional repressors (239-241). One of the consequence of
heterodimerization is altered interaction with the co-repressor complex. For example, the VDR does not interact with SMRT when expressed alone, however, co-expression of RXR promotes a strong VDR-SMRT interaction. Similarly, RXR binds very weakly to SMRT and RAR shows a somewhat stronger but nevertheless weak interaction with SMRT when expressed alone. However, co-expression of RAR and RXR results in synergistic interaction of the receptors with the co-repressor (242). Thus heterodimerization may play an important role in determining the transcriptional repression properties of the heterodimeric species.

Finally, as with other transcription factor families, recent data has shown that nuclear receptors have dominant negative heterodimeric partners. Short heterodimeric partner (SHP) is an orphan nuclear receptor that lacks a conventional DNA binding domain. SHP has been shown to interact with and inhibit transactivation by TR, RAR, RXR (243) and more recently ER (244). Thus SHP, like Id of the bZIP transcription factor family, can regulate the activity of some nuclear receptors by acting as a dominant negative inhibitor.

7. Mechanism of DNA Binding by the Glucocorticoid Receptor

Members of the nuclear receptor superfamily are capable of binding to DNA in three fundamentally different ways (monomer vs. homodimer vs. heterodimer, Table 1) (181). While dimerization is involved in regulating the binding of co-repressors, ligand and DNA by class II receptors, it plays a more restricted but nevertheless essential role in regulating the function of the steroid hormone receptors. An important contribution of dimerization to steroid hormone receptor action is in DNA binding.
To gain insight into the molecular basis for sequence specific DNA binding by GR and other steroid receptors, investigators took advantage of the fact that the isolated GR DBD is competent to bind DNA (144, 161). Subsequent to the identification of palindromes as binding sites for GR, a number of experiments showed the GR bound DNA as a dimer. First, studies show a stoichiometry of two receptor molecules bound per GRE. Second, footprinting techniques showed a dyad symmetry for receptor contacts with DNA. Third, site directed mutagenesis showed that residues within the DBD made base specific contacts with both half sites. The results of these in vitro studies were all confirmed when the crystal structure of the GR DBD-DNA complex showed two DBD molecules bound per GRE (152).

Using the isolated DBD, usually amino acids between 407 to 525 (numbering based on the rat GR sequence), numerous studies have demonstrated cooperative binding of the isolated GR DBD to DNA. At low protein concentrations, only one half site is occupied. However, at higher concentrations, DBD molecules bind to the second half site in a cooperative manner, i.e. the binding of the first monomer increases the affinity of the second by two orders of magnitude (245, 246). GRE recognition is dependent upon two regions of the GR DBD. The proximal or P-box composed of three amino acids located in the amino terminal Zn$^{2+}$ finger, is responsible for discrimination of HRE sequences. Changing the three amino acids of the P-box of GR to that of ER was sufficient to completely change the specificity such that the mutant GR transactivated from an ERE driven reporter (247-249). The second region, the distal or D-box, found in the knuckle of the C-terminal zinc finger restricted the GR DBD to response elements in which the two half sites were separated by three base pairs (152-155).
Mutation of five amino acids in the C-terminal zinc finger, the D-box, was sufficient to abolish cooperativity but not DNA binding. Further, conversion of the GR D-box to that of TR (250) or VDR (251) abolished cooperative binding to GRE. Conversely, replacing the TR D-box with that of GR enhances cooperative binding of TR to DNA (250).

Cooperative binding and the ability to functionally discriminate between HREs was postulated to be the result of dimerization of GR DBD as data showed that the distance and the relative orientation of the two half sites but not the integrity of the DNA backbone affected cooperativity in vitro and abolished transactivation in vivo (133, 251, 252). Definitive proof for the involvement of the D-box in cooperative binding was again obtained from the GR DBD crystal structure (152). Two complexes were crystallized. In one, the consensus high affinity binding site was employed and in the other the spacing between the two half sites was increased from three to four nucleotides. In both cases, D-box residues formed a dimerization interface. However, in the complex with the non-consensus DNA, one subunit was forced out of alignment with the recognition sequence (152). Naturally occurring GREs are not perfectly palindromic (147). Deviations from consensus may be a means of regulating affinity and activity of the response element. The crystal structure showed that dimers can still form even when one subunit is forced to make non-specific contacts (152). Obviously the binding in such a complex would not be as great as one in which the target DNA is a consensus GRE. Thus D-box dimerization may be a means of regulating target affinities and transcriptional responsiveness.
Cooperative binding to DNA appears to be a general mechanism for steroid receptors. Swapping experiments between ER and TR (253) and the crystal structure of the ER DBD complexed to DNA (254) also highlight the importance of the D-box dimerization interface in cooperative binding and HRE recognition by ER.

While the intact receptor and the isolated DBD recognized and bound the same DNA sequences and had the same protein:DNA contact points (143, 154, 255-257), the intact GR bound DNA with a ten fold higher affinity than the DBD alone (154, 257). This suggested that regions outside the DBD were required for maximal DNA affinity by GR. Three possible ways in which regions outside the DBD may increase the affinity of the receptor for DNA are as follows:

(i) regions in addition to the DBD mediate direct contact with the DNA. The increase in the number of protein:DNA contacts would result in a more stable receptor:DNA complex;

(ii) regions outside of the DBD increase binding affinity by causing structural changes in the DBD resulting in a more favorable DNA binding surface

(iii) regions outside the DBD dimerize resulting in enhanced affinity of the receptor for DNA.

Since the DBD is the only region of GR and other nuclear receptors shown to have DNA binding capabilities, it was unlikely that regions other than the DBD were making direct contact with the DNA.

The second scenario requires that either the amino or carboxy terminal portions of GR induce structural modification to the DBD. In the absence of x-ray crystallography data to support such an idea, the only evidence presented to date to support this idea
comes from biochemical studies with a N-terminally truncated GR mutant (GR$_{\text{ANH}}$). GR$_{\text{ANH}}$ has increased affinity for non-specific DNA relative to wild type GR and GR mutants which lack the LBD, a difference that suggested a role for the amino terminus in DNA binding (142, 143). In light of the fact that only the DBD makes contact with DNA, one interpretation of this finding was that the amino terminus may be required for proper conformation of the DBD. In an attempt to offer a molecular basis for the increased non-specific binding by the mutant GR, Eriksson and Wränge (143) proposed that the amino terminus, perhaps through protein:protein contact, was required to keep GR monomers in precise arrangement relative to each other in order to gain optimal DNA binding specificity. They suggested that with the amino terminus, GR was a more rigid protein. These conclusions were based on the observations that under their experimental conditions, GR$_{\text{ANH}}$ bound as a dimer to a GRE which had been mutated in one half site while the intact receptor did not (143). They interpreted these results to mean that deletion of the amino terminus resulted in a more flexible protein with greater DNA binding flexibility and this was proposed as a possible mechanism by which domains outside the DBD may indirectly influence DNA binding. These findings would seem to suggest a dimerization interface in the amino terminus of the receptor. In contrast, Danielsen et al (142) having done studies with a similar GR deletion mutant postulated that the increased affinity for non-specific DNA by GR$_{\text{ANH}}$ mutants resulted from the more positive net charge of an N-terminally deleted GR. Data to conclusively determine whether one or both of these theories are correct is still missing. Further, whether the amino terminus indirectly enhances DNA binding by GR or contributes to its dimerization is still debated.
Several studies have shown the importance of dimerization for DNA binding \textit{in vitro} and transcriptional activation \textit{in vivo}. In 1990, Chalepakis \textit{et al.} (255) demonstrated that GR homodimerization was important for high affinity DNA binding. This was followed by a study from Drouin \textit{et al.} in 1992 (218) which suggested not only that homodimerization may be a requirement for high affinity DNA binding, but put forward the notion that it was the rate limiting step in DNA binding by GR (218). A number of subsequent studies have since confirmed the importance of GR homodimerization for DNA binding. While there are conflicting ideas as to how this occurs, i.e. whether this dimerization occurred before or after DNA binding, all these studies concurred in showing a requirement for homodimer GR binding to GRE for hormone dependent activation. It is now generally accepted that dimerization by full length GR is responsible for its enhanced DNA binding affinity.

Thus, a third explanation for the enhanced DNA binding affinity seen with full length GR relative to the DBD alone, is the proposal that full length GR dimerizes through domain(s) other than the DBD. One possible way in which dimerization could enhance specific DNA binding may be that dimerization fixes the DBDs of the two receptor monomers in a conformation optimal for DNA either because it induces conformational changes in the receptor which made DNA binding more favorable or because it creates a larger interaction surface for protein:DNA contacts. This dimerization would necessarily be mediated by regions other than the DBD as the DBD alone was not sufficient for optimal DNA binding and the DBD does not dimerize in solution even at high protein concentrations (144, 146).
8. Evidence for Solution dimerization of GR

(i) *From DNA Binding studies of GR*

Once it became accepted dogma that the enhanced affinity of the intact receptor for DNA was a result of receptor homodimerization, the question became whether GR bound DNA as preformed homodimers or whether the intact receptor bound in a stepwise fashion as is the case with the DBD. In the latter instance, the receptor:DNA complex would be stabilized by protein:protein contacts in addition to the D-box interface following DNA binding. Current data on GR dimerization can be interpreted as being consistent with either possibility.

The proponents of the stepwise binding theory cite experiments done with wild type and mutant GREs as evidence in support of this model. In these studies, GR monomer:GRE complexes can be detected by EMSA *in vitro* using either wild type GRE or a GRE in which one of the half sites was mutated (258). Further, full length GR monomers complexed with GRE have also been detected by glycerol gradient centrifugation (143) in one study and by gel shifts in others (218, 255). The detection of the GR monomer:DNA complex is taken as evidence that the full length receptor binds DNA similar to the isolated DBD, with additional protein:protein contacts being made after DNA binding which stabilizes the dimer. It is argued that if binding resulted from a preformed homodimer, the GR monomer:DNA complexes should not be seen. As further proof, the detection of intact GR:GR DBD heterodimers under certain experimental conditions is given (255, 256). Since the isolated DBD is unable to dimerize in solution, it is reasoned that the heterodimers resulted from stepwise binding. In these experiments, however, the dimeric form of the full length GR:DNA complex predominated.
On the other hand, the GR:DNA monomeric complexes appeared to be inherently unstable and their detection depended on the experimental conditions used (218, 257). This together with the fact that monomeric full length GR:DNA complexes were not detected under conditions where both the dimeric full length receptor:DNA complex and the monomeric DBD:DNA complex was observed, was taken as evidence that binding of the full length receptor resulted from the binding of preformed homodimers. Advocates of this theory support a model of GR binding to DNA that includes a solution dimerization step prior to DNA binding. As evidence they cite the following: in 1989, Wränge et al presented data showing that the partially purified activated GR homodimerized in the presence as well as the absence of DNA (259). Glutaraldehyde treatment of ligand activated GR induced a 4 to 6S shift in sedimentation coefficient of the receptor in the absence of DNA. The 6S form was different from the hsp 90 associated 8S form and the hsp 90 free 4S form (259). They concluded that the glutaraldehyde-stabilized receptor was a homodimer for the following reasons. First, the 6S complex had the same sedimentation velocity as a GR:DNA complex shown to contain two GR molecules and second, in gel filtration experiments, the complex had a size similar to what would be predicted for a GR homodimer (259). In addition Cairns et al failed to observe heterodimers between full length GR and the GR DBD on DNA in contrast to other reports (260). Further, they showed that the glutaraldehyde stabilized 6SGR identified by glycerol gradient centrifugation bound specifically and with high affinity to a GRE while the 4S monomeric has very little DNA binding activity (260). In further contrast to other studies (143, 255, 256) which showed only the monomers of intact GR complexed to GRE with a mutant half site, Cairns et al saw no difference in the
mobilities of the complexes formed between full length GR and a wild type GRE and full length GR with a GRE mutated in one half site in vitro (260). They interpreted this to mean that GR dimerized prior to DNA binding and a stable complex formed between one monomer of the dimerized receptor and the non-mutated half site of the mutant GRE.

The debate about which form of GR binds to DNA (a preformed homodimer vs. a stepwise binding of monomers) continues because there has not been a comprehensive study of GR solution dimerization.

(ii) From studies of the nucleocytoplasmic shuttling of GR

In the absence of hormone, the hsp complexed GR is predominantly localized to the cytoplasm of target cells (58, 60-62). Hormone treatment results in dissociation of the GR from the hsp complex which leads to a rapid ($t_{1/2} = 4-5 \text{ min}$) and complete transfer of the receptor to the nucleus where it remains as long as ligand is present (58, 61, 62). Following the withdrawal of hormone, GR re-associates into the hsp complex but redistributes to the cytoplasm rather slowly (12-24 h) (62, 261).

The nuclear envelope (NE) represents the barrier to the passage of large proteins into and out of the nucleus. Therefore, GR must necessarily traverse the NE as it shuttles between the nucleus and the cytoplasm. Our laboratory has previously shown the association of GR with NE from rat liver using immunocytochemistry (262), binding studies (263, 264), immunoblotting and affinity labeling (265). Further, another group has shown immunoreactive GR present on NE from the liver of intact but not adrenalectomized rats (266). These results suggest that in the presence of hormone, at least part of the cellular pool of GR is localized to the NE, most likely during transport of
the receptor. One of the phenotypes of glucocorticoid resistance is an impairment of nuclear translocation. As GR has been shown to interact with the NE, it was possible that the defect in nuclear import in glucocorticoid resistant cell lines was at the level of the nuclear envelope binding. Our laboratory, in collaboration with Dr. Tony Antakly from the University of Montreal characterized the interaction of GR with nuclear envelopes isolated from three glucocorticoid target systems in the presence or absence of hormone: rat liver, steroid-sensitive or resistant human leukemic cell lines and steroid-sensitive or resistant mouse lymphoma cell lines (Appendix 1). During the course of experimentation, we observed that in response to hormone GR associated with the NE as dimers and multimers. One interpretation of these results is that GR may traffic between the nucleus and cytoplasm as a dimer and that solution dimerization is an important step in the mechanism of action of GR, not only as it pertains to DNA binding but also in regulating the nucleocytoplasmic trafficking of the receptor.

(iii) From Studies done with other steroid hormone receptors

Full length PR (267-271) and ER (134, 272, 273) have been shown to form stable dimers in solution in the absence of DNA in vitro and in vivo and the AR (274-276) has been shown to harbor a solution dimerization activity. Moreover, for ER there was a direct correlation between the ability of the receptor mutants to dimerize in solution and their ability to bind DNA with high affinity (134). The many structural and functional similarities among the different members of the steroid receptor subgroup of nuclear receptors predicted a similar activity for full length GR.
9. Project Goal

To evaluate the role of solution dimerization in GR action, I analyzed the nature of GR solution dimerization and mapped the receptor domain(s) involved in this activity.
II. MATERIALS AND METHODS

1. Receptor ligands, enzymes, chemicals

The synthetic GR ligand 1,4-Pregnadien-9α-fluoro-16α-methyl-11β, 17, 21-triol-3,20-dione (dexamethasone), (dex) was obtained from Steraloids Inc. (Wilton, NH, USA). Deacylcortivazol (DAC) and RU486 were kindly provided by Roussel Uclaf (France). Bovine serum albumin (BSA) and trizma base were purchased from Sigma Chemicals Co. (Oakville, ON, Canada). Acrylamide, N,N'-methylene bis acrylamide and all other chemicals for electrophoresis were from BDH (Quebec, Canada). All tissue culture reagents were obtained from Gibco BRL (Burlington, ON, Canada). Deoxyribonuclease I (DNase I) was from Boeringher Manheim (Laval, QC, Canada). All restriction enzymes and DNA modifying enzymes were from New England Biolabs (Mississauga, ON, Canada). Protein A Sepharose CL-4B beads was obtained from Sigma and Glutathione Sepharose was purchased from Pharmacia (Baie d'Urfé, Canada).

2. Expression Constructs

Glutathione-S-Transferase (GST) fusion proteins were expressed from plasmids containing fragments of the rat glucocorticoid receptor fused C-terminally to GST (277) and under the transcriptional control of a T7 RNA polymerase promoter. GST alone was expressed from the pGEX-3X vector (Pharmacia). To generate pGST-GR22-437, a 1.26 kb MscI/XcmI fragment encompassing amino acids 22 to 437 of the rat GR was excised
from pT7N556 (278). This fragment was ligated into GST vector pGEX-2T (Pharmacia) which had been digested with SmaI. pGST-GR<sub>X568</sub> (amino acids 407-568 of rat GR with an N-terminal GST tag) has been described previously (156). pGST-GR<sub>542C</sub> was made by excising the 759 base pair PstI/BamHI fragment (containing amino acids 542 to 795 of rat GR) from pRDN93 (279) and generating blunt end by treating the isolated fragment with T4 DNA Polymerase. The blunted fragment was then ligated into pGEX-3X vector which had been digested with SmaI. pGST-GR<sub>505C</sub> (amino acids 505 to 795 of rat GR fused C-terminally to GST) was made by PCR amplification of amino acids 505-795 using primers P2 (5'-GGAATTCGGGATCCGATGAAACCCTTGAAGCTCG-3') and P3 (5'-GGCGGGATTCCATATGGATCCTCACCCTGCTGCTCGAGCTTTTGGATGAAACA GAAGCCTTTGAT-3'). The PCR product was digested with SmaI and BsaAI and subcloned into pGEX-3X digested with SmaI. pGST-GR<sub>X550PKA</sub> (GST fusion protein of amino acids 407 to 550 of rat GR with a protein kinase A recognition site in the C-terminus) was constructed by insertion of an oligonucleotide encoding the protein kinase A recognition motif, LARRASYP, into the Styl (amino acid 550)/EcoRI sites of pGST-GR<sub>X568</sub>. pGST-GR<sub>505-550</sub> (amino acids 505 to 550 of rat GR with an N-terminal GST tag) was constructed by insertion of an oligonucleotide into the Styl/EcoRI sites of the pGST-GR<sub>505C</sub> plasmids.

The plasmids pAS2 and pGAD424 (Clontech) are yeast expression vectors for generating GAL4 DNA binding domain (GALDBD) and GAL4 transactivation domain (GALTA) fusion proteins respectively. The construct pAS540C which expresses GALDBD-GR<sub>540C</sub> fusion protein and pGAD540C which encodes the GALTAGR<sub>540C</sub> were made by PCR amplification of amino acids 540 to 795 of rat GR using primers P1 (5'-ATCCGTCGACATATGCTCGCAGCATTACCA-3') and P3 (5'GGCGGGATTCCATATGGG
digesting the PCR product with BamHI and subcloning the digested product into pAS2 and pGAD424 vectors which had been digested with BamHI. pAS505C (GALDBD-GR505C) and pGAD505C (GALTA-GR505C) were made by PCR amplification of amino acids 505 to 795 of rat GR using primers P2 (5' - GGAATTCGGGGATCCGATGAAACCATCTTTTTGAT-3') and P3 (5' - GCACGAGCTCGTTTGCATCAGCGGCG-3').

The PCR product wasdigested with BamHI and subcloning the digested product into pAS2 and pGAD424 which had been digested with BamHI. pACTX556 (GALTA-GRX556) (amino acids 407-556 of rat GR fused C-terminally to the GAL4 transactivation domain) was provided by R. Walther (University of Ottawa). pASX556 was made by PCR amplification of amino acids 407-556 using primers P4 (5' - CCGGAATTCATATGCTTTTCTAATGGA-3') and P5 (5' - CGGATCTCGAGGTACCCACCTCCAGTGAACC-3'). The PCR product was digested with NdeI and BamHI and then ligated into pAS2 which had been previously digested with NdeI and BamHI. pAS505-540 and pGAD505-540 (amino acids 505-556 fused to the DBD and AD of GAL4) were created by first PCR amplifying of amino acids 505-540. Next the PCR fragment was doubly digested with either NdeI/BamHI or EcoRI/BamHI. Finally, the digested fragment was ligated into either pAS which had been doubly digested with NdeI/BamHI or pGAD which had been doubly digested EcoRI/BamHI. For all PCR reactions, the plasmid pRdN93 (279) was used as a template.

For in vitro translation, the full length GR was expressed from the pRDN93 plasmid (279) which lacks the 21 consecutive glutamine residues in the N-terminal portion of the receptor at amino acids 75-96. Deletion of these residues did not affect
receptor activity. The X-receptor derivatives X795, X781, X768, X671, X616, X568, and XΔ509-631 described previously (278), express the first three codons of HSV thymidine kinase (TK) fused to amino acid 407 of GR and are C-terminally truncated up to the indicated amino acid (i.e. X781 contains amino acids 407-781). Following the indicated C-terminal receptor amino acid the peptides are extended by 6-34 non-receptor amino acids from the plasmid multiple cloning site (pSP64) or within an inserted synthetic oligo, adding 5-7 non-receptor amino acids. No differences were attributable to the extended non-receptor amino acids (278, 280). Derivatives denoted with Δ lack the receptor amino acids delineated by the numbered position within rat GR. For example, XΔ509-631 encodes amino acids 407 to 795 of rat GR with an internal deletion of amino acids 509 to 631. pT7N556, also described previously, expresses amino acids 1 to 556 of rat GR terminating in the plasmid multiple cloning site followed 7 non-receptor amino acids (281). These in vitro translation constructs were graciously provided by Dr. K. Yamamoto (UCSF, CA, USA). pGEM 505C was created by PCR amplification of GR fragment encoding amino acids 505 to 795 using primers P2 (5'-GGAATTCGCCGATCCGTATGAAACCTTGAGCTCG-3') and P3 (5'-GCGCGGATCCATATGGGATCTACTCACGCCACGTGCAGCTTTTTGATGAAACGAAAGCTTTTGAT-3') and subcloning into the pGEM-7Z vector (Promega). The firefly luciferase protein from the Promega coupled in vitro transcription-translation kit (pSP6luciferase) was used as a non-specific protein.

Where indicated below, DNA was restriction enzyme digested prior to in vitro transcription-translation at a plasmid DNA concentration of 0.05 mg/ml with the appropriate restriction enzyme and reaction buffer. The GR T7N556 plasmid (281) was completely digested with PstI or SphI prior to transcription-translation to produce the C-
terminal truncations N523 and N494, respectively. The DNA was phenol/chloroform extracted and ethanol precipitated in 0.3 M sodium acetate. The pellet was washed at least two times with 70% ethanol at room temperature. The DNA pellet was resuspended in a volume of double distilled water to a concentration of 0.5 μg/μl. The restriction digests were >98 % complete as judged by agarose gel electrophoresis and visualization with ethidium bromide staining.

To make pTLGR_{WT}, first p6RGR (282) was digested with BamHI to generate the 2.4 Kb BamHI GR fragment. Next, pTLFTZ-F1β (283) was doubly digested with BglII and BamHI and the 4.1 Kb vector backbone was gel purified. The GR fragment was then ligated into the pTL vector to generate pTLGR_{WT}. pTLGR_{NL1-} was similarly created from p6RGR_{NL1-} (61). The vector expressing GR_{NL1-} with a N-terminal myc tag was created by subcloning the MscI/BamHI GR fragment from p6RGR_{NL1-} (61) into pTLMTG (156). p6RGR_{NL1-} was created by mutating amino acids 513KKK515 of WT rat GR to 513NNN515 by site directed mutagenesis. This results in a GR protein with an inactivated NL1 and whose import is solely dependent on NL2 (61).

3. Plasmid preparation

Plasmid DNAs were transformed by a CaCl₂ heat-shock method (284) into a competent *Escherichia coli* -DH5α strain and plated on liquid broth (LB)/agar plates containing 150 μg/ml of ampicillin. Colonies were allowed to grow overnight and single colonies were used to inoculate 5 ml overnight cultures (LB/ampicillin). This in turn was used in the large scale inoculation of 500 ml of LB and grown overnight. The plasmid DNA was prepared using the alkaline-lysis maxi-preparation procedure (285, 286)
followed by 2 sequential cesium chloride gradients (287). Using this procedure, 80-95% of plasmid DNAs were supercoiled, as estimated by agarose gel electrophoresis and ethidium bromide staining.

4. Tissue culture

Sf7 cells which stably express WT GR with an N-terminal myc tag (156) were maintained in DMEM (GIBCO BRL) supplemented with 10 % heat inactivated fetal bovine serum (GIBCO BRL) (DMEM-10 % FBS) in the presence of 50 μg/ml G418 (FFF) in a tissue culture incubator at 37 C and a humidified atmosphere of 95% air/5 % CO₂. The parental cell line was grown in DMEM-10 % FBS in the absence of G418. COS7 cells (ATCC, Rockville, MD) were maintained in DMEM-10% FBS under the same incubation conditions. The hybridoma cell line that produced the anti c-myc antibody, 9E10 (288, 289), was maintained in RPMI 1640 supplemented with 10% FBS.

5. Preparation of 9E10 Antibody from Hybridoma Supernatant

9E10 hybridoma cells were grown in RPMI 1640-10% FBS. The cells were split and expanded until there was about 2L of 30-40% confluent cells, after which they were allowed to grow to confluence. The cells were removed by centrifugation (2 000 xg for 5 min), the supernatant collected and re-innected with a small portion (~ 5%) of the cell pellet. The cells were then allowed to grow to saturation and death as normal. Note that as cells reached saturation, glucose to 1% and HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) to 25 mM was added to the media. The cells were removed by centrifugation (3000 xg for 30 min at room temperature) and the supernatant transferred to a flask with a stir bar. Saturated ammonium sulfate solution (0.5:1, v/v)) was slowly
added and the flask was incubated at 4 C overnight with gentle stirring. After centrifugation at 3000 xg for 30 min the supernatant was transferred to another container, the pellet resuspended in a small amount of phosphate buffered saline pH 7.4 (PBS; 137 mM KCl, 2.7 mM NaCl, 4.3 mM Na₂HPO₄·7H₂O, 1.4 mM KH₂PO₄) and stored overnight at 4 C. Saturated ammonium sulfate solution was slowly added to the collected supernatant to 50% saturation and the flask incubated overnight at 4 C with gentle stirring. Following centrifugation as before, the pellet was resuspended in PBS (0.01:1, v/v). This was mixed with the first pellet/PBS solution and the combined suspension was dialyzed in 2 L PBS overnight at 4 C with 3 changes in the dialysis buffer. The dialysed antibody preparation was buffered by the addition of 1 M Tris pH 8.0 (0.05:1, v/v). Following the addition of sodium azide to 0.02 % the antibody preparation was stored at -20 C in 1 ml aliquots until used. Aliquots were thawed as needed and after thawing the antibody was stored at 4 C.

6. Coupled in vitro transcription-translation

The indicated plasmid DNA was in vitro transcribed and translated with either T7 or SP6 RNA polymerases using the Promega TNT coupled rabbit reticulocyte lysate system (290) according to manufacturer's instructions. Translations included 10 mCi/ml of translation grade ³⁵Sulfur methionine ([³⁵S]-met)(1,000 Ci/mmol, Amersham). Each translation reaction was performed for 90-120 min at 30 C in a 50 μl volume according to the manufacturer's protocol with the addition of 0.1 mM dithiothreitol (DTT) and 0.4 mM ZnOAc. Translations were stopped by adding excess (5 mM) unlabelled L-
methionine. DTT (1 μl of 5 mM stock) and glycerol (final concentration of 5 % (v/v)) were added and translations were stored at 4 C for a maximum of 5 days prior to use.

7. Untransformed and transformed steroid receptor

In vitro translated GRs were transformed (heat shock protein (hsp)-dissociated) from their native untransformed state (hsp-associated state) (168, 291, 292) by adding dex to 10⁻⁶ M or NaCl to 0.4 M to the translation mixture and incubating for 2 hours at 4 C followed by a 30 min incubation at 25 C (293). This yielded liganded (dex associated) or unliganded (NaCl treated) receptors free of hsp. The untransformed receptor/hsp complex was stabilized by the addition of sodium molybdate to 20 mM (294).

8. Co-immunoprecipitation Assay

Preparation of cytosolic extract

Cells (SF7 stably expressing myc GR or the parental cell line) were plated onto 10 cm² plates 2 days prior to the preparation of cytosolic extracts and grown to 95 % confluency. To prepare cytosolic extracts, cells washed once with PBS and harvested in PBS (0.5 ml per 10 cm² plate). The cells were pelleted (2 000 RPM for 2 min in a microfuge), the PBS aspirated and the cells resuspended (100 μl per 10 cm² plate) in TEGD buffer (10 mM Tris-HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF)). The cells were allowed to swell on ice for 10 min and lysis was accomplished by 3 freeze-thaw cycles (dry ice-methanol/37 C) with vortexing after each thaw. The lysates were centrifuged in a microfuge at maximum RPM for 5 min and the resulting supernatant was used as cytosolic extract.
Co-immunoprecipitation

To dissociate the heat shock proteins, cytosolic extracts were treated with either $10^{-6}$ M dex or 0.4 M NaCl as described above. After transformation, dex-treated cytosolic extracts (400 μg protein/assay) were then diluted 3 fold with 1X binding buffer (25 mM HEPES, pH 7.9, 60 mM KCl 0.5 mM EDTA, 12% glycerol, 0.1% NP-40, 0.2 mM DTT, 0.2 mM PMSF) while the salt-treated extracts were diluted with binding buffer without KCl. The myc-GR was immunoprecipitated by incubating the extracts with the anti-myc antibody, 9E10 (1:25), for 2 h at 4 C. Protein A sepharose which had been pre-blocked overnight at 4 C with 50 mg/ml BSA in ImmunoPrecipitation buffer (IP buffer; 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, .05% NP-40, .02% NaN₃) was added and the tubes incubated for a further 30 min at 4 C. The protein A beads were washed once with 1 ml ice-cold wash buffer (30 mM HEPES, pH 7.5, 250 mM NaCl, 5 mM EDTA, 1 % TritonX-100, 0.5 % sodium deoxycholate) and 2X with binding buffer. The beads were then pre-blocked on ice for 2 min with 5 μL rabbit reticulocyte lysate in 150 μL binding buffer, centrifuged, the supernatant removed and the beads resuspended in 150 µL 0.6X binding buffer. Equal amounts (as determined by SDS-PAGE and phosphorImage analysis) of [35S]-labeled in vitro translated protein products were incubated with the immunopurified myc GR for 2 h at 4 C with gentle mixing. Following binding, the protein A beads were washed 3X with 500 μL ice-cold 0.6X binding buffer and the bound proteins eluted by boiling for 5 min in SDS sample buffer (120 mM Tris-HCl pH 6.8, 20 % (v/v) glycerol, 4% (w/v) sodium dodecylsulfate (SDS), 10 % beta-mercaptoethanol (v/v), and 0.2 mg/ml of bromophenol blue). The eluted proteins were
analyzed by SDS-PAGE and either fluorography or phosphoimaging (BioRad, model GS-525) as described below.

For the DNaseI experiments, both the cytosolic extract and the \textit{in vitro} translated proteins were treated with 20 units of DNaseI in DNaseI buffer for 30 min at 25 C prior to co-immunoprecipitation.

\textbf{9. Expression and purification of GST fusion proteins}

The expression and purification of GST or GST-fusion proteins were carried out as previously described (156). The BL21pLys (DE3) strain of \textit{E.coli} (gift from Dr. M. Ekker, University of Ottawa, ON) were transformed with expression vectors for either GST alone (pGEX-3X) or GST fusion proteins. Single colonies were picked and grown in 5 ml cultures overnight. The 5 ml cultures were then added to 500 ml media and cultures were grown to an OD of 0.8 (\lambda 600). The cultures were then induced with 0.1 mM isopropyl \textbeta-D-thiogalactopyranoside (IPTG) overnight at room temperature. The IPTG induced the expression of the stable integrate of the bacteriophage gene 1 product (295) to produce T7 RNA polymerase unique to the BL21 (DE3) strain, which in turn initiated high level of expression of the target gene encoded by the plasmid. Cells were harvested by centrifugation at 5000 X g for 15 min at 4C and resuspended in lysis buffer (25 mM HEPES, pH 7.9, 100 mM KCl, 20% glycerol, 0.1% NP-40, 2 mM EDTA, 2 mM DTT, .2 mM PMSF). Lysozyme was added to a final concentration of 100 \mu g/ml and the suspension incubated on ice for 10 min. The bacterial suspension was then sheared sequentially by passing it through 18, 20 and 25 gauge needles (3 times each). The extract was then sonicated 10 times (40 sec pulses) using the small probe at 35 \% duty
cycle (Fisher Sonic Dismembrator-Model 300). The insoluble material was pelleted by centrifugation at 28,000 rpm for 15 min in a Beckman Ti-60 rotor. The supernatant was immediately added to glutathione sepharose beads which had been previously washed three times with 15 bead volumes of Binding Buffer (0.6 X lysis buffer containing 0.1 % NP-40). Washing comprised resuspension of the sepharose beads in buffer, followed by centrifugation at 1000 rpm for two minutes and removal of the buffer. The fusion protein was allowed to bind the affinity matrix for 90 min at 4 C on a rotating wheel. Following binding, the beads were washed 5 times with 0.6X binding buffer. After the final wash, excess buffer was removed and the beads resuspended in 1 bead volume 0.6X lysis buffer containing 1 mM PMSF and 0.02 % sodium azide. The suspension was called 50 % slurry and it was stored at 4 C for up to 6 weeks.

10. GST Pull Down Assays

A quantity of 0.5 µg of GST or GST-fusion protein coupled to glutathione sepharose was used in all binding assays. Equal amounts (as determined by SDS-PAGE and phosphoimage analysis) of 35S-labelled, *in vitro* translated protein product were added to the binding assay. The volume of rabbit reticulocyte lysate added was normalized with unprogrammed lysate. The binding reaction (fusion protein, labelled protein and binding buffer to 200 µl) was allowed to continue for 90 min at 4 C with gentle agitation. Following binding, the sepharose beads were washed 3X with 500 µl of binding buffer and the bound proteins were eluted by boiling the beads for 5 minutes in SDS sample buffer (204). The proteins which had been bound by the affinity matrix were analyzed by SDS-PAGE and visualized by fluorography or phosphorimaging. Ten percent of the labeled protein added to each binding assay was also loaded onto the SDS-
PAGE gels to demonstrate the amount of labeled proteins added to the incubations and to use as a comparison for the amount of binding.

11. **Direct Binding Assay**

For the direct binding assay, GST-GR$_{X550PKA}$ was expressed in and purified from bacteria as described above. The GST fusion protein immobilized on glutathione and resuspended in Sepharose in TEGz50 buffer (50 mM Tris, pH 7.5, 50 mM NaCl, 10% glycerol, 0.5 mM EDTA, 50 mM ZnCl$_2$ and 0.5 mM PMSF) + 1% Triton X-100 was then labeled with $^{32}$P-ATP by kinasing with the catalytic subunit of PKA (Sigma) in HMK buffer (20 mM Tris, pH 7.5, 100 mM NaCl, 12 mM MgCl$_2$, 1 mM DTT) for 30 min at 30 C. The reaction was terminated by the addition of 1 ml of stop buffer (10 mM NaPO$_4$, 10 mM Na$_4$P$_2$, 10 mM EDTA, 2 mg/ml bovine serum albumin (BSA)). The labeled GR portion of the GST-GR$_{X550PKA}$ fusion protein was eluted in TEGz50 buffer containing 1% Triton X-100 by thrombin (Sigma) treatment which cleaves the fusion protein between the GST and GR portions. Approximately 5 ng of the $^{32}$P-labeled GR peptide (amino acids 407 to 550) was incubated with immobilized GST fusion proteins in binding buffer in the presence of 2 mg/ml BSA and 1 mM PMSF. Following extensive washing, the bound peptide was eluted in SDS sample buffer, resolved by SDS-PAGE and visualized by autoradiography.

12. **SDS-Polyacrylamide gel electrophoresis**

The SDS-PAGE was carried out as previously described (296). Protein content throughout studies reported in this thesis was determined by the Bradford protein assay
using BSA as a standard. Protein samples were diluted in SDS-sample buffer and
denatured by boiling for 5 min at 95 °C. The samples were loaded on a 8%-15%
separating gel (296) with a 4% stacking gel at a thickness 0.75 mm. The gels were
poured using a mini-protein gel apparatus (BioRad) and run at 160 volts for 60-75 min in
electrophoresis buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1% (w/v) SDS).
Gels used for resolving radiolabeled proteins were fluorographed or phosphoimaged
while those containing non-radiolabeled samples were visualized using Coomassie Blue
staining or Western immunoblotting. Prestained molecular weight protein standard
markers (Amersham) were run on all gels.

13. Fluorography

Gel fluorography was used to enhance the low beta emitting property of \(^{35}\)Sulfur
atoms by incorporating a fluor directly into the polyacrylamide gel (297). SDS-PAGE
gels was dehydrated by soaking in glacial acetic acid for 15 minutes and the fluor was
incorporated by incubation in 20 % (w/v) 2,5-diphenyloxazole (PPO) in glacial acetic
acid for 20 minutes. Gels were then washed extensively with water to allow the
incorporated PPO to precipitate into the gel. Finally, gels were dried under vacuum with
heat. Once dried, the gels were exposed to either Kodak XAR-5 film at -80 °C or a
Phosphoimaging screen.

14. Coomassie Blue Staining

After electrophoresis, gels were incubated in staining solution (0.1% (w/v)
Coomassie blue, 40% (v/v) methanol, 10% (v/v) glacial acetic acid, 50% (v/v) H₂O) for
30 min at room temperature with gentle shaking. Gels were then destained by incubation
in destain solution (40% (v/v) methanol, 10% (v/v) glacial acetic acid, 50 % H₂O) with gentle shaking at room temperature.

15. **Western Immunoblotting**

After separation in SDS PAGE, proteins were transferred electrophoretically (298) from the gel in Transblot buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 0.1% (w/v) SDS, 20% (v/v) methanol) onto an Immobilon P membrane (Millipore Corp., Bedford, MA) which had been presoaked in methanol. The transblot cell was run for 30-45 min at room temperature at 100 V in a BioRad transblot apparatus. The membrane was removed, rinsed in washing buffer, Tris Buffered Saline-Tween (TBS-T, 20 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.1% (w/v) Tween-20) and blocked for 1 h at room temperature with gentle shaking in 10% (w/v) skim milk made up in TBS-T. The membrane was rinsed briefly using 2 changes of TBS-T, then incubated overnight at 4°C with primary antibody diluted in TBS-T. The mouse monoclonal anti-GR antibody, BuGR2 (Affinity BioReagents, Inc., Golden, CO, USA) and the mouse monoclonal anti-GAL4 DBD antibody (Santa Cruz, CA) were diluted 1:2 000 (v/v) to give final working concentrations of 0.5 ng/μl and 0.2 μg/ml respectively while the monoclonal anti-c-myc antibody, 9E10, and the rabbit polyclonal anti-GR LBD antibody (Santa Cruz, CA) were used at a 1:400 (v/v) dilution. The membrane was rinsed using 2 changes of TBS-T, then incubated once for 5 min and twice for 20 min with fresh changes of TBS-T at room temperature with gentle shaking. The membrane was then incubated for 1 h at room temperature with horse radish peroxidase (HRP) conjugated sheep anti mouse or donkey anti rabbit IgG antibody (1:50 000 v/v in TBS-T) with gentle shaking. After washing as before, immunodetection was by enhanced chemiluminescence (ECL) according to
instructions in the Amersham ECL western blotting kit (Amersham Life Science Inc. Oakville, ON, Canada)

16. Yeast Two Hybrid Assay

Growth and transformation of yeast

The Saccharomyces cerevisiae strain, Y190 (MATα, gal4 gal80 his3 trp1-901 ade2-101 ura3-52 leu2-3,-112 +URA3::GAL → lacZ, LYS2::GAL → HIS3 cyhR) (299) which contains two GAL1→lacZ fusions integrated at unknown locations was grown on YEPD (1 % (w/v) yeast extract, 2 % (w/v) peptone and 2 % (w/v) dextrose) agar (2% (w/v) plates. To prepare competent cells, 100 ml of YEPD was inoculated with a single yeast colony and grown overnight at 30 C. One L of YEPD was inoculated with the overnight stock and the culture was grown to mid-log phase, O.D. 0.5 to 0.8 (λ600). Cells were harvested by centrifugation at 5 000 RPM in a JA-10 rotor for 5 min, resuspended in 200 ml sterile LiAcTE (100 mM LiAc, 10 mM Tris-HCl pH 8.0, 1 mM EDTA) and re centrifuged for another 5 min. The cells were then resuspended in 50 ml sterile LiSORB (LiAcTE with 1 M sorbitol) and incubated at 30 C for 15-30 min. Following centrifugation, the cells were resuspended in 2.5 ml LiSORB and either used immediately for transformation or stored at -80 C in 50 μl aliquots with 5 % (v/v) dimethyl sulfoxide (DMSO) as cryo-protectant for later use. Transformation was carried out using the lithium acetate method with plasmid DNA (300). For transformation, the DNA-carrier mix preparation (10 μg plasmid DNAs (5 μg each of the DBD and AD fusion constructs); 10 μl of 10 mg/ml sheared salmon sperm DNA which had been boiled at 95 C for 5 min) was added to 50 μl competent yeast suspension and the tubes
incubated for 10-30 min at 30 C. Polyethylene glycol 4000 (PEG) (450 µl, 40% (w/v) in LiAcTE) was added and following mixing by pipetting, the cells were heat shocked for 10 min at 42 C. The cells were transferred to 5 ml of synthetic dropout (SD) media, incubated at 30 C for 3-4 h, centrifuged, plated onto SD agar plates and incubated at 30 C for 5 days. SD (1.79% (w/v) bacto yeast nitrogen base without amino acids, 0.5% (w/v) NH₄SO₄, 20% (w/v) dextrose) is a minimal media used in yeast transformations to select for specific phenotypes. One or more essential nutrients (leucine and trptophan in these experiments) are omitted to select for transformants carrying the corresponding nutritional gene. SD media was prepared as a 10X stock, filter sterilized and stored at 4 C. Dropout solution (10X, sterilized by autoclaving) contained the following: isoleucine (300 mg/ml), valine (1500 mg/ml), adenine hemisulfate salt (200 mg/ml), arginine HCl (200 mg/ml), histidine HCl monohydrate (200 mg/ml), lysine (300 mg/ml), methionine (200 mg/ml), phenylalanine (500 mg/ml), threonine (2000 mg/ml), tyrosine (300 mg/ml) and uracil (200 mg/ml). To prepare 1L of 1X SD, 800 ml water was autoclaved and cooled to room temperature. 100 ml each of 10X SD and 10X dropout was added to the water and the solutions mixed. To prepare SD plates 800 ml of water and 20 g of agar were autoclaved and cooled to ~ 55 C. 100 ml each of 10 SD and 10X dropout was added to the water/agar and the solutions mixed. Yeast colonies transformed with fusion constructs were grown in synthetic media lacking either leucine or tryptophan or both. For yeast transformed with a single construct, either leucine (1000 mg/ml stock) or tryptophan (200 mg/ml) was added to the media. Single colonies from the transformed yeast were selected and cultured overnight 1.5 ml SD media lacking the appropriate amino acid. The yeast cultures were then subcultured (1:10) in fresh selective media that
contained either ethanol or the synthetic glucocorticoid, DAC at a concentration of 10^{-6} M and grown for a further 16 h. The O.D. at λ = 600 nm was determined and the cultures were then assayed for β-galactosidase activity.

**β-galactosidase Assay**

Yeast cells were harvested from 0.5 ml culture by low speed centrifugation (4000 RPM in a microfuge for 1 min) and resuspended in 100 μl 1X Z-buffer (10 mM KCl, 1 mM MgSO4, 50 mM β-mercaptoethanol, 100 mM NaPO4· pH 7.0). Z-buffer is prepared and stored as a 5X stock without β-mercaptoethanol. β-mercaptorethanol is added fresh each time after dilution. Chloroform (50 μL) was added and the suspension was mixed by vortexing vigorously for 30 sec. The reaction was started by the addition of 700 μl of 2 mg/ml o-nitrophenyl β-D galactopyranosidase (ONPG) in 1X Z buffer (pre warmed to 30 C) and the tubes incubated at 30 C until a yellow color developed. The reaction was stopped by the addition of 500 μl of 1 M Na2CO3 and centrifugation for 2 min in a microfuge at maximum RPM. The upper phase was removed and the absorbance read at λ = 420 nm. β-gal units were calculated using the following equation: (1000*OD_{420})/(t*v*OD_{600}) where t = reaction time at 30C in min and v = initial volume of culture used in ml (280). Transformations were repeated 3-5 times and 3 individual colonies from each transformation was tested for β-galactosidase activity. All error bars represent the standard error of the mean.

17. **Preparation of yeast extract**

A single yeast colony was used to inoculate a 1 ml SD media and grown overnight at 30 C. Additional media (4 ml) was added to the culture tubes and the cultures were grown for further 24 h. The cells were centrifuged at 2000 Xg for 5 min,
the media removed and the cells resuspended by vortexing vigorously in 100 µl SDS-sample buffer. After heating at 95 C for 5 min, and centrifugation at maximum RPM in a microfuge for 5 min to clear cell debris, 25 µl of the extract was resolved by SDS PAGE and subjected to western immunoblotting.

18. Transfections and Semi Quantitative Indirect Immunofluorescence (IIF)

Sixteen hour prior to transfections, COS7 cells were seeded onto 60 mm plates. Transfections were performed using Lipofectamine® (Life Technologies, 10 µl per plate) according to manufacturer's instructions with an 8-10 h incubation time. Cells were transfected with 250 ng of either pTLGR<sub>WT</sub> or pTLMTGGR<sub>NLI</sub> singly or together. Transfections were stopped by adding serum to 10%. Sixteen hours after transfections were stopped, the media was replaced with DMEM supplemented with 10% charcoal treated FBS. Eight h later cells were replated onto poly-L-lysine (500 µg/ml) coated glass coverslips (each 60 mm dished made 8 coverslips) and each coverslip was incubated overnight in 35 mm tissue culture plates in DMEM containing 10 % charcoal stripped FBS. Cells were synchronized to G<sub>0</sub> by incubation in serum free DMEM for a further 21 h prior to the initiation of hormone treatment. Dex was added to a final concentration of 10<sup>-6</sup> M in serum-free medium for the indicated times followed by indirect immunofluorescences (62).

Following hormone treatment, cells on coverslips were fixed for 30 min at room temperature (RT) in 3% (w/v) paraformaldehyde in PBS. Intrinsic fluorescence due to fixation was quenched by incubation for 5 min at RT in 0.1 M glycine in PBS. The cells
were permeabilized with Triton X-100 (0.5% (v/v) in PBS) for 30 min at RT and non specific sites were blocked with 5% (v/v) normal goat serum (NGS) in PBS for 1 h at RT. The NGS was aspirated and primary antibodies were added to the coverslips overnight at 4°C. The primary antibodies were either the anti-GR antibody BUGR-2 (Affinity BioReagents, Inc 10 μg/ml)) for detection of GR<sub>WT</sub> or the anti-myc antibody, 9E10, for detection of MTGGR<sub>NL1</sub>. After extensive washing in PBS (2 ml PBS added, coverslips incubated at RT for 2 min, PBS aspirated and the procedure repeated 5 times), anti-mouse fluorescein-isothiocyanate-conjugated IgG secondary antibody (Amersham, 0.6 μg per ml in PBS) was added at room temperature for 1 h with gentle shaking. The washing procedure was repeated and coverslips were mounted in glycerol/PBS (1:1 v/v). Slides were examined for subcellular localization of GRs on a Zeiss Axiophot photomicroscope.

Cells were classified into one of five categories from exclusively nuclear to exclusively cytoplasmic as described previously (62): N, cells showing exclusively nuclear immunofluorescence; N>C, cells where the immunofluorescence from the nucleus is clearly higher than the cytoplasm; N=C, cells where the immunofluorescence is equally distributed between the nucleus and cytoplasm; C>N, cells where the immunofluorescence from the cytoplasm is clearly higher than the nucleus and C, cells with entirely cytoplasmic staining. Quantification was performed using double-blind encryption and at least 250 stained cells were counted for each experimental condition.
III. RESULTS

1. A Co-immunoprecipitation Assay for analysis of the Dimerization of GR in solution

To analyze the solution dimerization activity in rat GR, I first used a variation of a co-immunoprecipitation assay. My assay is summarized in Figure 3. I used an Sf7 cell line (provided by Dave Rodda, University of Ottawa) which had been stably transfected with an expression plasmid for full-length rat GR fused N-terminally to a myc tag. I took advantage of the fact that these cells express large quantities of tagged GR protein which can be easily and efficiently immunopurified from cellular extracts with the anti-myc antibody, 9E10. As a control, immunoprecipitates using 9E10 are also generated from the parental Sf7 cell line not transfected with myc GR. Both immunoprecipitates were immobilized on protein A sepharose, a solid affinity support to which the heavy chain of the antibody binds. The loaded protein A sepharose beads were used as an affinity matrix for the binding of radiolabeled in vitro translated GR and GR deletion mutants. After extensive washing, the bound proteins were eluted by boiling in SDS-sample buffer, resolved by SDS-PAGE and visualized by either fluorography or phosphoimaging. In all Figures and Tables which follow, "bound" refers to labeled proteins recovered from immunoprecipitates of extracts of cells which express myc GR while "control" refers to labeled proteins recovered in immunoprecipitates from the parental Sf7 cells lacking myc GR.
Figure 3. Schematic representation of the co-immunoprecipitation assay used to study solution dimerization of rat GR.

Cellular extracts prepared from an Sf7 cell line stably transfected with myc GR or from the parental Sf7 cells were first treated with either a GR ligand (10⁻⁶ M dex), salt (0.4 M NaCl) or antagonist (10⁻⁶M RU486) followed by the addition of the anti-myc antibody, 9E10. Protein A sepharose was used to precipitate the antibody-complexes. Radiolabeled GR in binding buffer was incubated with the protein A complex from both extracts. After extensive washing, the protein A sepharose was boiled in SDS-sample buffer and the eluted proteins analyzed by SDS-PAGE and either fluorography or phosphoimaging.
Sf7 cells stably transfected with mycGR

Parental Sf7 cells

Cellular extracts

Treatment of both extracts with either:
1. Agonist
2. Salt
3. Antagonist

Add α-myc Ab 9E10

Add protein A sepharose

Add GR

Add GR and Mg

Add Mg

WASH

ANALYSIS BY SDS-PAGE
2. Interaction between two GR monomers in vitro requires dissociation of the heat shock protein complex.

To begin to evaluate the dimerization of rat GR in solution, I assessed the ability of immunopurified myc tagged rat GR to co-precipitate in vitro translated \textsuperscript{15}S-labelled full length GR (Fig. 4). To do this, I incubated dex-treated in vitro translated GR with dex-treated immunopurified myc GR and assayed for interaction between the two GR monomers in the modified co-immunoprecipitation assay. Liganded in vitro translated GR was co-precipitated by liganded, immunopurified myc GR (Fig. 4A, lane 6) but not by control immunoprecipitates (Fig. 4A, lane 9), indicating that the in vitro translated GR associated specifically with the myc GR in the protein A sepharose complex. Comparing the amount of \textsuperscript{15}S-labeled GR specifically retained by myc GR (Fig. 4A, lane 6) to an amount of labeled GR that represents ten percent of the total amount of labeled GR added to the assay (Fig. 4A, lane 3) shows that slightly more than ten percent of the labeled input protein was co-precipitated. This is comparable to the amount of binding seen with many other proteins in similar assays (156, 301). Furthermore, when the unrelated firefly luciferase protein was similarly incubated, it was not retained by either myc GR (Fig. 4A, lane 4) or the control precipitates (Fig. 4A, lane 7) even though the amount of labeled luciferase protein added to the assay (Fig. 4A, lane 1) was comparable to the amount of GR used (Fig. 4A, lane 3). This again demonstrates this assay was able to detect a specific interaction between the two liganded GR molecules. Note that the lower molecular weight bands seen in lanes 2, 3 and 6 (Fig. 4A) may represent GR degradation products as they were specifically retained by the myc GR complex (Fig. 4A, lane 6) but
Figure 4. Interaction between GR monomers in solution requires dissociation of the heat shock protein complex.

A. $^{35}$S-labeled *in vitro* translated full length GR treated with either dex alone (lanes 6 and 9) or with dex in the presence of molybdate (lanes 5 and 8) or firefly luciferase (lanes 4 and 7) were incubated with myc tagged GR immunopurified from cellular extracts that had been treated with either dex alone (lanes 4 and 6) or with dex in the presence of molybdate (lanes 5 and 8). Immunoprecipitates of cell extracts from the parental cell line similarly treated with dex (lanes 7 and 9) or dex and molybdate (lane 8) were used as controls. Bound proteins were resolved by SDS-PAGE and fluorography. Ten percent of the *in vitro* translated proteins added to the assay is shown in lanes 1 to 3. B. myc GR immunoprecipitated from cell extracts treated with either dex alone (lane 1) or dex in the presence of molybdate (lane 2) was subjected to western immunoblotting with the anti-myc antibody, 9E10.
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**Western Immunoblot**

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not the control (Fig. 4A, lane 9). Thus, I was able to detect a specific interaction between two GR molecules in this assay.

Following translation in vitro in rabbit reticulocyte lysate but prior to exposure to steroid, $^{35}$S-labeled GR is packaged into an hsp-containing complex similar to the unliganded GR complex found in the cytoplasm of target cells (137, 168, 278, 291, 292). In addition to maintaining the GR LBD in a conformation that allows GR to bind ligand with high affinity, it has been proposed that the hsp complex masks other receptor functions such as the NLS, DNA binding and transcriptional activation, all of which are activated by ligand binding (77).

I was interested in determining whether the observed interaction between the liganded GRs can occur with GRs that are complexed to heat shock proteins. The GR-hsp complex can be disrupted simply by dilution (302, 303), so in those samples in which I wished to ensure that GR remained associated with the hsp for the duration of the co-immunoprecipitation assay, sodium molybdate, to a final concentration of 20 mM, was added to all buffers used in the immunopurification of myc GR. Molybdate was also added to the labeled GR after in vitro translation. Molybdate stabilizes the GR-hsp complex and inhibits its dissociation even in the presence of ligand (294, 304-307). Thus, in these assays both the in vitro translated GR and the myc GR were stably maintained in heat shock protein complexes even after hormone treatment. Under these conditions, when an amount of molybdate and dex treated labeled GR (Fig. 4A, lane 2) similar to that was used in the presence of dex alone (Fig. 4A, lane 3) was added to molybdate and dex treated myc GR, no interaction was detected between the two GR molecules (Fig. 4A, lane 5). As before, no binding was seen with the control precipitates.
(Fig. 4A, lane 8). These results indicated that under these experimental conditions, two untransformed GR molecules are unable to interact with each other. The presence of molybdate in the assay buffers did not adversely affect the immunoprecipitation of myc GR as western immunoblots show similar amounts of the myc GR protein immunopurified both in the absence (Fig. 4B, lane 1) and presence (Fig. 4B, lane 2) of molybdate.

3. Dimerization of GR is independent of residual DNA

Like other steroid hormone receptors, GR has a well-characterized DNA-dependent dimerization interface in the DBD (133, 152, 250). I was interested only in detecting GR:GR interactions that occurred independent of DNA binding. Although no exogenous DNA was being added to the co-immunoprecipitation assay, it remained formally possible that the observed interaction between in vitro translated GR and immunopurified myc GR was occurring through the interaction of the GR DBDs with contaminating DNA resulting from either the cellular extracts or the in vitro translation reaction. To ensure that the interaction between the two GRs was DNA-independent and was not being stabilized by contaminating DNA, prior to the binding reaction, the loaded protein A sepharose beads were incubated with either DNaseI buffer alone (Fig. 5A, lanes 3 and 6) or buffer with DNaseI (Fig. 5A, lanes 4 and 7), while the in vitro translated reactions were treated with DNaseI. DNaseI, an enzyme that non-specifically cleaves DNA, is commonly used as a tool to distinguish DNA-independent protein-protein interactions from those that are stabilized by contaminating DNA. With the addition of DNaseI, DNA stabilized interactions would be abolished because DNaseI would cleave
Figure 5. Solution dimerization is resistant to DNase I treatment.

A. Liganded, immunoprecipitated myc GR (lanes 2 to 4) or control immunoprecipitates (lanes 5 to 7) were treated (lanes 4 and 7) or not (lanes 2, 3, 5 and 6) with DNase I. The DNase I treated immunoprecipitates were incubated with $^{35}$S-labeled in vitro translated GR pretreated (lanes 4 and 7) or not (lanes 2, 3, 5 and 6) with DNase I as described in the methods. Bound proteins were resolved by SDS-PAGE and phosphorimaging. Lane 1 shows 10% input. B. Immunoprecipitated myc GR before (lanes 1 and 2) and after (lane 3) DNase I treatment was analyzed by western immunoblotting with the anti-myc antibody, 9E10. C. (left panel) Results of the co-immunoprecipitation assay shown in A quantified as the ratio of the $^{35}$S-labeled GR bound to the myc GR immunoprecipitates relative to that bound to the control precipitates. (right panel) The binding shown in A corrected for the relative amounts of myc GR protein used under the various experimental conditions.
any residual DNA and thereby disrupt any protein:protein interactions being stabilized by DNA. DNaseI treatment did not abolish the interaction between the two GR monomers (Fig. 5A, lanes 2 to 4). Moreover, the amount of labeled GR bound to the myc GR under the various experimental conditions was equal to (Fig. 5A, lane 4) or slightly more than (Fig. 5A, lanes 2 and 3) ten percent of the amount labeled protein added to the assay (Fig. 5A, lane 1). The apparent decrease in the binding in the presence of DNaseI (Fig. 5A, compare lanes 2 and 4) correlated with a similar decrease in the amount of protein A sepharose associated myc GR following DNaseI treatment (Fig. 5B, lanes 1 and 3). This was probably due to the presence of trace amounts of contaminating proteases in the DNase I sample. Note that the buffer required for the DNaseI enzyme also had no effect on the interaction between the two GR monomers (Fig. 5A, lane 3). In all instances, no binding to the control precipitates was seen (Fig. 5A, lanes 5 to 7).

When binding was quantified and expressed as fold binding above background, i.e. binding to immunopurified myc GR divided by binding to control immunoprecipitates, there again appeared to be a decrease in the presence of DNaseI (Fig. 5C, left panel, compare bars 1 and 3). However, replotted the data corrected for differences in the amounts of myc GR used (Fig. 5B, obtained by phosphoimage analysis) shows that there was no difference in the amount of binding seen in the presence or absence of DNaseI (Fig. 5C, right panel, bars 4 and 6). Again, incubation with DNase I buffer was not detrimental to binding (Fig. 5C, lanes 2 and 5). In fact, it appeared that the pre-incubation with DNaseI buffer alone may have enhanced the binding (Fig. 5C, lane 5). Thus, the interaction between the two GR molecules was not dependent on the presence of DNA. Therefore, I have shown a specific interaction
between two GR molecules in solution that is DNA independent. These results are all consistent with homodimerization of rat GRs in solution.

4. **Solution dimerization of GR is ligand independent**

The solution dimerization interface of PR (42, 268) and ER (134) maps to their LBDs. The structural similarity among steroid receptors raised the possibility that the GR LBD may have a similar activity. Ligand binding by GR is extremely sensitive to C-terminal truncations. In fact a deletion of only 14 C-terminal amino acids is sufficient to severely compromise ligand binding (278, 308). Further deletion into the LBD results in mutant GRs that associate with hsp but are unable to bind ligand (278). In the event that the solution dimerization interface mapped to the LBD of rat GR, it was necessary to establish conditions under which dimerization of C-terminal deletion mutants could be monitored in the absence of ligand binding.

An alternative to ligand treatment to cause hsp dissociation *in vitro* is salt treatment (73, 74, 309). In order to determine whether solution dimerization by GR had a strict ligand requirement, the dimerization of two ligand treated GRs was compared to dimerization between two GRs whose hsp had been dissociated by salt treatment. In the latter case, the cellular extract from which the myc GR was immunoprecipitated was treated with salt prior to the addition of the antibody. Likewise salt was added to the labeled GR after *in vitro* translation. Under my experimental conditions, when similar amounts of salt (Fig. 6A, lane 4) or ligand (Fig. 6A, lane 1) treated labeled GR were incubated with similarly treated myc GR, the salt treated receptors dimerized as
Figure 6. Solution dimerization does not have a strict ligand requirement.

A. $^{35}$S-labeled in vitro translated GRs treated with either dex (lanes 1 to 3) or salt (0.4M NaCl, lanes 4 to 6) were incubated with immunoprecipitated myc GR treated with either dex (lane 3) or salt (lane 6) and control precipitates similarly treated with either dex (lane 2) or salt (lane 5) as described in the methods. Bound proteins were resolved by SDS-PAGE and phosphorimaging. Binding is compared to 10% of the input (lanes 1 and 4).

B. The results of three repetitions of the experiment shown in A were quantified and expressed graphically. Error bars represent the standard deviation of the three repetitions.

C. Immunoprecipitated myc GR prepared after dex and salt treatment was analyzed by western immunoblotting.

D. Liganded myc GR was incubated with salt-treated $^{35}$S-labeled GR (lane 1) while dex-treated $^{35}$S-labeled GR was incubated with salt-transformed immunoprecipitated myc GR (lane 2). Binding was quantified by phosphorimaging and expressed graphically as described previously.
**A**

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**B**

![Bar graph showing fold binding for Dex and Salt](image)

**C**

**Western Immunoblot**

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<td>^{35}S-GR + salt</td>
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efficiently as liganded receptors (Fig. 6A, compare lanes 3 and 6) and (Fig. 6B). Western immunoblotting (Fig. 6C) showed that the amount of myc GR used in both assays was the same. In both cases there was no significant binding to the control immunoprecipitates (Fig. 6A, lanes 2 and 5).

Similar results were obtained in mixing experiments in which one partner was liganded while the other was salt treated (Fig. 6D). In these experiments, the amount of binding seen was the same irrespective of which partner was ligand or salt treated. Thus the differences in the conformation of the C-terminus of GR that may result from ligand or salt treatment do not appear to markedly affect GR solution dimerization.

Immunoprecipitation assays were then done in the presence of the GR antagonist RU486. RU486-bound GRs dissociate from heat shock proteins but activate transcription poorly. The poor transcriptional activity of RU486-bound GRs is ascribed to an altered GR LBD conformation (310, 311). To see whether RU486 treatment would affect the ability of rat GR to dimerize in vitro, I performed a co-immunoprecipitation where transformation of both the myc GR and the in vitro translated GR was accomplished by RU 486 treatment (Fig. 7). Binding was similar regardless of the presence of ligand or antagonist (Fig. 7, lane 2 and 3). A comparison of the amount of labeled protein retained by the myc GRs (Fig. 7, lanes 2 and 3) to a sample representing ten percent of the total amount of labeled protein added to both assays (Fig. 7, lane 1) showed that in both cases approximately ten percent of the labeled GR was retained by the myc GR (Fig. 7, compare lanes 1 with 2 and 3). The lower molecular weight band seen in lanes 1 to 3
Figure 7. **RU486 treatment does not affect solution dimerization of rat GR.**

$^{35}$S-labeled GRs treated with either dexamethasone (lane 2 and 4) or RU486 (lane 3 and 5) were incubated with immunopurified myc GR (lanes 2 and 3) or control precipitates (lanes 4 and 5) which had been pretreated with either dexamethasone (lanes 2 and 4) or RU486 (lanes 3 and 5). Bound proteins were resolved by SDS-PAGE. Ten percent of the *in vitro* translated GR added to the assay is shown in lane 1.
<table>
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</tbody>
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- kDa
  - 97
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1 2 3 4 5
most likely represents GR degradation products or late translation starts as it was specifically retained by myc GR (Fig. 7, lanes 2 and 3). Again, there was no binding to the control precipitates under either conditions (Fig. 7, lanes 5 and 6). Therefore, the conformational changes induced by ligand within the LBD of GR are not a requirement for solution dimerization although as shown above dissociation of the hsp complex appears to be a prerequisite.

5. Mapping of the solution dimerization interface in rat GR

To begin a preliminary mapping of the regions of the rat GR mediating solution dimerization, selected GR deletion mutants were produced in vitro in the presence of $^{35}$S and tested for their ability to dimerize with full length, immuropurified myc GR. The deletion mutants used are summarized in Figure 8A. As before, full length GRs dimerized with each other (Fig. 8B, lane 6). Removal of the amino terminus up to amino acid 407 (Fig. 8B, lane 9) did not appear to significantly affect the ability of the receptor to dimerize. This indicated that under these experimental conditions, the N-terminus of GR was not required for its solution dimerization activity. Surprisingly, given the location of the solution dimerization domain in other steroid receptors, deletion of the C-terminus to amino acids 556 (Fig. 8B, lane 7) also failed to significantly alter solution dimerization by GR, implying tha the N-terminus and the LBD, or at least the most C-terminal part of the LDB, were not required for this activity. These conclusions were validated by demonstrations that a GR deletion mutant encompassing the LBD alone, 547C (Fig. 8B, lane 10) failed to bind, while the X568 deletion mutant, which lacks most
Figure 8. Amino acids 407 to 568 of rat GR are sufficient for mediating solution dimerization in a co-immunoprecipitation assay.

A. At the top is a schematic diagram of rat GR with the DNA binding and ligand binding domains highlighted. The position of the amino acid that marks the beginning and the end of each domain is shown above the diagram. Below is a summary of the receptor fragments used in B. B. Anti-myc antibody immunoprecipitates of dex treated cell extracts from Sf7 cells with (lanes 6 to 10) or without (lanes 11-15) stably expressed myc GR were incubated with the indicated $^{35}$S-labeled GR receptor derivatives treated with either dex (WT, X795, 547C) or salt (X568). The bound proteins were resolved by SDS-PAGE and phosphorimaging. Ten percent of the labeled proteins added to the assay is shown in lanes 1 to 5.
of both the N-terminus and the LBD, bound strongly to myc GR (Fig. 8B, lane 8). These finding are in marked contrast to PR and ER where the LBD of these receptors have been shown to either mediate solution dimerization as is the case for ER (134) or at least contribute to the solution dimerization activity such as with PR (268).

The results presented above also suggest that amino acids 407 to 568 were sufficient for mediating GR solution dimerization. However, taken together with the result that there was binding of N556 to myc GR and no binding by the 547C mutant, it appeared that amino acids between 547 and 568 were dispensable for this activity. In an attempt to do a finer mapping of the C-terminal boundary of the dimerization interface of GR, I made two more C-terminal deletions, N523 and N494 (Fig. 9A). Deletion of amino acids 524 to 556 consistently reduced but did not eliminate GR dimerization (Fig. 9B, lane 7). However, further deletion to amino acids 494 completely abolished solution dimerization (Fig. 9B, lane 8). Thus, it appears that at the C-terminus, sequences between amino acids 494 and 523 of rat GR are necessary for solution dimerization while sequences between 523 and 547 (combining the results of Fig. 8 and 9) are required for optimal dimerization. Therefore amino acids 407 to 547 appeared to be involved in GR solution dimerization.

6. Binding of in vitro translated GR to recombinant GR peptides expressed in bacteria

GST Pull-Down Assay

Another method used extensively to identify protein-protein interactions in vitro is the GST-pull down assay (61, 156, 301, 312-316). In this system, the protein of interest is
Figure 9. Mapping of the C-terminal boundary of the solution dimerization interface in rat GR.

A. Schema of rat GR and the GR deletion mutants used in B. B. Liganded $^{35}$S-labeled GR (lanes 5 and 9) or the C-terminally truncated mutants N556 (lanes 6 and 10), N523 (lanes 7 and 11) and N494 (lanes 8 and 12) were incubated with dex treated immunopurified myc GR (lanes 5 to 8) or control precipitates (lanes 9 to 12) as described previously. Bound proteins were visualized by SDS-PAGE followed by fluorography. Ten percent of the input proteins is shown in lanes 1 to 4.
fused to GST. Generally, GST-fusion proteins, usually expressed in bacteria, are soluble and are easily purified from cell lysates under non-denaturing conditions as the GST component allows the fusion protein to bind an appropriate affinity resin such as glutathione sepharose with high affinity (277). The glutathione sepharose beads with the adsorbed fusion protein are in turn used as an affinity matrix for the target protein. In my case the GST system offered the opportunity to do two things: first, it allowed me to confirm the results of the co-immunoprecipitation assay. In the GST system, fragments of GR are fixed to a matrix while the full-length receptor is the target protein, essentially the reverse of the co-immunoprecipitation assay. Second, it allowed for the examination of potential interactions between the different receptor domains. The N- and C-terminal portions of AR for example have been shown to interact (274, 275). This system permitted me to test for similar activities in GR.

Different regions of the rat GR were expressed as GST fusion proteins (Fig. 10A). The GST-GR_{22-437} is a fusion protein of amino acids 22-437 of rat GR (the amino terminus) with an N-terminal GST tag. Similarly, GST-GR_{X568}, GST-GR_{305C}, GST-GR_{542C} encode amino acids 407 to 568, 505 to 795 and 542 to 795 of rat GR respectively fused to the C-terminus of GST (Fig. 10A). The GST-GR_{305C} construct was made to begin to map the N-terminal boundary. Previous results (Fig. 9B) showed sequences between amino acids 494 and 523 to be critical for GR solution dimerization. Amino acids 494 to 523 encompass the end of the DNA binding domain and the beginning of the hinge region (Fig. 2). It was unlikely that the DBD portion was involved as it has been shown to dimerize only on DNA even at high protein concentrations (146). Further the hinge region of PR was shown to be required for its solution dimerization activity (268).
Figure 10. Amino acids 505 to 568 of rat GR mediate solution dimerization in a GST pull-down assay.

A. Schematic representation of rat GR (top) and the GST-GR fusion proteins (below).
B. The GST-GR fusion proteins shown in A (B, lanes 2 to 5) or GST alone (B, lane 1) were expressed in and purified from E. coli as described in the Methods. A sample of each fusion protein was eluted from the glutathione sepharose by boiling in SDS-sample buffer. The eluted proteins were resolved by SDS-PAGE and visualized by Coomassie blue staining as shown in B or subjected to western immunoblot analysis with antibodies against the LBD (α-LBD) or the amino terminus (amino acids 404-423, BuGR2) regions of GR (C). D. in vitro translated, 35S-labeled, dexamethasone-treated full-length GR was incubated with either GST alone (lane 2) or the indicated GST-GR fusion proteins (lanes 3 to 6) as described in the Methods. Bound proteins were resolved by SDS-PAGE and visualized by fluorography. Ten percent of the input is shown in lane 1.
Therefore, I extended the 542C construct to amino acid 505 to include the hinge region while excluding DBD sequences.

Figure 10B shows an SDS-PAGE of the fusion proteins stained with Coomassie blue. As is often seen with GST-fusion proteins, there are several bands in each of the lanes due to either protein degradation during the purification of the fusion proteins or to bacterial proteins that bound non-specifically to the glutathione sepharose. However, a western immunoblot with antibodies specific for the various regions of GR show specific bands at the predicted molecular weight for each of the fusion proteins (Fig. 10C).

I therefore used the fusion proteins in a binding assay with *in vitro* translated $^{35}$S-labeled liganded full length GR. Full length GR interacted strongly with the GST-GR$_{558}$ (Fig. 10D, lane 4) and GST-GR$_{505}$ (Fig. 10D, lane 5). These results imply that amino acids between 505 and 568 (the hinge) were required for solution dimerization of rat GR in a GST pull down assay. Moreover, the GR DBD was dispensable for this activity as GST-GR$_{505}$, which lacked a DBD, was able to efficiently retain full length GR. In further corroboration of the co-immunoprecipitation assay results (Fig. 8B and Fig. 9B), no significant binding was seen with the LBD alone (GST-GR$_{542}$, Fig. 10D, lane 6) while a small amount of the labeled GR bound to the GST-GR$_{22-437}$ fusion protein (Fig. 10D, lanes 3). Binding was specific as the labeled GR was not retained by GST alone (Fig. 10D, lane 2). When compared to a sample representing ten percent of the total amount of label added to the assay (Fig. 10D, lane 1), the amount of labeled GR bound in this assay (Fig. 10D, lanes 5 and 6) was greater than the ten percent generally seen in the co-immunoprecipitation assay. In fact, almost 80% of the input label was retained by the GST-GR fusion proteins. This is considerably stronger than the binding seen with GR in
other GST based assays (156, 301). The difference in the amount of binding seen with the GST and co-immunoprecipitation assays can be attributed to the fact that, relative to the amount of myc GR used in a co-immunoprecipitation assay, the amount of GST-GR fusion proteins used in this assay is approximately 50 times more (Fig. 11). Also the amount of binding seen with the GST fusion proteins varied with different protein preparations. Nevertheless, in both a co-immunoprecipitation assay, where full length GR was immobilized to the matrix, and a GST pull down assay where the GR deletion mutants were tethered to the matrix, solution dimerization between two GR monomers or a GR monomer and a GR deletion mutant is observed. The combined results of the two assays would map the dimerization interface between amino acids 505 and 547, the hinge region of GR.

7. Sequences between amino acids 509 to 631 are necessary for solution dimerization of rat GR

To verify the GST-GR<sub>505C</sub> and to confirm that sequences in the hinge region were necessary for GR solution dimerization, I performed a co-immunoprecipitation assay with two additional GR deletion mutants, 505C and XΔ509-631 that was available in the lab. XΔ509-631 contains amino acids 407 to 795 of GR with an internal deletion of amino acids 509 to 631, most of the hinge and the N-terminal part of the LBD (Fig. 12A). WT GR and the X795 deletion mutant bound to myc GR (Fig. 12B, lanes 6 and 7) while the 547C mutant did not (Fig. 12B, lane 9) in agreement with previous results (see Fig. 8). In contrast to 547C, the 505C deletion mutant dimerized with myc GR (Fig. 12B,
Figure 11. Western immunoblot analysis of immunoprecipitated myc GR and GST-GR50SC.

Samples of immunoprecipitated myc GR (lane 1) and GST-GR50SC (lane 2) equal those used in typical co-immunoprecipitation and GST pull down assays were resolved by SDS-PAGE and subjected to immunoblot analysis with an GR LBD antibody. The lower band in lane 1 that migrates to a similar position as GST-GR50SC represents the heavy chain of the 9E10 antibody used to immunoprecipitate myc GR.
validating the results of the GST pull down assay and supporting the idea that hinge sequences were required for GR solution dimerization. Further support came from the finding that the X\Delta 509-631 bound only weakly to myc GR (Fig. 12B, lane 10). This confirmed that sequences in the hinge region were necessary for GR solution dimerization. Moreover, the results from this experiment clearly proved that the GR DBD is dispensable for GR solution dimerization while the hinge was required for the following reasons: first 505C which lacked the DBD dimerized efficiently with myc GR while the X\Delta 509-631 which had a intact DBD did not. Second, simply extending 547C to amino acid 505 (to include the hinge) was sufficient to restore dimerization activity to this mutant. Third, deletion of amino acids 509 to 631 in the context of X795 severely compromised solution dimerization.

8. Direct interaction between GR monomers

To examine whether the observed interaction between GR monomers was direct as had been shown for other steroid receptors or if a third bridging protein was involved, a binding assay was done with purified components that had been expressed in bacteria. Amino acids 407 to 550 with a protein kinase A (PKA) recognition sequence at the carboxy terminus was expressed as a GST fusion protein, labeled with \(^{32}\)P using PKA then separated from the GST moiety by thrombin cleavage. The labeled, purified GR peptide was then tested for binding to GR fragments expressed as GST fusion proteins (Fig. 13A) immobilized on glutathione sepharose. The labeled peptide bound strongly to
Figure 13. Direct binding of GR fragments.

A. Schematic representation of the GST-GR fusion proteins used in B. B. GST-GR_{X550} with a protein kinase A recognition site expressed in and purified from bacteria was labeled ^{32}P using PKA. The labeled peptide was cleaved from the GST moiety with thrombin and incubated with either GST alone (lane 2) of the GST-GR fusion proteins (lanes 3-6) shown in A. Ten percent of the ^{32}P-labeled GR peptide added to the binding reaction is shown in lane 1.
GST-GR\textsubscript{X550} (Fig. 13B, lane 4) and GST-GR\textsubscript{505-550} (Fig. 13B, lane 5). Binding to GST-GR\textsubscript{22-427} (Fig. 13B, lane 3) and GST-GR\textsubscript{542C} (Fig. 13B, lane 6) was comparable to that seen with GST alone (Fig. 13B, lane 2). These results show amino acids 505 to 550 of rat GR, shown above as being necessary for solution dimerization, was sufficient to mediate a direct interaction between purified GR fragments.

9. Physical interaction between the amino- and carboxy-terminal domains of GR

Molecular interactions between the N- and C-terminus of AR have been demonstrated (274, 275). Further, recent evidence suggests that an amino-terminal-carboxy-terminal interaction contributes to PR solution dimerization (268). To see whether GR had a similar activity, I used GST-GR\textsubscript{32-437}, (the amino terminus), GST-GR\textsubscript{X568} (the DBD) and GST-GR\textsubscript{542C} (the LBD) in GST pull down assays with the GR deletion mutants shown in Figure 14A.

GST-GR\textsubscript{22-437} was observed to efficiently interact with a GR fragment containing the LBD alone, 547C (Fig. 14B, lane 7). Extending the LBD to include the hinge region and the DBD (X795) inhibited its interaction with the amino terminus (Fig. 14B, lane 8). The X568 deletion mutant failed to bind to the amino terminus (Fig. 14B, lane 6) while N556, which lacks most of the LBD, bound to GST-GR\textsubscript{22-437} (Fig. 14B, lane 5) but with less efficiency than the LBD alone (Fig. 14B, lane 7). These findings provide some evidence for an interaction between the amino and carboxy terminus of GR.
Figure 14. Interaction between the amino and carboxy terminals of GR.

A. Schematic representation of GR deletion mutants used in B. B. \textit{in vitro} translated $^{35}$S-labeled GR deletion mutants were incubated with either GST-GR$_{22-437}$ (lanes 5 to 8), GST-GR$_{X568}$ (lanes 9 to 12), GST-GR$_{542C}$ (lanes 13 to 16) or GST alone (lanes 17 to 20) as described in the methods. Bound proteins were resolved by SDS-PAGE and visualized by fluorography. Ten percent of the input is shown in lanes 1 to 4.
In contrast to the results obtained with GST-GR_{22-437}, when GST-GR_{X568} was used as the affinity matrix (Fig. 14B, lanes 9 to 12), all the receptor fragments tested bound with the X568 fragment having the strongest interaction (Fig. 14B, lane 10), and the 547C mutant having the weakest (Fig. 14B lane 11). These data are consistent with results shown above demonstrating that determinants for solution dimerization of rat GR reside in amino acids 505 to 547.

Finally, when the GST-GR_{542C} fusion protein was tested for its ability to bind the GR deletion mutants, there was an interaction between it and the 547C protein fragment (Fig. 14B, lane 15). This is in contrast to previous results, which showed the LBD to be dispensable for GR solution dimerization (see Figs. 8 and 10). Further when the LBD was extended to include the hinge region and the DBD (X795), the efficiency of the interaction decreased (Fig. 14B, lane 16) similar to what was seen with GST-GR_{22-437} (Fig. 14B, lanes 7 and 8). N556 bound with a similar efficiency as X795 (Fig. 14B, lanes 13 and 16) while the X568 mutant failed to bind GST-GR_{542C} (Fig. 14B, lane 7).

These interactions were all specific as none of the mutant GRs bound to GST alone (Fig. 13B, lanes 17 to 20).

10. Solution Dimerization of rat GR in vivo

YEAST TWO HYBRID ASSAY

I have shown by two in vitro assays a solution dimerization activity in rat GR. I next needed to demonstrate solution dimerization by GR in vivo and to show that the sequences identified as being important for this activity by co-immunoprecipitation and GST-pull down assays were also required in vivo. To achieve these goals I made use of
the yeast two-hybrid system. The two-hybrid system, widely used in studying protein-protein interactions in vivo, uses transcriptional activity as a measure of protein-protein interaction (317-319). It is based on the observation that many transcription factors including the yeast GAL 4 protein are composed of physically separable and functionally independent domains responsible for DNA binding (DBD) and transcriptional activation (TA) (320-322). In the case of a yeast GAL 4 transcription factor, the DBD binds specific enhancer like sequences called upstream activator sites (UAS). The TA domain directs transcription of the gene downstream of the UAS. Both the DBD and the TA are required to activate transcription normally, however, they do not need to be covalently linked. When physically separated, the DBD and TA peptides do not activate transcription because they do not interact with each other. However, if they can be brought into close proximity with each other in the promoter region of target genes, via the interaction of any two proteins, transcriptional activation function can be restored.

The application of this system in yeast requires that fusion proteins of the GAL DBD and GAL TA domains with proteins that potentially interact with each other be constructed. The GAL DBD fused to protein X for example (Fig. 15) is able to bind in a sequence specific fashion to DNA but since it lacks a TA, it is unable to activate transcription. In contrast, the GAL TA fused to protein Y has the ability to activate transcription but cannot bind DNA. However, when the two fusion proteins are co-expressed in yeast with a reporter gene driven by UAS for the GAL 4 protein, if the X and Y components of the fusion proteins interact they reconstitute a functional activator by bringing the TA domain into close proximity of the DBD. This factor activates the reporter gene driven by the GAL 4 responsive elements in the promoter and the
Figure 15. Schematic representation of the yeast two-hybrid assay.

A general description of a two hybrid assay for studying protein-protein interactions in yeast. Gal DBD X represents a fusion of the DNA binding domain of the GAL4 transcription factor fused to protein X. Similarly, Gal TA Y is representative of the transactivation domain of the GAL4 fused to protein Y. Gal UAS (upstream activating sequences) symbolize specific binding sites for the GAL4 DNA binding domain in the promoter region of the reporter gene (lacZ).
• The DBD fusion protein binds (Gal DBD X) to the GAL UAS but is unable to activate transcription because it lacks an activation domain.

• In the absence of a DNA binding domain, the activation domain fusion protein (Gal TA Y) cannot bind the GAL UAS and is therefore unable to activate transcription.

• However, when both fusion proteins are present, interaction between X and Y in vivo results in activation of the reporter gene.
interaction is detected as expression of the reporter gene. If the two proteins do not interact with each other, the reported gene will not be transcribed. Note that in this assay yeast strains are used such that the native GAL 4 proteins do not bind to and activate the reporter gene.

11. Expression of GR fusion proteins in yeast

To test for solution dimerization of rat GR in a two hybrid assay in yeast, I made use of the GAL DBD and GAL TA fusion proteins schematically illustrated in Figure 16A. The GAL TA-GR<sub>X556</sub> construct was kindly provided by Rhian Walther (University of Ottawa). I first checked to ensure that the fusion proteins were being expressed. To do this the DBD and TA fusion protein constructs were separately transformed into yeast. Western immunoblots of soluble protein extracts with antibodies that specifically recognize the GR portion of the fusion protein are shown in Figure 16B. All the fusion except those encoding amino acids 505 to 540 of GR (Fig. 16B, lane 7) proteins were expressed. The next step was to verify that the individual hybrids alone do not activate transcription of the reporter gene. To do this, each construct was independently transformed into Y190 yeast strain. Y190 has a lacZ reporter driven by GAL 4 responsive elements stably integrated into its genome (299). I assayed for the activation of the of lacZ by doing a β-galactosidase (β-gal) assay on liquid yeast cultures which had been grown in the presence and absence of hormone. The rat GR LBD is known to associate in yeast with the yeast hsp51 into complexes similar to those that occur in mammalian cells (323, 324). These complexes are responsive to the synthetic
Figure 16. GAL DBD and GAL TA fusion proteins expressed in yeast.

A. Schematic representation of rat GR and the GAL DBD- and GAL TA-GR fusion proteins used in B. B. Extracts prepared from yeast transformed with the plasmids for the various fusion proteins shown in A were analyzed by western immunoblotting with either the anti-GR LBD antibody (lanes 1 to 4), the anti-GR antibody BuGR2 (lane 5) or an anti-GAL DBD (lanes 6 and 7).
glucocorticoid hormone deacetylcortivosol, DAC (324). Table 2 shows that no significant autonomous activation of the lacZ gene was seen with any of the fusion protein constructs either in the presence or absence of hormone. The small amount of activity seen with the GAL DBD-GR$_{505C}$ in response to hormone has been reported previously (324). However, this activity was sufficiently small that it was not expected to hinder measurement of GR-GR interactions.

12. Dimerization of rat GR in a Yeast Two Hybrid Assay

To test the hybrid proteins for interaction, I co-transformed yeast with the two hybrid proteins (DBD fusion protein and the TA fusion protein) and assayed the transformants for activation of the lacZ gene before and after hormone treatment. No hormone independent activity was seen with any of the co-transformants (Fig. 17). However, hormone led to an increase in β-gal activity in yeast co-transformed either with the two 505C fusion protein constructs (Fig. 17) or the GAL DBD-GR$_{505C}$ and the GAL TA-GR$_{X556}$ (Fig. 17). Co-transformation of GAL DBD-GR$_{X556}$ with its TA counterpart or the GAL TA-GR$_{505C}$ was not informative as the GAL DBD-GR$_{X556}$ was toxic to the yeast. No β-gal activity was detected with the two 540C constructs even in the presence of hormone, or when the empty vectors were co-transformed. The absence of β-gal activity in the 540C co-transformants was not due to a lack of expression of the fusion protein as western immunoblots show both proteins to be expressed (Fig. 16B). These data show a ligand dependent dimerization activity in rat GR in vivo and determinants for solution dimerization of GR in vivo reside between amino acids 505 to 540.
Table 2. GAL DBD-GR and GAL TA-GR fusion proteins expressed individually in yeast do not activate transcription of the lacZ reporter.

Plasmids for the fusion proteins shown in Fig. 16A were individually introduced into yeast. Liquid yeast cultures grown in the presence or absence of hormone were analyzed for β-galactosidase activity. β-galactosidase activity represent data from at least 5 independent transformations assayed in duplicate. Values shown are the mean ± S.E.M.
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</table>
Figure 17. Amino acids 505 to 540 are necessary for solution dimerization of rat GR in vivo.

Yeast were co-transformed with the indicated fusion protein plasmids or the empty vectors alone. Liquid cultures grown in the presence and absence of hormone were analyzed for β-galactosidase activity. Values represent the average of three independent transformations assayed in duplicate. Error bars are the S.E.M.
\[ \beta\text{-galactosidase Activity (Arbitrary Units)} \]

<table>
<thead>
<tr>
<th>FUSION PROTEINS</th>
<th>GAL DBD</th>
<th>( GR_{540C} )</th>
<th>( GR_{505C} )</th>
<th>( GR_{505C} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAL TA</td>
<td>-</td>
<td>( GR_{540C} )</td>
<td>( GR_{505C} )</td>
<td>( GR_{x556} )</td>
</tr>
</tbody>
</table>
To demonstrate the sufficiency of amino acids 505 to 540 for mediating solution dimerization of rat GR \textit{in vivo}, I attempted to express these amino acids as GAL DBD and GAL TA fusion proteins in yeast. However, for unknown reasons I was unable to detect expression of these proteins by western immunoblotting (Fig. 16B, lane 7) and therefore could not determine whether these amino acids were sufficient in this system.

In summary, I have shown by three independent assays solution dimerization of rat GR. In addition, I have mapped by all three assays this activity to a sub-domain of the receptor that included the hinge region (Table 3). While the exact C-terminal boundary of the dimerization interface was not precisely determined in these assays, note that all the results are consistent with the solution dimerization domain being localized to the same region of the receptor.

13. \textbf{Dimerization of full length GR monomers \textit{in vivo}}

I have demonstrated by three assays (two \textit{in vitro} and one \textit{in vivo}), solution dimerization by rat GR and I have mapped the activity to amino acids 505 to 540. The results of the yeast two hybrid assay demonstrate a ligand dependent interaction between two GR deletion mutants \textit{in vivo}. My next objective was to test for interaction between two full length receptors in the mammalian cell. The observation of GR dimers and multimers at the nuclear pore of nuclear envelopes isolated from target tissue treated with hormone suggested that GR monomers may interact during nuclear import. In the absence of hormone, GR is localized primarily to the cytoplasm of target cells. Hormone treatment provokes a rapid ($t_{1/2}$ 4-5 min) and complete transfer of the protein to the
Table 3. Summary of the results of the co-immunoprecipitation, GST pull-down and yeast two-hybrid assays.
**Table 3.** Summary of the results of experiments designed to map the solution dimerization interface in rat GR

<table>
<thead>
<tr>
<th>ASSAY USED</th>
<th>AMINO ACIDS IN RAT GR SHOWN TO BE REQUIRED FOR SOLUTION DIMERIZATION</th>
<th>REFERENCE FIGURE</th>
</tr>
</thead>
<tbody>
<tr>
<td>CO-I MMUNOPRECIPITATION ASSAY</td>
<td>505 TO 523</td>
<td>Figures 9, 10 and 13</td>
</tr>
<tr>
<td>GST PULL-DOWN ASSAY</td>
<td>505 TO 568</td>
<td>Figure 11</td>
</tr>
<tr>
<td>YEAST TWO-HYBRID ASSAY</td>
<td>505 TO 540</td>
<td>Figure 17</td>
</tr>
</tbody>
</table>
nucleus. I took advantage of the fact that GRs' nuclear import can be regulated by hormone to study GR homodimerization in vivo.

Previously, dimerization of PR in vivo was demonstrated by co-transfection experiments (42). A progesterone receptor with a mutation in its NLS was found to localize to the cytoplasm even in the presence of hormone. However, co-transfection of wild type PR with the mutant receptor caused the mutant PR to shift to the nucleus in response to hormone as a result of dimerization between the two receptors (42, 325). I was interested in determining whether co-transfection of GR_{WT} with GR_{NLI-} could, through dimerization, alter the subcellular distribution of GR_{NLI-} in response to hormone.

Nuclear accumulation of GR is due to two NLSs, a classical, basic sequence located in the hinge region, NL1, and an as yet undefined sequence in the LBD, NL2 (Fig. 2) (60). Using a semi-quantitative indirect immunofluorescence assay to monitor the subcellular localization of GR, we have previously shown that mutating three amino acids in the NL1 sequence (Fig. 18A) dramatically impairs its nuclear localization (61). Whereas hormone treatment of wild type GR (GR_{WT}) prompts the rapid nuclear import of GR_{WT}, GR with a mutation in NL1 (GR_{NLI-}) is imported into the nucleus more slowly with a $t_{1/2} > 30$ min. Furthermore, GR_{NLI-} never completely localizes to the nucleus even after prolonged hormone treatment. The ability to efficiently transfer to the nucleus is the only property affected by this mutation as it is fully competent to bind hormone, DNA and activate transcription (61).

First, I performed a co-immunoprecipitation assay to see whether GR_{WT} and GR_{NLI-} can dimerize in vitro. I in vitro translated GR_{NLI-} in the presence of $^{35}$S and
Figure 18. Co-expression of GR\textsubscript{WT} and myc GR\textsubscript{NL1}- in COS7 cells.

A. (left) Schematic representation of GR\textsubscript{WT} and myc GR\textsubscript{NL1}- showing the position and sequence of the amino acids mutated in GR\textsubscript{WT} to generate GR\textsubscript{NL1}-. (right) A summary of the properties of the two receptor types. B. Whole cell extracts prepared from COS7 cells transiently transfected with GR\textsubscript{WT} (lane 1), myc GR\textsubscript{NL1}- alone (lane 2) or GR\textsubscript{WT} and myc GR\textsubscript{NL1}- (lane 3) were subjected to western immunoblotting with the anti-GR antibody BuGR2. Abbreviations used: NL, nuclear localization; DNA, DNA binding; TA, transcriptional activation.
A

1 440 505 547 795
Ligand

GRWT

RKTKKKIK517

22 440 505 547 795
Ligand

mycGRNL1−

RKTNMK517

B

Western Immunoblot

kDa

97− 66−

1 2 3

← mycGRNL1−

← GRWT
assessed its ability to dimerize with myc GR<sub>WT</sub> in a co-immunoprecipitation assay. Figure 19 shows that mutation in the NL1 sequence of GR<sub>NL1</sub>- did not prevent this receptor from interacting with myc GR (Fig. 19, lane 2). No significant binding was seen with the control precipitates (Fig. 19, lane 3). Thus GR<sub>NL1</sub>- was able to dimerize with GR<sub>WT</sub> <i>in vitro</i>.

To see whether GR<sub>WT</sub> could alter the subcellular localization GR<sub>NL1</sub>- when they were co-expressed in cells, I constructed a GR<sub>NL1</sub>- with an N-terminal myc tag (Fig. 18A). The myc tag would allow for the differential monitoring of the subcellular localization of GR<sub>NL1</sub>- in the presence of GR<sub>WT</sub>. Figure 18B is a western immunoblot probed with the anti-GR antibody BuGR2 showing the expression levels of GR<sub>WT</sub> and mycGR<sub>NL1</sub>- when they are introduced either separately or together in COS7 cells. It was desirable to have an excess of GR<sub>WT</sub> relative to GR<sub>NL1</sub>- when the two were co-expressed to shift the ratio of homodimers of GR<sub>NL1</sub>- to heterodimers of GR<sub>WT</sub>:GR<sub>NL1</sub>- toward the formation of heterodimers. In my case there was approximately four times as much GR<sub>WT</sub> as GR<sub>NL1</sub>- (Fig. 18B, lane 3). This ratio is comparable to what has been previously reported for studies done with PR in a similar assay (325).

The subcellular localization of GR<sub>WT</sub> and GR<sub>NL1</sub>- was monitored after transfection of the various GR expression plasmids into COS7 cells. The anti-GR antibody BuGR2 was used to detect GR<sub>WT</sub> while the anti-myc antibody 9E10 detected GR<sub>NL1</sub>- either when it was expressed alone or together with GR<sub>WT</sub>. Before hormone treatment, more than 95% of the GR<sub>WT</sub> expressing cells contained cytoplasmic GR (Fig. 20, lane 1). Similarly, GR<sub>NL1</sub>- was found in the cytoplasm of 100% of the of the cells whether it was expressed alone or together with GR<sub>WT</sub> (Fig. 20, lanes 2 and 3).
Figure 19. Interaction between GRWT and GRNL1- in vitro.

Liganded, immunoprecipitated myc GR (lane 2) or control immunoprecipitates (lane 3) were incubated with in vitro translated dex-treated 35S-labeled GRNL1-. Bound proteins were resolved by SDS-PAGE and visualized by fluorography. Ten percent of the input is shown in lane 1.
<table>
<thead>
<tr>
<th>Sample</th>
<th>1</th>
<th>2</th>
<th>3</th>
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<tbody>
<tr>
<td>myc GR&lt;sub&gt;WT&lt;/sub&gt;</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>&lt;sup&gt;35&lt;/sup&gt;S-GR&lt;sub&gt;N1&lt;/sub&gt; &lt;sup&gt;-&lt;/sup&gt;</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>10% input</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
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</table>

kDa
97<sup>-</sup>
66<sup>-</sup>

1 2 3
Figure 20. myc GR<sub>NL1</sub>- localizes to the cytoplasm of transiently transfected COS7 cells in the absence of hormone when co-expressed with GR<sub>WT</sub>.

COS7 cells were transiently transfected with plasmids for GR<sub>WT</sub> alone (lane 1), myc GR<sub>NL1</sub>- alone (lane 2) or GR<sub>WT</sub> together with myc GR<sub>NL1</sub>- (lane 3). Subcellular localization of GR<sub>WT</sub> (lane 1) or myc GR<sub>NL1</sub>- (lanes 2 and 3) was evaluated by IIF. Detection of GR<sub>WT</sub> was with BuGR2 antibody (lane 1) while myc GR<sub>NL1</sub>- was detected with the anti-myc antibody, 9E10 (lanes 2 and 3).
Cytosolic Immunofluorescence (% fluorescent cells)

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<th>1</th>
<th>2</th>
<th>3</th>
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</thead>
<tbody>
<tr>
<td>GR&lt;sub&gt;WT&lt;/sub&gt;</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>myc GR&lt;sub&gt;NL1&lt;/sub&gt;</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Antibody α-GR α-myc
To evaluate the effect of co-expressing GR\textsubscript{WT} with GR\textsubscript{NL1-} on the subcellular distribution of GR\textsubscript{NL1-} following hormone treatment, COS7 cells transiently transfected with the appropriate expression plasmids were treated with dex. The results are summarized in Table 4. One hour after the addition of dex, cells expressing GR\textsubscript{WT} had nuclear or mainly nuclear fluorescence in 100% of the cells (Table 4). In contrast, GR\textsubscript{NL1-} expressing cells only 32% of the cells had mostly nuclear or completely nuclear staining after 1 h in the presence of hormone. However, when the two forms of GR were co-expressed in the same cells and the subcellular localization of GR\textsubscript{NL1-} monitored after a 1 h dex treatment, GR\textsubscript{NL1-} is localized to the nucleus in 74% of the cells. This is more than twice the number that is seen when GR\textsubscript{NL1-} is expressed alone. These results show that GR\textsubscript{WT} is able to change the subcellular distribution of GR\textsubscript{NL1-} in response to hormone most likely through the formation of GR\textsubscript{WT}:GR\textsubscript{NL1-} heterodimers.

14. **Heterodimerization between rat GR and other nuclear receptors**

GR and MR have been shown to heterodimerize on DNA (220, 221). A similar activity was demonstrated for GR and AR (224). However, solution dimerization between GR and other nuclear receptors has not been reported previously. I decided to test whether GR can heterodimerize with other nuclear receptors in solution. To do this I \textit{in vitro} translated the various nuclear receptors shown in Figure 21 in the presence of \textsuperscript{35}S methionine, treated with the appropriate ligand as required and then assessed their ability to interact with dex-treated myc GR. Dex-treated \textsuperscript{35}S-labeled GR was used as a positive
Table 4. Summary of the subcellular distribution of GR\textsubscript{WT} and myc GR\textsubscript{NL1-} after a 1h dex treatment.

COS7 cells were transiently transfected with plasmids for GR\textsubscript{WT} and myc GRNL1- alone or together. Dex treatment (10\textsuperscript{-6} M for 1h) was initiated 16 h after serum withdrawal and the subcellular distribution of the GRs evaluated by IIF. GR\textsubscript{WT} was detected with the BuGR2 antibody and the anti myc antibody 9E10 was used to identify myc GR\textsubscript{NL1-}.
Table 4. Subcellular distribution of GRs in response to a 1 h hormone treatment

<table>
<thead>
<tr>
<th>Constructs Transfected</th>
<th>Protein Monitored</th>
<th>% Subcellular Distribution after 1 h dex treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>N</td>
</tr>
<tr>
<td>GR&lt;sub&gt;WT&lt;/sub&gt;</td>
<td>GR&lt;sub&gt;WT&lt;/sub&gt;</td>
<td>86.8</td>
</tr>
<tr>
<td>mycGR&lt;sub&gt;NL1&lt;/sub&gt;-</td>
<td>mycGR&lt;sub&gt;NL1&lt;/sub&gt;-</td>
<td>9.6</td>
</tr>
<tr>
<td>GR&lt;sub&gt;WT&lt;/sub&gt; and mycGR&lt;sub&gt;NL1&lt;/sub&gt;-</td>
<td>mycGR&lt;sub&gt;NL1&lt;/sub&gt;-</td>
<td>20.8</td>
</tr>
</tbody>
</table>

Transfected cells were classified into one of five categories according to the distribution of the immunofluorescence: N, exclusively nuclear staining; N>C, cells with higher immunofluorescent signal from the nucleus than the cytoplasm; N=C, cells with uniform staining; N<C, cells with higher immunofluorescent signal from the cytoplasm than the nucleus; C, cells with exclusively cytoplasmic staining (39, 58, 61, 62).
Figure 21. Solution heterodimerization between GR and several members of the nuclear receptor superfamily.

\(^{35}\)S-methionine labeled GR (lane 9), PR (lane 10), AR (lane 11), ER\(\alpha\) (lane 12), RAR\(\alpha\) (lane 13), RXR\(\gamma\) (lane 14), TR (v-erbA, lane 15), and the Drosophila nuclear receptor Ftz-f1\(\beta\) (lane 16) were incubated with dex-treated immunoprecipitated myc GR (lanes 9 to 16) or control precipitates (lanes 17 to 24) as described in the Methods. Bound proteins were resolved by SDS-PAGE and fluorography. Ten percent of the input proteins are shown in lanes 1 to 8. Ligand treatments were as follows: GR-10^{-6} M dex; PR-10^{-6} R5020; AR-10^{-6} DHT and ER-10^{-6} M DES. RAR\(\alpha\), RXR\(\gamma\), TR and Ftz-f1\(\beta\) were not treated with ligand.
control (Fig. 21, lane 9). Liganded AR (Fig. 21, lane 11), ERα (Fig. 21, lane 12) and TR (Fig. 21, lane 15) bound equally well to myc GR. PR (Fig. 21, lane 10), RARα (Fig. 21, lane 13), and the Drosophila nuclear receptor Ftz-f1β (Fig. 21, lane 16) also interacted with myc GR albeit to a lesser extent than AR, ER and TR. RXRγ (Fig. 21, lane 14) was the only receptor tested that did not bind to myc GR. These interactions appeared to be specific as none of the receptors bound to the control precipitates (Fig. 21, lanes 17 to 24). These results demonstrate that GR has the potential to interact with several members of the nuclear receptor superfamily.
IV. DISCUSSION

I have demonstrated solution dimerization of the rat glucocorticoid receptor by three independent assays: co-immunoprecipitation, GST-pull down and yeast two-hybrid assays. My results provide the first detailed characterization of the solution dimerization activity in rat GR. My data show solution dimerization by rat GR requires prior dissociation of the heat shock proteins but does not have a strict ligand requirement in vitro as dimerization occurred with liganded as well as with salt and antagonist transformed GRs. Further, I have mapped a solution dimerization interface to amino acids 505 to 547 in vitro and provide evidence which show possible interactions between the GR carboxy and amino terminals. I have also demonstrate by two in vivo assays solution dimerization of GR. Using the yeast two-hybrid assay, I have shown that solution dimerization of GR is ligand dependent in vivo and that amino acids 505 to 540 are necessary for this activity. In co-transfection experiments in COS7 cells, with wild type GR and a GR with a mutation that abolished the activity of the nuclear localization signal in the hinge region, I have demonstrated dimerization between two full length GRs in response to hormone treatment. Finally, I have provided data to show that GR has the potential to heterodimerize with several members of the nuclear receptor superfamily.
1. Assays used to monitor study solution dimerization of GR

(a) Co-Immunoprecipitation Assay

The co-immunoprecipitation assay employed in this study proved to be a valuable tool in characterizing the solution dimerization activity in rat GR. One of the major advantages of this technique was that it allowed for the rapid testing of deletion mutants in the absence of hormone binding. Further, it was easy to manipulate experimental conditions such as various modes of GR transformation (Figs. 6 and 8). Usually, four major criteria are used to validate the authenticity of an interaction detected by co-immunoprecipitation. First, it has to be established that the antibody does not recognize the co-precipitated protein. This is demonstrated in my experiments by the lack of binding of the in vitro translated GR and GR deletion mutants to the control precipitates containing the anti myc antibody. Second, it has to be established that the co-precipitated protein is actually being co-precipitated and not pulled down by a contaminating antibody. This problem is usually avoided with the use of a monoclonal antibody and thus in my experiments I used the monoclonal anti-myc antibody, 9E10. Third, it is usually desirable to determine if the interaction is direct or if it proceeds through a third bridging protein. This aspect is usually addressed with purified proteins. My results (Fig. 13) show a direct interaction between two GR fragments encompassing the identified solution dimerization interface expressed in and purified from bacteria. Finally, it is important to determine that the interaction observed in vitro takes place in the cell. I have provided data from two in vivo assays, which show solution dimerization by rat GR. Thus, by these criteria the results of the co-immunoprecipitation assay are validated.
(b) GST Pull-Down Assay

The GST system was useful for investigating solution dimerization of GR as it is a sensitive method which, at high concentrations of the immobilized protein, has the ability to detect weak interactions. One potential drawback of the system is the detection of interactions \textit{in vitro} that are not physiologically relevant \textit{in vivo}. This problem is addressed by demonstrating the interaction in an \textit{in vivo} assay. In my case I show the interaction in two \textit{in vivo} assays.

Another consideration when using this assay is the fact that many protein-protein interactions depend on the modification state of one or both of the partners. Protein-protein interactions that require a post-translational modification such as phosphorylation for the interaction are not detected if the proteins are expressed in cells in which the proteins are not properly modified. Bacteria lack many of the modification enzymes found in mammalian cells. Further, in the case of the GST-GR fusion proteins, bacteria also lack the hsp complex required for the proper folding of the GR LBD. Thus, these fusion proteins were not expected to be properly folded or post-translationally modified. However, consistent with the results of the co-immunoprecipitation assay showing that the tertiary structure of the LBD was not required for the GR homodimerization (Figs. 6, 7 and 8), GST-GR fusion proteins dimerized efficiently with \textit{in vitro} translated GR and GR deletion mutants (Figs.11D and 14B). Thus, the data from this assay was consistent with and validated by the results obtained from the other assays used.
(c) Yeast Two-Hybrid Assay

The third assay employed in this study was the two-hybrid assay in yeast. This assay was used to demonstrate solution dimerization \textit{in vivo} and to confirm the results of the two \textit{in vitro} assays. Initially, I attempted to do this assay in mammalian cells. However, the autonomous activation obtained with the GALDBD-GR constructs due to AF-2 activity (Fig. 2), was so strong that it precluded detection of any GR-GR interactions. I switched to the yeast for three reasons: first, it has been shown previously that AF-2 is a weak transactivation domain in yeast (324); second, yeast hsp homologues can and do replace mammalian hsp68s in the GR-hsp complex (85, 323) and third, the yeast components required for activity of the mammalian receptor are present and competent. Thus the glucocorticoid receptor signaling system in yeast closely mimics the mammalian process (324).

The two-hybrid system has several features that makes it useful for analysis of protein-protein interactions, the most important of these being that it is highly sensitive and can detect interactions that are often beyond the limits of \textit{in vitro} detection methods such as co-immunoprecipitation (326). The remarkable sensitivity of this assay is a result of a number of factors: (i) the hybrid proteins are generally over-expressed from vectors with strong promoters favoring complex formation, (ii) the signal measured is approximately proportional to the equilibrium concentration of the heterodimeric complex and (iii) the detection assay, in my case \textbeta-gal activity, amplifies the signal.

There are some limitations to this assay. First, the hybrid proteins are targeted to the nucleus. Therefore, interactions that depend on post-translational modifications that take place in the endoplasmatic reticulum such as glycosylation may not be detected.
Second, proteins that require modification such as phosphorylation or acetylation by non-yeast proteins for association, may not be detected. Finally, some hybrid proteins are harmful or lethal when expressed in yeast. This proved to be the case when I attempted to express GALDBD-GR$_{X556}$. However, I had no trouble expressing other GALDBD-GR fusions (Fig. 16B). Note that they were responsive to hormone treatment. Further, the results from this assay correlated with those obtained from the two in vitro assays in that (i) they showed a requirement for dissociation of the heat shock proteins for homodimerization and (ii) the dimerization interface delimited by the in vitro assays corresponded with the region of the receptor shown to be required for solution dimerization in vivo (Table 3).

Finally, it is noteworthy that this system has been used extensively to study homo- and heterodimerization of nuclear receptors (273, 274, 327-330). Specifically, ER (273), PR (271) and AR (274) homodimerization as well as heterodimerization between RAR (328, 330), TR (327, 328, 330), VDR (328, 330) and RXR have been demonstrated using the two-hybrid assay.

2. Solution dimerization of GR in vitro requires dissociation of heat shock proteins

The only requirement for dimerization of GR that I was able to detect in vitro was the release of GR from the hsp complex. This was demonstrated in experiments where dex-, salt-, and RU486 transformed GRs but not hsp-complexed GRs receptor dimerized in the co-immunoprecipitation assay in the absence of DNA (Figs. 4, 6, 7 and 8). These
results are consistent with two possibilities: the conformational change and ligand binding in the LBD does not affect dimerization or alternatively, that the dimerization interface is outside of the LBD core.

That hsp dissociation appears to be a prerequisite for GR solution dimerization contrasts with solution dimerization of the dioxin receptor. The dioxin receptor (DR) is a ligand activated transcription factor that belongs to the basic helix-loop-helix (bHLH)-PAS family of transcription factors. The PAS domain is a region of homology conserved between the *Drosophila* circadian rhythm regulatory protein Per, the *Drosophila* single-minded gene, Sim, and the aryl hydrocarbon receptor, Arnt (331). Like GR, the DR is present in the cytoplasm of target cells in an hsp complex in the absence of ligand (332, 333). By further analogy to GR, the hsp complex is required for maintaining the receptor in a non-activated, high affinity ligand binding conformation (334). Following ligand binding, DR heterodimerizes with the PAS factor, Arnt, and is imported into the nucleus where it activates transcription (332). The interesting aspect of dimerization between DR and the PAS factor Arnt, is the fact that following ligand binding, dimerization with Arnt is required for dissociation of the hsp5 (335) as it has been shown that the interaction between DR and Arnt only occurs with liganded DR (335). Thus the activity of DR appears to be controlled by a pattern of protein-protein interactions resulting in either negative or positive regulation. Given the similarities between GR and DR, it was possible that a similar mechanism is functioning to control GR activity.

One difference between GR and DR is the observation that in the absence of Arnt, DR fails to dissociate from the hsp5 upon exposure to ligand (335). No such factor appears to be required for dissociation of the GR-hsp complex following hormone
treatment (75-77). It was therefore possible that GR molecules promoted hsp
dissociation following ligand binding by homodimerizing. To examine for the possibility
of interaction between hsp-complexed GRs, it was necessary to establish conditions
under which the GR-hsp complex would be stable. Molybdate is a compound that
prevents dissociation of the complex even in the presence of ligand (294, 304-307). Note
that molybdate treated receptors are still able to bind ligand with high affinity. My
results show that there is no dimerization between hsp associated GRs (Fig. 4) suggesting
that, unlike the dioxin receptor where heterodimerization with Arnt is required for
dissociation of the hsp's, GR homodimerization occurs subsequent to the dissociation of
heat shock proteins.

Alternatively, molybdate artificially stabilizes the GR-hsp complex. Thus, it
remains possible that molybdate treatment locks the complex into a conformation which,
while allowing ligand binding is not favorable for homodimerization. Ligand binding
may induce small conformational changes in the receptor that permit dimerization.
Dimerization may then be the trigger for hsp dissociation. In the presence of molybdate,
ligand can still bind, however, the subsequent conformational change may not take place
because molybdate has locked the complex in an unfavorable conformation.

The observation that interaction between GR molecules in the yeast two-hybrid
assay was seen only in the presence of hormone appeared to support the conclusion that
hsp-complex dissociation, prior to dimerization, was also required in vivo. However, an
alternative, and perhaps more likely explanation for these data is that the hsp complex
prevents the GAL DBD from binding to DNA and that hormone treatment removes this
hindrance by causing dissociation of the hsp complex. Thus the inability to detect an
interaction between the hsp-complexed GRs in this assay does not necessarily confirm
that hsp dissociation in required in vivo.

The GR LBD is in fact sufficient for conferring hormone regulation onto a fusion
protein. The fusion of heterologous proteins to the LBD of GR has long been used as a
tool to control protein activity (60, 336-341). In each of these examples, the LBD of
GR confers hormonal control of fusion proteins by conferring hormone-regulated binding
of hsp.

3. Tertiary structure of the GR LBD that results from the binding of
agonist is not required for solution dimerization

The observation that GR did not have a strict ligand requirement for dimerization
implied that solution dimerization was not specifically dependent on the tertiary structure
of the LBD that results from agonist binding.

The hsp-GR complex maintains the receptor in an open conformation capable of
binding ligand with high affinity (83-88). The binding of hormone agonist not only
releases the receptor from the complex, but evidence also supports an agonist-dependent
conformational change within the LBD such that the receptor is transcriptionally active
(342-345). While the high affinity binding site in the LBD appears to be the same for
both agonists and antagonists (346) and both can cause the release of hsp, the
agonist-receptor complexes are generally transcriptionally inactive. X-ray
crystallography studies of unliganded (347-349), agonist-bound (267, 350, 351) and
agonist bound (350) receptors have shown that the extreme C-terminus tail, AF2 AD,
can exist in two alternative conformations that are induced by agonists and antagonists. Poor transcriptional activation in the presence of antagonist results from improper folding of the LBD in the presence of antagonists (310, 311).

Solution dimerization of salt and RU486 transformed receptors provide strong evidence that agonist-induced conformational changes in the C-terminal domain of GR were not specifically required for this activity. Salt and RU486 transformed GRs dissociate from the hsp-complex, bind DNA but are poor activators of transcription (310). However, neither of these treatments appeared to affect homodimerization of GR.

Transcriptional activation is not the only receptor function that specifically requires the binding of agonist. Our laboratory has recently shown that nuclear import of GR, mediated solely by the NLS in the LBD is agonist dependent (61). Using a full length GR with a mutation in its NL1 sequence that abrogates nuclear import mediated by this NLS, we have shown that nuclear import by NL2 specifically requires the presence of agonist and does not occur with antagonist treated mutant receptors (61). Moreover, the observation that the NL1 mutant was still able to dimerize in vitro (Fig. 19) and in vivo (Table 4) showed that NL1 was not involved in dimer formation even though the identified dimerization interface includes NL1.

The physiological relevance of the observed ligand independent solution dimerization of GR (i.e. dimerization of salt-transformed GRs) is not yet clear. PR (352), ER (353, 354) and AR (355) can be activated in a ligand-independent manner. Recent data now suggest that under some circumstances GR may also be similarly activated in a ligand independent fashion (356). Since dimerization of GR is an important step in the
mechanism of action of GR, ligand independent dimerization therefore becomes important for ligand independent signaling by GR.

The conclusion that ligand-induced conformational changes in the LBD are not required for GR solution dimerization is further supported by the observation that GR fragments expressed as GST fusion proteins in bacteria were able to dimerize with in vitro translated GR. Bacteria lack the hsp complex and by extension any protein folding activity associated with the complex. Thus, the GR fragments would not be expected to be to be in a proper conformation. That GST-GR fusion proteins still dimerized efficiently supported the idea that GR LBD conformation was not specifically required for homodimerization. Thus, my data support the idea that primary amino acid sequence rather than secondary or tertiary structure forms the GR solution dimerization interface.

Moreover, these results are consistent with studies which show solution dimerization of PR in the presence of both agonist and antagonist (271). However, they contrast with ER where antagonists appear to inhibit receptor dimerization (357).

Finally, another possible explanation for my observations of a lack of agonist binding for solution dimerization, is that different dimerization interfaces are utilized depending on whether GR is bound to agonist or another agent used to cause hsp dissociation. This seems less likely for the following reason: I performed mixing experiments in which one receptor molecule was treated with ligand while the other was salt treated. In these experiment the two receptors were still able to dimerize (Fig. 6D).
4. The solution dimerization interface of rat GR maps to the hinge domain

The solution dimerization interface of rat GR mapped to amino acids 505 to 547 by co-immunoprecipitation assay, 505-546 by GST pull down assay and 505 to 540 by the yeast two-hybrid assay (Table 3). These amino acids all fall within the hinge region of GR. These results were somewhat surprising because solution dimerization interfaces in other nuclear receptors (discussed in detail below) have generally mapped to the LBD. The only nuclear receptor for which the hinge region has been implicated in solution dimerization is PR (268). The results of that study were consistent with PR solution dimerization being mediated by multiple receptor domains. However, that study did not determine whether the hinge contributed directly to the dimerization interface or indirectly by affecting the structural conformation of the other domains of PR (268). The results of my studies show the hinge region contributes directly to the solution dimerization interface of rat GR. This conclusion is supported by the following observations: first, deletion of either the amino terminus and the DBD to amino acid 505 (505C, Figs. 10D, 12 and 13) or the C-terminus amino acids to 547 (Figs. 8, 10D, 12 and 13) did not significantly affect dimerization as would be expected if these domains were directly involved in the formation of the interface. Second, an internal deletion of the hinge region severely compromised the ability of the receptor to dimerize (Fig. 12B). Third, in both the GST pull-down assay (Fig. 10) and the two-hybrid assay in yeast (Fig. 17), extending the LBD, which failed to dimerize on its own, to include the hinge region was sufficient to confer dimerization activity. This observation on its own does not
eliminate the possibility that the hinge contributes indirectly by affecting the structural conformation. However, when it is coupled with the fact that the GR LBD in the absence of the hinge is capable of carrying out other properties ascribed to this domain such as hsp complex association and ligand binding, the failure of the LBD to mediate solution dimerization does not appear to be due to misfolding of the domain. Thus, I conclude from these results that the hinge region of the rat GR is required for solution dimerization.

Finally, two previous studies offer clues as to the location of the solution dimerization interface in GR. In 1992, Dahlman-Wright et al showed that addition of the hinge and LBD to the DBD of GR increased the DNA binding affinity of the DBD to wild type levels (358). This increase in binding was attributed to solution dimerization of the GR through the LBD (358). However, this study did not evaluate the contribution of each domain individually.

Segard-Maurel et al using a mathematical model to assess the influence of receptor domains other than the DBD on DNA binding affinity posited that the presence of both the N- and C-terminal domains were necessary for optimal dimer formation in the absence of DNA (257). The results of my experiments showing the hinge region as harboring the dimerization interface are consistent with the finding from both of these studies.

While my data suggests that the hinge forms the core solution dimerization in GR, I have no evidence as to the sufficiency of the hinge for solution dimerization. An attempt to show the sufficiency of the hinge in the two-hybrid assay failed because the fusion proteins were not expressed. Two alternative ways to demonstrate sufficiency are:
to show that the amino acids making up the dimerization interface are sufficient for
mediating an interaction in another system. Another option is to show that the GR
solution dimerization domain is able to confer onto a protein that is functionally active as
a monomer the ability to homodimerize.

5. Comparison of the GR dimerization with dimerization interfaces
in other nuclear receptors

The identification of the hinge region as the solution dimerization interface for
GR was interesting, because to date, identified solution dimerization interfaces in nuclear
receptors have all mapped to the LBD (134, 347, 349-351). The structural similarity of
nuclear receptors, particularly the steroid receptor subgroup, therefore, predicted a LBD
dimerization interface for GR.

Mutagenesis studies (134) first identified a solution dimerization domain in the
ER LBD. It contained a heptad repeat of hydrophobic residues resembling a leucine
zipper and was conserved in members of the nuclear receptor superfamily (Fig. 22).
Leucine zipper motifs are often involved in transcription factor dimerization (359). The
ER interface was confirmed when the crystal structure of the ER LBD was resolved
(350). In contrast to ER, deletion of the homologous region in GR failed to disrupt
solution dimerization (Fig. 8) demonstrating that these two classical steroid receptor were
likely to have distinct solution dimerization interfaces.

Interestingly, the position of the solution dimerization interface in the ER LBD
was similar to the dimerization interface seen in the crystal structure of the RXR
Figure 22. Sequence alignment of nuclear receptor proteins.

(top) Schema of rat GR with boxes that highlight the DNA and ligand binding domains. Selected amino acids positions, primarily those for the boundaries of the major domains are indicated above the diagram. DM represents the position of the solution dimerization motif identified in rat GR. (below) The protein sequence of helices 9 and 10 of the LBD from selected members of the nuclear receptor family were aligned. This region is involved in solution dimerization of several nuclear receptors. Grey boxes highlight the dimerization interface of nuclear receptor identified either by mutagenesis or crystallography. To the left are the abbreviated names of the nuclear receptor.
H9

H10

GR 698 LKSQELFDEI RMTYIKELGK AIVKREGNSS QNWQRFYQLT KLIDSMHEVV ENLNYCFQT FLDKTMSIE
MR LKSQAFFEM RTNYIKELRK MVTKCPNNSG QSWQRFYQLT KLLDSDMLDV SDELLECFYET FRESHALKVE
PR LRSQTQFEEM RSSYIRELIK AIGLRQKGVV SSSQRFYQLT KLLDNLHDLV KQLHLYCLNT FIQSRALSVE
AR LKNQKFFDEL RMNYIKELDR IIACKRNPT SCSR RFYQLT KLLDSVQPIA RELHQFTFDDL LIKSHMVSVD
ERa LSSTLKSL EE KDHIHRVLDK ITDTHILLMA KAGLT[QQH QR]LAQLL[IL] SHRHMNSKGM EHLYSMKCK
RARa LEQ[DRVDM]LQEP[LLEALKV YVRRR... ] .PSR[PH... MFFKMLMKI TDLRSISAKG AERVITLKM.
PPAR LLNIGYIEKL QEGIVHV LKL HLOSN... ] .PD DT FLFPKLIQKM VDLRQ[LV]TEH AQLVQVIIK.
(347, 350, 360), the RAR (351) and the TR (349) LBD. These structures, as well as that of the ER LBD, have a common fold that is described as an α-helical anti-parallel sandwich composed of twelve α-helices (H1-H12) (346). In each structure, the LBD is packed as a dimer in which the monomers interact through a rotationally symmetrical dimerization interface formed primarily by H9 and H10 and to a lesser extent by H7 and H8. These interfaces overlap almost perfectly with the regions of the ER (134), RAR (169, 330) RXR (328, 330, 361) and TR (169, 361, 362) LBD shown by mutagenesis to be important for dimerization. The similarity of the crystal structures of ER LBD, a classical steroid hormone receptor, to that of RAR, RXR and TR, non-steroidal ligand dependent receptors, suggested that all nuclear receptors were likely to have a similar structural organization and a model for the folding of the GR LBD has been proposed based on this hypothesis (346).

However, the resolution of the crystal structure of the PR LBD proved that this predicted similarity did not extend to all nuclear receptors. Like ER, RAR, RXR and TR LBDs, the PR LBD crystallized as a dyad symmetric dimer (267). However, the PR LBD dimer interface in the crystal structure was substantially different from the one predicted by the model. Not only was the dimerization interface in the PR LBD smaller than that of the ER and RXR LBDs (267, 360), but the location of the interface in PR was different from that of ER and RXR (360). Whereas helices 7 to 10 were involved in the formation of the dimerization interface for ER, RAR, RXR and TR, the PR dimerization interface was composed predominantly of helices 11 and 12 as well as the extreme C-terminal tail (267, 360). The smaller PR LBD dimerization interface suggested a less stable dimer
than ER and RXR and it was noted that the PR LBD fragment used for the crystal structure elutes as a monomer in gel filtration experiments (267, 360). This is entirely consistent with the in vitro studies of Tetel et al. showing that the PR LBD requires additional sequences, primarily in the hinge region but also the amino terminus, for the formation of stable dimers in solution (268).

The GR solution dimerization interface appears to be more closely related to the PR interface than to that of ER. The location of the core of the GR interface to the hinge region of GR is consistent with the work of Tetel et al showing a role for the hinge in solution dimerization by PR (268) and contrasts with studies done with ER in which there appeared to be no requirement for the hinge (134). Moreover, my results suggest that relative to the dimerization interface in ER (350), a smaller sequence makes up the core of the GR dimerization interface. Finally, while my data show the core of the dimerization interface being located in the hinge domain, there is evidence for protein:protein contacts with other receptor domains (Fig. 14). These observations are consistent with studies done with the PR (268) and AR (274, 275) showing interactions between the carboxy and amino terminal domains. Further, it is possible that interaction between two LBD fragments in GR (Fig. 14) may correspond to the interaction seen between helices 11 and 12 of the PR LBD (267). The crystallization of the GR LBD will show whether this is true or not.

Since both GR and PR seem to have a different dimerization interface than ER and RAR, it is possible that nuclear receptors may fall into two groups based on their solution dimerization interfaces. Those receptors such as RAR, TR, RXR and VDR may form ER/RAR like dimers, while others such as GR, PR, AR and MR may form PR like
dimers. The proof of this hypotheses awaits crystallography data from the other nuclear receptors. However, there is some data available in the literature to suggest that this hypothesis is not unreasonable.

First, the residues that contribute to the ER/RAR like dimerization interface are highly conserved in these receptors and have been implicated in dimerization by mutagenesis. That ER, with respect to solution dimerization, behaves more like a class II type receptor rather than like a class I classical steroid hormone receptor is not entirely inconsistent. Its’ DNA binding site is more closely related to those of the class II receptors.

Second, PR (268), AR (274, 275) and potentially GR, all seem to have multiple receptor domains contributing to solution dimerization. For PR and GR, the major dimerization interface appears to be centered around the hinge region, however, in vitro studies have shown protein-protein contacts can be made with the amino terminus.

6. Contribution of other GR domains to solution dimerization

While my data showed that the hinge region makes a major contribution to the solution dimerization interface of rat GR in vitro and in vivo, it also indicated that amino and carboxy terminal sequences may play a role in dimerization (Fig. 14B). In a GST pull-down assay, the amino terminus interacted with the LBD (Fig. 14B, lane 7) and with itself (lane 5). Further, two LBD peptides, in the absence of other receptor sequences, were able to interact with each other (Fig. 14B, lane 15). These results are consistent with protein-protein contacts between two amino termini, between the amino terminus
and the LBD and between two LBDs. Whether any of these interactions are physiologically relevant is called into question by the observation that two GR LBDs failed to interact in vivo in the yeast two-hybrid assay despite the fact that they were expressed at high levels (Fig. 16B, lanes 1 and 2) and this assay is noted for its high sensitivity.

The reason for the apparent discrepancy between the GST and the yeast two-hybrid assays is not known. One consideration is, as mentioned above, the large amount of purified GST fusion protein used in the GST assay (0.5 μg/assay). This is both a benefit and a potential drawback. On the one hand, it permits detection of weak protein-protein interactions. On the other hand, it may detect physiologically irrelevant interactions. Alternatively, it may be that dimerization via the hinge domain either positions the two GR molecules in a configuration that allows further protein-protein contacts between other domains or induces conformation changes within the receptor that creates other interaction surfaces.

The hypothesis of multiple contact sites between the two monomers in the GR homodimer is supported first by studies done with an N-terminally truncated GR deletion mutant. Deletion of the N-terminus of GR results in a receptor with reduced DNA binding specificity. In a series of cross linking experiments designed to investigate the role of N-terminal domain on receptor:DNA interaction, Eriksson and Wrange concluded that deletion of the amino terminus changed the contact points within the GR dimer. The altered protein contacts was proposed as the reason for the reduced DNA binding specificity seen with N-terminally deleted GRs. Their data suggested that protein:protein
contacts in the N-terminus of GR was required for optimal steric arrangement of the two DNA binding domains (143).

Second, Segard-Maurel et al, endeavored to clarify the role of regions outside the DBD in GR dimerization by analyzing the binding of a series of GR deletion mutants to GREs in gel shift assays. Binding parameters for GR mutants where either the amino or carboxy terminal domain had been deleted were determined and compared to the binding of the DBD alone. The conclusion was that both the N- and C-terminal domains were required for efficient dimerization (257).

Moreover, these results are also consistent with observations from other steroid hormone receptors. Interactions between separately expressed amino terminal domains and between the amino- and carboxy terminal domains of the AR have been detected (274, 363). The authors of the study proposed anti-parallel interactions between the amino and carboxy termini in the AR homodimer (274, 363). Similarly, the amino and carboxy termini of the ER were shown to interact (364). Recently, Tetel et al demonstrated an interaction between two amino terminal fragments of PR and provided evidence to suggest that the amino terminus of PR interacted with its carboxy terminus (268).

Nuclear receptors are transcription factors that are generally thought of as modular proteins with discrete function domains whose regulatory regions operate independently (365). This viewpoint was based on the results of domain swapping experiments in which activation or repression domains demonstrate intrinsic activity when tethered to a heterologous DNA binding domain. The yeast two-hybrid assay is based on this observation. However, recent evidence suggests that the regulatory
domains in some transcription factors may not behave as autonomous modules. Lefstin and Yamamoto (366), for example, have proposed that transcription factors may be modified in an allosteric manner by the DNA sequence to which they bind. In like manner, it may be that the intra/inter domain interactions shown in this study for GR and demonstrated previously for PR (268) and AR (275) may be necessary for allosteric modifications of these domains.

The observation that binding of both the amino terminus (GST-GR$_{22-437}$) and the LBD (GST-GR$_{542C}$) to the LBD peptide when the LBD was expressed in the absence of other receptor sequences (547C, Fig.14B, lanes 7 and 15) was severely compromised when the LBD was extended to include the hinge and the DBD (Fig. 14B, lanes 8 and 16) would seem to support the idea of an induced conformational change. The LBD with the hinge and DBD may be folded such that while the hinge dimerization domain is exposed, other protein interaction surfaces may be occluded or not created. Dimerization via the hinge domain may result in refolding of the other domains such that other protein-protein interaction surfaces are exposed or created.

My data, as well as the studies with PR (268) and AR (274, 275) are consistent with the notion that multiple receptor domains contribute to the receptor homodimerization. However, whether these interactions contribute to the dimerization interface, or are part of the intermolecular interactions within a monomer or both remains to be determined. To confirm the significance of the hinge for GR solution dimerization, it will be necessary to generate a GR receptor with a mutation in the hinge that only abolishes the solution dimerization activity. The fact that NL1 activity was disrupted without any apparent adverse effect on solution dimerization (Fig 19) suggests that such a
mutation is possible even though there appears to be multiple overlapping functions in the hinge. Such a dimerization mutant will allow for the evaluation of the contribution and relevance of the intra/inter domains interactions to solution dimerization of GR.

7. Other receptor functions identified in the hinge region

The hinge region found between the DBD and LBD of steroid receptors (Fig. 2) has traditionally been thought of as a flexible link between these two domains. Sequences within this region of the receptor are not as well conserved as other regions of the receptor such as the DBD. However, there is now evidence that the hinge domain sequences possess important functions.

The first identified and best-characterized function of the hinge is the nuclear localization function (39, 42, 60, 367). Basic sequences characterized as classical NLSs are present in the hinge of all steroid receptors. However, even these sequences show some variability (39, 42, 60, 367). While the GR NLS and solution dimerization interface co-localizes to the hinge, my results show that the two activities are being mediated by different amino acid sequences.

The hinge region has also been reported to have variable influence on the ligand binding affinity of GR. Ligand binding by GR is dependent upon the association with heat shock proteins (83-88). The GR LBD with the hinge expressed in vitro bound hormone with high affinity (368) as did the LBD alone. In other studies, however, when the LBD without the hinge was expressed in mammalian cells, the protein appeared to be highly unstable (369). However, fusion of the LBD to an unrelated protein such as β-
galactosidase resulted in stable polypeptides that bound ligand with high affinity (369). Whether the hinge could substitute for β-gal was not investigated in the study. These results suggest that the hinge region of GR may indirectly influence ligand binding by stabilizing the LBD in vivo. In contrast, for ER, there appears to be no requirement for the hinge in hormone binding as the ER LBD expressed in bacteria bound hormone with affinity equal to that of the full length receptor (91). Similar results were obtained with PR (268).

Mutagenesis studies have shown that amino acids from different parts of the LBD are involved in ligand binding by GR (see (346)). None of these amino acids are found in the region of the hinge required for solution dimerization. Thus it appears unlikely that dimerization would have a direct effect on ligand binding. Further, the high affinity ligand binding form of GR is a GR monomer in a hsp complex.

Recently, the hinge domain has been identified as a binding site for co-repressors, proteins that function to inhibit transcription by receptors in the absence of ligand, in RAR and TR (236, 370). Further, in ER, the binding site for the transcription factor TAFII30 was mapped to the hinge (107). However, unlike RAR and TR, GR has not been reported to bind co-repressors. Rather, the unliganded GR cannot bind DNA because it is sequestered in the hsp complex.

8. GR solution dimerization interface overlaps with the binding site for the NLS receptor, importin α
Examination of the amino acid sequence that forms the solution dimerization interface of GR reveals no obvious motifs such as a leucine zipper that may mediate dimerization (Fig. 23). The only prominent feature in this region is the nuclear localization signal, NL1. GR has two NLSs, NL1 and NL2, a ligand dependent NLS in the LBD whose sequence has not been delimited (60, 135). Nuclear import of proteins bearing NL1 type NLS is dependent on two cellular factors termed importin α and importin β (371-373). Importin α functions as the NLS receptor and also binds to importin β. It thus mediates the formation of an import complex that includes the NLS-containing protein and importin β. The heterocomplex is targeted to the nuclear pore complex (NPC) due to the interaction of importin β with nucleoporins, proteins that make up the NPC. The complex then translocates through the NPC into the nucleus by an energy dependent process that requires the activity of the Ran GTPase and a Ran cofactor termed p10. Thus, the first step in the import of proteins into the nucleus is the binding of the NLS to importin α.

We have recently shown that NL1 of GR binds to importin α (61). It is interesting that this binding site overlaps with the solution dimerization interface. One possible explanation for the apparent ability of one sequence to mediate both dimerization and nuclear import is that different interfaces formed by the tertiary structure of the hinge sequence of GR mediate each function. This idea is supported by the fact that mutation of residues critical for the nuclear localization function of NL1 (61), apparently leaves the dimerization function intact (Fig. 19). Alternatively, a finer mapping of the solution dimerization interface may show that different amino acids are involved in nuclear localization and dimerization as suggested by the findings in the co-
Figure 23. Sequence alignment of the GR solution dimerization domain with the homologous regions of other nuclear receptors.

*(top)* Schema of rat GR with boxes that highlight the DNA and ligand binding domains. Selected amino acids positions, primarily those for the boundaries of the major domains are indicated above the diagram. DM represents the position of the solution dimerization motif identified in rat GR. *(below)* The primary amino acid sequence of the identified solution dimerization domain in rat GR aligned with the homologous region of several members of the nuclear receptor superfamily. To the left are the abbreviated names of the nuclear receptor.
transfection experiments. Finally, receptor dimerization may create the importin α interaction surface.

9. Dimerization of full length GR monomers during nuclear transport in vivo

To demonstrate solution dimerization of full-length GR monomers in vivo, I utilized a GR mutant in which the core NL1 sequence was mutated, GR$_{NL1^-}$ (Fig. 18A) and I took advantage of the fact that nuclear import of GR can be regulated by hormone treatment (58, 61, 62). Nuclear import of GR$_{NL1^-}$ is mediated by the NL2 of GR and occurs with kinetics that are drastically different from import of the wild type receptor (61). The goal of these experiments was to, through dimerization with GR$_{WT}$, alter the kinetics of GR$_{NL1^-}$ import. Similar studies have been previously used to show solution dimerization of PR monomers in vivo (42).

In co-transfection experiments with GR$_{WT}$ and GR$_{NL1^-}$ the addition of hormone caused a shift in the subcellular distribution of GR$_{NL1^-}$ towards the nucleus (Table 4, 74% of cells with mostly nuclear staining) relative to when GR$_{NL1^-}$ is expressed alone (32% of cells with mostly nuclear staining). The simplest explanation for this is that hormone provoked an interaction between GR$_{WT}$ and GR$_{NL1^-}$ allowing the form capable of rapid nuclear transfer (GR$_{WT}$) to carry along the form initially localized to the cytoplasm (GR$_{NL1^-}$) thus accounting for the increase in the amount of GR$_{NL1^-}$ localized to the nucleus.
Another mechanism could theoretically be compatible with the present experimental data: GR\textsubscript{WT} and GR\textsubscript{NL1}- may both bind to a third protein which would help facilitate the import of GR\textsubscript{NL1}- in the presence of GR\textsubscript{WT}. Such a bridging protein would most likely be involved in GR import, and, an obvious candidate for the bridging activity is the NLS binding protein, importin α. However, it is unlikely that this mechanism is responsible for the increased nuclear presence of GR\textsubscript{NL1}- in the presence of GR\textsubscript{WT} given that we have shown that GR\textsubscript{NL1}- lacks the ability to interact with importin α (61). My present data, however, does not rule out the possibility that such an interaction with a third protein is not occurring through other GR domains such as the NLS in the LBD.

That all the GR\textsubscript{NL1}- did not localize to the nucleus even in the presence of GR\textsubscript{WT} was not unexpected. Co-transfection of GR\textsubscript{WT} and GR\textsubscript{NL1}- should result in the formation of three dimeric complexes: GR\textsubscript{WT}:GR\textsubscript{WT}; GR\textsubscript{WT}: GR\textsubscript{NL1}- and GR\textsubscript{NL1}:-: GR\textsubscript{NL1}-. The slower import of the GR\textsubscript{NL1}:-: GR\textsubscript{NL1}- complexes were not expected to be influenced by the presence of GR\textsubscript{WT}.

10. **Cytoplasmic Dimerization of GR**

In previous studies (Appendix A), we detected GR dimers on nuclear envelopes isolated from glucocorticoid treated cells. While the data was consistent with the interaction between two GR monomers during transport and independent of the DNA binding, the design of the experiments in those studies did not allow us to determine whether the observed dimers were seen on their way into or out of the nucleus. Thus we were not able to make any conclusions as to where in the cell, i.e. whether in the nucleus
or the cytoplasm, homodimerization of GR occurred. Data from co-transfection studies done with PR are clearly consistent with cytoplasmic homodimerization (42). I propose that my present results are also consistent with a cytoplasmic dimerization mechanism for GR.

One possible reason for cytoplasmic dimerization by GR is to prevent reassociation of the hsp complex following ligand binding and prior to nuclear import. Dimerization of monomers may occlude the hsp binding site in GR and thereby prevent its inactivation by the hsp complex.

It is theoretically possible that the GR<sub>WT</sub>:GR<sub>NL1</sub>- dimer forms in the nucleus after import. In this scenario more GR<sub>NL1</sub>- is retained in the nucleus because one of the monomers (in this case GR<sub>WT</sub>) was able to bind DNA or some other nuclear components. DNA binding is an important determinant for complete nuclear localization of GR (58, 62). However, we found that GR<sub>NL1</sub>- was able to bind DNA as well as GR<sub>WT</sub> in a gel shift assay (61). Therefore, the decrease nuclear import of GR<sub>NL1</sub>- in the presence of hormone was not likely to be due to reduced affinity of GR<sub>NL1</sub>- for DNA.

The fact that the binding site for the NLS receptor, importin α, overlaps with the solution dimerization interface in GR, would suggest that cytoplasmic homodimerization and nuclear import of GR are two mutually exclusive events. However, I have no evidence to suggest that the same amino acids are involved in the two activities. On the contrary, the observation that mutation of the NL1 sequence did not dramatically affect dimerization (Fig. 19) supports the idea that different amino acids are required for the two activities.
11. **Heterodimerization between GR and other nuclear receptors**

Unlike class II and class III receptors (Table 1) which heterodimerize with RXR in solution and on DNA, solution heterodimerization among the steroid hormone receptors has not been reported previously. The DBDs of GR and MR and GR and AR heterodimerizes on DNA. My results show, for the first time, that GR has the ability to heterodimerize with several nuclear receptors in solution (Fig. 21). While there are potential benefits in the heterodimerization of GR with other steroid hormone receptors such as Ar and MR, the physiological relevance of heterodimerization between GR and other nuclear receptors is not immediately obvious.

Whether the same sequence mediating GR homodimerization in solution also mediates solution heterodimerization with other nuclear receptors cannot be determined from my data. There is no conserved motif between GR and the other nuclear receptors in the identified solution dimerization interface in GR (Fig. 23). However, the sequences shown to mediate solution dimerization of ER, RAR and TR are somewhat conserved in GR (Fig. 22). Thus it is possible that heterodimerization between GR and the other nuclear receptors occurs through a GR sequence that is different from the one involved in GR homodimerization.

12. **A hypothesis for solution dimerization of GR**

My results are consistent with the idea that homodimerization of GR in solution is not mediated by a single discrete domain, but that multiple regions of GR contribute to its
dimerization. In particular, GR solution dimerization seems to require the hinge with further protein:protein contacts in other domains of the receptor. Three models can potentially explain my data on GR solution dimerization. While each model is anchored by dimerization through the dimerization interface identified in the hinge, they differ in the possible additional contacts made by the two monomers. In the first, further contacts are made between the amino and carboxy terminal domains of the monomers. The second model features, in addition to the hinge dimerization, intra-molecular interactions between the amino and carboxy terminals of a GR monomer. Such interactions would not necessarily exclude intermolecular interaction other than that of the hinge domain. Finally, in the third model, solution dimerization is made up of the hinge region dimerization with anti-parallel contacts between the amino and carboxy terminal domains of the monomers.
V. CONCLUSION

I have characterized a solution dimerization activity that localized to the hinge region of the GR. The most striking feature of the dimerization interface is that it appears to overlap with NL1, the binding site for the NLS receptor, importin α. However, my data suggests that dimerization and importin α binding are mediated by different sequences in the hinge. Future studies are needed to address the role of dimerization in the mechanism of action of GR. These studies will help provide answers to the following questions. What role if any does solution dimerization play in the nucleocytoplasmic trafficking of GR? What is the contribution of solution dimerization to DNA binding and transcriptional activation by GR? These questions will be answered when a mutation which abolishes solution dimerization without affecting the other physiological properties of the receptor is identified. A GR solution dimerization mutant would also allow one to determine whether the same sequence that mediates solution dimerization of GR is involved in the heterodimeric interaction between GR and other nuclear receptors.

The glucocorticoid receptor function can be faithfully reconstituted in yeast. Given the ability to couple random mutagenesis with phenotypic screens in this organism, the tools for creating a dimerization defective GR are available.
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VII. APPENDIX A
Nuclear Envelope Glucocorticoid Receptor: Localization to Nuclear Pores and Regulation by Glucocorticoids

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Running Title: Nuclear Envelope GR
ABSTRACT

Glucocorticoid receptor (GR) trafficking between the cytoplasm and nucleus is a regulated process governed by cytoplasmic interactions, import and export processes and nuclear interactions. To better understand nucleocytoplasmic trafficking of GR, we have further characterized the interaction of GR with the nuclear envelope, the barrier to free transport of molecules into and out of the nucleus and a reported nuclear site of interaction of GR. Receptor molecules were directly visualized and quantified by immunoelectron microscopy. In rat hepatocytes and CEM leukemic cell lines GR immunoreactivity on the nuclear envelope was found primarily in association with nuclear pores, the conduits for passage of molecules across the nuclear envelope. Furthermore, most of the GR was detected as dimers and multimers, suggesting that trafficking of GR across this membrane system may be preceded by homodimer formation. In the rat liver and steroid-sensitive human leukemic cells exposed to dexamethasone, there was an increase in receptor labelling on the pores. In contrast, in steroid-resistant human leukemic cells, the GR level was up-regulated upon exposure to dexamethasone. This contrasting effect of glucocorticoid on nuclear envelope-associated GR in steroid-sensitive versus steroid-resistant cell lines was also observed in the well-studied mouse lymphoma cell lines, by western blotting and affinity labelling of cellular subfractions. Interestingly, a doublet of GR present on nuclear envelopes prepared from intact animals was detected. In summary, these studies are consistent with a highly regulated hormone-dependent interaction of GR during passage of the receptor across the nuclear envelope and suggest that homodimer formation and modification of GR may precede its association with nuclear envelope components.
Introduction

Accumulation of small proteins in the nucleus can be attributed to selective retention after entry by diffusion. In contrast, for larger proteins (above 65 kDa) there is ample evidence of selective entry into the nucleus (for reviews see (1-3)). The barrier to unrestricted passage of larger proteins into the nucleus is the nuclear envelope which is a complex membrane system consisting of two layers of membrane lined on the inside with lamins and perforated by nuclear pores, which are formed where the two membranes come into contact with one another (4-6). Studies of several transcription factors have revealed that their presence in the nucleus is conditional upon stimulation by various stimuli including hormones, viral infections and developmental cues. Examples include the α-interferon-regulated factor, ISGR-3 (7), the nuclear v-jun oncogenic counterpart of the AP-1 transcription complex member c-jun (8, 9), the yeast SW15 (10), the D. melogaster morphogen dorsal (11, 12) and the nuclear factor NFκB (13, 14). The cytoplasmic localization of these transcription factors until exposed to the appropriate stimulus allows for exquisite control of their nuclear activity.

One of the first examples of regulated intracellular compartmentalization of transcription factors was provided by the glucocorticoid receptor (GR), a member of the steroid/nuclear receptor superfamily of transcription factors (reviewed in (15, 16)). Nuclear hormone receptors have a similar primary structure which is characterized by a highly conserved centrally located DNA binding domain, a hormone binding domain at the carboxy terminus and a more variable transactivation domain at the amino terminus. Whereas some steroid receptors are constitutively present in the nucleus, there is general agreement that, in the absence of steroid, the GR is localized mostly in the
cytoplasm, as originally shown by subcellular fractionation (reviewed by (17, 18)). Immunocytochemical studies confirmed earlier work and clearly demonstrated that the GR translocated to the nucleus following binding of the steroid (19-26). The dependence on intracellular translocation may not be restricted to glucocorticoid receptors and may include other members of the superfamily of nuclear receptors, namely the mineralocorticoid (27) and androgen (AR) (28) receptors, and the viral counterpart of the thyroid receptor (TR) (29), although evidence in these cases is not as abundant as for GR. Importantly, a deficiency in the ability of GR to translocate to the nucleus has been associated with steroid resistance in human and mouse leukemic cells (reviewed in (21)).

However, once translocated to the nucleus, GR does not reside there in a static state, but rather continues to traffic between the nucleus and cytoplasm (30). Nucleocytoplasmic trafficking, the process whereby a molecule is transported between the nuclear and cytoplasmic compartments may be regulated by at least four mechanisms: 1. retention in the cytoplasm, 2. import into the nucleus, 3. retention within the nucleus, and 4. export from the nucleus. Cytoplasmic retention has been described for several transcription factors (for review see (1)), including GR, whose cytoplasmic retention in the absence of glucocorticoids is generally acknowledged to be mediated via binding to heat shock proteins ((31), see (32)). Most proteins, including GR, that are imported into the nucleus contain short basic peptide sequences that function as nuclear localization sequences (NLSs) (33). In the cytoplasm, the NLS is recognized by the NLS receptor complex importin , which docks the NLS-containing protein to the cytoplasmic site of the nuclear envelope, at nuclear pore complexes. Subsequently, the docked complex is translocated across the nuclear envelope into the nucleus by a process that requires multiple
interactions with components of the pore and the hydrolysis of GTP by the Ran GTPase (34, 35). Exposure of the protein's NLS is thus essential to its nuclear translocation. Masking of the NLS is a mechanism which has been shown to regulate transfer to the nucleus. The p105 precursor of NFκB appears to retain the NFκB p50 subunit in the cytoplasm through intramolecular masking of its NLS (36-38). Antibodies specific to the NLS recognize p50 but not p105, implying that the NLS is inaccessible in the larger precursor (37). The mechanism of unmasking of the NLS appears to be through proteolysis of the p105 COOH-terminal (39, 40). It has been reported that the NLS of cytoplasmic GR is inaccessible to an anti-NLS peptide antibody (41) and it is speculated that interaction with the heat shock proteins is responsible for the masking of the GR NLS.

Less is known about export from the nucleus. Recently, specific nuclear export signals, (NESs) have been identified for the PKA specific inhibitor protein PKI (42) and the HIV-1 protein (43), and it has been suggested that proteins functioning as NES receptors analogous to importins in nuclear import are required (44). To date, specific NESs for GR have not been identified although regions homologous to the aforementioned NESs are found in GR (our unpublished observations). It is also possible that export is a default pathway such that nuclear retention is the limiting factor.

We have recently demonstrated that DNA binding of GR is an important determinant of both nuclear localization and tight nuclear binding of this nuclear receptor (45). However, while we estimated that DNA binding is the major nuclear retention site, other nuclear binding sites of GR have been identified. Their role in nuclear localization and retention remains to be elucidated. These include protein-protein interactions with other nuclear transcription-regulatory factors (46-48), the nuclear matrix (reviewed in (49);
(50)] and the nuclear envelope (51). The nuclear envelope binding sites would seem to be particularly important in consideration of transport, and in earlier work, we identified and characterized GR binding to nuclear envelopes prepared from rat liver (52). In this paper, we confirm the physiological significance of this sub-population of nuclear GR by demonstrating its correlation with glucocorticoid responsiveness in several glucocorticoid regulated cells. Further, we show by electron microscopy that the nuclear envelope GR is associated with nuclear pores, thus substantiating the involvement of this interaction in translocation of GR to the nuclear envelope. Unexpectedly, the GR associates with the nuclear envelope in dimers and tetramers, suggesting that translocation across the nuclear envelope occurs after dimer formation.
Materials and Methods

Chemicals

Unlabelled dexamethasone (dex) was from Steraloids, Inc. (Wilton, New Hampshire, U.S.A.). [3H]-dex (41 Ci/mmol), [3H]-dexamethasone mesylate (dexam-mes) (38-48.9 Ci/mmol), and unlabelled crystalline dex-mes were from New England Nuclear (Montreal, Quebec, Canada). Sucrose, ultra-pure, was from Schwartz-Mann (Cambridge, Massachusetts). Deoxyribonuclease I (DNase I; RNase free; shipped on dry ice), DTT, bovine serum albumin (BSA), TAPS (N-tris-(hydroxymethyl) methyl-amino-propanesulfonic acid) and trizma base were purchased from Sigma Chemical Co. (St. Louis, Missouri). Acrylamide, N,N'-methylene bis acrylamide, the reagents for the Bradford assay and all other chemicals for electrophoresis were from BIO-RAD (Mississauga, Ontario, Canada). Immobilon-P was obtained from Millipore (Mississauga, Ontario, Canada). The monoclonal anti-GR antibody BuGR2 was kindly donated by Dr. RW. Harrison, Rochester NY. Prestained protein standards (high range) for immunoblotting, enhanced chemiluminescence (ECL) kits and horseradish peroxidase-conjugated sheep anti-mouse IgG antibody were obtained from Amersham (Oakville, Ontario, Canada).

Animals

Male Sprague-Dawley rats weighing 200-250 g were obtained from Charles River Canada Inc. (Montreal, Quebec, Canada) and maintained on a diet of Wayne Lab. Chow (Allied Mills, Chicago, Illinois) and tap water ad libitum. Animals were housed and treated according to the guidelines of the Medical Research Council of Canada. The rats were killed by decapitation and the livers quickly removed and placed on ice. Adrenalectomy was performed by Charles
River Canada under ether anesthesia, and rats were subsequently maintained with 0.9% saline solution. Adrenalectomized animals were used for experiments 7 days after surgery. Another group of adrenalectomized rats received glucocorticoid replacement commencing 7 days after surgery. These rats were injected with dex phosphate (Decadron, Merck Frosst, Montreal, Quebec, Canada) for the indicated time (see figures and their legends) at a dose of 4 mg/kg, sc. The last injection was given 30-45 min before death.

Tissue Culture

The CEM cells were originally supplied by E.B. Thompson, University of Texas, Galveston, and were grown as stationary suspension cultures in RPMI 1640 medium (GIBCO, Burlington, Ontario, Canada) supplemented with complete (non-absorbed) 5% fetal calf serum (GIBCO). Cells were maintained in a tissue culture incubator at 37°C and a humidified atmosphere of 5% CO2/95% air at a density of 0.5 - 1.5 x 10^6 cells/ml. Where indicated, cells were pre-treated with 10^-6 M dex for 30 min in the tissue culture incubator (37°C). The cells were collected by low speed centrifugation, washed once with PBS (PBS, 140 NaCl, 3 mM KCl, 10 mM Na2HPO4 and 15 mM KH2PO4, pH 7.2) and finally fixed for electron microscopy (see below). After several rinses with PBS, the cells were processed for immunocytochemistry as described below. The wild-type (wt) S49.1 cell line (ATCC TIB 28) was obtained from the American Type Culture Collection (Rockville, Maryland). The nuclear transfer deficient S49 22R (nt-) was the kind gift of K. R. Yamamoto, University of California, San Francisco. The cells were grown in suspension in Dulbecco's Modified Eagles medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine calf serum in a humidified atmosphere containing 5% CO2/95% air at
37°C. Where indicated, cells were pretreated with 10⁻⁶ M dex for 1 h in the tissue culture incubator.

*Isolation of cytosol, nuclei and nuclear envelopes*

Nuclei from S49 cell lines were isolated by a modified procedure of Widnell and Tata (53) and nuclear envelopes were prepared from purified nuclei of S49 cell lines by a modification of the procedure of Kay et al. (54). All procedures were carried out as rapidly as possible at 0-4°C. The cells (1-3 x 10⁹) were harvested at 800 x g and washed two times in homogenization buffer (0.32M sucrose containing 3 mM MgCl₂.6H₂O and 1 mM DTT). The pellets were then resuspended in lysis buffer and the cells lysed by one cycle of freeze-thawing. The lysed cells were diluted 3-fold with homogenization buffer and homogenized in a Dounce homogenizer with 8 strokes of pestle A. The homogenate was used as the starting material for either cytosol or nuclei and nuclear envelopes.

Cytosol was prepared by centrifugation of the homogenate in a microfuge for 15 min. The recovered supernatant was the cytosol.

Nuclei from S49 cell lines were isolated and nuclear envelopes were prepared from purified nuclei by modification of procedures used previously (55). To isolate nuclei the homogenate was made up to 2.0 M sucrose using 2.4 M sucrose containing 1 mM MgCl₂.6H₂O and 1 mM DTT pH adjusted to 7.2 with NaHCO₃ and nuclei recovered after centrifugation at 50,000 x g for 65 min. Nuclei were resuspended in ice cold digestion buffer (10 mM Tris-HCl pH 8.5, 0.25 M sucrose, 0.1 mM MgCl₂.6H₂O and 0.5 mM β-mercaptoethanol). DNase I was added to a final concentration of 0.01 mg/ml. The digestion was carried out at room temperature until complete as judged by phase contrast microscopy (3-5 min), and was stopped by centrifugation at 33,000 x g for 6 min. The crude nuclear envelopes were resuspended in 10 mM Tris-HCl pH 7.4
containing 1 mM DTT and centrifuged at 33,000 x g for 10 min. The pellet was resuspended in 0.5 ml 10 mM Tris-HCl pH 7.4, 1 mM DTT and this suspension was layered onto a discontinuous sucrose gradient composed of 9 ml each of 2.0, 1.8 and 1.5 M sucrose and 4.5 ml of 0.25 M sucrose all prepared in 10 mM Tris-HCl pH 7.4 and 1 mM DTT. The gradients were centrifuged at 100,000 x g for 95 min. The band at the 1.8/1.5 M sucrose interface contained the purified nuclear envelopes. The nuclear envelopes were then washed by resuspension in 10 mM Tris, pH 7.4 containing 1 mM DTT and centrifuged at 33,000 x g for 10 min. Purified nuclear envelopes were then resuspended in a small volume of 10 mM Tris pH 7.4 containing 1 mM DTT. To investigate possible degradation during nuclear envelope preparation, the protease inhibitors leupeptin (10 \( \mu \)g/ml), soybean trypsin inhibitor (100 \( \mu \)g/ml) and benzamidine (10 mM) were included in the homogenization buffer and leupeptin (10 \( \mu \)g/ml) was added to the 10 mM tris buffer, pH 7.4, containing 1 mM DTT, used in later stages of preparation.

_Electron microscope immunogold labelling._

After excision, tissues were immediately fixed in ice-cold 1% glutaraldehyde or 4% paraformaldehyde containing 0.1% glutaraldehyde in 0.1 M phosphate buffer for 120 min, then dehydrated and embedded at -25°C in Lowicryl K4M. Ultrathin sections were stained with one of the steroid receptor antibodies against GR as indicated in the Results section and reacted with anti-rabbit or anti-mouse IgG labelled with colloidal gold 10 nm particles (Janssen, Amersham, United Kingdom). After counterstaining with uranyl acetate, the sections were examined by electron microscopy and photographed. Morphometric measurements and quantitation of gold particles was performed by using a PC Computer and Sigma Scan software (Jandel Sci., San Diego,
California). The specificity of the immunocytochemical staining was verified by absorbing the antibody with purified receptor or by substituting the receptor antibodies with pre-immune serum or non-specific IgG. Another type of control has been reported earlier in which the BuGR1 antibody was pre-absorbed with purified GR resulting in near complete absence of immunocytochemical signal. The data summarized in this paper represent approximately 80 tissue preparations.

**Chemical characterization of the nuclear envelope preparations**

DNA, 5'-nucleotidase and glucose-6-phosphatase were assayed as described previously (52). Protein content was determined by the Bradford protein assay (BIO-RAD) using BSA as a standard.

**Gel electrophoresis and immunoblotting**

SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described by Laemmli (56) in vertical slab gels consisting of a 4% acrylamide stacking gel and a 7.5% running gel. Cytosol from adrenalectomized rat liver was run as a positive control for staining of GR by the monoclonal anti-GR antibody BuGR1 (57). Pre-stained molecular weight protein standards were run on gels for blotting. The proteins were transferred electrophoretically from the gel in transblot buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 20% (v/v) methanol and 0.1% (w/v) SDS) onto an Immobilon-P membrane (58). The transblot cell was run for 4 h at room temperature at 26 V. The membrane was removed, rinsed in washing buffer, Tris buffered saline-Tween (TBS-T, 20 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.1 % (wt/vol) Tween-20) and blocked for 1 h at room temperature with 100 ml of 10% skim milk (59) made up in TBS-T. The membrane was rinsed briefly using 2 changes of washing buffer (TBS-T), then
incubated once for 15 min and twice for 5 min with fresh changes of washing buffer at room temperature with shaking. The membrane was then incubated overnight at 4°C with the primary antibody BuGR2 diluted 1:2,000 in TBS-T. After washing as before, the membrane was incubated for 1 h at room temperature with a horseradish peroxidase-conjugated sheep anti-mouse IgG antibody (1:50,000 in TBS-T). The immunodetection procedure was carried out according to instructions in the Amersham ECL western blotting kit.

*Quantification of crossreactivity on ECL western blots.*

The quantity of GR in subcellular fractions was estimated by quantification of GR crossreactivity on immunoblots using densitometry and from total volumes of each subfraction as described previously (60).

*Affinity labelling of nuclear envelopes*

Purified nuclear envelopes (100 µg protein/tube) were labelled with [³H]-dex-mes by incubation overnight at 0-4°C. Non-specific binding was determined by the addition of the affinity label to samples incubated with 1000-fold excess unlabelled dex for 16 h. The incubation was terminated following the addition of 1 ml of excess buffer by centrifugation for 1 min in a microfuge and aspiration of the supernatant. The washing was repeated twice. The pellets were dissolved by boiling for 5 min in 150 µl SDS-sample buffer and run on 7.5% SDS-PAGE. After staining, the gels were cut into 2 mm slices and dissolved in 150 µl of 30% hydrogen peroxide: 0.85 ammonia solution (19:1; v/v) by incubation for 2 h at 60-70°C. Following the addition of 10³ Units of catalase and 250 µl of 10% ascorbic acid (v/v) to the cooled tubes, the samples were counted in 10 ml of Ready Solv (Beckman, Canada).
Results

Association of GR with nuclear envelopes is hormone-dependent

We have previously demonstrated hormone-dependent localization of GR on rat liver nuclear envelopes (51). To confirm the correlation between steroid responsiveness and the interaction of GR with nuclear envelope in another glucocorticoid responsive system, we first investigated the presence of GR on nuclear envelopes and its regulation by glucocorticoid in subcellular fractions of the mouse lymphoma S49 cell lines. These cell lines were chosen for initial studies as they had been well characterized in relation to nuclear translocation of GR. The S49 wt cell undergoes cytolysis in the presence of glucocorticoid, an effect which as been shown to be mediated by GR. In contrast, a steroid-resistant S49 sub-line known as the nuclear transfer decreased mutant (nt⁻) contains GR that binds hormone but cannot bind to DNA with high affinity (61, 62). Like the wt, nt⁻ GR has a Mr of 94,000. Another S49 clone which proved useful in these investigations was the nuclear transfer increased (nt⁺) cell line which has a GR that binds hormone normally but which binds more tightly to DNA than wt receptor (61). The nt⁺ GR is a truncated receptor fragment of Mr 40,000 identical to the wt carboxyl terminus fragment which contains the hormone and DNA binding regions (63). In addition to these hormone binding GRs, each of the three cell lines also expresses a GR (nhb GR) with a mutation of Gly for Cln at position 546 in the hormone binding domain, which renders this mutant allele non-hormone binding (64).

Since specific interactions of wt GR with nuclear envelope components occurs after exposure of GR to hormone (51), concomitant with its gaining the ability to translocate into the nucleus, we reasoned that a defective interaction of the mutant nt⁻ GR with nuclear envelope might be correlated with its
defective nuclear translocation. The possibility of aberrant nuclear envelope association of the mutant nt\(^-\) GR was therefore investigated. An isolation scheme optimal for nuclear envelope preparations from wt, nt\(^-\) and nt\(^+\) cell lines was devised and the nuclear envelopes were characterized. Although the tumour cell lines varied greatly in the ease with which they were disrupted, the isolation procedure resulted in similar yields from all three cell lines. The chemical and biochemical properties of the preparations were also similar: they each contained DNA but in quantities less than 1% of the total cellular DNA and had undetectable amounts of the plasma membrane marker enzyme, 5'-nucleotidase. The total activity and specific activities of the endoplasmic reticulum marker, glucose-6-phosphatase, was insignificant (results not shown).

Immunoblots of S49 cell line subfractions before and after short-term exposure to dex detected the GR at approx 94 kDa (Fig. 1). Rat liver cytosol was used as the positive control for GR. It was not possible to reduce background labelling by immunoprecipitation of GR prior to western blotting, as attempts to detergent-solubilize nuclear envelope GR resulted in loss of detectable cross-reactivity. To establish whether any of the cross-reactive bands in the nuclear envelopes were degradation products, nuclear envelopes from the three cell lines were prepared in the presence of protease inhibitors (see Materials and Methods), but no differences were apparent in the immunoblot patterns. It is observable that in wt cells, exposure to dex caused a shift from cytoplasm to nucleus of the GR immunoreactive at 94 kDa, while in the nt\(^-\) cells, dex caused no such redistribution. However, in both cell lines, there was some association of GR with the nuclear subfraction in the absence of dex.
The results of densitometric scanning of the bands in Fig. 1 followed by normalization for the total protein of each fraction as described in Materials and Methods allowed quantification of the relative abundance of GR the nuclear envelope fractions (Fig. 2). Importantly, the relative abundance of wt GR in nuclear envelope subfractions increased by approx 2-fold in response to a 1 h exposure to dex. In contrast, in nt\(^{-}\) cells, in which some GR was also associated with the nuclear envelope in the absence of hormone, no increase of immunoreactive GR was observed in nuclear envelopes after dex treatment (Fig. 2).

Of note, is the prominent band detected by the ant-GR antibody at approximately 45 kDa in the nuclear subfractions on the nt\(^{-}\) cell line (Fig. 1B) and barely detectable in nuclear subfractions of the other cell lines (Fig. 1A and 1C) and in the control blot (Fig. 1D) which as mentioned above was not altered by protease inhibitors. Interestingly, in the nt\(^{-}\) cell line the antigen at this Mr responds to hormone identically as the 94 kDa band, i.e. the relative abundance is not increased in response to dex. In fact, like the 94 kDa band in this line, the relative abundance was decreased by approximately 2-fold in response to dex treatment (results not shown). Thus, it may be that this band represents a degradation product which is in greater abundance in the nt\(^{-}\) cell line because the mutation in this GR is more susceptible to protease degradation. We are currently investigating this possibility in experiments in which the allele is transfected singly into COS cells. However, since the response to hormone is identical to the 94 kDa entity, the formation of this degradation product does not alter the conclusion, i.e. that the nt\(^{-}\) GR associated with the nuclear envelope does not respond appropriately to hormone treatment.
Further, in the absence of dex, there was detectable GR associated with the S49 cell nuclear envelopes in both wt and nt⁻ cell lines. To determine whether the immunoreactive GR noted in the absence of dex was the nhb allele of GR, which would not be expected to be responsive to dex manipulation but which may be associated with the nuclear envelope, we first investigated nt¹ cell lines, in which the only immunoreactive band at 94,000 is the nhb GR. Calculations made from densitometric scanning (Fig. 2) revealed that a much higher proportion of the immunoreactive 94 kDa GR was associated with the nuclear fraction of the nt¹ in the absence of dex. Furthermore, nuclear envelope-associated GR was detected in the absence of hormone (Fig. 1). However, in contrast to the wt GR and as expected no up-regulation of this nhb GR occurred in response to hormone treatment (Fig. 2).

Finally, to confirm that the association of GR with the nuclear envelopes from the nt⁻ cell line in the absence of hormone is due to signal produced from the nhb allele, nuclear envelopes from wt and nt⁻ cell lines prepared after exposure to hormone were incubated with the affinity label [³H]-dex-mes. If the only GR being measured in the immunoblots of the nuclear envelopes from the nt⁻ cell line was the nhb GR, radioactive dex-mes should not be able to detect any GR in the nt⁻ cells. In preliminary experiments, it was established that cytosolic GR from nt⁻ cells bound dex-mes (results not shown). A typical profile of affinity labelled nuclear envelopes from the S49 cell lines after resolution of polypeptides by SDS-PAGE is shown in Fig. 3. The pattern of specific labelling of [³H]-dex-mes for the nuclear envelopes isolated from the wt cell line shows a peak of radioactivity (doublet) in the 90-100 kDa range which corresponds to the Mr of the GR. An identical pattern of labelling has been reported previously for rat liver nuclear envelopes (51). The labelling at lower molecular weights may be due to lower affinity dex binding sites which we also have previously
characterized in the rat liver (51). In marked contrast to the patterns obtained from affinity labelling of the wt cell line, there was no specific binding of dex- 
mes to nuclear envelopes isolated from the nt- cell line, thus confirming that lack of association of the nt- mutant GR and that the immunoreactive GR detected on nuclear envelopes from the nt- cells was the n hb GR.

In sum, these studies firmly established that glucocorticoid responsive GR associates with nuclear envelopes upon exposure to hormone. Two non-
glucocorticoid responsive GRs, the nt- and n hb GR, do not respond to glucocorticoid appropriately by increasing their association with the nuclear envelope. In fact, our data indicates that the only GR present on the nuclear envelopes from the nt- cell line is the n hb GR.

**GR is localized to nuclear pores of the nuclear envelope**

To further localize GR on nuclear envelopes we examined GR in rat liver by electron microscopy using colloidal gold particles. Figure 4 illustrates immunogold localization of GR in liver from a hormone replete animal. In cytoplasm, GR labelling was observed both as diffuse or in association with RER/polysomes and mitochondria. In the nucleus, labelling was mostly present in the loose chromatin and on the periphery of condensed chromatin. However in addition to the expected labelling of the cytoplasm, labelling of the nuclear envelope was also detectable. Most, but not all of the labelling of the nuclear envelope was on areas identifiable morphologically as the nuclear pores of the nuclear envelope. Structural criteria used to identify nuclear pores were based on previous established procedures (6). It should be noted that in the present study, due to the use of lowicryl, which favours antigen preservation rather than morphology, the structure of the pores is not as conspicuous as in classical electron microscopic morphological studies. However, this did not
prevent reproducible identification of nuclear pore sites. Gold particles were clearly associated with typical pores, either on the cytoplasmic or nuclear sides and sometimes within the pores themselves. In most cases one to four particles and rarely four or more, can be seen. In fact they are often in pairs.

*Nuclear pore associated GR is regulated by hormone*

We then investigated the hormonal regulation of nuclear pore associated GR in two systems: first, the rat liver, the classical glucocorticoid responsive tissue which expresses a high level of GR (17) and with which we had previously demonstrated glucocorticoid-regulated association of GR (51); second, the human leukemic, CEM cell lines where glucocorticoid-sensitive and -resistant subclones have been established ((65, 66); reviewed in (67). We attempted to examine S49 cells but this proved technically difficult probably due to low numbers of detectable GR molecules in these cells. We therefore investigated the CEM cell lines which we had extensively characterized with respect to nuclear translocation. Furthermore, these cells contain relatively high receptor levels (21).

In liver tissue, a quantitative assessment of labelling was obtained from micrographs of hepatocytes from intact and adrenalectomized as well as adrenalectomized animals to whom dex had then been administered (Fig. 5). With both antibodies, a shift of GR molecules to the cytoplasm was observed upon adrenalectomy. However, a redistribution to the nucleus occurred upon administration of ligand (Fig. 5). Both of these observations have been made previously (21). However, since quantitative assessment of a steroid receptor by electron microscopy is shown here for the first time it is relevant to note several observations. First GR levels are reduced by about half in the cytoplasm of adrenalectomized rats, and dex injection restores much of this. These effects
are essentially the same whether the BuGR1 or 14B2 antibodies are used. In the nucleus, the effect of adrenalectomy is more dramatic (4.4 versus 1.6 and 7.7 versus 2.8). Surprisingly, following dex injection, the receptor number is slightly higher than in intact rats (5.7 versus 4.4 and 11.8 versus 7.7). Glucocorticoid regulation of nuclear pore GR was clearly shown: the effect of adrenalectomy and of adrenalectomy followed by re-injection of hormone was more like that followed by nuclear GR. We extensively tested the specificity of labelling either by use of preimmune sera or by pre-absorption of GR antibodies with purified GR (68). In other experiments we omitted the primary antibody. In controls, labelling was either greatly reduced or absent. Quantitative gold particle counting revealed that controls displayed significantly less signal (p<0.001) than immune labelling.

Finally, glucocorticoid regulation of nuclear envelope labelling was evaluated in the CEM cells following a short exposure to dex (Fig. 6). The CEM-C7 cell line has two GR species: a normal and a mutant allele, while the 4R4 cell line has only the mutant allele (67, 69). The defective allele has two single point mutations: one in the GR steroid binding domain (leu753-phe753) (67, 69) and another in the proximal finger of the DNA binding domain (67, 69). the 4R4 GRs are 'activation labile' since they rapidly lose steroid during attempted activation (65, 70) The histograms show that already, after 1 h of dex treatment, a significant (p<0.01) increase in GR labelling was noted in the case of the steroid sensitive C7 cells. In contrast, in the steroid-resistant 4R4 cells, labelling was decreased following exposure to dex.

Again, it is important to note that in both of the systems used for electron microscopy, there is association of GR with nuclei (rat liver) and with nuclear envelopes (rat liver and CEM cell lines) in the absence of hormone. A non-hormone binding GR, GRβ, is known to be present in most mammalian cells
and this form may be responsible for the nuclear immunoreactivity in the absence of steroid. In the CEM-C7 cell lines, the defective Gr allele accounts for the nuclear envelope-associated GR in the dex-deprived state (compare wt and 4R4 nuclear associated GR in Fig. 6). The increase observed in the CEM-C7 cell line upon dex treatment may thus be an underestimate as the immunoreactive allele decreases its association with the nuclear envelope in response to dex. This would mask some of the increases association of the wt GR.
Discussion

Two issues basic to the mechanism of steroid receptor action have been addressed in this paper: first, the subcellular localization to nuclear pores of the nuclear envelope, and second, the glucocorticoid regulation of the GR-associated with the nuclear envelope, which argues for the importance of the interactions with this membrane system during the intracellular actions of glucocorticoids. Our findings of the tight control by hormone of nuclear envelope GR argues for the importance of GR interactions with this membrane system during the intracellular actions of glucocorticoids. Much debate and controversy have surrounded the topic of the exact location of steroid receptors (see [21, 24-26]). In this study, we have mapped the nuclear envelope GR binding site to specific subcellular domains, namely the nuclear pores. As the nuclear pores are the conduits for passage of molecules between the cytoplasm and nucleus, it seems likely that the nuclear envelope-associated GR represents a sub-population of GR captured in the process of trafficking between these two compartments. From findings reported here, this is a highly regulated process.

Very few studies (68) have examined the in situ distribution of steroid receptors (or any other transcription factors) by electron microscopy, a technique that can achieve the highest resolution and sensitivity, and these provided only a qualitative assessment of receptor levels. The present manuscript reports the localization of GR on nuclear pores. We found that approximately 6-10% of the cellular GR is associated with the nuclear envelope in the liver from an intact rat. Quantitative immunoelectron microscopic studies showed that upon adrenalectomy and then upon dexam administration to adrenalectomized rats, a redistribution of GR occurs. Similar effects were observed with two different GR antibodies. We have previously documented by Western blotting and affinity labelling with dex-mes (51), a dramatic decrease of
GR on nuclear envelopes isolated from adrenalectomized animals compared to nuclear envelope-associated GR from intact rats and a reinstatement of nuclear envelope GR following glucocorticoid administration to adrenalectomized animals (51). Thus, the electron microscope studies of the rat liver confirm these earlier studies of dex regulation of nuclear envelope associated GR in the rat liver and extend them to localized much of the nuclear envelope-associated GR to the nuclear pores.

Glucocorticoid regulation of nuclear envelope-associated GR was also studied in steroid responsive and steroid unresponsive cell lines. We first investigated nuclear envelope associated GR in subfractions of S49 cell lines by immunoblotting and binding of $[^3H]$-dex-mes. In the absence of dex, GR at 94,000 kDa is detectable on nuclear envelopes from all three cell lines. These results are consistent with a constitutive association of the nhb GR with the nuclear envelopes. Others have reported the constitutive nuclear association of a non-hormone binding GR in a mouse lymphosarcoma cell line (71). Importantly, in the wt, but not in the nt cell line, there was up-regulation by dex of nuclear envelope associated GR. Second, nuclear envelope associated GR was investigated in CEM-C7 cell line by immunoelectron microscopic studies. As for S49 cells, in CEM-C7 cells which had not been exposed to steroid, GR was detectable on the nuclear envelope. Since we detect nuclear envelop association in the absence of translocation, our observations are consistent with models of nuclear import in which separate mechanisms govern docking and translocation through pores (35). It is possible that the mutation in the non-hormone binding allele causes one or both of the GR NLSa to be exposed sufficiently to allow binding by the NLS binding complex for docking at the pore, but that the energy-requiring translocation across the nuclear envelope is not achievable.
Interestingly, we observed not only an inability of the nuclear envelope GR to increase in response to dex but a dex dependent decrease in association of the CEM-C7 4R4 mutant allele and of the S49 nhb allele with the nuclear envelope. As 4R4 GRs are able to bind hormone but are not capable of nuclear translocation (20, 21, 70), the decrease suggests that this mutant GR when bound to hormone, in the absence of productive nuclear translocation, is shifted away from the nuclear envelope and into the cytoplasm. In the case of the S49 nhb allele, we have shown that when transfected singly into COS cells which possess no GR, the subcellular distribution of the nhb allele is unresponsive to dex treatment (45). This finding suggests that the dex-dependent decrease in nuclear envelope associated nhb GR is the result of either a direct or indirect interaction between the nhb and hormone binding allele after dex treatment. The easiest model to test is one which involves a direct interaction between the two receptor forms. Although as yet unproven conclusively for GR, there is evidence for a DNA independent dimerization interface in nuclear receptors. Since the nhb allele docks at the nuclear envelope, this may suggest that the dimerization interface in that domain is also exposed. Like 4R4 cell line, in the absence of productive translocation, the nhb GR may shift to the cytoplasm.

In contrast to organelle-free cytoplasm, the nuclear envelope associated receptors are seen as clusters of two or more; the ratios of momomers: dimers/multimers in the cytoplasm compared to the nuclear envelope are respectively 5:1 and 1:8. The observation by immunogold electron microscopy of dimers or multimers of GR at the nuclear pores suggests further that GR may enter or exit the nucleus as a dimer. It is clear that formation of GR homodimers are required for high affinity binding to DNA (72, 73) and furthermore that homodimer formation can occur in solution in the absence of
DNA binding (74). However, the site of dimer formation within the cell remains unknown. Milgrom and his co-workers showed that the progesterone receptor is capable of forming dimers in the cytoplasm enabling a nuclear translocation deficient mutant receptor to gain access to the nuclear compartment by 'piggybacking' with a translocation competent progesterone receptor molecule (75). Our studies suggest that translocation competent receptors may also traverse the nuclear envelope as dimers. We and others have found a constitutive association of mutant GR with nuclear envelope. To date the physiological relevance of these interactions are unknown. Given our observations of nuclear envelope association of nhb alleles of GR, together with cytoplasmic and nuclear envelope multimers of GR, it would be possible to speculate that heterodimerization between hormone binding and nhb GRs may allow entry of the nhb GRs into the nucleus and more importantly, their modulation of transcriptional regulation by wt GR. This would be particularly interesting in the case of the human GR isoform β.

A final interesting observation in this study was the doublet of dex-mes labelled GR in the S49 cells. This doublet may be the result of a chemical modification of the receptor. GR is known to be a phosphoprotein ((76) and reviewed in (77)). Interestingly, phosphorylation has also been proposed a protein modification involved in nuclear import (see (1) and in light of the identification of a GR doublet in association with the nuclear envelope, phosphorylation needs to be considered as a requirement for nuclear entry of the of the receptor.

In summary we have verified that an important nuclear site of interaction of GR is the nuclear pore of the nuclear envelope. Further investigations of the role of this GR nuclear retention site should shed light on the mechanism of
nucleocytoplasmic trafficking of GR and could provide further insight into mechanisms of steroid resistance.
Acknowledgments

This work was supported by grants to T.A. and Y.A.L. from the Medical Research Council of Canada and the National Cancer Institute of Canada. We also thank Xudong Wang, Alice Parissi and Margaret Connors for technical assistance and Patricia Ciesla for secretarial help.
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Legends to Figures

Fig. 1. Hormone-dependent subcellular distribution of immunoreactive GR in subcellular fractions of S49 mouse lymphoma cell lines. Confluent wt, nt\textsuperscript{-} and nt\textsuperscript{1} cells were grown in serum-free media for 16-18 h and then incubated for 1 h at 37°C in the presence or absence of 10\textsuperscript{-6} M dex. Cytosolic, nuclear and nuclear envelope fractions were isolated as described in Materials and Methods. Protein (100 μg/lane) from each fraction was resolved by 7.5% SDS-PAGE and transferred to an Immobilon-P membrane. The membrane was incubated with the monoclonal anti-GR antibody BuGR2 (1:2,000) overnight at 4°C. After washing, the bound antibody was detected with a horseradish peroxidase-conjugated sheep anti-mouse IgG antibody by ECL. Panels A, B and C are immunoblots of fractions from wt, nt\textsuperscript{-} and nt\textsuperscript{1} cells respectively, incubated in the absence (lanes 2-5) or presence (lanes 6-9) of dex. Lane 1, cytosol from adrenalectomized rat liver used as a positive control; lanes 2 and 6, homogenate; lanes 3 and 7, cytosol; lanes 4 and 8, nuclei; lanes 5 and 9, nuclear envelope. Panel D is a control blot of Panel A in which no primary antibody was used.

Fig. 2. Hormone dependent increase in nuclear envelope-associated GR in glucocorticoid responsive cells but not in glucocorticoid unresponsive cell lines. Values for nuclear envelope associated GR prepared from wt, nt\textsuperscript{-} and nt\textsuperscript{1} S49 cells lines before (-) and after (+) dex treatment were determined from crossreactivity on immunoblots using densitometry and from total volumes of each subfraction as described previously (60). Three identical experiments were performed. These results were obtained from the representative experiment shown in Fig. 1.
Fig. 3. Affinity labelling of nuclear envelopes from the S49 mouse lymphoma cell lines. Nuclear envelopes (100 µg) were incubated with 167 nM [3H]-dex-mes for 16-18 h at 0-4°C in 25 mM TAPS, pH 8.6, to determine total binding. Non-specific binding was determined by the addition of [3H]-dex-mes to nuclear envelopes incubated for 16-18 h with 167 µM unlabelled dex-mes. The assay was terminated and the nuclear envelopes were dissolved in SDS-sample buffer and resolved on a 7.5% gel. The gel was cut into 2-mm slices, dissolved and the radioactivity was determined.

Fig. 4. Immunogold localization of GR in ultrathin sections from hepatocytes. Immunolabelling was performed as described in Materials and Methods. Note several gold particles associated with the nuclear pores (P) (arrows). (A) is a general view showing labelling on the pores as well as in the cytoplasm and nucleus. N, nucleus; rer, endoplasmic reticulum. (B)-(D) display higher magnification of nuclear pore areas showing several gold particles located on either side of the pore structure. Arrow heads in panel (B) point to the double layer of the nuclear envelope. (x 120,000 for B-D). (B')-(D') are line drawings of (B)-(D). (E) is a control preparation in which the GR antibody was absorbed by pure GR (see Materials and Methods). Note near complete absence of labelling. An occasional gold particle is shown by arrow. (x 80,000)

Fig. 5. Quantitative analysis of GR associated with nuclear envelopes using immunogold labelling. Gold particles were counted from electron micrographs of rat liver as described in Materials and Methods. Twenty micrographs of each experimental condition were analysed at a final magnification of 47,000 X. Two different antibodies were tested, BuGR1 (57) and 14B2 (21). Numbers
shown represent gold particles per 10 μm of nuclear envelope length. Data are means ±S.E.M. of counts from the 20 micrographs.

Fig. 6. Histograms demonstrating the effect of dex treatment on nuclear envelope labelling in CEM-C7 and CEM-4R4 cell lines. Quantification of gold particles in nuclear envelopes in electron micrographs magnified at 40,000 X was performed using a morphometric device (see text) and data expressed as number of gold particles per 10 μm nuclear envelope. Data is displayed as mean ± S.E.M. and statistical significance is indicated (* = p < 0.05, ** = p < 0.01, *** = p < 0.005) as compared to controls (n=20 micrographs).
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