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Selective Binding of Steroid Receptors to Octamer Transcription Factors Determines Transcriptional Synergism at the Mouse Mammary Tumor Virus Promoter: A Molecular Mechanism for Transcription Factor Recruitment to Promoter DNA.

Gratien G. Préfontaine

Thesis submitted to the Department of Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry.

University of Ottawa

Ottawa, Ontario, Canada

May, 2001

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ABSTRACT

The glucocorticoid receptor (GR) and octamer transcription factors -1 and -2 (Oct -1/-2) function synergistically to activate gene transcription from the Mouse Mammary Tumor Virus (MMTV) promoter. Mechanisms responsible for the transcriptional synergy have not been characterized. I demonstrated a protein-protein interaction between rat GR and human Oct-1/-2 *in vivo*, and showed the interaction was sensitive to GR point mutations C500Y and L501P. This interaction correlated with the recruitment of Oct-1/-2 to promoter DNA and appeared to contribute at least in part to the transcriptional synergy observed. Based on this observation, a molecular mechanism was proposed that would be expected to restrict gene transcription to regulatory regions containing binding sites for both factors.

The direct protein-protein interaction with GR and Oct-1/-2 mapped to the Octamer factor homeodomains suggesting the potential for a broadly based interaction for homeodomain proteins. Previously, *in vitro* binding studies had identified several nuclear hormone receptors with the potential to bind to the POU domain of octamer factors. However *in vivo*, only the GR, the progesterone receptor (PR) and the androgen receptor (AR) appeared to have the potential to interact with octamer factors physically and functionally through their DNA-binding and hinge domains. In contrast, the mineralocorticoid receptor (MR) failed to interact. These steroid receptors can activate transcription through common hormone response elements (HREs) but they perform distinct physiological functions by regulating unique target genes. In transient transfection assays, I demonstrated that these steroid receptors could activate transcription from the MMTV promoter to similar levels. However, differential modes of

gene regulation were employed by individual steroid receptors. Transcription mediated by GR and PR was dependent on the octamer motifs while that mediated by MR and AR was octamer motif independent. The configuration of the MMTV HREs was restricted to GR- and PR- mediated transcription but octamer factor recruitment to DNA permitted gene transcription from the MMTV promoter. These results suggest that the configuration of the HREs on the MMTV promoter determine steroid receptor-specific transcriptional responses.

DEDICATION

This thesis is dedicated to my parents Guy and Alice for their unconditional love and support through what seems to have been a long time. I would like to give a special mention to all my friends in Ottawa who became a very large part of my family over the years.

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ABBREVIATIONS

11-β-HSD-II .... 11-beta-hydroxy steroid dehydrogenase II

aa .... amino acids

ACF .... ATP-ulitizing chromatin assembly and remodelling factor

ACTH .... adrenocorticotropic hormone

ACTR .... activator of TR and RAR

AF .... activation function

AIB1 .... steroid receptor co-activator amplified in breast and ovarian cancer

ald .... aldosterone

aldosterone 18-aldocorticosterone

AML-1 .... acute myelogenous leukemia-1

AP-1 .... activator protein-1

AR .... androgen receptor

ARA .... AR-associated protein

ARC .... activator recruited complex

ATF .... activating transcripton factor

ATP .... adenosine triphosphate

AVP .... arginine vasopression

bHLH .... basic helix-loop-helix

Brg1 .... brahma related gene 1

BRL .... Bethesda Research Laboratories

BSA .... bovine serum albumin

bZIP .... basic leucine zipper

CAT .... chloramphenicol acetyltransferase

CBP .... CREB binding protein

CHO .... Chinese Hampster ovary
CHRAC .... chromatin accessibility complex
cHRE .... complex HRE
CoA .... cofactor A
cpm .... counts per minute
CREB .... cyclic AMP response element binding protein
CRF .... corticotropin releasing factor
CT .... calf thymus
C-terminus .... carboxy terminus
dATP .... deoxyadenosine triphosphate
DBD .... DNA-binding domain
dd .... doubly distilled
DEAE .... diethylaminoethyl
dex .... dexamethasone
dexamethasone .... 1,4-Pregnadien-9α-fluoro-16α-methyl-11β,17,21-triol-3,-20 dione
DHT .... dihydrotestosterone
dihydrotestosterone 17 beta-hydroxy-5alpha-androstan-3-one
DMEM .... Dulbecco's modified eagle medium
DMSO .... dimethyl sulfoxide
DNA .... deoxyribonucleic acid
DNA-PK .... DNA-dependent protein kinase
dNTP .... deoxynucleotide triphosphate
DR .... direct repeat
DRIP .... vitamin D receptor interacting proteins
DSE .... distal sequence element

DTT .... dithiolthreitol

ECL .... enhanced chemiluminescence

EDTA .... ethlyenediaminetetraacetic acid

EMSA .... electrophoretic mobility shift assay

enh2 .... enhancer region 2

ER .... estrogen receptor

ERE .... estrogen response element

exo .... exonuclease

FBS .... fetal bovine serum

FRAP .... fluorescence recovery after photobleaching

FT .... freeze-thaw

ftz .... fushi tarazu

GFP .... green fluorescent protein

GnRH .... gonadotropin releasing hormone

GR .... glucocorticoid receptor

GRIP .... GR-interacting protein

GST .... glutathione S transferase

HA .... flu hemagglutanin

HAT .... histone acetyltransferase

HBS .... HEPES buffered saline

HCF .... host cell factor

HDAC .... histone deacetylase

HEPES .... 4-(2-hydroxylethyl)-1-piperazine ethanesulfonic acid
HMGI .... high mobility group protein I
HP1 .... heterochromatin protein 1
NP40 .... nonidet P-40
HPA .... hypothalamo-pituitary-adrenal axis
HRE .... hormone response element
NRE .... negative regulatory element
HRP .... horse radish peroxidase
hsp .... heat shock protein
HSV .... herpes simplex virus
IAP .... intracisternal A particle

^{35}S-Met .... ^{35}Sulfur methionine

NR .... NR

NURF .... nucleosome remodelling factor

OCA-B .... octamer transcription factor co-activator-B

Octs .... octamer transcription factors

Oct-1 .... octamer transcription factor-1

Oct-1/-2 .... octamer transcription factors -1 and -2

Oct-2 .... octamer transcription factor -2

ONPG .... o-nitrophenyl-β-D-galactose

p/CAF .... p300/CBP associated factor

p/CIP .... p300/CBP interacting protein

PAGE .... polyacrylamide gel electrophoresis

PBS .... phosphate buffered saline

PCR .... polymerase chain reaction

PGC-1 .... PPAR-γ co-activator -1

PKA .... protein kinase A

plfG .... proliferin gene

PMSF .... phenylmethylsulfonyl fluoride

pol II .... polymerase II

POMC .... pro-opiomelanocortin

POU .... Pit, Oct and Unc

POUhd .... POU-homeodomain

POUsp .... POU-specific domain

PPAR .... peroxisome proliferator-activated receptor

PPO 2,5-diphenyloxazole

PR .... progesterone receptor

PSE .... proximal sequence element

R .... repression domain

RAC .... receptor associated coactivator

RAR .... retinoic acid receptor

RBCC .... RING finger, B boxes, coiled-coil

RIP .... receptor interacting protein

RNA .... ribonucleic acid

RSV .... Rous Sarcoma Virus

RT-PCR .... reverse transcriptase-PCR

RXR .... retinoid X-related receptor

SC .... synergy control

SDS .... sodium dodecylsulfate

SDS-PAGE .... sodium dodecylsulfate-polyacrylamide gel electrophoresis

SMCC .... SRB/mediator cofactor complex

SMRT .... silencing mediator of retinoid and thyroid receptors

sn .... small nuclear

SNAPc .... snRNA activating protein complex

SNF2 .... sucrose non-fermentable 2

SPR .... surface plasmon resonance

SRC .... steroid receptor co-activator

STAT .... signal transducers and activators of transcription

SV40 .... simian virus 40

SWI2 .... switch 2

TAF1 .... transactivation function -1

TAFs .... TBP-associated factors

TBE .... tris-boric acid EDTA

TBP .... TATA-binding protein

TBS .... Tris based saline

TBS-T .... Tris buffered saline-Tween

TCRα .... T-cell receptor α

TF .... transcription factor

TIF .... transcriptional intermediary factor

TK .... thymidine kinase

TLC .... thin layer chromatography

TR .... thyroid hormone receptor

TRAM-1 .... thyroid hormone activator molecule

TRAP .... thyroid hormone receptor associated proteins

TRIP-1 .... thyroid hormone interacting protein

VDR .... vitamin D receptor

VP16 .... viral protein 16

α-MEM .... α-minimal essential media

β-gal .... β-galactosidase

SWI2 .... switch 2

TAF1 .... transactivation function -1

TAFs .... TBP-associated factors

TBE .... tris-boric acid EDTA

TBP .... TATA-binding protein

TBS .... Tris based saline

TBS-T .... Tris buffered saline-Tween

TCRα .... T-cell receptor α

TF .... transcription factor

TIF .... transcriptional intermediary factor

TK .... thymidine kinase

TLC .... thin layer chromatography

TR .... thyroid hormone receptor

TRAM-1 .... thyroid hormone activator molecule

TRAP .... thyroid hormone receptor associated proteins

TRIP-1 .... thyroid hormone interacting protein

VDR .... vitamin D receptor

VP16 .... viral protein 16

α-MEM .... α-minimal essential media

β-gal .... β-galactosidase

I. INTRODUCTION

1. Overview

Gene transcription is controlled by complex transcriptional regulatory regions in and around genetic units that contain multiple binding sites for sequence-specific transcription factors (1). Sequence-specific transcription factors act directly and indirectly to recruit the general transcriptional machinery, including RNA polymerase II (pol II) to promoter DNA (2). The regulatory potential of these transcription factors and general transcriptional machinery is determined by chromatin structure (3, 4). Sequence-specific transcription factors may be ligand inducible like the GR (5), a NR, or may act constitutively like octamer transcription factor-1 (Oct-1) (6), a homeodomain containing protein.

Members of both the NR family and homeodomain proteins can function together to modulate gene transcription. Both synergistic (7-20) and inhibitory (15, 16, 21-26) interactions have been observed. The rate of gene transcription is determined by the combination of synergistic and inhibitory interactions among regulatory proteins brought directly or indirectly to DNA (2). Many inhibitory interactions interfere with the events leading up to the assembly and elongation events by RNA pol II and act by decreasing the rate of gene transcription. Synergistic transcriptional regulation is defined as the transcriptional activity contributed by two factors being more than the sum of the activity of each factor individually (27). Events leading to transcriptional synergy rely on the precise arrangement of sequence-specific transcriptional activators to their recognition

sites at enhancer/promoter regions to generate a network of protein-protein and protein-DNA interactions.

Activation of gene transcription can be divided into 2 separate events that appear to happen sequentially. First, sequence-specific transcription factors are assembled on enhancer/promoter regions (28). They may act by recruiting factors to manipulate or remodel DNA in a manner that facilitates, permits or restricts access of other sequence dependent transcription factors to their DNA recognition sites (29, 30). The binding of sequence-specific transcription factors to DNA aligns multiple protein-protein interaction surfaces complementary to the interaction surfaces of the general transcriptional machinery (27). In a second event, the general transcription machinery is recruited and positioned at the promoter through interaction surfaces (2). Transcription complex assembly is initiated by the binding of TATA-binding protein (TBP) to promoter DNA. Each sequence-specific transcription factor alone bound to DNA may display some protein-protein interaction surfaces acting to recruit the general transcriptional machinery or induce chromatin remodelling. Only the precise combination of transcription factors bound to DNA will optimally activate gene transcription and these are dictated by the DNA sequences of enhancer/promoter regions.

There are several examples of protein-protein interactions between two sequence specific transcription factors that can result in cooperative DNA-binding and transcriptional synergy (31-35). Some of these events occur through individual DNA-binding domains of heterologous transcription factors (35). Moreover, inducible transcription factors can modulate the DNA-binding activity of constitutive transcription factors, converting their constitutive activity into an induced response (7).

The MMTV long terminal repeat (LTR) has been utilized extensively as a model system for studying gene transcription (17, 36-43). In my work, I have employed the MMTV LTR as the enhancer/promoter system for studying the role of the GR and Oct-1 cooperativity in the induction of gene transcription. Synergistic activation of MMTV transcription by steroid hormone correlates with octamer motif occupancy on the viral promoter (17, 43). The minimal requirement for transcriptional synergy was identified as the juxtaposition of binding sites for GR and Oct-1. The juxtaposition of the DNA binding sites appears to be the sole requirement for the transcriptional synergy because the synergy could be reproduced on a synthetic promoter containing binding sites for both GR and Oct-1 (16).

GR is a ubiquitous, hormone-activated sequence-specific transcription factor and the founding member of the NR superfamily (5). Oct-1 is a constitutively active sequence-specific, ubiquitously expressed transcription factor and a founding member of the POU subfamily of homeodomain-containing developmental regulators (44).

One of the first events in gene activation is the assembly of sequence-specific transcription factors on enhancer/promoter DNA (28). Often the assemblies of multiple factors to enhancer/promoter DNA can be facilitated by direct protein-protein interactions between individual transcription factors and have been shown to stabilize the DNA binding function of each factor. Alternatively, in the absence of a direct protein-protein interaction, one factor binds to DNA which indirectly results in modifying DNA structure to accommodate the binding of a second factor (39).

During the course of my Master's thesis (45), I demonstrated that GR could interact with Oct-1 *in vitro*, through a protein-protein interaction. This protein-protein

interaction mapped to the highly conserved DNA binding domain of GR and to the highly conserved POU domain of Oct-1. I have hypothesized, for the present study, that the protein-protein interaction between GR and Oct-1 promotes the cooperative binding of these factors to the MMTV promoter and is responsible for at least a major component of transcriptional synergy between GR and Oct-1 in activating the MMTV LTR, *in vivo*.

The main objectives of this study were to characterize this specific protein-protein interaction between GR and Oct-1 *in vivo* under near physiological conditions, and to determine the extent to which the protein-protein interaction was responsible for promoting transcriptional synergy between GR and Oct-1, *in vivo*. Following the observation that the GR/Oct-1 protein-protein interaction mapped to highly conserved regions within each protein, I also sought to determine whether the interaction could be broadly based between other family members of the NR family and other POU/homeodomain containing factors.

2. Gene transcription by RNA pol II

(a) Basal transcription

Transcriptional activation of eukaryotic genes during development or in response to extracellular signals is regulated by complex interactions between a large number of sequence-specific transcription factors that act in a highly specific manner to recruit RNA polymerases and associated factors to transcribe specific genes (2). The basic RNA pol II transcriptional machinery is composed of two components, the TBP complex and the RNA pol II holoenzyme (46).

In most cases the assembly of the transcription complex is initiated by the binding of TATA binding protein (TBP) to TATA box containing promoters, an AT-rich element usually located within promoters, –32 to –25 base pairs relative to the transcription initiation site (47). TBP may function alone or within the transcription factor (TF) IID complex, which consists of TBP and at least eight TBP-associated factors (TAFs) (48). TBP is required for basal levels of gene transcription, while the TAFs are required for activated levels of transcription. Using purified factors, RNA transcription by RNA pol II can be reproduced *in vitro* when assembled in a step-wise fashion (2, 49). TFIIA and TFIIB bind to and stabilize the complex between TFIID and the TATA-containing promoter. Pre-formed TFIIF-RNA pol II complex is recruited by the TFIIF moiety to the TFIIA-TBP-TFIIB –complexed promoter. Finally TFIIE and TFIIH are recruited into the complex. TFIIH contains two helicase subunits and a kinase–cyclin complex (50). The assembly of these components on promoter DNA in a "closed" (inactive) complex is considered the minimum requirements in the formation of the preinititation complex. In an adenosine triphosphate (ATP)-dependent fashion, the catalytic activity by TFIIH converts the preinitiation complex into an inititation complex by the formation of an "open" (active) conformation with synthesis of the first phosphodiester bond of the nascent transcripts (51). Following the initiation of transcription RNA pol II moves away from the general initiation factors after synthesizing 10-15 nucleotide long transcript in a step referred to as promoter escape. The carboxy terminus (C-terminus) of RNA pol II becomes hyperphosphorylated and promotes RNA transcriptional elongation. In total, these factors constitute the minimum requirements for accurate transcription *in vitro*.

(b) RNA pol II holoenzyme

In vivo, RNA pol II is thought to occur in high molecular weight holoenzyme forms that are composed of the core RNA pol II, most of the general transcription factors (e.g. TFIIB, TFIIF and TFIIH) and many other factors including a subcomplex termed the mediator complex (52). Alone, RNA pol II holoenzyme is incapable of promoter recognition because it lacks TFIID. Holoenzyme preparations contain most of the transcription factors necessary for accurate initiation of transcription when assembled on core promoters together with TFIID.

Related holoenzyme preparations have been described that contain some or all of the general transcription factors, mediator complexes, DNA-remodelling complexes, DNA repair proteins and splicing and polyadenylation factors (53-61). The mediator complex has been shown to relay biological signals from activators to the polymerase in order to activate transcription (62). Independently the mediator complex, containing over 20 peptides, was identified by several independent groups and therefore has been alternatively named thyroid hormone receptor associated proteins (TRAP) (63), vitamin D receptor interacting proteins (DRIP) (64), activator recruited complex (ARC) (65) and SRB/mediator cofactor complex (SMCC) (66).

(c) Sequence-specific transcription factors

Sequence-specific transcription factors act through binding to specific DNA sequences that occur in promoter proximal and distal transcriptional regulatory regions (enhancers) (1). They act in at least two ways. First, they can interact directly with multiple interaction surfaces of TFIID and RNA pol II holoenzyme to recruit these complexes directly to promoters (67). Alternatively, sequence-specific transcription

factors modulate gene transcription indirectly through recruitment of intermediary chromatin remodelling factors (29) or co-activator/co-repressor molecules (30). It is presently hypothesized that the correct combination of activators and co-activators will recruit basal transcription factors to provide an efficient docking area for RNA pol II, although the precise details of these interactions remain to be resolved.

Some activators can act through multiple levels including recruitment of TFIID and the RNA pol II holoenzyme. For example, multiple copies of the transcription factor viral protein 16 (VP16) can act together by stabilizing the assembly of the initiation complex whereby one molecule stabilizes TFIID binding while the other enhances the stability of TFIIB binding (68-71). In addition, it is believed that multiple contacts formed between general transcriptional machinery and transcriptional activators stabilize the binding of the sequence-specific activators within transcriptional regulatory regions (72). Studies show that the complex of TFIIA, TFIIB, TFIID and other co-activators had reciprocal effects on sequence-specific activators' stability, enabling them to survive challenge by competitor oligonucleotides (73).

Most transcriptional regulatory regions are complex and contain binding sites for many sequence specific transcription factors. These factors interact with each other in multiple ways to determine transcriptional responses to generate a network of protein-protein and protein-DNA interactions (27). Viral induction of the interferon-β gene provides one of the best-characterized examples of combinatorial interactions of factors binding to distinct regulatory elements (74). The enhancer region contains four distinct positive regulatory domains including binding sites for the sequence specific transcription factors nuclear factor -κB (NF-κB), interferon regulating factor (IRF) -1, activating

transcription factor (ATF) -2/cJun and high mobility group (HMG) I. Individually, neither of these positive regulatory elements functions to transmit the viral induction signals. Two or more regulatory elements encourage cooperative binding of activators in response to induction signals, but only the precise alignment of all factors on the DNA allow for the optimal level of transcriptional induction.

(d) Chromatin remodelling factors

The regulatory potential of individual sequence-specific transcription factors is determined by chromatin structure. Chromatin may positively influence gene transcription by bringing distantly spaced binding sites closer to the core promoter or by facilitating the assembly of multiple transcription factors on DNA through beneficial alterations in DNA structure that can occur when DNA is folded into a nucleosome (43, 75). However, usually chromatin acts to repress transcription due to the occlusion of transcription factor binding sites as a result of folding DNA around the nucleosome core and through compression of nucleosomes into heterochromatin structures, a transcriptionally silent condensed form of chromatin (4, 76). Some sequence-specific transcription factors interact directly with chromatin remodelling factors and recruit them to enhancer/promoter regulatory regions. In an ATP dependent manner, chromatin remodelling factors manipulate chromatin in a way that alters DNA accessibility to sequence-specific and general transcription factors (77). Thus, chromatin remodelling can directly promote the transcription factor loading required for increased probablility of gene transcription (78, 79).

Genetic studies in S. *cerevisiae* have identified a chromatin remodelling complex, SWI2 (switch 2) /SNF2 (sucrose non-fermentable) that is essential for transcription by

many sequence-specific transcription factors (80). The SWI2/SNF2 complex contains 11 known subunits that form a stable complex of two MDa in size (81) with intrinsic ATPase activity that functions at least in part by using energy from ATP hydrolysis to remodel chromatin (82). The first link of the SWI/SNF complex to chromatin remodelling with gene transcription originated from the observation that mutations in SWI factors could be rescued by mutations that alter SIN1 or SIN2 genes (83), encoding an HMG-1 like protein and histone H3, respectively. Likewise, deletion of the HTA1 and HTA2 cluster, which encodes one of the two copies of the histones H2A and H2B, restores the expression of the SUC2 gene in the absence of SWI2/SNF2 (84). Mammalian cells have homologues of these factors that are also essential for many transcriptional events. The human brahma related gene (*Brg1*) is an example of a gene-specific and cell-type-specific factor implicated in chromatin remodelling (85). Exogenous expression of *Brg1* in cells potentiates the activity of transcription factors that recruit it to DNA while having marginal effects on other factors that do not interact with Brg1. More recently ISWI, a homologue of brahma has been identified in *Drosophila* (86) and two homologues identified in human, termed hSNF2H and hSNF2L (87, 88). The ISWI ATPase acts as a component of at least three different chromatin complexes in *Drosophila* with distinct chromatin remodelling activities termed nucleosome remodelling factor (NURF) (89), ATP-utilizing chromatin assembly and remodelling factor (ACF) (90) and chromatin accessibility complex (CHRAC) (91). By and large, the gene specificity and complementarity of these distinct chromatin remodelling activities remains to be determined.

(e) Co-activators and co-repressors

Sequence-specific transcription factors also modulate gene transcription by recruiting co-activator and co-repressor molecules (30). Co-activators are believed to be components of protein complexes that contain activity which covalently modify elements of chromatin or other transcription factors through acetylation, phosphorylation, ADP-ribosylation, methylation and ubiquitination (92).

SRC-1, is a member of the p160 family of co-activators identified by their ability to reverse the effects of transcriptional squelching between two steroid hormone receptors (93). At least three distinct members of the p160 family have been identified including steroid receptor coactivator-1 (SRC-1) (93)/NR coactivator (NCoA)-1 (94), SRC-2/TIF2 (95)/ GR interacting protein (GRIP1) (96)/NCoA-2 (97) and SRC-3/ p300/CBP interacting protein (p/CIP) (97)/ activator of thyroid hormone receptor (TR) and retinoic acid receptor (RAR) (ACTR) (98) / receptor associated co-activator (RAC)-3 (99) / steroid receptor co-activator amplified in breast and ovarian cancer (AIB1) (100) / thyroid hormone activator molecule (TRAM)-1 (101). Each can also exist as multiple splice variants (102). Through intrinsic histone acetylase activity p160 co-activator modifies chromatin structure by acetylating or deacetylating specific lysine residues in the N-terminal tails of histones (103). Acetylation is believed to lead to a more open chromatin structure by relaxing the interaction of histone tails with DNA (104). Alternatively, acetylated lysine tails could serve as recognition sites for chromatin remodelling factors. For example, acetylated lysines of histones have been shown to interact directly with the bromodomain of brahma (105).

The p160 family of co-activators also recruits other co-regulatory molecules with Histone acetyltransferase (HAT) activity including p300/C/EBP binding protein (CBP) (94) and p300/CBP associated factor (p/CAF) (106). The closely related p300/CBP proteins can also bind independently from p160 to sequence-specific transcription factors (107). Together p300/CBP and p160 co-activators can enhance transcriptional activation synergistically (108). Furthermore, p300/CBP and SRC-1 can interact independently with P/CAF, suggesting they have the capacity to form large complexes (106). Gene activation by specific transcription factors utilizes the HAT activity from specific co-activators. For example, although both SRC-1 and P/CAF are recruited to the promoter by the RAR, P/CAF HAT activity is required for gene activation while the SRC-1 HAT activity is dispensable (109). By contrast CBP acetyltransferase activity may be required for the modification of other non-histone factors like p53 (110) and GATA-1 (111).

Some of these co-activator molecules are involved in the transcriptional regulation of multiple signaling pathways. The CBP/p300 family of transcriptional integrators can interact physically and functionally with various transcription factors such as activator protein -1 (AP-1), Myb, signal transducers and activators of transcription (STAT)-1 and NRs (94, 112-114). However, because the number of co-integrator complexes are limited, simultaneous induction of multiple signaling pathways utilizing these complexes results in cross-talk between pathways (94, 112).

The counterpart to co-activators are co-repressors, molecules that can act to recruit histone deacetylases (HDACs) to deacetylate histone lysines residues (115). Two classes of co-repressor molecules have been identified: the nuclear hormone receptor co-repressor (N-CoR) (116), including the silencing mediator of retinoid and thyroid

receptors (SMRT) family of proteins (SMRT (117), SMRTer (118), SUNCoR (119), and Alien (120)); and transcriptional intermediary factor-1 (TIF-1) protein family (121-123). The N-CoR/SMRT co-repressors exist as complexes with HDACs. At least two different complexes containing N-CoR/SMRT co-repressor molecules have been identified in mammalian cells, N-CoR-1 and N-CoR-2 (124). N-CoR-2 contains HDAC 3, chromatin remodelling factors including components of the SWI2/SNI2 complex and other factors typically associated with heterochromatin, like Krab-associated protein -1 (KAP-1). N-CoR-1 contains different HDAC components including HDAC 1, 2 and Sin3A. Co-repressors function by modification of chromatin structure, specifically N-CoR/SMRT function has been correlated with the recruitment of HDAC complexes and histone deacetylation.

The function of the TIF-1 family of proteins is poorly understood. These proteins contain two conserved regions: an N-terminal RING finger, B boxes, coiled-coil (RBCC) motif and a C-terminal region containing a PHD finger and a bromodomain (122). TIF-1β has been shown to associate with members of the heterochromatin protein -1 (HP1) family of heterochromatin-mediating silencing proteins in *Drosophila* (121). Thus to date, the co-repressors that have been characterized appear to function at the level of chromatin to promote more stable DNA/histone interactions and formation of heterochromatin acting directly to counter the effects of the HAT co-activators.

3. Transcriptional synergy

Transcription from most eukaryotic promoters is low, likely because basal transcription factors have only a relatively weak affinity for promoter sequences. Typically strong transcriptional responses require the convergence of the multiplicative

action of several activators bound at distinct sites in the enhancer/promoter (27). This amplification of signals through multiple interactions is referred to as transcriptional synergy. Precise mechanisms responsible for transcriptional synergy are complex and involve many levels of regulation. However, although the precise mechanisms underlying these events are unknown, hypotheses that may explain transcription synergy are beginning to be proposed.

Transcriptional synergy can be subdivided into two classes based on inherently distinct mechanisms. In Class I, transcriptional synergy is proposed to occur in two steps, first the binding of one factor to DNA initiates events leading to the recruitment of chromatin modifying factors which manipulate the DNA in a manner that influences the binding of subsequent factors. This mechanism is independent of a direct protein-protein interaction between individual transcription factors. One of the most convincing examples to date is the cooperativity between steroid receptors (including GR) and nuclear factor 1 (NF-I) on the MMTV promoter (39). In the absence of steroid, NF-I binding to MMTV is undetectable. In the presence of steroid, GR binding to MMTV is proposed to induce changes in chromatin structure indirectly, in a way that exposes the NF-I binding site to facilitate NF-I, *in vivo*. The changes in chromatin structure are dependent upon ATP-hydrolysis by the chromatin remodelling activity of ISWI on MMTV mininucleosomes.

In Class II, protein-protein interactions between individual sequence-specific transcription factors to cooperative DNA-binding (31-34, 125-127). The result of the cooperative DNA-binding is stabilized DNA-binding of both factors and can result in a change in recognition of specific DNA sequence. For example, Oct-1 acting alone binds

to the DNA sequence ATGCTAAT, however upon association with host cell factor (HCF) and VP16 the sequence recognition is altered to TAATGARAT (R=purine) (128).

There are many examples of protein-protein interactions between individual DNA binding domains of transcription factors that correlate with cooperative DNA binding leading to transcriptional synergy (31-34). Some ligand inducible transcription factors function by cooperating with constitutive transcription factors (7). In some instances, multiple transcriptional activation functions are required for transcriptional synergy in addition to the interaction between DNA-binding domains (DBDs) (35).

Transcriptional synergy can by divided into sequential events. First the sequence-specific transcription factors are assembled on the enhancer/promoter (27, 28). Once these multiple activators are precisely positioned by determinants in the DNA sequence of the enhancer region, specific activation surfaces are displayed that are chemically and spatially complementary to surfaces of co-activators, chromatin remodelling factors, TFIID and RNA pol II holoenzyme (129). Transcriptional synergy results when the sequence specific transcription factors bind to DNA cooperatively and recruit co-activators, chromatin remodelling factors, TFIID and RNA pol II holoenzyme with efficiency that is higher than when each factor is acting alone .

For the activation to be more than additive of the activity of each factor alone, the activators can independently target a common complex, sub-complex or distinct complexes involved in gene transcription. Activators working through a single target is best illustrated with TFIID (130). Two sequence-specific transcription factors, bicoid (Bcd) and hunchback (Hb) interact with individual components of a TFIID subcomplex containing TBF, $TAF_{II}110$, $TAF_{II}60$ and $TAF_{II}250$. Individually Bcd and Hb weakly

recruit the subcomplex. However when present together, the subcomplex is recruited to the promoter with high affinity. This effect translates into a 100-fold increase in transcriptional activation.

Alternatively, synergy may arise from interactions through multiple targets. For example, VP16 has been shown to target several proteins residing in distinct complexes *in vivo*, including TFIID (TBP or $TAF_{II}40$) and the RNA holoenzyme (TFIIB and TFIIH) (2). In a promoter containing multiple binding sites for Gal4/VP16 one molecule would target TFIID while the other may target TFIIB (68-71).

Enhancer regions are usually formed on short segments of DNA (100-300 bp) and their assembly is sometimes facilitated by the bending of DNA by specific proteins to create a more favorable geometry and spatial organization of binding sites (131). In addition, chromatin remodelling events allow access of all the necessary sequence-specific and other architectural proteins to enhancer DNA, thus facilitating the assembly of sequence-specific transcription factors that promote transcriptional synergism. For example, lymphoid enhancer binding factor-1 (LEF-1) and HMG I are sequence-specific DNA-bending proteins that each contain a conserved 79 amino acid HMG domain. The HMG domain functions by binding DNA in the minor groove and intercalates a hydrophobic amino acid between adjacent base pairs in the site. The HMG domain of LEF-1 bends and twists the DNA to fit the contour of the protein, resulting in a 120° bend. On the T-cell receptor α (TCRα) promoter the effects of LEF-1 on DNA structure are key to the cooperative binding of ATF, acute myelogenous leukemia-1 (AML-1) and Ets-1 (132).

Thus, transcriptional synergy can reflect coalescent actions at many levels including cooperative DNA binding of sequence-specific transcription factors, chromatin reorganization, architectural rearrangement of DNA and recruitment of the general transcriptional machinery.

4. Identification and cloning of NRs- A historical perspective.

The GR belongs to the NR superfamily of proteins that are regulated by small lipophilic molecules (133). Ligands for NRs include steroids (glucocorticoids, mineralocorticoids, progestins, androgens and estrogens), retinoids, thyroid hormones, vitamin D_3, ecdysone, other cholesterol derivatives, bile acids and amino acid derivatives (134). These lipophilic hormones have been identified, based on their abilities to affect development, differentiation, metamorphosis and physiology and to signal through specific receptor proteins (133). Overproduction or the lack of some of these substances are associated with human diseases, while some can be used as therapeutic agents. Therapeutically, the ability of glucocorticoids to suppress inflammation has made them valuable for treatment of chronic inflammatory diseases such as asthma (135, 136), rheumatoid arthritis (137) and systemic lupus erythematosus (138, 139). Further their ability to induce apotosis in the immune system has made them valuable tools for the treatment of certain leukemias and lymphomas (140, 141).

Clues linking lipophilic hormones directly to gene transcription arose from the observation that ecdysone, an insect molting steroid, induced chromosomal puffs at specific sites in the *Drosophila* polytene chromosome (142-144). This was the first indication that hormones had an effect on gene transcription in the nucleus. The development of radiolabeled steroids facilitated the isolation of binding proteins for

steroid hormones (145). It was then necessary to identify, clone and characterize hormone receptors to provide a better understanding of the molecular mechanisms involved.

The development of synthetic radiolabeled ligands that covalently bound to a receptor protein facilitated the purification of the GR protein (145). Monoclonal antibodies specifically recognizing immunogenic GR peptides were used to probe cDNA expression libraries which eventually led to the cloning of cDNAs from rat (146), human (147) and mouse (148). Shortly thereafter, the estrogen receptor (ER) was cloned in a similar fashion (149). The high sequence similarity observed within the NR DNA and ligand binding domain (LBD) has lead to the identification of many novel NRs by low stringency hybridization and polymerase chain reaction (PCR) (133). To date, approximately 300 NRs have been identified in mammals, *D. melanogaster* and *C. elegans* species (150, 151).

Receptors for which a ligand has yet to be identified are termed orphan receptors (133). Orphan receptors are potentially exciting pharmacological targets as they may prove to bind a variety of small molecules, which can be accessible to drug design (152). To date, drugs acting as ligands for various NRs have been used to treat and control functions associated with major pathologies including cancer and diabetes. The development of synthetic ligands for ER (153), RAR (154, 155) and peroxisome proliferator-activated receptor (PPAR) -γ has led to pharmacological applications for cancer and type II diabetes (156).

5. Structure/function of NRs

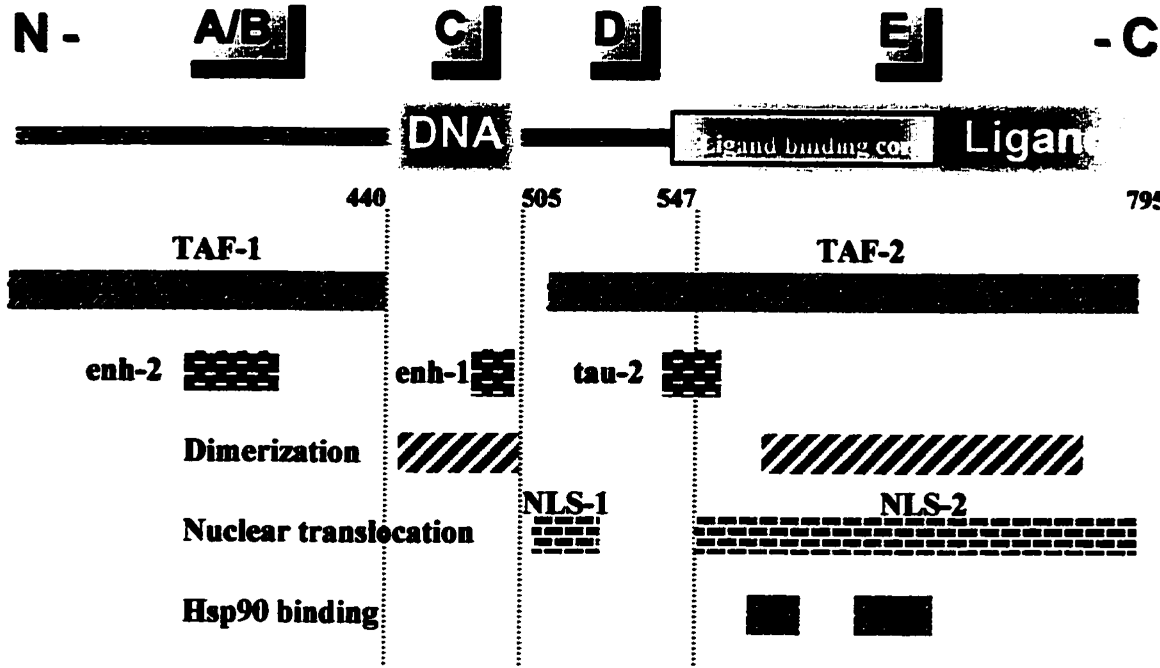
NRs are modular proteins that can be separated into four discrete domains labeled A/B, C, D and E (157). The modular structure of NRs is schematized in Figure 1, using rat GR (158), which is the major subject of this thesis. The three major functional domains of NRs are the N-terminal activation domain (activation function -1 [AF-1], A/B domain), the central DBD (C domain), and the C-terminal hormone-binding domain which contains a second activation domain (AF-2, E domain). A fourth domain (D) consists of a hinge or a flexible linker separating the DBD and LBD. The modularity of these domains is emphasized by domain swapping experiments using GR and ER, where their individual LBDs determined the ligand specific regulation of transcription (159).

The N-terminal region of NRs often contains a hormone independent transactivation domain referred to as transactivation function-1 (TAF1), AF-1 or enhancer region 2 (enh2) as for GR (160). Enh2 mapped to aa 237-218 of the rat receptor, as determined by peptide deletion analysis. The N-terminal A/B region is highly variable in sequence and in length among individual NRs ranging in length from less than 100 aa up to 600 aa (161, 162).

The DBD displays the region of highest similarity among receptors (162). Most NRs can bind to DNA as homo- and hetero-dimers. Invariably, the DBD contains two arrays of four cysteine residues, each tetrahedrally coordinating one zinc atom to form what has been termed two cysteine-four zinc finger DNA binding motifs (see also Figure 2a) (163, 164). Interestingly each finger motif is encoded by separate exons of the receptor genes (165-167).

Figure 1. Function related to structural domains of the rat GR.

Schematic of the rat GR protein represented by a solid horizontal line and boxes to highlight the DNA and ligand binding domains (adapted from S. Simmons, 1994 [158]). The functional domains common to most nuclear receptors are labeled A-E above the main schema. Below the schema, numbers represent the amino acid position assigned to the residues found in the rat GR. Some functional properties are indicated below the main schema. Major transactivation functions TAF-1 and TAF-2 map to the N- and C-terminal domains of GR, respectively (160). Under physiological conditions, the TAF-1 domain is constitutively active, while TAF-2 activity is ligand dependent. The enh-2 (aa 237-318) has been identified by deletion mutational analysis, as a region containing most of the activity of the rat GR TAF-1 domain. The enh-1 region (aa 484-492) is a transcriptionally active domain, defined by truncated receptors in yeast and CV-1 cells. Some point mutations in this region affected transactivation but not DNA binding to HREs. The tau2 motif was originally identified by its ability to enhance transcription, despite overlapping with the ligand binding core, mutations distinguishing ligand binding and transactivation have been identified in tau2. The centrally located, dimerization domain is required for cooperative DNA binding of receptor monomers to DNA, physiologically a point mutation in this region abolished transcriptional regulation of target genes requiring the DNA binding function of GR, while those transcriptional regulation does not require DNA binding were normal. The second dimerization domain located in the E region has been proposed based on homology with ER. The nuclear localization signal -1 (NLS-1) is located between 497 and 524 and contains a bipartite localization sequence of basic amino acids separated by 10 spacer amino acid (173). A second NLS termed NLS-2, has been localized to amino acids 540-795. The smallest peptide shown to stably associate with hsp90 has been localized between 537-673, although multiple other sites may exist (179).

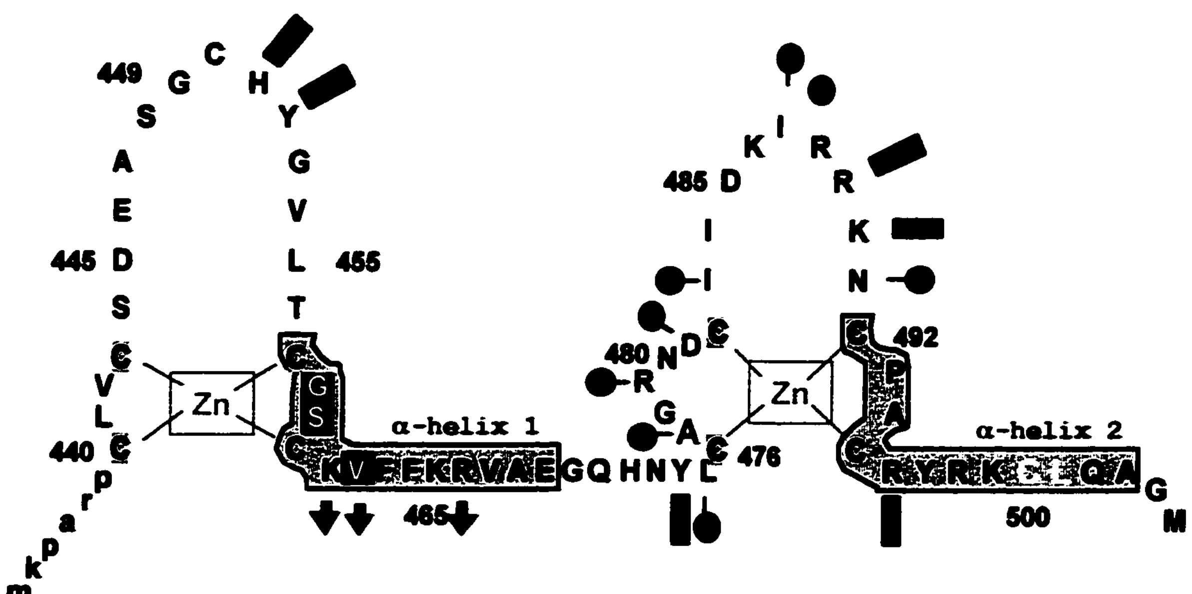


N -
A/B
C
D
E
- C
DNA
Ligand binding
440
505
547
795
TAF-1
TAF-2
enh-2
enh-1
tau-2
Dimerization
NLS-1
NLS-2
Nuclear translocation
Hsp90 binding

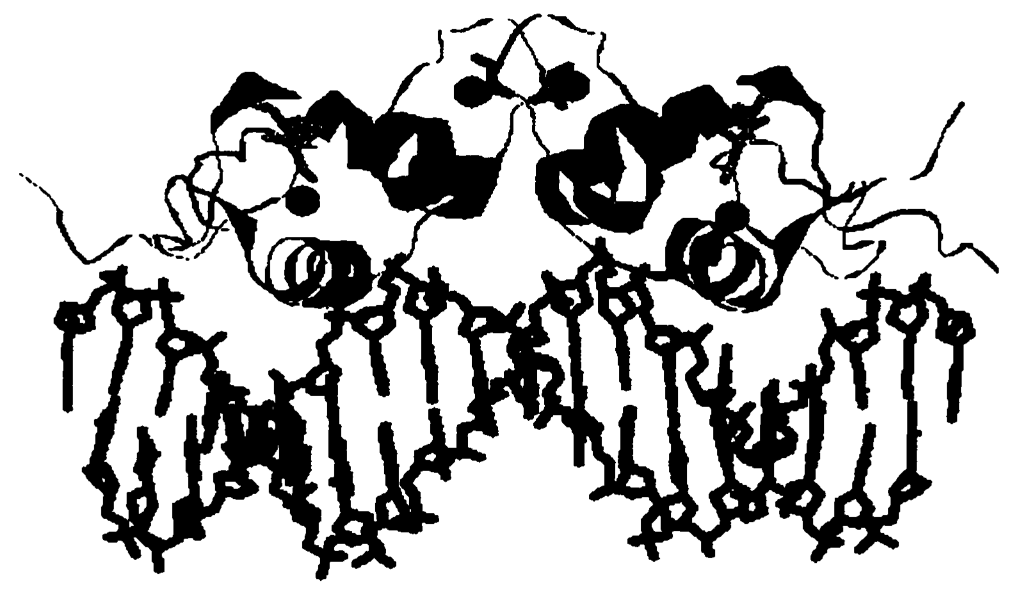
Figure 2. The GR DNA binding domain.

a. A schematic representation of the GR DBD (aa 440-505, uppercase) coordinated by two zinc atoms (cyan) was adapted from a summary of the crystal structure of rat GR (169). The lower case letters represent 6 leader amino acids. The solid rectangles represent the amino acid residues that make phosphate specific contacts with the DNA phosphate backbone while the solid arrows represent the residues that make base-specific contacts. The residues marked with a solid circle make protein-protein contacts in the DNA bound receptor, A477 required for binding of GR to HREs under physiological conditions is highlighted in magenta. The boxed residues form α-helical secondary structures. The residues highlighted in white (G, S, V) determine the specificity of the DNA binding domain. The residues C500 and L501 are highlighted in yellow to represent the residues required for the direct protein-protein interaction with Oct-1. b. Graphical illustration of the GR dimer bound to a classical HRE, constructed from coordinates derived from the crystal structure of rat GR with the rasmol v2.6 software. The DNA is shown in grey wire frame (lower) bound by two individual molecules of GR as a ribbon diagram (upper), one GR molecule was colored in red and the other in green. The α-helices are represented by wide ribbons wound in the shape of cylinders, with helix-1 lying in the major groove of DNA and helix-2 lying perpendicular to the first helix leading into the dimerization interface between individual GR monomers. The 4 zinc atoms are shown using the space fill function in cyan. The C500 and L501 residues, in yellow as sticks, are required for binding to Oct-1, notice that each of these residues point to the core of each GR monomer, making it unlikely that they would be involved in contacting Oct-1, directly. In magenta, A477 essential for DNA binding, *in vivo* is located at the protein-protein interface between each GR monomer.

a.



b.



Biochemical analysis along with X-ray crystallography and nuclear magnetic resonance (NMR) have revealed the molecular structure and the mechanism of DNA binding of GR and other NRs (Figure 2b) (164, 168, 169). Determinants, in α-helix 1 of the DNA bound NR dimer, were found to be responsible for the specificity of DNA binding through base specific contacts with the major groove of DNA. α-helix-2 located in the second zinc finger makes extensive phosphate contacts with the DNA and lies perpendicular to α-helix-1. The second zinc finger also contains a number of amino acid residues that are directly involved in forming a DNA dependent homodimerization interface. When bound to DNA, helix-2 has an exposed surface with the potential to make additional protein contacts (170) while helix-1 lies across the DNA major groove and is almost completely engaged in DNA binding.

The P-box, a short amino acid motif (5 aa in length) in α-helix-1 of the first zinc finger motif is responsible for the recognition of specific bases on DNA. For example, the GR P-box sequence, GSxxV (Figure 2a, highlighted in white within a black background, xx = CK), recognizes specific bases in a GR half site 5'-AG<u>AA</u>CA, in contrast the ER P-box residues EGxxA (xx= CK) recognizes specific bases in an ER half site 5'-AG<u>GT</u>CA. Often the term "half site" which represents the DNA binding site for one receptor molecule, usually a hexamer, is used for the purpose of simplification. Indeed substitution of these three residues in GR for those of ER make the modified GR recognize with similar affinity an ERE (171).

The D domain separates the DNA binding domain from the C-terminal LBD (Figure 1). This highly variable region, which appears to function at least in part as a flexible hinge, may be crucial for the spatial configuration of the receptor. It also usually

contains a basic nuclear localization signal (NLS) motif (172). This motif is at least partly responsible for nuclear transfer of GR (173, 174). More recently, we identified a solution homodimerization domain in the hinge region of the receptor (175), similar to one identified for the PR (176).

The C-terminal E domain has several overlapping functions including transcriptional activation, dimerization, hormone binding and for some receptors, a second NLS (158, 174). In addition, the LBD contains determinants that mediate association of unliganded steroid receptors into heat shock protein 90 (hsp90) and immunophilin containing protein complexes (177-179). Generally, upon ligand binding, NRs are transformed from a transcriptionally inactive to an active state (180). X-ray crystallography comparing unliganded to liganded receptors, specifically for TR have shown that the LBD is maintained in an open conformation allowing access of the ligand to the hydrophobic core of the ligand binding pocket.

While the crystal structure of the GR LBD remains to be determined, the LBDs of several NRs have been shown to adopt a highly conserved overall structure. Structurally, the NR LBDs generally consist of 12 α-helices and 3 short β-strands assembled in three layers with the helix-6 and helix-7 in the middle layer to form the binding pocket (180). Following insertion of the ligand, multiple contacts are formed with the ligand in the ligand binding core that allows compaction of the ligand-binding domain. As a result, the C-terminal α-helix or α-helix-12 reorients over the ligand binding pocket resulting in a conformational change in the LBD that configures protein-protein interaction surfaces for effector proteins. The significant change in conformation following ligand binding correlates with protease digestion studies showing distinctly

different conformations for unliganded receptors and ligand-bound receptors (181-184). Generally, this switch induces a transcriptionally active NR.

However, for at least one NR, CAR, ligand has the opposite effect (185). In the absence of ligand, CAR is maintained in an activated state. Following ligand binding, the NR changes to a conformation that is transcriptionally inactive. The molecular basis for this difference remains to be determined.

6. Genome sequencing and NRs

The genome sequencing project of the *C. elegans* and *D. melanogaster* indicates the total number of NRs ranges from over 225 for the *C. elegans* (151, 186) to only 20 in *D. melanogaster* (150). In *C. elegans,* NRs make up nearly 1.5% of the entire coding sequence (186) with individual receptors having been implicated in functions such as odorant response, sex determination, embryonic development and metabolic control (187).

Sequence analysis indicates that many of the divergent NR genes in nematodes have arisen from extensive gene duplication and diversification events (186). Of the 28 potential genes tested, 21 genes show mRNA expression, suggesting that a large fraction of the predicted genes encode functional genes (187). Although some NR gene expression is restricted to distinct cell-types, many are expressed in overlapping patterns. Spatial and temporal analyses have revealed that NRs showing higher degrees of similarity are also expressed in overlapping patterns. This, together with the observation that overexpression of the NRs that are expressed in overlapping patterns result in a similar phenotype, suggests many NRs in *C. elegans* have not evolved sufficiently to acquire distinct functions in the nematode.

Nematodes show unprecedented diversity in the P-box of many NRs (151, 186). By analogy with GR and ER sequence recognition (188), it is assumed that these novel NRs will exhibit different DNA binding specificity from other previously characterized NRs. One of these novel P-box sequences, RAxxA (xx=CA) is found in as many as 64 genes (151). This suggests that further studies of these NRs may reveal novel types of NRs in higher organisms.

Phylogenetic analysis of the DNA binding domains (DBDs) and LBDs has yielded an evolutionary tree defining 6 subfamilies of unequal size (189, 190). The genomic sequence project in yeast and an extensive PCR survey in fungi, plants or other unicellular organisms do not identify NR signatures suggesting that they are specific to metazoans (191). It is believed that diversification of the superfamily arose from two waves of gene duplication. An early wave, during the emergence of the main metazoan phyla, has led to the present subfamilies and groups of receptors. Following the split in the lineage leading to arthropods and vertebrates, a second wave occurred specifically in vertebrates, producing the paralogous groups within each family. Most NRs appear to be ancient since these receptors have homologues in both arthropods (including insects) and vertebrates. In *D. melanogaster* and *C. elegans*, no homologues of the classical steroid hormone receptors (AR, GR, PR, ER and MR) have been identified, suggesting that they were formed after the arthropod-vertebrate split and are consequently specific to vertebrates.

The current hypothesis is that the NRs have gained rather than lost the ability to bind ligand (191). Wide spread distribution of orphan receptors in the phylogenetic tree shows no correlation between the evolutionary relationships of NRs and the nature of

their ligands. For example TRs, RARs, PPARs and VDRs are phylogenetically closely related within their DBDs, but each binds ligands from different biosynthetic pathways. In contrast RAR and retinoid X-related receptor (RXR), which are not as closely related phylogentically, bind similar ligand. Moreover, evidence suggests that ligands existed long before their receptors. Steroids, for example, exist not only in metazoans but also in plants and fungi.

Many orphan receptors were named prior to the establishment of their precise function or the identification of their specific ligand. Hence, a unified nomenclature system has been devised for the NR superfamily based on the evolution of the two well-conserved domains: the DBD and the LBD (190). Briefly, subfamilies are defined as the last most internal branches of the evolutionary tree with robustness ("bootstrap") values above 90%. Gene subfamilies were designated by arabic numerals, groups by capital letters and individual genes by arabic numerals. In the case of functionally and structurally distinct variants derived from the same gene a lower case letter was added at the end of the name. A complete description of the alignment procedures, tree reconstruction methods and the evolutionary implications can be found in Laudet et al. (1997) (161), while a more complete description can be found at a web site devoted to the regular implementation of this nomenclature http://www.ens-lyon.fr/LBMC/LAUDET /nomenc.html. For example, the human GR gene has been assigned 3C1 while the human MR gene was termed 3C2.

7. NRs and their interaction with HREs

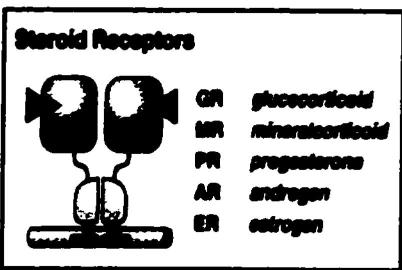
The NR superfamily can been divided into 4 types based on DNA binding and dimerization properties (Figure 3) (192). Steroid receptors, including GR, bind to DNA as homodimers (Type I) in a head to head configuration, with each monomer recognizing hexameric half-sites organized as inverted repeats separated by 3 base pairs. As the steroid receptor binds to DNA each monomer becomes juxtaposed in a head to head configuration and makes DNA dependent protein-protein contacts through aa located in the second zinc finger motif of the DNA binding domain. GR, PR, AR and MR recognize the same 5'AGAACA-3' half site while ER recognizes 5'RGGTCA-3' (R=purine).

Type II receptors heterodimerize with the RXR. These heterodimers characteristically bind to direct repeats (DRs) of the core 5'AGGTAC-3' half-site with variable spacing in what has been termed the 1 to 5 rule (although some bind to inverted and everted repeats) (192, 193). The spacing between the DRs determines the RXR heterodimerization partners, which include RARs, RXRs, TRs, PPARs, and VDRs. Biochemical studies show RARs regulate transcription preferentially through DRs spaced by two or five nucleotides, whereas the vitamin D receptor (VDR) and the TR regulate through DRs spaced by three and four nucleotides respectively. RXR-PPAR heterodimers as well as RXR homodimers regulate through DRs spaced by one nucleotide; thus all spacing options from one to five nucleotides are used by distinct dimeric complexes. Type III orphan receptors bind to DNA DRs as homodimers while type IV orphan receptors bind as monomers to a single core binding site with extended flanking sequences (133).

Figure 3. Four types of nuclear receptor/DNA interactions.

Schematic representation or nuclear receptor types based on DNA and dimerization properties (adapted from Mangelsdorf et al. 1995 [133]). Beside each illustration are abbreviations representing nuclear receptor belonging to that type with their activating ligand (to the right). Type I steroid receptors, like GR, bind as homodimers to hexamer consensus sequences (AGAACA for GR, MR, PR and AR, but AGGTCA for ER, below) arranged as inverted repeats separated by 3 non discrete bases (nnn). Type II nuclear receptors can bind as heterodimers with unliganded RXR to direct repeats of the consensus AGGTCA sequence with variable spacing (indicated by ?) usually 1-5 bps. Type III nuclear receptors can bind as homodimers to direct repeats separated by variable spacing. In constrast, Type IV nuclear receptors bind as monomers.

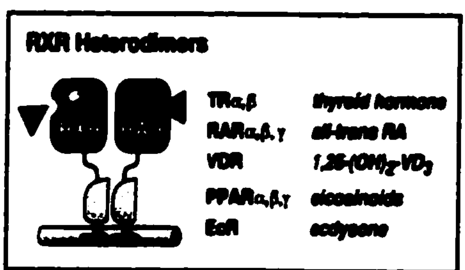
Type I



HRE AGAACAnnnTGTTCT

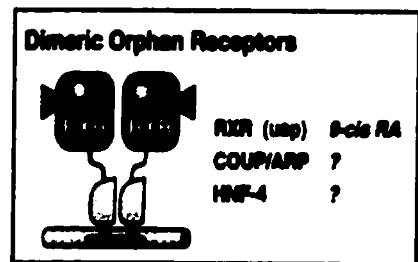
ERE AGGTCAnnnTGACCT

Type II



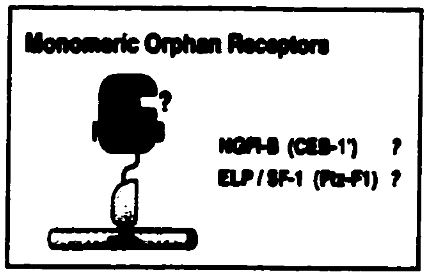
DR AGGTCA..? AGGTCA

Type III



DR AGGTCA ...? AGGTCA

Type IV



AGGTCA

8. Mechanisms of transcriptional regulation by NRs

Sequence-specific regulators like NRs, regulate transcription through interaction with molecules that act directly or indirectly to recruit TFIID, RNA pol II holoenzyme, co-activators/co-repressors (194, 195). These protein-protein interactions result in chromatin remodelling, changes in DNA architecture and in the case of gene activation recruitment of the general transcription machinery to the promoter.

NRs have been shown to interact physically with components of the general transcription machinery, like TF-IIB (196, 197), TBP (198, 199) and TF-IID (200, 201). Studies examining the ligand independent transcriptional activities in the N-terminus of GR, AF1, have shown that it could enhance the formation of stable preinitiation complexes at target promoters *in vitro* (202). Further it has been suggested through squelching assays and protein-protein interaction studies, that GR can contact the TFIID complex directly through interaction with TBP (201, 203). The physiological relevance of these interactions is unclear, but has been proposed to contribute to transcriptional activation by recruiting the general transcriptional machinery to the promoter or by positioning rate limiting factors through direct protein-protein interactions.

In the absence of ligand, most receptors are nuclear and remain bound to DNA to constituitively regulate transcription. Typically in the absence of hormone, co-repressor molecules including N-CoR/SMRT remain bound to the NR and act by negatively regulating gene transcription (116, 204). The negative regulation is due at least in part to chromatin condensation because of the recruitment of HDAC activity (205). For example, RAR-RXR DR-5 mediated transcriptional repression in the absence of ligand requires HDAC 1, 2 and mSin3 because nuclear microinjection studies with antibodies

recognizing these factors abolish transcriptional repression (206). Furthermore, measuring transcription in this system in the presence of HDAC inhibitors, trichostatin A or butyrate, relieves transcriptional repression. Co-repressors repress transcription by binding to NRs in the absence of ligand as described for RAR and TR, or in the presence of antagonists as exemplified with ER (207). Studies have identified a region within the hinge or D domain of NRs required for association with co-repressors (116).

Following ligand binding, the co-repressor molecule is displaced from the DNA bound NR and replaced with a co-activator molecule (208). Recently several, co-factors for NRs have been identified. These include thyroid hormone interacting protein (TRIP)-1 (123) (a homologue of yeast SUG-1 (p46) (209)), TIF1 (122), receptor interacting protein (RIP) 140 (210), RIP 160 (also known as ERAP 160) (204, 211), SRC-1 (93)/N-CoA-1 (94), SRC-2 /N-CoA-2 (97)/ TIF2 (95)/GRIP1 (96), TRAP (63)/DRIP (64), TRIP230 (212), AR-associated protein 70 (ARA70) (213), SRC-3 (214)/pCIP (97)/RAC3 (99)/ACTR (98)/AIB1 (100)/TRAM-1 (101), PPAR-γ co-activator (PGC-1) (215) and CBP (94)/p300 (107).

Some of the first histone acetyl transferase (HAT) co-activators (e.g. p160) were identified on the basis of their interaction with the LBD of NRs through the NR box motif (216). The NR box is represented by the consensus LXXLL sequence. NR boxes play an important role in mediating the interaction between co-activators and receptors by associating with critical residues in the co-activator interface region of the receptor LBD (217-220).

While many NRs, such as RXR, RAR and TR, have been shown to interact directly with the p300/CBP integrator no steroid receptor has been shown to function

directly through CBP/p300. In turn, steroid receptors like GR can interact directly with chromatin remodelling factors, like the Brg1 ATPase and no reports exist of a direct interaction with other NRs.

9. Steroid hormone action

By contrast to most NRs that are constuitively nuclear and DNA bound, steroid receptors experience an additional level of control through the reversible association with chaperone proteins that maintain them in a mostly inactive state in the absence of steroid (221). In this state, steroid receptors appear to form similar hsp-protein complexes sedimenting at 8S, in contrast to the 4S sedimentation coefficient of the hsp-free receptor (222, 223). Later the composition of the hsp-complex was shown to include 2 molecules of hsp 90, hsp70, p23 and imunophilins, Cyp-40 and FK506-binding proteins (FKBP51 and FKBP52) (224, 225).

For certain classical steroid receptors, hsp/immunophilin-association influences the subcellular distribution in a receptor-specific fashion. For example, GR-hsp association promotes localization of the receptor to the cytoplasm (226). By contrast, it appears that PR and ER are always nuclear (227, 228). Then for AR and MR, the precise nuclear/cytoplasmic localization is less clear and may be distributed thoughout the cell in the absence of steroid (229, 230). For GR, it is hypothesized that the receptor-specific localization is achieved either by masking an NLS or by revealing a nuclear export signal following association with hsp/immunophilins (221).

Steroid receptors are phosphoproteins (231). *In vivo*, transcriptionally active steroid receptors are generally hyper-phosphorylated. For GR, the naive receptor is hypophophorylated but following exposure to steroid the transcriptionally active receptor

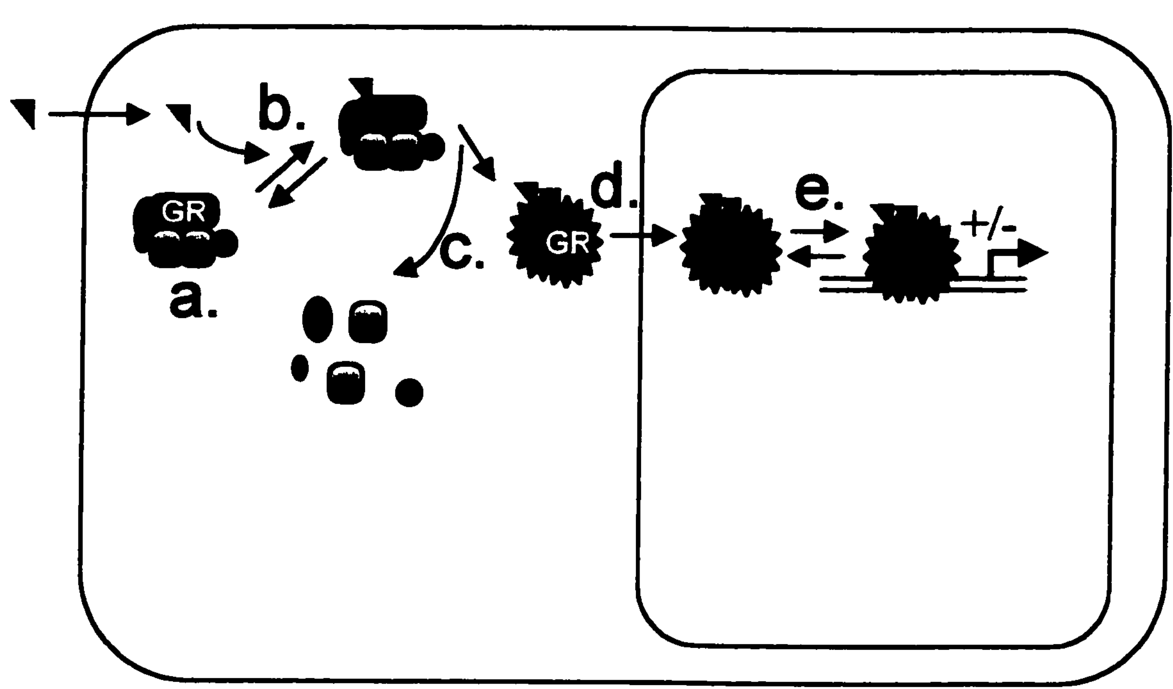
becomes phosphorylated (232). However, production of a receptor that can not be phosphorylated has only modest effects in relation to gene transcription (233). Thus the precise role of GR phosphorylation remains to be determined. In contrast, ER can be activated in the absence of steroid by specific phosphorylation events in the AF-1 region that are induced by growth factors (234).

Steroid hormones act in similar ways with their cognate receptors (235). The effects of steroid treatment of GR are illustrated in Figure 4. The hsp/immunophilin-association maintains the receptor in an open conformation that allows the receptor to bind to ligand with high affinity (236, 237), for GR this occurs in the cytoplasm. Following ligand binding, the receptor dissociates from hsps/immunophilins and rapidly translocates to the cell nucleus. The free ligand bound receptor can dimerize in solution and acts in the cell nucleus to regulate gene transcription through protein-protein and protein-DNA interactions (5).

The transcription factor DNA binding activity is a dynamic process with a descreet half-life. Recently it has been established that ligand influences the kinetics of DNA binding of steroid receptors to DNA. For example, without changing the overall affinity of GR for HREs, the "on rate" of hsp-free GR for DNA containing HREs was accelerated by 2-5 X while the "off-rate" was accelerated 10 to 20 X by treatment with hormone agonist (238). With this in mind, it has been proposed that following hormone activation, the receptor interacts transiently with a response element recruiting a secondary set of factors that in turn form a stable complex at the regulatory site. This mechanism has been termed “hit-and-run” (239, 240).

Figure 4. Signal transmission by glucocorticoids.

Schematic representation of steroid hormone action using the GR as a model (235). a. Unliganded, the GR is inactive, associated with 2 molecules of hsp90 (yellow squares), hsp70 (red oval) and others (blue circle, small grey oval grey). b. Steroid ligand diffuses freely across the cellular membrane and binds to the GR complexed with hsps in the cytoplasm. c. Upon ligand binding the hsps dissociate, GR becomes activated (star shaped) and d. transferred to the cell nucleus as a dimer. e. Once in the nucleus, GR modulates gene transcription by interacting with DNA directly or indirectly through protein-protein interactions.


b.
GR
a.
c.
GR
d.
e.
+/-

For ER, real time interaction analysis using surface plasmon resonance methodology (SPR) show that agonist (estradiol) bound ER accelerates receptor DNA interactions by 50 X compared to unliganded receptor (241). In contrast, antagonist (ICI-182,780) bound receptor decreases receptor DNA interactions by 1000X compared to the agonist bound receptor. Therefore, a correlation can be observed between gene transcription and the rate of receptor-DNA exchange induced by ligand.

More recently, this hypothesis of rapid exchange was validated in live cells with the direct binding of GR to multiple HREs located in the MMTV promoter, in the context of chromatin (242). The green fluorescent protein fused to GR acts as a fluorescent tag to follow GR in live cells. Photobleaching techniques are used to irreversibly modify the green fluorescent protein (GFP) or GFP conjugated protein for the purpose of studying protein kinetics in real time. In this specific example, GR was fused to GFP and following hormone treatment could be observed bound to the genomically integrated MMTV promoter. Using a technique called fluorescence recovery after photobleaching (FRAP) at this genomic locus, the fluorescent signal was lost following photobleaching, but fluorescence recovered within seconds through the renewal of GR molecules at the photobleached site. This demonstrated that GR molecules bound to the genomic locus at the time of photobleaching irreversibly lost their ability to fluoresce but could be rapidly replaced by new GR-GFP molecules in the nucleoplasm. Thus, GR rapidly exchanges between the HRE and the nucleoplasm and its localization in the nucleus is not static.

10. Glucocorticoid hormone

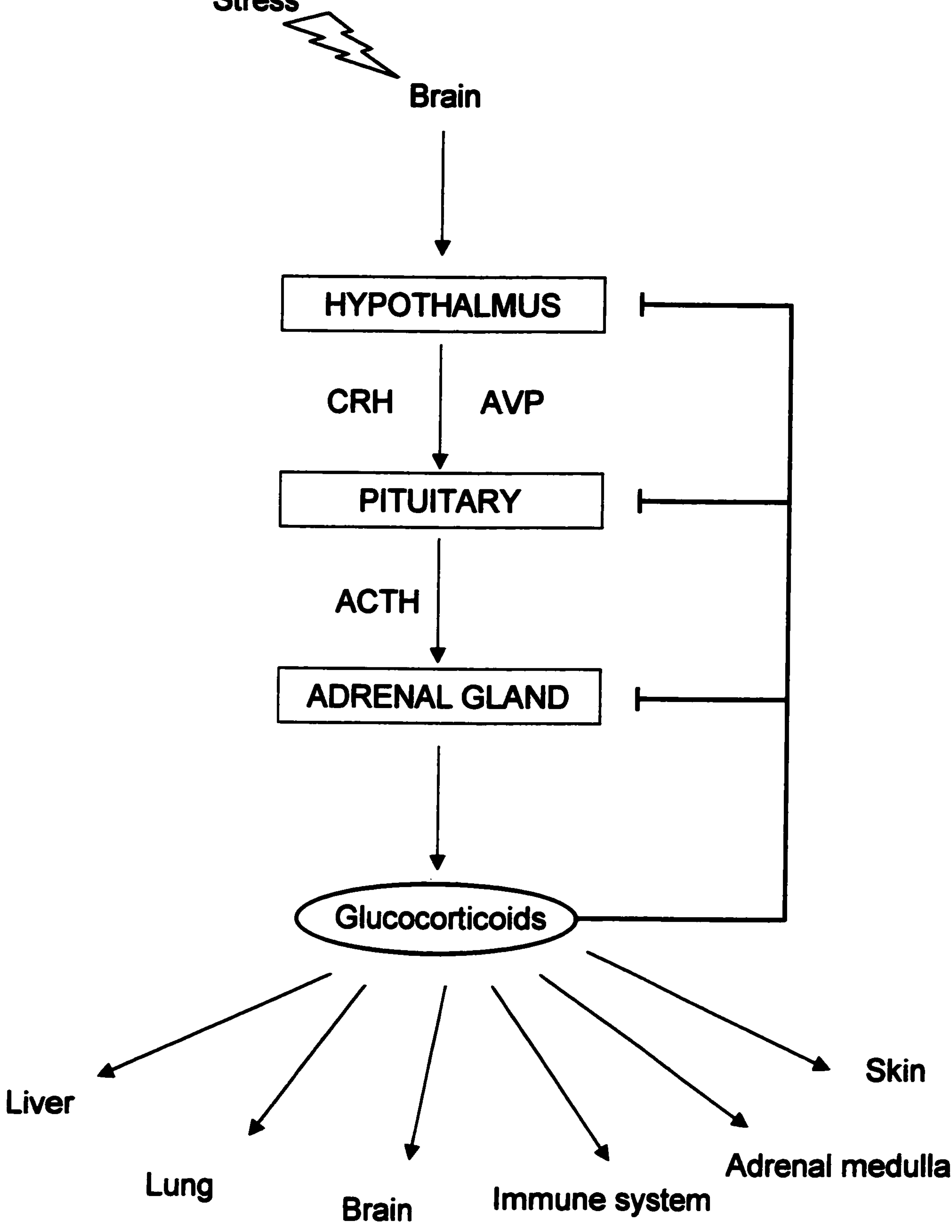
The GR transmits the signal of circulating glucocorticoids to changes in specific gene expression (243). Glucocorticoid hormone (cortisol in humans and corticosterone in rats)

are steroids synthesized in the adrenal gland (244). Glucocorticoids have a wide range of functions including regulation of glucose, fat and protein metabolism, anti-inflammatory and immuno-suppressive actions and has effects on mood and cognitive functions. Circulating glucocorticoid levels are tightly controlled by a hormonal cascade: the hypothalamo-pituitary-adrenal axis (HPA) (Figure 5) (245). The hypothalamus secretes arginine vasopression (AVP) and corticotropin releasing factor (CRF) under the control of the hippocampus and the amygdala. These neuropeptides stimulate the synthesis of adrenocorticotropic hormone (ACTH) from anterior pituitary cells, which in turn stimulate the production of glucocorticoids by the adrenal cortex. Equilibrium of this system is maintained by a negative feedback mechanism in which glucocorticoids directly inhibit the synthesis and secretion of AVP, CRH and ACTH. The HPA axis can be stimulated following physical stress due to infection or injury or emotional stress resulting in the release of glucocorticoids that coordinate immune, nervous and endocrine responses.

Glucocorticoids evoke the mobilization of energy resources. They enhance transcription of gluconeogenic enzymes including phosphoenolpyruvate carboxykinase, glucose-6-phosphatase, serine dehydratase and tyrosine aminotransferase in hepatocytes, increase lipolysis in adipocytes, limit glucose uptake in peripheral tissues (244, 246). In addition, glucocorticoids act in the central nervous system to stimulate feeding behavior (247). Glucocorticoids also act on several cell types to protect the body from over-reaction to infection, due in part to the ability of GR to repress the production of pro-inflammatory cytokines by repression of activator protein-1 (AP-1) and NF-κB (248).

Figure 5. Glucocorticoid secretion is tightly regulated.

Glucocorticoid secretion can be triggered by stress signals in the brain which signal synthesis and secretion from the adrenal gland through a signaling cascade involving the hypothalamus, pituitary and adrenal gland (HPA) (243). Glucocorticoids can regulate gene transcription in many tissues including the lung, liver, skin, adrenal medula, brain and the immune system. Glucocorticoid synthesis and secretion can be regulated through a negative feed back loop at many signaling levels in the HPA axis (represented by the bar ends). The negative feedback loop is achieved by the binding of glucocorticoids to GR in target tissues to downregulate the synthesis of signaling peptides. The signaling peptides include CRF: corticotropin releasing hormone, AVP: arginine vasopressin, ACTH: adrenocorticotropin hormone.


Stress
Brain
HYPOTHALMUS
CRH
AVP
PITUITARY
ACTH
ADRENAL GLAND
Glucocorticoids
Liver
Lung
Brain
Immune system
Adrenal medulla
Skin

Glucocorticoids act by binding to the two closely related NRs, GR and MR (249). GR is ubiquitously expressed meaning that it can be found in nearly all cell types. In contrast MR protein expression is limited to tissues involved in sodium absorption, such as the epithelial cells of the collecting ducts of the kidney and large intestine, heart and in neurons of the limbic system (147). MR can also be activated physiologically by mineralocorticoids, which is an exclusive property of MR because the affinity of GR for mineralocorticoids is low. Specificity of the mineralocorticoid response can also be achieved by a mechanism independent of the receptor. In aldosterone-responsive cells of the kidney and intestine, MR is protected from binding glucocorticoids by the presence of 11-beta-hydroxy steroid dehydrogenase II (11-β-HSD-II) (250). This enzyme acts by converting the glucocorticoid corticosterone into an inactive form, 11-dehydrocorticosterone. Although, glucocorticoids bind both GR and MR, they bind to MR with higher affinity. In cells of the limbic system where both receptors are expressed, it has been hypothesized that in response to hormone, MR is activated first followed by GR due to the higher affinity of MR for glucocorticoids (251).

GRβ is an alternatively spliced minor, species specific form of GR found in rat and human but not mouse (reviewed in (252, 253)). The two receptor isoforms, α and β, are identical up to aa 727 (human sequence) but diverge beyond this position, where hGRα has an additional 50 aa and GRβ an additional 15 non-homologous aa (147). In contrast to its human genomic counterpart, the murine gene encoding GR does contain a putative splice site in front of exon 9β, a consensus sequence motif required for splicing the message to produce the β isoform (254). Furthermore using RT-PCR no evidence of a GRβ mRNA was found in mouse. The GRβ receptor is defective in steroid binding and

can mildly inhibit GRα activity when overexpressed. Studies measuring the relative cellular mRNA and protein levels of GRα and GRβ show GRα expression levels far exceeding those of GRβ in most normal tissues (255-258). Relative expression levels of the GR isoforms coupled with the lack of the mRNA GRβ isoform in mice raises serious questions about the biological significance of the GRβ isoform.

11. Mechanisms of GR mediated transcriptional regulation

GR knockout mice illustrate the GR DNA-dependent and DNA-independent biological effects on gene transcription. The $GR^{-/-}$ mice die a few hours following birth, presumably because of a defect in lung maturation (259). In order to study further the biological function of GR, conditional knockout mice were produced (260, 261). Using the cre/lox system it was possible to produce live mice while studying the effects of deleting GR in a tissue specific manner. GR was specifically deleted in the nervous system by targeting the GR gene with neuronal specific expression of Cre recombinase. These mice displayed impaired emotional behavior with decreased responses to stress and anxiety, confirming GR's role in mediating stress responses.

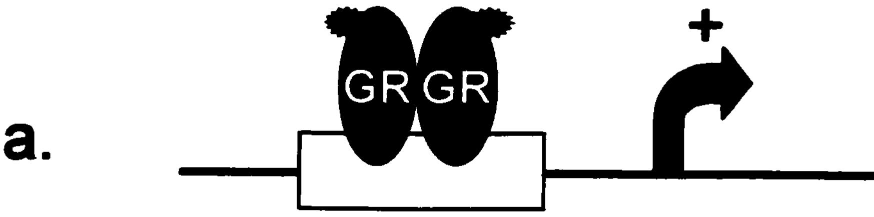
In order to distinguish the DNA-independent from the DNA-dependent biological activities of GR, homozygous mice for GR A458T (threonine residue replacing an alanine residue normally at position 458 for mouse, A476T for rat GR) were produced (262). GR A476T produces a mutation in GR that disrupts its DNA binding function. Surprisingly, these mice survived past birth, correcting the lung defect. The survival of $GR^{A458T/A458T}$ mice demonstrates that some GR functions independent of sequence-specific DNA binding are very important to the physiological role of GR.

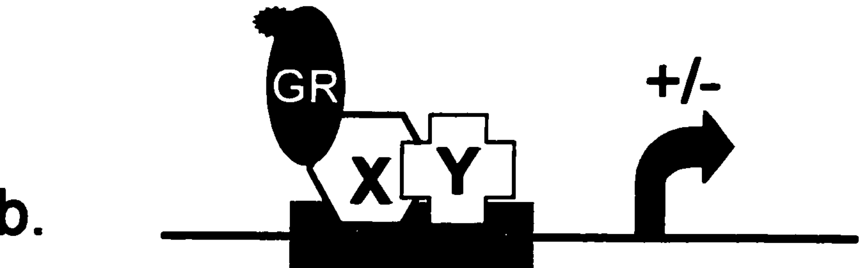
As discussed previously, although the HRE acts as a common binding site for GR, PR, AR and MR, glucocorticoids, progestins, androgens and mineralocorticoids function in distinct biological programs (263-269). The differences in response can be attributed to at least four different properties: (1) differential protein expression, (2) the sequence of the HRE to promote receptor specific responses, (3) differential co-factor binding properties and (4) differential interactions with non-receptor factors interacting with sequences surrounding the HREs of complex promoters. Subsequent to the identification of multiple co-activator molecules for NRs it has been shown that individual receptors bind preferentially to specific co-activator family members. For example, AR has been shown to bind preferentially to GRIP-1 over SRC-1 (270). Further, individual steroid receptors bind to different surfaces within the same co-activator molecule. The central domain of SRC-1 which contains NR boxes I-III preferentially binds the LBD of ER and PR while the C-terminal, NR box IV strongly binds AR and GR (270, 271). Alternatively, GR and MR have been shown to differentially repress transcription through interaction with AP-1 and NFκB (272-274). On the proliferin promoter, functional interaction of GR with AP-1 was attributed in part to determinants in the N-terminus of the receptor, whereas MR had no effect on transcription (275). In another example, AR specifically activated transcription from the sex-limited promoter, whereas GR was unable to activate transcription significantly (263, 276). In this example, it has been proposed that GR contains a repressive function in the N-terminus of the receptor that requires specific determinants in the GR-DBD.

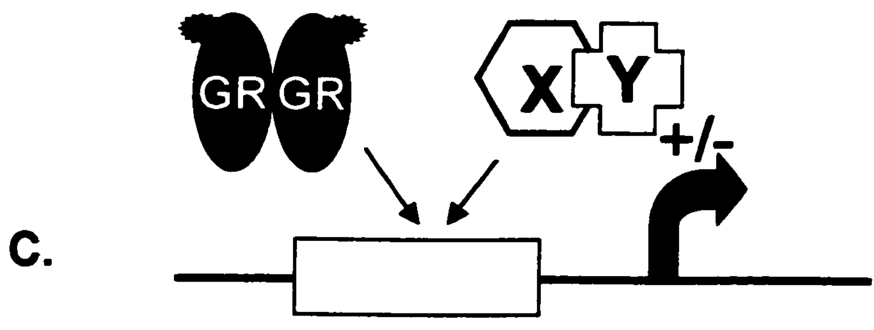
Depending on the promoter context, DNA response elements for GR function can be classified into 4 categories (Figure 6) (28): (a) simple and tandem HREs, where GR

Figure 6. Modes of GR mediated transcriptional regulation.

Schematic illustrations of mechanism in which GR can regulate gene transcription at the level of promoter DNA (adapted from [28, 244]). a. Simple HRE. Ligand bound (as indicated by the red jagged oval GR homodimers (green ovals) bind cooperatively to a classical GRE (open rectangle) to activate gene transcription (as indicated by +). b. Tethering HRE. Interaction of GR with a second transcription factor (represented by a heterodimer of factors X and Y) may result in transcriptional repression or activation (indicated by +/-) in a manner that does not require GR DNA binding. The binding site of the heterologous transcription factor is represented by the solid rectangle. c. Complex HRE. In the event that binding sites for GR and a second transcription factor overlap to produce a complex response element transcription may be positively or negatively influenced. In one example GR may bind cooperatively with a second factor to activate gene transcription, alternatively GR may compete for the binding site with a second positively acting transcription factor to negatively regulate gene transcription. d. Paired HRE. GR homodimers bind cooperatively with a second transcription factor to activate transcription (+). In the absence of binding sites (represented by the open and solid rectangles) for either transcription factor, cooperative binding and synergistic activation are lost.


a.
GR GR
+


b.
GR
X Y
+/-


c.
GR GR
X Y
+/-

d.
GR GR
X Y
+

can bind directly to DNA, including single or multiple response elements in tandem, (b) tethering HREs, where GR binds to DNA indirectly through a heterologous transcription factor, (c) complex HREs, where the HRE overlaps with a binding site for a heterologous transcription factor, (d) paired HREs, where a HRE is near to but not overlapping with a heterologous transcription factor; often the DNA binding activity of both factors is cooperative. Depending on the promoter context GR can act through the same transcription factor in multiple mechanisms involving more than one of the categories described above as exemplified by GR with AP1.

(a) Simple and tandem HREs

GR binds as a homodimer to a hexamer consensus organized as an inverted DR with 3 bp spacing to activate gene transcription (5). This requires interaction of aa within the D-loop of the DNA binding domain of GR (277). As GR binds to DNA, protein-protein interactions between each GR monomer are necessary and are believed to be important for transcriptional activation. Applying this classical model to natural promoters like those driving the expression of the tyrosine aminotransferase, it is hypothesized that GR alone functions by recruiting rate limiting subunits of the general transcription machinery.

In many promoters, HREs are found in multiple tandem copies or overlapping one another (278, 279); however, the transcriptional activation is not additive. Synergy control (SC) motifs, located in the N-terminal TAF1 motif, restrain synergy on promoters containing multiple HREs, while having no effect on promoters containing only one copy of the HRE (280). Indeed multiple unrelated transcription factors in addition to most receptors contain SC motifs.

(b) Tethering HREs

The mechanisms of action involving GR transcriptional regulation from tethering HREs are independent of DNA binding and have been shown to be essential for proper development and survival in mice (281). Tethering HREs involve direct protein-protein interactions between GR and a heterologous transcription factor bound to DNA and can result in transcriptional repression. Examples of this include GR interactions with Nur77 (282), AP-1 (283-286) or NF-κB (272, 274, 287-289). They are termed tethering elements because the repression does not require the binding of GR to DNA directly, but requires only direct interaction of GR with the target factor (243, 277). This activity can be distinguished from the classical activation function of the receptor bound to simple HREs by a point mutation in the DBD binding domain of the receptor, A476T in rat GR. The point mutation abolishes the DNA dependent dimerization function of the receptor but allows the receptor to regulate transcription through protein-protein interactions.

The first demonstration of transcriptional repression by GR was through the direct protein-protein interaction with AP-1 (283, 284, 286). Physiologically, glucocorticoids were shown to repress transcription from the collagenase promoter (290, 291). The repression was mapped to the AP-1 site, the binding site for c-Jun and c-Fos. Initially, it was proposed that the consequence of the protein-protein interaction between GR and AP-1 resulted in mutual inhibition of both GR and AP-1 mediated transcription (283, 284). It was hypothesized that the mutual inhibition was the result of a reduction in DNA binding of both factors. More recent results, demonstrate GR does not influence AP-1 DNA binding activity. Taken together, these results indicate that GR mediated repression of AP-1 occurs by a direct effect on transcriptional activation. Similar observations have

been described between GR with NFκB and Nur77. Surprisingly, of the protein-protein interactions domains that have been mapped on GR, (GR with AP-1 and GR with NFκB) the protein-protein interaction motif maps to a region including the DBD of GR.

Recently, GR has been shown to inhibit phosphorylation of the C-terminal domain of RNA pol II through a negative HRE tethering element (292). Lack of phosphorylation of the C-terminal domain represses transcription by preventing proper elongation of RNA pol II resulting in production of only short RNA transcripts.

(c) Complex HREs

Complex HREs (cHREs) are response elements that overlap with binding sites for heterologous transcription factors. GR may act by repressing gene transcription. In one case, complex HREs can be found in regions of promoters or enhancers that are transcriptionally active in the absence of hormone but are repressed in the presence of glucocorticoids. On these enhancer/promoter elements, cHREs often overlap with binding sites for other transcription factors that normally positively regulate transcription. GR repression function through this type of element requires its DNA binding activity. It is hypothesized that GR is in competition with other activating factors for this site or that GR neutralizes their positive effects.

One major physiological function of glucocorticoids is the negative feedback loop of the HPA axis by repression of CRH and ACTH expression. Glucocorticoids have been shown to repress transcription of the ACTH precursor gene, pro-opiomelanocortin (POMC) (293). The POMC promoter is bound by multimers of GR that are responsible for transcriptional repression. This region overlaps with a region required for basal and activated transcription (294). It appears as though GR repression is due to overlapping

binding sites with the TFIID complex thus disrupting the assembly of the preinitiation complex (295). A similar example has been described for the osteocalcin promoter (296).

In another example, GR acts to either repress or activate transcription at cHREs. A cHRE has been described for the proliferin promoter containing binding sites for GR and AP-1. The proliferin cHRE has juxtaposed binding sites for GR and AP-1 family members. The cHRE consists of only short stretches (up to 3 bp) of the consensus HRE and a near consensus AP-1 site (5 of 7 match) (297). The binding sites for GR and AP-1 are separated by less than 20 bps from the center of each binding site and have been found in promoters for genes encoding proliferin (297), alpha-fetoprotein (298) and papilloma virus (299). The composition of the AP-1 dimer determines whether the receptor represses or activates transcription. When AP-1 is comprised of c-Jun-c-Jun homodimers, the receptor activates transcription from a linked gene, and when AP-1 is comprised of c-Jun- c-Fos heterodimers the receptor represses transcriptional activity (300). When the spacing is increased above 26 bps the transcriptional responses reflected those observed on paired HREs (301).

(d) Paired HREs

Paired HREs are sites in which HREs are paired with binding sites for other factors often resulting in synergistic transcriptional activity. Cooperative functional interactions between HREs and binding sites for constitutive and inducible factors are numerous. The precise mechanisms responsible for the cooperative binding leading to transcriptional synergy have not been elucidated. It has been proposed that synergy is the result of cooperative binding through a recruitment mechanism involving protein-protein

interactions, or through a bimodal process involving a chromatin remodelling event due to the binding initiated by the first transcription factor followed by binding of the second factor to DNA.

In reference to the example of the proliferin gene (plfG) element previously described, when binding sites for GR and c-Jun/c-Fos were separated by more than 26 bp, previous the repression signal was converted into an activation signal (301). Moreover the activation of transcription became independent of the composition of the AP-1 dimers (i.e c-jun-homodimers or c-jun-c-fos heterodimers). The transcriptional synergy on these paired elements was highly dependent on the specific DNA binding of both factors, because elimination of DNA binding site for either factor resulted in loss of transcriptional synergy.

The transcriptional synergy between GR and Stat-5 is another example of a paired HRE with a Stat-5 binding site on the β-casein promoter (302). Initially the hormone dependent transcriptional synergy was reported as the first example of GR acting as a co-activator while being tethered through another sequence specific transcription factor, because of the lack of an identifiable HRE. More detailed analysis of the promoter identified a HRE half site in the β-casein promoter which was required for the GR mediated transcriptional synergy observed with Stat-5 (303, 304). It is now accepted that this interaction resembles a paired type HRE. The transcriptional synergy with Stat5 can also be observed with the MR and PR but not with ER or AR (305). Therefore the transcription by paired GR and Stat5 mediated effects is restricted to selected steroid receptors including GR, MR and PR.

12. POU transcription factors

POU transcription factors belong to the homeodomain family of transcription factors. The homeodomain is a 60 amino acid conserved DNA binding motif found in a large superfamily of proteins involved in development, differentiation and maintainance of cellular homeostasis (306). The homeodomain is composed of a helix-turn-helix motif that is highly conserved for homeodomain proteins (307-313). A complete listing of homeodomain protein structures can be found at http://genome.nhgri.nih.gov/homeodomain/structure (314). Usually, homeodomain proteins bind to DNA response elements with a core TAAT motif with some specificity dictated by flanking DNA sequences. Originally, homeodomain proteins were identified based on their ability to control patterning during *Drosophila* development (306). Mutation of certain homeobox genes produce a homeotic transformation or transformation of a body part into the likeness of another. One classic example is when the antennapedia gene is misexpressed in the head region, the antenna develop into legs (315).

Although most homeodomain proteins are thought of as transcriptional activators, their transcriptional activity can be influenced through N-CoR (316). N-CoR contains three repression domains (RI [aa 92-393], RII [aa 751-1,016], RIII [aa 1,035-1,460]) (116, 204). Using RIII to probe a λgt11 library revealed that 1/3rd of the positive clones contained a sequence encoding a homeodomain (316). A negative regulating homeodomain protein, Rpx, revealed a tight association with N-CoR. Transcriptional repression by Rpx proved to be dependent on association with N-CoR and HDAC activity.

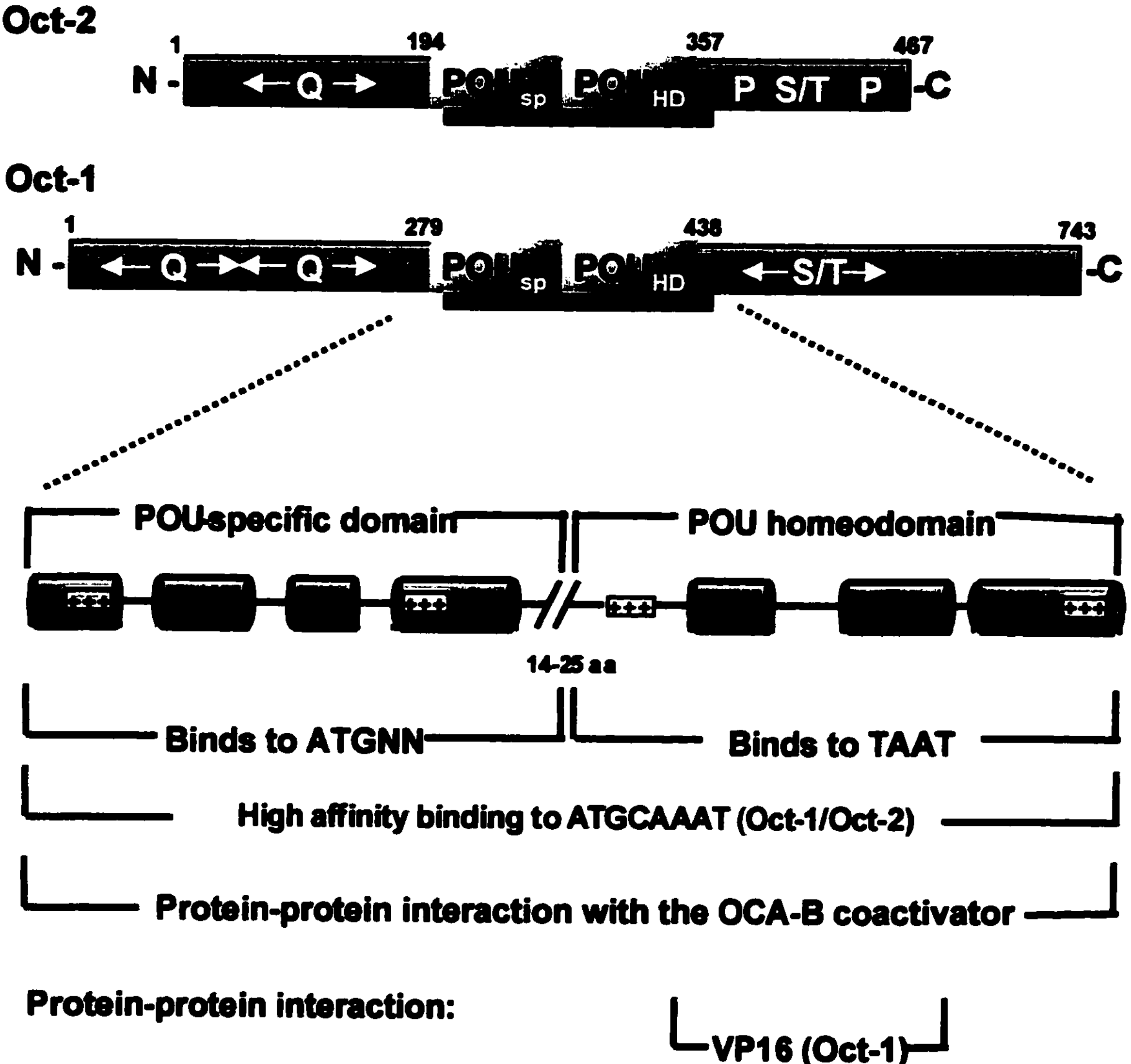
The octamer transcription factors, Oct-1 (also termed, OTF-1, NF-A1, NFIII and OBP-100) (6) and Oct-2 (317), are homeodomain proteins named for the length of their core DNA binding sites, which is 8 nucleotides long. They were originally identified by screening a λ gt11 phage fusion protein expression library with a radiolabeled probe containing a consensus octamer motif, 5'-ATGCAAAT, which was known to be a response element for both ubiquitous and B cell specific factors. The pituitary specific protein, Pit-1 (318, 319), along with Oct-1 and -2 and the *C. elegans* protein Unc-86 (320) are the founding members of the POU subfamily.

In addition to the homeodomain, POU factors have a second centrally located, highly conserved domain called the POU-specific domain (POUsp, Figure 7) (319, 321-323). The POUsp domain (80 aa in length) has remarkable similarity in structure to the λ and 434 repressors and 434 Cro protein, consisting of four α-helices, with the second and third helices forming a helix-turn-helix motif (324-328). The POUsp domain specifically recognizes the 5' half of the octamer motif ATGNN (N=any nucleotide).

In contrast to most of the homeodomain proteins that contact DNA through their homeodomain alone, POU factors combine the DNA-binding activity of the homeodomain and that of the POUsp domain. Consequently, POU factors can bind with higher affinity to extended recognition motifs (308, 321). The POUsp and the POU-homeodomain (POUhd) form modular structures that are separated by a short linker sequence (15-27 aa in length) which has no apparent structural properties (329). The three dimensional structure of the POU domain bound to an octamer motif has been solved using X-ray crystallography (308). Each subdomain binds to DNA in the major groove making base and phosphate specific contacts through helix-turn-helix motifs

Figure 7. Schematic representation of the Oct-1 and Oct-2 transcription factors.

Human Oct-1 and Oct-2 proteins are represented as rectangles with numbers corresponding to amino acid positions (diagram adapted from [382]). Highlighted through elevation, is the centrally located bipartite POU domain flanked by glutamine (Q), serine/threonine (S/T) and proline (P)-rich activation domains. Below, the POU domain is expanded to show the α-helical secondary structures represented by 3 dimensional cylinders. Regions containing basic residues are indicated by +++. The POU specific (sp) and POU homeodomain (hd) are separated by a short linker sequence in various POU family members (14-25 aa in length). Various functional properties of the POU domain are illustrated below the schema. The homeodomain, like most homeodomain proteins recognize A/T-rich sequences with a TAAT core. Alone the POUsp domain recognizes 5 bp DNA motifs with an ATGNN core (N= any nucleotide). Together the POUsp and POUhd cooperate to bind to DNA with very high affinity to sequences with a certain degree of degeneracy. Oct-1 and Oct-2 bind to sequences that resemble ATGCAAAT. The POU domain can form protein-protein interaction with OCA-B and VP-16 which have been shown to selectively activate transcription from specific DNA sequences ATGC<u>A</u>AAT (an adenine must be at position 5) and TAATGARAT (R=purine), respectively.



Oct-2
1
194
357
467
N -
Q
sp
HD
P S/T P
-C
Oct-1
1
279
438
743
N -
Q
Q
sp
HD
S/T
-C
POU-specific domain
POU homeodomain
14-25 aa
Binds to ATGNN
Binds to TAAT
High affinity binding to ATGCAAAT (Oct-1/Oct-2)
Protein-protein interaction with the OCA-B coactivator
Protein-protein interaction:
VP16 (Oct-1)

(330). The N-terminal arm of the POUhd makes additional contacts with the DNA minor groove, whereas the linker region does not appear to contact DNA directly.

POU factors play key roles in developmental programs during early embryogenesis and specify cell-type specific terminal differentiation events (331-337). POU factors have been categorized into at least 6 classes, based on primary amino acid sequences of their POU domains and the conservation of the variable linker regions (44). Beyond the POU domain there are no structural characteristics shared between POU factor family members. In addition to the POU domain itself the N- and C- terminal regions appear to carry distinct gene transcription regulatory functions.

Insights into mechanisms of gene transcription by POU proteins are beginning to emerge. The modulation of transcription by Pit-1 is regulated by co-repressor complexes (N-CoR, mSin 3A/B, HDAC2) and co-activator complexes (CBP/pCAF) (316). The constitutive gene regulation of Pit-1 is activated through signal transduction pathways by forskolin or insulin/epidermal growth factors that stimulate Pit-1 association with CBP/PCAF.

The octamer DNA binding motif is found in a large number of gene regulatory regions including constitutive, cell-specific and viral promoters recognized by either RNA pol II or RNA pol III. The ubiquitous 100 kDa Oct-1 protein is cell cycle regulated and is a primary determinant for the expression of the histone H2B gene (338-340), the constitutive expression of small nuclear (sn) RNA genes (341-346) and the expression of many viral genes. Octamer motifs have been identified in the viral promoters of the MMTV (17, 347, 348), the simian virus 40 (SV40) (349, 350) and the herpes simplex virus (HSV) (351). Oct-1 has also been reported to participate in adenovirus replication,

presumably through the recruitment of the DNA polymerase complex through a direct protein-protein interaction with the Oct-1 POUhd (352-355).

In addition to binding directly to octamer motifs, Oct-1, but not Oct-2, is able to form a ternary complex on DNA with the HSV protein 16 (VP-16 transcriptional activator, also referred to as Vmw65, alpha-TIF, VF65 and ICP25 (356, 357) and a host cell factor (HCF, also termed C1, VCAF and CFF) (358). The ternary complex has an altered DNA binding specificity of its recognition sequence than Oct-1 alone, binding with high affinity to the 5'-TAATGARAT-3' DNA motif (with R=Purine) (128). This redirected complex is required for the expression of immediate-early (IE) genes of HSV. The POUhd of Oct-1 is sufficient to induce formation of a complex with VP-16 (359). Furthermore, a single amino acid in the homeodomain distinguishes the ability of Oct-1/Oct-2 to associate with VP-16 (360). When the amino acid at position 22 of the homeodomain is a glutamine (E) residue, corresponding to the primary amino acid structure of Oct-1, the octamer factor is able to associate with VP-16. However, if residue 22 of the homeodomain of Oct-1 is an alanine (A) as in Oct-2, the octamer factor is unable to associate with VP-16 (360). Once the complex is bound to the HSV enhancer, VP-16 directs transcriptional activation through a potent carboxyl-terminal acidic activation domain (361).

Oct-2 (~60 KDa) is expressed in B lymphocytes and cells of the central nervous system (6, 333, 362) and is involved in the lymphoid-specific expression of immunoglobulin light and heavy chains (363). Octamer response elements are found in both promoter and enhancer regions of these genes (364-371). Oct-2 is required for B-cell maturation and postnatal survival of mice, but not in early B-cell development (372).

In homozygous Oct-2 null mutant mice, cells which phenotypically resemble B-cells appear to develop normally; however, upon external stimulation (T-cell derived signals) these B-cells do not increase their immunoglobulin expression. The mutant B-cells are functionally defective and unable to secrete immunoglobulins.

Both Oct-1 and Oct-2 mediated transcription in B cells depends on their association with a cell-type specific co-activator, Octamer transcription factor -B (OCA-B) (OBF-1, Bob1) which is essential for high levels of immunoglobulin expression (373-377). OCA-B acts together with Oct-1 and Oct-2 to selectively activate transcription from response elements with an A at position 5 of the octamer motif (i.e. 5'-ATGCAAAT) (375, 378). OCA-B will not bind to protein-DNA complexes containing a T at position 5. Biochemical analysis and crystallography of the ternary complex formed by DNA, octamer factors and OCA-B, show that OCA-B contacts DNA directly (adenine 5 and its complementary base) (374, 375, 379-381). OCA-B has been proposed to stabilize the DNA binding activity of octamer factors and may act as a scaffold for building transcriptionally active complexes. Its role in relation to activities associated with chromatin remodelling factors, co-activators with HAT activity, or others remains to be established.

While Oct-1 and Oct-2 bind the same DNA sequence motifs, they differentially regulate transcription through promoter selective activation domains (382) (see Figure 2). Immunoglobulin genes have octamer motifs in both the promoter and enhancer regions and both regions are required for their expression in a B cells (383-385). Although both Oct-1 and Oct-2 can activate transcription from the promoter proximal motif, Oct-2 was found to be required for the activation through the enhancer regions. Moreover, Oct-2 is

generally a stronger activator of mRNA promoters than Oct-1 (382, 386, 387). The activation of mRNA promoters from remote sites by Oct-2 has been attributed to the unique C-terminal activation domain (386).

Lastly, Oct-1 but not Oct-2 is able to selectively activate snRNA promoters (341, 387). The selectivity is dictated by determinants in the POUsp domain (387, 388). Basal transcription of RNA pol II snRNA promoters requires a proximal sequence element (PSE) recognized by snRNA activating protein complex (SNAPc) a core promoter-binding factor consisting of five subunits: SNAP190, SNAP50/PTFb, SNAP45/PTFd, SNAP43/PTFg and SNAP19 (reviewed in (389)). SNAPc and TBP bind cooperatively to their targets to nucleate the assembly of an RNA pol II transcription initiation complex. Enhanced transcription requires a distal sequence element (DSE) and is present in the snRNA promoter. The DSE contains a binding site for Oct-1 and when coupled with the PSE results in the cooperative binding of Oct-1 with SNAPc (390, 391). The cooperative binding is a result of a direct protein-protein interaction involving the Oct-1 POUsp domain and a 40 amino acid region within the carboxy terminal region of SNAP190. It has been hypothesized that in the absence of Oct-1, the carboxy terminus of SNAP190 imposes an inhibition of the DNA binding activity of SNAPc. The interaction of Oct-1 with SNAP190 relieves the inhibition of DNA binding by SNAP190 and promotes transcriptional activation (390, 392).

13. Homeodomain protein functional interactions with other transcription factors.

Many homeodomain proteins bind individually to DNA sequences containing a core homeodomain binding site with moderate affinity, *in vitro* (306, 393, 394). *In vivo*, selectively and specificity of homeodomain protein-DNA binding appears to come in

large part from interactions with co-factors or with other transcription factors on promoters containing binding sites for multiple transcription factors in addition to homeodomain binding sites (395, 396). Cofactors and other sequence specific transcription factors appear to function by increasing the affinity and the specificity of homeodomain proteins for their response elements (19, 397-401). Following numerous observations of these interactions, it is now generally accepted that homeodomain proteins combine with other factors to elicite transcriptional responses.

For POU factors both the POUsp domain and the homeodomain contribute to increased DNA binding selectivity and affinity when compared to other homeodomain proteins (321). POU factors also have been observed to interact with many sequence-specific transcription factors to regulate gene transcription. Several examples exist of POU factors interacting functionally with other transcription factors including GATA factors (402, 403), Ets factors (35, 404), HMG-box containing proteins (34, 405) and others (400, 406, 407). Interaction of Oct-2 with HMG-2, a non-histone chromatin associated factor with non-sequence specific DNA binding activity has been shown to stabilize the sequence-specific DNA binding activity of Oct-2 (405). The direct protein-protein interaction between Oct-2 and HMG-2 requires the POU domain and results in increased levels of gene transcription.

In addition many NRs and POU factors interact functionally to modulate gene transcription. Both synergistic (7-20) and inhibitory (15, 16, 21-26) interactions have been observed. Genetic and functional interactions have been described between Ftz, a homeodomain protein, and the Ftz-F1 orphan receptor in the developing *Drosophila* embryo (19, 20). Ftz-F1 facilitated the binding of Ftz to a weak binding site located in an

enhancer element upstream of the ftz gene and this was required for formation of the proper number of segments during development of the drosophila larvae.

Functional interaction has also been described for ER and Brn 3a/Brn3b (15). This interaction requires the DNA binding domain of ER and the POU domain of Brn 3a/3b. The direct ER and Brn 3a/3b interaction is proposed to result in an increased affinity of ER for an ERE and the type of response was dictated by either Brn 3a or Brn 3b. For example, the ER mediated transcriptional response was synergistically activated by Brn 3b, while no effect or a mild repression effect was observed by Brn 3a. It was proposed that Brn 3a/3b acts through a trimeric complex including the POU factor, ER and an ERE.

The POU factor, Pit-1, has been shown to interact with TR to activate transcription from the growth hormone gene (408) and with ER to modulate gene transcription from the prolactin promoter (12, 18). The RXR has been shown to interact with Oct-1 and Oct-2 (409). This interaction mapped to the DBD and hinge region of RXR and the homeodomain of Oct-1. The interaction of Pit-1 with Oct-1 and Oct-2 has shown to repress gene transcription of promoters regulated by RXR/TR heterodimers. *In vitro*, the POU domain of Oct-1 was shown to interfere with the binding of RXR/TR heterodimers to DNA using EMSAs. Therefore, it was proposed that Oct-1 may repress gene transcription by inhibiting heterodimerization of RXR and TR. Note that the protein-protein interaction described between the NRs and POU factors have consistently mapped to DBD/hinge region (12, 15, 409) and region including the POU domain, respectively.

In contrast, GR can repress transcription in response to hormone agonist on promoters with consensus octamer motifs but lacking functional HREs such as those derived from the histone H2B (21), prolactin (23) and gonadotropin releasing hormone (GnRH) (24-26, 410) genes. Comprehensive studies by several groups have shown that the repression was a result of a direct protein-protein interaction between GR and Oct-1. Initially, this protein-protein interaction was shown by co-immunoprecipitation (IP) and crosslinking assays (21) and later the complex was demonstrated to be tethered to DNA through Oct-1 on both the prolactin and GnRH promoters by gel shift assays with GR specific antibodies (23, 26). Alternatively, using super stoichiometric amounts of GR to Oct-1, GR was shown to disrupt Oct-1 binding to DNA, *in vitro*. Using domain swapping experiments, the homeodomain of Oct-1 was proposed to be important for mediating the interaction with GR (21). Other experiments showed that GR could mediate transcriptional repression through both Oct-1 and Oct-2 (16), in addition to the heterodimer consisting of Oct-1/pbx (pbx, a homeodomain protein) (23).

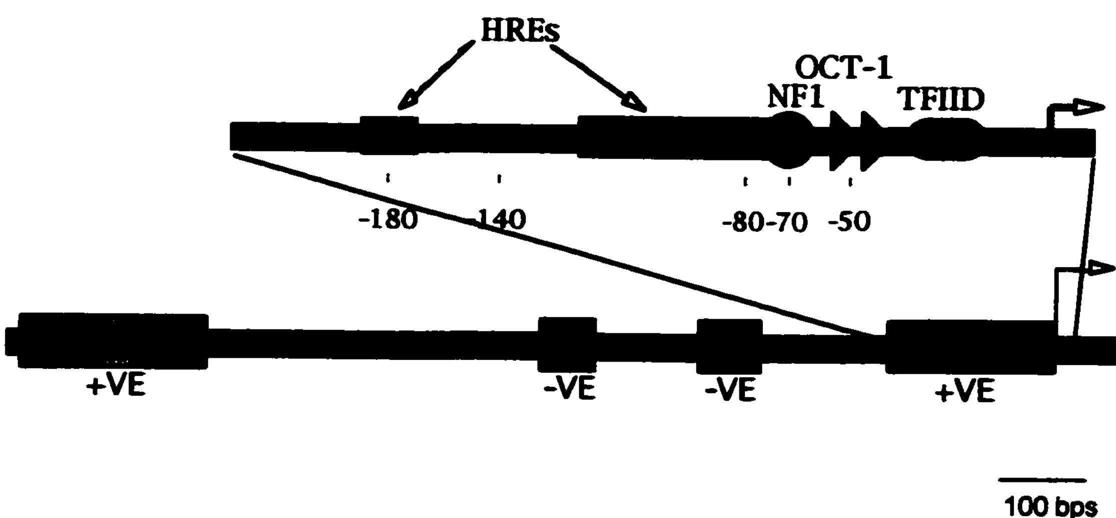
14. GR and Oct-1 converge to synergistically activate transcription on the MMTV promoter

MMTV is a retrovirus that functions by integrating into the murine genome upstream of cellular proto-oncogenes and specifically activates these genes in the breast epithelium during lactation in response to elevated levels of steroid hormone (411, 412). The MMTV LTR contains complex multiple regulatory regions dispersed throughout the LTR that function to either activate or repress gene transcription (Figure 8a) (413). The proximal 190 bps upstream from the transcriptional start site (nucleotides -190 to 1) harbor the necessary information for mediating induction by steroid hormones. In

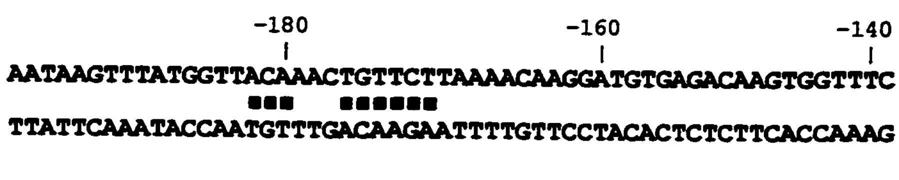
Figure 8. MMTV LTR and its nucleosome structure.

a. A schematic representation of the MMTV LTR with enlarged boxes representing regions conferring positive (+VE) or negative (-VE) gene regulation, indicated below the boxes of the main schema. A scaled bar representing the length of 100 bps of DNA (bottom right). The promoter proximal region is enlarged to demonstrate the position of the HREs and binding sites (green rectangle) for NF-I (olive circle), Oct-1 (orange triangle) and TFIID (blue rounded bar). The numbers below the enlarged schema indicate the nucleotide position relative to the transcriptional start site (bent arrow). b. The region of the LTR encompassing -197 to +15 is shown, with the arrow indicating the viral transcriptional start site at +1. Immediately upstream from the start site is the TATA box (✚) at -28 which is the assembly point of the transcription initiation complex. The binding sites for relevant transcription factors are highlighted. The ■ outlines the sequences within the 4 binding sites for GR that are identical to the consensus receptor half site. The core NF-I binding sites are highlighted by (✗) and two degenerate octamer motifs are represented by (✪). c. Three dimensional organization of the MMTV LTR DNA (-221 to +123) around 2 histone octamer cores, labeled A and B (adapted from Collingwood, et al. 1999 [195]). Below the schema are numbers representing the amino aid positions of the nucleosome boundaries. Above the main schema is a key for understanding the location of the binding sites relative to the nucleosome boundaries. The solid bar represents the DNA binding sites for GR, the hatched bar represents the binding site for NF-I, the crossed bar, the binding sites for octamer factors and the speckled bar the binding site for TFIID, DNA binding component of RNA pol II. The hooked arrow indicates the transcriptional start site position. Note the octamer motifs are situated in the linker region between 2 nucleosomes and should be accessible in the context of ordered chromatin.

a.



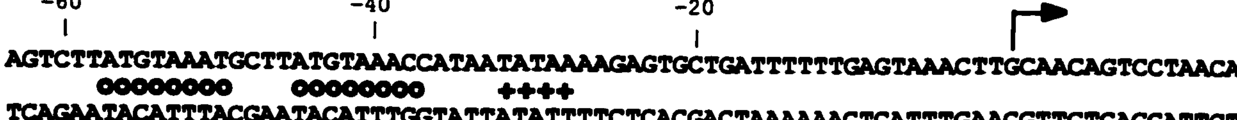
b.



-120 -100 -80

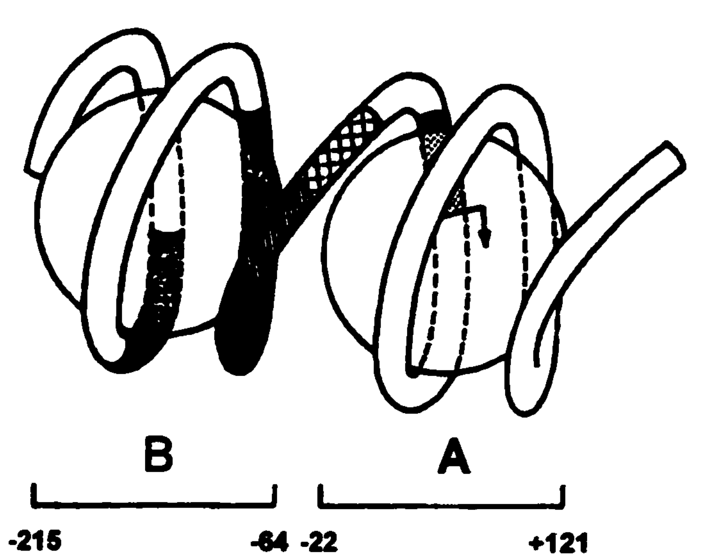
CTGAGTTGGTTTGGTATCAAATGTTCTGATCTGAGCTCTTAGTGTTCTATTTTCCTATGTTCTTTTGGAATCTATCCA

GACTCAACCAAACCATAGTTTACAAGACTAGACTCGAGAATCACAAGATAAAAGGATACAAGAAAACCTTAGATAGGT



C.

Trans-acting Factor	GR	NF1	OCT	TFIID	Transcription Start Site
cis-Acting Element					



addition to containing multiple copies of HREs (-185 to -79) (414), binding sites for NF-I (-75 to -64) (41), Oct-1 (-57 and -36) and a core promoter element (centered at nucleotide -30) (415) are found in the MMTV promoter proximal region. The MMTV promoter proximal region responds to GR, PR, AR, and MR (265, 416-419). Although together the HREs represent high-affinity binding sites for steroid receptors (166, 416, 420, 421) *in vitro,* the sequences are highly degenerate (Figure 8b, solid squares) from a consensus HRE represented by TGTTCT arranged as an inverted repeat separated by three nucleotides.

The promoter proximal negative response element (nucleotides -421 to -364) can act to repress glucocorticoid-mediated gene activation of the MMTV LTR (422). This negative regulatory element is a binding site for the Ku autoantigen and DNA-dependent protein kinase (DNA-PK). Gene regulation is dependent on kinase activity of Ku/DNA-PK and may occur through direct phosphorylation of GR (423).

The chromatin structure of the MMTV LTR reflects the precise positioning into six nucleosomes, A to F and comprise both translational and rotational phasing (424). Translation phasing describes the entry and the exit points of the DNA from the histone octamer, the protein component of a nucleosome. The rotational phasing is the curvature of the DNA around nucleosomal histones and is usually found in 10.5 bp increments representing one turn of the DNA helix.

Nucleosome B (nucleotides -215 to -64 +/-20 bps) and A (nucleotides -22 to +121) occur over the promoter proximal and transcriptional initiation sites, respectively (425-427) (Figure 8b, c). Nucleosome B encompasses the 4 HREs and at least part of the NF-I binding site while the octamer motifs are located predominantly in the linker region

between nucleosomes B and A. High-resolution mapping studies at single nucleotide resolution, show nucleosome A adopts one major translational phase and encompasses nucleotides -22 to +121 (40). By contrast, nucleosome B adopts 3 major translational phases separated by twenty bps, B5 (nucleotides -235 to -86), B1 (nucleotides -215 to -64) and B4 (nucleotides -193 to -47). Even though three major translational phases are adopted by nucleosome B, the rotational phase is unchanged for all three translational phases with a 10 bp periodicity located at the same position. This means the orientation of response elements relative to the nucleosomal histones is conserved in all three phases.

In the context of chromatin and in the absence of hormones, transcription mediated by the MMTV LTR is strongly repressed (428). Despite their constitutive presence in the nucleus, neither NF-I nor Oct-1 was observed bound to the chromatin template (36, 43). The NF-I binding site is blocked by nucleosome B, because the major groove of the DNA binding site is oriented towards the histone octamer. It is less obvious why Oct-1 is not bound in the absence of hormone because the octamer motifs are located predominantly in the linker between nucleosome B and A.

Progestins and glucocorticoids induce events leading to chromatin remodelling of nucleosome B of the MMTV LTR *in vivo* (39, 429) and result in occupancy of all promoter proximal cis-acting elements in chromatin including NF-I and Oct-1 binding sites (39, 43). Following DNA binding, functional synergy is observed between the corresponding transcription factors. For NF-I, GR/PR binding induces chromatin remodelling potentially through recruitment of an ISWI remodelling complex (39). Binding of NF-I and Oct-1 is coincident with remodelling of chromatin induced by GR/PR.

In contrast to the situation on chromatin, NF-I and GR act independently of one another on naked DNA (41). On transiently transfected plasmid DNA, NF-I is constitutively bound to the MMTV promoter proximal region. Moreover, experiments on linear DNA, show GR and NF-I actually compete for DNA binding to the promoter proximal region. On transiently introduced templates, NF-I is responsible for mediating high levels of basal transcriptional activity and is independent of steroid induced transcription (17). Together, this suggests that chromatin and chromatin remodelling are required for cooperative binding of GR and NF-I to the MMTV promoter proximal region.

By contrast, DNA binding and activation of MMTV transcription by GR/PR and Oct-1 have been shown to be highly cooperative on naked DNA (17). The octamer motifs located in the MMTV promoter proximal region are somewhat degenerate from a consensus octamer motif and Oct-1 binds with low affinity. With transiently transfected reporter genes driven by the MMTV promoter, transcriptional activity contributed by the octamer motifs was synergistic in response to glucocorticoids and progestins. Deletion of both octamer motifs or the distal octamer motif alone resulted in loss of most of the hormone dependent transcriptional activity while having a mild effect on the basal level of transcription (17). Using synthetic promoters with juxtaposed binding sites for GR and Oct-1, demonstrated that these were sufficient for transcriptional synergy and that both binding sites were required (16).

For cooperative binding studies *in vitro*, the addition of purified GR or purified PR reduced the concentration of purified Oct-1 required to saturate the octamer motifs on the MMTV LTR (17). This parallels results *in vivo*, showing Oct-1 binds cooperatively

with PR/GR (43). Cooperative binding of purified factors suggests that GR directly facilitated binding of Oct-1 to DNA. Indeed, it has been proposed that a protein-protein interaction between GR and Oct-1 existed (21). Thus a GR/Oct-1 protein-protein interaction may be a major component for transcriptional synergy on the MMTV LTR mediating transcription both on chromatin and naked DNA while actual chromatin and chromatin remodelling events may contribute to a lesser extent.

15. GR full-length binds to the POU domain of Oct-1/-2 *in vitro* and the interaction is sensitive to GR point mutations C500Y and L501P.

In my Master's thesis, I demonstrated that GR bound through its DBD/hinge region to the POU domain of Oct-1/-2 *in vitro* and I identified 2 point mutations in the GR DBD that abrogated binding (45).

My initial binding studies showed that steroid-treated *in vitro*-translated-GR bound efficiently to the POU domain of Oct-1 expessed as a glutathione S transferase (GST) fusion protein. However, the hsp-associated naive GR did not. Ligand binding *per se* was not required to make GR competent for binding to the POU domain of Oct-1 but dissociation of the hsp from the receptor were important. This was demonstrated by treating the receptor with heat and high salt or with RU486, an antagonist, to dissociate the hsps from the receptor to produce a receptor competent for Oct-1POU binding. Further, the GR /Oct-1 interaction was shown to be independent of DNA because the binding was resistant to 400 µg/ml of ethidium bromide. At this concentration, ethidium bromide has been shown to disrupt protein-protein interactions stabilized by DNA.

Mapping studies showed that this protein-protein interaction occurred between the POU domain of human Oct-1 (aa 280-439) and the DBD/hinge region of rat GR (aa 407-

556). Further, the binding was sensitive to point mutations at C500Y and L501P located in helix 2 of the GR DBD (see Figure 2a and b). As the GR DBD mapped to a highly conserved motif and that several reports demonstrated a protein-protein interaction between the NR DBD/hinge and the POU domain of POU factors, it was possible that other NRs could interact with POU domains. Indeed at least *in vitro*, I found that PR, AR, ERα, RARα, RXRα, TRα, and the distantly related Ftz-F1α bound to the POU domain of Oct-1. For the steroid receptors tested (PR, AR and ERα), binding was hormone dependent. Further the binding of a series RARα C-terminally deleted peptides showed the interaction was lost at the same location observed with GR C-terminally deleted peptides. Together, these results suggested the potential for a broadly based interaction between NRs and POU factors in the cell.

16. Objectives

The goal of my Ph. D. project has been to explore the contribution of the protein-protein interaction observed *in vitro* to the transcriptional synergy between GR and Oct-1 on the MMTV promoter proximal region *in vivo*. Additionally I have explored whether broadly based *in vitro* binding between NRs and Oct-1 also occurs in the cell.

Specific aims

1. Determine if GR and Oct-1/-2 associate *in vivo* and to dissect the nature of the binding.
2. Explore the determinants on Oct-1 and highly related Oct-2 required for interaction with GR.
3. Determine the role of the GR/Oct-1 interaction in cooperative binding and transcriptional synergy at the MMTV enhancer/promoter.
4. Explore the potential interaction of other NRs with POU factors, *in vivo*.
5. During the course of experiments, I determined GR not MR could interact with Oct-1/-2 *in vivo*. I sought to use a chimeric receptor strategy to explore determinants for the trancriptional synergy on the MMTV enhancer/promoter.

II. MATERIAL AND METHODS

1. Materials (list and suppliers)

The synthetic GR ligand 1,4-Pregnadien-9α-fluoro-16α-methyl-11β,17,21-triol-3,-20 dione (dexamethasone, dex) was purchased from Steraloids; R5020 was a gift from Roussel Uclaf, France. The natural MR ligand 18-aldocorticosterone (aldosterone, ald) and 17 beta-hydroxy-5alpha-androstan-3-one (dihydrotestosterone, DHT) were obtained from Sigma. All restriction enzymes and DNA modifying enzymes were purchased from New England Biolabs, with the exception of λ exo, which was purchased from Life Technologies BRL. The *in vitro* coupled transcription translation kit, TNT® Quick Coupled Transcription/Translation System was obtained from Promega. All tissue culture reagents including fetal bovine serum (FBS) and Geneticin ® (G418) were obtained from Life Technologies, Bethesda Research laboratories (BRL). Lipofectamine® transfection reagent was obtained from Life Technologies, BRL.

2. Plasmids acquired externally

(a) pGSTOct-1POU also know as (a.k.a.) pET11c.G.POU-1: An inducible bacterial expression vector (pET11c, Novagen) that expresses a glutathione S transferase (GST) tag derived from pGEX2T (Pharmacia) fused N-terminally to the POU domain of human Oct-1 (aa 280-439) (321). Complete cDNA Oct-1 accession number is X13403. The plasmid was a gift from Dr. W. Herr, Cold Spring Habor Laboratories, N.Y.

(b) pRDN93-GR: An *in vitro* translatable plasmid (SP6 RNA pol) that expresses full-length rat GR, lacking the 21 consecutive Q residues in the N-terminus of GR (aa 75-96)

(430). GR complete cDNA accession number M14053. The plasmid was a gift from Dr. K.R. Yamamoto, UCSF, CA.

(c) pET11dCREB: An *in vitro* translatable vector (Novagen) (T7 RNA pol) expressing rat cyclic AMP response element binding protein (CREB) protein (431). A gift from Dr. P. Sassone-Corsi, Strasbourg, France.

(d) pT7luc: Control DNA for *in vitro* translation (T7 RNA pol) expresses firefly luciferase (Promega).

(e) pSV2-Neo: The plasmid contains a neomycin resistant gene down-stream of the SV40 early promoter has been described elsewhere (432). The plasmid was a Gift from Dr. M. Beato, Marbourg, Germany.

(f) pGEX2T: A bacterial expression cloning vector that expresses GST. Plasmid accession number U13850 (Pharmacia).

(g) pGEX3X-dlx2: An inducible bacterial expression vector (pGEX-3X, Pharmacia) that expresses GST fused N-terminally to full length zebrafish dlx2 (433). A gift from Drs. T. Zerucha and M. Ekker, University of Ottawa, ON.

(h) pGEX2T-PrdHD: A bacterial expression vector (pGEX2T, Pharmacia) that expresses GST fused N-terminally to the homeodomain of *Drosophila* Prd protein (434). A gift from Dr. D.S. Wilson, The Rockefeller University, N.Y.

(i) pGEX2T-OtdHD: Unpublished bacterial expression vector (pGEX2T, pharmacia) that expresses GST fused N-terminally to the homeodomain of *Drosophila* orthodenticle

(Otd). In addition to the homeodomain 11 non-homeodomain aa were included immediatly upstream of the homeodomain as well as 4 non-homeodomain aa at the C-terminus. A gift from Dr. D.S. Wilson, The Rockefeller University, N.Y.

(j) pGEX3X-msxB: An inducible bacterial expression vector (pGEX3X, Pharmacia) that expresses GST fused N-terminally to the aa 135-196 of zebrafish msxB (433). A gift from Drs. T. Zerucha and M. Ekker, University of Ottawa, ON.

(k) pGEX2T-FtzHD: Unpublished bacterial expression vector (pGEX2T, pharmacia) that expresses GST fused N-terminally to the homeodomain of Drosophila fushi-tarzu (Ftz). In addition 4 non-homeodomain aa were included immediately upstream of the homedomain and 11 aa at the C-terminus. A gift from Dr. D.S. Wilson, The Rockefeller University, N.Y.

(l) pTL2-dlx2: A mammalian expression vector (pSG5 derivative (435)) expressing full-length zebrafish dlx2 protein (433). A gift from Drs. T. Zerucha and M. Ekker, University of Ottawa, ON.

(m)pTL2-hoxD4: A mammalian expression vector (pSG5 derivative (435)) expressing full-length zebrafish hoxD4 protein (433). A gift from Drs. T. Zerucha and M. Ekker, University of Ottawa, ON.

(n) pGalO: A mammalian expression vector expressing aa 1-147 of the yeast Gal4 protein (436). A gift from Dr. G. F. Tomaselli, Johns Hopkins Oncology Center, Maryland.

(o) pNL-VP16: A mammalian expression vector expressing the first 11 aa of the yeast Gal4 protein followed by the SV40 Large T- antigen NLS (PKKKRKVD) and aa 411-455 of the HSV protein (VP16) (436). Also a gift from Dr. G. F. Tomaselli, Johns Hopkins Oncology Center, Maryland.

(p) pG5E1BCAT: The plasmid (pSP72 derivative, Promega) contains a chlorampenicol acetyltransferase (CAT) reporter gene whose expression is driven by 5 tandem Gal4 binding sites immediately upstream of the E1B adenovirus minimal promoter (437). The plasmid was a gift from Dr. G. F. Tomaselli, Johns Hopkins Oncology Center, Maryland.

(q) pCGN-Oct-2: A mammalian expression vector expressing hemagglutinin tagged (HA) human Oct-2 protein (386). Oct-2 complete cDNA accession number M36653. A gift from Dr. W. Herr, Cold Spring Habor Laboratories, N.Y.

(r) pSG5-CREB: A mammalina expression vector expressing full length rat CREB (438). A gift from Dr. P. Sassone-Corsi, Strasbourg, France.

(s) p6RGR: A mammalian expression vector expressing full length rat GR (275). GR complete cDNA accession number M14053. A gift from Dr. K.R. Yamamoto, UCSF, CA.

(t) pSVPR a.k.a. pKSV10-rPR: Mammalian expression vector expresses the complete rabbit PR gene under constitutive expression of the SV40 enhancer/promoter, the plasmid has been described elsewhere (439). In addition to carrying genes for antibiotic resistance to ampillicin, pSVPR is also resistant to tetracyclin. The plasmid was obtained

from Dr. E. Milgrom, Le Kremlin-Bicetre, France. Rabbit PR accession number is M14547.

(u) pSV40AR: A mammalian expression vector expressing rat AR (440). AR complete cDNA accession number M23264. A gift from Dr. Matusik, University of Manitoba, MB.

(v) p6RMR: A mammalian expression vector expressing full length rat MR (275). MR complete cDNA accession number M36074. A gift from Dr. K.R. Yamamoto, UCSF, CA.

(w) HRE-TKCAT: A CAT reporter gene construct with the HSV-thymidine kinase (TK) promoter (-109 to +51) whose expression is driven by two copies of a HRE (GGG ACA CAG TGT CCT) (441). A gift from Dr. M. Beato, Marburg, Germany.

(x) ERE-TKCAT: A CAT reporter gene construct with the HSV-TK promoter (-109 to +51) whose expression is driven by two copies of an estrogen response element (ERE) (GGG TCA CAG TGA CCT) (441). A gift from Dr. M. Beato, Marburg, Germany.

(y) phGR, aka $phGR_{NX}$: A mammalian expression vector (pRS (442)) expressing full length human GR with NotI and XhoI restriction site immediately flanking the DNA binding domain (DBD) (443). The creation of the NotI site resulted in a point mutation of P416R, whereas the introduction of the XhoI site produced a silent mutation. hGR complete cDNA accession number M10901. A gift from Dr. R.M. Evans, The Salk Institute, CA.

(z) phMR, aka $phMR_{NX}$: A mammalian expression vector expressing full length human MR with NotI and XhoI restriction sites flanking the DBD. The creation of the NotI site resulted in a point mutation at S600P and the creation of the XhoI resulted in a point mutation at G671E (166, 443). Complete MR cDNA accession number M16801. A gift from Dr. R.M. Evans, The Salk Institute, CA.

(aa) phGMG: A mammalian expression vector expressing full-length human GR aa 1-777 with aa 420-479 substituted with aa 602-661 of human GR, in addition to a point mutation at residue P416R (443). A gift from Dr. R.M. Evans, The Salk Institute, CA.

(bb) phMGM: A mammalian expression vector expressing full-length human MR aa 1-984 with aa 602-671 substituted with aa 420-489 of human GR in addition to a point mutation at S600P (443). A gift from Dr. R.M. Evans, The Salk Institute, CA.

3. Plasmids constructed onsite

The plasmids listed below describe briefly the strategy employed for construction followed by the name of the collaborator, who constructed the plasmid. In the event that no name is listed at the end of the plasmid description the plasmid was created by myself.

(a) pTL2mtg-GR: The mammalian expression plasmid which expresses 6 tandem copies of the c-myc epitope fused N-terminally to rat GR aa 21-795 was cloned using a 2 step cloning strategy. First the plasmid termed pTL2mtg was created by insertion of the 350 bp BglII/SmaI fragment from pCRIIMTG (a gift from Dr. J. Bell, University of Ottawa, ON) into pTL2 Ftz-F1α (439) gift from Dr. M. Petkovich, Queen's University, ON) digested with the same restriction enzymes replacing the sequences encoding for the

Drosophila Ftz-F1α gene. In step 2, the MscI/BamHI 2.4 kB fragment derived from p6RGR (275) was inserted into the SmaI/Bam HI polycloning sites of pTL2mtg. The rat GR accession number is M14053.

(b) pTL2mtg-GR_{L501P}: The plasmid was cloned exactly as described for pTL2mtgGR except the plasmid used in step 2 was p6RGR_{L501P}. p6RGR_{L501P} was cloned by L. Pope employing a site directed mutagenesis stratagy (Sculptor mutagenesis kit by Statagene).

(c) pSP64-Oct-2: The plasmid can be used for *in vitro* translation and expresses sequences encoding the entire human Oct-2 protein. The plasmid was created by excising the 1715bp XbaI/BamHI fragment from pCGN-Oct-2 (386) and inserting it into the same sites of pSP64 (Promega). Dr. M. Traykova-Andonova constructed the plasmid. The human Oct-2 cDNA Accession number is M36653.

(d) pGEM7Z-Oct-1: The plasmid was created by insertion of the HindIII/BamHI 2584 bp fragment from pBSOct-1 (6) (a gift from Dr. W. Herr, Cold Spring Habor Laboratories, NY) into pGEM 7Z restricted with the same enzymes. Dr. M. Traykova-Andonova constructed the plasmid.

(e) pGEX2T-GR_{X568}: The plasmid expresses GST fused N-terminally to rat GR aa 407-568. The plasmid was cloned by inserting the ~500 bp BamHI/EcoRI fragment from pSP64X568 (444) into the same sites of pGEX2T (Pharmacia). Dr. M. Traykova-Andonova constructed the plasmid.

(f) pGEX2T-GR_{C500Y} The plasmid expresses GST fused N-terminally to rat GR aa 407-556 with a point mutation at C500Y. The plasmid was constructed by inserting the ~500

bp BamHI/SmaI fragment from pT7C500Y (444) into the same sites of pGEX2T. Dr. M. Traykova-Andonova constructed the plasmid.

(g) pTL2HA-Oct-2: The mammalian expression plasmid expresses the full length human Oct-2 sequence with an N-terminal flu hemagglutanin (HA) epitope . The plasmid was cloned in 2 steps. In the first step, the Oct-2 sequence was excised with XbaI (blunted)/BamHI from pCGN-Oct-2 (386) and inserted into the SmaI/BamHI site of the yeast expression vector pACT-2 (Clonetech) to acquire the HA epitope and was termed pACT-2HAOct-2. Second, pACT-2HAOct-2 was digested with BglII/XhoI and the fragment corresponding to Oct-2 with an HA tag was inserted into the same sites of TL2 Ftz-F1α (a gift from Dr. M. Petkovich, Queen's University,ON) replacing the sequences encoding the Ftz-F1α gene. Dr. M. Lemieux constructed the plasmid.

(h) pTL2HA-Oct-2ΔPOU: The plasmid was created using a two step cloning strategy strategy. First, a Bsi WI site was created by site directed mutagenesis (Sculptor mutagenesis kit, Stratagene) on single-stranded DNA with the 5' G CTG CAC CCC AGC CG<u>T</u> AC<u>G</u> GACT GGA GGG GGT GG oligonucleotide producing silent mutations at aa R384 and T385 with a novel restriction site, BsiWI, creating a plasmid termed pTL2HAOct-2 (Bsi WI). Second, the pTL2HAOct-2 (Bsi WI) was digested with XmaI/BsiWI and self ligated. Dr. M. Lemieux created the plasmid.

(i) pTL2HA-Oct-2ΔSP: The plasmid was constructed by digesting the plasmid pTL2HAOct-2 (Bsi WI), described for pTL2HA-Oct-2ΔPOU (i), with EagI and XmaI and filled in with Klenow DNA polymerase I and self ligated. The plasmid restored the

XmaI site and expresses the Oct-2 protein lacking aa 152-286. The plasmid was constructed by L. Pope.

(j) pTL2HA-Oct- 2ΔHD: The plasmid with pTL2HAOct-2 (Bsi WI), described in pTL2HA-Oct-2ΔPOU, was first digested with EagI, filled in with Klenow DNA polymerase I and then digested with PstI and blunted with mung bean nuclease. Finally, the plasmid was self-ligated. The plasmid expresses the Oct-2 protein lacking aa 285-350. The plasmid was constructed by L. Pope.

(k) pGEX2T-GR_{X550}PKA: The plasmid expresses GST fused N-terminally to aa 407-550 of rat GR with a protein kinase A (PKA) recognition and phosphorylation site (LARRASYP). The plasmid was constructed by insertion of a pre-hybrizated double stranded linker DNA consisting of 5'C TTG GCT CGT CGT GCA TCT TAT CCG G and 5'AA TTC CGG ATA AGA TGC ACG ACG AGC, into pGEX2TGR_{X568} digested with StyI/EcoRI. Insertion of the linker produces a unique BspEI site (underlined).

(l) pGEX2T-GR_{L501P}PKA: The plasmid was constructed as described for pGEX2T-GR_{X550}PKA, except that the plasmid used was pGST-GR_{L501P}. pGST-GR_{L501p} was constructed by subcloning the BamHI/SmaI fragment from pT7L501P (445) into pGEX2T (Pharmacia) digested with the same restriction enzymes.

(m)pGEX2T-Oct-2POU: The plasmid expresses aa 185-367 of human Oct-2 fused C-terminally to GST. The plasmid was constructed by PCR amplification using pCGN-Oct-2 (386) as template DNA with the primers, 5'-CG GGA TCC AAA TGC TTG GAG CCA CC and 5'-GAA TTC TGG GCT GGG CAG CAT GG creating a linear DNA

fragment containing the POU domain of Oct-2 with flanking Bam HI and EcoRI sites that was inserted into pGEX2T (Pharmacia) digested with the same enzymes.

(n) pGEX2T-Oct-2HD: The plasmid was constructed by digesting pGEX2T-Oct-2POU with BamHI and EagI and adding the linker oligonucleotides, 5'-GA TCC GGT ACC GGC and 5'- G GCC GCC GGT ACC G, using the linker tailing method (446). Cloning was verified through the creation of a unique KpnI site (underlined).

(o) pGEX2T-HoxC4HD: A DNA fragment encoding aa 148-227 of human HoxC4 was prepared as a blunt ended fragment derived from pGAD HoxC4 and inserted into the SmaI sites of pGEX2T (Pharmacia) eliminating the SmaI sites in the process. The HoxC4 sequence was originally identified in a yeast screen of a human Jurkat T-cell cDNA library (Schild-Poulter C. et al. submitted). The plasmid was created by Dr. C. Schild-Poulter.

(p) pGal-GR_{X556}: The plasmid is a mammalian expression vector that expresses aa 1-147 of the yeast Gal4 protein fused N-terminally to aa 407-556 of rat GR. The plasmid was cloned by inserting the NdeI/SacI fragment from pT7X556 (164) into the same sites of pGalO (436).

(q) pGal-GR_{L501P}: The plasmid was cloned exactly as described for pGal-GR except that the NdeI/SacI GR fragment was excised from pT7L501P (445).

(r) pOct-1POU VP16: The plasmid expresses aa 411-455 of HSV VP16 fused N-terminally to aa 265-444 of human Oct-1 containing the POU domain. Sequences encoding the POU domain were PCR amplified with the upper primer, 5'-CGC TGCTCG

AGC CAC AGA GCC ACT G, and the lower primer, 5'-GCC GTC TAG ACC CAC CAC TGC TTG G, creating a DNA fragment with XhoI and XbaI sites flanking the POU domain. The PCR product was digested with XhoI/XbaI and inserted into the same sites of the pNLVP16 polylinker (436).

(s) pRSV β-Gal: The plasmid expresses the yeast LacZ gene, β-galactosidase (β-gal) from the Rous Sarcoma Virus (RSV) promoter derived from pRSVCAT (447) and inserted in a plasmid termed pGA 307. The plasmid was created by and obtained from M. Walker, UCSF, CA.

(t) p5XGAL-4Oct$_{WT}$E1BCAT: Preannealed oligonucleotides consisting of the Octamer motif distal, 5'-AGC TTG CTT ATG CAA ATA AGG TG and 5'- GAT CCA CCT TAT TTG CAT AAG CA, flanked by BamHI and HindIII half sites were preligated, the ends blunted and the fragments inserted into pG5E1BCAT (437) digested with XbaI and blunted. A plasmid containing four copies of the octamer motif was sequenced and revealed to contain a unique HindIII site. The plasmid was constructed by L. Pope.

(u) p5XGAL-4xOct$_{mut}$E1BCAT: The plasmid was created by insertion of a double stranded oligonucleotide consisting of, 5'CTA GAC ACC TTA TTT GCC GAA GCC ACC TTA TTT GCC GAA GCT AND 5'-CTA GAG CTT CGG CAA ATA AGG TGG CTT CGG CAA ATA AGG AGT, into the XbaI site of pG5E1BCAT (437). The plasmid was constructed by L. Pope.

(v) pMMTV(-237 to +125) CAT: Contains MMTV LTR sequences (-237 to +125) inserted BamHI/PstI, upstream of the CAT gene in pBLCAT3. The plasmid was constructed by H. Safrin.

(w)pBSHREOct: The plasmid was constructed by insertion of the 65 bp HindIII fragment from pTKCAT-GREOct into the HindIII site of pBluescript II KS. The DNA fragment consisted of a single HRE (5' GGG ACA CAG TGT CCT) adjacent the human histone H2B consensus octamer motif (5'-ATG CAA AT). pTKCAT-GREOct was created by Dr. Wei Huang. Preligated Octamer motif oligonucletides, 5'-AGC TTG CTT ATG CAA ATA AGG TG and 5'- GAT CCA CCT TAT TTG CAT AAG CA were inserted into pTKCATGRE restricted with BamHI to create pTKCATGREOctwt.

(x) pBSGalOct: The plasmid was constructed by insertion of a double stranded oligonucleotide consisting of 5'-CGG AGT ACT GTC CTC CGG TAC CTG TAT GCA AAT AAG GT and its complementary strand into the pBluescript KS digested with EcoRV. The plasmid was constructed by L. Pope.

(y) pBSGalIAP: The plasmid was constructed by insertion of a double stranded oligonucleotide consisting of 5'-CGG AGT ACT GTC CTC CGG TAC CCT GCG CAT GTG CCA AG and its complementary strand into the pBluescript KS digested with EcoRV. The plasmid was constructed by L. Pope.

(z) pGAL-$PR_{A\text{-}DBD}$: A DNA fragment containing the human PR_A aa 535-688 was prepared by PCR with the forward, 5'-CAGG ATC CAG GTC TAC CCG CCC TAT C, and reverse, 5' GCT CTA GAG GTT GAT CAG TGG TGG AAT CAA C, primers from

pT7PRA (181) and digested with BamHI/XbaI. The 462bp fragment was inserted into pGalO digested with the same restriction enzymes. The plasmid was constructed by L. Pope.

(aa) pGAL-AR$_{DBD}$: The DNA fragment containing rat AR aa 515-671 was prepared by PCR with the following primers, forward 5' CAGG ATG CCT TAT GGG GAC ATG CGT TTG G and reverse 5' GGT CTA GAT GTC CGG CAC ACA CCA CTC C, using pGEM4ZrAR (440). Following PCR the DNA was digested with BamHI/XbaI and inserted into pGALO digested with the same enzymes. The plasmid was constructed by L. Pope.

(bb) pGAL-MR$_{DBD}$: The DNA fragment encoding rat MR aa 577-709 was prepared by PCR with the following primers, forward: 5' G CGT CGA CTC CTA GAG TAC ATT CCA G and reverse: 5' GCT CTA GAT CGG AGC GAT GTA TGT GG using 6RMR (275) as a DNA template. The DNA fragment was digested with SalI/XbaI and inserted into pGalO digested with the same enzymes. The plasmid was constructed by L. Pope.

(cc) pGAL-ERα_{DBD}: The DNA fragment encoding human ERα aa 164-299 was prepared by PCR amplifiction with the following primers, forward: 5'-C AGG ATC CCC AGT ACC AAT GAC AAG G and reverse primer: 5'-GCT CTA GAC GTT TGA TCA TGA GCG GG, from pHEO (149). The PCR product was digested with BamHI/XbaI and inserted into pGALO digested with the same enzymes. The plasmid was constructed by L. Pope.

(dd) pGAL-RXRα_{DBD}: The DNA fragment encoding aa 118-236 of mouse RXRα was prepared by PCR amplification with the following primers, forward: 5' C GGG ATC CTC AAT GGC GTC CTC AAG G and reverse: primer: 5' GCT CTA GAG GCA TGT CCT CGT TGG CAC, from pSG5RXRα (448). The PCR product was digested with BamHI/XbaI and inserted into pGALO digested with the same enzymes. The plasmid was constructed by L. Pope.

(ee) pGAL-RARα_{DBD}: The DNA fragment encoding aa 60-160 of mouse RARα was prepared by digesting the PCR products with PvuI (blunted) and then BamHI. The DNA fragment was prepared by PCR with the forward: 5'-ACG CCA CCA TCG GGA TCC AGA GCA, reverse: 5'-CAG GCG TCA GCG AGT AG primers using pGEM4ZmRARα' (449) and inserted into pGALO digested with ClaI (filled-in with Klenow DNA polymerase I)/BamHI. The plasmid was constructed by M. Lemieux.

(ff) pGAL-FtzF1α_{DBD}: The DNA fragment encoding aa 478-610 of *Drosophila* Ftz-F1α was prepared by PCR with the following primers, forward: 5'-C GGG ATC CAC AAC TCC GGT CCC GGC AAT C and reverse: 5'-G CTC TAG ATG CCG CTG CCG CAT CAC TTG, from pTL2Ftz-F1α. The PCR product was digested with BamHI/XbaI and inserted into pGALO digested with the same enzymes. The plasmid was created by L. Pope.

(gg) pGal-GR$_{C500Y}$: The plasmid is a mammalian expression vector that expresses aa 1-147 of the yeast Gal4 protein fused N-terminally to aa 407-556 of rat GR. The plasmid was cloned by inserting the NdeI/SacI fragment from pT7C500Y (445) into the same sites of pGal-O (436). L. Pope constructed this plasmid.

(hh) pMMTV(-188 to -36)E1BCAT: The MMTV LTR sequences (-188 to –36) were prepared by PCR amplification with the MMTV –188 forward, 5'-ACA TGC ATG CGT TAC AAA CTG TTC TTA AAA CAA GG and reverse, 5'-GCT CTA GAG GTT TAC ATA AGC ATT TAC primers from pBLMMTV(-631 to +125)CAT. The PCR product was digested with SphI/XbaI and inserted into pG5E1BCAT (437) digested with the same enzymes, effectively replacing the 5 tandem Gal4 DNA binding sites.

(ii) pMMTV(-188 to -36)Oct_{mut}E1BCAT: The plasmid was constructed exactly as described for pMMTV(-188to-36)E1BCAT except for the PCR reaction, the reverse primer used was: 5'GCT CTA GAG GTG GTA CCA AGC TGA TCA ATA AGA CTT GGA TAG. The plasmid was constructed by L. Pope.

(jj) $6RGR_{R498G}$: The plasmid was created by site direct mutagenesis based on the Excite PCR based strategy (Stratagene). Oligonucleotides 5'-GG AAA AAC TGC CCA GCA TGC CGC TAT GGC AAA TGT CTT CAG GCT GG with its complementary strand and p6RGR (275) were used for PCR amplification with Pfu DNA polymerase (Stratagene). The PCR product was digested with DpnI to select against the parental plasmid DNA. The DNA was transformed directly into Bacteria and the positive clones were identified by the creation of a BglI restriction site (underlined in the PCR primers) in p6RGR.

(kk) $6RGR_{R498Q}$: The plasmid was created by site directed mutagenesis using as described above (see $6RGR_{R498G}$). Oligonucleotides 5'-GG AAA AAC TGC CCA GCA TGC CGG TAC CAA AAA TGT CTT CAG GCT GG with its complementary strand and p6RGR (275) were used in the PCR reaction. Positive clones identified by the creation of a KpnI site in p6RGR.

4. Additional oligonucleotides

(a) MMTV+74 to +52 primer 5'(GAAGG ATAAG TGACG AGCGG AGA)3'

(b) T3 universal primer 5'(ATTAA CCCTC ACTAA AGGGA)3'

(c) T7 universal primer 5'(TAATA CGACT CACTA TAGGG)3'

(d) HRE 5'(ACAGT TCGAC ATAGA ACAAA CTGTT CTTAA AAGGT ACCCA)3'

(e) intracisternal A particle (IAP) 5'(CTGCG CATGT GCCAA GGGTA TCTTA TGACT)3'

(f) negative regulatory element (NRE) l_{C3H} 5'(AGCTT GTCTC AAGAA GAAAA AGACG AGAG)3'

(g) GalOct 5'(CGGAG TACTG TCCTC CGGTA CCTGT ATGCA AAT)3'

(h) GalIAP 5'(CGGAG TACTG TCCTC CGGTA CCCTG CGCAT GTG)3'

(i) GalOct$_{mt}$ 5'(CGGAG TACTG TCCTC CGGTA CCTGT CGGCA AATAA GGT)3'

5. Mini-scale plasmid preparation for clone screening

Plasmid DNAs were transformed using a $CaCl_2$ heat-shock method (450) into a competent *Escherichia coli* –DH5α strain and plated on bacterial agar plates containing 150 μg/ml of ampicillin. The bacteria were allowed to grow overnight and single colonies were subsequently used to inoculate 5 ml overnight cultures. Plasmids were prepared using an alkaline lysis exactly as described by Maniatis et al. (451). Following

ethanol precipitation and washing with 70% ethanol, the pellet containing the plasmid DNA was dried using a speed vacuum system and resuspended in 50 µl of T.E. (10 mM Tris, pH 7.9 and 1 mM ethlyenediaminetetraacetic acid [EDTA]). The DNA in T.E was treated with RNAse A (0.2 mg/ml) for a minimum of 15 min at 50°C prior to screening either by restriction enzyme digest, by PCR or by DNA sequencing using standard protocols.

6. Large scale plasmid DNA preparation

Plasmids were introduced in *E. coli* as described above (I. 5). The 5 ml overnight culture was used to inoculate 500ml of Luria broth (LB) that was grown again overnight. Plasmid DNA was prepared using an alkaline-lysis maxi-preparation procedure (452, 453) followed by double cesium chloride gradient centrifugation in the presence of 200 µg/ml of ethidium bromide to visualize the plasmid DNA (454). Following extraction of the ethidium bromide from the plasmid DNA with water and salt saturated isopropanol, the DNA was dialyzed 2X in 4L of 10 mM Tris-HCl, pH 7.9 and 1 mM EDTA at 4°C for a minimum of 4 hours. Using this procedure, routinely 80-99 % of plasmid DNAs was supercoiled, as estimated by agarose gel electrophoresis and ethidium bromide staining.

7. Linker-tailing method for insertion of short oligonucleotides into plasmid DNA

This protocol was used to clone double-stranded oligonucleotides into various plasmids and was adapted from Lathe et al. (1984) (446). The protocol insured that only one copy of the double stranded oligonucleotide had been inserted into a plasmid. Double-stranded linkers (100X in excess of the DNA ends, usually 200 ng in total) were ligated using standard ligation conditions into either compatible 5' or 3' overhangs or

blunt DNA ended plasmid DNA (200ng total plasmid/reaction). Samples were heated to 75°C to dissociate linkers not covalently bound to the plasmid DNA and then cooled rapidly on ice. Excess linkers were removed by ethanol precipitation in 300 mM sodium acetate and 2 volumes of ethanol at -20°C followed by a 70% ethanol wash. The linker-tailed DNA was then resuspended in T.E., and rehybridized by heating to 80°C and cooled slowly to 4°C. Finally, the plasmids were transformed into bacteria for screening.

8. Expression and purification of GST fusion proteins

The expression and purification of GST or GST-fusion proteins was carried out as previously described (360). The *E.coli* BL21 pLysS (DE3) strain of bacteria (Novagen) was transformed with expression vectors for either GST alone (pGEX2T or 3X) or GST fusion proteins and plated on bacterial agar plates containing 150 μg/ml of ampicilin. Single colonies were selected and grown in 5 ml cultures overnight, which were then induced to express protein with 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) over a second night at room temperature. The IPTG induced the expression of the stable integrate of the bacteriophage gene 1 product (455) to produce T7 RNA polymerase unique to the DE3 strain, which in turn initiated high levels of expression of the target gene encoded by the plasmid. Cells were harvested by centrifugation at 5000 X g for 15 minutes at 4°C and resuspended in lysis buffer (25 mM 4-(2-hydroxylethyl)-1-piperazine ethanesulfonic acid [HEPES], pH 7.9, 100mM KCl, 20% glycerol, 0.1 % nonidet-P 40 [NP40], 2 mM EDTA, 2 mM dithiolthreitol [DTT] and 0.2 mM phenylmethylsulphonyl fluoride [PMSF]). Cellular extracts were prepared by sonication on ice (10 X 20 second pulses) using a small probe at 35% duty cycle (Fisher Sonic Dismembrator – Model 300). The insoluble material was pelleted by centrifugation at 28 000 rpm for 15 minutes at 4°C

in a Beckman Ti-60 rotor. The supernatant was immediately added to glutathione Sepharose beads (Amersham-Pharmacia), that had been previously washed 3X with 15 bed volumes of binding buffer (0.6X lysis buffer containing 0.1% NP40). Washing consisted of resuspending the beads in buffer followed by centrifugation at 1000 X g for 2 minutes and removal of the buffer. The fusion protein was allowed to bind to the affinity matrix for 90 minutes at 4°C on a rotating wheel. The beads were then washed 5X with ice cold binding buffer. After the final wash, excess buffer was removed and the beads resuspended in 1 bed volume of binding buffer containing 1 mM PMSF and 0.02% sodium azide. The suspension or "slurry" could be stored at 4°C for up to 4 weeks.

9. Coupled *in vitro* transcription-translation

Plasmid DNA containing the cDNA encoding for protein was *in vitro* transcribed with T7, SP6 or T3 RNA polymerase using Promega's TNT coupled rabbit reticulocyte lysate system (456) according to the manufacturer's protocol. Translations included 10 mCi/ml of translation grade ^{35}Sulfur methionine (^{35}S-Met, 1,000 Ci/mmol, Amersham). Each translation reaction was incubated at 30°C for 90-120 minutes in a 50 μl reaction volume containing 0.1 mM DTT. All translation reactions were stopped by the addition of an excess (5mM) of unlabeled L-methionine at room temperature. The samples were used immediately, or stored at 4°C in the presence of 1mM DTT for up to 2-3 days before use.

10. GST pull down assays

0.5 μg of GST alone or GST-fusion protein coupled to 8-20 μls of glutathione Sepharose slurry was used in all binding assays. Equal amounts of ^{35}S-labeled, *in vitro*

translated protein were added to the binding assay (usually between 1-8 μl) as determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The volume of rabbit reticulocyte lysate added was normalized by the addition of unprogrammed lysate. The binding reaction containing the immobilized fusion protein and labeled protein were continued for 90 minutes at 4°C in binding buffer with gentle agitation. Subsequently, the Sepharose beads were washed 3X with 500 μl of binding buffer and the bound proteins were eluted from the beads and prepared for SDS-PAGE by boiling for 2 minutes in 2 X sodium dodecylsulfate (SDS)-sample buffer (0.125mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol and 0.0025 %(w/v) Bromophenol Blue). The bound proteins were resolved by SDS-PAGE and visualized by fluorography and/or phosphorimager analysis. Ten percent of the labeled protein added to each binding assay was also loaded onto the SDS-PAGE gels to show the amount of labeled protein added to each binding reaction. Typically, approximately 5-10% of the total protein added to the assay bound specifically to the affinity matrix loaded with 0.5 μg GST-fusion protein.

For oligonucleotide competition experiments, following the incubation of the immobilized GST fusion protein with the rabbit lysate containing the *in vitro* translated GR protein, samples were washed 3 X with binding buffer. Next the protein-protein complexes were challenged with 100 ng of double stranded DNA consisting of oligonucleotides containing a HRE (5'-ACAGT TCGAC ATAGA ACAAA CTGTT CTTAA AAGGT ACCCA-3') (457), an IAP enhancer core element (5'-CTGCG CATGT GCCAA GGGTA TCTTA TGACT-3') (458), a Ku antigen DNA binding site from the C3H strain of MMTV (C3H) (5'-AGCTT GTCTC AAGAA GAAAA AGACG AGAG-

3') (457), or highly sheared calf thymus (CT) DNA for 20 minutes at room temperature. The beads were washed once with binding buffer and the proteins resisting the DNA challenge were eluted in SDS-sample buffer, resolved by SDS-PAGE, and visualized by fluorography.

11. Polyacrylamide gel electrophoresis (PAGE)

(a) Sodium dodecyl sulfate (SDS)-PAGE

Protein samples were diluted with 2X sample buffer and denatured by heat at 100°C for 2 minutes. Samples were loaded on 8-15% separating gels (Tris-HCl, pH 8.8) with a 4% stacking gel (Tris-HCl, pH6.8) at a thickness of 0.75 –1.5 mm (459). The gels were poured sequentially using a mini-protean gel apparatus (Biorad) and run at 150 volts in electrode buffer (25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS). Gels used for resolving ^{35}S labeled proteins were visualized by fluorography, those labeled with ^{32}P were simply dried and visualized by autoradiography and those containing non-radiolabeled proteins were visualized using Coomassie blue staining.

(b) Non-denaturing PAGE

For the resolution of the protein-DNA complexes, 4% polyacrylamide (39.5:1, acrylamide: bis-acrylamide) gels were prepared in 0.5X Tris borate-EDTA (TBE). The gels were run at 150 volts for 225 Volt X Hours. The gels were dried and the samples visualized by autoradiography, exposing the gel to Kodak XAR-5 film at -80°C.

(c) 8 M urea DNA sequencing gels

For resolution of the PCR amplified products from the DNA footprinting assays, 48 g of urea was dissolved in 6 % polyacrylamide solution (final concentration, 19:1, acrylamide: bis-acrylamide) and 0.5 X TBE (final concentration) to a final volume of 100 ml. Once the urea was dissolved, the solution was filtered through 3 mm Whatman paper under vacuum suction. The solution was briefly cooled on ice, then 500 µl of 15% APS and 50 µl of TEMED were added to induce polymerization. Immediately, the gel solution was poured in between two glass plates clamped together with 0.15 mm spacers. An appropriate comb was inserted and the gel was allowed to polymerize for at least one hour. Next, the sequencing apparatus was assembled and 0.5 X TBE buffer added to the upper and lower chambers. The comb was removed and the wells flushed extensively with the 0.5X TBE buffer. The gel was pre-run at 55 watts (W) for at least 30 minutes prior to loading the samples. The samples were heated to 80°C and the wells washed a second time prior to loading 3 µl of sample. The gels were run at 55 W for an appropriate time (typically for 2-4 hours). After electrophoresis, the gel was place on Whatman paper and dried using a BioRad vacuum dryer under heat (80°C) for 1 hour. Finally, the gel was placed in an autoradiography cassette and exposed to Kodak XAR-5 film.

12. Coomassie blue staining of proteins in SDS-polyacrylamide gels

Immediately following PAGE, gels were incubated in staining solution (0.1% Coomassie Blue, 40% methanol, 10% (v/v) glacial acetic acid and 50% Millipore filtered water) for 30 minutes at room temperature with gentle shaking. Gels were then destained

by incubation in destaining solution (40% (v/v) methanol, 10% (v/v) glacial acetic acid and 50% Millipore filtered water) with gentle shaking at room temperature.

13. Fluorography

Gel fluorography was used to enhance the low beta emitting property of ^{35}S atoms by incorporating a fluor directly into the polyacrylamide gel (460). Gels were dehydrated by soaking in glacial acetic acid for 15 minutes and then placed in 20% (w/v) 2,5-diphenylyoxazole (PPO) in glacial acetic acid for 20 minutes. The incorporated PPO was precipitated in the gels by extensive washing with distilled water. Finally the gels were dried under vacuum with low heat (<65°C) so as not to damage the capacity of the fluor to emit light. Once dry, the gels were autoradiographed with Kodak XAR-5 film exposed at –80°C or a phosphorimager screen (Biorad). Typical exposure times ranged from 4-36 hours.

14. Tissue culture

Sf-7 murine fibroblasts were maintained in Dulbecco's modified eagle medium (DMEM, Life Technologies BRL) supplemented with 10% heat inactivated fetal bovine serum (Life Technologies BRL) in a tissue culture incubator at 37°C, 95% humidity, 5% CO_2. The SF-7 cells stably transfected with pTL2mtgGR$_{WT}$ (D. Rodda, University of Ottawa) and pTL2-mtgGR$_{L501P}$ were maintained in DMEM-FBS in the presence of 400 μg/ml G418 (Life Technologies BRL). Chinese hampster ovary (CHO) -K1 cells (American type culture collection, ATCC, Rockville, MD) were maintained in α-minimal essential medium (α-MEM, Life Technologies BRL) supplemented with 10% FBS (Life Technologies) in the same incubator conditions. Cos7 cells (ATCC, Rockville, MD)

were grown in DMEM with 10% FBS from Hyclone under the same incubator conditions. The hybridoma cell line that produces the anti-myc antibody, 9E10 (461, 462), was maintained in RPMI 1640 (Life Technologies BRL) supplemented with 10% FBS (Life Technologies BRL).

15. Transfections

(a) Stable transfections

Sf-7 murine fibroblasts were selected for stable transfections to allow for high levels of expression of both WT and L501P GRs with N-terminal c-myc epitope tags. The Sf-7 cell line stably transfected with the pTL2mycGRWT was obtained from David Rodda, University of Ottawa. For the pTL2mycGR$_{L501P}$ transient transfection, cells were seeded into 15 cm dishes and allowed to grow to 20-30 % confluency and then transfected with a DNA mixture containing 0.5 μg of pSV2Neo and pTL2mycGR$_{L501P}$ (3.5 μg) with 10 μl of lipofectamine (Life Technologies BRL) in media for 6 hours as described by the manufacturer. The transfection was stopped by the addition of FBS to 10%. At 24 hours following transfection the cells were treated with 400 μg/ml of Geneticin (Life Technologies BRL) to select for transfected cells. Drug selection was continued for 14-21 days or until individual colonies were visible without the aid of microscope magnification (432). Individual colonies were picked by using 3mm Whatman paper soaked in 10X trypsin and replated individually in 24 well plates. Each colony was then expanded until two confluent 60 mm plates of cells were obtained. One plate was used to propagate the cell line while the other plate was tested for GR protein expression. Whole cell extracts were made from one of the 60 mm plates and 50 μg of

whole cell extract was western blotted using the anti-myc antibody, 9E10. The $mtgGR_{L501P}$ cell line that expressed the highest and nearly equal level of protein to the $mtgGR_{WT}$ cell line GR was identified and only that line was further propagated for use in the co-IP binding assay.

(b) DEAE dextran transient transfections

CHO-K1 cells used for transient transfection and CAT assays were transfected at a 50-70% confluency with a total of 10 μg of DNA (2 μg of reporter gene, 3 μg pRSV-βgal, varying amounts of expression vector described below and highly sheared salmon sperm (ss) DNA to a total of 10 μg. The DNA was mixed with 500 μl of Tris buffered saline (TBS, 25 mM Tris HCl, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$ and 0.5 mM $MgCl_2$) and diethylaminoethyl (DEAE)-dextran (0.5 mg/ml) in TBS was added to each sample. Plates that were divided into groups of four were washed once with TBS and the DNA-DEAE mix was added in drop-wise fashion to each set (of 4) at 5 minute intervals. Each plate was tilted 10° (3X at minute intervals) to make sure the cells did not dry out and that the DNA was evenly distributed across the plate. α-MEM (5 mls) with 15% dimethyl sulfoxide (DMSO) was then added to each plate at 30 second intervals and maintained for 2 minutes. The media was aspirated, the cells were washed once with 5 ml of TBS, then replaced with 10 ml of fresh medium containing 0.1 mM chloroquine and incubated for three hours at 37°C. Following the 3 hours, a full media change was made. For the mammalian two hybrid assay (Figures 19, 20), a ratio of 5:1,VP16 of DNA expression vector to Gal4DBD expression vector was used. For example, in the mammalian two hybrid-assay, transfections were carried out with 2.5 μg pNLVP16 or pVPOct-1POU plasmid and 0.5 μg of pGALO, $pGal\text{-}GR_{WT}$ or $pGalGR_{L501P}$.

Transcriptional activity was assessed by co-transfecting plasmid DNA containing a CAT reporter gene construct, pG5E1BCAT or pG54XOctE1BCAT(2 μg/plate) and the transfection efficency was monitored by co-transfecting a β–galactosidase (β-gal) construct whose expression was driven by a RSV promoter, pRSV-βgal (3 μg/plate).

(c) Calcium phosphate transfections

CHO K1 cells were plated on 10 cm plates split (1:15) the day before transfection. The cells were fed with fresh α-MEM supplemented with 10% FBS 2 to 4 hours prior to transfection. The DNA was mixed in a total of 450 μl of sterile double-distilled (dd) H_20 followed by addition of 50 μl of 2.5 M $CaCl_2$. Next, 500 μl of filter-sterilized 2X HEPES buffered saline (HBS, 0.05 M HEPES, 0.28 M NaCl, 1.5 mM Na_2HPO_4, pH 7.05) was added in a dropwise fashion to the DNA/$CaCl_2$ mix. As the HBS solution was slowly added the sample was mixed by bubbling air into it with a mechanical pipettor. The samples were immediately vortexed for 5 seconds and precipitated for 20 minutes at room temperature. The precipitate was applied evenly over the cells and incubated for 16 hours in complete media. The next day, cells were washed twice with phosphate buffered saline (PBS, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, 137 mM KCl and 2.7 mM KCl, pH 7.4) and fed with fresh complete medium. Twenty-four hours later the cells were treated with hormone (1 X 10^{-6} M), 15 minutes prior to harvest.

Transfections for the *in vivo* footprinting assays (Figures 21, 22, 23, 28 and 29) employed 1.5 μg of footprinting template (pMMTVCAT, pBSGREOct, pGalOct or pGalIAP) with 1.5 μg of expression plasmid (pCGN-Oct2, with pGRGR, pGalGR, $pGalGR_{L501P}$, $pGal\text{-}GR_{C500Y}$, $pGal\text{-}PR_A$, pGal-AR, pGal-MR, pGal-ERα, pGal-RXR, pSV40-PR, pSV40AR or p6RMR) and 5 μg of highly sheared calf thymus DNA.

(d) Lipofectamine transfections

One day prior to transfection, cells were seeded on 60 mm tissue culture plates to achieve 80-90 % confluency prior to transfection. DNA was diluted in 50 μl (per plate) α-MEM in a 15 ml polystyrene test tube while the lipofectamine (10 μl for transcription assays (CAT assays) and 20 μl for the coimmunoprecipitation assays) was diluted to 50 μl with α-MEM and prepared in a separate tube. Next, the two samples were combined by adding the lipofectamine solution to the DNA solution and allowed to form complexes over 1 hour at room temperature. The DNA lipofectamine mixes were added to cells pre-washed with serum-free α-MEM, and incubated under standard cell growth conditions for 5 hours in the absence of serum and antibiotics. Transfections were stopped following the addition of α-MEM plus FBS to 10%. After 8 hours the media was replaced with fresh α-MEM and incubated for 24 hours.

All transfections were performed in 60 mm tissue culture dishes with CHO-K1 or Cos7 cells at 80 to 90% confluency. Specific quantification of plasmids and lipofectamine used for the transfections are shown in Table I. Cellular extracts

(a) Whole cell extracts prepared for the western and IP assays

Prior to harvest where indicated, cells were treated for 15 minutes with 1 μM Dex. Sf-7 cells, or the stable lines expressing $mycGR_{WT}$ and $mycGR_{L501P}$ were washed 2 times with PBS and removed from the tissue culture plate in 0.5 ml PBS with a rubber policeman. The cells were collected by centrifugation at 6000 rpm in a microfuge, resuspended in binding buffer containing 0.1% Triton X-100 and sonicated 5 X in 10 second pulses using a small probe at 35% duty cycle (Fisher Sonic Dismembrator – Model 300); all steps were performed at 4°C. The whole cell extracts were separated

Table 1. Quantity of reagents used for lipofectamine mediated transfections.
Quantities of plasmid DNA and lipofectamine used in various combinations for transient transfection assays in CHO-K1 and COS7 cells. The combination plasmids employed can be found in the legend of each figure. The transfections are listed in order by Figure number.

Figure number /cell type	Plasmids					Lipofect -amine (µl)
	25 ng	50 ng	100 ng	400 ng	1 µg	
18a /CHO-K1					pGAL-GR pGAL-GR$_{L501P}$	20
19 /CHO-K1	pCGN-Oct-2		pTL2-HoxD4 pTL2-dlx2 pSG5-CREB pGALO pGAL-GR pGAL-GR$_{L501P}$	pG5E1BCAT		10
26 /CHO-K1	pCGN-Oct-2		pGAL-GR pGAL-GR$_{L501P}$ pGAL-PR$_A$ pGAL-AR pGAL-MR pGAL-ERα pGAL-RARα pGAL-RXRα pGAL-Ftz-F1α	pG5E1BCAT		10
27 /CHO-K1					pGAL-GR pGAL-PR$_A$ pGAL-AR pGAL-MR pGAL-ERα	20
30 /CHO-K1		p6RGR p6RMR pSV40PR pSV40AR	pMMTV (188 to -36) E1BCAT pMMTVOct$_{mut}$ (188 to -36) E1BCAT			10
31 /CHO-K1		p6RGR p6RMR pSV40PR pSV40AR	pTKCAT-HRE pTKCAT-ERE			10
33 /COS7		p6RGR p6RMR p6RGR$_{R489G}$ p6RGR$_{R489Q}$	pMMTV (188 to -36) E1BCAT pMMTVOct$_{mut}$ (188 to -36) E1BCAT			6
34 /COS7		phGR phMR phGMG phMGM	pMMTV (188 to -36) E1BCAT pMMTVOct$_{mut}$ (188 to -36) E1BCAT			6

from the insoluble cellular debris by centrifugation at 13,000 rpm in a microfuge for 5 minutes. The supernatant was transferred and used immediately for IP or aliquoted and stored at -80°C for use at a later time.

(b) Nuclear extracts prepared from mammalian cells

Nuclear extracts were prepared as outlined by Andrews and Faller (1991) (463). This protocol was designed for nuclear extract preparations for between 5 X 10^5 to 1 X 10^7 cells. Cells were removed from the plate in 0.5 ml of PBS using a rubber policeman and centrifuged at 6000 rpm in a microfuge for 2 minutes. The cells were gently resuspended in a low salt buffer (10 mM HEPES-KOH, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM PMSF) and the nuclei were allowed to swell on ice for 10 minutes. The cells were hypotonically lysed by vortexing for 10 seconds and the nuclei collected by centrifugation for 10 seconds at high speed. The nuclei were gently resuspended in 2 pellet volumes of high salt buffer (20 mM HEPES-KOH, pH 7.9, 420 mM NaCl, 1.5mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF and 25% glycerol) and extracted for 20 minutes on ice before centrifugation at high speed for 2 minutes. The nuclear extract supernatant was divided into aliquots for storage at –80°C.

16. Protein determination assay

All protein concentrations were determined using the BioRad microassay procedure using bovine serum albumin (BSA) as a standard. A series of 6 standards (0-16 μg) including a water blank were used to construct a standard curve. Samples (typically at a volume of 2-8 μl) were diluted into a total of O.8 ml of H_2O to which 0.2 ml of dye reagent concentrate (BioRad) was added. The samples were mixed and their

OD_{595} read 5 to 60 minutes later. OD_{595} was plotted against protein concentration using the standards and the unknown samples were read from the standard curve. For subsequent protein determinations, a formula was derived from the standard curve and used to calculate the protein concentration of the unknown samples.

17. Preparation of the 9E10 antibody from hybridoma supernatant

The 9E10 antibody, which recognizes the myc epitope, was prepared essentially as described by Harlow and Lane, 1988 (464). The hybridoma cells were grown and expanded in DMEM-10%FBS until there was 2L of 30-40% confluent cells, after which they were allowed to grow until fully confluent. The supernatant was collected after removal of the cells by centrifugation (2000 X g for 5 minutes) and the same supernatant re-inoculated with only 5% of the original cell pellet. The cells were allowed to grow to saturation for a second time until death. As cells reached saturation, the media was supplemented with glucose (1%, final concentration) and HEPES pH 7.3 (25 mM, final) to provide an energy source for the live cells and to maintain normal pH levels, respectively. Again the cells were removed by centrifugation and the supernatant transferred to a flask. While stirring continuously, 0.5 volumes of a saturated solution of ammonium sulfate was added slowly and incubated overnight at 4°C. The solution was centrifuged at 3000 X g for 30 minutes. The pellet was discarded and the supernatant transferred to another flask. Again a saturated solution of ammonium sulfate (0.5 volumes) was added to make the supernatant 50% with saturated ammonium sulfate solution and incubated overnight with gentle stirring. The protein precipitate was collected by centrifugation and the pellet was resuspended in phosphate buffered saline (PBS, 1:100 of the original supernatant volume) and dialyzed against the same buffer.

Following the addition of sodium azide to 0.02%, the antibody preparation was stored in 500 µl aliquots at –20°C for long term storage. Once thawed, the antibody was stored at 4°C for up to 6 months.

18. Western Immunoblotting

Proteins separated on SDS-polyacrylamide gels were transferred electrophoretically to Immobilon P membranes (Millipore, Bedford, MA) in transfer buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% (w/v) SDS and 20% methanol) using a Biorad mini transfer apparatus (465). The membrane was prepared by presoaking in methanol for 1 minute prior to equilibration with transfer buffer. The transfer was carried out at 100 volts (or 1 ampere) for 45 minutes in a transfer chamber cooled with ice. The membrane was removed, rinsed once in Tris buffered saline-Tween (TBS-T, 20 mM - HCl, pH7.5, 500 mM NaCl, 0.1% (w/v) Tween-20) and then blocked with 10% (w/v) skimmed milk in TBS-T for 1 hour at room temperature. The membrane was briefly rinsed once with TBS-T before incubating with a primary antibody diluted in TBS-T overnight with rocking at 4°C.

Following the incubation with the primary antibody, the membrane was rinsed 3 times (once for 5 minutes and twice for at least 20 minutes) with a large volume of TBS-T at room temperature. The membrane was subsequently incubated for 1 hour at room temperature with a horse radish peroxidase (HRP) conjugated sheep anti-mouse or Donkey anti-rabbit IgG (1:50,000 v/v) in TBS-T with gentle shaking. Once again the membrane was extensively washed as described above and protein signals were visualized by autoradiography using enhanced chemiluminescence (ECL, Amersham) according to the manufacturers protocol.

19. Protein A Sepharose preparation

Dehydrated Protein A Sepharose (0.5 mg) was presoaked with a large excess of ddH_2O overnight with gentle shaking at 4°C. The beads were washed once with 1 mM HCl and then equilibrated with ~20 bed volumes of 1X IP buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA and 0.1% Triton X-100). They were blocked with 50 mg/ml of BSA in IP buffer at least overnight at 4°C, washed with 2-5X with IP buffer and stored as a slurry (1:1, beads to solution) with 5 mg/ml of BSA in IP buffer with 0.02% sodium azide for up to 4 weeks at 4°C.

20. IP binding assay.

Whole cell extracts were prepared from $mycGR_{WT}$ and $mycGR_{L501P}$ as well as the parental Sf-7 cell line and incubated with ammonium sulfate precipitated 9E10 antibody prepared from hybridoma cells diluted 1:50 in IP buffer for 90 minutes at 4°C. Pre-blocked Protein A Sepharose was added and the samples incubated for a further 30 minutes at 4°C. The beads were washed 3X with ice cold IP buffer and once with binding buffer (25 mM HEPES, pH 7.9, 60mM KCl, 0.5 mM EDTA, 12% glycerol, 0.1% NP40, 0.2 mM DTT and 0.2 mM PMSF). After washing, beads were collected by centrifugation at 1000 rpm for 2 minutes in a microfuge. The immunoprecipitates were resuspended in 150 µl of binding buffer containing ^{35}S-labeled *in vitro* translated test proteins and incubated for 90 minutes at 4°C. Following binding the beads were washed 3X with 500 µl of ice cold binding buffer and the bound proteins eluted by boiling for 2 minutes in SDS-sample buffer. The eluted proteins were resolved by SDS-PAGE, visualized by fluorography and quantified by phosphorimager analysis (BioRad, model GS-525), where indicated.

21. Phosphorimager analysis

For phosphorimager analysis, dried gels were exposed to Biorad CS (carbon/sulfur) or CH (Chemiluminescence, for western analysis) screens for a minimum of 12 hours. The screens were scanned at 200 micron resolution using a Biorad GS-525 scanner. All values were corrected for background radiation by choosing a region of the scanned screen away from the gel and were expressed as relative units.

22. Direct Protein-Protein Binding Assay

GST-GRX_{550}PKA or GSTGR_{L501P}PKA was expressed in, and purified from BL21pLysS. The GST X-PKA (X=GR) fusion proteins were immobilized on Sepharose beads and resuspended in TEGz50 buffer (50 mM Tris, pH 7.5, 50 mM NaCl, 10% glycerol, 0.5 mM EDTA, 50 μM $ZnCl_2$ and 0.5 mM PMSF) + 0.1% Triton X-100. The proteins were labeled with 1 μCi/μl of γ^{32}P-ATP (10 mCi/ml, 300 Ci/mmol, Amersham-Pharmacia) by kinasing with the catalytic subunit of PKA (Sigma) in HMK buffer (20 mM TrisHCl, 100 mM NaCl, 12 mM $MgCl_2$, 1 mM DTT) for 30 minutes at 30°C. The reaction was terminated by the addition of 1 ml of stop buffer (10mM $NaPO_4$, 10 mM $Na_4O_7P_2$, 10 mM EDTA and 1 mg/ml BSA). The PKA labeled peptides were eluted from the beads in TEGz50 buffer containing 0.1% Triton X-100 by thrombin (Sigma) cleavage between the GST and GR portion of the peptides with the GST moiety left on the beads. This yielded a labeled GR with a specific activity to approximately 23 X 10^6 counts per minute (cpm) / mg. Approximately 20 ng of the ^{32}P labeled peptide was incubated with immobilized GST fusion proteins in binding buffer in the presence of 2 mg/ml BSA and 1 mM PMSF for 30 minutes at room temperature. Following extensive washing, the

bound peptide was eluted in SDS-sample buffer, resolved by SDS-PAGE and visualized by autoradiography.

23. Preparation of cell extracts for CAT and β-gal assays

Cells were transfected 48-72 hours prior to harvest with a plasmid expressing lacZ and were then washed once with PBS. Cells were scraped with a rubber policeman into 1 ml of PBS and collected by centrifugation (2 min, 6000 rpm) at 4°C. The cells were resuspended in freeze-thaw (FT) Buffer (10 mM Tris HCl, pH 7.4, 0.25 M sucrose, 10 mM EDTA) and lysed by 3 cycles of rapid freezing in dry ice/methanol and thawing at 37°C (466). Cell debris was removed by high speed centrifugation in a microfuge for 5 minutes. The supernatants were transferred to separate tubes and processed immediately or stored at –80°C.

24. βgal assays

5-50 μl of cellular extract was mixed with Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ and 50 mM β-mercaptoethanol (added fresh), pH 7.0 final) to a final volume of 200 μl. The reaction was started by the addition of 40 μl of ONPG (o-nitrophenyl-β-D-galactose, Sigma, 4 mg/ml in 100 mM phosphate buffer) and incubated at 30°C until a faint yellow color was observed. The reaction was stopped with 100 μl of 1M Na_2CO_3 and 200 μl H_2O and the incubation time was recorded. The sample color was allowed to develop for at least fifteen minutes prior to reading the absorbance at λ=420. β–gal activity was calculated as follows:

$$\beta\text{-gal units /ml} = \frac{A_{420}/0.0045}{\text{reaction time (minutes) x volume of cell extract (ml)}}$$

and the β-gal transfection efficiency estimated with β-gal units /mg =

$$\frac{A_{420}/0.0045}{\text{reaction time (minutes) x volume of cell extract (ml) x protein concentration (mg/ml)}}$$

25. CAT assays

CAT assays were performed essentially as described by Gorman et al. 1982 (467). Cytoplasmic extracts were heat treated at 65°C for 10 minutes to inactivate endogenous deacetylase activity (468) (note that the β-gal activity was assessed prior to the CAT assays as heat treatment inactivates β-gal activity). The extracts were centrifuged at high speed in a microfuge for 2 minutes to remove any denatured proteins and the supernatant transferred to fresh tubes.

2-50 µl of cell extract was incubated in the prescence of 1.2 µCi/ml of CAT assay grade ^{14}C chloramphenicol (Amersham Pharmacia, at 50 mCi/mmol and 0.05 mCi/ml to a final concentration of 25 µM chloramphenicol in a reaction) and 0.5 mg/ml of acetyl coenzyme A (CoA) or n-butyryl CoA in 0.25 M Tris HCl, pH 7.8 in a total volume of 125 µl. Incubations were carried out for a fixed time, usually for 2 hours (the incubation period can vary between 30 minutes to 20 hours, as required). The reactions were terminated by the addition of 800 µl of ethyl acetate for the thin layer chromatography assay (TLC) or 300 µl of mixed xylenes (Aldrich Cat#24-764-2) for the liquid scintillation counting assay (LSC).

(a) Thin layer chromatography method

The CAT enzyme catalyzes the transfer of the acetyl or the n-butyl moiety from CoA to chloramphenicol. Chloramphenicol can be acetylated or butyrylated at two sites. These two forms migrate faster than the unmodified chloramphenicol substrate on a TLC

plate. Once the CAT enzyme reaction was terminated with ethyl acetate the samples were vortexed for 1 minute and centrifuged at high speed for 5 minutes. The organic upper phase (a volume representing 90 % of the organic phase) was carefully extracted, transferred to a new tube and evaporated until dry in a speed vac (Eppendorf). The residue was resuspended in 8 μl of ethyl acetate and the sample spotted onto a pre-run silica gel TLC plate. The TLC plate was inserted into a developing chamber pre-equilibrated with chloroform/methanol (9:1) for one hour or until the liquid phase reached 3 cm from the top of the plate. The silica plate was removed, dried at room temperature and then exposed to a CS phosphorimager screen (BioRad) for at least 4 hours and quantified. The CAT activity was expressed as a percentage of chloramphenicol acetylated or n-butyrylated divided by the efficiency of transfection, as determined by the β-gal assay and expressed as relative CAT units corrected for transfection efficiency.

(b) Liquid scintillation counting method

In the latter half of this work, CAT assays were quantified using the more sensitive LSC method (469). This method differs from the TLC method in the procedures used to separate the modified radiolabeled chlroamphenicol from the unmodified molecule. The LSC method uses a phase extraction procedure with xylenes to separate the butyrylated chroamphenicol from the unmodified substrate, which is only soluble in aqueous solution. N-butyryl CoA was used in place of acetyl-CoA because it provides a bulkier group which makes the butyrylated product more soluble in the xylene phase. The reactions were stopped by the addition of 300 μl mixed-xylenes and vortexed for 30 seconds. The sample was centrifuged for 3 min at full speed in a microfuge and

250 µl of the original 300 µl of mixed xylenes transferred to a separate tube. 200 µl of 0.25 M Tris HCl pH 8.0 was added to the mixed xylene sample and back-extracted by vortexing for 30 seconds and then centrifuged at maximum speed for 3 minutes. 200 µl of the mixed xylene phase was transferred to a scintillation vial containing 4 ml of scintillation fluid (Ready Safe scintillant) and the samples were counted in a scintillation counter. The cpm were calculated after substraction of the counts obtained with negative control reactions containing no cell extract to determine the specific level of n-buyrylated chloramphenicol products in each sample. The CAT activity was expressed as relative CAT units after correction for the efficiency of transfection as determined using the β-gal assay.

26. λ exonuclease (exo) footprinting

Nucleus and λ exo protection were performed essentially as described by Mymryk et al. 1994 (470). Cells transiently transfected by $CaPO_4$ precipitation were treated with the appropriate hormone as indicated for 15 minutes prior to harvest. The cells were scraped in 1 ml of PBS and washed once with the same buffer. The cell pellet was resuspended in 5 ml of homogenization buffer (10 mM Tris HCl (pH 7.4), 15 mM NaCl, 60 mM KCl, 1 mM EDTA, 0.1 mM EGTA, 0.1% NP40, 0.15 mM spermine, 0.5 mM spermidine, 5% sucrose), transferred to a 7 ml dounce homogenizer and the cell membranes lysed by 3 strokes with an "A" pestle. Lysates were overlaid on a 1 ml sucrose pad (10 mM Tris HCl (pH 7.4), 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 10% sucrose). The intact nuclei were pelleted by centrifugation at 1400 X g, resuspended in 4 ml of wash buffer (10 mM Tris HCl (pH 7.4), 15 mM NaCl, 60 mM KCl, 0.15 mM spermine, 0.5 mM spermidine) and re-centrifuged at 700X g for 5

minutes. Purified nuclei were resuspended in 0.4 ml of digestion buffer (50 mM Tris HCl (pH 9.0), 10 mM NaCl, 2.5 mM $MgCl_2$, 5% glycerol, 1 mM DTT) to a final concentration of 1.5 X 10^6 nuclei/ ml. 0.1 ml aliquots were digested with 100 units of restriction enzyme and 15 units of λ exo (Life Technologies) simultaneously for 15 minutes at 30°C. Nuclei from cells transfected with pHCWT were digested with BamHI, while those transfected with pBSHREOctd were digested with SmaI. Nuclei from cells transfected with pBSGALOct and pBSGalIAP were digested with XhoI. Reactions were stopped by the addition of 0.9 ml of stop buffer (10 mM Tris HCl (pH 7.6), 10 mM EDTA, 0.5% SDS, 0.2 mg/ml proteinase K). Samples were incubated at 37°C for at least 4 hours, phenol/chloroform extracted, and ethanol precipitated. The samples were digested with a second restriction enzyme located outside the region between the restriction enzyme used as an entry point and the area for footprinting to reduce sample viscosity and provide an internal standard for loading equivalent amounts of DNA following linear PCR.

Linear PCR extensions to reveal exo pausing were performed with denaturing gel purified, end-labeled primers (2ng) extending from +74 to +52 for pHCWT, a T3 polymerase promoter primer for pBSHREOct and a T7 polymerase promoter primer for pBSGalOct and pBSGalIAP. DNA samples (10 μg) were analyzed by linear PCR (25 cycles) using Taq polymerase (Perkin-Elmer) in 50 μl of Taq buffer (10 mM Tris HCl (pH 8.3) 50 mM KCl, 5 mM $MgCl_2$, 0.5% Tween 20, 2 mM of each dNTP). After an initial cycle of 4 minutes at 94°C, 2 minutes at 55°C, and 3 minutes at 72°C, 25 additional cycles were performed at 2 minutes at 94°C, 2 minutes at 55°C and 3 minutes at 72°C, followed by a 15 minute extension at 72°C. The reaction products were

phenol/chloroform extracted and ethanol precipitated. The samples were dissolved in formamide loading buffer (1X TBE, 80% formamide) and resolved on a 6% sequencing gel. The A sequencing tracks were prepared using a standard sequencing kit (Sequenase 2.0, Amersham-Pharmacia) but using the end-labeled primer as indicated in the linear PCR.

27. Electrophoretic mobility shift assays (EMSA)

Radiolabeled oligonucleotides were prepared either by filling-in a pre-hybridized double stranded oligonucleotide with the Klenow fragment of DNA polymerase I or by end-labeling with T4 polynucleotide kinase, and then hybridizing to an excess of the complementary oligonucleotide. Labeling of the oligonucleotides was achieved by following the manufacturer's protocol in the presence on α^{32}P-dATP (3000 Ci/mmol, 10 mCi/ml, with Klenow) or γ^{32}P-dATP (3000 Ci/mmol, 10 mCi/ml with Kinase). In each case the oligonucleotide was gel purified prior to use in the mobility shift assay. Nuclear extracts were prepared from transiently transfected CHO-K1 cells to over express Oct-2, $GalGR_{L501P}$, or GalGRWT alone. The nuclear extracts were preincubated alone or in combination for at least four hours at 4°C. Incubation was followed by the addition of 2 ng of labeled oligonucleotide (between 20,000 -50,000 cpms), 1 μg poly (dI-dC), 1μg of BSA and 100 ng of competitor oligonucleotides, as indicated in Figure 19, for 20 minutes at room temperature in EMSA buffer (12mM HEPES (pH 7.9), 12% glycerol, 60 mM NaCl, 0.12 mM EDTA). The protein-DNA complexes were resolved on 4% non-denaturing polyacrylamide gels in 0.5X TBE.

III. RESULTS

1. Point mutation at L501P in the GR DBD prevents the binding of GR to full-length Oct-1 and Oct-2 *in vitro*.

Initially, the interaction between GR and Oct-1 was characterized by GST pull-down assays (45, 471). These assays employed the POU domain of Oct-1 as an affinity matrix to map the protein-protein interaction with the GR. Full-length GR was shown to interact with the POU domain of Oct-1 in a ligand-dependent fashion. In addition, this system established a region including aa 407-523 of rat GR that was sufficient for the protein-protein interaction with the POU domain of Oct-1. Furthermore, two point mutations within the GR DBD, C500Y and L501P, could disrupt the protein-protein interaction.

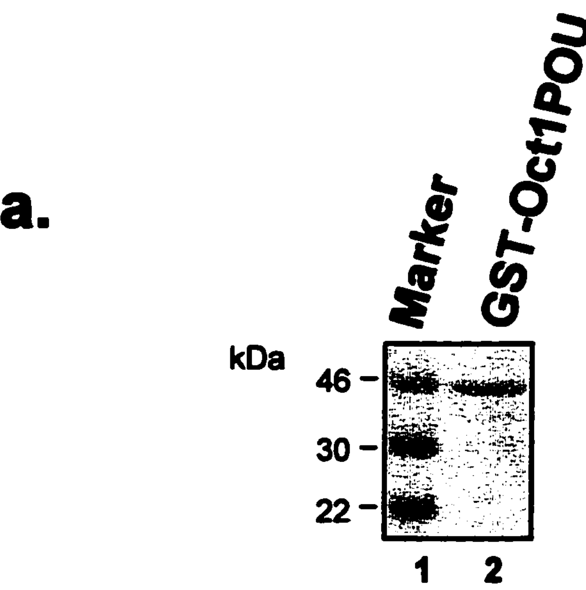
First, to illustrate the interaction *in vitro*, a GST-pull down experiment was performed with the POU domain of Oct-1. This technique takes advantage of producing abundant quantities of GST-Oct-1 POU protein from *E. coli* and purifying the target protein by immobilization to a solid matrix (glutathione Sepharose) to subsequently be used as an affinity matrix for interacting proteins. The interacting proteins labeled with ^{35}S-Met-labeled, rat GR, rat CREB and firefly luciferase, were transcribed and translated *in vitro* from plasmid DNA template using a rabbit reticulocyte lysate system (Promega). The sample containing GR was treated with synthetic glucocorticoid for 4 hours on ice followed by incubation at 30°C for 30 minutes to ensure proper dissociation of the hsps (472). Protein samples were then incubated with glutathione Sepharose beads loaded

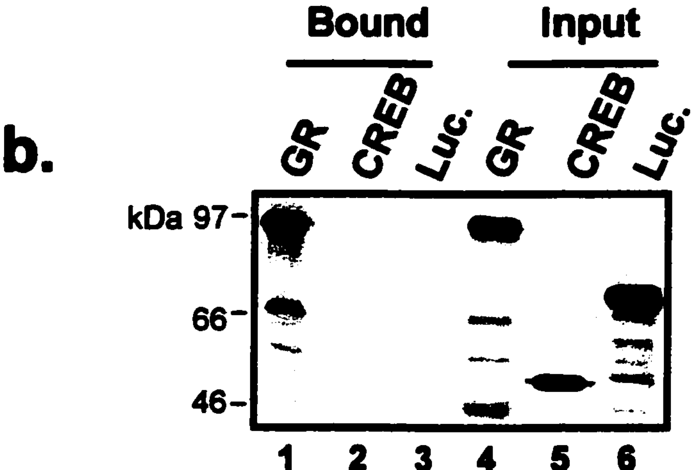
with 0.5 μg of GST-Oct1-POU (Figure 9a) (45, 360). Following an incubation period, the affinity matrix was extensively washed and the bound proteins eluted in SDS-sample buffer. The bound proteins were resolved by SDS-PAGE and visualized by fluorography. The proteins were termed "bound" if a band appeared and migrated at the same relative position as the input protein, represented as 10% of the total added to the binding reaction (Figure 9b, lanes 4-6). Approximately 10% of the ligand-treated GR bound (lane 1) to the affinity matrix compared to the input protein (lane 4). Under these conditions, neither a basic leucine zipper (bZIP) transcription factor, CREB nor firefly luciferase bound to GST-Oct-1POU (lanes 2 and 3, respectively). The binding of ligand treated GR to GST-Oct-1POU was determined to be specific because under the same conditions binding of at least 2 other unrelated proteins could not be detected.

To explore this interaction further, I developed more stringent assays under more physiological conditions to assess the binding of GR to Oct-1 and/or Oct-2 (Oct-1/-2). To do this, cell lines were established and selected expressing GR-WT or GR-L501P. A myc epitope was added to the N-terminus of the GRs to facilitate quantititative comparison of binding. A mouse lung fibroblast cell line, Sf-7, stably transfected with a plasmid expressing full-length rat GR tagged N-terminally with a myc epitope had been established by D. Rodda. This cell line provided a source for producing a large quantity of GR protein which could be rapidly and efficiently immunopurified from cellular extracts with the anti-myc antibody, 9E10 and protein A Sepharose. In addition, the extract from the parental cell line could be used as a negative control. I repeated the selection of GR expressing Sf-7 cells with a plasmid encoding myc GR with a point mutation at L501P. The cell line was produced by co-transfection of a plasmid

Figure 9. Hormone treated GR binds specifically to GST Oct-1POU, *in vitro.*

a. Bacterially expressed GST-Oct-1POU was immobilized to glutathione Sepharose. Following 4 washes with binding buffer, a sample (lane2) equal to that used in the assay described in b. was eluted in SDS-sample buffer, resolved by SDS-PAGE, visualized by Coomassie Blue staining and imaged using a computer desktop scanner. The position relative to the molecular weight markers (lane 1) is indicated to the left of the panel (KDa). b. ^{35}S-labeled in vitro translated GR (lane 1), CREB (lane 2) or firefly luciferase (lane 3) were incubated with immobilized GST-Oct-1POU in binding buffer. Following 4 washes with binding buffer, the bound proteins were eluted by boiling in SDS-sample buffer and resolved by 8% SDS-PAGE with 10% of the input proteins shown in lanes 4-6. The proteins were visualized by fluorography. The position of the molecular weight markers (kDa) is indicated to the left.


a.
Marker
GST-Oct1POU
kDa
46
30
22
1
2


b.
Bound
Input
GR
CREB
Luc.
GR
CREB
Luc.
kDa 97
66
46
1
2
3
4
5
6

conferring antibiotic resistence to G418, pSV2Neo. Following transfection and a period of drug selection, resistant cells were isolated and the cell lines expanded. Whole cell extracts from several lines (Figure 10) were prepared and the levels of $mycGR_{L501P}$ expression were compared to the WT mycGR (lane 2) by western blot analysis with the 9E10 antibody. The extract derived from the 2D1 line (lane 5) showed a level of $mycGR_{L501P}$ that more closely resembled the level of the WT mycGR line (lane 2). The 2D1 clonally derived cell line was selected, renamed $mycGR_{L501P}$ and used in subsequent experiments.

Extracts from these stable cell lines provided a source of both GR wild-type and L501P full-length protein, in addition to a control represented by the parental cell line that could be used subsequently in attempts to co-immunoprecipitate full-length octamer factors. At the time these experiments were performed Oct-1 and Oct-2 antibodies were not available in sufficient quantity or quality for detection following western analysis of whole cell extracts or extracts prepared following over-expression by transient transfection.

An IP binding assay system was developed to overcome the inability to detect the expression of endogenous or exogenous octamer factor proteins. In this assay, ^{35}S labeled *in vitro* translated Oct-1 or Oct-2 was incubated with GRs immunoprecipitated from Sf-7 nuclear extracts. Prior to harvest, cells were treated for 15 minutes with dex to activate the GRs . Western blot analysis with a GR specific antibody (BuGR2, Affinity Bioreageants) showed that equal levels of GR WT and GR_{L501P} were used in each binding assay (Figure 11 a, lanes 1 and 2). The control extract prepared from the parental cell line had no detectable immunoprecipitated GR (lane3). Immunoprecipitates were

Figure 10. Selection of the stably transfected Sf-7 cell line expressing GR_{L501P}.

Sf-7 cells were stably co-transfected with a plasmid expressing myc-tagged full-length GR with a point mutation at L501P and pSV2Neo. Following drug selection, individual colonies were isolated and individually expanded. Whole cell extracts were prepared from individual cell lines (indicated above the panel) and equal protein quantity analyzed by western blot with the myc specific antibody 9E10 (lanes 3-9). Samples were compared to extracts from the parental Sf-7 cell line (lane 1) and an established cell line expressing mycGR WT protein (lane 2). *denotes the extract from the 2D1 cell line (lane 5) having myc-tagged protein with an expression level that more closely resembled GR WT expression levels and was renamed $mycGR_{L501P}$.

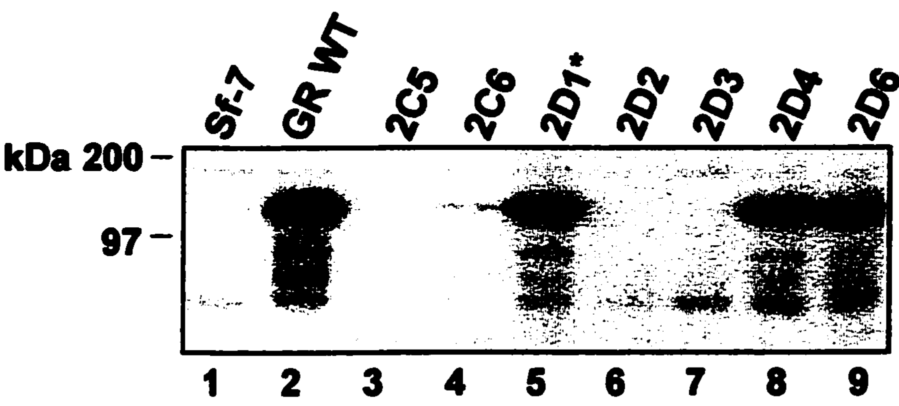
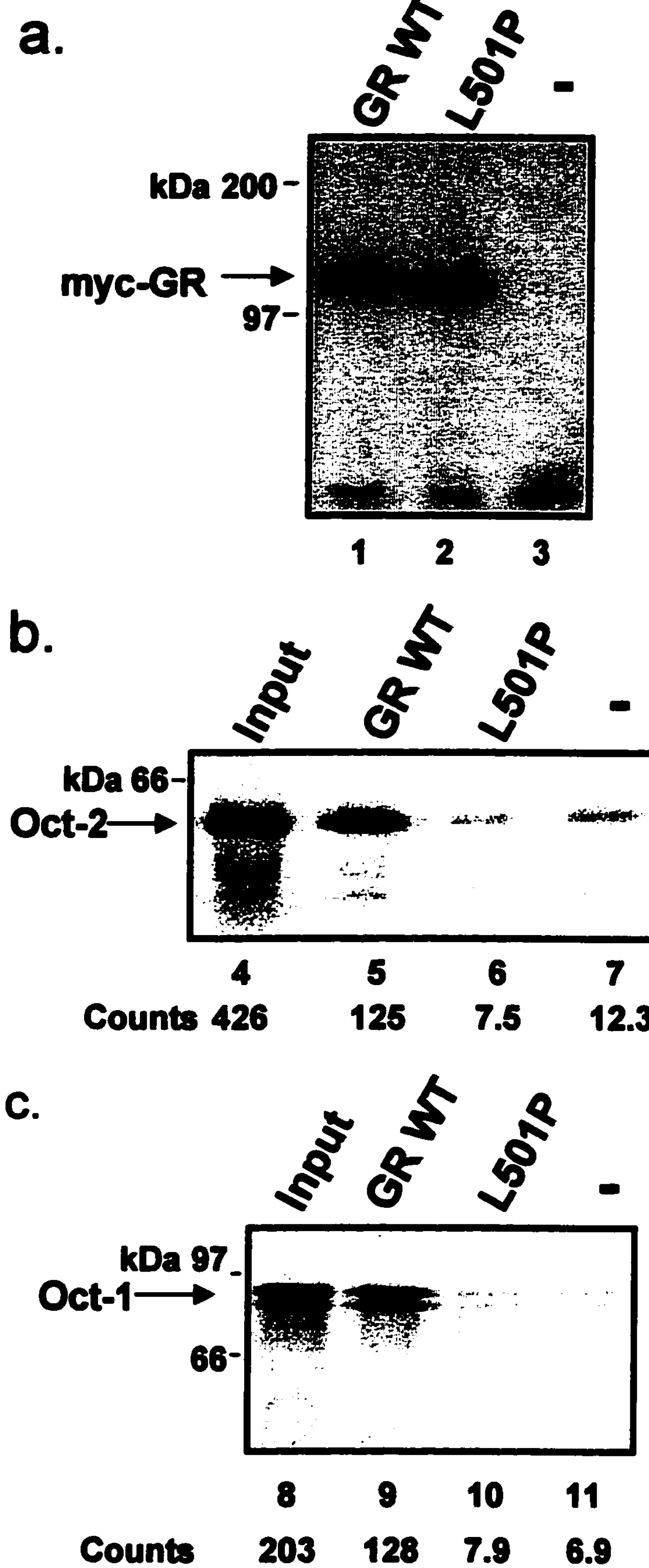

Sf-7
GR WT
2C5
2C6
2D1*
2D2
2D3
2D4
2D6
kDa 200
97
1
2
3
4
5
6
7
8
9

Figure 11. GR_{WT} but not GR_{L501P} binds to full length Oct-1 and Oct-2.

a. Western analysis (BuGR2 antibody) of anti-myc (9E10) immunoprecipitates used in the binding assays described below. The immunoprecipitates were prepared from nuclear extracts of stably transfected Sf-7 cell lines expressing GR_{WT} (lane 1) or GR_{L501P} (lane 2) fused N-terminally to 6X myc epitope tags or control extracts from the Sf-7 parental line (lane 3). Prior to harvest, the cells were treated for 15 minutes with 1 μM dex. b. and c. The individual immunoprecipitates were incubated with ^{35}S-labeled, *in vitro* translated Oct-2 (panel b) or Oct-1 (panel c) in binding buffer for 90 minutes at 4°C. Following 4 washes with binding buffer, bound proteins (GR WT, lanes 5,9; GR L501P, lanes 6 and 10; or control samples, lanes 7, 11) were eluted in SDS-sample buffer. Each sample and 10% of the input *in vitro* translated proteins (lanes 4 and 8) were resolved by SDS-PAGE, visualized by fluorography. Each band representing full-length Oct-2 or Oct-1 was quantified by phosphorimager analysis with the relative counts, corrected for background radiation levels, displayed below each lane. The position of the molecular weight markers (kDa) is indicated to the left of each panel.


a.
GR WT
L501P
-
kDa 200
myc-GR
97
1
2
3
b.
Input
GR WT
L501P
-
kDa 66
Oct-2
4
5
6
7
Counts 426
125
7.5
12.3
c.
Input
GR WT
L501P
-
kDa 97
Oct-1
66
8
9
10
11
Counts
203
128
7.9
6.9

prepared simultaneously and were tested for binding to *in vitro* translated ^{35}S-Met-labeled Oct-1 and Oct-2 (Figure 11 b and c). The binding was quantified by exposure to a CS (Carbon-Sulphor) screen, analysed using a BioRad GS-525 phosphorimager and quantified using NIH image software. Numbers corresponding to the intensity of individual bands were assigned and labeled below each lane in b and c. Binding of Oct-1 (lane 9) and Oct-2 (lane 5) were compared to 10% of the total input proteins in lanes 8 and 4, respectively. Oct-1 bound with approximately twice the intensity of Oct-2. Neither Oct-1 (lane 10) nor Oct-2 (lane 6) bound to GR_{L501P} above the level of control immunoprecipitates (lanes 11 and 7, respectively). Note that the *in vitro* translation products produced by the Oct-1 DNA template produced two major forms of the protein. Most likely these represent either late transcriptional starts or early transcriptional termination products; none the less, both products bound to similar levels (lane 9). Together, these results showed that Oct-1 and Oct-2 full length proteins bound to liganded, full-length GR and this binding was sensitive to a point mutation at amino acid L501P.

2. Octamer factor homeodomain is required for the C500Y- and L501P- sensitive GR DBD binding *in vitro*.

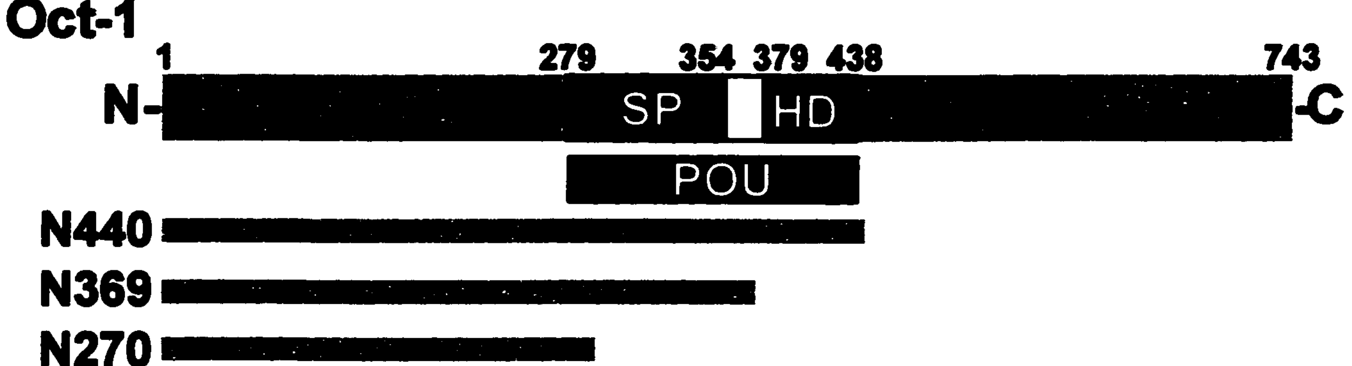
In a first step towards determining the requirements for the binding of the full-length POU proteins, Oct-1 and Oct-2, to GR, we examined the binding of C-terminally truncated forms of Oct-1 to the GR DBD, including the hinge region (aa 407-568). The binding of full-length Oct-1 was compared to the binding of the GR DBD with a point mutation at C500Y (aa 407-556). To do this the GR DBDs were expressed as a GST fusion protein purified on glutathione Sepharose and used in a GST pull down assay

(Figure 12). *In vitro* translated ^{35}S-labeled Oct-1 full-length protein bound specifically to GST-GR DBD (lane 2) as it did not bind to a GST- fusion protein with a point mutation at C500Y (lane1). C-terminal deletion of Oct-1 up to the end of the POU domain decreased binding considerably (lane 3) whereas deletion into the POUhd abrogated binding completely under these conditions (lane 4). These results suggested that the POUhd was required for binding to a region encompassing 407-568 of rat GR *in vitro*. A Coomassie blue stained SDS-polyacrylamide gel (lanes 10, 11) was used to confirm that the level of binding observed between lanes 1 and 2, was not due to the level of GST-fusion protein immobilized to the beads.

Following the observation that both Oct-1 and Oct-2 bound to full-length GR in a co-IP binding assay (refer to Figure 11) and that the binding of Oct-1 required the homeodomain of Oct-1 to interact with the GR DBD and hinge region in a GST pull down assay (refer to Figure 12), I sought to determine if the homeodomain of Oct-2 was also required to interact with full-length GR. Several plasmids encoding Oct-2 internal deletion mutants were cloned into vectors from which this could be *in vitro* translated. Three deletion mutations were constructed: Oct-2ΔPOU which lacked the entire POU domain, Oct-2ΔSP, which lacked only the POU specific domain and Oct-2ΔHD which was missing only the POU homeodomain region. The full-length Oct-2, the three Oct-2 internal deletion mutants and firefly luciferase as a control were tested for binding to mycGR immunoprecipitates from nuclear extracts prepared from stably transfected Sf-7 cells, previously described. To ensure the mycGR immunoprecipitate was the same in each individual binding reaction, a scaled-up version of the initial IP was used. The IP

Figure 12. Oct-1 binding to the GST-GRDBD is lost upon C-terminal deletion into the POU_{HD}.

(Top) Schematic representation of the human Oct-1 protein with the amino acid positions of the POU_{SP} and the POU_{HD} clearly indicated. Below the main schematic the position each of the C-terminally deleted peptides was illustrated. (left panel) Binding of in vitro translated, ^{35}S-Met labeled Oct-1 full-length (lane 2) or C-terminally deleted peptides (lanes 3-5) to immobilized GST-GR_{WT} (aa 407-568) or GST-GR_{C500Y} (aa 407-556) as indicated. (right panel) 10% of the input proteins (lanes 6-9). Each sample was resolved by 10% SDS-PAGE analysis with the positions of the molecular weight markers indicated on the left. The bound and input proteins were visualized by fluorography. The plasmid DNAs were truncated with a restriction enzyme, gel purified prior to in vitro translation to yield C-terminally deleted Oct-1. (Below) GST-GR_{WT} (lane 10) and GST-GR_{C500Y} (lane 11) samples equal to those used in the binding assays described above. Samples were eluted from glutathione Sepharose by boiling in SDS-sample buffer, resolved by 15% SDS-PAGE prior to Coomassie Blue staining. The gel was vacuum dried onto 3mm Whatman paper and then photographed using a digital computer scanner. In all gels the position of the molecular weight markers (kDa) is indicated to the left on each panel.



Bound

N743 N743 N440 N369 N270

GST-GR WT - + + + +

C500Y + - - - -

97 -
69 -
46 -
30 -

1 2 3 4 5

Input

N743 N440 N369 N270

6 7 8 9

GST-GR

WT C500Y

46 —
30 —

10 11

was then divided into five equal samples and incubated with the *in vitro* transcribed-translated radiolabeled protein.

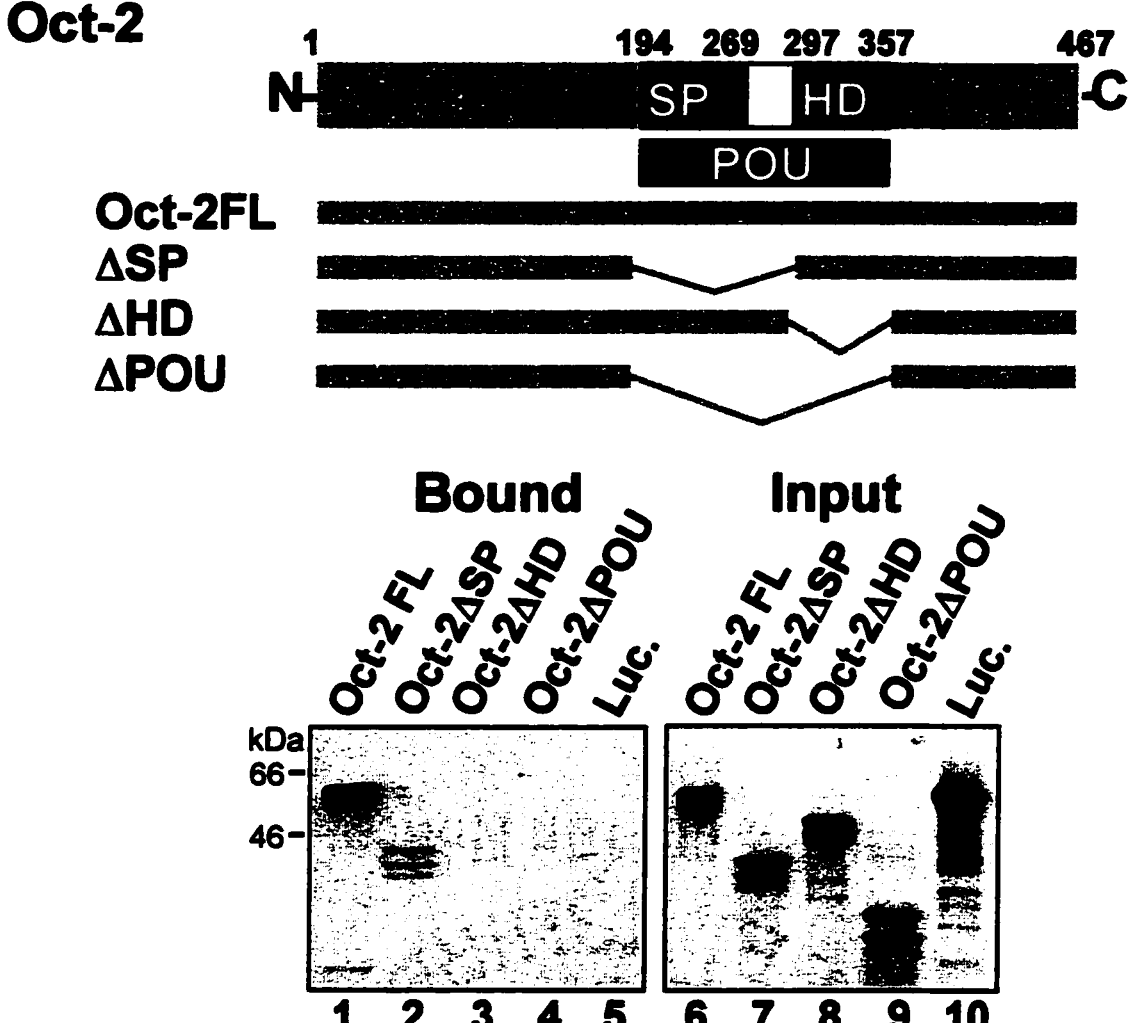
Full-length Oct-2 bound strongly to immunoprecipitated GR (Figure 13, lane 1), deletion of the whole POU domain or the homeodomain alone, eliminated all detectable binding (lanes 4 and 3, respectively). Deletion of the POUsp domain decreased but did not eliminate all binding (lane 2). The binding appeared to be specific because a control protein, firefly luciferase did not bind to affinity purified GR (lane 5). These results indicated that the POUhd was required for the binding to full length GR and that the POUsp domain may play a subordinate role. However it is also possible that the POU factors lacking the POUsp domain may have decreased binding due to alterations in the folding of the protein that could have decreased access of GR to the homeodomain.

3. GR can bind directly to several homeodomains and homeodomain containing proteins both *in vitro* and *in vivo*.

In all the binding assays described in this work, protein-protein interactions have been analyzed in the presence of rabbit reticulocyte lysates. Lysate preparations contain numerous proteins that may influence protein-protein interactions. Studying the binding under those conditions suggested the interaction between GR and Oct-1/-2 may be direct or might also require auxiliary factors in the lysate. To show that the protein-protein interaction between GR and Oct-1 was directly, I designed an assay to test the binding using completely purified components. Immobilized, recombinant GST-Oct-2 POU was used in a GST pull down assay with a recombinant peptide consisting of the GR DBD/hinge ^{32}P-labeled *in vitro*. Plasmids encoding GST-GR-WT and GST-GR_{L501P} with C-terminal PKA phosphorylation sites were constructed in GST vectors (pGEX2T,

Figure 13. The Oct-2 POU_{HD} is required for binding to full-length GR.

(TOP) Schematic illustration of the Oct-2 proteins used in the binding assay described below. The amino acid positions flanking the POU_{SP} and the POU_{HD} are shown above. The folding hinge indicates the portion of each deleted peptide. In vitro translated ^{35}S-labeled Oct –2 full-length, ΔSP (lacks aa 152-286), ΔHD (lacks aa 296-359), ΔPOU (lacks aa 152-349) or a control protein firefly luciferase (lanes 1-5, respectively) were incubated in binding buffer for 90 minutes at 4°C with myc-tagged GR immunoprecipitate. The immunoprecipitate was prepared from a nuclear extract of stably transfected Sf-7 cell lines as described previously (see Figure 10). Following 4 washes in binding buffer the bound proteins were eluted in SDS sample buffer. The bound proteins and 10% of the input proteins (lanes 6-10) were resolved by 10% SDS-PAGE and visualized by fluorography. The position of the molecular weight markers are indicated to the left of the first panel (in kDa).


Oct-2
1
194
269
297
357
467
N
SP
HD
C
POU
Oct-2FL
ΔSP
ΔHD
ΔPOU
Bound
Input
Oct-2 FL
Oct-2ΔSP
Oct-2ΔHD
Oct-2ΔPOU
Luc.
Oct-2 FL
Oct-2ΔSP
Oct-2ΔHD
Oct-2ΔPOU
Luc.
kDa
66
46
1
2
3
4
5
6
7
8
9
10

Pharmacia) containing a thrombin protease site between the GST and the GR fusions (Figure 14a). To prepare the ^{32}P-labeled GR peptide, the recombinant protein was expressed in bacteria

and purified on glutathione Sepharose, labeled with ^{32}P-γ-ATP and the catalytic subunit of PKA *in vitro* and the ^{32}P-GR peptides liberated by thrombin digestion.

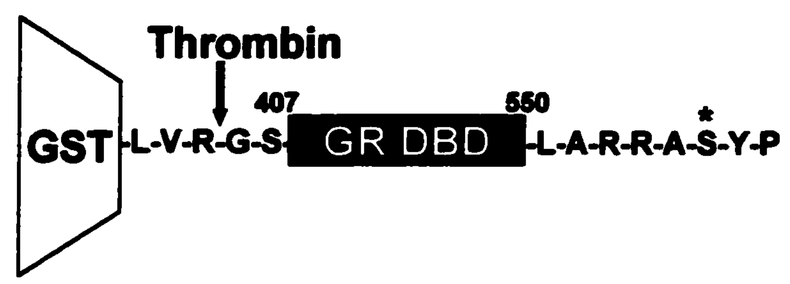
First, in order to test the efficiency of thrombin protease digestion, GST-GRWT-PKA and GST-GR_{L501P}PKA were expressed as GST fusion proteins in bacteria, immobilized to glutathione (lanes 2 and 3) and samples of each of the unlabeled peptides were digested with thrombin (lanes 4 and 5). The supernatent was discarded from each of the thrombin treated samples. Proteins were eluted in SDS-sample buffer and SDS-PAGE. Coomassie Blue staining of an analytical SDS-polyacrylamide gel (Figure 14b), showed the untreated GST-GR WT and GST-GR_{L501P} samples (lanes 2 and 3, respectively) compared to the samples digested with thrombin (lanes 4 and 5). A major peptide corresponding to the full-length GST-GRPKA (both wild-type and L501P) was observed at 45 kDa (lanes 2, 3). Following treatment with thrombin (lanes 4 and 5), the major GST-GR protein band was lost (~45 kDa) and replaced with two lower molecular weight forms. Based on the relative length of each peptide, the 30 kDa peptide represented the GST moiety while the peptide migrating at a similar level as the 21.5 kDa marker represented the GR moiety (407-550). The positive identification of each peptide in this assay could have been verified by western analysis.

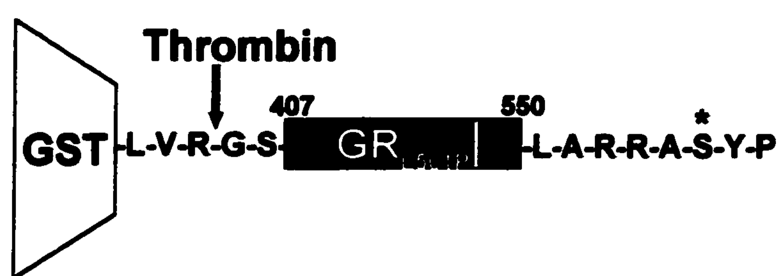
A lower, minor peptide of ~30 kDa in size (lanes 2, 3) most likely represented degradation products that co-purify on the glutathione beads. An experimental observation from another group that has performed similar GR DBD expression and

Figure 14. Preparation of recombinant purified GR and GR_{L501P} DBD peptides.

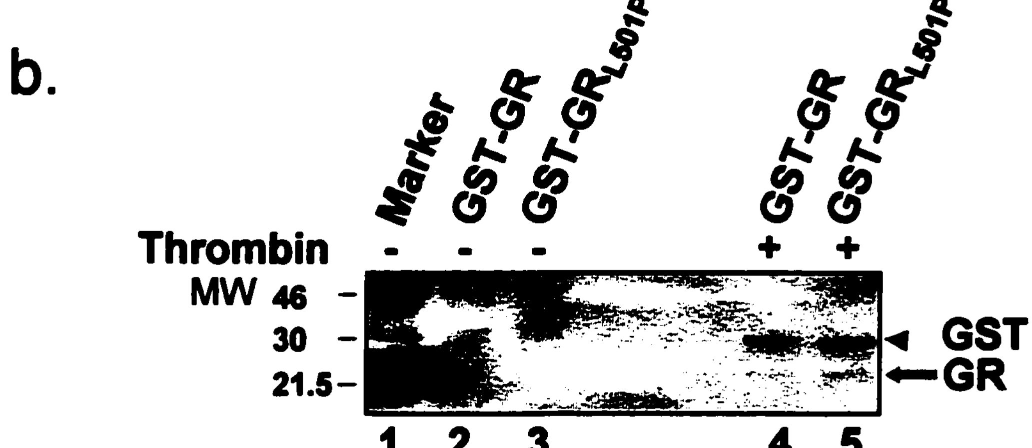
a. Schematic illustration of the GST-GR and GST-GR_{L501P} proteins engineered to test direct binding to GST-Oct2, GST-Oct2-HD fusion protein used in the experiment shown in Figure 15. Represented are the GST-GRPKA and GST-GR_{L501P}PKA proteins. The GST (trapezoid) and GR (aa 407-550, black rectangle) are shown separated by a thrombin cleavage recognition sequence (primary amino acid sequences and dark arrow). The vertical white line distinguishes the GR WT and L501P motifs. At the C-terminus is the recognition sequence required for efficient in vitro phosphorylation by protein kinase A. The site of phosphorylation is indicated by the asterisk. Following protease treatment the GR moieties would be released from the affinity matrix for subsequent use in binding assays. b. Cell lysates were prepared from *E. coli* expressing either GST-GRPKA or GST-GR_{L501P}PKA and samples were affinity purified on glutathione Sepharose. Following an incubation period, the Sepharose was washed 3 times with binding buffer and once with thrombin cleavage buffer. Samples were resuspended in thrombin cleavage buffer and each sample was divided into two: one set was incubated in the presence of thrombin (+) the other set in the absence of thrombin (-). The supernatant was discarded and SDS-sample buffer was added directly to each sample, eluted by boiling and resolved by SDS-PAGE. The gels were stained with Coomassie Blue and vacuum dried onto Whatman paper prior to photographing using a computer desktop scanner. 25% of the untreated GST-GR and GST_{L501P} samples were shown in lanes 2 and 3, while the 100% of the treated samples were shown in lanes 4 and 5, respectively. The molecular weight markers were shown in lane 1 with the size (kDa) to the left. The GST moiety was indicated by the arrowhead and the GR moiety by the dark arrow.

a.





b.



purification studies in *E. coli,* have demonstrated that the peptide that runs at ~30 kDa represents the GST moiety because it was identified by western blot analysis with an anti-GST antibody but not detected by a GR specific antibody (473). In that same study, only the full-length version of the GST-GRDBD/hinge retained sequence-specific DNA-binding function. Here, the point mutation at L501P did not influence the stability of the protein because under identical expression and purification conditions the quality or quantity of the proteins produced and isolated were similar for wild-type and L501P peptides (lanes 2, 3).

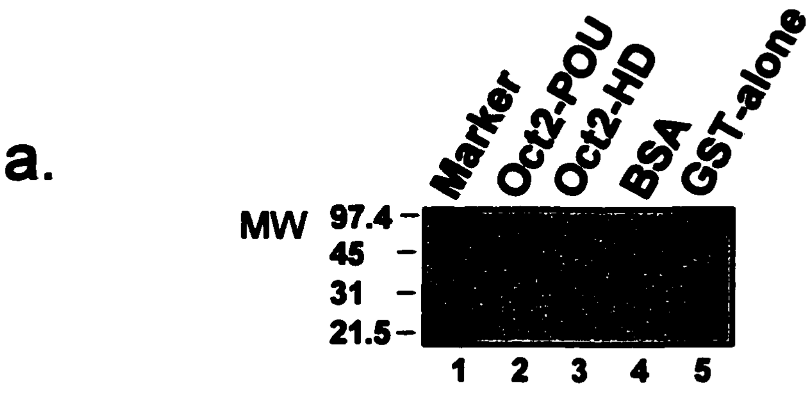
To determine if the binding of GR to GST-Oct-2POU was direct and if the POUhd of Oct-2 was sufficient for binding to GR, purified factors were prepared prior to testing the binding in a GST pull down assay. A comparison of the immobilized GST fusion proteins were shown in a Coomassie-blue stained SDS-polyacrylamide gel in Figure 15a. Shown are samples equal to that used in the binding assays described below compared with 0.5 μg of BSA (lane 4).

The GR peptide probes, ^{32}P-GRDBD and ^{32}P-GR$_{L501P}$ were produced essentially as described previously, except prior to thrombin cleavage the immobilized GR peptides were labeled with γ^{32}P-ATP and with the catalytic subunit of PKA (Sigma). The labeling reactions were stopped by the addition of 10 mM sodium pyrophosphate and the free ^{32}P-ATP removed by two washes in stop buffer. The labeled GR moiety was released into solution by the addition of thrombin protease and a fraction of the sample quantified by scintillation counting. The peptides were labeled to a specific activity of 23 X 10^6 cpm/ mg. The cpms were determined by scintillation counting and the total protein labeled

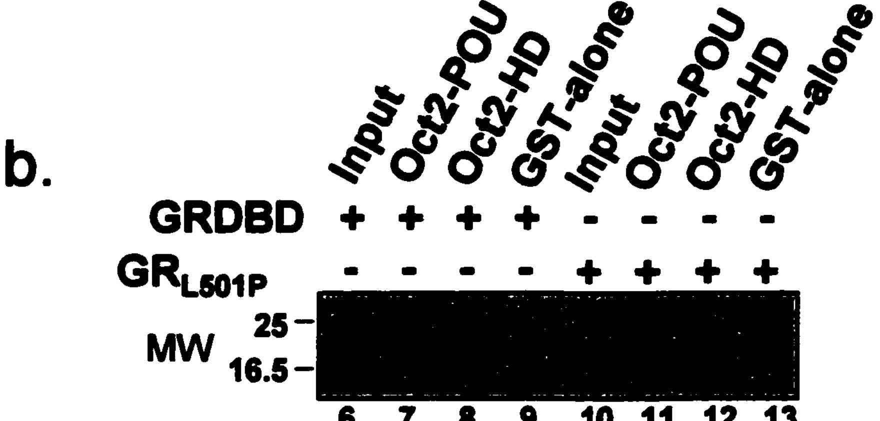
Figure 15. The GRDBD can bind directly to the Oct-2 homeodomain.

a. Coomassie Blue stained SDS gels of the proteins used in the direct binding assay described below. Extracts from bacteria expressing GST-Oct-2POU (aa 195-377, lane 2), GST-Oct-2HD (aa 294-377, lane 3) or GST alone (lane 5) were immobilized to glutathione Sepharose, washed 4 times and eluted by boiling in SDS-sample buffer. The samples were resolved by SDS-PAGE and visualized by Coomassie staining and photographed using a computer desktop scanner. The samples were compared to 0.5 μg of BSA (lane 4) and protein molecular weight markers (lane 1), with their size (kDa) indicated to the left of the panel. b. Individually, the GST-GRDBD (aa 407-550) or the GST-$GRDBD_{L501P}$ were immobilized and purified to near homogeneity on glutathione Sepharose beads. The GRDBD and $GRDBD_{L501P}$ each containing a consensus PKA phosphorylation site, were ^{32}P-labeled with protein kinase A, cleaved with thrombin and released into solution effectively removing the GST moiety. The labeled GRDBDs were tested for binding to immobilized Oct-2 POU (lanes 7, 11), Oct-2 POUhd (lanes 8, 12) or GST alone (lanes 9, 13), as a negative control. Following incubation in binding buffer for 90 minutes at 4°C, the bound proteins were washed 3 X with binding buffer and eluted by boiling in SDS-sample buffer. The samples were resolved by 18% SDS-PAGE. Input proteins (10%) are shown in lanes 6 and 10. The position of the molecular weight markers (kDa) was indicated on the left.

a.



b.



estimated by a Coomassie blue staining of a polyacrylamide gel using BSA as a protein standard.

In this assay, it was essential to perform the binding under conditions that inactivated the thrombin protease because the immobilized GSTOct-2-POU and GST-Oct-HD fusion proteins both have a thrombin protease site separating the fused proteins. To do this the incubation time and temperature were adjusted to 30 minutes at 21°C and the binding reactions performed at pH 7.9 with 1mM PMSF under conditions that allowed for the half-life of PMSF in aqueous solution to be extended to 35 min (474). Since an effective working concentration of PMSF required for thrombin inhibition is 0.1 mM PMSF (475), thrombin protease activity should have been completely blocked.

Labeled GR moieties, WT and L501P (20 ng) were incubated with immobilized GST-Oct2-POU, GST-Oct2-HD or to GST alone, as a control, in the presence of 100X non-specific competitor protein, BSA and 1mM PMSF for 30 minutes at room temperature. Following incubation, the samples were washed 3 X with binding buffer and resolved by SDS-PAGE (Figure 15b). Wild-type ^{32}P-GR-PKA peptide bound to GST-Oct2-POU and GST-Oct2-HD (lanes 7 and 8). The level of binding to GST-Oct2-POU and GST-Oct2-HD was equal suggesting that the POU homeodomain was sufficient for the direct binding to the GR-PKA. The binding was specific because the GRWT was barely detectable to GST alone (lane 9). Binding of the GR_{L501P} peptide to immobilized GST-Octs were barely detectable at this exposure (lane 11 and 12) and only slightly higher than that observed with GST alone (lane 13). These results taken together, suggest that the binding of GR (aa 407-550) to Oct-2 was direct and that the homeodomain alone (aa 294-377) was sufficient for the direct interaction *in vitro*.

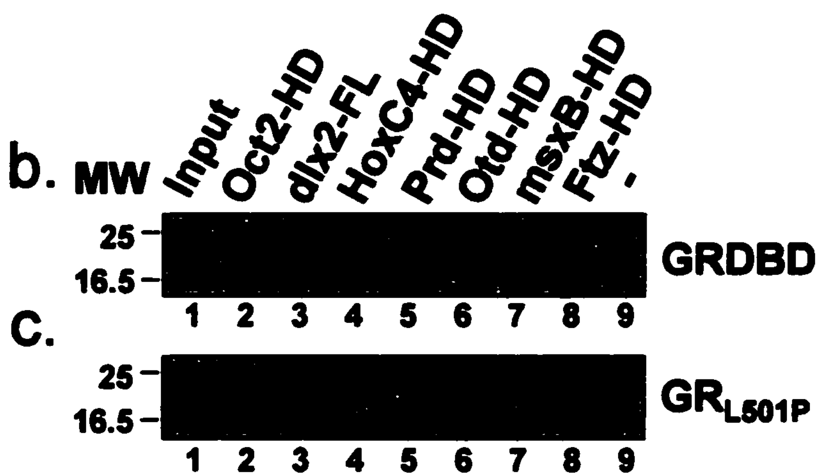
As the homeodomain is well conserved and represents a signature motif for the homeodomain family (476), it seemed possible that GR binding to the homeodomain may be a broadly conserved function of this gene family. To test this hypothesis, I examined the binding of the GR DBD/hinge (aa 407-550) to the homeodomains of a number of homeodomain containing proteins: human Hox C4; *Drosophila* paired (Prd); *Drosophila* orthodenticle (Otd); zebrafish msxB; *Drosophila* fushi-tarazu (Ftz). Plasmids encoding the homeodomains of these proteins were obtained and expressed in bacteria as GST-fusion proteins. In addition, full length zebrafish dlx2 as a GST fusion protein was available and also expressed in bacteria. A Coomassie-blue stained SDS-polyacrylamide gel of immobilized GST fusion proteins equal to that used in the direct binding assay described below are shown in Figure 16a. Beneath the full-length peptides are degradation products generated in bacteria that generally have no effect on the outcome of the binding assays.

^{32}P-labeled GR DBD-PKA or GR_{L501P}-PKA peptides were tested for direct binding to immobilized GST homeodomain fusion proteins as previously described. The GR DBD/hinge peptide bound to all of the GST-homeodomains tested including GST-full length dlx-2. The GR WT peptide bound to the immobilized proteins with variable intensity shown in Figure 16 b, lanes 2-8. Binding was specific because no binding was observed to mock transformed purified bacterial extracts (b, lane 9). As expected, little binding was observed to the homeodomains using the GR_{L501P} peptide (c, lanes 2-8). Binding was strongest to full length dlx-2 (lane 3) and the homeodomain of HoxC4 (lane 4) and the weakest binding to the homeodomain of Paired (Prd, lane 5), a homeodomain

Figure 16. The GRDBD binds directly to several homeodomain proteins.

a. Homeodomains of a several proteins or full length dlx2 (dlx2-FL) were expressed in E.coli and purified as GST fusion proteins on glutathione Sepharose. A quantity equal to that used in the binding assay described in (b) and (c) is shown on a Coomassie blue stained 15% SDS polyacrylamide gel. GST-Oct-2 homedomain (Oct-2-HD,lane 2), full length zebrafish dlx2 (dlx2-FL, lane 3), GST fused to the homeodomains of human Hox C4 (HoxC4-HD, lane 4), *Drosophila* paired (Prd-HD, lane 5), *Drosophila* orthodenticle (Otd-HD, lane 6), zebrafish msxB (msxB-HD, lane 7), *Drosophila* fushi tarazu (Ftz-HD, lane 8) or from mock extract derived from the parental bacteria BL21(plysS) (lane 9). Bovine serum albumin (0.5 μg) was shown in lane 1 as a control for protein loading. b. and c. ^{32}P labeled GRDBD WT (b) or $GRDBD_{L501P}$ (c) produced and purified from bacteria (as described in Figure 13) were tested for binding to various GST fusion proteins immobilized to glutathione Sepharose. Following incubation in binding buffer for 90 min at 4°C, the bound proteins were washed 3 times with binding buffer and eluted in SDS-sample buffer. The bound proteins (lanes 2-9) and 10% of the input proteins (lanes 1) were resolved by 18% SDS-PAGE and visualized by autoradiography. As a control for binding, lanes 9 showed the binding to glutathione Sepharose blocked with extract from mock transformed bacteria. The position of the molecular weight markers (kDa) was indicated to the left of each panel.

a.
MW
BSA
Oct2-HD
dlx2-FL
HoxC4-HD
Prd-HD
Otd-HD
msxB-HD
Ftz-HD
-
62
47.5
32.5
25
1 2 3 4 5 6 7 8 9


b.
MW
Input
Oct2-HD
dlx2-FL
HoxC4-HD
Prd-HD
Otd-HD
msxB-HD
Ftz-HD
-
25
16.5
GRDBD
1 2 3 4 5 6 7 8 9
c.
25
16.5
GRL501P
1 2 3 4 5 6 7 8 9

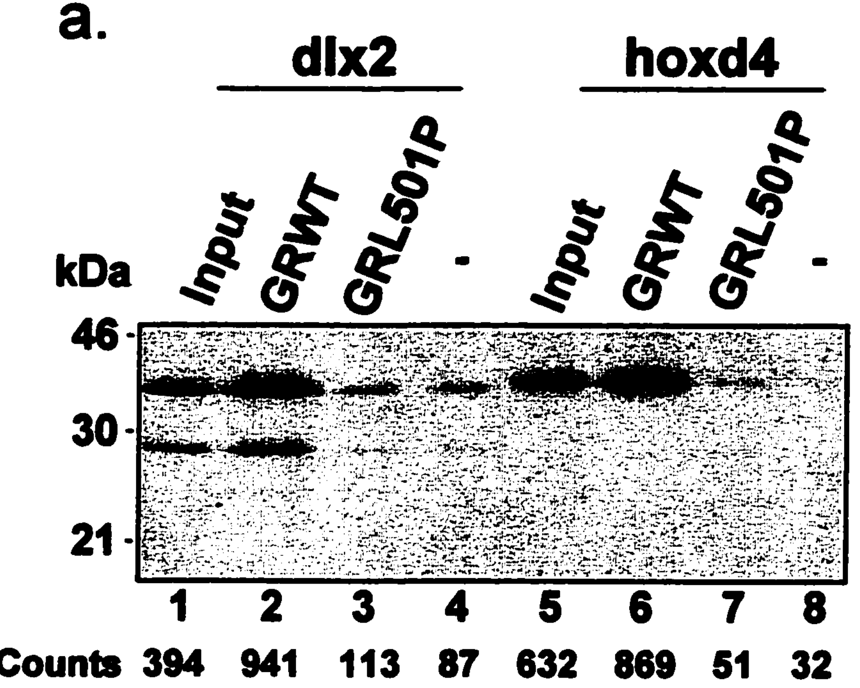
of the paired class. These results suggest that the GRDBD/hinge (407-550) has at least the potential to bind several homeodomains *in vitro*.

To examine the potential for full-length GR to interact with full length homeodomain proteins, *in vitro* translated ^{35}S-labeled dlx2 and hoxD4 were tested for their ability to bind to immunoprecipitates from ligand treated nuclear extracts prepared from stably transfected Sf-7 cells (mycGR-WT and mycGR$_{L501P}$) or the control cell line, Sf-7 (17). The bound proteins were quantified by phosphorimager analysis and compared to the input proteins. The dlx2 protein bound more strongly (23%, compare lanes 1 and 2) than hoxD4 (14%, compare lanes 5 and 6) to full length GR compared to the input proteins. This trend was consistent in duplicate binding assays. Binding was specific because both dlx2 and hoxD4 bound to GR$_{WT}$ (a, lanes 2 and 6, respectively) but not to affinity purified mycGR$_{L501P}$ (a, lanes 3, 7) or affinity purified extracts prepared from cells lacking mycGR (a, lanes 4, 8). Figure 17 b shows that the myc-tagged GRs, GR$_{WT}$ and GR$_{L501P}$ were immunoprecipitated to similar levels from these cell extracts. The immunoprecitation binding experiment showed that full-length GR has the potential to interact with several homeodomain-containing proteins in an L501P-sensitive manner.

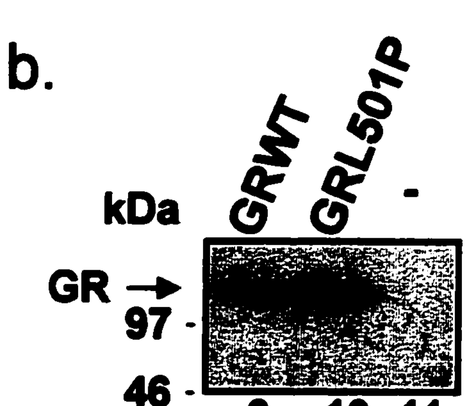
To test the GR interaction with POU factors *in vivo*, I perfomed a mammalian two-hybrid assay. The two-hybrid assay was originally developed in yeast by Fields and Song, 1989 (477) and later adapted to mammalian cells (436). The two-hybrid system is based on the observation that the DNA binding and activation domains are modular and that they do not necessarily need to be covalently linked but can be brought together by interaction of any two proteins. The DNA-binding domain serves to target the activation domain to specific genes and the activation domain contacts other proteins of the

Figure 17. Full-length GR binding to dlx2 and hoxD4 is sensitive to L501P substitution in the GR DBD.

a. ^{35}S- labeled *in vitro* translated dlx2 (lanes 2-4) or hoxD4 (lanes 6-8) were tested for binding to 9E10 immunopurified myc-tagged GR (lanes 2, 6) and GR_{L501P} (lanes 3, 7) from nuclear extracts of clonally derived Sf-7 cells lines or control immunoprecipitates from parental Sf-7 fibroblasts (lanes 4, 8). Bound proteins were resolved by 12% SDS-PAGE and visualized by fluorography. Input proteins (10%) are shown in lanes 1 and 5. To the left of the panel the position of the molecular weight markers are indicted (kDa). b. Western blot analysis with a GR specific antibody (BuGR2) of the immunoprecipitates used in the experiments described above. Each band corresponding to the full length protein was quantified by phosphorimager analysis with the value indicated below each lane, expressed as arbitrary units (counts) corrected for background.



Western



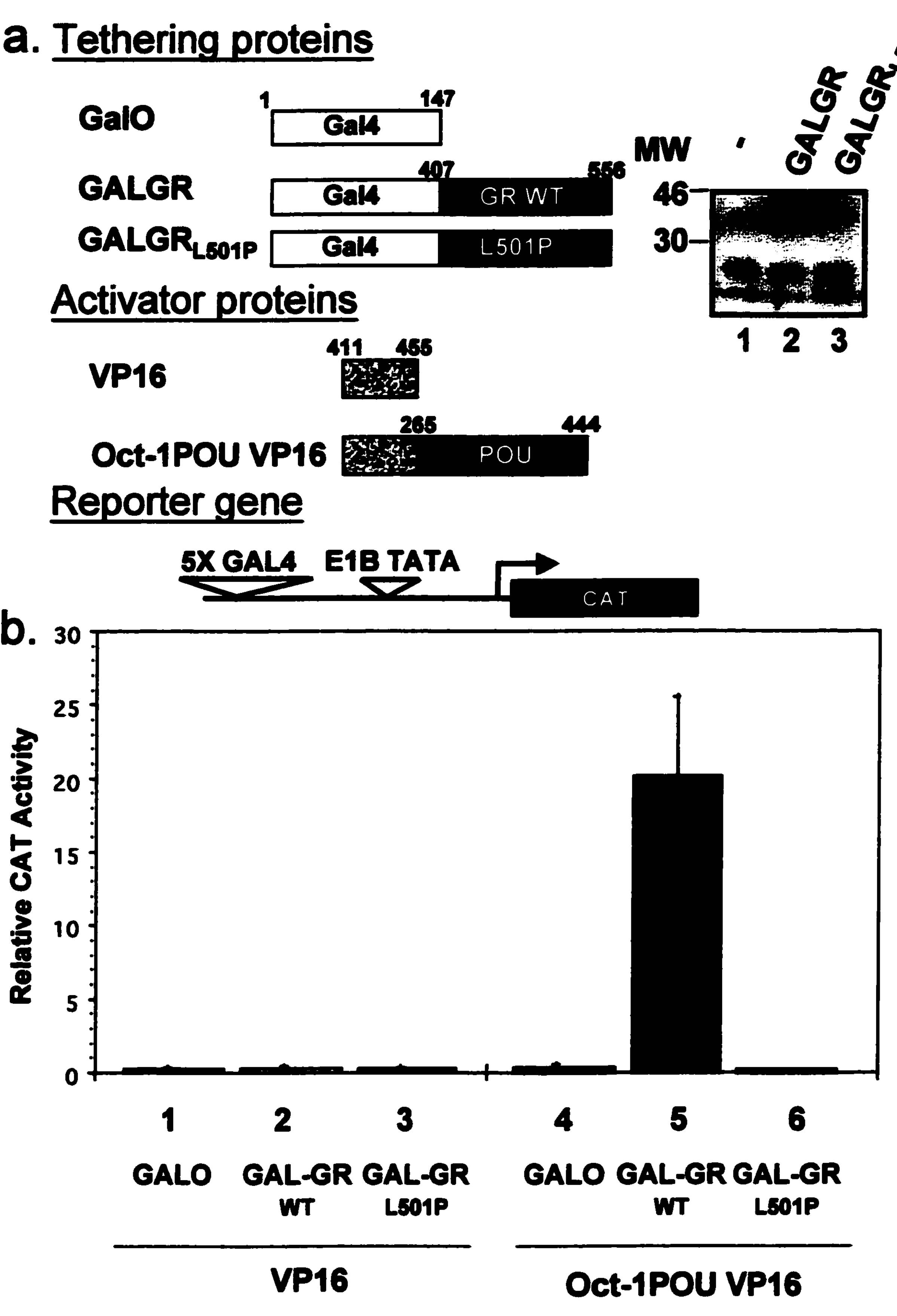
transcriptional machinery to enable transcription to occur. To test for two hybrid interactions a DBD (i.e. Gal4 DBD) is fused to the "bait" protein, and a transcriptional activation domain (i.e. VP16 activation domain) is fused to the "prey" protein. When these fusion proteins are expressed in the same cell with a reporter gene containing specific binding sites for the DNA binding domain and if bait and prey proteins interact a functional activator is created. The protein-protein interaction is measured by reporter gene activation. This technique has been used to study leucine zipper interactions of c-myc, c-Fos and c-jun in mammalian cells (436).

The mammalian two-hybrid assay employed in this work used the yeast Gal4 DNA binding domain (aa 1-147) fused N-terminally to the DNA-binding domain and hinge region of GR (aa 407-556, Gal-GR). The acidic activation domain of VP16 (aa 411-455) was fused N-terminally to the POU domain of Oct-1 (aa 265-444, Oct-1POUVP16). The Gal4DBD contains intrinsic NLSs while the SV40 dominant NLS (478) was inserted into the VP16 clone, to ensure both fusion proteins would be localized to the cell nucleus following expression (436). The reporter gene included 5 copies of a consensus Gal4 DNA binding site immediately upstream of the adenovirus E1B minimal promoter (436) driving the expression of the bacterial CAT gene (479).

Plasmids expressing the Gal4 DBD alone (GALO), Gal DBD fused to GR (aa407-556, Gal-GR_{WT}) or Gal-GR with a point mutation at L501P (Gal-GR_{L501P}) were cotransfected with plasmids expressing VP16 alone or Oct-1POU-VP16 in CHO-K1 cells (Figure 18a). Following transfection (48 hours), the cells were harvested and assayed for transcriptional activity by measuring CAT activity corrected for transfection efficiency (Figure 18b). Co-transfection of Gal-GR with Oct-1POU VP16 displayed an induction of

Figure 18. Mammalian two-hybrid analysis of the interaction of the GRDBD with the Oct-1 POU domain in CHO-K1 cells.

a. Schematic illustration of the proteins expressed following co-transfection with the G5E1BCAT reporter plasmid used in the co-transfection assay described below. Gal4DBD containing plasmids expressed the GalDBD alone, GalO or the DBD fused N-terminally to the rat GRDBD (aa 407-556), GALGR, or the same GRDBD with a point mutation at L501P (indicated by the white bar), $GALGR_{L501P}$. The activator proteins shown represent the VP16 activation domain (aa 411-455, marble filled bar) fused to the POU domain of human Oct-1 (aa 265-444), Oct-1POU-VP16 (black bar) or the VP16 activation domain alone, as a control. Above the representation of each fusion protein was the amino acid positions corresponding to individual protein motifs. The G5E1BCAT reporter gene shows 5 Gal4 DBD binding sites are located immediately upstream of a minimal promoter (E1B TATA) driving the expression (bent arrow) of the CAT reporter gene. (Top right) Western blot analysis of nuclear extracts prepared from transiently transfected CHO cells with equal quantity of plasmid expressing GalO, Gal-GR or $GalGR_{L501P}$ (lanes 1-3, respectively). The membrane was probed with BUGR2, a GR specific antibody. b. Levels of transcription from pG5E1BCAT in response to co-expression of GALGR with Oct-1POU-VP16 were represented by relative CAT activity as a result of co-transfection of plasmids expressing Gal-GRWT, $GalGR_{L501P}$ or the Gal4 DBD alone with the VP16 activation domain alone (left) or Oct-1POU VP16 (right) into CHO-K1 cells. The results represented the mean and standard errors of the mean of at least 5 independent transient transfections performed in duplicate.



a. Tethering proteins
GalO
1
147
Gal4
GALGR
407
556
Gal4
GR WT
GALGR L501P
Gal4
L501P
MW
GALGR
GALGR L501P
46
30
1 2 3
Activator proteins
VP16
411 455
Oct-1POU VP16
265 444
POU
Reporter gene
5X GAL4
E1B TATA
CAT
b.
Relative CAT Activity
0
5
10
15
20
25
30
1 2 3 4 5 6
GALO
GAL-GR WT
GAL-GR L501P
GALO
GAL-GR WT
GAL-GR L501P
VP16
Oct-1POU VP16

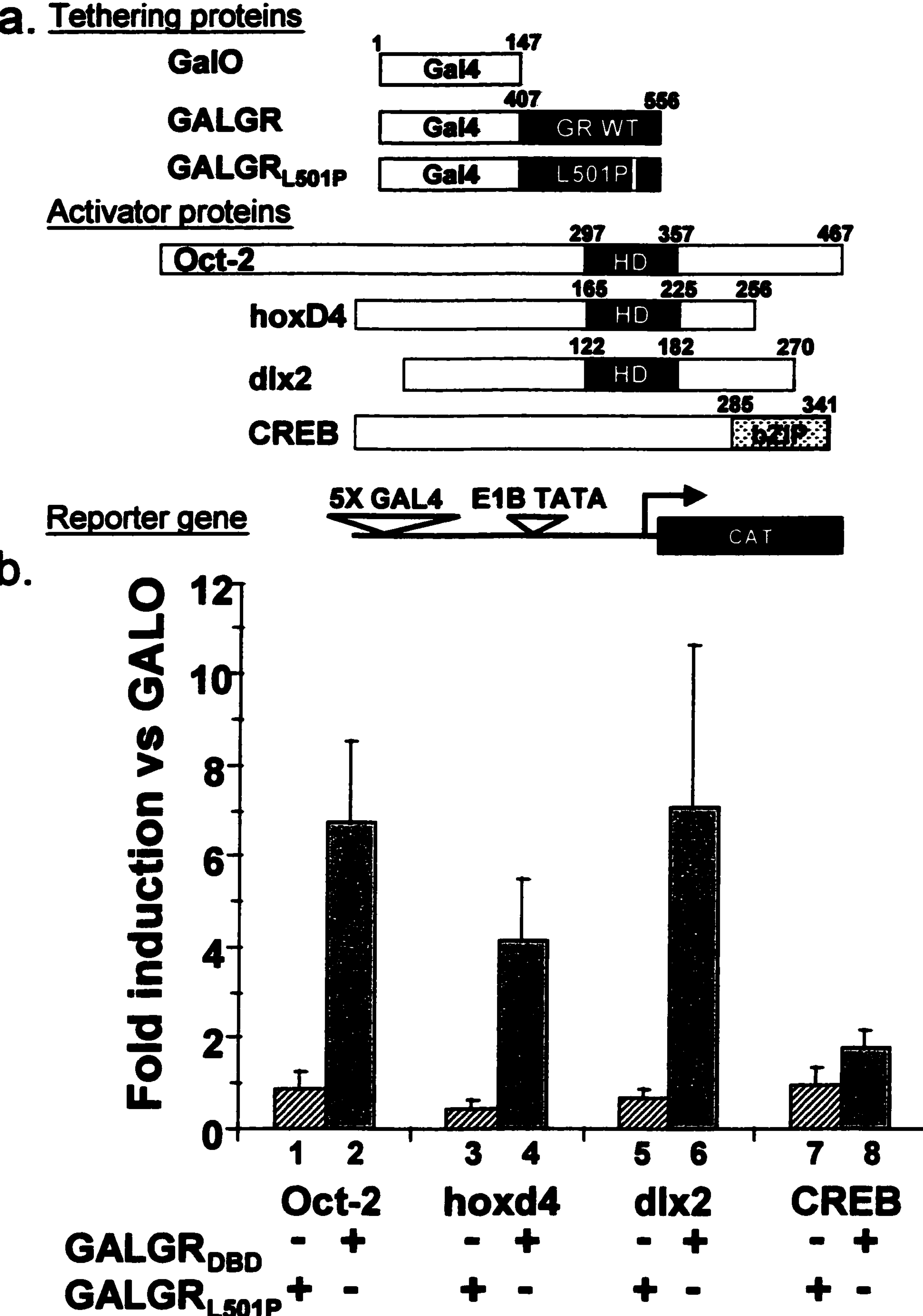
transcription at least 50 X (lane 5) above the level in control lanes (lanes 2 and 4). These results demonstrated that Gal-GR interacted with Oct-1POU-VP16 in the nucleus of mammalian cells. Transcription was dependent on the GR (aa 407-556) and Oct-1POU because removal of just one of these motifs was sufficient to abolish gene transcription under these conditions (lanes 2 and 4). No transcription following coexpression of $GalGR_{L501P}$ with Oct1POU-VP16 (lane 6) was observed above the level of control samples (lanes 3 and 4), demonstrating transcription was sensitive to point mutation at L501P. It is unlikely that the difference in levels of transcription was due to levels of DNA-binding domain hybrid proteins because western analysis of transfected cells with equal quantity of plasmid showed the levels Gal-GR and $Gal\text{-}GR_{L501P}$ protein were similar (Figure 18a, lane 2 and 3, respectively).

The GR DBD was shown to bind directly to a number of homeodomain containing proteins *in vitro*. To determine if the interaction between GR and homeodomain proteins occurs more broadly in the cell, a mammalian one-hybrid approach was employed. This assay was similar to the two-hybrid assay described above but instead of using the activation domain of VP16 to activate transcription, the activator proteins use their intrinsic activation function (Figure 19a). The activators tested were full-length human Oct-2, zebrafish hoxD4, zebrafish dlx2. An unrelated transcription factor rat CREB was also used as an activator to test for the specificity in the assay. The DNA binding hybrid Gal-GR was compared with the control GalO and $Gal\text{-}GR_{L501P}$ on the G5EIBCAT reporter plasmid.

Plasmids expressing the Gal DBD alone (GALO), Gal-GRDBD (aa 407-556) or $GalGR_{L501P}$ were co-transfected in CHO cells together with plasmids expressing Oct-2,

Figure 19. Mammalian one-hybrid analysis of the L501P sensitive interaction of the GR DBD with full length Oct-2, hoxD4 and dlx-2.

a. Schematic illustration of the proteins expressed with the G5E1BCAT reporter plasmid in a transient transfection transcription assay described below. At the top, the tethering Gal4DBD fusion proteins are illustrated. The Gal4 DBD alone (GalO) encodes aa 1-147 of the yeast transcription factor and includes the DNA binding domain, dimerization and a nuclear localization signal. This protein domain was also fused N-terminally to the GR DBD (aa 407-556), GalGR or $GalGR_{L501P}$, the same region with a point mutation at L501P (indicated by the white bar). The activator proteins are illustrated below the tethering proteins. The length of each protein is indicated by the total amino acid count on the far right of each illustrated protein along with the aa position flanking the homeodomain for human Oct-2 and zebrafish hoxD4 and dlx-2. As a control for specificity, CREB, a basic leuzine zipper protein (bZIP), is also illustrated. The G5E1BCAT reporter gene contains 5 Gal4 binding site immediately upstream of a minimal promoter (E1B TATA) driving the expression of the CAT reporter gene. b. CHO-K1 cells were co-transfected with various combinations of plasmids expressing DNA binding and activator proteins, as indicated, along with the G5E1BCAT reporter plasmid. Following transfection (48 hours), cells were harvested, assayed for CAT activity and corrected for transfection efficiency. The data was expressed as fold induction of GalGRDBD (solid bars) and $GalGRDBD_{L501P}$ over the GalDBD alone (GALO). The error bars represented the S.E.M. of at least 3 independent experiments performed in duplicate ($P<0.02$).



a. Tethering proteins
GalO
1
147
Gal4
GALGR
407
556
Gal4
GR WT
GALGR L501P
Gal4
L501P
Activator proteins
297
357
467
Oct-2
HD
165
225
256
hoxD4
HD
122
182
270
dlx2
HD
285
341
CREB
bZIP
Reporter gene
5X GAL4
E1B TATA
CAT
b.
Fold induction vs GALO
0
2
4
6
8
10
12
1
2
3
4
5
6
7
8
Oct-2
hoxd4
dlx2
CREB
GALGR DBD
- + - + - + - +
GALGR L501P
+ - + - + - + -

hoxD4, dlx2 or CREB. Forty-eight hours post transfection the cells were harvested, extracts prepared and tested for CAT activity (Figure 19b). The level of transcription obtained with pGALO varied to a small extent when co-transfected with each test construct; therefore, the values were expressed as fold induction of the $GalGR_{DBD}$ and $GalGR_{L501P}$ over the GALO. The homeodomain- containing proteins, but not the unrelated basic leucine zipper protein, CREB, co -expressed with GalGR (lanes 2, 4, 6, 8) activated transcription significantly above the level obtained by co-expression of Gal-GR_{L501P} (lanes 1, 3, 5, 7). When the GAL-GR was co-expressed with Oct-2, hoxD4 or dlx2 the induction level varied between 4 and 7 fold (compare lanes 4 and 6 with 3 and 5, respectively). Please note that as heterologous activation domains are used in each construct for this assay, the relative activation is not a quantitative reflection of the strength of the interaction.

These results confirmed that in addition to Oct-1 and Oct-2, at least two other homeodomain containing proteins, dlx2 and hoxD4 and perhaps many others interact with the GR DBD-hinge in the cell nucleus. Taken together, these results showed in three independent assays (GST-pull-down direct binding, co-IP binding assay and the mammalian two-hybrid assay) that GR had the potential to interact broadly with homeodomain- containing proteins and the binding was sensitive to a point mutation at amino acid L501P of the rat GR *in vivo*.

4. Octamer factor recruitment to DNA potentiates transcriptional activation.

GR and Oct-1 synergistically activate transcription when response elements for both factors are juxtaposed on both natural and synthetic promoters (16, 17). Both Oct-1POU-VP16 and full-length Oct-2 can activate transcription with Gal-GR through two-

hybrid and one-hybrid assays, respectively, suggesting that the GR DBD (407-556) can activate transcription by bringing either the POU domain of Oct-1 or Oct-2 to the proximity of the promoter. One possible explanation for the transcriptional synergy observed between GR and Oct-1/Oct-2 was that GR may act through a protein recruitment mechanism.

In order to test the contribution of the protein-protein interaction to synergistic transcriptional activation, two reporter genes were constructed: one with five Gal4 response elements juxtaposed to four high-affinity octamer motifs, $pG_54XOct_{WT}CAT$ ($p5XGAL\text{-}4Oct_{WT}$ E1BCAT), and the other a control reporter with five Gal4 response elements with mutant octamer motifs, $pG_54XOct_{mut}CAT$ ($p5XGAL\text{-}4Oct_{mut}$ E1BCAT). Multiple copies of transcription factor binding sites were employed to magnify transcriptional effects. The low level of transcriptional activity upon expression of Gal-GR_{WT} fusion protein alone allowed for specific focus on the transcriptional activity contributed by either the Oct-2 or the Oct-1POU-VP16 fusion protein. In addition, using the L501P-substituted Gal-GR fusion protein, which does not bind to octamer factors in the cell nucleus, allowed for the specific evaluation of the transcriptional activation contributed by the protein-protein interaction between GR and octamer factors. Co-expression of Gal-GR with either Oct-1POU-VP16 or Oct-2 on the $G_54XOct_{WT}CAT$ reporter would result in a level of gene transcription represented by the sum of two separable events: the 2 hybrid effect and the effect of GR recruitment of Oct-1POU-VP16 or Oct2 to the promoter.

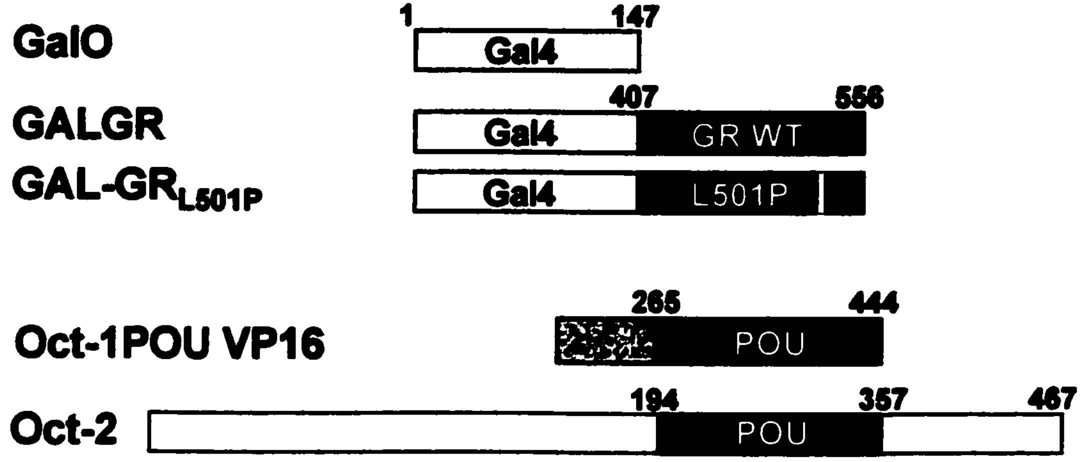
CAT activity from the mutant reporter, $pG_54XOct_{mut}CAT$, reflected the two hybrid effect. Plasmids expressing the Gal DBD alone, Gal-GR or $GalGR_{L501P}$ were

cotransfected with plasmids expressing either Oct-1POU-VP16 (hatched bars) or Oct-2 (stippled bars) with the $G_5$4XOct$_{mut}$CAT reporter. Forty-eight hours post-transfection the cells were harvested and cellular extracts tested for CAT activity (Figure 20a). When Gal-GR was co-expressed with Oct-1POU-VP16 and Oct-2 (lane 3), transcription was increased by 4 and 14 fold respectively above control samples (lane 2) representing the transcriptional activity from the contribution of the two hybrid effect. As expected, the two-hybrid effect was sensitive to Gal-GR$_{L501P}$ (lane 4). This represented an experiment almost identical to those described in Figures 18 and 19, respectively, with the exception that the five copies of the Gal4 response elements were separated from the E1B minimal promoter element with a 95 bp spacing element consisting of the mutant octamer motifs. The fold activation was substantially reduced from the activation in the previous two-hybrid experiments lacking the spacer element (compared to 7 and 50 fold, respectively). This reduction indicated that the proximity of the Gal4 DNA binding sites to the E1B promoter was important for translating the two-hybrid GR to Oct-1POU-VP16 binding into a signal for activating transcription.

The reporter containing 4 consensus octamer motifs juxtaposed to the 5 Gal4 DNA binding sites, p$G_5$4XOctCAT, driving the expression of the CAT gene resulted in a level of transcription substantially above that obtained with the reporter with mutant octamer motifs and is shown on a separate graph (Figure 20b). Co-expression of GalGR$_{WT}$ with Oct-2 or Oct-1POU-VP16 increased transcription 30 and 9 fold respectively, above the level obtained by co-expressing Oct-2 or Oct-1POU-VP16 with the Gal DBD, lacking the GR moiety (compare lane 7 and lane 6). Gal-GR$_{L501P}$, which does not interact with the POU domain of octamer factors, did not increase transcription

Figure 20. The GR DBD dramatically potentiates Oct-2 and Oct-1POU-VP-16 mediated transcription in an octamer motif dependent fashion.

(Top) Schematic representations of the proteins expressed from plasmids co-transfected with the reporter genes in the experiments described below in a. and b. The Gal4 DBD fusion proteins express amino acids 1 -147 of yeast Gal4 protein alone (GalO), fused to GR amino acids 407-556 (GalGR), or fused to the same GR peptide but with an L501P point mutation ($GALGR_{L501P}$, vertical white bar). The Gal4 fusion proteins were co-expressed with plasmids encoding Oct-1POU-VP16, the POU domain of human Oct-1 fused N-terminally to the acidic activation domain of VP16 (aa 411-455) or the full length Oct-2 protein. Above each illustration are numbers corresponding to the amino acid position of individual protein motifs. a. and b. Reporter genes containing multiple consecutive Gal4 binding sites flanking 4 consensus octamer motifs (b) or 4 mutated octamer motifs (a) upstream of a minimal E1B promoter driving the expression of a CAT gene illustrated above each graph. Each reporter gene was co-transfected into CHO-K1 cells with plasmids co-expressing the GAL DBD alone (GALO, lanes 2, 6), $Gal\text{-}GR_{WT}$ (lanes 3, 7) or $Gal\text{-}GR_{L501P}$ (lanes 4, 8), with either Oct-2 (stippled bars) or Oct-1POU-VP16 (hatched bars). As a control for transcription by endogenous octamer factor the reporter genes in a (lane 1) and b (lane 5) were transfected alone. Following transfection (48 hours), cells were harvested, cell extracts prepared and CAT assays were performed. Results were expressed as arbitrary relative CAT units corrected for transfection efficiency. Transfection efficiency was determined using a β-gal liquid assay of cellular extracts following co-transfection with a βgal expression plasmid. The results represented the average and S.E.M. of at least 3 independent transfections in duplicate. The black bar, in lanes 1 showed the basal promoter activity in the absence of ectopically expressed proteins.



a. pG$_5$4XOct$_{mut}$CAT

4XOCT$_{mut}$

Relative CAT Activity

0
1
2
3
4

1 -
2 GALO
3 GAL-GR WT
4 GAL-GR L501P

b. pG$_5$4XOct$_{WT}$CAT

Relative CAT Activity

0
10
20
30
40
150
200
250
300
350

5 -
6 GALO
7 GAL-GR WT
8 GAL-GR L501P

above that obtained by co-expression with the GAL4 DBD (lane 8). The transcriptional activation was highly dependent on the presence of DNA-binding sites for Oct-2 and Oct-1POU-VP16, because mutation of the octamer motifs reduced transcription by at least 100 fold (compare lanes 3 and 7). The results showed that GR-Oct-1/-2 binding strongly increased octamer-motif-dependent transcription.

5. GR recruits octamer factors to adjacent octamer motifs, *in vivo*.

In vitro footprinting assays with DNAse I on the MMTV promoter, showed that incubation of purified GR with increasing amounts of Oct-1 facilitated octamer motif occupancy (17); in the absence of GR little or no protection was observed. Thus, the amount of Oct-1 required for full occupancy of the octamer motifs on the MMTV promoter was decreased in the presence of GR. *In vivo* footprinting assays also indicated the level of Oct-1 occupancy correlated with GR binding to the MMTV HREs. The hormone-dependent octamer motif occupancy has been observed on the genomically integrated MMTV promoter (43). However GR also induces chromatin rearrangement of promoter DNA *in vivo* (38, 39). Therefore it has not been possible to distinguish whether Oct-1 promoter occupancy *in vivo* was a consequence of cooperative DNA binding with GR or a chromatin remodelling event induced by GR which resulted in a more open DNA conformation to allow access of Oct-1 to the octamer motif.

It has been established for plasmid DNA, that the chromatin structure is not ordered and is not properly phased in the absence of DNA replication (480). Differences of NF-I DNA-binding properties have been described using the MMTV promoter, on replicating and non-replicating plasmid DNA (36). NF-I bound constitutively to non-replicating plasmid DNA. In contrast, NF-I DNA-binding was hormone-dependent on

replicating plasmid DNA. The hormone-dependent binding of NF-I required chromatin remodelling prior to occupancy of the MMTV promoter (39). Therefore, in an attempt to minimize the effects of chromatin remodelling events from GR and Oct-2 cooperative binding, experiments with transiently transfected non-replicating DNA templates were employed.

To assess the binding of transcription factors to DNA, a DNA footprinting assay was used (481). Exo fooprinting is a positive assay in that it can efficiently detect certain transcription factors bound to template DNA by the appearance of a band representing a protein/DNA boundary impeding the progress of an exo (470, 482). The MMTV promoter is a complex promoter that contains binding sites for GR, NF-I and Oct-1. A number of sequence specific transcription factors including NF-I and TFIID, but not GR can be detected by exo digestion on cell nuclei (470, 481, 482). Up to this point no *in vivo* footprinting of Oct-1 or Oct-2 has been reported using either λ exo or exo III. This type of assay has been termed *in vivo* because following the isolation of nuclei from live cells, the reaction conditions are mild, keeping the nuclear matrix and nuclei intact for the duration of a brief digestion reaction (15 min at 30°C).

Two types of exonucleases can be used to map transcription factor boundaries exo III (Exo III) or λ exo (λ exo) (470). Mapping of transcription factor binding sites is done similarly for both nucleases. Both exonucleases can be activated by providing an entry point on the promoter of interest. Usually the entry point is created by an endonuclease with its restriction endonuclease site near the location of the footprint. Exo III digests 3' to 5' while λ exo has a 5' to 3' exo activity removing 5' phosphomononucleotides. Some DNA binding proteins can impede progression of exo digestion. λ exo provides an

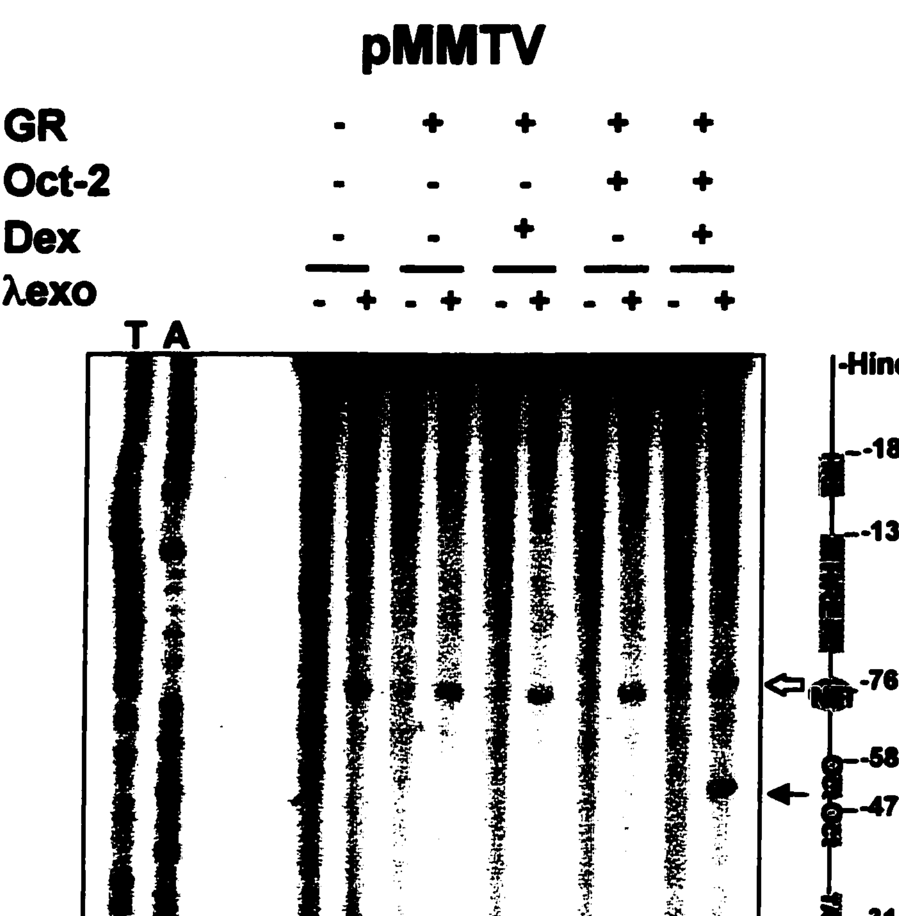
advantage over exo III in that the progressive DNA activity is independent of base composition allowing for more uniform analysis (483).

The digested DNA serves as a template for linear PCR to build an oligonucleotide from a predetermined end labeled primer location to the pause points generated by λ exo. The length of these labeled oligonucleotides can be resolved by denaturing polyacrylamide gel electrophoresis and compared to a DNA sequencing tract with the same primer to determine the position of the pause point from the predetermined DNA promoter sequence at single nucleotide resolution.

First, the binding of transcription factors to the MMTV promoter (-237 to +125) in the pHCWT plasmid was examined. CHO-K1 cells were transiently transfected by calcium phosphate precipitation with pHCWT template DNA along with expression plasmids for GR and Oct-2, as indicated. Prior to harvest (15 minutes) the cells were treated with 1 μM dex to activate GR. Following cellular lysis, the nuclei were collected by centrifugation through a sucrose pad. Then, the nuclei were digested simultaneously with 100 U of *Bam* HI and 15 U λ exo for 15 minutes at 30°C. Finally, the reaction was stopped by the addition of SDS/EDTA and the DNA purified by proteinase K treatment, phenol/ chloroform extraction and ethanol precipitation. Complete digestion with a second restriction enzyme, *Hind* III, was used as an internal control for loading. The DNA was amplified by linear PCR with an end-labeled primer complementary to specific MMTV DNA sequence (+74 to +52, 5' to 3'). The products were resolved on a 6% sequencing gels and visualized by autoradiography (Figure 21). The products were compared to T and A sequencing tracts (lanes 1 and 2, respectively) and used to create the schematic diagram to the right of each panel. A λ exo dependent footprint was

Figure 21. Hormone treated GR induces an Oct-2 dependent footprint on the MMTV promoter.

CHOK1 cells were transiently transfected by calcium phosphate precipitation with pHCWT (reporter plasmid containing MMTV promoter sequences -237 to +105), GR and/or Oct-2 expression vectors, 24 hours later the cells were treated with 1μM dex or not treated. The cells were harvested and the nuclei were isolated by centrifugation through a sucrose pad. The nuclei were then incubated at 30°C for 15 min in the presence of Hind III and λ exonuclease, as indicated. The reactions were stopped and the DNA purified and digested with *Bam* HI. The exonuclease pause sites were revealed by linear PCR using a ^{32}P end-labeled oligonucleotide corresponding to the complementary DNA strand of the MMTV DNA sequence at position +74 to +52 from the transcriptional start site. Samples were resolved on a denaturing 6% sequencing gel and visualized by autoradiography. The unique pause site in lane 12 represent Oct –2 bound to the promoter DNA and is highlighted by a solid arrow. A constitutive pause site represents a protein barrier as a result of NF-I binding and is highlighted with the open arrow. To the right, a schematic representation of the position relative to the GR, NFI, Oct, and TBP binding sites of the MMTV as determined by the T and A sequencing tracks (lanes 1, 2) using the same primer.


pMMTV
GR - + + + +
Oct-2 - - - + +
Dex - - + - +
λexo - + - + - + - + - +
T A
1 2 3 4 5 6 7 8 9 10 11 12
-Hind III
-187
-132
-76
-58
-47
-31
+52
+74
MMTV Primer

observed upstream of the NF-I binding site on the MMTV promoter and was independent of ectopic expression of GR and Oct-2 (lanes 4, 6, 10). As shown previously by Mymryk and Archer, 1994 (470), the intensity of binding by NF-I was independent of hormone treatment (lanes 4, 6, 8, 10, 12). Under these conditions no footprint was observed adjacent to the octamer factor binding sites in the absence of steroid hormone. However, upon hormone treatment a strong Oct-2 dependent footprint was observed flanking the octamer binding sites (lane 12). Unexpectedly, the pause site mapped exactly between the two octamer motifs at –49 rather than near –60, adjacent upstream of the octamer binding sites. However, the location of the pause site was consistent with previous published results (38, 470) that suggested the pause site reflected the binding of TFIID to DNA. The results showed a steroid induced binding of a factor to the MMTV promoter near the octamer motifs that required the ectopic expression of Oct-2 and GR, *in vivo*.

To determine whether the pause site indeed reflected the binding of Oct-2 and not the binding of other transcription factors, like TFIID, a footprinting experiment was performed using a pBluescript template DNA lacking a functional mammalian basal promoter element or TATA box, and for which no transcription would be expected. A single consensus octamer motif was inserted next to a consensus HRE into the pBluescript plasmid DNA, pBSHREOct and used as an *in vivo* DNA fooprinting template.

Expression vectors for GR and Oct-2 were transfected as indicated into CHO K1 cells with pBSHREOct. Forty-eight hours following transfection, the cells were treated with steroid as indicated for 15 minutes, harvested, and the nuclei isolated, and treated essentially as previously described in Figure 21, except that the nuclei were digested with

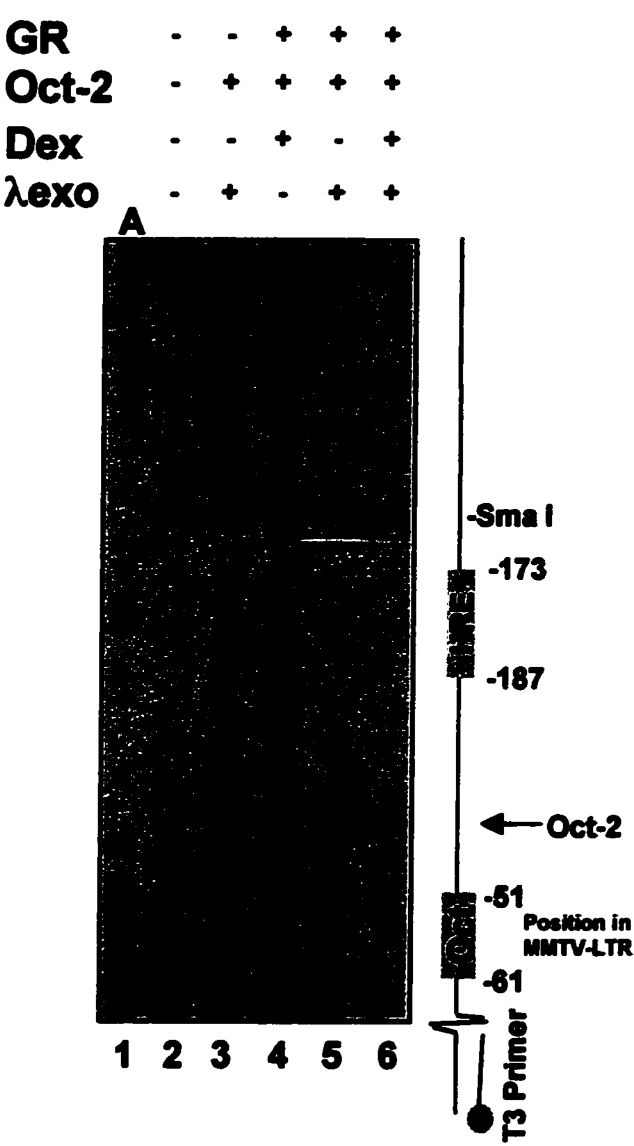
SmaI rather than *Bam* HI to introduce an entry point for λ exo. The λ exo pause points were revealed by linear PCR amplification with an end-labeled universal T3 primer and resolved by running the samples on a 6% sequencing gel.

A λ exo, Oct-2, steroid and GR dependent single pause site was detected flanking the octamer factor binding site following linear PCR (Figure 22, lane 6). Importantly, no footprint was observed by expression of Oct-2 in the absence of ligand-treated GR (lane 5). This again supported the hypothesis, that hormone-treated GR promoted the binding of Oct-2 and not other factors, like TFIID, to DNA templates containing binding sites for both factors in the cell nucleus. In addition, GR facilitated the binding of Oct-2 to DNA independent of spacing between the binding sites or orientation, because the position and orientation of the binding sites have been altered in the pBSHREOct construct relative to HREs in the MMTV promoter.

To establish whether Oct-2 recruitment to the MMTV promoter was required because the octamer motif represented a weak binding site, the footprinting was repeated using a consensus octamer motif derived from the histone H2B promoter. To show that the recruitment of Oct-2 was dependent on the L501P sensitive GR-Oct-2 protein-protein interaction described in this work, the footprinting assay was performed on a DNA template with a single Gal4 DNA-binding site juxtaposed to a high affinity octamer motif, pBSGalOct. To control for specificity, a template plasmid DNA construct was cloned containing a single Gal4 DNA binding site adjacent to a single binding site for an unrelated transcription factor, IAP, pBSGalIAP. With the knowledge that the L501P mutation in the GR DBD also disrupted DNA binding (445) and to have comparable

Figure 22. Liganded GR induces an Oct-2 dependent footprint in the absence of basal or other flanking cis- acting elements.

Nuclei were isolated from transiently transfected CHO-K1 cells with pBluescript containing a single binding site for both GR (relative to MMTV promoter sequences -187 to –173 from the transcriptional start site) and OCT –1/-2 (relative to the promoter sequneces -61 to –51 from the transcriptional start site), pBSHREOct, and expression vectors for GR and/or Oct-2 and treated with 1μM Dex for 15 min prior to harvest. The nuclei were digested simultaneously with Sma I and λ exonuclease, as indicated, at 30°C for 15 minutes. The DNA was purified and subjected to linear PCR amplification with a ^{32}P end-labeled universal T3 primer. The amplified DNA samples were resolved by a denaturing 6% sequencing gel and visualized by autoradiography. The solid arrow indicated the pause point that results when hormone treated receptor and Oct-2 were coexpressed. (right) Location of GR and Oct-2 binding sites were determined by the A sequencing tract using the same primer (lane 1).


pHREOCT
GR - - + + +
Oct-2 - + + + +
Dex - - + - +
λexo - + - + +
A
-Sma I
-173
-187
Oct-2
-51
Position in MMTV-LTR
-61
1 2 3 4 5 6
T3 Primer

DNA targeting of the WT and L501P GR peptides, a fusion with a heterologous DNA binding domain, Gal4 DBD, was used. Following transfection with the appropriate template and the expression plasmids as indicated, CHO-K1 cells were harvested and the nuclei collected. The nuclei were digested with 100U of *Xho*I and 15 U λ exo and the DNA prepared as previously described (Figure 21). Following linear PCR with an end-labeled universal T7 primer, samples were resolved on a DNA sequencing gel and visualized by autoradiography (Figure 23).

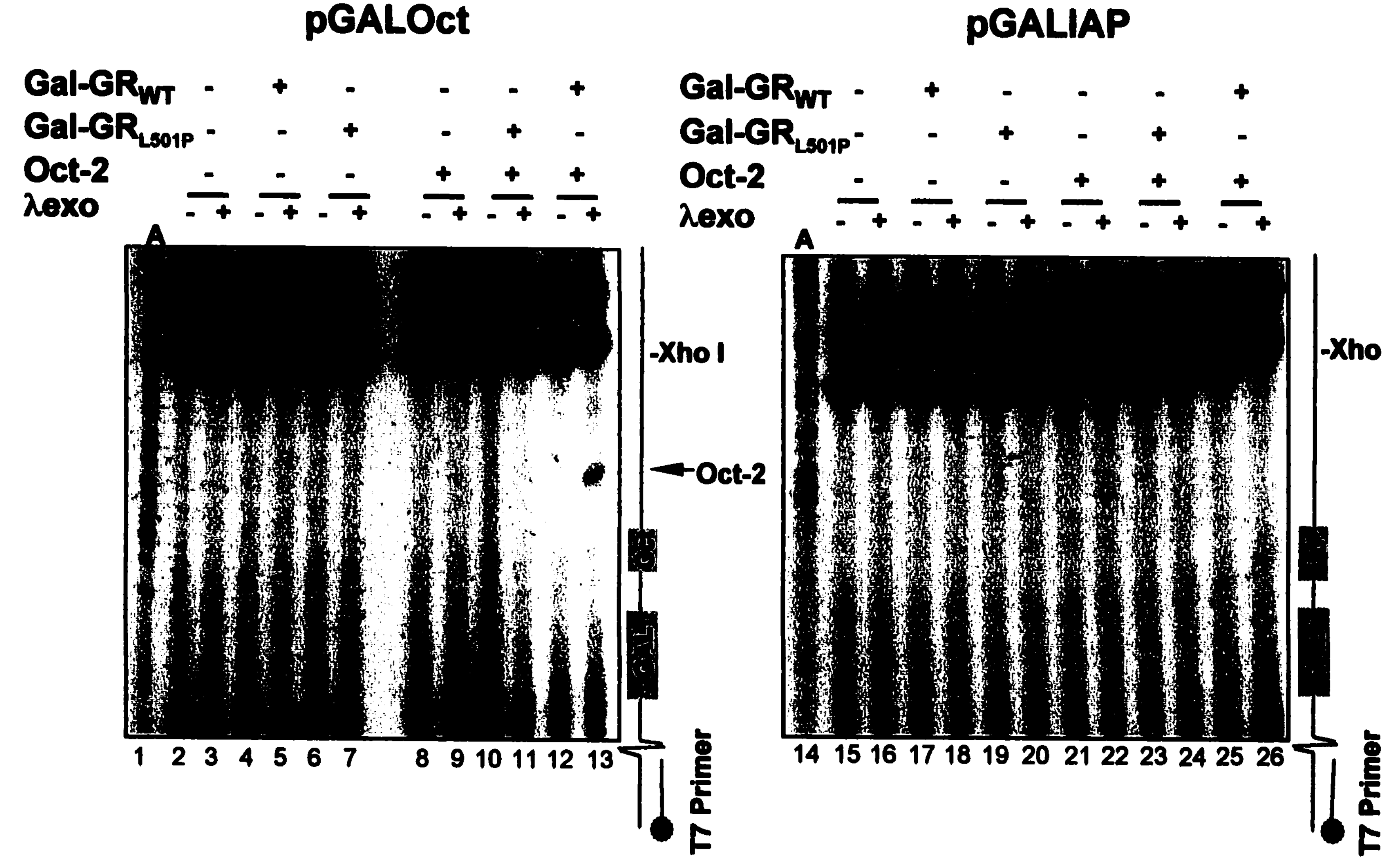
No pause sites were observed flanking the Gal4 DNA binding sites following expression of the Gal4 fusion proteins (lanes 5, 7, 18, 20). This suggested that DNA binding of Gal4, like GR, does not impede the progress to λ exo digestion. However, coexpression of both $GalGR_{WT}$ with full-length Oct-2 revealed a pause adjacent to the octamer motif (lane 13). The Oct-2 dependent footprint was completely sensitive to the L501P mutation (lane 11). By contrast, no pause site was revealed with template DNA containing a binding site for an unrelated transcription factor in place of the octamer motif (lane 26) confirming the dependence on the octamer motif. Together, these results showed GR promoted the binding of Oct-2 to octamer motifs adjacent to binding sites that recruited GR. Oct-2 facilitated binding by GR correlated with the protein-protein interaction because mutation at L501P that prevented this interaction, also abolished Oct-2 binding to DNA.

6. The GR/Oct-1 protein complex formed *in vitro* is disrupted by a HRE.

Strong transcriptional activation was observed as a result of the two-hybrid effect of Oct-1 and Oct-2 being tethered to DNA through a Gal4 DNA-binding site by

Figure 23. The recruitment by the GR DBD is sufficient to induce an Oct-2 dependent footprint.

CHO-K1 cells were co-transfected with pBluescript containing a single binding site for Gal4 and an octamer motif (pBSGalOct) or a binding site for an unrelated transcription factor, interciternal A particle (IAP), (pBSGalIAP) along with GalGRDBD or $GalGRDBD_{L501P}$ and Oct-2 expression plasmids. The nuclei were isolated and digested at 30°C for 15 min with XhoI and λ exonuclease, as indicated. The digestions were revealed by linear PCR extension using a ^{32}P end-labeled T7 universal primer. The DNA samples were resolved on a denaturing 6% sequencing gel. The same primer was used in an A sequencing reaction and run in lanes 1 and 14, respectively to position the GAL4 and Oct-2 or IAP core binding sites illustrated schematically to the right of each panel. The Oct-2, GalGRDBD and octamer motif specific footprint (lane 13) was indicated by the solid arrow.


pGALOct
pGALIAP
Gal-GR$_{WT}$
Gal-GR$_{L501P}$
Oct-2
λexo
A
-Xho I
Oct-2
T7 Primer
GAL
1 2 3 4 5 6 7 8 9 10 11 12 13
14 15 16 17 18 19 20 21 22 23 24 25 26

$GalGR_{WT}$. Others have demonstrated that Oct-1 and Oct-2 do not influence transcription through a HRE in the absence of octamer motifs (16). These results suggested the likelihood that the binding of GR to DNA might be incompatible with the continued interaction with Oct-1 and Oct-2.

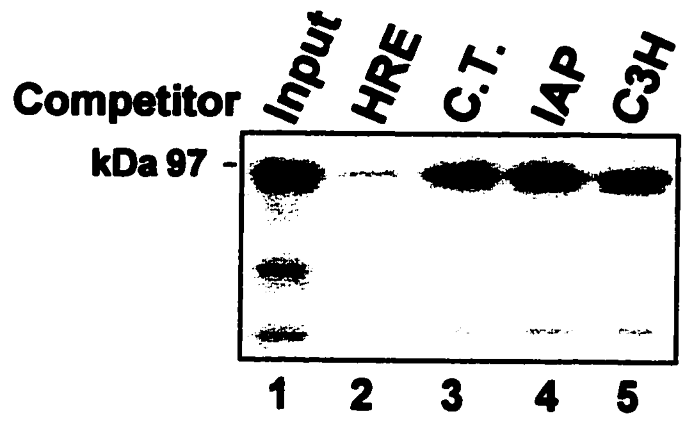
To examine the effect that GR binding to DNA might have on the GR/ Oct-1 complex, the ability of DNAs to disrupt the GR/ Oct-1 complex was tested in a GST pull-down experiment (Figure 24). ^{35}S labeled dexamethasone-treated GR was preincubated with GST-Oct-1POU immobilized to glutathione Sepharose under standard binding conditions to allow complex formation. The preformed GR/ Oct-1 complex was then challenged with various DNAs. Following 4 washes, the bound proteins were resolved by SDS-PAGE. The results showed the GR/ Oct protein complexes were unaffected by the addition of 100 ng of highly sheared calf thymus DNA or non-specific DNA oligonucleotides added to the binding assay (lanes 3 – 5, compare with the binding with Figure 9). However, addition of a consensus HRE disrupted the GR/ Oct-1 complex (lane 2). These results indicate that a HRE can disrupt GR/ Oct-1 binding and that the GR/Oct-1 complex was still competent to specifically recognize a HRE.

The DNA footprinting studies proposed that GR facilitated the binding of Oct-2 implying that GR recruited Oct-1/-2 for cooperative DNA binding. The disadvantage of the exo footprinting assay was that GR could not be detected bound to DNA using that technique. Therefore to examine whether GR and Oct-2 bound to the same molecule of DNA cooperatively, electrophoretic mobility shift assays (EMSAs) were employed.

To do this CHO-K1 cells were transiently transfected with plasmid DNAs overexpressing Oct-2, Gal-GR or $GalGR_{L501P}$. Post-transfection (24 hours), nuclear

Figure 24. The GR/Oct complex formation in vitro, may be specifically disrupted by a HRE oligonucleotide.

^{35}S-labeled in vitro translated GR was preincubated with 0.5 μg of immobilized, bacterially expressed Oct-POU domain fused to GST. The preformed complex was challenged with 100 ng of double stranded oligonucleotides containing binding sites for GR (HRE, lane 2), intracisternal A particle (IAP, lane 4), Ku autoantigen (C3H, lane 5) or highly sheared calf thymus DNA (C.T., lane 3). The competition for GR binding to GST Oct-POU by these oligonucleotides were assessed by resolving the bound proteins by SDS-PAGE and visualized by fluorography. The binding was compared to 10% of the input protein in lane 1. The position of the 97 kDa molecular weight marker are shown to the left.

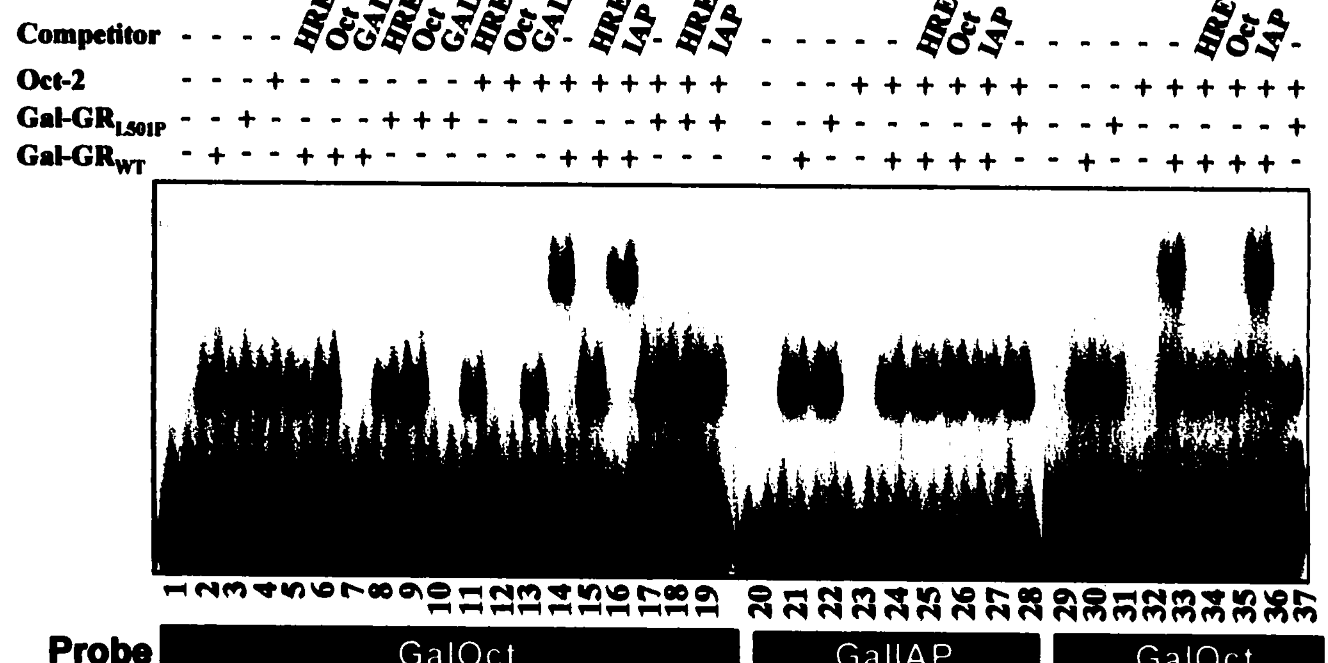

Competitor
Input
HRE
C.T.
IAP
C3H
kDa 97
1
2
3
4
5

extracts were prepared from these cells and control cells. The nuclear extracts were preincubated alone or in combination, prior to the addition of ^{32}P labeled oligonucleotide and competitor oligonucleotides. When looking for evidence of cooperative binding it was important to shift no more than 50% of the labeled probe following incubation with the protein extract. The Gal-GRs and Oct-2 were overexpressed to levels well above the level of endogenous Oct-1 in these cells which did not yield a shifted complex in this experiment (Figure 25, lanes 1, 20, 29). The Gal-GR proteins and Oct-2 formed complexes with an oligonucleotide containing binding sites for both the Gal4 protein and octamer factors (lanes 2 to 4, 21, 22, 30, 31). The binding of the Gal-GR proteins were specific because the protein/DNA complexes were competed by the addition of Gal4 competitor DNA (lanes 7, 10) but not an octamer motif (lanes 6, 9). The Oct-2/DNA complexes were specific because the Oct-2-dependent shift was competed by an excess of octamer motif DNA (lane 12) but not an excess of oligonucleotides containing a HRE or Gal4 binding site (lanes 11, 13, respectively). Coincidentally, the Gal-GR_{WT}, Gal-GR_{mut} and Oct-2 shift all migrated at the same position following non-denaturing gel electrophoresis. This likely occurred because the charge-to-size ratio of the Gal-GR/DNA and Oct-2/DNA complexes were similar. The Gal-GR-dependent protein/DNA complex (lane 2) was not disrupted by the addition of HRE competitor DNA (lane 5), suggesting that the GR moiety was not participating in the DNA-binding function of the hybrid protein.

When Gal-GR_{WT} was preincubated with nuclear extract containing Oct-2 prior to the addition of labeled oligonucleotide to the incubation, only a single slower mobility protein/DNA complex was observed (lane 14). In the presence of an excess competitor

Figure 25. GR and Oct cooperative binding to DNA may be disrupted by a competitor HRE.

EMSA using nuclear extracts from untransfected CHO-K1 cells or cells transfected with plasmids expressing either Oct-2, $GalGRDBD_{WT}$ or $GalGRDBD_{L501P}$. Nuclear extracts prepared from untransfected or transfected cells were preincubated for 4 hours prior to the addition of a radiolabeled oligonucleotide containing a single binding site for Gal4 coupled with an octamer motif (ATGCAAAT) (GalOct), coupled with a binding site for a none related protein (GalIAP) or coupled with a mutant octamer motif (CGGCAAAT) ($GalOCT_{mt}$) and competitor DNAs, as indicated. Incubation was extended for a further 20 minutes at room temperature prior to non-denaturing PAGE. Individual combinations of nuclear extracts and competitor DNAs (100 fold excess) are indicated above the panel.


Competitor
HRE
Oct
GAL
IAP
Oct-2
Gal-GR$_{L501P}$
Gal-GR$_{WT}$
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37
Probe
GalOct
GalIAP
GalOct

HRE-containing oligonucleotide (lane 15) the slower mobility complex was lost, suggesting a role for the GR DNA-binding function in formation of the complex. This competition was specific because there was no competition by non-specific competitor DNA (IAP, lane 16). Even though this complex had a slower rate of mobility, it still only contained less than 5% of the total labeled oligonucleotide, suggesting that most of the Gal-GR and Oct-2 were associated in this heterocomplex and that GR and Oct-2 bound coordinately to DNA. As expected, when $GalGR_{L501P}$, a form of GR that could not bind to Oct-2, was preincubated with Oct-2 and tested for the ability to form a higher shifted heterocomplex, only the complexes representing $GalGR_{L501P}$ and Oct-2 individually bound to the DNA probe were observed (lane 17). The higher heterocomplex in lane 14 included Oct-2, because the heterocomplex was not observed when the octamer motif in the probe was replaced with a binding site for an unrelated transcription factor, IAP (lane 24).

Further, under the same conditions a higher heterocomplex was observed using a $GalOct_{mut}$ probe (lane 33), although this protein/DNA complex appeared to be less stable than that formed with the probe containing the WT octamer motif because the lower complex representing individually bound GALGR and Oct-2 to DNA was also observed. The formation of the GalGR and Oct-2 heterocomplex bound to DNA was specifically sensitive to the addition of an excess of unlabeled oligonucleotide containing a HRE or an octamer motif (lanes 15, 34 and 35). By contrast, addition of a non-specific oligonucleotide had no detectable effect on the formation of the higher heterocomplex. (lanes 16, 36). Together these results suggest that specific interaction by either GR or

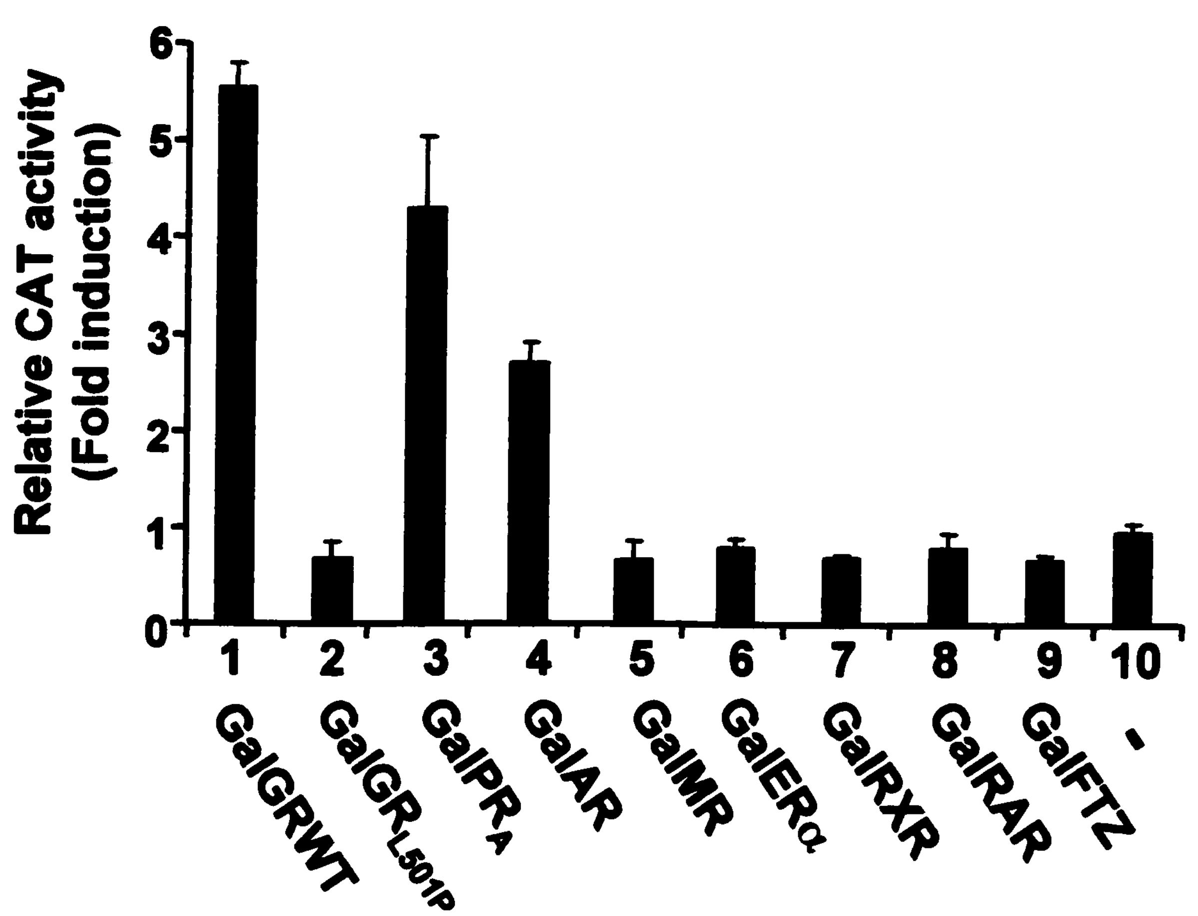
Oct-2 with DNA containing individual binding sites was incompatible with protein-protein interaction.

7. Oct-2 can bind to the DBDs of GR, PR and AR, but not other NR in the cell nucleus.

The protein-protein interaction between GR and Oct-1/-2 mapped to the DNA binding domain of GR, the most highly conserved region among members of the NR superfamily (162). In previous studies using GST pull down assays (45, 471), several NRs were *in vitro* translated and tested for binding to GST Oct-1POU. The NRs included four steroid receptors (GR, ERα, AR and PR), RAR and RXR isoforms (RARα and RXRα), a thyroid hormone receptor (TR α2) and a *Drosophila* orphan receptor (Ftz-F1α). The results showed that all the NRs bound to the POU domain of Oct-1 in this assay. All the steroid receptors tested required ligand to make them competent to bind to the immobilized POU domain while the non-steroidal receptors bound constitutively. A closer examination of RARα binding to the POU domain using C-terminally a deleted RAR, revealed that truncation into the C-terminus of the RAR DBD abolished binding to the POU domain (45, 471). This was exactly consistent with the C-terminal boundary determined using GR truncations. Thus, at least several NRs bound specifically to the POU domain of Oct-1 *in vitro*. Most likely, the motif important for protein binding was located in a similar location in these NRs.

To determine whether this *in vitro* binding result reflected the ability of NRs to associate through their DBDs with Oct-2 *in vivo*, the binding was assessed using a mammalian one-hybrid assay (Figure 26). In this assay, CHO cells were transfected with the $pG_5E1BCAT$ reporter in addition to an expression plasmid for Oct-2 and one of a

Figure 26. Mammalian one-hybrid analysis of the interaction of nuclear receptor DBDs with Oct-2.
Mammalian expression vectors expressing several nuclear receptor DBDs (rat GR aa 407-556, lane 1; rat GR_{L501P}, same peptide but with a point mutation at L501P, lane 2; human PR_A aa 535–688, lane 3; rat AR aa 515–671, lane 4; rat MR aa 577–709, lane 5; human ERαaa 164–299, lane 6; mouse RXRα aa 118–236, lane 7; mouse RARα aa 60–160, lane 8; *Drosophila* Ftz-f1α aa 478–610, lane 9) fused to the yeast Gal4 DBD (aa 1-147) or none (lane 10) were co-transfected in CHO-K1 cells with an expression vector for Oct-2 and a CAT reporter gene driven by 5 Gal4 response elements upstream of a minimal promoter. Following transfection (24 hours), the cells were harvested, cellular extracts were prepared and assayed for CAT activity. The results were displayed as fold induction of relative CAT activity by co-expression of Oct-2 and each Gal4-nuclear receptor fusion protein individually over the activity of expressing Oct-2 alone with the reporter. Each transfection was corrected for transfection efficiency, with each value representing the mean and the S.E.M. of at least 3 independent transfections in duplicate.


Relative CAT activity
(Fold induction)
0
1
2
3
4
5
6
1
2
3
4
5
6
7
8
9
10
GalGRWT
GalGR$_{L501P}$
GalPR$_A$
GalAR
GalMR
GalERα
GalRXR
GalRAR
GalFTZ
-

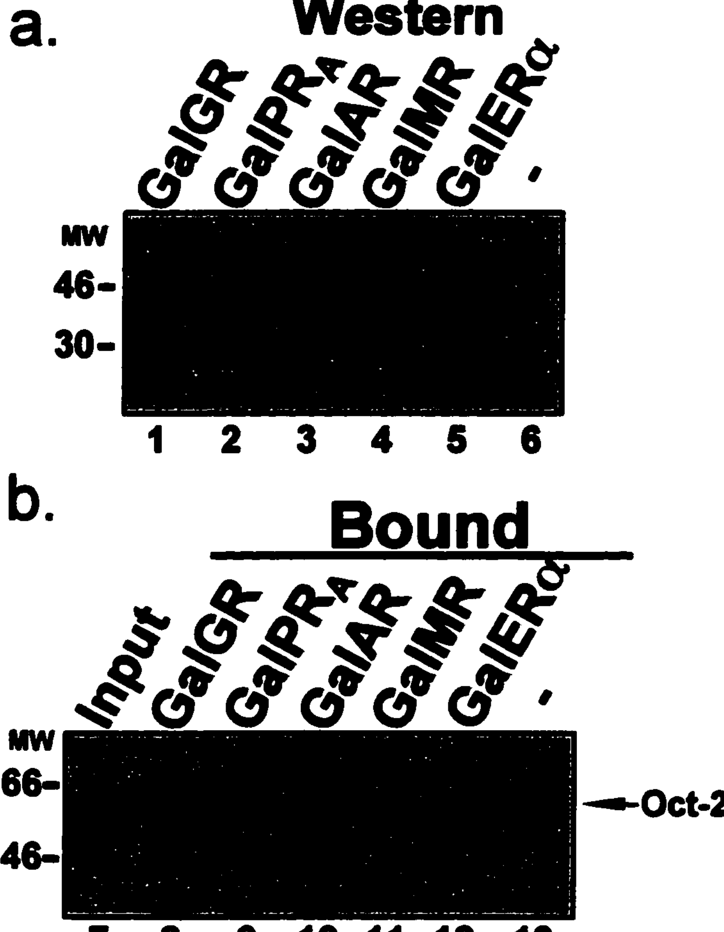
variety of Gal-NR fusion proteins including rat GR, human PR_A, rat AR, rat MR, human ER α, mouse RXR α, mouse RAR α and *Drosophila* Ftz-F1α. CAT enzyme activity was expressed as a function of the fold activation over the activity obtained by expressing the Gal4 DBD alone. Expression of Oct-2 alone had no effect on the transcription of the G_5E1BCAT reporter gene (lane 10). However, coexpression of Gal-GR, Gal-PR and Gal-AR with Oct-2 induced CAT activity 3-6 fold (lanes 1, 3 and 4, respectively). The activity was abolished by coexpression of GR with point mutation at L501P (lane 2). By contrast to the GST-pull-down assay (45, 471), Gal fusion proteins containing the same regions of MR, ERα, RXRα, RARα and Ftz-F1α, co-expressed with Oct-2 were unable to stimulate CAT gene transcription (lanes 5-9).

Most of the Gal fusion proteins tested were expressed to similar levels in CHO-K1 cells, as determined by western blot analysis with a GalDBD antibody (Santa-Cruz, CA) (Figure 27a). Cells were transfected with equal quantities of plasmids expressing Gal-GR, Gal-PR_A, Gal-AR, Gal-MR and Gal-ER. The cells were harvested 48 hours post-transfection and analyzed by western blotting with a Gal4DBD-specific antibody. Each of the NRs varied slightly in levels of protein expression but levels of protein expression did not correlate directly with the levels of transcription in Figure 26. For example, the slightly lower level of transcription by Gal-AR was not due to a reduced level of protein expression (compare Figure 26, lanes 4 and 1 with Figure 27a, lanes 3 and 1, respectively).

To test the Gal-NR/Oct binding more directly, an IP binding assay was employed. The Gal-NR proteins were overexpressed in CHO-K1 cells and immunoprecipitated with a Gal4 antibody from nuclear extracts and the immunoprecipitates tested for binding to *in*

Figure 27. Gal-GR, Gal-PR, and Gal-AR can interact with Oct-2, in vitro.

a. A western blot analysis with a Gal4 antibody of nuclear extracts prepared from CHO-K1 cells transiently transfected with mammalian expression vectors for the Gal4 (aa 1-147)-nuclear receptor fusion proteins (rat GR aa 407-556, lane 1; human PRA, aa 535–688 lane 2; rat AR, aa 515–671, lane 3; rat MR aa 577–709, lane 4; human ERα, aa 164–299, lane 5) shown above or mock transfected cells (lane 6). The bands were visualized using a secondary antibody linked to HRP and visualized using ECL. Note that each protein was expressed to similar levels. b. Binding of ^{35}S-labeled, *in vitro* translated Oct-2 protein was tested for binding to GalDBD fusion protein immunoprecipitates from nuclear extracts prepared from CHO-K1 cells transiently transfected with plasmids expressing GalGR, GalPR$_A$, GalAR, GalMR and GalERα. Following incubation in binding buffer for 90 minutes at 4°C, the bound proteins were washed 3 times with binding buffer. The bound proteins were eluted in SDS-sample buffer, resolved by SDS-PAGE and visualized by fluorography (lanes 8-13). 10% of the Oct-2 input protein is shown in lane 7. The positions of the molecular weight markers are shown to the left of each panel (kDa). Note that the level of binding of Oct-2 in lanes 8-13 did not reflect the protein loading of the Gal4-nuclear receptors in lanes 1-6.


a.
Western
GalGR
GalPRA
GalAR
GalMR
GalERα
-
MW
46-
30-
1
2
3
4
5
6
b.
Bound
Input
GalGR
GalPRA
GalAR
GalMR
GalERα
-
MW
66-
46-
Oct-2
7
8
9
10
11
12
13

vitro translated ^{35}S-labeled Oct-2. This assay was performed with comparable amounts of Gal-NR fusion protein, as shown in the western blot analysis (Figure 27, a). The immunoprecipitates were incubated with ^{35}S-labeled Oct-2 for 90 minutes at 4°C in binding buffer. The samples were washed three times with binding buffer, eluted in SDS-sample buffer and resolved by SDS-PAGE. *In vitro* translated Oct-2 bound strongly to Gal-GR, Gal-PR_A and Gal-AR (Figure 27, b, lanes 8-10). By contrast, Gal-MR or Gal-ERα fusion proteins (lanes 11-12) bound to levels barely above background (lane 13). Together, these results demonstrated that GR, PR and AR but not MR or other NRs tested bound to Oct-2, *in vivo*.

8. GR, PR and AR promote Oct-2 recruitment to DNA.

GR, PR, AR and MR can recognize the same response elements with similar affinity. The observation that GR, PR, AR but not MR bound to octamer factors *in vivo* suggested the intriguing possibility that these differential interactions could markedly affect hormone responsiveness on the MMTV LTR. First, it would be important to determine if MR had the ability to promote the recruitment of Oct-2 to DNA.

Previously, it was shown that Gal-GR facilitated the binding of Oct-2 to template DNA containing a single binding site for Gal4 and an octamer motif (see Figure 23). To determine whether the interaction of Oct-2 with AR and PR could similarly promote the occupancy of octamer motifs adjacent to binding sites that recruit NR peptides, λ exo footprinting assays were performed on the same transiently transfected plasmid DNAs as described in Figure 23.

Nuclei prepared from CHO cells transfected with the Gal4-NR fusion proteins, Oct-2 and pBSGalOct, were digested with *Xho*I and λ exo. The DNA was isolated and

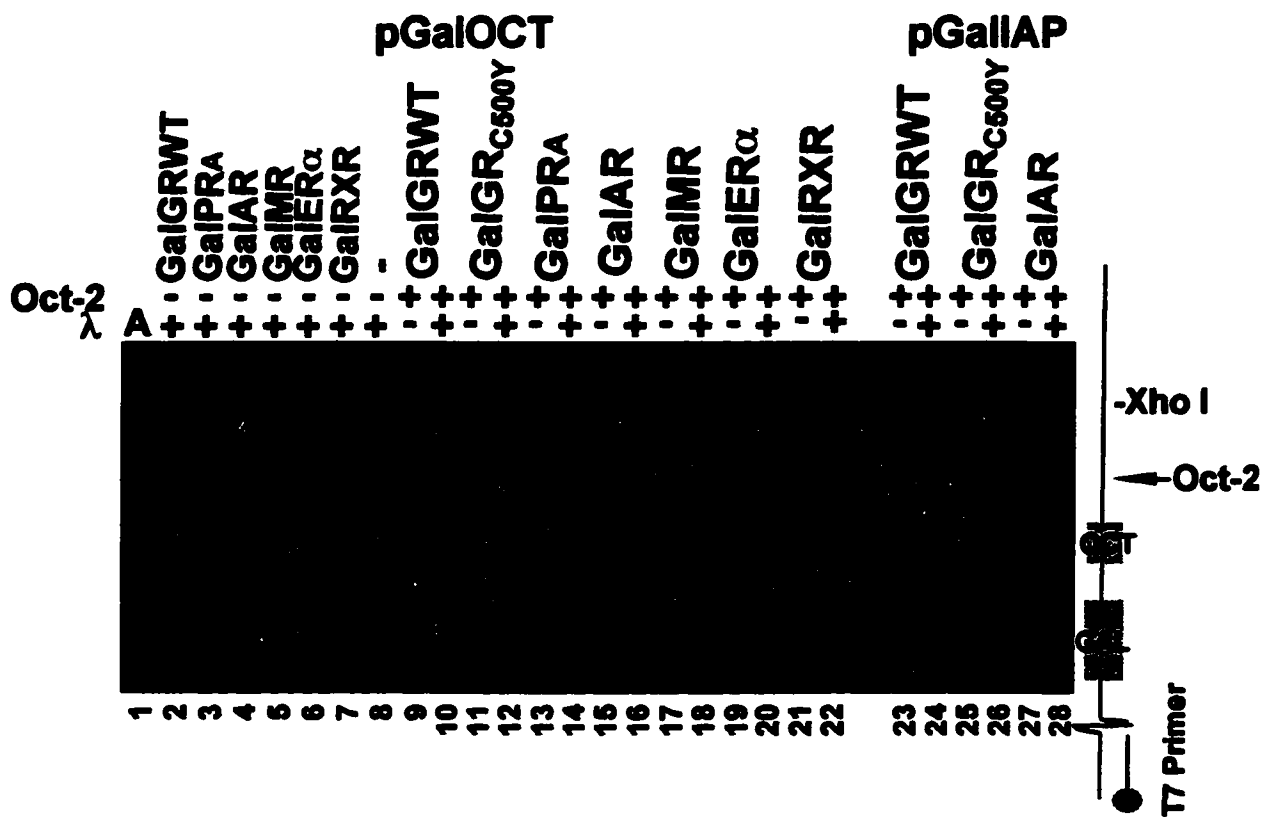
analyzed by linear PCR and denaturing gel electrophoresis. As before, co-expression of Gal-GR_{WT} and Oct-2 induced an Oct-2 dependent footprint (Figure 28, lane 10). As expected the C500Y GR mutation, that results in a form of GR that does not bind to the Oct-1 POU domain (45), did not induce an Oct-2 dependent pause site (lane 12). Like Gal-GR, the pause site was also promoted by presence of Gal-PR and Gal-AR (lanes 14, 16). However, neither Gal-MR, Gal-ERα nor Gal-RXR were able to promote an Oct-2 dependent footprint (lanes 18, 20, 22), consistent with previous observations that showed each lacked functional interaction with Oct-2 in the one-hybrid assay. The promotion of Oct-2 binding by Gal-GR and Gal-AR was dependent upon the presence of an octamer motif (lanes 23-28). These results showed that coexpression of Gal-PR or Gal-AR like Gal-GR with Oct-2 produced an Oct-2 dependent λ exo footprint, consistent with their ability to interact functionally with Oct-2 *in vivo.*

Next, I examined the ability of full-length receptors to recruit full-length Oct-2 to the MMTV promoter. Following transient transfection of pMMTVCAT with expression vectors for rat GR, rabbit PR, rat AR and rat MR with Oct-2, cells were treated for 15 minutes with 1 μM of the appropriate hormone (dex, R5020, DHT, ald, respectively) prior to harvest, as indicated (Figure 29). The nuclei were isolated and the samples treated exactly as described in Figure 21.

Under these experimental conditions, a pause site at position –49 from the MMTV transcriptional start site, reflected the binding of Oct-2. In the absence of hormone, no pause site was observed near position –49, however, a constitutive pause site near –75, reflecting the binding of NF-I, was observed at similar levels for all steroid receptors

Figure 28. GR, PR and AR DBDs fused to the Gal4 DBD induce an Oct-2 dependent footprint on transiently introduced plasmid.

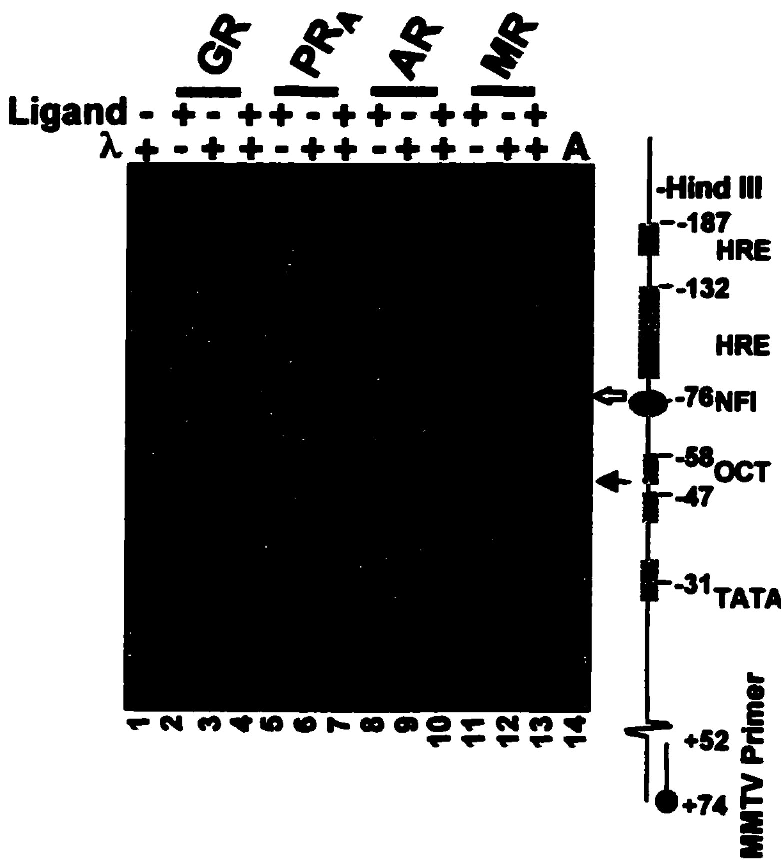
Nuclei from CHO-K1 cells cotransfected with pGalOct (lanes 2-22) or pGalIAP (lanes 23-28) in addition to plasmids expressing Gal4-nuclear receptor DBD fusion proteins and Oct-2 were digested with Xho I in the presence (+) or absence (-) of λ exonuclease. The DNA was purified and amplified using linear PCR with a ^{32}P end-labeled universal T7 primer. The DNA samples were resolved using a 6% sequencing polyacrylamide gel and visualized by autoradiography. The Oct-2 and Gal-nuclear receptor induced pause in exonuclease digestion is indicated by the arrow. Using an A sequencing track (lane 1) with the same primer, a schematic representation of the position of the core Gal4 DNA binding site and the octamer motif were illustrated on the right.



pGalOCT
pGalIAP
GalGRWT
GalPRA
GalAR
GalMR
GalERα
GalRXR
GalGRC500Y
Oct-2
λ
A
-Xho I
Oct-2
T7 Primer
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28

Figure 29. Binding of Oct-2 to the MMTV promoter in transiently transfected cells is induced by GR, AR and PR but not MR.

CHO-K1 cells cotransfected with MMTV reporter plasmid pMMTVCAT (-237 to +125) and expression plasmids for full length GR, PR, AR, MR and Oct-2 were treated with dex, R5020, DHT, or ald, as indicated, for 15 minutes followed by isolation of the cell nuclei. The nuclei were digested with Hind III in the presence (+) or absence (-) of λ exonuclease at 30°C for 15 minutes. The samples were amplified using linear PCR with a complementary ^{32}P-end-labeled MMTV (+74 to+52) primer, resolved on a sequencing polyacrylamide gel and visualized by autoradiography. A constitutive pause site for exonuclease digestion flanking the NF-I binding site was indicated to the right with an open arrow and the hormone inducible pause site flanking the octamer motif was indicated by the solid arrow. Using the A sequencing track (lane14) with the same primer, the positions of the core transcription factor binding sites were determined and schematized on the right.



GR
PRA
AR
MR
Ligand - + - + + + - + + + - + + + - +
λ + - + + - + + - + + - + + A
-Hind III
-187
HRE
-132
HRE
-76 NFI
-58 OCT
-47
-31 TATA
+52
+74
MMTV Primer
1 2 3 4 5 6 7 8 9 10 11 12 13 14

tested independent of ligand (lane 3, 6, 9, 12). Following treatment with hormone, the cells expressing GR, PR and AR revealed an additional pause site at –49, indicative of Oct-2 binding to DNA induced by hormone induction by GR, PR and AR (lane 4, 7, 10). By contrast, the treatment of hormone on cells expressing MR did not produce an Oct-2 dependent footprint to the promoter DNA (lane 13). These results showed the inability of MR to bind to Oct-2 correlated with the lack of MR to induce a detectable Oct-2 footprint. The major implication of these results is that steroid activation of the MR should be independent of the octamer motifs on the MMTV LTR.

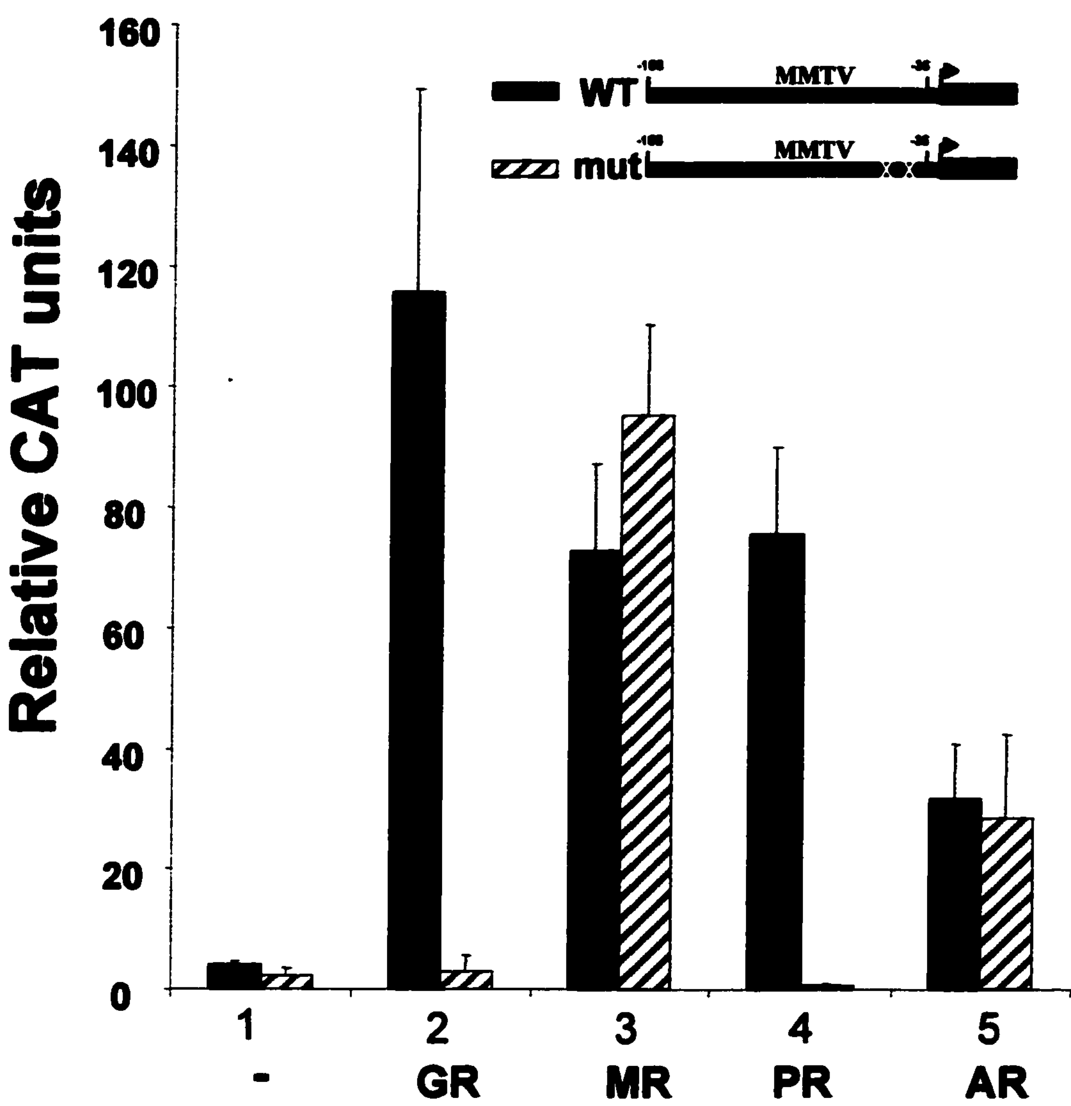
9. MR mediated transcription is octamer factor independent.

The role of the octamer motifs in GR-, PR-, AR- and MR- mediated hormone induction of the MMTV promoter proximal regulatory region was examined by comparing reporter gene transcription by MMTV in the presence or absence of octamer motifs. To do this CHO-K1 cells were transfected with plasmids expressing GR, PR, AR or MR and a plasmid containing a MMTV reporter gene, pMMTVCAT, or one lacking functional octamer motifs, pMMTVOct$_{mut}$CAT. Twenty fours following transfection the cells were treated with the appropriate hormone as indicated for 4 hours prior to harvest. Cellular extracts were prepared and assessed for CAT activity (Figure 30).

In the absence of co-transfected steroid receptor expression plasmids, mutation of the octamer motifs decreased hormone-independent MMTV transcription by less than 2 fold compared to the wild-type reporter (lane 1, compare the solid bar with the striped bar). The low level of transcription in the absence of coexpressed receptor and hormone represents the basal level of transcription by the endogenous octamer factor and NF-I (17). In the presence of the octamer motifs, GR and PR increased transcription at least 30

Figure 30. Hormone induced MMTV transcription mediated by GR and PR is dependent on octamer motifs while MR and AR mediated transcription is octamer motif independent.

The MMTV WT reporter (solid bars) or the reporter with mutant octamer motifs (hatched bars) (schematized above the graphic) were co-transfected with plasmids expressing GR (lane 2), MR (lane3), PR (lane 4), AR (lane 5) or no expression plasmid (lane 1) into CHO-K1 cells. The cells, 16-24 hours later were induced with the appropriate hormone (dex, ald, R5020, DHT, no hormone, respectively) as indicated for 24 hours before harvest. The transcriptional effects were recorded by CAT assay and expressed as relative CAT units corrected for transfection efficiency. Transfection efficiency was determined by liquid β-gal assay by cotransfection with β-gal expression plasmid. The values in the graph represented the average and the standard error of the mean (S.E.M.) of three independent experiments performed in duplicate.



Relative CAT units
160
140
120
100
80
60
40
20
0
WT
MMTV
mut
MMTV
1
-
2
GR
3
MR
4
PR
5
AR

fold (lanes 2 and 4, black bar). Strikingly, mutation of the MMTV octamer motifs almost completely eliminated the transcriptional response by GR and PR (lanes 2 and 4, hatched bar). This is consistent with the proposal that GR and PR mediated transcription was almost entirely dependent upon adjacent octamer motifs.

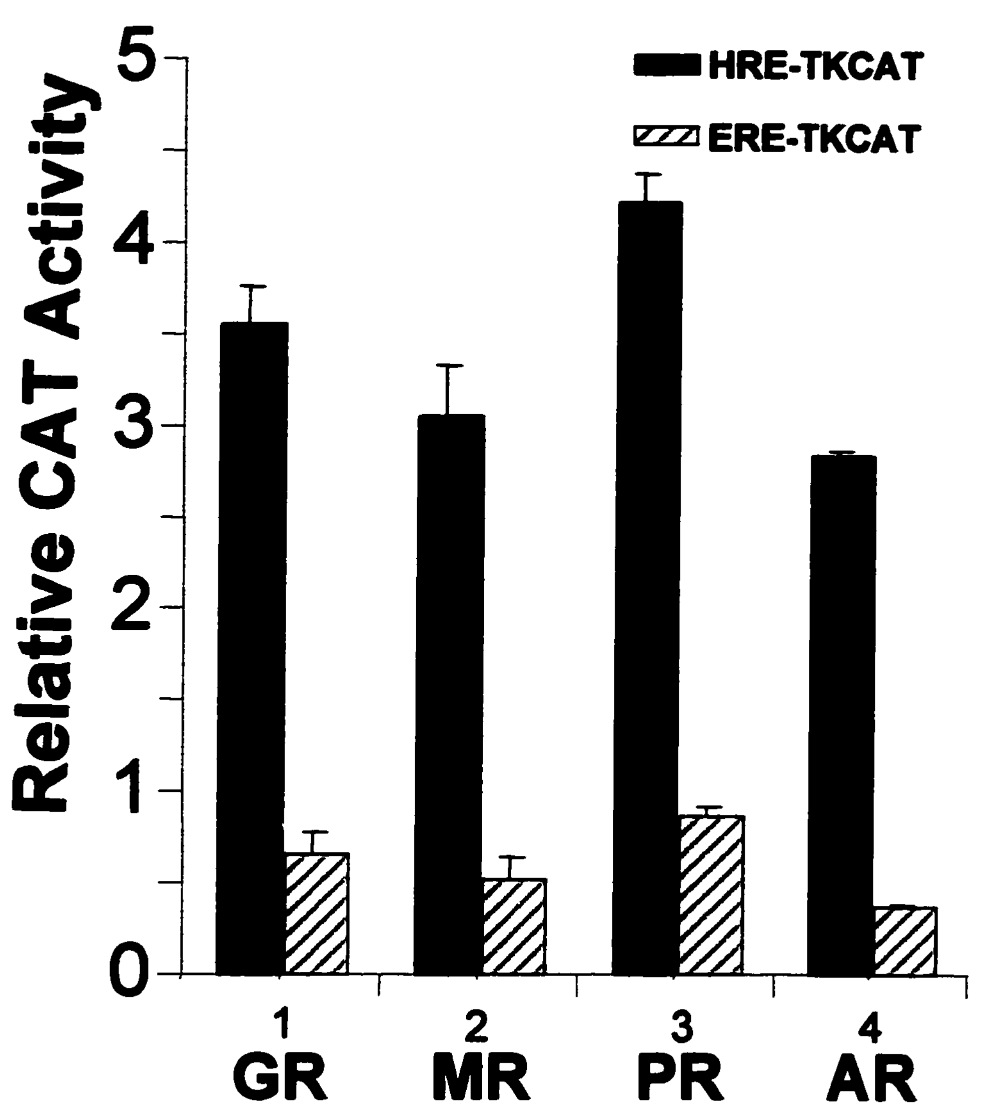
MR mediated transcription was almost entirely independent of the presence of octamer motifs in the MMTV promoter proximal region (lane 3). However, despite being unable to recruit octamer factors to the promoter, MR induced transcription of the reporter was almost as strong as the level obtained with GR. Unexpectedly, transcriptional responsiveness by AR, which bound to and recruited Oct-1/-2 to the promoter was also completely independent of adjacent octamer motifs (lane 5).

The differences in steroid transcriptional responsiveness on the MMTV promoter could have been due to inherent properties of the steroid receptor and the unique property of the MMTV HREs. To show the difference in transcriptional control was specific to the MMTV HREs, transcriptional activation by PR, GR, AR and MR was assessed using a gene reporter system driven by two copies of a HRE directly upstream of the HSV-TK minimal promoter, pHRE-TKCAT. To do this, plasmids expressing PR, GR, AR and MR were cotransfected with either pHRE-TKCAT or pERE-TKCAT, a control reporter containing EREs in place of the HREs. 24 hours following transfection the cells were treated with the appropriate hormone for 4 hours prior to harvest. The cell extracts were prepared and tested for CAT activity.

The results showed that each steroid receptor activated transcription approximately to the same level above the control reporter (Figure 31). This indicated that the difference in the induction of transcription by the steroid receptors from the

Figure 31. GR, MR, PR and AR activate transcription similarly from a consensus HRE.

The TK-CAT reporter plasmid with 2 copies of consensus HRE (solid bars) or 2 copies of a consensus ERE (hatched bars) were transiently transfected with expression plasmids for GR, MR, PR or AR into CHO cells. Cells were treated with 1 μM of the appropriate hormone (dex, ald, R5020 or DHT, respectively) 24 hours prior to harvest. The transcription was measured using a standard CAT assay and expressed as relative CAT units corrected for transfection efficiency. Transfection efficiency was determined by liquid β-gal assay by cotransfection with β-gal expression plasmid. The values in the graph represented the average and the standard error of the mean (S.E.M.) of three independent experiments performed in duplicate.



HRE-TKCAT
ERE-TKCAT
Relative CAT Activity
5
4
3
2
1
0
1
GR
2
MR
3
PR
4
AR

MMTV promoter was an inherent property of the HREs and the HREs paired with octamer motifs in the promoter context coupled with a difference in the activation potential of individual steroid receptors.

10. Sequences flanking the GR DBD are important for directing octamer factor dependent transcription.

The three dimensional structural information derived from NMR and X-ray crystallography data places residues C500Y and L501P in the core of the three dimensional structure of the GR (refer to Figure 2) (168, 169). Based on these observations it would seem unlikely that these residues (C500 and L501) would be contacting octamer factors directly. Rather the mutations would be expected to alter the overall conformation of the receptor in a way that abolished binding.

The C500Y and L501P mutated receptor DBDs were originally obtained from a screen for GR DNA binding mutants (445) and effectively when these residues are mutated, the GR is unable to bind to its DNA response element with high sequence-specific activity. These point mutations created a receptor that has at least two functions that have been disrupted: (1) Octamer factor -1/-2 binding and (2) DNA binding. In the recruitment model proposed these two events (Octamer factor binding and DNA binding) most likely happen sequentially for cooperative DNA-binding and transcriptional synergy to occur. Therefore to study further the contribution of the protein-protein interaction to gene transcription, it would be necessary to produce a GR that functions like the wild-type receptor with DNA-binding and transcriptional activity from simple HREs equivalent to the wild-type receptor but with a specific inability to bind to Octs.

Co-IP and mammalian two-hybrid assays used for identifying protein-protein interactions in the cell, suggested that the minimal domain required to make a productive association with octamer factors was aa 407-556. Amino acid alignment identifies a single amino acid substitution at position R498 that is present in GR, PR and AR but not MR (Figure 32) or other NRs that did not interact with the POU domain *in vivo*. This observation and evidence provided by at least one other group suggests that mutation of this residue in the context of the full-length receptor reduced transcriptional activity to about 25% of the full-length WT receptor on the MMTV promoter (484), consistent with the transcriptional potency mediated by hormone-treated GR on the MMTV promoter with mutated octamer motifs in Cos7 cells (Figure 33, lane 1). Testing the R498G and R498Q mutations for octamer motif sensitivity under my conditions revealed that neither of these point mutations reduced transcription significantly from the wild-type activity (lanes 2, 3). In addition, transcription was sensitive to the loss of the octamer motifs suggesting that the R498G and R498Q mutations might still recruit octamer factors to DNA and cause an increase in Oct dependent gene transcription. Transcription of hormone treated MR was compared in this transcription assay with a less than two fold effect on mutating the octamer motifs probably due to the high basal level of transcription mediated by endogenous NF-I activity (17) in Cos7 cells (lane 4).

Lastly, to determine if the octamer dependent transcriptional synergy was directed by the DBD alone of GR, human GR/MR chimeric receptors were obtained from Dr. R. Evans, The Salk Institute, La Jolla, CA. The DNA-binding domains of either GR or MR were substituted in the context of either full-length MR or GR, respectively, to produce mammalian expression plasmids expressing proteins encoding MGM and GMG. These

Figure 32. Alignment of the primary amino acid sequences of GR, PR, AR and MR of sequences from second zinc finger of the DBD to the C-terminus of the hinge region.

Sequences from rat (rat) GR, AR MR and human (hum) PR_A were aligned using the pile-up program (GCG software package). Illustrated are steroid receptor sequences corresponding to aa 476-556 of rat GR. Identical amino acids were substituted with a period. Gaps in the sequence alignment were indicated by an underscore. Above the sequence alignment are numbers representing the amino acid position corresponding to rat GR with the position of α-helix-2 highlighted by the solid bar. The amino acid position within the open rectangle highlighted a candidate residue common in GR, PR and AR but different in the MR primary aa sequence.

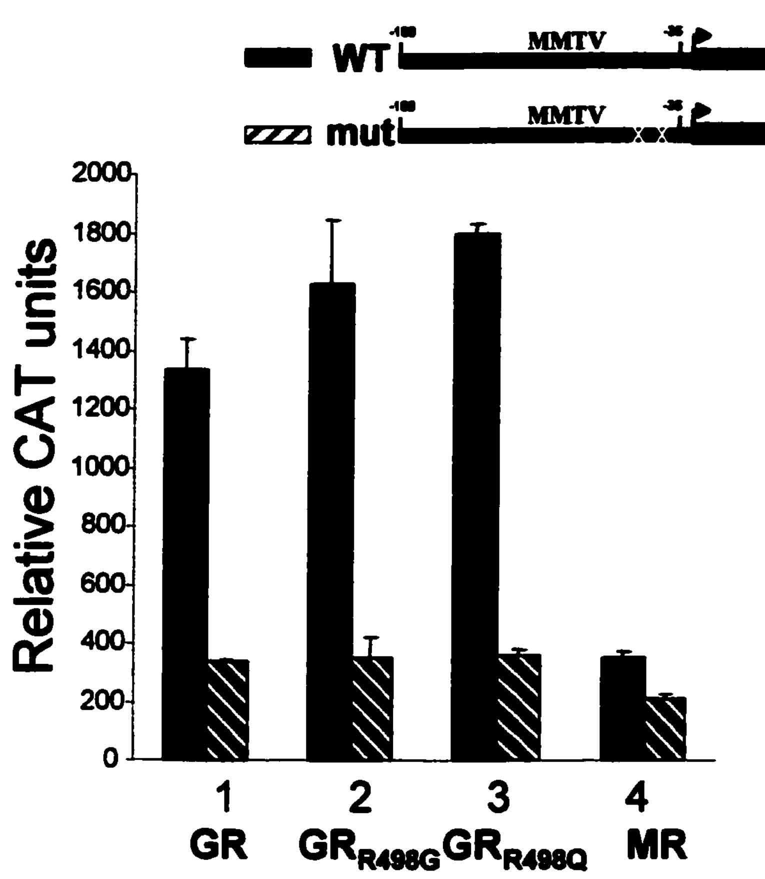
α Helix 2

```
rat_GR  CAGRNDCII DKIRRKNCPA CRYRKCLQAG MNLEARKTK K__KIKGI__
hum_PR  ........V .......... ..L...C... .V.GG..F. .FNKVRVVRA
rat_AR  ..S....T. ..F......S ..L...YE.. .T.G...L. .LGNLK____
rat_MR  ......... .......... ..LQ...... ...G...S. .LGKLKGLHE

rat_GR  ___QQATAGV SQDTSEN___ __PNK__TIV PAALPQ____ ___LT_PTLV SLLEV
hum_PR  L_________ _DAVALPQPV GV.NESQAL_ SQRFTFSPGQ DIQ.I_.P.I NL.MS
rat_AR  L_________ _QEEG..SSA GS.TEDP___ SQKMTVSHIE GYECQ_.IFL NV..A
rat_MR  EQP..PPPPP PQSPEEGTTY IA.T.EPSVN S.LV..LTSI THA.._.SPA MI..N
```

Figure 33. GR_{R498G} and GR_{R498Q} are unable to alleviate the dependence on the octamer motifs for transcription.

Cos7 cells were transfected with plasmids expressing GR, GR_{R498G}, GR_{R498Q} or MR with a wild-type MMTV reporter or the same reporter with mutated octamer motifs. Schematic diagrams of the reporter plasmids are shown above the graphic. Following transfection, the cells were treated with hormone (1 μM dex for GR and 1μM ald for MR), harvested 24 hours later and assayed for CAT activity. CAT activity was displayed as relative CAT units corrected for transfection efficiency. Transfection efficiency was determined by liquid β-gal assay by cotransfection with β-gal expression plasmid. The results represent the mean of at least 2 independent transfections in duplicate with S.E.M. The solid bars represent the wild-type reporter and the hatched bars the same reporter with mutant octamer motifs.

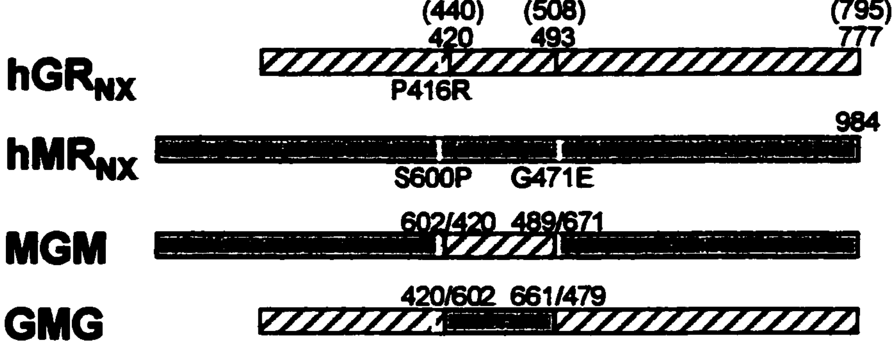


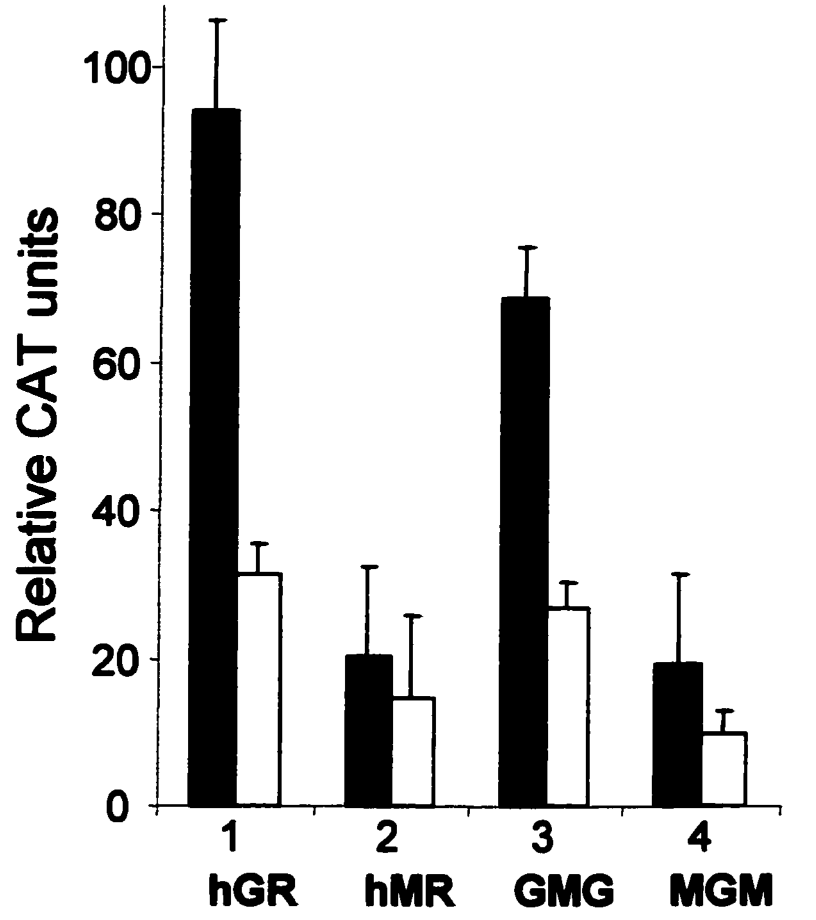
WT
MMTV
mut
MMTV
Relative CAT units
2000
1800
1600
1400
1200
1000
800
600
400
200
0
1
GR
2
GR$_{R498G}$
3
GR$_{R498Q}$
4
MR

plasmids along with their WT human counterparts were co-transfected into Cos7 cells and tested for octamer dependent transcriptional synergy by the MMTV promoter (Figure 34). The results showed that the transcriptional synergy was independent of determinants in the GR DBD because when the DBD was inserted into the corresponding position of MR, MGM, transcriptional synergy was not significantly altered (compare lanes 2 and 4). Similarly when the MR DBD was substituted into the GR, GMG, octamer motif dependent transcriptional synergy was only slightly reduced when compared to GR (lanes 1 and 3). Therefore, this suggests that some determinants for octamer factor binding and transcriptional synergy must be located outside the minimal DNA binding domain (aa 440-508).

Figure 34. Determinants within the GR DBD are not sufficient for directing octamer motif dependent transcription.

(Top) Schematic of the human GR and MR or GR/MR chimeric receptors used in the transcription assay described below. The white vertical bars represent point mutations created (substitutions indicated below the bar diagram) following insertion of the NotI and XhoI sites. The chimeric receptors have the DNA binding domains substituted at the amino acid residue positions as indicated above the bar diagrams. The numbers in brackets correspond to the amino acid position using the rat GR numbering scheme and all other numbers represent the amino acid position according to the human GR and MR numbering scheme. (Bottom) Cos7 cells were transiently transfected with expression plasmid encoding GR, MR, GMG or MGM along with the WT or octamer motif mutant MMTV promoter as previously described. The WT MMTV reporter was represented by the solid bars and the mutated octamer motifs by the hatched bars. 24 hours prior to harvest, the cells were treated with 1 μM dex (for GR and GMG) or 1 μM ald (for MR and MGM). The levels of transcription were expressed as relative CAT units corrected for transfection efficiency. Transfection efficiency was determined by liquid β-gal assay by cotransfection with β-gal expression plasmid. The results represented the mean of at least 3 independent transfections performed in duplicate with the S.E.M.


(440)
420
(508)
493
(795)
777
hGR$_{NX}$
P416R
984
hMR$_{NX}$
S600P
G471E
602/420
489/671
MGM
420/602
661/479
GMG


Relative CAT units
100
80
60
40
20
0
1
hGR
2
hMR
3
GMG
4
MGM

IV. DISCUSSION

I have characterized the ability of GR to bind directly to Oct-1/-2 and investigated the consequences of this protein-protein interaction for steroid mediated transcriptional activation by Oct-1/-2. I have described a DNA-independent protein-protein interaction between GR and Oct-1/-2 that is sensitive to a point mutation at either C500Y or L501P of rat GR, *in vitro* and *in vivo*. Many examples of protein-protein interactions have been described between DBD/hinge region of NRs and the POU domain of POU factors (12, 15, 45, 409). My results indicate that for Oct-2 protein-protein interactions with NRs, *in vivo*, were limited to GR, PR and AR. For the first time it was shown that the binding of the steroid receptor DBD/hinge to Oct-2 dramatically potentiated the ability of the octamer factor to occupy octamer motifs adjacent to HREs *in vivo*. For GR and PR, MMTV-mediated transcription was dependent on the octamer motifs for steroid induced transcription. In contrast, MR did not bind to octamer factors or recruit them to DNA *in vivo*. As a result, MR-mediated transcription of the MMTV promoter was independent of octamer motifs for steroid induced MMTV transcription. This suggests that steroid receptor protein-protein interactions with other sequence-specific transcription factors result in differential steroid gene regulation. The interaction between GR and Oct-1/-2 appeared to be incompatible with binding to a HRE. This led me to propose that transcriptional cooperativity between GR/PR and Oct-1/-2 is restricted to MMTV and other putative promoters containing DNA binding sites for both factors. The GR/Oct-1/-2 interaction mapped to the homeodomain of Oct-1/-2 and GR was shown to interact directly to multiple other types of homeodomains *in vitro* and to at least two homeodomain containing proteins including dlx-2 and hoxD4 *in vivo*.

1. GR interaction with the POU homeodomain of Oct-1 is direct and sensitive to GR point mutations at C500Y and L501P, *in vivo.*

The protein-protein interaction between GR and Oct-1/-2 was demonstrated in this work using several types of protein binding studies *in vitro* and *in vivo*. The *in vitro* methods included GST pull down assays using GST-fused recombinant GR and Oct-1/-2 peptides with *in vitro* translated proteins (Figure 9). The direct binding studies utilized recombinant purified GR and Oct-2 peptides (Figure 15). The protein-protein interaction of full-length GR protein with full-length Oct-1 and Oct-2 proteins was revealed by an IP binding assay (Figure 11). *In vivo*, the interaction was demonstrated with the GR DBD/hinge (407-556) and either full length Oct-2 or the POU domain of Oct-1 in a mammalian one- or two- hybrid assay, respectively (Figures 19 and 18, respectively). In each assay system the specificity of the interaction was demonstrated by comparing the binding to rat GR with point mutations at either C500Y or L501P, which abolish octamer factor binding, or other non-specific proteins like firefly luciferase, GST, or CREB.

Mapping of the protein interaction domain of GR with the POU domain of Oct-1 showed that the minimal region required for interaction *in vitro* was aa 407-523 of rat GR, including the DBD and at least part of the hinge region of the receptor (45). The characterization of the mapping studies of the POU domain of Oct-1 with GR were detailed in my master's thesis (45). GR-Oct-1 binding occurred in a way that was independent of zinc coordination because neither point mutations of the zinc coordinating cysteine residues nor removal of zinc from the DNA binding domain of GR interfered with binding to Oct-1 *in vitro*. The first indication of the importance of residue C500 of rat GR for Oct-1 binding came from the observation that modification of free cysteine

residues in the GR DBD/hinge with MMTS (methyl methane-thiolsulfonate) abolished binding to Oct-1 (45). The GR DBD contains in total 10 cysteine residues, 8 of which are involved in zinc coordination, one located in the first zinc finger while the second is found in helix 2 of the DBD at amino acid 500. Further analysis of other GR DNA binding point mutations revealed that two residues located next to one another C500Y and L501P abolished Oct-1 binding.

Full length GR interacted with Oct-1 and Oct-2 in an L501P sensitive fashion. A co-IP binding assay was used to evaluate the binding of *in vitro* translated Oct-1 and Oct-2 to full length GR in an L501P sensitive fashion in an assay that closely resembled physiological conditions (Figure 11). The results showed the binding of immunoprecipitated full-length myc-tagged GR to full-length Oct-1 and Oct-2 and the binding was abolished using myc GR_{L501P} immunoprecipitates.

A mammalian one- and two-hybrid assay was employed to show that the interaction between GR with Oct-2 and the POU domain of Oct-1 occurred in the cell nucleus (Figures 19 and 18). The results demonstrated the GR DBD (aa 407-556) fused to the GalDBD (Gal-GR) acted as bait to bring the Oct-1 POU (aa 265-444) fused to an acidic activation domain (VP16 Oct-1POU) near the promoter in a manner that increased gene transcription. GR-mediated transcription was increased by 30 fold when coexpressed with VP16 Oct-1POU above the level of the Gal DBD alone or a Gal DBD fusion protein with a GR point mutation at L501P. Again this demonstrated the interaction was completely sensitive to point mutation at GR L501P. Further, the interaction of GalGR in an L501P-sensitive fashion was shown to interact with full-length Oct-2 in a mammalian one-hybrid assay (Figure 19). Together, these assays

represented the first convincing identification of the interaction of GR with the POU domain of Oct-1 and full-length Oct-2 in the cell nucleus. In addition, GR mutations C500Y and L501P are the first point mutations shown to disrupt any NR interactions with POU proteins.

C500Y and L501P were originally identified in a saturation mutation screen to find GR mutations that abolish GR activation in a HRE dependent fashion in yeast (444). Next, the DNA-binding activity of each mutant was characterized in EMSAs (444). The results presented here and elsewhere (45, 471) suggested a Oct-1/-2-binding surface in the GR DBD plus hinge that is distinct from, but overlaps with, the DNA-binding interface. The overlap appears to be limited, because several point mutations that interfered with GR DNA-binding (C460Y, C492R, C495Y) by disrupting the overall finger structures, and others that disrupted DNA-dependent dimerization (R479K, R489K and N491S), had no effect on Oct-1 POU binding *in vitro* (45, 471).

Although the mutations C500Y and L501P interfered with POU-domain binding, crystallographic analysis of the GR DBD (169) places these aa on the surface of the DNA-phosphate contact helix and oriented inwards, towards the core of the DBD (Figure 2b). Therefore it would be unlikely that either amino acid would contact the POU domain directly. Providing functional support for this statement, substitution of C500 to an alanine or serine residue each of which had no effect on GR-mediated gene transcription on the MMTV promoter (485). Mutations C500Y and L501P appear to function similarly, forcing a change in secondary (α-helix-2) and possibly the tertiary structure of the GR DBD, that alters the configuration of the POU binding surface in a way that results in loss of binding. Typically, proline residues inserted into α-helical

structures act by breaking the structures suggesting the integrity of the helix was an important component for the protein-protein interaction. Cysteine residues are considered non-polar and are relatively hydrophobic; in contrast tyrosine residues are bulky polar groups. Therefore, it would be expected that replacing the cysteine residue at position 500 with a tyrosine would distort helix 2 away from the DBD core. In most experiments the L501P GR mutation was used because it was considered a more targeted mutation structurally, however on occasion C500Y was used interchangeably in some experiments.

Ideally, to study the function of GR with octamer factors, it would be important to identify a GR that abolished interaction with octamer factors but had no effect on GR DNA-binding function or other functions. Attempts have been made to identify point mutations in GR that disrupt binding to the POU domain of Oct-1 while maintaining HRE-dependent gene transcription using a yeast two-hybrid system (Walther, R., Lemieux, M.E. and Haché, R.J.G. personal communication). However, these efforts have been unsuccessful because co-expression of Gal4DBD-GRDBD and GalAD-Oct-1POU (AD=activation domain) appears to be toxic to the yeast strain.

The protein-protein interaction domain of Oct-1 and Oct-2 with the GR DBD mapped to the homeodomain of Oct-1 and Oct-2. The homeodomain of Oct-1 was mapped using a GST pull down assay with GST-GR DBD/hinge (aa 407-568) and *in vitro* translated C-terminally deleted Oct-1 (Figure 12). The results showed strong binding in the presence of full-length Oct-1 (aa 1-743) and binding to C-terminally deleted Oct-1 up to the C-terminus of the homeodomain. Truncation into the homeodomain abolished detectable binding altogether. IP binding assays with full-length

Oct-2 bearing internal deletions demonstrated that the homeodomain of Oct-2 was required for the interaction (Figure 13). Further, direct binding studies demonstrated that recombinant purified GR DBD (407-550) peptide bound equally to recombinant purified Oct-2 POU domain and the Oct-2 homeodomain alone (Figure 15).

My results suggested that both Oct-1 and Oct-2 interact with GR in identical ways. Indeed, in all instances where both Oct-1 and Oct-2 were tested they were found to be indistinguishable. In the latter half of this work, I employed Oct-2 for two reasons. First, Oct-2 protein expression is limited to B cells and cells of the central nervous system (333, 362) and therefore in assays performed in ovarian cells (CHO-K1) exogenous Oct-2 could be differentiated from endogenous octamer protein. Second, it has been established that Oct-2 is generally a stronger activator because its activity can be influenced over longer distances whereas Oct-1 activity is functional only when octamer motifs are near the promoter (382, 386, 387).

2. The GR/Oct-1 is distinct from other protein-protein interactions described with GR

Many protein-protein interactions with the GR have been described to date including GR interaction with sequence specific transcription factors like Stat5, AP-I, NF-κB and Nur77. In most cases, these interactions were also mediated by the DBD of the heterologous partner as well. As a result, GR complexed with the heterologous factors modulate gene transcription of both GR and its partner. For example, GR binding with AP-1 (c-jun homodimers) represses gene transcription of each factor from simple response elements but activated gene transcription from paired elements (300). Similarly, GR binding to NF-κB, and Nur77 repressed transcription mediated by both

factors individually. In addition, *in vitro* the protein-protein interaction results in reduced levels of DNA-binding of each factor (282, 300). Stat-5 activated gene transcription with GR from paired elements, while the protein-protein interaction between GR and Stat-5 blocked GR DNA-binding (302).

The consequence of the protein-protein interaction between GR and Oct-1/-2 appears to be distinct from other protein-protein interactions described for GR in that association with Oct-1/-2 did not appear to influence GR DNA-binding activity. Following the observations that preformed GR/Oct-1 complexes were specifically disrupted by a HRE and that GR could recruit Octs to HRE-containing promoters suggested that in the protein-protein configuration GR can still recognize a HRE. Furthermore it appears that GR/Oct-1 binding was exclusive to interaction with a HRE. To date, no functional observations of POU factors influencing GR mediated transcription or DNA binding have been reported. Indeed most results suggested that Oct-1 has no effect on GR DNA-binding or other functional consequences for GR-mediated transcription from a HRE in the absence of a juxtaposed octamer motif (16, 21).

The mutations C500Y and L501P have not been reported to disrupt the binding of GR to other factors. However, no direct comparison has been made. A more detailed analysis of GR-Oct-1/-2 binding requirements will need to clarify the distinction between this interaction and other protein-binding events mediated by the GR DBD. None of the mutations that have been shown to disrupt GR interaction with Stat5, AP-1 or NFκB have been tested directly for interaction with Oct-1/-2. It is likely that the interaction of GR with Oct-1 is distinct from the interaction described with AP-1 because GR with a double point mutation Y498L/R479G abolished GR mediated AP-1 transrepression while having

no effect on MMTV-mediated transcription (484). Furthermore, GR DBD truncated to amino acid 509 could inhibit AP-1 DNA-binding activity (285) whereas the same GR DBD peptide could not bind to the POU domain of Oct-1 (471).

Most GR interactions with non-sequence-specific transcription factors, including chromatin remodelling factors, co-activators and hsps are mediated by protein-protein interactions outside the DBD/hinge. For example, chromatin remodelling factors, like the SWI/SNF complex, can interact with the N-terminal TAF1 region of the receptor (486). The co-activators with HAT activity like SRC-1 (93), GRIP-1 (96), and CBP (94, 107) mediate interaction with the C-terminal TAF2 region of GR, while hsp 90 proteins mediate interaction with GR through its C-terminual LBD (179).

3. The functional consequence of the GR/Oct-1/-2 interaction is distinct from other protein-protein interactions described for Oct-1/-2

Protein-protein interactions with the POU domain of octamer factors can stabilize the binding of Oct-1/-2 to DNA through a mechanism that involves the direct participation of an auxiliary factor. In one example, the interaction of Oct-1 with VP16 and HCF stabilizes DNA binding to a TAATGARAT motif (R=purine) and involves the direct contact of VP16 with DNA. Interaction of Oct-1 or Oct-2 with OCA-B stabilizes binding to octamer motifs with an adenine residue at position 5 (ATGCAAAT) and involves the direct contact of OCA-B to DNA (379, 381). Therefore it seems that while the presence of the POU specific domain increases the specificity and affinity of DNA sequence recognition by POU homeodomain proteins, auxiliary factors through additional protein-protein interactions may be required to direct and increase binding of these factors to octamer motifs in the cell.

A substantial structural transition occurs upon Oct-1 binding to DNA (487). Protease digestion profiles have revealed significant differences before and after DNA binding. Calorimetric assays, which studied the binding of Oct-1 to DNA, showed a negative potential value for DNA binding suggesting energy was required for DNA binding. Therefore it is conceivable that the protein-protein interactions may act to produce a structural transition in the octamer factor that facilitates DNA binding.

Many non-receptor protein-protein interactions with Oct-1 and/or Oct-2 have been identified that are mediated with the POU domain. These include interactions with HMG-2, OCA-B, $SNAP_C$, and VP16. In some cases interactions were specific to Oct-1 (i.e. VP16) (360) while in others interactions occurred with both Oct-1 and Oct-2 (i.e. OCA-B and HMG-2) (375, 405). Some protein-protein interactions were DNA-independent like that of HMG-2, while others were dependent on DNA for interaction like that observed with OCA-B.

The interaction of octamer factors with HMG-2 is most similar to that described for GR-Oct-1. HMG-2 is a non-histone chromatin associated protein with non-specific DNA binding activity (488). HMG-2 interacts with the homeodomain of Oct-2 and the interaction could also be extended to Oct-1, Oct-4 and Oct-6 proteins in a DNA independent manner *in vitro* and *in vivo* (405, 489). HMG-2 acted by increasing the sequence-specific DNA binding activity of Oct-2 (405). In contrast to the interaction with GR-Oct-1/-2, which appeared to facilitate the DNA binding of octamer factors through cooperative DNA binding, the HMG-2-Oct-2 interaction appears to increase the DNA binding activity of Oct-2 without the direct binding of HMG-2 to DNA.

The interaction of SNAPc with the POU of Oct-1 results in cooperative DNA binding (390, 392). In contrast to GR-Oct-1 binding, which was mediated by the homeodomain of Oct-1, the interaction with SNAPc was mediated by the POU specific-domain. Point mutation E7S in the POUsp domain abolished interaction with SNAPc. It has been proposed that the protein-protein interaction acted by relieving the inhibitory effects imposed by the C-terminus of SNAPc on its DNA binding activity. Together both factors bound cooperatively to DNA, which in part contributed to increased gene transcription.

The interaction of Oct-1 with VP16 is distinct from that observed with GR. GR interacted with both Oct-1 and Oct-2, in contrast VP16 interaction was limited to Oct-1. Glutamine at position 22 of the homeodomain of Oct-1 dictated the specificity of binding to VP16, whereas for Oct-2 this amino acid is an alanine and precluded interaction with VP16 (360). These same mutations, E22A in Oct-1 POU and A22E in Oct-2 POU, were tested for binding to GR and were shown not to influence GR-Oct-1 binding (45). Furthermore, VP16 interaction with Oct-1 changes the DNA binding specificity of Oct-1 to produce a complex with HCF that recognizes a TAATGARAT (R= purine) motif to positively activate transcription (128).

The interaction of OCA-B with Oct-1 and/or Oct-2 is distinct from the interaction with GR. The OCA-B interaction is specific for Oct-1 and Oct-2 because it forms a ternary complex with DNA and either Oct-1 or Oct-2 but not other POU factors like Oct-4, Oct-6 or Pit-1 (381). Biochemical (381) and x-ray crystallography analysis (379) showed that OCA-B contacts both the POUsp and the homeodomain of octamer factors in addition to the octamer motif DNA, directly.

4. Oct-1/-2 recruitment to DNA.

In vivo, octamer motif occupancy correlates with co-occupancy of GR/PR and NF-I-binding to the MMTV promoter on chromatin (43). *In vitro*, footprinting studies with purified factors demonstrated that GR reduced the amount of Oct-1 required to saturate the MMTV octamer motifs (17). My results indicate GR has a similar ability to promote the binding of Oct-2 to the MMTV octamer motifs on plasmid DNA *in vivo* (Figure 21). They also indicate that the recruitment of Oct-2 to the MMTV LTR was dependent upon the L501P-sensitive protein-protein interaction between GR and Oct-1.

Although the MMTV promoter proximal region of the LTR contains four HREs, one NF-I binding site and two octamer motifs upstream of a core promoter element, exo fooprinting revealed that the minimal DNA requirements for Oct-2 recruitment to DNA was a single HRE adjacent to an octamer motif (Figure 22). Co-expression of liganded GR with Oct-2 resulted in a λ exo pause site flanking a consensus octamer motif on plasmid DNA also containing a consensus HRE but lacking other mammalian promoter elements. Therefore, additional sequence-specific or general transcription factors were not required for GR-facilitated DNA binding of Oct-2. In addition this also suggests that the recruitment of Oct-2 was independent of on-going transcription. Furthermore, these results suggested the precise composition of the HRE or the octamer motif was not essential for GR dependent recruitment of Oct-2.

When tethered to DNA through the Gal4DBD, the GR DBD offered the same potentiation of Oct-2 binding to plasmid DNA *in vivo* (Figure 23). Binding to the octamer motif was sensitive to GR_{L501P}, suggesting a protein-protein interaction between

GR and Oct-1/-2 was required for GR-mediated recruitment of Oct-2 to plasmid DNA *in vivo*.

The implication of these results is that the steroid-dependent occupancy of the octamer motifs *in vivo* on chromatin is going to depend at least in part on the association of GR with Oct-1/-2. The mechanism of MMTV occupancy of Oct-1 is in striking contrast to the apparent mechanism utilized by GR for recruitment of NF-I to MMTV which seems to be almost entirely dependent on chromatin and chromatin remodelling (36, 39, 43). Further, no direct interaction between NF-I and GR has been recorded.

Oct-1/-2 recruitment to DNA by hormone-treated GR is independent of spacing and orientation of the DNA-binding sites for each factor. The promoter proximal region of the MMTV LTR contains HREs and octamer motifs separated by 21 nucleotides encompassing the NF-I binding site. Together, synthetic consensus HRE and consensus octamer motif were inserted into a plasmid spaced by 29 nucleotides and oriented with the 3' end of the octamer motif nearest the HRE, the opposite orientation to what is observed on the MMTV promoter. The Gal4DBD was separated by 7 nucleotides from the consensus octamer motif. In all the examples above, octamer factor recruitment by GR demonstrated the versatility of GRs ability to promote octamer motif occupancy on plasmid DNA *in vivo*. The implication for this versatility is that a similar mechanism may occur on other naturally occurring promoters containing juxtaposed binding sites for both factors. It would be interesting to test the limits of the ability of GR to recruit octamer factors to DNA and determine the maximum separation between the binding sites that supports recruitment.

The Oct-2 and GR-dependent footprint was located at –49 on the MMTV promoter, mapping exactly between the two octamer motifs. The location of the exo pause site was consistent with previous reports demonstrating a hormone-dependent pause site on the MMTV promoter (38). In these reports, the pause site was ascribed to TFIID, a component of the general transcription machinery. The footprinting experiments presented here suggest the pause site most likely reflects the binding of Oct-2 to promoter DNA, exemplified by the strict dependence on Oct-2 in the footprinting experiments using promoter-less DNA templates (Figure 22, 23).

The results presented here agree with most if not all *in vitro* and *in vivo* data on GR and Oct-1 DNA-binding function. In one previous report, exo footprinting on the MMTV promoter for detection of transcription factor DNA-binding following steroid treatment did not reveal a pause at -49 (490). However, in these footprinting experiments the processive enzyme used was exo III, not λ exo. In a more recent report, Oct-1/-2 binding was demonstrated with a pause site at nucleotide position –49 that could only be detected by the more sensitive λ exo, not exo III (470).

λ exo is more sensitive than exo III in the detection of protein/DNA boundaries because the nuclease-treated DNA template can be used directly for linear PCR (470). λ exo digests the single stranded DNA in a 5' to 3' direction so that template DNA can be amplified directly with an end labeled DNA primer. In contrast, exo III digests the DNA in a 3' to 5' manner and therefore requires an additional step prior to linear PCR. An S1 nuclease digestion reaction is required to remove the extended single-stranded DNA on the opposite strand. Then the strand opposite to that digested with exo III is amplified in the linear PCR step. Further, it has been shown that λ exo digests the DNA in a sequence

independent fashion while the digestion pattern by exo III may be influenced by DNA sequence (483).

The major disadvantages of the exo footprinting assay are that they cannot detect the binding of more than one protein to the same template DNA. Once the exo progression ceases, other proteins bound 3' to the pause site would not be revealed. Further, some DNA-binding proteins bind to DNA in a way that does not present a barrier for exo digestion. Specifically GR and yeast Gal4 bind to DNA in a way which is not detected by exo digestion and therefore DNA-binding cannot be revealed using the exo footprinting assay (Figures 21 and 23).

To show cooperative DNA-binding of proteins to DNA *in vivo*, sequential chromatin IP assays could be used. In this assay, proteins would be reversibly cross-linked to DNA and the DNA template immunoprecipitated sequentially with antibodies to specific DNA-binding proteins. Subsequently, PCR would reveal only DNA template doubly immunoprecipitated.

5. Facilitated octamer factor DNA-binding by GR potentiates transcription from an octamer motif.

Octamer-motif-dependent transcription by Oct-2 and VP16OCT-1POU was potentiated by a GR peptide and was dependent on the protein-protein interaction between GR and Oct-1/-2 (Figure 20). The GR peptide fused to the Gal DBD was able to activate reporter gene transcription only when coexpressed with either the POU domain of Oct-1 fused to the VP-16 activation domain or the full-length Oct-2. The reporter gene consisted of five Gal4 DNA binding sites juxtaposed to four octamer motifs. The level of gene activation was increased almost two orders of magnitude and directly correlated

with an increase in activation potential by octamer factors because the majority of the activation was dependent on the octamer motifs and co-expression of VP16-Oct-1 POU or Oct-2. Furthermore, most of the transcriptional activation was attributed to the GR/Oct-1/-2 protein-protein interaction because the GR-mediated activation of gene transcription was completely sensitive to GR L501P. In the absence of octamer motifs and Oct-1 POU or Oct-2, Gal-GR was transcriptionally inert.

These results suggested that octamer factor-mediated transcription alone from octamer motifs was poor. Most likely, the transcription was poor because *in vivo* octamer factors bound to promoter DNA poorly in the absence of recruitment by GR. From the $G_5$4XOCTWT reporter gene, in the absence of co-expressed GAL-GR peptide, Oct-2 and VP16Oct-1POU-mediated transcription increased from 2 units to 4 and 37 units, respectively. The variation in gene transcription from Oct-2 and VP16Oct-1-POU are most likely attributable to the differences in activation domains. The activation domains of Oct-2 are relatively uncharacterized while the VP16 activation domain has been shown to be able to interact with multiple subunits of the general transcription machinery (69-71). Following coexpression of GAL GR, Oct-2 mediated transcription was increased at least 35 fold while the transcription by VP16Oct-POU was increased approximately 10 fold. Therefore it appears that GR potentiated the transcription by Oct-2 and the VP16 Oct-1 POU fusion protein primarily by recruiting the POU-containing factors to DNA.

6. The consequence of GR/Oct-1 binding for selective gene transcription.

On viral and synthetic promoters containing paired HREs and octamer motifs, gene transcription is activated synergistically in response to glucocorticoids that is dependent on the binding sites for both factors (16, 17). No reports have been observed

in which octamer factors influence the activity of promoters containing HREs in the absence of octamer motifs. By contrast, on the prolactin, GnRH and histone H2B promoters which contain octamer motifs but lack a HRE, glucocorticoids repress gene transcription, presumably by active repression by GR tethering to DNA through octamer factors (21, 26, 491).

One striking feature of the preformed GR/Oct-1 complex is that it was disrupted by a HRE in a GST pull-down experiment (Figure 24). Therefore, the binding of GR to Oct-1 appeared to be exclusive of GR binding to a HRE. The capacity of GR for sequence-specific DNA recognition did not appear to be affected when complexed to Oct-1 because these complexes were competed by a HRE and not other DNA, like highly sheared calf thymus DNA or DNA containing binding sites for other transcription factors. Preformed GR fusion protein and Oct-2 complexes bound cooperatively to oligonucleotides harboring paired DNA response elements in EMSAs (Figure 25). In these assays, simultaneous challenge with competitor oligonucleotides containing a single HRE resulted in loss of cooperative DNA binding.

The implications of these results are that the GR/Oct-1/-2 complex can cooperatively bind to DNA and activate gene transcription synergistically from promoters containing paired response elements while having limited or no effect on promoters containing HREs but lacking an octamer motif. The observation that in the absence of hormone no octamer factor was observed bound to DNA suggested that in the absence of GR, octamer factors were unable to bind to octamer motifs with high affinity *in vivo*. The ability of GR, in an L501P-sensitive fashion, to promote octamer factor binding to DNA through protein-protein interactions was supported by *in vivo* footprinting

experiments on transiently transfected plasmids (Figure 23). Furthermore, EMSAs showed that both factors bound cooperatively to DNA in a way that depended on the protein-protein interaction (Figure 25).

The protein-protein interaction between GR and Oct-1 induces cooperative DNA binding of Oct-1 on the same DNA molecule *in vitro*. The cooperative DNA binding of a Gal-GR and Oct-2 *in vitro* using an EMSA showed that GR can induce complex formation with slower mobility containing GR and Oct-2. The complex was dependent on the protein-protein interaction because no slower shifted complex was observed following incubation of GR_{L501P} with Oct-2. Both factors were determined to be part of the slower migrating complex because an excess of unlabeled HRE DNA or octamer motif DNA disrupted the complex, but not other competitor DNAs.

Cooperative DNA-binding of GR and Oct-1/-2 was abolished by the presence of a HRE or Oct binding site (Figure 25). The use of oligonucleotides to disrupt cooperative DNA-binding of transcription factors has not been reported elsewhere. I propose that this would provide a tighter level of control of gene expression through HREs by reducing the potential of octamer factors to influence GR responsive genes lacking octamer motifs. Further, this suggests that it is unlikely that Oct-1 and Oct-2 can act as co-activators for GR through consensus HREs, despite their ability to interact in solution.

Although GR appears to function similarly with octamer factors through a consensus octamer motif (5'-ATGTAAAT) and the MMTV distal octamer motif (5'-ATGCAAAT), it would be interesting to determine the precise base composition of the octamer motif required for cooperative DNA-binding, especially following previous reports showing that the degenerate octamer motif, 5'-<u>CG</u>GCAAAT, disrupted octamer

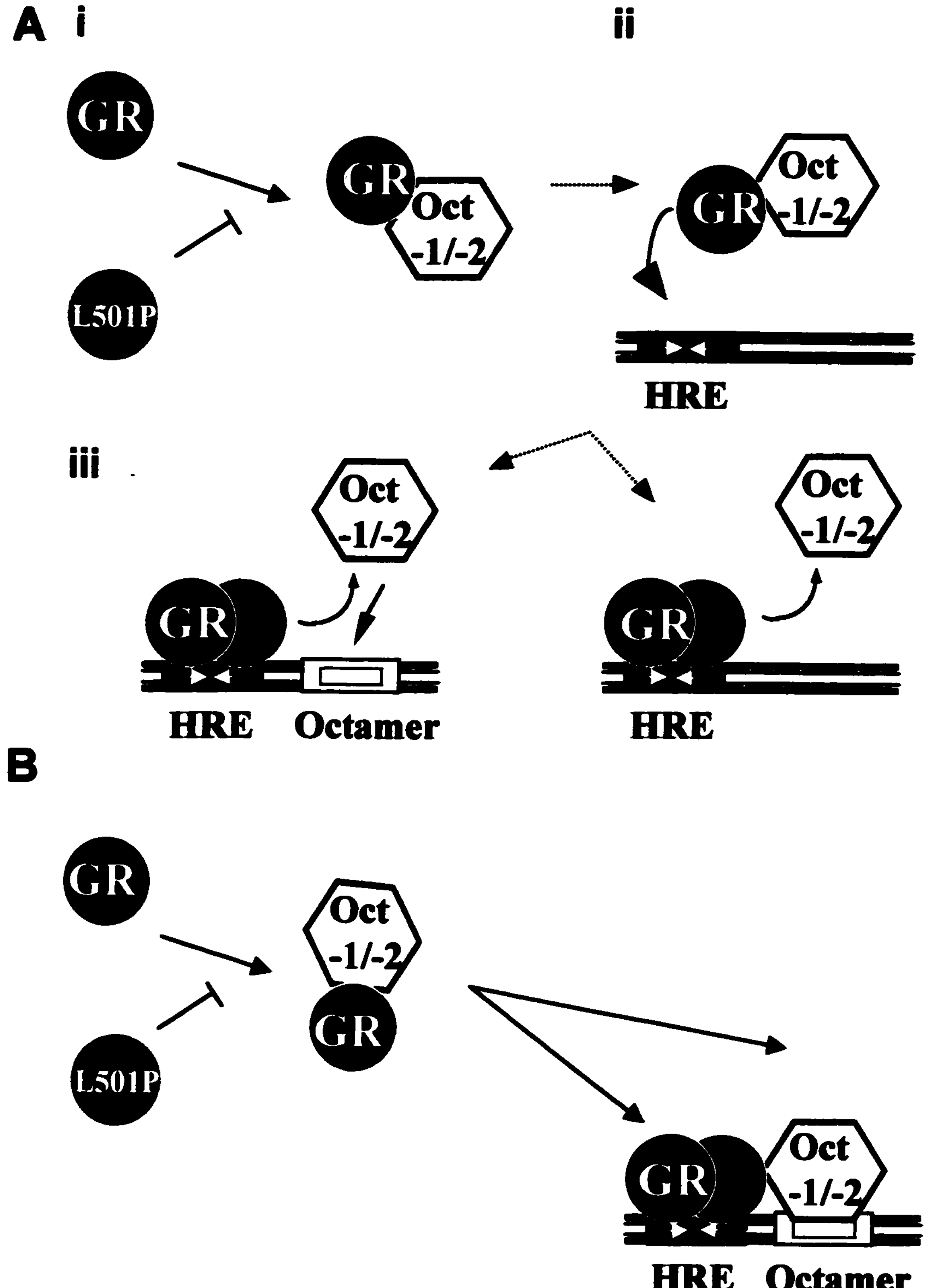
factor DNA-binding activity in the context of a single response element (492). However, when the same response element was paired with a binding site for the Gal-GR fusion protein, cooperative DNA binding of both factors ensued in an L501P-sensitive manner (Figure 25). This suggests that GR was able to recruit octamer factors even to extremely degenerate octamer motifs *in vitro*. However, the level of cooperative binding was lower than that observed with a paired consensus octamer motif. As the POUsp domain recognizes the 5' half of the octamer motif, these results suggest that the DNA-binding function of POUsp domain may play a subordinate role in the cooperative DNA-binding between GR and Oct-1/-2.

Two potential mechanisms are proposed to explain ways in which the GR/Oct-1/-2 complex in solution could result in cooperative binding of Oct-1/-2 to DNA and are illustrated in Figure 35a. In the first model, association of GR and Oct-1/-2 through their respective DBDs occurs in solution (step i) in a manner that allows GR to specifically recognize a HRE initiating DNA binding (step ii). The binding of GR to a HRE is exclusive to interactions with Oct-1/-2. Upon GR DNA binding the octamer factor is released (step iii). If the octamer factor is liberated in transcriptional regulatory regions with octamer motifs in the proximity, Oct-1 and Oct-2 DNA binding is facilitated by an increase in their local concentrations. In the absence of an octamer motif the octamer factor is not retained by DNA binding and is simply released. The advantage of this mechanism is that it is not dependent of the precise juxtaposition or the orientation of the DNA binding sites relative to one another.

In the second model, it is proposed that GR and Oct-1/-2 bind simultaneously to DNA (Figure 35b). The GR-Oct-1/-2 complex would somehow alter the overall

Figure 35. Models for cooperative DNA binding by GR/Oct-1/2 complexes.

a. Upon treatment with steroid, liganded GR localizes to the cell nucleus where it can form a protein-protein complex with Oct-1 or Oct-2 (Oct-1/-2) through the respective DNA binding domains of each transcription factor. (step i). Mutations L501P or C500Y in the GR disrupt the protein binding. The GR/Oct complex can still recognize with specificity the GRE (step ii). As, a result the protein-protein complex is destabilized and GR releases Oct –1/-2 (step iii). In the presence of an adjacent octamer motif, binding of Oct-1/-2 to the octamer motif is promoted. However, in the absence of a nearby octamer motif Oct-1/-2 is released. b. The second mechanism is similar to that proposed in A., except the GR/Oct-1/-2 complex binds coordinately to regulatory regions containing GREs and Octamer motifs linked in cis. Whether a continued interaction is maintained when both GR and Oct-1/-2 are bound is not known.



A
i
ii
iii
GR
L501P
Oct
-1/-2
HRE
Octamer
B

structures of their respective DBDs in a way that would enhance the DNA-binding function of each factor. This model is favored by the observation that the Gal-GR fusion proteins also promoted Oct-2 DNA binding *in vivo* when an octamer motif is juxtaposed to a GAL4 binding site. The second model does not exclude the possibility of continued interactions following DNA binding. It would be expected that if continued interactions were maintained following cooperative binding that the precise juxtaposition of the paired elements would influence this.

Studying the influence of GR on Oct-1/-2 DNA binding kinetics may provide additional insight into the mechanisms for cooperative DNA binding. For example, if GR contributed to the stability of DNA binding through a continued protein-protein interaction between GR and Oct-1, this would be expected to decrease the rate of dissociation of Oct-1/-2 from octamer motifs. By contrast, if the GR/Oct-1/-2 complex resulted in an altered conformation of Oct-1 in a way that increased its recognition of the octamer motif, one might expect to increase the on-rate for Oct-2 to DNA. In the recruitment release model, increasing the local concentration of octamer factors near the promoter harboring octamer motifs, one might expect to increase both the on and off rates of Oct-1/-2 binding to an octamer motif.

In this study, I have not addressed directly the ability of GR to repress gene transcription from octamer motifs derived from the Histone H2B, prolactin or GnRH promoters. In an EMSA, the cooperative DNA binding of GR/Oct-2 was challenged with an octamer motif and as a result cooperative DNA binding was lost (Figure 25, Lane 35). This suggested that the continued interaction of GR DBD with Oct-2 is incompatible with Oct-2 DNA binding. This together with other published observations, that showed full-

length GR but not the GR DBD/hinge was able to block DNA binding of Oct-1 to an octamer motif, suggest determinants outside the GR DBD/hinge are important for blocking DNA binding (21). I propose that the interaction of GR with Oct-1 through tethering elements (prolactin and GnRH octamer motifs) would most likely occur through alternative protein-protein interactions stabilized in the presence of DNA that occurs through determinants outside the GR DBD/hinge.

7. Only GR, PR and AR through their DBD/hinge regions interact with Oct-2, *in vivo.*

Several lines of investigation suggest that NRs may interact broadly with POU factors (7-26, 45). NRs share a highly conserved structure and sequence within their DBD region (162). *In vitro* binding studies using GST pull-down experiments have suggested that several NRs had the potential for binding to the POU domain of Oct-1 (45). Previously, I demonstrated that several NRs bound to the POU domain of Oct-1 *in vitro*. Further RARα' bound to the Oct-1 POU domain within a region that required the DBD of RAR and mapped to approximately the same position as that described for GR.

More recently, others have shown that some NRs through their DBD/hinge region interacted with POU factors. Kakizawa et al. (1999) (409), demonstrated RXR interacted with the homeodomain alone of Oct-1 and required the DNA binding domain of RXR in addition to at least part of the hinge region. Further, ERα has been shown to interact directly with two POU factors, Brn 3a and Brn 3b (15). Again the protein-protein interaction mapped to the DNA binding domain of ERα. Characterization of the protein-protein interaction of GR with Oct-1/-2, RXR with Oct-1, ERα with Brn 3a/3b and ERα with Pit-1 (12), suggests these NRs interacted with POU factors through common motifs,

in vitro. However, the functional results from these interactions vary depending on the promoter context with both positive (12, 15-17) and negative (15) transcriptional outcomes described. It is quite clear in the event that both factors bind to the same promoter, that gene transcription can be activated synergistically.

In vivo, not all NRs bound via their DBD/ hinge regions to Oct-2 in mammalian one-hybrid and co-IP assays (Figures 26 and 27). In these assays, the DBD/hinge of various NRs: human PR_A (aa 535-688), rat AR (aa 515-671), rat MR (aa 577-671), human ERα (aa 164-294), mouse RXRα (aa118-236), mouse RARα (aa 60-160) and *Drosophila* Ftz-F1α (aa 478-610) were fused to the Gal4 DNA binding domain and tested for activation while being co-expressed with Oct-2 protein in CHO-K1 cells. Of these NRs, only GR, PR and AR were shown to activate transcription in the presence of Oct-2. Analysis using a co-IP assay showed directly that the Gal DBD/hinge steroid receptor fusion proteins bound to Oct-2. These results showed *in vivo* GR, PR and AR but not MR or ERα can bind to Oct-2, *in vivo*.

The binding of Oct-2 correlated precisely with the promotion of the binding of Oct-2 to DNA templates with paired elements (Figures 28 and 29). *In vivo* footprinting assays showed the DBD/hinge region of GR, PR and AR recruited Oct-2 to paired synthetic response elements and the full length receptors recruited Oct-2 to the MMTV promoter in a hormone dependent manner. These results are consistent with *in vitro* footprinting studies published elsewhere, showing GR and PR decreased the concentration of Oct-1 required to saturate the octamer motifs (17). MR lacked the ability to interact with Oct-2 and promote the occupancy of the octamer motifs adjacent to the HREs on both synthetic paired response elements and the MMTV promoter.

8. The MMTV HREs determines steroid receptor specific responses and dependence on octamer motifs for communicating transcriptional effects

GR, PR, AR and MR are closely related in amino acid sequence and in transcriptional activation function from consensus HREs (5). They can bind as homodimers to the same primary sequence of the AGAACA arranged in an inverted repeat separated by three non-discrete base pairs with each receptor monomer recognizing one half of the site. Indeed from a reporter gene whose expression was regulated by two copies of a HRE, GR, PR, AR and MR activated transcription to equal levels (Figure 31). However *in vivo*, many HREs are divergent from consensus HREs and AR, GR, PR and MR elicit very different physiological responses to their cognate ligands by activation of unique sets of genes (263-268). These differential effects can be achieved through specific responses unique to individual steroid receptors.

The results presented here and elsewhere (269) showed that the target DNA HRE can dictate receptor specific responses. The MMTV promoter can be activated to similar levels of gene transcription by AR, GR, PR and MR, therefore it was assumed that the MMTV HREs failed to discriminate between steroid receptors. The results presented here show individual steroid receptors employ differential modes for activating transcription on the MMTV promoter (Figures 30 and 31). Gene transcription directed by the MMTV HREs showed, in the absence of the octamer motifs, that GR- and PR-mediated gene transcription was severely impaired. Moreover, transcriptional activation was reduced to near basal levels. These results were exactly consistent with results in Hela cells (17). For GR and PR, the presence of functional octamer motifs on the promoter resulted in a striking increase in gene transcription to levels equal to or

exceeding those observed by MR and AR (Figure 30). In the absence of the octamer motifs, AR and MR mediated transcriptional activation was strong and almost completely refractory to the presence of the octamer motifs on the promoter. Therefore it appeared that the MMTV HREs did indeed determine steroid receptor specific transcriptional responses. For the first time, I showed that GR, PR, MR and AR have differential modes for inducing gene transcription from the MMTV LTR on transiently introduced reporter plasmids.

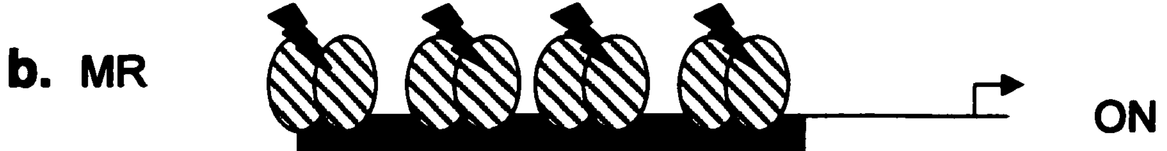
GR and PR mediated transcription was almost entirely dependent on the MMTV octamer motifs. These results suggested that octamer factors were instrumental for glucocorticoid- and progesterone- mediated gene transcription. I demonstrated for PR/GR that the dependence on octamer motif-mediated transcription on the MMTV LTR correlated directly with binding to the POU domains of Oct-1/-2 and with octamer factor recruitment to promoter DNA. I present a model in which GR/PR mediated transcription by the MMTV HREs in the absence of octamer factors was completely ineffective for activating gene transcription (Figure 36a). However, in the presence of octamer motifs gene transcription is activated at least in part due to the recruitment of octamer factors to DNA (Figure 36c). The level of transcription due to the direct recruitment and transcriptional synergy remains to be determined.

In contrast for MR and AR, MMTV mediated transcription was completely independent of the octamer motifs (Figures 36b and d). For MR it is clear how mineralocorticoid-mediated transcription is independent of the octamer motifs because no octamer factors were recruited by MR to the promoter. Surprisingly, MR appeared to have a compensatory mechanism within the MMTV HRE that produced a similar total

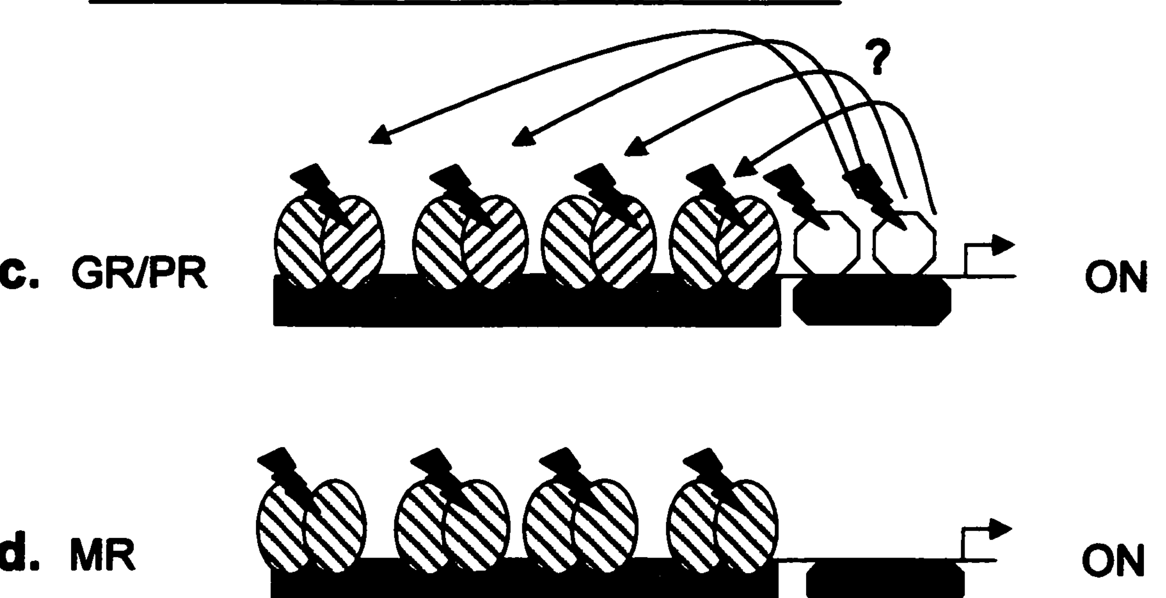
Figure 36. Differential modes of transcriptional activation employed by GR/PR and MR from the MMTV promoter.

A summary through schematic illustration of the results obtained from studying the induction of gene transcription mediated by GR, PR and MR on the MMTV promoter. The promoter DNA is represented by a solid thin line with a bent arrow and the HREs highlighted by the solid rectangle. The steroid receptors are represented as striped ovals. The octamer factors are represented as empty octagons and the octamer motifs as the solid horizontally stretch octagons. The lightening bolt denotes the active transcription factors. a. In the absence of octamer motifs, GR and PR are bound to DNA and in a conformation that does not permit the induction of gene transcription (Off). b. In the absence of octamer motifs, MR is bound to DNA in a transcriptionally active conformation resulting in the induction of gene transcription (ON). c. On the MMTV promoter in the presence of octamer motifs GR and PR can recruit octamer factors to the promoter DNA and function cooperatively to induce gene transcription (ON). Currently the level of transcription due to transcriptional synergy between individual activation domains of the steroid receptor and the octamer factors remain to be determined (indicated by the ?). d. In the presence of functional octamer motifs, MR is illustrated transcriptional active with no octamer transcription factors bound to DNA because MR could not recruit octamer factors to the promoter DNA.

MMTV HREs



MMTV HREs paired with octamer motifs



level of transcriptional responsiveness compared to GR, at least in CHO-K1 cells on a transiently introduced DNA reporter gene. Although AR interacted with Oct-2 in co-IP binding assays and one hybrid experiments and promoted octamer motif occupancy, AR mediated transcription was completely independent of the octamer motifs on the MMTV promoter. Therefore, at least for AR one other level of control exists.

The interaction of Oct-1 with AR appeared to be different than that described for GR. For GR the interaction with Oct-1/-2 appeared to be independent of DNA in the peptide contexts of both the DBD/hinge and the full-length receptor (45). More recently, the interaction of the AR DBD/hinge with Oct-1 has been shown to be independent of DNA (493). However in the context of the full-length AR the interaction with Oct-1 was DNA-dependent because binding, in the absence of DNA resulted in the loss of AR/Oct-1 binding. The protein-protein interaction could be enhanced by the presence of a specific enhancer DNA from the sex-limited protein promoter containing binding sites for both AR and Oct-1. The increased interaction was attributed to steroid receptor DNA binding signals communicated by the N-terminus of AR. However the interaction of full length AR with another POU factor, Brn, was completely DNA independent, an interaction more reminiscent of that described for GR and Oct-1/-2 (45, 493).

It remained possible that AR interacted differentially with Oct-1 and Oct-2. Although it remains to be tested directly, it is possible that AR functions like GR and PR to recruit Oct-2 to the promoter and activate transcription but functions more like MR with Oct-1. The co-IP, one hybrid assay and *in vitro* footprinting experiments (Figures 26, 27, 28, 29) showed AR interactions with Oct-2, whereas the octamer motif sensitivity transcriptional assay used endogenous octamer factors including Oct-1 from CHO-K1

cells (Figure 30). More complete assays comparing the interaction of AR with Oct-1 and Oct-2 will be required to confirm this hypothesis.

Although the interaction between NRs and the POU domain of octamer factors required the NR DBD and the extreme N-terminus of the hinge for interaction *in vitro* (45), the precise determinants for the protein-protein interaction, *in vivo*, have not been finely mapped. It is clear that determinants outside the immediate GR DBD were required for interpreting the signals from the MMTV HREs for octamer motif dependent transcription. The use of chimeric GR/MR proteins demonstrated that the immediate GR DBD was not sufficient to confer octamer motif sensitivity in gene transcription to the MR (Figure 34). Therefore I proposed determinants were localized outside of the GR DBD.

In order to show some determinants for interaction with the Oct-1 POU domain were localized to the GR hinge region, experiments were performed with a GR DBD/hinge peptide, excluding major transcriptional activation domains (Appendix A). Preliminary experiments showed that when the GR DBD/hinge was coexpressed with Oct-1POU VP16 a two fold increase in transcriptional activation could be observed from a reporter plasmid containing the MMTV HREs but lacking functional octamer motifs. However when the MR DBD/hinge was tested under the same conditions, no increase in transcriptional activation was observed. Testing a series of GR/ MR chimeric peptides revealed that MR gained the ability to increase transcription when coexpressed with Oct-1POU VP16 only when a region including GR aa 522-556 was fused to the corresponding positions of the MR peptide. Further, the GR peptide lost the ability to act functionally with Oct-1POU VP16 when aa 522-556 were substituted with MR

sequences. These results suggest that some determinants for functional interaction with the POU domain of Oct-1 were located in the GR hinge region between aa 522-556. However, these results remain to be confirmed in the context of the full-length receptor.

Surprisingly, GR aa 522-556 map to a region of the receptor that contains determinants for solution homodimerization interfaces (175). These determinants for homodimerization are lacking in the MR. Further a similar homodimerization motif has recently been described in the hinge region of the PR (176).

I propose that the MMTV HREs determine the ability of individual steroid receptors to assume an active conformation. In the absence of DNA, it has been proposed that GR adopted an inactive conformation and following DNA binding a conformational change occurs that causes the receptor to assume an active conformation (494). The active conformation would presumably result in an differential GR association with coactivator or coregulatory factors. A mutation in the DBD of the receptor, K461A, converts the receptor into an active conformation even from the plfG promoter, whereas the wild-type receptor acts to transrepress gene transcription. The results presented in this work showed that the MMTV HREs somehow limited the ability of GR and PR to activate transcription in the absence of octamer motifs, potentially by restricting the receptor to interact with coregulatory factors, chromatin remodeling factors or other factors that remain to be identified. Therefore, it may be interesting to test the ability of GR K461A to activate MMTV gene transcription in the absence of octamer motifs.

Studies with hormone antagonists such as RU486 on the MMTV promoter suggest that receptor interaction with coregulatory proteins are likely to be important for

transcriptional synergy between GR and octamer factors. RU486 functions in a way that permits GR to bind to transcription regulatory regions but blocks the ability of GR to interact with coactivator molecules (495). In contrast, the protein-protein interaction of Oct-1 with RU486 treated GR is maintained (45). On the MMTV promoter, RU486 treated GR can act to block transcriptional activity (484), although further experiments are required to confirm that RU486 treated GR can function to recruit octamer factors, *in vivo*.

The dependence on the octamer motifs was not cell-type specific, however the cellular context did indeed influence the degree to which gene transcription depended on the octamer motifs. In CHO-K1 cells, mutation of the octamer motifs led to 50 fold reduction in the level of gene transcription. Similarly, but not to the same extent, transcription decreased approximately 5 fold in Cos7 cells. The degree to which the level of transcription was increased in the presence of octamer motifs may be a combination of effects but most likely are due to an increased basal level of transcription conferred by the NF-I binding site in the MMTV LTR.

NF-I binding sites may be recognized by proteins produced from four genes (NF-I -A,-B, -C, -X) with several different isoforms present in unequal ratios in various cell lines (496). Some act as activators while others act by repressing gene transcription. This hypothesis could be verified be repeating the gene transcription experiments with the MMTV LTR with a mutation of the NF-I binding site. Alternatively, the difference in the level of transcription among cell lines may be due to the presence of various co-activator and co-repressor complexes or general transcription factors that function by differentially amplifying signals induced by the sequence specific transcription factors.

9. The GR DBD can interact with the homeodomain of several homeodomain proteins.

The homeodomains of Oct-1 and Oct-2 were required for interaction with GR. Using GST-GR in pull down-assays with *in vitro* translated C-terminally deleted Oct-1 revealed an Oct-1 peptide truncated immediately following the homeodomain, N-440, retained binding (Figure 12). However binding was substantially reduced when compared to the full-length Oct-1. Further C-terminal deletion into the linker region, N369, abolished detectable binding. These results suggested that the Oct-1 homeodomain was required for interaction with GR.

Using co-IP binding experiments with affinity purified full-length GR and *in vitro* translated Oct-2 with internal deletion mutants, revealed that the Oct-2 homeodomain was required for the binding to immunoprecipitated GR (Figure 13). In contrast, deletion of the POUsp domian retained binding, albeit at lower levels when compared to the binding of full-length Oct-2 protein. These results confirmed that the Oct-2 homeodomain was required for interaction with GR.

Further examination of the binding between GR and Oct-2 with GST-Oct-2 homeodomain in direct binding GST-pull down assays revealed the Oct-2 homeodomain was sufficient in mediating the direct interaction with GR (Figure 15). The reduced binding observed for Oct-1 N440 and the Oct-2Δsp constructs was most likely caused by improper folding of the proteins in the *in vitro* translation reactions by peptide regions outside the homeodomain. This hypothesis is supported by direct binding experiments that showed the homeodomain alone fused to GST retained the same binding properties as the full POU domain. Together, these results show that Oct-1 and Oct-2 interacted

directly with GR *in vitro* and the homeodomain was required and sufficient in mediating the interaction with GR.

The homeodomain is a highly conserved DNA binding domain found in a large number of transcription factors that affect development (497). Homeodomain proteins represent a large transcription factor family characterized by 60 aa core DNA binding domain and can act redundantly to mediate developmental events. hoxA3 and hoxD3, have been shown to have identical biological function and that their unique roles contribute to biological function as a result of quantitative modulations in gene expression (498).

Alone, homeodomains act by binding to A/T rich DNA elements (TAAT), but only with relatively low affinity (306). In many instances, homeodomain protein targeting to specific DNA elements in the cell has been shown to be dependent on protein-protein interactions with other transcription factors (44, 397, 398, 434, 499-503). For example, some Hox homeodomain proteins gain DNA-binding specificity and affinity through cooperative binding with the divergent homeodomain protein Pbx1 (504), and some abd-B, like Hox proteins, DNA binding activity is stabilized by its association with Meis1 (505, 506). Moreover, Pbx1 and Meis1 can dimerize which results in distinctive DNA binding specificity (507).

Homeodomain protein DNA binding activity can also be modulated by non-related transcription factors. The cardiac factor, Nkx-2.5, can be recruited by the serum response factor to activate α-actin gene transcription in murine fibroblasts (508). Nkx-2.5 can also cooperate with the zinc finger protein, GATA-4, to activate transcription of both cardiac α-actin and atrial natriuretic factor genes (403, 509). In these two examples,

the protein-protein interaction has been mapped directly to the homeodomain of Nkx-2.5 (403, 508, 509).

Following the observation that the homeodomain alone of Oct-1/-2 was required and sufficient for interaction with the GR DBD, it was interesting to assess whether other homeodomain proteins interacted similarly. All of the homeodomains tested bound similarly to ^{32}P-labeled GR DBD in GST pull down assays in an L501P sensitive manner (Figure 16). By comparing the quantity of GST fusion protein used in the assay, to the amount of binding in the pull down assay, suggested that some of the proteins bound with slightly different efficacies. For example, binding to the hoxC4 homeodomain and to full-length dlx2 was strongest while binding was weakest to the Prd homeodomain. Under these conditions it would be difficult to make conclusions on the efficacies of protein binding and extending them to protein affinity without carrying out protein-protein affinity binding studies. For this, it would be important to test the structural integrity of each protein purified from bacteria. The binding information obtained from the pull-down studies was supported in the co-IP studies (Figure 17).

The mammalian one-hybrid studies showed that full length Oct-2 and two other homeodomain containing proteins hoxD4 and dlx2, but not an unrelated transcription factor, increased transcription significantly above the level obtained with the Gal4 DBD in the absence of a GR DBD fusion (Figure 19). The binding of hoxD4 and dlx2 to full length GR in an L501P sensitive way were verified using a co-IP binding assay (Figure 17). These results suggest the high degree of conservation within the homeodomain may permit broadly based binding to the GR DBD *in vitro* and *in vivo*. The extent of GR-homeodomain binding remains to be determined.

Glucocorticoids are teratogenic (510) and most frequently correlated with cleft palate following embryonic exposure (511). At the start of Xenopus embryo development GR mRNA is abundant in Xenopus oocytes but is rapidly degraded during early cleavage stages of the embryo. GR transcripts are re-expressed prior to the completion of gastrulation and become localized to the dorsal ectoderm. In mammals, mRNA expression of the GR has been detected beginning at embryonic day 9.5 (512, 513). Down regulation of GR mRNA at final stages of tissue differentiation in which it is expressed is suggestive of a morphogenic role (513).

Several homeodomain-containing proteins are involved in the first steps of the establishment of vertebrate body axis during gastrulation. In the initial events of development, proteins are expressed in gradients across the embryo believed to establish the anterior-posterior and ventral-dorsal orientations of the embryo. Prior to the onset of zygotic gene expression, some homeodomain containing proteins are expressed as maternal factors such as goosecoid (514), Otx2 (505, 515) and Xtwin (516) as well as a number of POU factors (331, 332, 517, 518).

Zygotic genes become activated between the midblastula transition and the onset of gastrulation. Homeobox genes such as goosecoid (519-521), floating head/Xnot (521, 522), GSX (523), Xlim-1 (524), Otx2 (515, 525), Xtwin (516) and Siamois (526-528) are expressed in the organizer. When some of these factors are misexpressed in the embryo, the embryo becomes dorsalized. Some factors like Xvent-1 and Xvent-2/Vox, have been shown to be important for ventral fate specification (529-531). Despite the obvious important functions that these genes play at the onset of gastrulation, little is known about

the role of maternal homeodomain proteins during cleavage of the vertebrate embryo and the role of the maternal and zygotic homeodomain proteins prior to gastrulation.

In experiments described in Wang et al. (1999) (532), we showed that the GR DBD disrupted development of zebrafish embryos in a manner that led to embryonic death during gastrulation. This effect was rescued by co-expression of the Oct-2 homeodomain. Injection of the mRNA encoding the GR DBD (aa 407-556) was lethal for at least 55% of the embryos and induced some type of visible embryonic malformation in approximately 80% of embryos. Microinjection of the same GR mRNA but with a point mutation at L501P in zebrafish embryos was indistinguishable from injection of control samples. The co-injection of mRNA encoding the Oct-2 HD with the GR homeodomain-binding motif resulted in an almost complete rescue of the phenotype induced by mRNA encoding the GR DBD and hinge region alone.

It was unlikely that the malformations were due to GR binding to a genomic locus because controls employed GR mRNA containing one of two point mutations, C460Y and K489R. The GR peptide product would have aberrant GR/DNA binding activity but retain the capacity for interaction with the POU domain of Oct-1. Further micro-injection of GR mRNA containing L501P in addition to C460Y showed the same level of embryonic disruption as control injections of either saline or antisense RNA.

Defects observed by overexpression of the GR DBD and hinge region in zebrafish developing embryos were similar to defects observed in overexpression studies with RXR and TR in *Xenopus* embryos (533). These results suggest that NRs may indeed interact broadly with homeodomain proteins. Our results highlight the potential role of

GR in DNA-independent effects during development of multiple organisms and that some of the effects may result from direct interaction with homeodomain proteins.

V. CONCLUSION

This thesis provides compelling evidence for a direct interaction between the GR, PR and AR with octamer factors. In contrast under the same conditions, the interaction of MR with octamer factors could not be observed. The contribution of the interaction appears to result in the recruitment of octamer factors to DNA when binding sites for both factors are juxtaposed. The MMTV HREs restricted the ability of PR and GR to induce gene transcription, however when juxtaposed to the MMTV octamer motifs, gene transcription was permitted. These results suggest mechanisms employed by GR and PR are different from those used by AR and MR for activating gene transcription from the MMTV promoter. The direct GR protein-protein interaction with octamer factors maps to the homeodomain and the homeodomaine of several proteins, *in vitro*, and at least two homeodomain proteins *in vivo*, dlx-2 and hoxD4. This suggests the interaction may be broadly based for homeodomain proteins. Specifically, I demonstrated that:

1. GR, PR and AR, in a rat GR L501P sensitive manner, bound to octamer factors, *in vivo*, using co-IP and mammalian one hybrid assays. Under these same conditions MR could not bind to octamer factors. I present preliminary data demonstrating some determinants for interaction with octamer factors may be localized to the GR hinge region.

2. Using *in vivo* footprinting assays of transiently transfected DNA, GR, PR and AR recruited octamer factors to DNA in a way that was dependent on the protein-protein interaction. Transfection assays showed the recruitment of octamer factors to DNA potentiated the ability of octamer factors to activate gene transcription. This

provided a demonstration of a ligand activated transcription factor modulating the transcriptional activity of a constitutive transcription factor.

3. On the MMTV LTR using transient transfection assays, the HREs determined the mode of steroid receptor regulation. GR and PR regulated gene transcription was almost entirely dependent on the octamer motifs for hormone induced gene transcription, while transcription induced by AR and MR was octamer motif independent.

4. The interaction of GR with octamer factors mapped to the homeodomain of Oct-1 and Oct-2 using GST-pull down and coimmunoprecipitation assays. Mammalian one hybrid and coimmunoprecipitation assays showed the interaction could be observed *in vivo* with other homeodomain proteins, suggesting the interaction is a broadly conserved property of homeodomain proteins.

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VII. APPENDIX A

Rational

The studies in this work suggested that determinants for functional interaction with the POU domain of Oct-1 were localized outside the immediate DBD (rat GR aa 440-508), (Figure 34). The mammalian two hybrid analysis (Figure 18) showed that aa 407-556 was sufficient for functional interaction, in vivo. In addition, MMTV HREs conferred determinants for octamer factor sensitivity for GR but not MR in transient transfection studies (Figures 30 and 34). Taken together these results suggested some determinants for octamer factor sensitivity were localized somewhere in the hinge region of GR between aa 508-556.

Aim

My specific aim was to map GR hinge determinants using GR/MR chimeric peptides conferred by the MMTV HREs for functional sensitivity to the POU domain of Oct-1.

Experimental procedure

Plasmids: For the GR and MR chimeric peptides a 2 step cloning procedure was utilized. An oligonucleotide encoding aa 407-414 was inserted into the BglII/XhoI sites of pTL2 to produce pTL2 BUGR1/2. Next a PCR-ligation-PCR strategy was employed to insert chimeric receptor derivatives into the XhoI/ EcoRI sites pTL2 BUGR1/2. The PCR products were produced using p6RGR and p6RMR as template DNAs. The pGR, pMR, $pG_{510}M$, $pM_{510}G$ and $pM_{522}G$ clones were screened using unique restriction sites created through silent mutagenesis and the resulting plasmids verified by DNA sequencing. The plasmids, pOct-1POU VP16, pMMTV Oct_{mut}EIBCAT, p6RGR and p6RGR have been described in the methods section of the thesis.

Cell maintenance: Hela cells (ATCC) were maintained in DMEM with 10% FBS (Hyclone) at 37°C in a water saturated atmosphere with 5% CO_2.

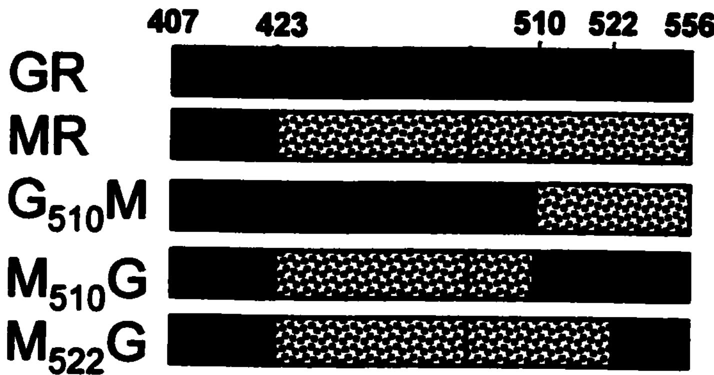
Transfection: Hela cells were seeded onto 60 mm tissue culture plates 24 hours prior to transfection. Cells were transfected using Exgen500 according to the manufacturer's protocol (MBI Fermentus). Briefly, for each plate Exgen500 (8μl) and a DNA/saline solution containing 500 ng pMMTVOct$_{mut}$EIBCAT, 250 ng pRSVβgal, 500 ng of plasmid expressing GR or GR/MR peptides and 2 μg pOct-1VP16 in various combinations were incubated for 15 minutes. Cells were washed with DMEM without FBS and then the exgen500/DNA/saline solution and DMEM lacking FBS was added and incubated for 3 hours. The transfection was terminated by the addition of DMEM with FBS to 10%. The cells were harvested 48 hours later, cellular extracts prepared and assayed for CAT and β-gal activity exactly as described the methods section of the thesis.

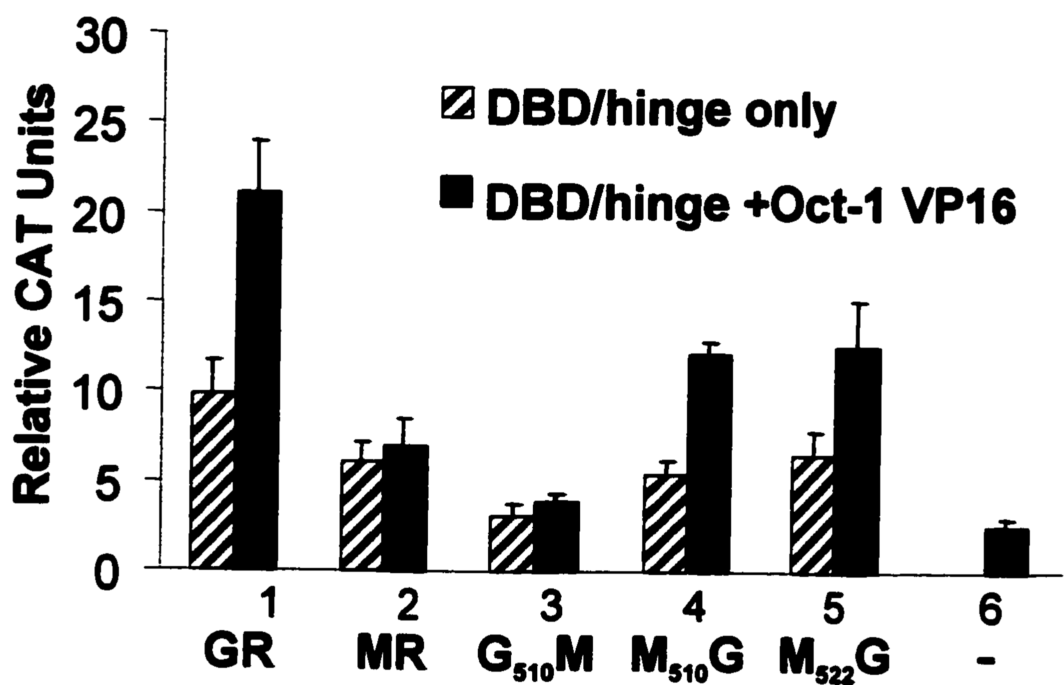
Results

The results showed when the Oct-1POU VP16 was coexpressed with the GR peptide transcription was induced about two fold (Figure A.1, lane 1). In contrast when Oct-1POUVP16 was co-expressed MR no significant increase in transcription was observed (lane 2). MR chimeric peptides containing aa 522-566 of GR increased transcription when co-expressed with Oct-1POU VP16 (lanes 4 and 5) to a similar fold induction observed with GR. Further when the aa 510-556 of rat GR was replaced with the corresponding region of MR the increased level of transcription was lost (lane 3). Most of the transcription was dependent on the expression of the receptor peptides because when Oct-1POU was expressed alone transcription was significantly reduced (lane 6). Taken together these preliminary results suggest some determinants conferred

Figure A.1. Determinants for Oct-1 sensitivity are localized to aa 522-556 of rat GR. (top) Schematic illustration of the reporter plasmid and the GR and MR chimeric proteins used in the experiment described below. The MMTV Oct_{mut} reporter plasmid (-188 to -36) is illustrated with Xs over the octamer motifs. The GR and GR/MR chimeric proteins are illustrated as rectangles filled solid representing the GR peptide regions or stippled representing MR peptide regions. The amino acid numbers above the schematic represent amino acids corresponding to the numbering scheme of rat GR. (Bottom) Hela cells were transiently transfected with the MMTV Oct_{mut} reporter plasmids and plasmids expressing GR, MR, $G_{510}M$, $M_{510}G$, $M_{522}G$ alone (striped bars) or with a plasmid expressing Oct-1POU VP16 (solid bars), as indicated. Following 48 hours cellular extracts were prepared and assayed for CAT activity. Transfection efficiency was determined by liquid β-gal assay by cotransfection with β-gal expression plasmid. The results were represented as the mean and S.E.M from one experiment performed in duplicate.

-188
MMTV
-36
Oct_mut


407
423
510
522
556
GR
MR
G510M
M510G
M522G


Relative CAT Units
30
25
20
15
10
5
0
DBD/hinge only
DBD/hinge +Oct-1 VP16
1
GR
2
MR
3
G510M
4
M510G
5
M522G
6
-

by the MMTV HREs for Oct-1 POU sensitivity by GR are localized to the hinge region, aa 522-556.

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Thesis title: Requirements for the Binding of the Glucocorticoid Receptor to Octamer Transcription Factor-1, *in vitro*.

1993 B.Sc. (Biochemistry, Honors)
University of Ottawa, Ottawa, Ontario

AWARDS:

2001-1004 Canadian Institutes of Health Research, Fellowship

1997-2000 Medical Research Council of Canada, Studentship.

1994 Gordon Research Conference, Travel Award.

1989 University of Ottawa, Entrance Award.

EXPERIENCE:

1994 –1995 Lab Demonstrator - BCH 3926 and BCH 2540
University of Ottawa.

PUBLICATIONS AND ABSTRACTS:

I. Papers (6):

1. Savory J.G., **Préfontaine G.G.**, Lamprecht C., Liao M., Walther R.F., Lefebvre Y.A., Hache R.J. G. (2001) Glucocorticoid Receptor Homodimers and Glucocorticoid-Mineralocorticoid Receptor Heterodimers Form in the Cytoplasm through Alternative Dimerization Interfaces. *Molecular and Cellular Biology 21:* 781-793.

2. **Préfontaine, G.G.**, Giffin, W., Walther, R.F., Lemieux, M.E., Pope, L. and Haché, R.J.G. (1999) Selective binding of steroid hormone receptors to octamer transcription factors determines transcriptional synergism at the MMTV promoter. *Journal of Biological Chemistry 274:* 26713-26719.

3. Wang, J.M., **Préfontaine, G.G.**, Lemieux, M.E., Walther, R.F., Pope, L., Akimenko, M.-A. and Haché, R.J.G. (1999) Developmental effects of ectopic expression of the glucocorticoid receptor DNA binding domain that interferes with homeodomain binding. *Molecular and Cellular Biology 19:* 7106-7122.

4 **Préfontaine, G.G.**, Lemieux, M.E., Giffin, W., Schild-Poulter, C., Pope, L., Lacasse, E., Walker, P. and Haché, R.J.G. (1998) Recruitment of octamer transcription factors to DNA by glucocorticoid receptor. *Molecular and Cellular Biology 18:*3416-3430.

5. Giffin, W., Kwast-Welfeld, J., Rodda, D.J., **Préfontaine, G.G.**, Traykova-Andonova, M., Zhang, Y., Weigel, N.L. Lefebvre, Y.A. and Haché, R.J.G. (1997) Sequence-specific DNA binding and transcription factor: Phosphorylation by Ku autoantigen/DNA-dependent protein kinase. *Journal of Biological Chemistry 272:* 5647-5658.

6. Giffin, W., Torrance, H., Rodda, D.J., **Préfontaine, G.G.**, Pope, L. and Haché, R.J.G. (1996) Sequence-specific DNA binding by Ku autoantigen and its effects on transcription. *Nature 380:* 265-268.

II. Abstracts (14):

1. Wang, J.-M., **Préfontaine, G.G.,** Liao, M., Akimenko, M.-A. and Haché, R.J.G. Development of a far western screen for proteins targetted by dominant negative glucocorticoid hormone receptor peptides that severely perturb early zebrafish development. The Endocrine Society Meeting 2000, Toronto, ON.

2. Walther, R.F., **Préfontaine, G.G.**, Savory, J.A., Reich, T., Haché, R.J.G., Lefebvre. Temporal regulatio of trancription responsiveness to glucocorticoid hormones is accompanied by reassiciation of the glucocorticoid receptor with a chaperone complex. The Endocrine Society Meeting 2000, Toronto, ON.

3 **Préfontaine, G.G.**, Giffin, W., Lemieux, M.E., Pope, L. and Haché, R.J.G. (1998) Selective binding of steroid hormone receptors to octamer transcription factors determines transcriptional synergism at the MMTV promoter. Hormone Action, Gordon Research Conference, Meriden, NH.

4 **Préfontaine, G.G.**, Lemieux, M.E., Wang, J.-M., Giffin, W., Schild-Poulter, C., Pope, L., Walker, P., Akimenko, M.-A. and Haché, R.J.G. (1997) Recruitment of homedomain proteins to transcriptional regulatory regions by nuclear hormone receptors. Mechanisms of Eucaryotic Transcription, Cold Spring Harbor, NY.

5 **Préfontaine, G.G.**, Lemieux, M.L., Schild-Poulter, C., Pope, L., Traykova-Andonova, M., Walker, P. and Haché, R.J.G. (1997) Gene targeting of homeodomain proteins by nuclear hormone receptors. EMBO Work Shop: Structure Function of NRs, International School of Pharmacoloy, Erice, Italy.

6 Lemieux, M.E., **Préfontaine, G.G.**, Walther, R.F., Pope, L., Huang, Walker, P. and Haché, R.J.G. (1997) Mechanisms of glucocorticoid receptor - octamer protein transcriptional synergy. The Endocrine Society, 79th Annual Meeting, Minneapolis, MN.

7 Kwast-Welfeld, J., **Préfontaine, G.G.**, Giffin, W., Zhang, Y., Weigel, N.L., Lefebvre, Y.A. and Haché R.J.G. (1996) DNA sequence-directed phosphorylation of rat glucocorticoid receptor by DNA dependent protein kinase. The Endocrine Society, 78th Annual Meeting, San Diego, CA.

8 **Préfontaine, G.G.**, Walker, P., Traykova-Andonova, M., Pope, L. and Haché, R.J.G. (1995) A motif in the DNA binding domain of the glucocorticoid receptor that mediates direct contact with the POU domain of octamer transcription factors *in vivo* is conserved throughout the NR superfamily. Mechanisms of Eucaryotic Transcription, Cold Spring Harbor, NY.

9 **Préfontaine, G.G.**, Walker, P., Traykova-Andonova, M., Pope, L. and Haché, R.J.G. (1995) A motif in the DNA binding domain of the glucocorticoid receptor that mediates direct contact with the POU domain of octamer transcription factors *in vivo* is conserved throughout the NR superfamily. The Endocrinology Society, 77th Annual Meeting, Washington, DC.

10. Torrance, H., Rodda, D.J., **Préfontaine, G.G.**, Giffin, W. and Haché, R.J.G. (1995) Phosphorylation of the glucocorticoid receptor on the MMTV promoter by DNA-PK is directed through a sequence-specific binding site for Ku autoantigen. The Endocrine Society, 77th Annual Meeting, Washington, DC. **Selected for an oral presentation.**

11. **Préfontaine, G.G.**, Walker, P., Traykova-Andonova, M., Pope, L. and Haché, R.J.G. (1995) A motif in the DNA binding domain of the glucocorticoid receptor that mediates direct contact with the POU domain of octamer transcription factors *in vivo* is conserved throughout the NR superfamily. Hormone Action, Gordon Research Conference, Meriden, NH.

12. Torrance, H. Rodda, D.J. **Préfontaine, G.G.**, Giffin, W. and Haché, R.J.G. (1995) Ku autoantigen is a transcription factor that represses the induction of Mouse Mammary Tumor Virus transcription by glucocorticoids. Hormone Action, Gordon Research Conference, Meriden, NH.

13. **Préfontaine, G.G.**, Walker, P., White, T.C., Huang, W., Pope, L. and Haché, R.J.G. (1994) Mapping of the physical association between the glucocorticoid receptor and octamer transcription factor 1. The Endocrine Society, 76th Annual Meeting, Anaheim, CA. **Selected for an oral presentation.**

14. **Préfontaine, G.G.**, Walker, P., White, T.C., Huang, W., Pope, L. and Haché, R.J.G. (1994) Mapping of the physical association between the glucocorticoid receptor and octamer transcription factor 1. Hormone Action, Gordon Research Conference, Meriden, NH. **Selected for a travel award.**

CONTRIBUTION OF COLLABORATORS

Madelaine Lemieux performed the mammalian one-hybrid assay described in Figure 19. Both Madelaine Lemieux and Louise Pope contributed to this worked by doing the transient transfection and CAT assay described in Figures 20 and 26. Louise also performed the Oct-1 and Oct-2 mapping studies described in Figures 12 and 13. I would like to thank Ward Giffin for his excellent technical support for the *in vivo* footprinting assays and the EMSA. I would also like to thank Maya Traykova-Andonova for her plasmid contributions.