REQUIREMENTS FOR THE BINDING OF GLUCOCORTICOID RECEPTOR TO OCTAMER TRANSCRIPTION FACTOR-1, *IN VITRO.*

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ABSTRACT

Nuclear receptors and POU transcription factors have been shown to regulate gene transcription through complex cis-acting elements located in upstream promoter regions of target genes. Regulation has been shown to occur through both synergistic and inhibitory mechanisms. Specifically, glucocorticoid dependent transcriptional synergism has been observed within complex regulatory elements on the mouse mammary tumor virus Long Terminal Repeat (MMTV LTR) that contain binding sites for both the glucocorticoid hormone nuclear receptor (GR) and the ubiquitous POU factor, octamer transcription factor -1 (Oct-1). By contrast, on promoters like that of the histone H2B gene that contains regulatory elements only for Oct-1, glucocorticoids actually appear to inhibit the activation of gene transcription by Oct-1. Previous experimental observations were suggestive of a direct physical interaction between the DNA binding domain of GR and the POU DNA binding domain of Oct-1.

In this work I have examined the requirements for the binding of *in vitro* translated, radiolabeled GR to the POU domain of Oct-1 expressed as a glutathione-S-transferase (GST) fusion protein and immobilized on glutathione sepharose. My results show that in this system: 1) GR and Oct-1 bind directly through their respective DNA binding domains; 2) Binding of GR to Oct-1 requires dissociation of the heat shock proteins from GR; 3) The Oct-1/GR interaction is distinguishable from a separate Oct-1/VP-16 interaction. 4) Point

mutations C500Y and L501P in the DNA binding domain of rat GR abrogate binding to the POU domain of Oct-1. 5) Oct-1 binding appears to be a broadly conserved property of the nuclear hormone receptor superfamily as several *in vitro* translated receptors bound the POU domain of Oct-1 with an avidity similar to that of GR. These experiments provide evidence that most if not all nuclear receptors may act through a common mechanism with Oct-1 to regulate gene transcription. They also may provide insight into mechanisms whereby inducible transcription factors act to modify the functional targeting of constitutive transcription factors.

DEDICATION

This thesis is dedicated to my parents, Guy and Alice, for their love and financial support, when I needed it most. I thank Alice for teaching me to be patient and considerate towards others and Guy for his inspiration to become successful and showing me that I am capable of overcoming many obstacles. A special dedication to my grandfather Philip Préfontaine who passed away earlier this year.

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ABBREVIATIONS

17β-estradiol .....1,3,5(10)-estratrien-3,17β-diol

^{35}S-Met.....^{35}sulfur labeled methionine

A.....alanine

aa.....amino acid

AP-1 .....activating protein-1

AR.....androgen receptor

Bob-1.....B cell specific coactivator

BSA .....bovine serum albumin

bZIP .....basic leucine zipper

CBP.....CREB-binding protein

cDNA.....complementary deoxyribonucleic acid

CHO.....chinese hamster ovary

CTD.....carboxyl terminal domain

cys .....cysteine

D/M .....dexamethasone 21-mes

dATP.....deoxyadenosine triphosphate

DBD .....DNA-binding domain

dCTP .....deoxycytosine triphosphate

Des.....17β-estradiol

dex.....dexamethasone

dex 21-mes.....1,4-Pregnadien-9α-fluoro-16α-methyl-11β,17,21-triol-3,20-dione 21-methanesulfonate

dexamethasone 1,4-Pregnadien-9α-fluoro-16α-methyl-11β,17,21-triol-3,20-dione

dGTP.....deoxyguanosine triphosphate

DNA.....deoxyribonucleic acid

DR.....direct repeat

DTT.....dithiolthreitol

dTTP.....deoxythymine triphosphate

E.....glutamate

ECL.....enhanced chemiluminescence

EDTA.....ethylenediaminetetraacetic acid

EMSA.....electrophoretic mobility shift assay

ER.....estrogen receptor

ERE.....estrogen response element

GnRH.....gonadotropin-releasing hormone

GR.....glucocorticoid receptor

GRE.....glucocorticoid response element

GST.....glutathione-S-transferase

GST-Oct.....GST-Oct fusion protein

HCF.....host cell factor

HEPES.....4-(2-hydroxylethyl)-1-piperazine ethanesulfonic acid

HLH.....helix-loop-helix

HRE .....hormone response element
hsp.....heat shock protein
HSV.....herpes simplex virus

IE.....intermediate-early
IPTG.....isopropyl β-D-thiogalactopyranoside

LB .....liquid broth
LTR .....long terminal repeat
luc.....firefly luciferase

MMTS.....methyl methane-thiosulfonate
MMTV.....mouse mammary tumor virus
MR.....mineralcorticoid receptor

NF-1.....nuclear factor-1
NLS.....nuclear localization signal
NMR.....nuclear magnetic resonance
NP-40.....nonidet-P 40

Oct-1.....octamer transcription factor -1
Oct-2.....octamer transcription factor-2
OD.....optical density

P.....proline
PIC.....preinitiation complex
Pit-1.....pituitary transcription factor-1
PMSF.....phenylmethylsulfonyl fluoride

pol II.....polymerase II
POU-hd.....POU homeodomain
POU-sp.....POU specific domain
PPO.....2,5-diphenlyoxazole
PR.....progesterone receptor

Q.....glutamine

R.....purine
RAR.....retinoic acid receptor
RNA.....ribonucleic acid
RRL.....rabbit reticulocyte lysate
RXR.....retinoid X-related receptor

S.....serine
SDS.....sodium dodecylsulfate
SDS-PAGE.....sodium dodecylsulfate-polyacrylamide gel electrophoresis
sn.....small nuclear
SV40.....simian virus 40

T.....threonine
TAF.....transactivation function
TBE.....tris-boric acid-EDTA
TBP.....TATA-binding protein
TF.....transcription factor
TK.....thymidine kinase
TPA.....12-O-tetradecanoylphobol-13-acetate
TR.....thyroid hormone receptor

Tris .....tris(hydroxymethyl)aminomethane

VP-16 .....virion protein 16

I. INTRODUCTION

Nuclear receptors and POU transcription factors have complex interactions that influence the expression of genes transcribed by RNA polymerase II (pol II) (1-17). Both synergistic (1-14) and inhibitory (3, 15-17) interactions have been reported between individual members of each family. Synergism reflects the simultaneous action of individual factors having a greater total effect on specific gene transcription than the sum of the effects of either factor, separately. Of particular interest for the present work, on promoters containing DNA recognition elements for both glucocorticoid receptor (GR) and the POU factor octamer transcription factor -1 (Oct-1), gene transcription is activated synergistically in response to glucocorticoid hormone (3, 4). By contrast, on promoters containing a DNA recognition element for Oct-1 and no glucocorticoid response element (GRE), gene transcription is repressed in response to hormone (4, 15, 16). The molecular mechanisms attributed to these transcriptional responses have not previously been identified, nor has the potential for a broadly based mechanism of regulatory interaction between nuclear receptors and POU factors been investigated.

1. GR and the nuclear receptor superfamily

Nuclear receptors are ligand-activated transcription factors that directly regulate gene expression by binding to specific *cis*-acting DNA sequences surrounding target genes (18-20). Their ligands are lipophilic hormones, including steroids, retinoids, thyroid hormones, vitamin D_3 and ecdysone, which differentially modulate gene expression through their cognate receptors. Initially, these lipophilic hormones were isolated, based on their abilities to affect development, cell differentiation and organ physiology (19). Following the development of radiolabelled ligands, nuclear binding proteins (nuclear hormone receptors) for these hormones were identified. The involvement of nuclear receptors in regulating gene transcription was realized when ecdysone, an insect metamorphic hormone, caused chromosomal puffing at specific locations in the polytene chromosomes of *Drosophila* (21).

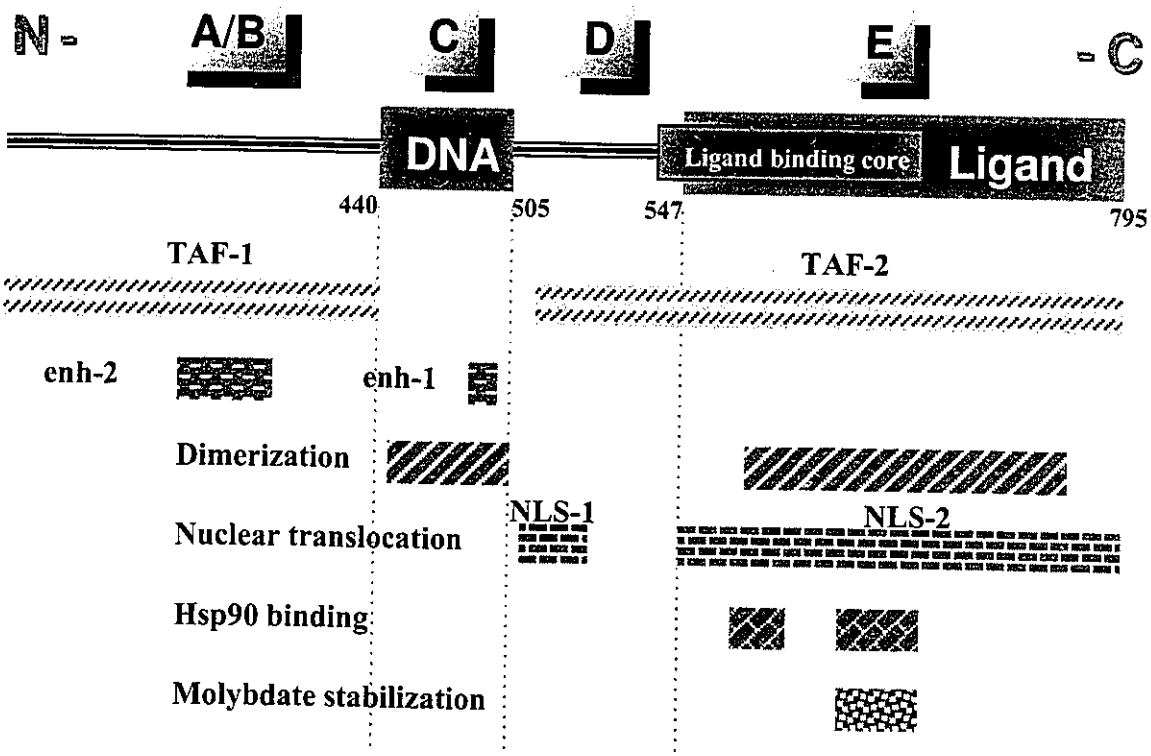
The first steroid receptor cDNAs identified, for glucocorticoid receptor (GR) and estrogen receptor (ER), were cloned using purified ligands and specific antibodies (22-24). This type of cloning together with low stringency cDNA library screening led to the identification of genes for other ligand-activated receptors. By 1990, 15 members of the nuclear receptor family of transcription factors were identified as the receptors for all the lipophilic ligands known at the time. By the end of

1995, low stringency hybridization of cDNA libraries has led to the identification of more than 150 different related genes in many animal species including worms and insects. Many of the proteins encoded by the genes are termed orphan receptors because ligands for these proteins are not yet apparent (25). It is expected that some of these receptors will interact with novel ligands, while others may be ligand independent transcription factors, as their receptor function is only an hypothesis based on their homology to ligand-activated receptors.

Nuclear receptors are modular in construction and can be divided into five discrete domains labelled A-E (summarized for GR in Figure 1) (19). The N-terminal A/B transactivation domain is highly variable between individual nuclear receptors and ranges from ~100-600 amino acids in length. By contrast, The central or C DNA binding and D/E carboxyl-terminal ligand binding domains are of similar length in all receptors (~70 and ~250 amino acids, respectively). The C domain contains two arrays of 4-cysteine residues. Each array tetrahedrally coordinates one zinc atom, to form what has been termed two cys-4 zinc finger DNA binding motifs (26, 27). The DNA binding domain is the region of highest homology among receptors (28). Biochemical analysis, X-ray crystallography and nuclear magnetic resonance (NMR) have revealed the molecular structure and the mechanism of DNA binding of GR and the other nuclear receptors (26, 29, 30). α-helix-1 in

Figure 1. Schematic representation of rat GR and its functional domains.

GR is represented by the bold line and the boxes highlight the DNA and ligand binding domains as indicated (adapted from S. Simons, 1994 (33)). The ligand binding core is encompassed in a 16kDa fragment, amino acids 537-673 identified as the product of a trypsin digest that retains considerable affinity and all the specificity of ligand binding seen with the intact receptor (33, 34). Above, the GR schema has been labeled A-E to represent functional domains common to all nuclear receptors (19). The amino acid positions are indicated by the numbers below the main diagram. Functional properties are summarized beneath the receptor schema. Transactivation functions TAF-1 and TAF-2 map to the N- and C-terminal domains of GR, respectively (35, 36). 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 analysis, as the region containing the major activity of the rat GR TAF-1 domain (36). The enh-1 (aa 484-492) domain is a transcriptionally active domain, defined by truncated receptors in yeast and CV-1 cells (37). Moreover, point mutations in this domain that affected transactivation but not DNA binding could be obtained (37). The centrally located, dimerization domain (aa 477-481) represents the DNA dependent dimerization domain that encourages the cooperative DNA binding of the receptors monomers to DNA (38, 39). The second dimerization domain located in the E region has been proposed to reside between amino acids 730-764 on the basis of homology with ER (1). 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 (40, 41). A second NLS termed NLS-2, has been localized to amino acids 540-795. The smallest peptide shown to stably associate with hsp 90 has been localized between 537-673, although multiple sites of hsp 90/receptor interaction may exist (42). Molybdate has the ability to stabilize receptors in the untransformed state, presumably by its interaction with cysteine residues in a region encompassing sequences 644-671 (43, 44)


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

the first finger motif was found to be responsible for the specificity of DNA binding through base specific contacts in the major groove of DNA, while α-helix-2 in the second finger makes extensive phosphate contacts with the DNA juxtaposed perpendicular to α-helix-1 (also see Figure 17). When bound to DNA, α-helix-2 has an exposed surface with the potential to make additional protein-protein contacts (31) while helix-1 lies across the DNA major groove and is almost wholly engaged in DNA binding.

The D domain separates the DNA binding domain from the carboxyl-terminal ligand binding domain. This region, which appears to function as a flexible hinge, may be crucial for the spatial configuration of the receptor. It also contains a motif with striking similarity to the SV40 T-antigen nuclear localization signal (32). This motif is at least partly responsible for nuclear transfer of GR (40).

The carboxyl-terminal E domain has several overlapping functions including transcriptional activation, dimerization, hormone binding and for some receptors, a second nuclear localization signal (33). In addition for steroid receptors, the ligand binding domain contains determinants that mediate association of unliganded receptors into heat shock protein 90 (hsp 90) containing protein

complexes (45, 46). In simple terms the E or ligand binding domain acts as a molecular switch in the regulation of specific gene expression. Upon ligand binding, steroid receptors are transformed from an inactive to a transcriptionally active state. In domain swapping experiments using GR and ER, the ligand binding domains determined the ligand specific regulation of transcriptional activation, while the DNA binding domains directed the response to specific promoter sequences (47, 48).

The nuclear receptor superfamily has been divided into four classes based on homology and their DNA binding and dimerization properties (20). Class I include the steroid hormone receptors which bind to DNA as homodimers with each monomer recognizing hexamer half-sites (49). These half-sites are organized as inverted repeats separated by 3 base pairs and are generally located in upstream promoter regions. Once the receptor is bound to DNA the receptor monomers are juxtaposed in a head to head configuration and make DNA dependent protein-protein contacts through the C-terminal cys-4 zinc-finger of the DNA binding domain. GR, progesterone receptor (PR), androgen receptor (AR), and mineralcorticoid receptor (MR) recognize the same 5'-AGAACA-3' half-site while estrogen receptor (ER) recognizes 5'-RGGTCA-3' half-site (R=purine).

Class II receptors heterodimerize with the retinoid X-related receptor (RXR) "master regulator" (25). These heterodimers characteristically bind to direct repeats (DRs) of the core 5'-AGGTCA-3' half-site with variable spacing in what has been termed the 1 to 5 rule (although some also bind to inverted or everted repeats with variable spacing) (50). In contrast to the steroid hormone response element which allows the receptor to bind only as homodimers, the nucleotide spacing between direct repeat motif sites determines the heterodimerization partner able to signal through individual hormone responsive elements (HREs). Class III orphan receptors bind as homodimers to DNA direct repeats while Class IV orphan receptors bind as monomers to a single core binding site with extended conserved flanking sequences.

GR is a classical target for studying steroid hormone action (51). *In vivo* the inactive or untransformed GR, is localized in the cytoplasm, is associated with heat shock proteins including 2 molecules of hsp 90, hsp 70, p60, p59 (hsp56) and potentially several other, less well characterized proteins (52). *In vitro*, untransformed GR can be stabilized by the presence of molybdate (43). The hsp-GR complex is proposed to maintain the receptor in an open conformation that allows GR to bind ligand with high affinity (53, 54). Ligand binding promotes the dissociation of the receptor-bound heat shock proteins to produce the

transcriptionally active, "transformed", free liganded receptor. This ligand activated receptor is rapidly transferred to the nucleus where it regulates transcription of cognate genes (51).

2. Oct-1/Oct-2 and the POU transcription factor family

POU factors, a subfamily of the homeodomain superfamily of transcription factors play key roles in developmental programs during early embryogenesis and specify cell type specific terminal differentiation events (55-61). The family name POU (62) is derived from the first letters of the names of the first family members identified Pit-1 (also termed GHF-1) (63, 64), Octamer factors -1 (65) and -2 (66) and the *Caenorhabditis elegans* protein *unc*-86 (67). The octamer transcription factors, Oct-1 (also termed, OTF-1, NF-A1, NFIII and OBP-100) and Oct-2 , are named for the length of their DNA binding sites, which is 8 nucleotides long. They were originally cloned by screening λgt11 phage library fusion proteins with a radiolabelled probe containing a consensus octamer motif, 5'-ATGCAAAT-3', which was known to be a response element for both the ubiquitous Oct-1 and B-cell specific Oct-2.

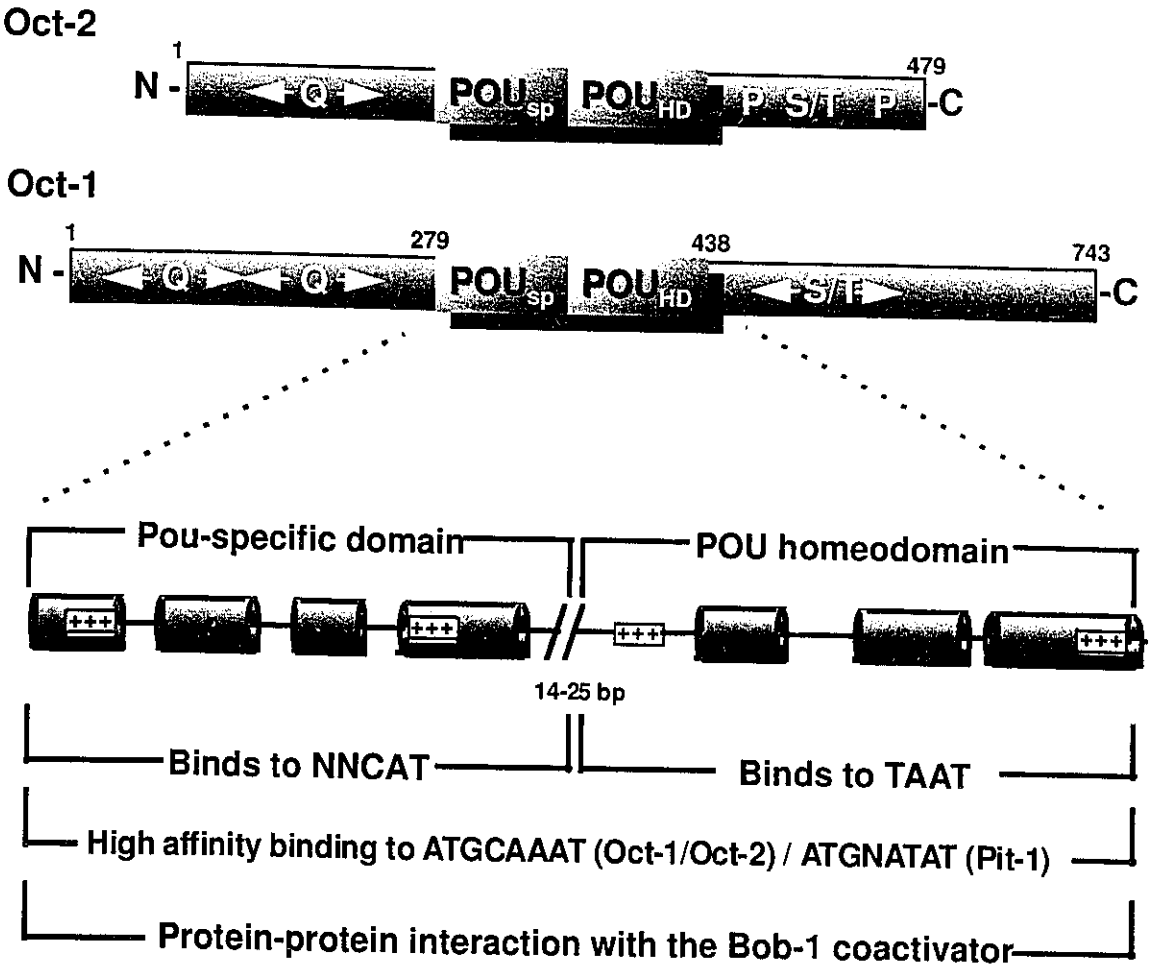
POU factors have a centrally located, highly conserved bipartite DNA binding domain that can be further divided into a POU-specific

domain (sp) and POU-homeodomain (hd) (68-71) (summarized in Figure 2 for Oct-1/Oct-2). The homeodomain is a conserved DNA-binding domain which is a common component of numerous proteins that regulate transcription during development (72). The POU-sp (60 aa in length) and the POU-hd (80 aa in length) form modular structures that are separated by a short linker sequence (15-27 aa in length) which has no apparent structural properties. The three dimensional structure of the POU domain has been solved using X-ray crystallography (73) and NMR (74, 75). The POU-sp domain has remarkable similarity in structure to the λ and 434 repressors and 434 Cro protein (83-87), while the POU-hd is similar in structure to the engrailed, MATα2, and Antennapedia homeodomains (88-90). Beyond the POU domain there are no structural characteristics in common between POU factor family members. The N- and C- terminal regions, in addition to the POU domain it-self, function in transcriptional regulation.

The Class I POU factors, Oct-1 and Oct-2, recognize the same DNA regulatory element 5'-ATGCAAAT-3' (octamer motif). The POU-sp domains contact the 5' half of the motif (ATGC), while the homeodomains contact the 3' octamer site (AAAT) (68, 70). Although each motif is capable of recognizing it's half site individually, together the POU subdomains bind cooperatively, with the sum of the 2 sets of

Figure 2. Schematic diagram of Oct-1 and Oct-2.

Human Oct-1 and Oct-2 are represented as indicated with the numbers corresponding to amino acid positions (diagram adapted from (76, 77)). The POU domain is centrally located in Oct-1 and Oct-2 and is flanked by glutamine (Q), serine/threonine (S/T) and/or proline (P) rich activation domains. Below the POU domains are further expanded to show details of the POU specific (sp) and POU homeodomains (hd) secondary structures. α-helices are represented by the cylinders and basic regions are highlighted by +s. The POU-sp and POU-hd are separated by a linker segment that is from 14 to 25 amino acids long in various POU family members. Various functional properties of the POU domains are illustrated below the schema. The homeodomains recognize A/T-rich sequences with a TAAT/ATTA-core. Alone, the POU-sp domain recognize longer DNA motifs with a consensus to NNCAT (where, N=any nucleotide). Together the POU-sp and POU-hd 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 the octamer ATGCAAAT motif (65, 66, 78), while Pit-1 binds to sequences that resemble ATGNATAT (79). The POU domain as a whole or in part has been implicated in forming protein-protein interactions with the Bob-1 coactivator (80) and VP-16 (81, 82) as indicated.


Oct-2
1
N -
Q
POU sp
POU HD
P
S/T
P
479
-C
Oct-1
1
279
438
743
N -
Q
Q
POU sp
POU HD
S/T
-C
Pou-specific domain
POU homeodomain
+++
14-25 bp
Binds to NNCAT
Binds to TAAT
High affinity binding to ATGCAAAT (Oct-1/Oct-2) / ATGNATAT (Pit-1)
Protein-protein interaction with the Bob-1 coactivator
Protein-protein interaction:
VP16 (Oct-1)

protein-DNA contacts leading to high affinity binding to the octamer motif (71, 73). Each subdomain binds to DNA in the major groove making base and phosphate specific contacts through helix-turn-helix motifs (91). The N-terminal arm of the POU-hd subdomain makes additional contacts into the DNA minor groove, whereas the linker region has no apparent structural features and does not appear to directly contact DNA.

The octamer motif DNA binding site is found in a large number of gene regulatory regions, including ubiquitous, cell specific and viral promoters recognized by either RNA pol II or RNA pol III. The ubiquitous, 100 kDa, Oct-1 is cell cycle regulated and is a primary determinant for the expression of the histone H2B gene (92-94), the constitutive expression of small nuclear (sn) RNA genes (95-100), and the expression of many viral genes. Octamer motifs have been identified in the viral promoters of the mouse mammary tumor virus (MMTV) (3, 101, 102), the simian virus 40 (SV40) (103, 104) and the herpes simplex virus (HSV) (105). The MMTV long terminal repeat (LTR) has tandem degenerate octamer motifs (see Figure 3) located in the promoter proximal region which are essential in mediating a full transcriptional response of the virus following induction with glucocorticoids or progestins, but which may not be necessary for basal transcription (3). 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 POU-hd (106-109).

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 Herpes Simplex Virus (HSV) protein 16 (VP-16, also referred to as Vmw65, α-TIF, VF65 and ICP25) (110, 111) transcriptional activator and a host cell factor (HCF, also termed C1, VCAF and CFF) (112). The ternary complex has an altered DNA binding specificity compared to Oct-1 alone, with high affinity binding redirected to the TAATGARAT DNA motif (with R=Purine) from the octamer motif (113). For example, the ternary complex, but not Oct-1 alone, is required for the expression of immediate-early (IE) genes of HSV. The POU-hd of Oct-1 is sufficient to induce formation of a complex with VP-16 (114). Further, a single amino acid in the homeodomain distinguishes the ability of Oct-1/Oct-2 to associate with VP-16 (81). When the amino acid at position 22 of the homeodomain is a glutamine (E) residue as in the primary structure of Oct-1, the octamer factor is able to associate with VP-16. However, if the residue is an alanine (A) as in the primary sequence of Oct-2, the octamer factor is unable to associate with VP-16 (81). Once the complex is bound to the HSV enhancer, VP-16 directs transcriptional activation through a

potent carboxyl-terminal acidic activation domain (115).

Oct-2 (~60 KDa) is expressed in B lymphocytes and cells of the central nervous system (57, 65, 116) and is involved in the lymphoid-specific expression of immunoglobulin light and heavy chains (117). Octamer response elements are found in both promoter and enhancer regions of these genes (78, 118-124). Oct-2 is required for B-cell maturation and postnatal survival of mice, but not early B-cell development (125). Normally in homologous Oct-2 null mutant mice, B-cells appear to have developed however, upon external stimulation (T-cell derived signals) B-cells do not increase immunoglobulin expression. These mutant B-cells are functionally defective and unable to form mature B cells that secret immunoglobulins.

While Oct-1 and Oct-2 bind the same DNA sequence motifs, they differentially regulate transcription through promoter selective activation domains (76) (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 (126-128). 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 (76, 129, 130). The activation of mRNA promoters from remote sites by Oct-2 has been

attributed to the unique C-terminal activation domain of Oct-2 (130). By contrast Oct-1, but not Oct-2 is able to selectively activate snRNA promoters (99, 129).

3. Multiprotein complexes mediate the activation of transcription by RNA polymerase II.

Transcriptional activation of eukaryotic genes during development or in response to extracellular signals involves the regulated assembly of multiprotein complexes on enhancers and promoters. Gene transcription by RNA polymerase II (pol II) requires an array of over 20 proteins to be assembled 25-30 bp upstream of a transcriptional initiator element (131, 132). The most common type of promoter element studied to date is an A/T rich element referred to as the TATA box, which usually marks the -25/-30 DNA site (133). At TATA containing promoters, preinitiation complexes (PIC) are assembled in a step wise fashion around the direct binding of the TF-IID protein complex to the TATA element through the TATA-binding protein (TBP) subunit (134). Several other transcription factors, including at least TF-IIB and TF-IIF (minimal requirements), directly recruit RNA pol II to the promoter region to form the PIC. In turn, the RNA pol II holoenzyme (SRB/Pol II complex) aids in the recruitment of at least TF-IIE and TF-IIH to form the complete initiation complex (135). Upon

initiation of transcription, the carboxyl terminal domain (CTD) of the large subunit of RNA pol II becomes partially phosphorylated following transcription factor rearrangement and releases SRB proteins from the RNA pol II holoenzyme. Hyperphosphorylation of the CTD ensues, allowing promoter clearance of the polymerase and release of TF-IIB, F, E and H .

Enhancer regions of genes remote from the promoters also promote the correct assembly of preinitiation and initiation complexes to provide a more favorable environment for transcription to occur, while silencer regions interfere with the process (136). The striking feature of these regulatory regions is that they are able to act on promoters from a distance independently of orientation. Both nuclear receptors and POU factors can act as promoter proximal and enhancer/silencer binding factors, depending on the context of their binding sites. From both types of elements, they may act directly with the initiation complex by recruiting or positioning a rate limiting initiation factor through direct protein-protein interactions; or they may act also indirectly through coactivators.

There is evidence that both octamer factors and nuclear receptors activate transcription by the recruitment of both basal transcription factors and coactivators. The POU domains of Oct-1 and Oct-2 have been shown to interact directly with TBP (137). Recently, a

B-cell specific Oct-1 and Oct-2 coactivator has been identified and cloned, variously termed Bob1, OCA-B or OBF-1 (138-141). That Bob-1 can form a complex on DNA with the POU domain of octamer factors without affecting DNA binding, suggests that Bob-1 helps to recruit the transcriptional machinery. The recent demonstration that Bob-1 can interact with TBP and TF-IIB provide strong evidence to support this coactivation model (80).

Nuclear receptors have been also shown to interact directly with the basal machinery. Steroid receptors have been shown to interact physically with TF-IIB (142), TBP (143) and $TAF_{II}30$, a subunit of TF-IID (144). The amino terminus of TR has been shown to directly interact with TF-IIB (145), while RXR has been shown to directly interact with TBP (146). The physiological relevance of these interactions is unclear, but have been proposed to contribute to transcriptional activation. Recently several, co-factors for nuclear receptors have been identified. These include TRIP-1 (147) (a homologue of yeast SUG-1 (p46) (148)), TIF1 (149), RIP 140 (150), RIP 160 (also known as ERAP 160) (151, 152), SRC-1 (153), GRIP1 (154), CBP and p300 (155). The importance of some of these factors for transcription has been highlighted in coexpression studies, including experiments where the coactivator, SRC-1, was shown to reverse the ability of the ER to squelch activation by PR (153).

(a) Specific mechanisms of transcriptional regulation by GR.

Over the past several years a question of particular interest to me has been to understand how individual transcription factors interact with each other on complex promoters to coordinate regulatory effects. Two possible mechanisms for functional intervention have been investigated. First as just described, it has been proposed that individual transcription factors binding to complex promoter or enhancer regulatory elements can act on initiation complex function through similar and/or different pathways to promote or interfere with initiation. Generally, steroid receptors have been shown to synergistically act on synthetic promoters containing HREs adjacent to DNA binding sites of constitutive transcription factors (2). However, it has also been recognized that transcription factors can modify each others activity directly through protein-protein interactions (156). For nuclear receptors and GR in particular, there are several examples of direct protein-protein interactions with heterologous transcription factors (15, 155, 157-159). Perhaps the most studied, and best understood of these interactions is that between GR and Activating Protein-1 (AP-1).

(b) Characterization of functional interactions between AP-1 and GR.

An intriguing series of positive and negative gene regulatory interactions between GR and AP-1 resulting from direct GR/AP-1 contacts have been described (for review see (31)). AP-1 proteins are a subfamily of transcription factors characterized by a basic leucine zipper (bZIP) DNA binding motif (160). These factors bind to DNA as dimers (161). The components of the AP-1 dimers arise from the pairing of members of the cJun and cFos subfamily of bZIP proteins. The cJun related proteins (i.e. JunB and JunD, etc.) homodimerize with each other while cFos related proteins (i.e. FosB and Fra1) form only heterodimers with cJun related factors (162). AP-1 factors have been shown to be activated through various signal transduction pathways, including those initiated by morphogens such as 12-O-tetradecanoylphobol-13-acetate (TPA) (163). GR/AP-1 regulatory interactions are complex. For example, in many cell lines transcription of the proliferin gene is positively activated by TPA through AP-1 and repressed by glucocorticoids (164). By contrast, in Drosophila S2 cells and human HeLa cells, glucocorticoids enhanced rather than repressed proliferin transcription (162, 165). The main determinant of these transcriptional responses of the proliferin promoter was identified as a 25-base pair composite response element, termed plfG. PlfG is

substantially different from simple GRE and AP-1 binding sites. By DNA footprint analysis, GR and AP-1 were shown to bind plfG simultaneously (162). Strikingly, cJun-cJun homodimers conferred hormonal stimulation of gene transcription in the presence of GR, whereas cells expressing cJun-cFos heterodimers repressed transcription in response to glucocorticoid (162). Thus, both the activation/repression and magnitude of the transcriptional response due to the co-occupancy of the plfG by GR and AP-1 was dependent on the ratio of cJun to cFos in the cell. Cross-linking, coimmunoprecipitation and functional assays have shown that a direct protein-protein interaction between GR and cJun contribute to the regulation observed (162, 166). The binding sites on each of these proteins have been mapped to their respective DNA binding domains. For cJun the bZIP domain was required (167) to interact with the GR zinc finger DNA binding motif comprising at least amino acids 440-533 of rat GR (166, 167).

Other reports have also shown that GR and AP-1 are capable of mutual inhibition in the absence of DNA binding (166). Originally it was postulated that direct GR/AP-1 binding inhibits binding to simple response elements. However, more recently it has also been shown that there may be redundant mechanisms of mutual repression between GR/AP-1. In addition to mutual inhibition of DNA binding,

GR/AP-1 appear to compete for a common co-activator, CREB-binding protein (CBP) (155).

(c) Complex regulatory interactions between GR and Oct-1.

Nuclear receptors and POU transcription factors are spatially and temporally expressed during development and differentiation (18, 19, 57-59, 168, 169). Separately, members of each family regulate transcription of specific genes through distinct DNA binding sites. However on many natural promoters, nuclear receptors and POU factors influence each others ability to regulate transcription (2-17). Numerous gene regulatory regions have been identified that show synergistic or inhibitory transcriptional responses in response to nuclear receptor ligands that also require the participation of POU factors. With one possible exception (170), molecular mechanisms attributed to these transcriptional responses have not been identified.

On promoters containing DNA recognition elements for both glucocorticoid receptor (GR) and octamer transcription factor -1 (Oct-1), activation of gene transcription is synergistic in response to hormone (3, 4). By contrast, on promoters containing DNA recognition elements for Oct-1 alone, gene transcription is repressed in response to glucocorticoids (3, 15, 16). Understanding the mechanism of these complex interactions between GR and Oct-1 will advance

understanding of ways in which transcription factors interact to regulate transcription. Further, it may also be expected to reveal a more general mechanism of how nuclear receptors and POU factors interact in the cell to coordinately regulate gene expression patterns.

4. A system for studying transcriptional synergism between GR and octamer factors.

Mouse Mammary Tumor Virus (MMTV) is a retrovirus that initiates a pathway leading to cellular transformation of mammary epithelial cells by activating transcription of cellular proto-oncogenes flanking viral integration sites (171, 172). In the absence of hormones, the stably integrated MMTV long terminal repeat (LTR) is precisely packaged into an array of at least six nucleosomes that appear to preclude the binding of the ubiquitous transcription factors Oct-1 and Nuclear Factor-1 (NF-1) (173, 174).

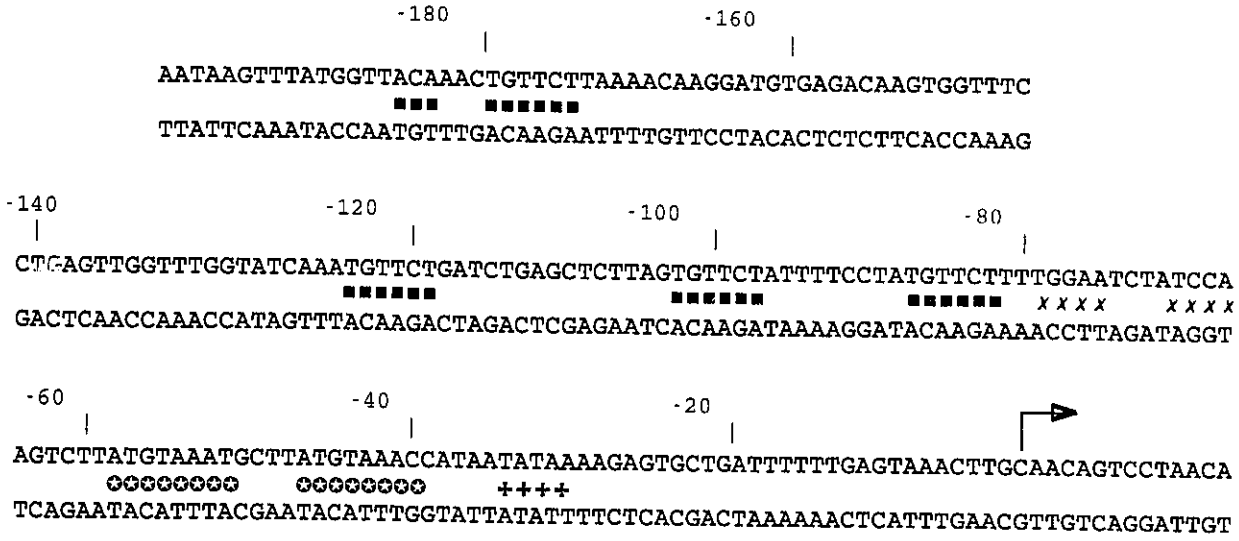
In particular, the promoter proximal nucleosome (-210 to-32) is rotationally packaged in a way that markedly decreases the binding of NF-1. GR binding however, is only minimally impaired (175). Following hormone treatment, GR appears to induce a structural transition in the promoter proximal MMTV nucleosome that renders the octamer motifs and NF-1 binding site accessible. The promoter proximal regulatory region of MMTV is organized as follows (Figure 3), GR

homodimers binds to a cluster of four sites located between -190 and -75 from the transcriptional start site (176, 177) (see Figure 3). Immediately downstream of the HREs are binding sites for an NF-1 dimer (-75 to -62) and two octamer motifs located between -56 and -37. All of these binding sites are required for optimal response to steroid (3, 178). Although the octamer motifs are degenerate when compared to the high affinity consensus motif 5'-ATGCAAAT-3', Oct-1 is able to bind these motifs with somewhat reduced affinity (3). *In vitro*, footprinting experiments reveal only weak protection over the Oct-1 binding sites at limiting concentration of purified Oct-1. However preincubation of the DNA template with purified GR at concentrations that saturate the GREs increase the occupancy of the octamer motifs (3). Thus GR appears to facilitate Oct-1 binding to the MMTV LTR. Further, this interaction is not limited to GR and Oct-1, as similar observations have been reported for PR.

The use of synthetic promoters has also provided insight into the mechanisms of interaction between GR and Oct-1 (4). Consistent with facilitated binding of Oct-1 on the MMTV LTR in response to activated GR, transcription from a promoter containing octamer motifs and HREs in *cis*, is activated synergistically by GR and Oct-1 following hormone treatment (4), but only very poorly by Oct-1 alone. Together, these data

Figure 3. Schematic representation of the promoter proximal regulator region of the MMTV LTR.

The region of the LTR encompassing -187 to +17 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 nuclear factor -1 binding sites are highlighted by (✗) and two degenerate octamer motifs are represented by (✪).


-180
-160
AATAAGTTTATGGTTACAAACTGTTCTTAAAACAAGGATGTGAGACAAGTGGTTTC
TTATTCAAATACCAATGTTTGACAAGAATTTTGTTCCTACACTCTCTTCACCAAAG
-140
-120
-100
-80
CTGAGTTGGTTTGGTATCAAATGTTCTGATCTGAGCTCTTAGTGTTCTATTTTCCTATGTTCTTTTGGAATCTATCCA
GACTCAACCAAACCATAGTTTACAAGACTAGACTCGAGAATCACAAGATAAAAGGATACAAGAAAACCTTAGATAGGT
-60
-40
-20
AGTCTTATGTAAATGCTTATGTAAACCATAATATAAAAGAGTGCTGATTTTTTGAGTAAACTTGCAACAGTCCTAACA
TCAGAATACATTTACGAATACATTTGGTATTATATTTTCTCACGACTAAAAAACTCATTTGAACGTTGTCAGGATTGT

suggest that transcriptional activation of MMTV by Oct-1 may be dependent, at least in part, upon the recruitment of Oct-1 to the MMTV LTR by GR (3).

Studies using promoters with consensus octamer motifs and no GRE such as the histone H2B gene revealed that GR inhibited transcription in response to hormone agonist (4). The mechanism by which this transcriptional repression was achieved has been controversial. Initially, it had been suggested that the repression was indirect and due to squelching of a rate limiting co-factor (4). However the molecular data to reinforce this statement was weak. Later, a more comprehensive study showed a direct interaction between GR and Oct-1 using coimmunoprecipitation and crosslinking assays (15). Moreover, *in vitro*, GR was able to compete for Oct-1 binding to DNA in gel mobility shift assays. Surprisingly, Oct-1 was unable to compete for the binding of GR to a GRE. The homeodomain of Oct-1 appeared to be important for interaction with GR as determined by domain swapping experiments (15). Further, other experiments showed that activation of transcription by Oct-1 and Oct-2 could also be repressed by GR (4).

Thus, it appears that a direct interaction between GR and Oct-1 modifies transcriptional regulation by both GR and Oct-1 or Oct-2.

Further, the lack of similarity between Oct-1 and Oct-2 outside the POU domain suggests the POU domains to be the most likely site of interaction with GR. Therefore, I have undertaken to determine the nature of GR/Oct-1/-2 binding and to map the interaction domain of GR responsible for interaction with Oct-1.

5. Physical methods of detecting protein-protein interactions

To understand the nature of protein-protein interactions between transcription factors in a cell, the interactions and the extent to which they take place in the cell must be identified. A direct protein-protein interaction between purified GR and Oct-1 has been observed (15). Physically mapping the interaction domains will provide insight into the nature and consequence of this interaction as it applies to the known functions of these proteins.

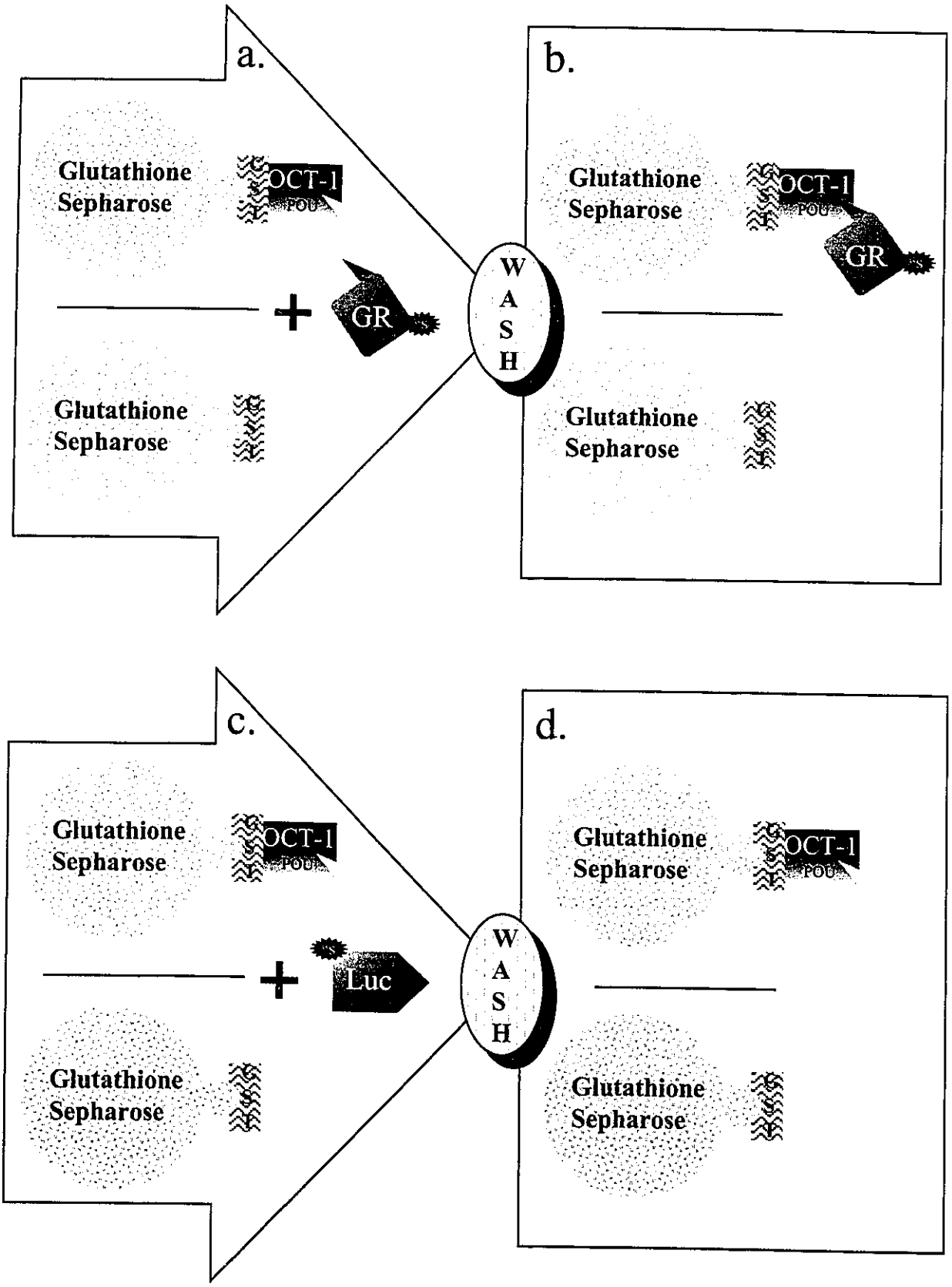
Library based methods are used to screen large libraries for genes or fragments of genes whose products interact with a protein of interest (179). The yeast two hybrid system is commonly used for these studies, but is not suitable for this work as the binding proteins have been identified. In addition, the yeast system is further complicated by the inability of Oct-1 to retain functional activity in

yeast either by its inability to fold properly or exist stably in the yeast cell.

Studies that have investigated protein-protein interactions implicating Oct-1 and VP16 and HCF have used a recombinant protein expression system in *E.coli*, where full length Oct-1 or partial domains of the protein are expressed as a fusion protein (81, 180). A fused selectable tag is used to decrease toxicity of proteins and to aid in their purification from bacteria. I have employed a glutathione-S-transferase (GST) fusion system to study GR/Oct binding, with GST fused to the N-terminus of octamer factor POU domains. This system allows purification of the fused protein under non-denaturing conditions on a glutathione affinity column. In turn, the loaded Sepharose beads is used as a binding matrix for the target protein, in this case nuclear receptors. The experimental scheme followed in the study described in this thesis, is shown in Figure 4. Various deletion mutants of GR were expressed and radiolabelled using an *in vitro* translation system. *In vitro* translated products were incubated with the matrix which was subsequently extensively washed. Specifically bound proteins are retained on the GST-Oct-1 beads while other factors are lost. As a control for specific binding the same proteins were tested with an affinity matrix containing GST alone.

Figure 4. Schematic representation of the assays for GR binding to the immobilized POU domain of Oct-1/Oct-2 used in this study.

a. Shows radiolabeled rat GR incubated with either immobilized GST-Oct-1 (upper) or GST alone (lower) in binding buffer. b. Following extensive washing (4 times each with 30 volumes of binding buffer), GR was retained by GST-Oct-1 but not GST alone. c. Shows the negative control, radiolabeled firefly luciferase (Luc) incubated with either immobilized GST-Oct-1 (upper) or GST alone (lower). d. Following extensive washing (4 times each with 30 volumes of binding buffer), luciferase was not retained to either affinity matrices.


a.
Glutathione Sepharose
OCT-1
POU
GR
Glutathione Sepharose
W A S H
b.
Glutathione Sepharose
OCT-1
POU
GR
Glutathione Sepharose
c.
Glutathione Sepharose
OCT-1
POU
Luc
Glutathione Sepharose
W A S H
d.
Glutathione Sepharose
OCT-1
POU
Glutathione Sepharose

Protein affinity chromatography is extremely sensitive. At high concentrations of immobilized protein, interactions with binding constants as weak as 10^{-5} M have been detected, while the weakest interaction to be physiologically relevant is estimated to be in the 10^{-3} M range (181). As a control against non-specific binding, firefly luciferase was used as a non-specific cytoplasmic protein in the association assays.

6. Objective

To map and assess the physical interaction between GR and the POU factors, Oct-1 and Oct-2 *in vitro*, as a first step towards understanding complex interactions between nuclear receptors and POU factors that affect the expression patterns of their common target genes.

II. MATERIAL AND METHODS

1. Receptor ligands

The synthetic GR ligands 1,4-Pregnadien-9α-fluoro-16α-methyl-11β,17,21-triol-3,20-dione (dexamethasone), 1,4-Pregnadien-9α-fluoro-16α-methyl-11β,17,21-triol-3,20-dione 21-methanesulfonate (dexamethasone 21-mesylate) and 1,3,5(10)-estratrien-3,17b-diol (17β-estradiol) were obtained from Steraloids.

2. Expression constructs

Plasmids that expressed Glutathione-S-Transferase - Octamer transcription factor (GST-Oct) fusion proteins were a kind gift from Dr. W. Herr (Cold Spring Harbor, New York). The GST-Oct-1 (71) plasmid expressed amino acids (aa) 280-439 of human Oct-1 (65) protein fused C-terminally to GST (182) and under the transcriptional control of a T7 RNA polymerase promoter. The GST-Oct-1 plasmid included a thrombin cleavage site between the tag and the POU domain of Oct-1. The mutant fusion proteins GST-Oct-1 E22A, GST-Oct-1/Ho2 and GST-Oct-1/Ho2 A22E (81) were expressed in the same context as GST-Oct-1. The Ho2 fusion proteins contained the Oct-1 POU domain with the 7 substitutions of amino acids contained in the Oct-2 homeodomain.

The point mutations were numbered according to their position relative to the homeodomain (183). The amino acid before the number represents the residue found in the wild type protein while the second represents the residue in the mutant protein. GST alone was expressed from the pGEX 3X vector (Pharmacia).

Rat GR expression plasmids were graciously provided by Dr. K. Yamamoto (UCSF, California). For *in vitro* translation the full length GR was expressed from the pRDN93 plasmid (23) which lacks the 21 consecutive glutamine residues in the N-terminal portion of the receptor at amino acids 75-96. The X-receptor derivatives (X795, X781, X768, X671, X616, X568, XΔ616-694, and XΔ616-632) (184), with the exception of X556, express the first three codons of HSV thymidine kinase (TK) fused to GR amino acid 407 and are C-terminally truncated up to the indicated amino acid (i.e. X556 contains aa 407-556). Following the indicated C-terminal receptor amino acid the peptides are extended by 6-34 non-receptor amino acids from the plasmid multiple cloning site (pSP64) or within an inserted synthetic oligo, adding 5-7 non-receptor amino acids. No differences were attributable to the extended non-receptor amino acids (184, 185). Derivatives denoted with Δ lack the receptor amino acids delineated by the numbered position within rat GR.

The N556 C-terminally truncated receptors encoded GR amino acids 1 to 556 terminating in the plasmid multiple cloning site adding 7 non-receptor amino acids (186). The point mutated GRs expressed in the context of the X556 (26) include C460Y, R479K, R489K, N491S, C492R, C495Y and L501P (37). The point mutant receptor derivatives C500Y and G504R were subcloned from a yeast (pG-1 (187)) and mammalian expression vector (pVARO (40)), respectively. A XhoI/SacI fragment, 465 bps in length from the G-C500Y yeast vector (37) was ligated into the XhoI/SacI digested pT7-X556 parental plasmid replacing the wild-type sequences with those containing the point mutation. The point mutation was confirmed by sequencing using a T7 DNA polymerase sequencing kit (Pharmacia). The GR sequence for the G504R point mutation was derived from the pVA-G504R mammalian expression vector (184). The XhoI/PstI (blunted) 327 bps fragment was inserted into the XhoI/SacI (blunted) pT7X556 plasmid creating vector expressing GR amino acids 407-523 with the desired point mutation. The point mutant derivatives may be *in vitro* transcribed-translated or bacterially expressed through the T7 RNA polymerase promoter (188) and the lac UV 5 promoter (189), respectively. The peptide product contains 10 amino acids of leader sequence from the bacteriophage T7 gene 10 product (189) and 13 non-receptor amino acids from the polylinker region (37).

Other nuclear receptors *in vitro* translated used in this study include the human estrogen receptor (ER) (24), the mouse dominant negative retinoic acid receptor α' (RAR) (190), the mouse retinoic X-related receptor α (RXR) (191), the rat thyroid hormone receptor (TR) - erb Aα-2 (192) and the drosophila FTZ-F1α orphan receptor (193). The firefly luciferase protein from the Promega coupled *in vitro* transcription-translation kit was used as a non-specific protein.

3. Plasmid preparation

Plasmid DNAs were transformed by a $CaCl_2$ heat-shock method (194) into a competent *Escherichia coli* -DH5α strain and plated on agar plates containing 150 mg/ml of ampicillin. Colonies were allowed to grow overnight and single colonies were used to inoculate 5 ml overnight cultures. This in turn was used in the large scale inoculation of 500 ml of liquid broth (LB) and grown overnight. The plasmid DNA was prepared using alkaline-lysis maxi-preparation procedure (195, 196) followed by 2 sequential cesium chloride gradients (197). Using this procedure, 80-99% of plasmid DNAs were supercoiled, as estimated by agarose gel electrophoresis and ethidium bromide staining

4. Restriction digests for *in vitro* translations

Where indicated below, DNA was restriction enzyme digested prior to *in vitro* transcription-translation at a plasmid DNA concentration of 0.05 mg/ml with the appropriate restriction enzyme (New England Biolabs) and reaction buffer. The GR T7N556 plasmid (186) was completely digested with Pst-I or Sph-I prior to transcription-translation to produce the C-terminal truncations N523 and N494, respectively. The RAR α' plasmid (190) was restricted using Bsu-36I, Pvu-I and Xmn-I prior to transcription-translation to produce the N181, N161 or N145 C-terminal truncations, respectively. The DNA was phenol/chloroform extracted and ethanol precipitated in 0.3 M sodium acetate. The pellet was washed at least two times with 70% ethanol at room temperature. It should be noted that DNA precipitation with alternative salts namely, ammonium acetate or sodium chloride yielded a DNA template with significantly lower yields of ^{35}S-Met incorporated into specific peptide following *in vitro* transcription-translation. The DNA pellet was resuspended in a volume of double distilled water to a concentration of 0.5 mg/ml. The restriction digests were >98 % complete as judged by agarose gel electrophoresis and visualization with ethidium bromide staining.

5. Expression and purification of GST fusion proteins from bacteria.

The expression and purification of GST or GST-fusion proteins were carried out as previously described (81). The BL21 (DE3) strain of E.coli (gift from Dr M. Ekker, University of Ottawa, ON) containing the pGST-Oct or pGEX-3X (GST only) plasmids were grown to late log phase to an OD (λ = 600 nm) of 0.8-1.0. The culture was induced to express individual fusion proteins with 0.01% (w/v) isopropyl β-D-thiogalactopyranoside (IPTG) (Sigma) for at least four hours at room temperature. The IPTG was induced to express the stable integrate of the bacteriophage gene 1 product (198) to produce T7 RNA polymerase unique to the BL21 (DE3) strain, which in turn initiated high level of expression of the target gene encoded by the plasmid.

The suspension was cooled on ice for ten minutes and centrifuged at 4000 X g for six minutes to pellet the bacteria. The bacterial pellet was resuspended in lysis buffer (25mM 4-(2-hydroxylethyl)-1-piperazine ethanesulfonic acid (HEPES), pH 7.9, 100 mM KCl, 20% glycerol, 2mM ethylenediaminetetraacetic acid (EDTA), 2mM dithiothreitol (DTT) and 0.2mM phenylmethylsulfonyl fluoride (PMSF). The bacterial suspension was sheared 3 times with a 25 gauge needle and syringe in the presence of 0.1% Nonidet-P 40 (NP-40)

(Sigma). The extract was then sonicated 10 times with 40 second pulses until the suspension became dull (grey) in color with a small probe at 35% duty cycle (Fisher Sonic Dismembrator-Model 300). The insoluble material was pelleted by centrifugation at 28,000 rpm for 30 minutes in the Beckman Ti-60 rotor.

The supernatant was promptly added to glutathione Sepharose 4B (Pharmacia) previously washed three times with 15 volumes of Binding Buffer (0.6 X lysis buffer containing 0.1 % NP-40). Washing comprised of resuspending the sepharose beads in 15 volumes of buffer, followed by centrifugation at 1000 rpm for two minutes and removal of the buffer. The fusion protein was allowed to bind the affinity matrix on a slowly rotating wheel for 90 minutes at 4 °C and then washed 4X with binding buffer. Finally, the sepharose beads were cleared of excess buffer and resuspended in 1:1 volume of sepharose beads to binding buffer termed, slurry. This allowed for precise pipetting of the affinity matrix used in the binding assays. The slurry was preserved for up to 6 weeks with 1mM PMSF and 0.02% Sodium Azide at 4 °C.

6. Coupled *in vitro* transcription-translation

The indicated plasmid DNA was *in vitro* transcribed and translated with T7 or SP6 RNA polymerases using the Promega TNT

coupled rabbit reticulocyte lysate system (199) including 10 mCi/ml of translation grade ^{35}-Sulfur methionine (1,000 Ci/mmol, Amersham). Each translation reaction was performed for 90-120 minutes at 30 °C in a 50 ul volume according to the manufacturer's protocol with the addition of 0.1 mM DTT and 0.4 mM ZnOAc. Translations were stopped by adding an excess (5mM) of unlabelled L-methionine. Before freezing, glycerol was added to a final concentration of 8 % (v/v), flash frozen, and stored at -80 °C.

7. Untransformed and transformed steroid receptor

GRs were transformed (heat shock protein (HSP)-dissociated) (200, 201) from their native untransformed state (HSP-associated) by adding of 0.5 μM of dexamethasone (Dex), dexamethasone mesylate (Dex 21-mes) or 0.4 M NaCl to the rabbit reticulocyte lysate (RRL) translated, radiolabeled GR and incubated for 1 hour on ice then heating at 25°C for 25 minutes. This yielded liganded/unliganded receptors free of HSPs. The untransformed receptor/HSP complex was stabilized by the addition of 20 mM Sodium Molybdate. The estrogen receptor (ER) was ligand transformed as previously described with the GR (above) but using 1 μM 17β-estradiol as ligand.

8. Binding assays

A quantity of 0.5 µg of GST or GST-fusion protein (equal to about 15 µl of slurry) was used as binding partner for *in vitro* translated radiolabelled protein product. Equal amounts of radiolabeled protein product were added to the binding assay as determined by SDS-PAGE and fluorography. The volume of rabbit reticulocyte lysate added was normalized for GR. Where indicated, ethidium bromide was added to the binding reaction at a concentration of 150 µg/ml to confirm DNA independent protein-protein interactions (202). For the DNA competition experiments, increasing quantities of the plasmids pTKCAT-ERE or pTKCAT-GRE (203) ranging from 10-1000 ng was added to the binding reaction. The binding was allowed to continue for 90 minutes at 4 °C with gentle agitation. Following association, the Sepharose beads were washed 4X with 500 µl of binding buffer and the bound proteins were eluted by boiling the beads for 3 minutes in Laemmli-sample buffer (120 mM Tris-HCl pH 6.8, 20 % (v/v) glycerol, 4% (w/v) sodium dodecylsulfate (SDS), 10 % beta-mercaptoethanol (v/v), and 0.2 mg/ml of bromophenol blue) (204). The proteins retained by the affinity matrix were analyzed by SDS-PAGE and visualized by fluorography. Ten percent of the protein added to each binding assay

was also loaded to demonstrate the amount of labeled proteins added to the incubations.

9. Sucrose gradients

Sucrose gradients (205, 206) were used to analyze the transformation status of the GR. The full length GR/heat shock protein (Hsp) complex has a sedimentation coefficient of 8S on a sucrose gradient while the transformed receptor runs as 4S. Stock solutions of 15, 20, 25 and 30 % (w/v) sucrose were prepared in gradient buffer (20 mM Tris-HCl pH 7.4, 1 mM EDTA, 10 % glycerol, 1 mM DTT) containing either 20 mM Na_2Mo_4 or 0.4 mM NaCl stored at 4 °C. Sodium chloride was preferred to potassium chloride because potassium cations precipitate SDS during gel electrophoresis. Each stock solution (1 ml) was added to a centrifuge tube (Beckman) beginning with the lowest concentration sucrose solution using a syringe and a spinal needle. The gradient was made by underlying the previously added solution, in order of increasing concentration of sucrose. The gradient was allowed to linearize for 12-16 hours at 4 °C. The protein samples consisting of 5-10 µl of ^{35}S-labeled *in vitro* translated GR derivatives (X795, X671 and X616) were layered on the surface of individual gradients and centrifuged at 55,000 rpm in a SW 55Ti rotor (Beckman) at 4°C for a minimum of 16 hours. Following

centrifugation, fractionated samples of two drops each (~50 µl) were collected from the bottom of a sealed tube by applying positive pressure using a peristaltic pump. The fractions were analyzed by SDS-PAGE and visualized by fluorography. The linearity of the sucrose gradient was monitored by measuring the refractive index of each fraction with a refractometer (Fisher Scientific) and by plotting the refractive index as a function of fraction number. Internal (rabbit hemoglobin) and external (^{14}C bovine serum albumin) protein standards (207) have sedimentation coefficients of 4.2 S and 4.6 S, respectively. The rabbit hemoglobin was identified visually while the presence of BSA was monitored by scintillation counting.

10. Partial purification of the DNA binding domain of GR from bacterial lysates.

The T7X556 plasmid (26) was transformed into the E. coli BL21/pLys strain using the $CaCl_2$ method (194). A colony was grown for 24 hours and used to inoculate a 5 ml overnight culture. In turn, the 5 ml culture was used to inoculate a 500 ml culture for large scale preparation. The culture was grown to late log phase OD (λ=600nm) of 0.8-1.0 units and induced to express the target protein by addition of 0.01% (w/v) IPTG overnight at room temperature.

The GR partial purification scheme was adapted from Freedman et al. (1988) (26). The bacteria was pelleted by centrifugation and resuspended in 3 volumes of TGEz50 buffer (50 mM Tris-HCl pH 7.5, 50 mM NaCl, 10 % glycerol, 0.5mM EDTA, 50 μM $ZnCl_2$, 5mM DTT and 0.5mM PMSF). The bacterial suspension was sheared and sonicated as previously described in the purification of the GST fusion protein. Sodium deoxycholate (Sigma) was added to a final concentration of 0.05% and the lysate was gently stirred for 30 minutes on ice. The supernatent was cleared of insoluble cellular debris by centrifugation at 60,000 X g for one hour (Beckman Ti60 rotor @ 28,000 rpm). The lysate was transferred to fresh tubes and brought to 0.2% polyethylenimine in a drop wise fashion from a 5% stock solution to precipitate bacterial DNA. The DNA was pelleted by centrifugation at 35,000 Xg for 30 minutes (Beckman Ti60 rotor @ 19,000 rpm). The supernatant was once again transferred to fresh centrifuge tubes and brought to 10% ammonium sulfate saturation with saturated solution of ammonium sulfate. The precipitate was removed by centrifugation at 12,000 Xg for 20 minutes. The supernatant was brought to 35 % saturation with saturated ammonium sulfate solution and centrifuged at 12,000 Xg for 20 minutes. The partially purified GR DBD protein pellet was resuspended in TEGz50 buffer and dialyzed against the same buffer overnight. The GR specific protein represented 5-10% of the

total protein as estimated from SDS-PAGE. The specific band was confirmed with the anti-GR antibody, BUGR 2 (208) using a standard western blot procedure (209). The GR was visualized using a horseradish peroxidase linked secondary antibody (Amersham) with an Enhanced Chemiluminescence (ECL, Amersham).

11. SDS-Polyacrylamide gel electrophoresis

The SDS-PAGE was carried out as previously described (204). Protein samples were diluted in Laemmli-sample buffer and denatured by boiling for 3 minutes. The samples were loaded on a 8%-15% separating gel (204) with a 4% stacking gel at a thickness 0.75 mm. The gels were poured using a mini-protein gel apparatus (Biorad) and run at 160 volts for 40 minutes. Gels used for resolving radiolabeled proteins were fluorographed while those containing non-radiolabeled samples were visualized using Coomassie Blue staining.

12. Fluorography

Gel fluorography was used to enhance the low beta emitting property of ^{35}Sulfur atoms by incorporating a fluor directly into a polyacrylamide gel (210). The gel was dehydrated by soaking in glacial acetic acid for 15 minutes and then placed in 20 % (w/v) 2,5-diphenlyoxazole (PPO) in glacial acetic acid for 20 minutes. The incorporated PPO was precipitated in the gel by extensive washing with

water. Finally, the gel was dried under vacuum with low heat. Heat was maintained below 65 °C not to damage the capacity of the fluor to emit light. Once the gels were dry, they were autoradiographed using Kodak XAR-5 film exposed at -80 °C. Typically, exposures ranged from 9-48 hours.

13. Removal of the zinc from the finger structure using reducing agents.

Bacterially expressed and *in vitro* translated, radiolabeled GR DNA binding domain were treated in duplicate reactions essentially as previously described to strip the receptor DBD of zinc coordination and then for one sample to restore its capacity to bind zinc (26, 211). Two sources of GR were used in this analysis. The bacterially expressed X556 was used as source of GR for the electrophoretic mobility shift assay (EMSA) while the *in vitro* translated GR X616 was used in the Oct-1 binding assay. The GR protein products were treated with 20 mM hydrogen peroxide in the presence of 50 mM EDTA for one hour on ice. Catalase (1300 units) was added to the reaction for 15 minutes on ice to inactivate the hydrogen peroxide by converting it to oxygen and water. This was followed by reducing the thiol groups with 0.1 M DTT in the presence or absence of 250 μM $ZnCl_2$. The *in vitro*-translated and bacterially expressed GR derivatives were tested for their ability to

bind to Oct-1 or a glucocorticoid responsive element (GRE), respectively. Zinc coordination was restored to 10% of its original binding capacity as judged by an EMSA.

The *in vitro* translated GR-receptor source was also treated with methyl methane-thiosulfonate (MMTS) to selectively reduce thiol groups in the DNA binding domain of GR (211-214). MMTS reacts in a highly specific and reversible manner with protein thiol groups by adding a thiomethyl group. Here GR was treated with 5 mM MMTS for one hour after which the MMTS was quenched by adding 10 mM L-cysteine for one hour at 4 °C. The X556 protein was tested for its ability to bind Oct-1 in a binding assay.

14. Electrophoretic mobility shift assays

A glucocortioid responsive element (GRE) was used as a probe to test the ability of the GR DBD to bind to its responsive element using a standard method (215). The 42 bp probe was excised from the pTKCAT GRE reporter construct (203) using Bam-HI and Hind-III. The vector was first linearized using Hind III and filled in with the Klenow fragment of DNA polymerase I (New England Biolabs) in the presence of 50 μCi of α-^{32}P-dATP (Amersham) and 2nM of each unlabeled dCTP, dGTP, dTTP followed by a Bam HI restriction digest. The 42 bp fragment was separated by electrophoresis using an 8 % polyacrylamide

non-denaturing TBE gel. The gel was exposed wet to XAR-5 film for 5 minutes at room temperature. The labelled probe was excised and crushed through a needle hole in the bottom of an eppendorf centrifuge tube. The labelled probe was eluted from the crushed gel pieces using a high salt buffer (0.5M ammonium acetate, 1mM EDTA) (216) on a rotating wheel, overnight at room temperature. The eluted probe was phenol/chloroform extracted, precipitated with ethanol and resuspended in TE (10 mM Tris-HCl pH 7.9, 1 mM EDTA).

Binding assays were performed essentially as previously described (215) using 0.5 ng of GRE probe in 12 mM HEPES pH7.9, 12% glycerol, 60 mM KCl, 0.12 mM EDTA, 500µg/ml bovine serum albumin (BSA), 2 µg of highly sheared salmon sperm DNA and 1 µl of partially purified recombinant GR-DBD. The binding assay proceeded for 20 minutes at room temperature before being loaded on a prerun 0.8-mm thick, 4% polyacrylamide gel with 37.5:1 ratio of acrylamide: bisacrylamide in 0.5 X TBE running buffer. The gels were run at 150 volts for 225 volt hours, dried at 80 °C under vacuum before being exposed to XAR-5 film (Kodak) using a Lightning Plus (DuPont) intensifying screen at -80 °C.

III. RESULTS

1. Binding GR to GST fusion proteins *in vitro*

In the following experiments, the main assay used was adapted from a protocol established by Lai et al. 1992 (81) to investigate protein-protein interaction between Oct-1 and the HSV VP-16 protein (81). My assay system used the immobilized POU domain of octamer transcription factors as an affinity matrix for the binding of radiolabeled nuclear receptor (summarized in Figure 4). The affinity matrix usually consisted of the POU domain of Oct-1 fused N-terminally to GST immobilized on a glutathione sepharose solid support system. *In vitro*-translated, ^{35}S labeled nuclear receptors were added to immobilized GST-Oct-1 in binding buffer and incubated for 90 minutes at 4°C. Following 4 washes with 30 volumes (hydrated sepharose beads or slurry) of binding buffer, bound proteins were eluted by boiling in SDS-sample buffer and resolved by SDS-PAGE. Following fluorography, the radiolabelled proteins were compared with a sample representative of the input proteins. Generally, approximately ten percent of the ^{35}S labeled protein added to the binding assay remained associated with the immobilized Oct POU domain.

2. The GR binds the POU domain of Oct-1 *in vitro.*

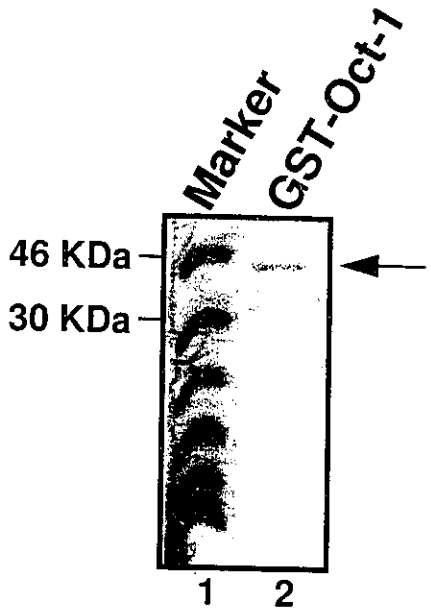
Previously, coimmunoprecipitation assays had demonstrated that purified Oct-1 immunoprecipitated with a GR specific antibody, but not with a control antibody in the presence of purified GR (15). This, together with the ability of GR to repress transcription by Oct-2 and the inability to detect immunoprecipitation of an Oct-1 chimeric protein with a region substituted in the homeodomain, suggested that the POU domain of Oct-1 may be sufficient to direct a protein-protein interaction with GR (3, 15).

In an attempt to determine whether GR could directly bind to the POU domain of Oct-1 *in vitro,* I compared the binding of ^{35}S labeled wild-type GR to immobilized GST-Oct-1 (Oct-1 aa 280-439, human) or GST alone (Figure 4). Typically, for each binding assay 0.5 ug of the 44 KDa GST-Oct-1 recombinant protein was used as determined by Coomassie Blue staining (Figure 5a). In the first experiment, ligand treated rat GR was retained by the immobilized GST-Oct-1 and not GST alone, suggesting that GR associated specifically with the Oct-1 moiety (Figure 5b, lanes 1 and 3). The association appeared to be specific as the radiolabeled protein in the reticulocyte lysate represented less than 1/20,000 of the total protein used in the binding assay, yet strong binding was observed. Further, when the cytoplasmic protein firefly luciferase was similarly incubated with GST-Oct-1 it was not retained

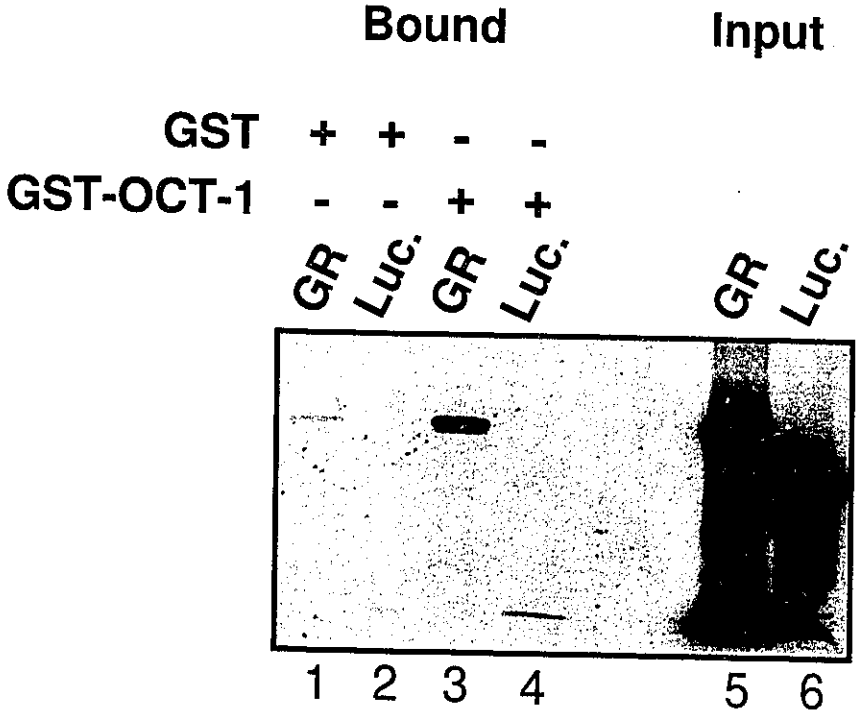
Figure 5. GR binds the POU domain of Oct-1 *in vitro*.

a. A sample of the quantity of GST-Oct-1 used for a typical binding assay. GST-Oct-1 was produced and purified from *E.coli* as described in the methods. A sample equal to that used in a typical binding assay was eluted from glutathione sepharose by boiling in SDS-sample buffer. The eluted protein was resolved by 15% SDS-PAGE and visualized by Coomassie Blue staining (lane 2, GST-Oct-1 is indicated by the arrow). A prestained molecular weight marker (Biorad) is shown in lane 1 with the molecular weights of the indicated proteins indicated to the left. The quantity of GST-Oct-1 used in each binding assay is estimated to be 0.5 μg as judged by Coomassie Blue staining using BSA as a protein standard. b. ^{35}S-Met labeled, Dex-treated GR (lanes 1 and 3) or firefly luciferase (Luc., lanes 2 and 4) *in vitro* translated in rabbit reticulocyte lysate were incubated with GST (lanes 1 and 2) or GST-Oct-1 (lanes 3 and 4) as described in the methods. Bound proteins were resolved by 8% SDS-PAGE and visualized by fluorography. Lanes 5 and 6 are examples of 100% of the input GR (lane 5) and luciferase (lane 6).

a.


Marker
GST-Oct-1
46 KDa
30 KDa
1
2

b.


Bound
Input
GST + + - -
GST-OCT-1 - - + +
GR
Luc.
GR
Luc.
GR
Luc.
1 2 3 4 5 6

by either GST-Oct-1 or GST alone (lanes 2 and 4). Thus the POU domain of Oct-1 was sufficient to extract full length ligand treated GR from rabbit reticulocyte lysate.

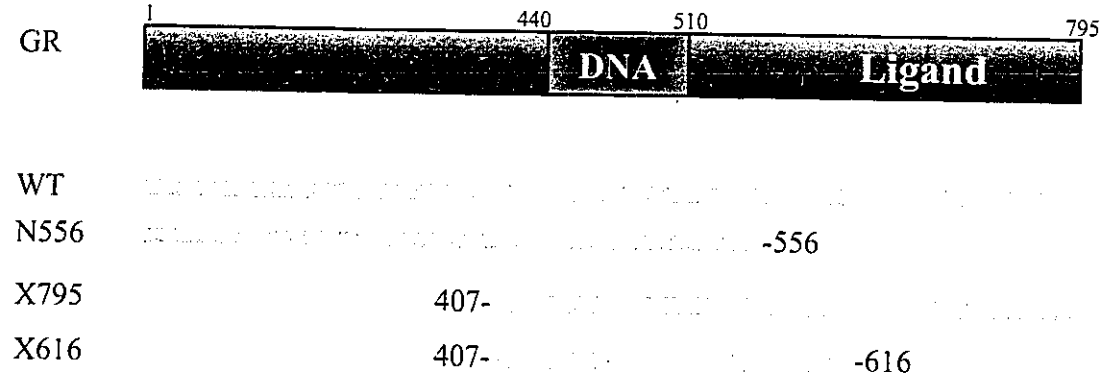
Following *in vitro* translation but prior to exposure to steroid, the ^{35}S-labeled GR is associated with heat shock proteins (217). Receptor derivatives containing the molybdate-treated C-terminal ligand binding domain in addition to the DNA binding domain were not appreciably retained by the immobilized GST-Oct-1 (Figure 6b, lanes 1 and 5), suggesting that the "untransformed" receptor was unable to associate with Oct-1. Both the full length GR and N-terminally deleted GR X795 (X=407) retained this property. Thus it appeared that heat shock protein associated GR was unable to associate with immobilized GST-Oct-1. However liganded GR, devoid of heat shock proteins (see below) bound GST-Oct-1 with the same avidity as receptor derivatives unable to bind heat shock protein (Figure 6, lanes 3 and 6).

As the biochemical assay demonstrated that the GR interaction with Oct-1 POU domain was specific, the minimal GR domain necessary to obtain the interaction was evaluated in a standard binding assay using GR deletion mutants (Figure 6, lanes 3 and 4). These data suggested that amino acids 407-556 encompassing the GR DBD were sufficient for binding to immobilized GST-Oct-1, indicating that the N-terminal domain (or A/B domain) and the C-terminal ligand

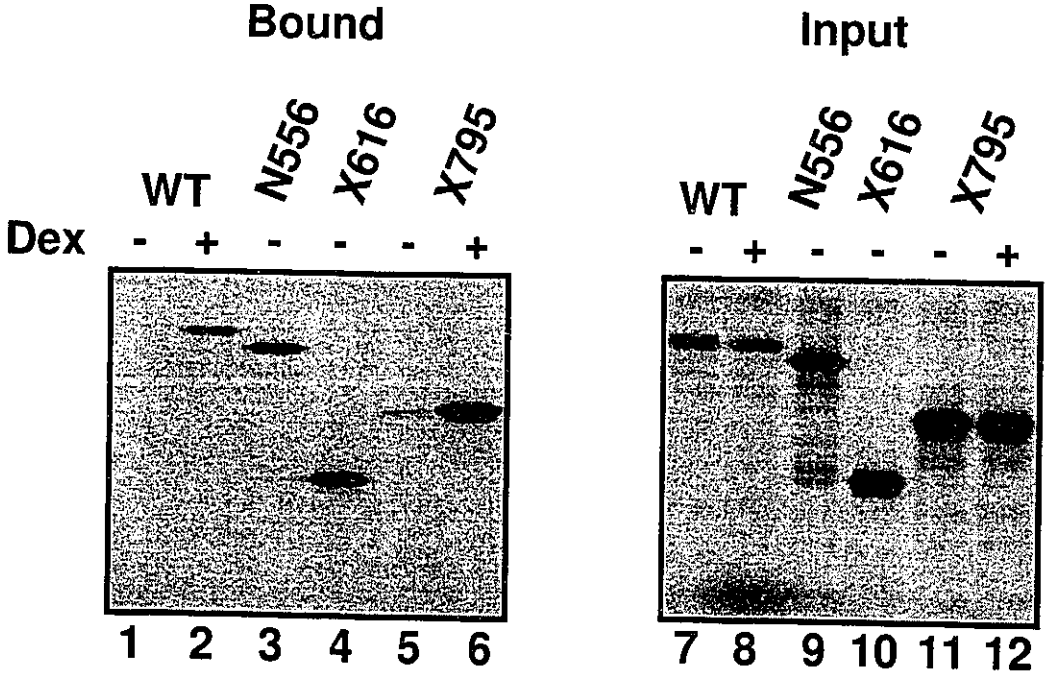
Figure 6. The DNA binding domain of GR is sufficient for binding to the POU domain of Oct-1.

a. At the top is a schematic diagram of the rat GR. The DNA binding and the ligand binding domains are highlighted, with the amino acid positions indicated above. Below is a summary of the ^{35}S-Met labeled, receptor fragments employed in b. b. ^{35}S-Met labeled, molybdate- (lanes 1 and 5) or Dex-treated (lanes 2 and 6) full length GR (lanes 1 and 2) and salt-transformed (lanes 3-5) receptor derivatives that were incubated with GST-Oct-1 as described in the methods. The bound proteins were visualized by 10% SDS-PAGE followed by fluorography. Ten percent of the input proteins are shown in lanes 7-12.

a.


GR
1
440
510
795
DNA
Ligand
WT
N556
-556
X795
407-
X616
407-
-616

b.


Bound
Input
WT
N556
X616
X795
Dex
- + - - - +
1 2 3 4 5 6
7 8 9 10 11 12

binding domain (or E-domain) were dispensable for Oct-1 binding. Moreover, GR deletion mutants N556 and X795 (lanes 3 and 6, respectively) did not show decreased binding to GST Oct-1 when compared to the full length GR (lane 2) (where X=amino acid 407).

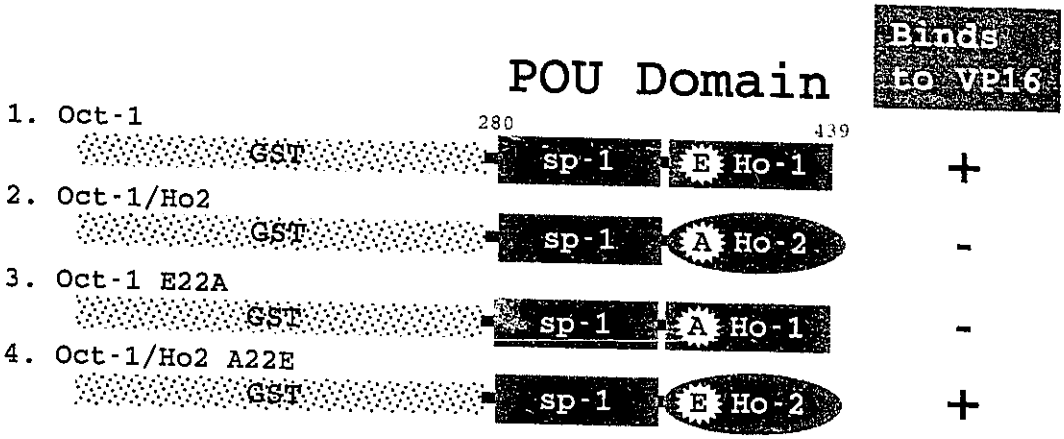
3. The interaction of Oct-1 with GR is distinct from the Oct-1/VP-16 interaction.

Previous experimental data showed that GR can physically interact with Oct-1 (15) and that GR can also repress Oct-2 dependent transcriptional activation (3). By contrast other data suggested that production of a chimeric protein that substituted the second α-helix of Oct-1 with the corresponding α-helix of Oct-2 (see Figure 2) resulted in a chimera that was unable to be coimmunoprecipitated with a GR specific antibody in the presence of purified GR (15). To determine whether GR can associate with both Oct-1 and Oct-2, an Oct-1/Oct-2 chimera was tested for association with ligand treated GR in a standard binding assay. Therefore, in contrast to Kutoh et al. 1992 (15), my results showed that GR was able to associate with GST-Oct-1 with its homeodomain substituted with that of Oct-2 (Figure 7b, lane 5). Only 9 amino acids are different between the POU domains of Oct-1 and Oct-2. 7 of these differences occur in the POU-hd while only two amino acids differ in the POU-sp domain.

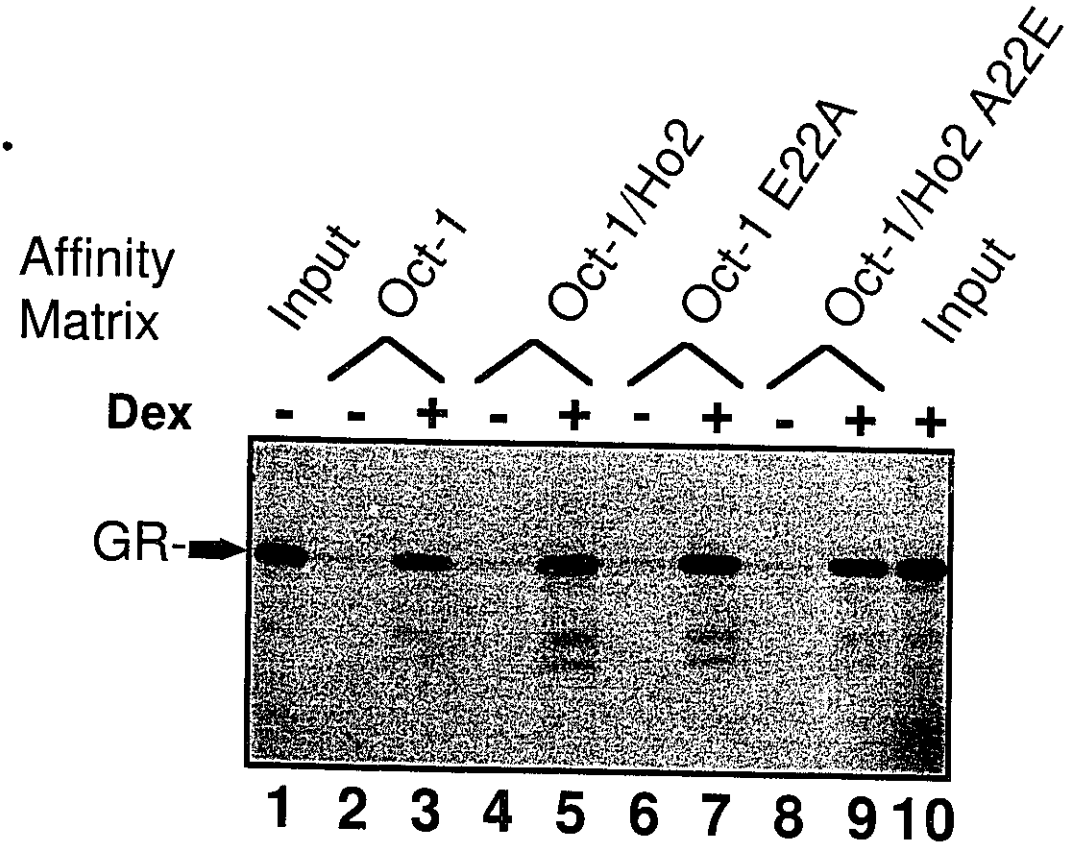
Figure 7. The interaction of GR with Oct-1 is distinct from the Oct-1/ VP-16 interaction.

a. A schematic representation of the GST-fusion proteins employed in b. The POU domains of human Oct-1 (amino acids 280-439) and octamer factor chimera are fused C-terminally to GST (labeled 1-4). Their ability to bind to VP-16 is indicated on the right by +. The POU domain is be subdivided into the POU-specific (sp) and POU-homeodomain (Ho) with the shaded rectangle (-1) representing the subdomains of Oct-1 and the shaded oval representing (-2) the subdomains of Oct-2. The POU domains of Oct-1 and Oct-2 differ by only 9 amino acids with 2 in the POU-sp domain and 7 in the POU-Ho. A single amino acid at position 22 of the Ho-domain is able to distinguish binding to VP-16. When this amino acid is a glutamate (E), the octamer is able to associate with VP-16, in contrast when amino acid at position 22 is an alanine (A), binding to VP-16 is undetectable (81). b. ^{35}S-Met labeled, Dex- (lanes 3, 5, 7, 9)or molybdate-treated (lanes 2, 4, 6, 8) full length GR were incubated with GST-Oct-1 or GST-Oct-chimeras (see a.) as described in the methods. The bound proteins were resolved by 8% SDS-PAGE and visualized by fluorography. Ten percent of the input GR proteins are represented in lanes 1 (unliganded) and 10 (liganded).

a.


POU Domain
Binds to VP16
1. Oct-1
280
439
GST
sp-1
E
Ho-1
+
2. Oct-1/Ho2
GST
sp-1
A
Ho-2
-
3. Oct-1 E22A
GST
sp-1
A
Ho-1
-
4. Oct-1/Ho2 A22E
GST
sp-1
E
Ho-2
+

b.


Affinity Matrix
Input
Oct-1
Oct-1/Ho2
Oct-1 E22A
Oct-1/Ho2 A22E
Input
Dex
- - + - + - + - + +
GR-
1 2 3 4 5 6 7 8 9 10

Oct-1 can form a multiprotein-DNA complex with VP16 and HCF-1 that changes the Oct-1 binding specificity to a TAATGARAT motif, a specific degenerate form of the octamer motif (218, 219). Oct-2 does not have the capacity to form multiprotein complexes involving VP16. Mapping the interaction domain between Oct-1 and VP16 identified the Oct-1 POU homeodomain as sufficient for VP16 binding (81). With only 7 of 60 amino acids that differed between the homeodomain domains of Oct-1 and Oct-2 (65), it was possible to identify a single amino acid residue at position 22 of the homeodomain that could distinguish binding to VP-16 (Figure 7a) (81). When amino acid 22 was a glutamate residue (E) as in wild-type Oct-1 (Figure 7a-1), VP-16 was able to bind to Oct-1 (81). However, when amino acid 22 was an alanine (A) as in wild-type Oct-2 (Figure 7a-2, Oct-1/Ho2), VP-16 was not able to bind to the octamer factor. Further, substitution of amino acid 22 with an alanine (A) in the context of the Oct-1 wild-type protein (Figure 7a-3, Oct-1 E22A) abolished binding to VP-16. Reciprocally, the Oct-1/Ho2 association with VP16 was restored by substituting the amino acid at position 22 to a glutamine residue (E) (Figure 7a.-4., Oct-1/Ho2 A22E). Thus, the glutamate residue at position 22 of the homeodomain expressed in the context of the POU domain was able to confer specific association with VP16. To determine whether GR binding to Oct-1 was similar in nature to the binding of VP-16, the POU domains of mutant

and wild-type Oct-1 were expressed as GST-fusion proteins as previously described, immobilized on glutathione sepharose. Equal quantities of recombinant fusion protein were used as an affinity matrix for the full length dex-treated GR. Interestingly, neither Oct mutation was able to disrupt the protein-protein interaction with radiolabeled GR *in vitro* (Figure 7b). Thus, it was concluded that the protein-protein interaction between GR and Oct-1 is distinct from the binding of Oct-1 to VP-16.

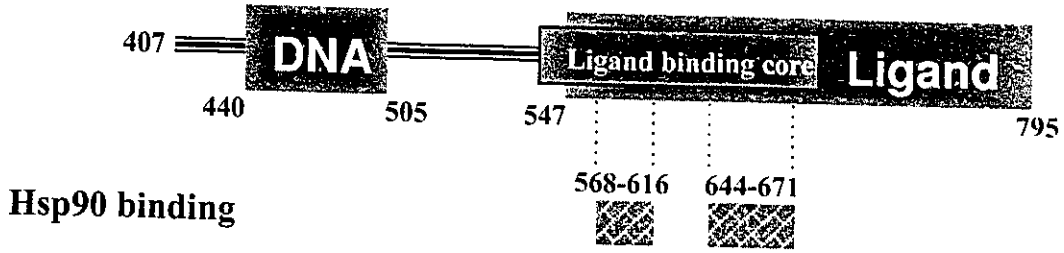
4. GR must be dissociated from heat shock proteins to interact with Oct-1.

As previously shown in Figure 6, ligand-treated GR but not the untransformed receptor was able to associate with Oct-1 (lanes 1 and 5). The untransformed GR is associated with a complex of heat shock proteins in reticulocyte lysate that block DNA sequence recognition by GR. Of these, hsp90 has been shown to directly interact with the C-terminal domain of GR (amino acids 547-795). (Figure 8a) (220, 221). GR, complexed with heat shock proteins maintains the C-terminal domain in an open conformation permitting GR to bind ligand with high affinity (222). Ligand bound GR promotes dissociation of the heat shock proteins to produce a receptor that is able to bind to DNA and activate transcription (for review see (223)). Another way the receptor

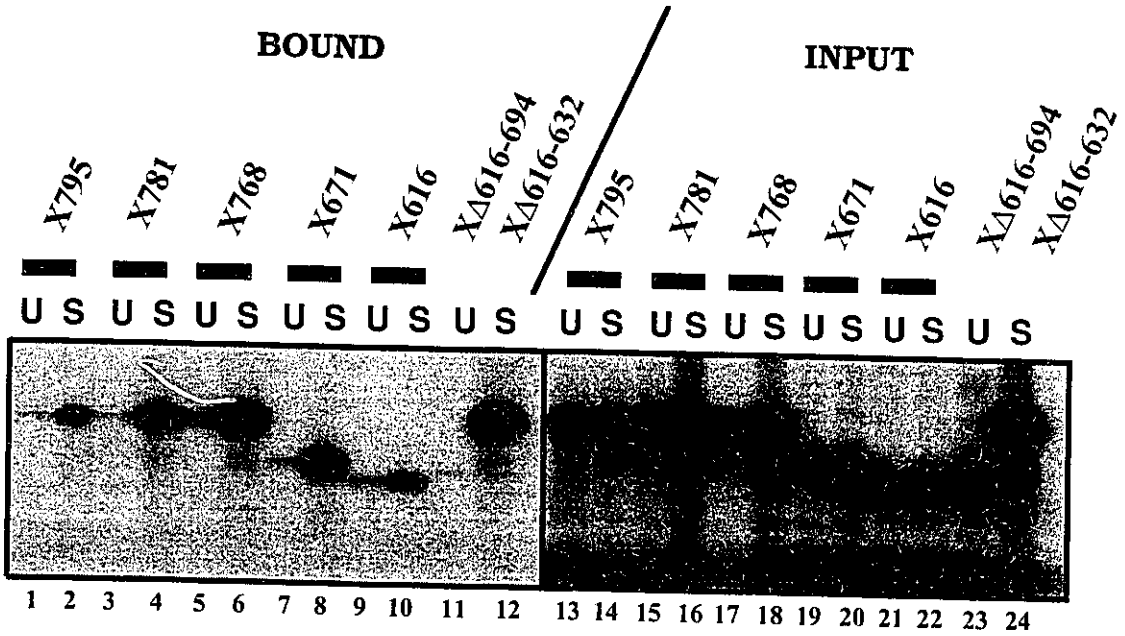
Figure 8. Salt transformed but not untransformed GR derivatives associate with Oct-1.

a. At the top, a schematic representation of amino acids 407-795 of the rat GR (X-795) summarizing the functional domains as in Figure 1. The corresponding amino acid positions are indicated below the schema. Below, a summary of the hsp90 binding domains identified. The hsp90 binding domain of the GR, amino acids 568-616, was identified by deletion experiments (220, 221), while amino acids 644-671 of rat GR were identified by homology with mouse GR, by specific peptides that blocked hsp90 association with GR (44). b. 35S-Met labeled, salt- (lanes 2, 4, 6, 8, 10 and 12) or molybdate-treated (lanes 1, 3, 5, 7, 9 and 11) receptor derivatives were incubated with the Oct-1 affinity matrix as described in the methods. The bound proteins were analyzed by 15% SDS-PAGE and visualized by fluorography. A quantity equal to 10 percent of the input proteins were run on a duplicate gel (lanes 13 - 24). (X = aa 407). U = Untransformed and S = Salt transformed.

a. X795


407
DNA
Ligand binding core
Ligand
440
505
547
795
568-616
644-671
Hsp90 binding

b.


BOUND
INPUT
X795
X781
X768
X671
X616
XΔ616-694
XΔ616-632
U S U S U S U S U S U S U S U S U S U S U S U S U S U S
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24

has been dissociated from heat shock proteins *in vitro* is through salt treatment (200, 201). Salt mediated transformation produces a receptor that is able to bind to DNA, but is only poorly able to activate transcription. Thus ligand transformed and salt transformed GR produce receptors with different conformations particularly in the ligand binding domain.

In order to determine whether GR binding to Oct-1 required bound steroid, both untransformed and salt transformed GR deletion mutants were compared in a standard binding assay with immobilized GST-Oct-1. All of the salt transformed receptor derivatives tested bound to immobilized GST-Oct-1 but none of the untransformed receptor derivatives (Figure 8b). It was concluded that the conformation of the C-terminal domain does not markedly influence the association with the POU domain of Oct-1. This conclusion is strengthened by the results of the binding assay in Figure 11, lanes 3 and 4. Both dex agonist and dex 21-mes (antagonist that produces an altered C-terminal GR conformation) transformed GR bound to GST-Oct-1 with similar avidity.

To provide evidence that the untransformed GR derivatives unable to bind GST-Oct-1 were complexed with heat shock proteins, the GR X795 and X616 were fractionated using sucrose gradient equilibrium centrifugation (205, 206). Sucrose gradient centrifugation

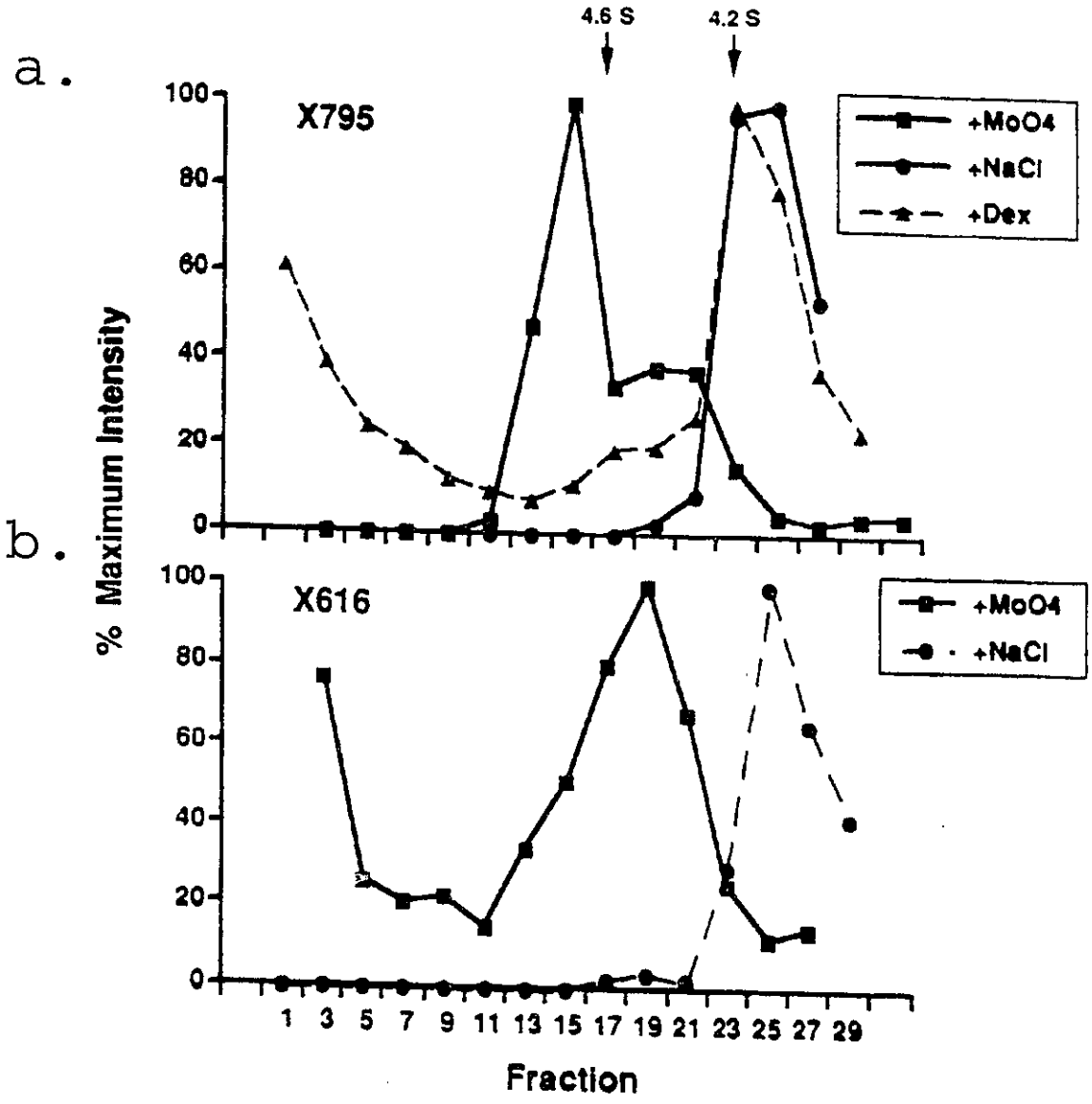
separates proteins according to their buoyant density. As each cellular lysate component moves down the density gradient, it eventually reaches a position where the density of the solution is equal to its own density. At this point, the component is in equilibrium and can move no further down the gradient. Proteins have sedimentation coefficients or S values, based primarily on the size and shape of the protein. Sucrose density gradients have provided a valuable tool for separating untransformed GR which runs as an 8S protein product, from transformed GR, which runs as a 4S protein product (206, 207).

In this study, it was important to provide evidence that untransformed GR X616 was unable to associate to Oct-1 because it was complexed with heat shock proteins. As I was working with truncated derivatives of GR when compared to the full length protein, the S values of these peptides were substantially lower than the 8S and 4S sedimentation coefficients normally obtained with full-length GR. The pattern of sedimentation however, remained the same

Figure 9a, shows the plot representing sedimentation of individually treated samples of radiolabeled receptor fragments (X795 and X616) through 15% to 30 % sucrose gradients in the salt transformed and untransformed states. Following equilibrium centrifugation, fractions were collected and individually resolved by SDS-PAGE. The *in vitro* translated and radiolabeled GRs were

Figure 9. Sucrose gradient analysis of the X795 and X616 rat GR derivatives.

^{35}S-Met labeled, GR derivatives in a., X795 or in b., X616 were treated with either 2 X 10^{-7} Dexamethasone (solid triangle), 0.4M NaCl (solid circle) or 20 mM sodium molybdate (solid square) before equilibrium centrifugation on a 15% to 30% sucrose gradient. Following centrifugation, 50 µl fractions were collected and odd numbered fractions were analyzed by 15% SDS-PAGE and fluorography. The fluorograph was analyzed by linear densitometry where the area under the curve was equal to the intensity (Fisher Scientific). For each sucrose gradient assay, the intensity of the band representing the radiolabeled protein was expressed relative to the band in the lane with the highest intensity, labeled 100%. The relative intensity of the ^{35}S-Met labeled GR derivatives were plotted as a function of the fraction number. The sedimentation coefficients 4.2S (rabbit hemoglobin) and 4.6S (BSA) are indicated on top. (X=amino acid 407)


a.
b.
4.6 S
4.2 S
X795
X616
+MoO4
+NaCl
+Dex
% Maximum Intensity
Fraction
0
20
40
60
80
100
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29

visualized by fluorography. Each band corresponding to GR was quantified by linear densitometric analysis (Ultrascan XL, LKB) which provides a printout of the absorbance as a function of distance. The area under the curve was proportional to the intensity of the band. The most intense band in each batch of collected fractions was assigned 100 % maximum intensity. Each subsequent band in the set was expressed as a percentage of the maximum intensity and plotted as a function the fraction number. The molybdate treated samples had a peak intensity shifted to the left, with a higher S value when compared to the salt or ligand treated samples. This is indicative of untransformed or heat shock protein associated GR (220). The salt treated samples had peak intensities lower than 4.2S. The peaks shifted to the right with the lower S values were indicative of transformed or heat shock protein dissociated GR. The dexamethasone treated X795 was used as a marker for the transformed receptor with its peak intensity migrating in the same fraction numbers as the salt transformed sample.

Together, Figures 8 and 9 demonstrate an inverse correlation between GR complexed with heat shock proteins and the binding of GR to GST-Oct-1. Thus, hsp90 might be an integral factor in the production of a form of GR that blocks binding to Oct-1. However, the exact

component of the heat shock protein complex responsible for this interference remains to be confirmed.

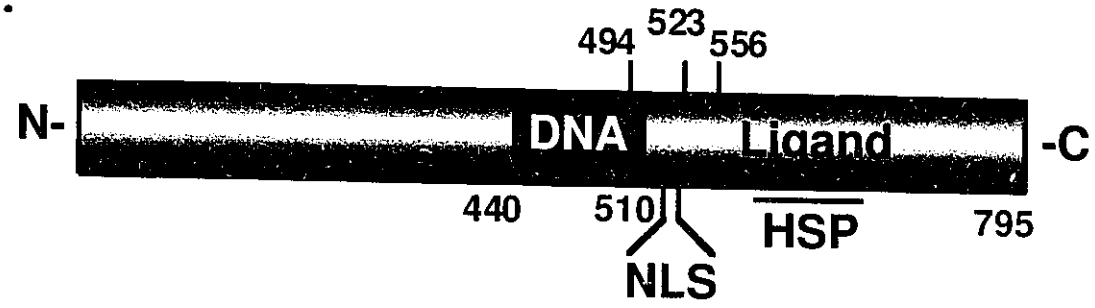
5. Fine resolution mapping the interaction domain of GR: N494 fails to associate with Oct-1.

To this point, it had been found that the DNA binding domain of GR (aa 407-556) may be sufficient to bind to Oct-1 (Figure 6). My next goal was to determine the structural motifs in the DNA binding domain of GR necessary to bind to immobilized GST-Oct-1. GR deletion mutants were produced by linearizing N556 DNA plasmid to produce DNA templates for *in vitro* transcription-translation in rabbit reticulocyte lysate. As a result GR receptor derivative proteins were produced lacking the C-terminal region of the N556 peptide (Figure 10a,b). A series of C-terminal GR deletion mutants were tested in a standard binding assay with immobilized GST-Oct-1. Figure 10c shows that radiolabeled GR lost its ability to interact with Oct-1 when truncated to amino acid 494 (lane 5), but still maintained its interaction when truncated to amino acid 523 (lane 4). This could mean one of two things. First, that the region residing between amino acids 494 and 523 could be a region that is required to maintain a stable complex with Oct-1. Second, that truncating into the zinc coordinating structure found between amino acids 476 and 495 resulted

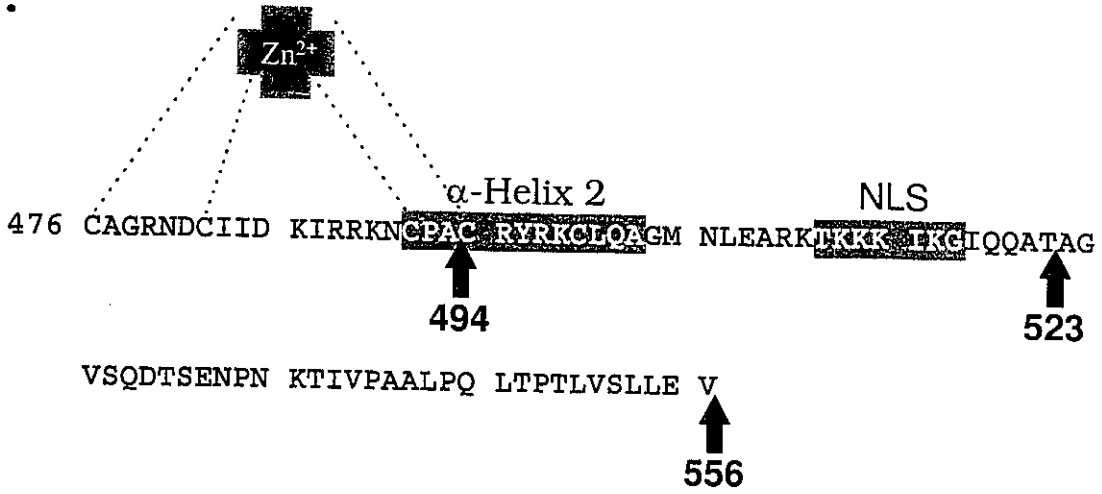
Figure 10. Fine resolution mapping of the interaction domain of GR: N494 fails to associate with Oct-1.

a. A schematic representation of the full length rat GR. The numbers represent the amino acid position within the rat GR with the DNA binding domain delimited between aa 440-510. A nuclear localization signal (NLS-1), the ligand and heat shock protein binding (HSP) regions are highlighted in the schematic diagram. b. The primary structure of rat GR C-terminal truncations used in c with the arrows indicating the position of truncation. Note that a truncation to N494 does not allow for the tetrahedral coordination of zinc in finger 2. c. The molybdate or Dex.-treated, ^{35}S-labeled, full length, GR (lanes 1 and 2) or C-terminal truncations, N556, N523 and N494 (lanes 3-5) were incubated with the GST-Oct-1 affinity matrix as described in the methods. The bound proteins were analyzed by 10 % SDS-PAGE and visualized by fluorography. Ten percent of the input proteins are shown in lanes 6-10.

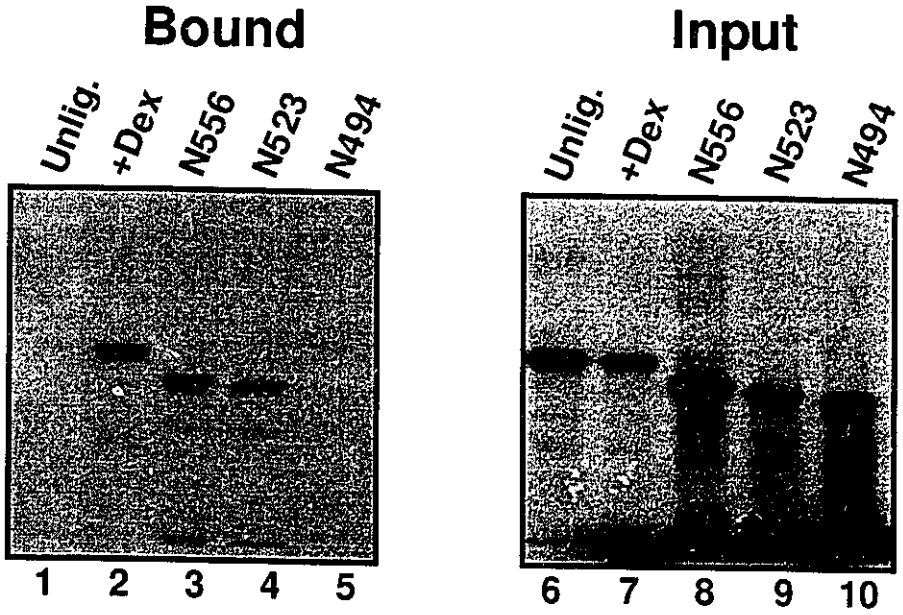
a.


494
523
556
N-
DNA
Ligand
-C
440
510
HSP
795
NLS

b.


Zn2+
α-Helix 2
NLS
476 CAGRNDCIID KIRRKNCPAC RYRKCLQAGM NLEARKTKKK IKGIQQATAG
494
523
VSQDTSENPN KTIVPAALPQ LTPTLVSLLE V
556

c.


Bound
Input
Unlig.
+Dex
N556
N523
N494
1 2 3 4 5
6 7 8 9 10

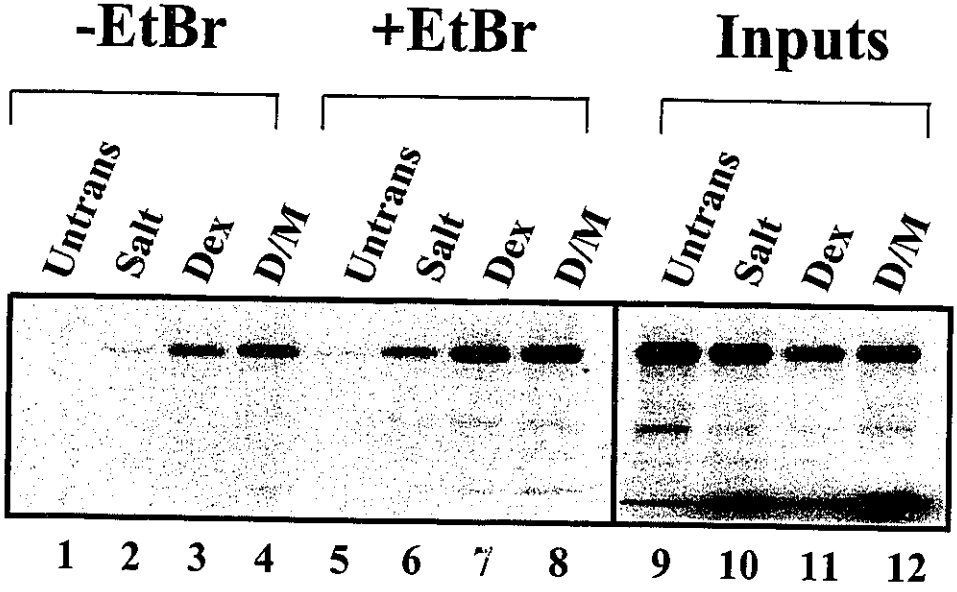
in a form of GR that is unable to bind Oct-1 because of the loss of the zinc finger structure.

6. GR binding to Oct-1 is increased in the absence of DNA.

The potential importance of the zinc finger structure for GR/Oct binding to DNA raised the question of how GR/Oct-1 association might be affected by DNA binding of GR. Ethidium bromide has been used as an agent for identifying protein-protein interactions stabilized by DNA (202). The purification of the recombinant GST-Oct-1 fusion protein is a one step protocol which can result in contamination of the fusion protein with bacterial genomic DNA. With the addition of ethidium bromide which intercalates DNA, DNA stabilized protein-protein interactions would be abolished. The binding of transformed GR in the presence of 150 mg/ml of ethidium bromide was used to assess the DNA dependence of GR/Oct-1 interaction in a standard binding assay with GST-Oct-1. Figure 11 shows that rather then interfering with GR/Oct binding salt, dex and dex 21-mes transformed GR binding to GST-Oct-1 was enhanced in the presence of ethidium bromide. Further the binding intensity increased approximately equally, independent of the method used to transform the GR. Thus, it appeared the interaction of GR with DNA may actually have been detrimental to

Figure 11. Ethidium bromide increases GR binding to Oct-1.

In vitro translated, ^{35}S-Met labeled, full length, GR was incubated with (lanes 1-4) or without (lanes 5-8) 150 μg/ml of ethidium bromide to the GST-Oct-1 affinity matrix. Prior to incubation, the GR was treated with either molybdate (lanes 1 and 5), salt (lanes 2 and 6), Dex. (lanes 3 and 7) or D/M (lanes 4 and 8). as described in the methods. Following extensive washing, the bound proteins were resolved by 8% SDS-PAGE and visualized by fluorography. Ten percent of the input proteins are shown in lanes 9-12. Dexamethasone mesylate (D/M) is a GR antagonist that acts by covalently modifying Cys-656 of rat GR (224).


-EtBr
+EtBr
Inputs
Untrans
Salt
Dex
D/M
Untrans
Salt
Dex
D/M
Untrans
Salt
Dex
D/M
1 2 3 4 5 6 7 8 9 10 11 12

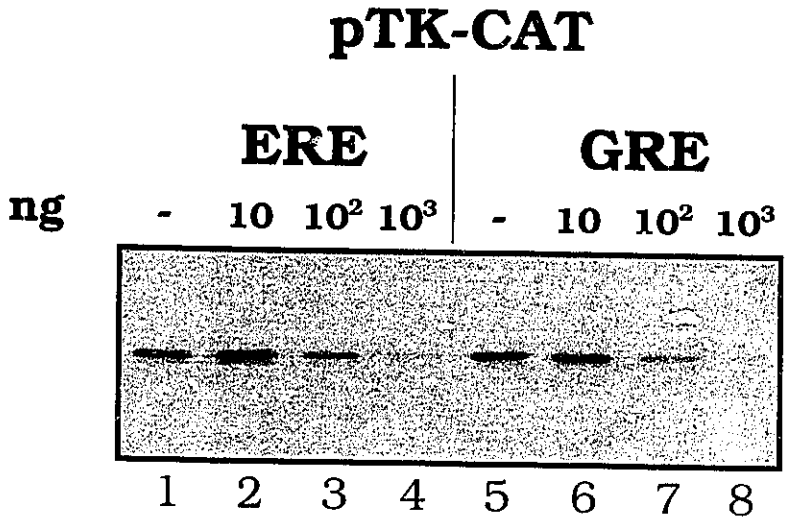
GR/Oct-1 binding.

(a) Both GREs and EREs containing plasmids inhibit GR binding to Oct-1.

Since the GR Oct-1 protein-protein interaction appears stabilized in the absence of DNA. I decided to test whether sequence specific GR binding sites could inhibit GR binding to Oct-1. Ligand transformed GR was tested in a standard binding assay with immobilized Oct-1 in the presence of GREs or control DNA to determine whether DNA binding by GR could destabilize or prevent the GR/Oct-1 interaction (Figure 12). The DNAs added consisted of plasmid containing either two copies of an estrogen responsive element (ERE), pTKCAT-ERE or two copies of a GRE, pTKCAT-GRE (203). Both plasmids competed for the binding of GR to Oct-1 with similar efficiency. Thus in the presence of DNA, GR was unable to form a stable protein-protein interaction with Oct-1. Surprisingly, the pTKCAT-ERE plasmid was able to compete as efficiently as the pTKCAT-GRE. However, it turns out that these plasmids both contain Bam HI sites which when methylated can act as a GRE (225). Since these plasmids were grown in an *E.coli* strain, DH5α which contain genes that encode for methylase enzymes, it is possible that the Bam HI sites were modified inappropriately, thus producing a pTKCAT-ERE plasmid containing

Figure 12. DNA inhibits GR binding to the POU domain of Oct-1.

Equal amounts of ^{35}S-Met labeled, full length GR was incubated alone (lanes 1 and 5) or with increasing amounts (10 ng, 100 ng and 1000 ng) of pTKCAT plasmids containing two copies of consensus EREs (lanes 2-4) or GREs (lanes 6-8) in a standard binding assay as described in the methods. The bound proteins were resolved by 8% SDS-PAGE and visualized by fluorography.


pTK-CAT
ERE
GRE
ng
-
10
10²
10³
-
10
10²
10³
1
2
3
4
5
6
7
8

binding sites for GR.

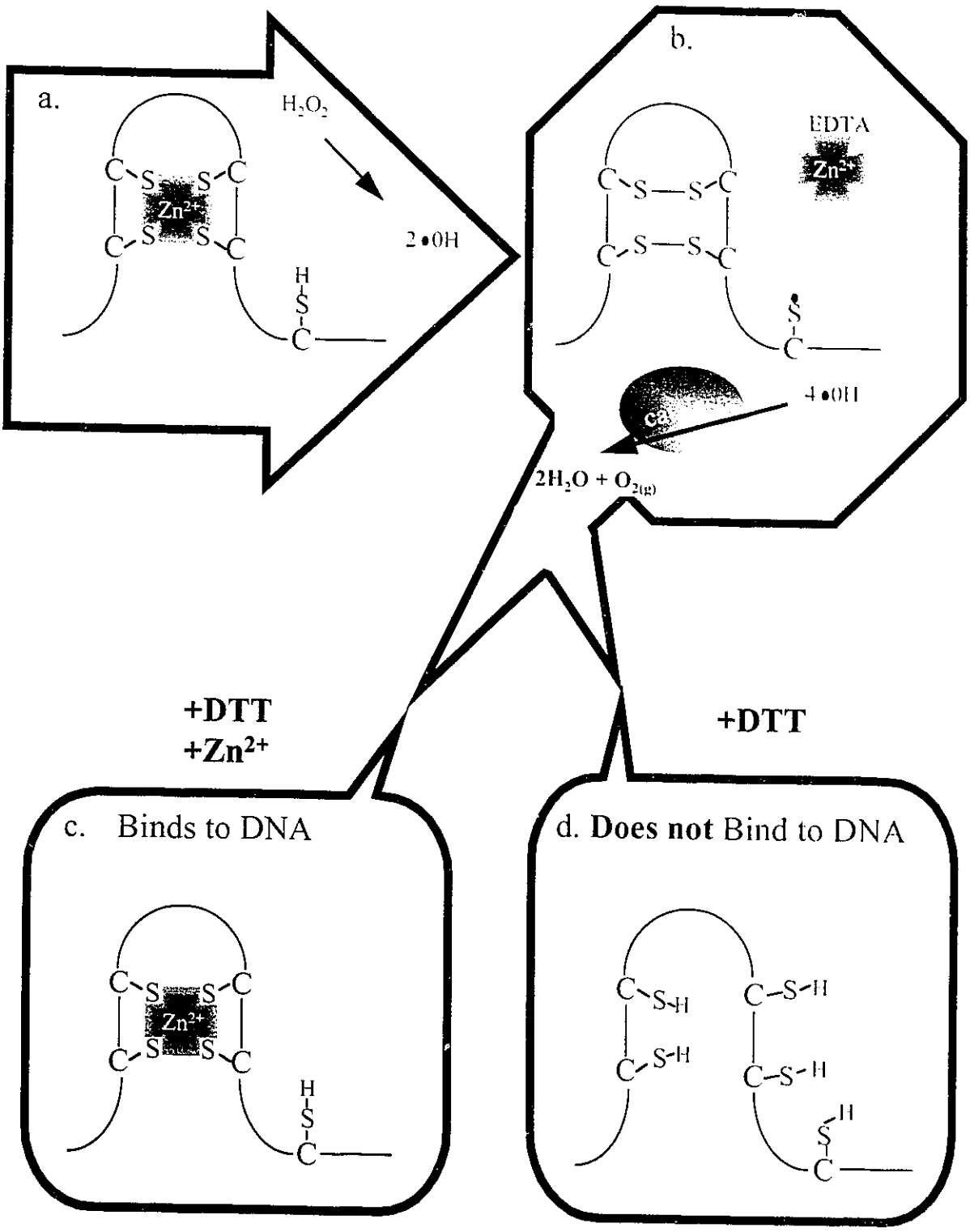
7. Zinc coordination of GR is not required for association with Oct-1.

C-terminal deletion to amino acid 494 of rat GR abolished the binding to immobilized GST-Oct-1 (Figure 10). Deletion to this amino acid removed cys 495 involved in wild-type coordination of zinc and it would severely affect the tertiary protein structure of GR. To address the role that zinc coordination played in GR/Oct-1 binding, the zinc atoms were removed from the GR finger structure and then the GR DNA binding domain was tested for its ability to bind to immobilized GST-Oct-1. The removal of the zinc from the finger structure of GR can be easily monitored by EMSAs as apo GR DNA binding activity (to a GRE) is compromised.

The schematic diagram in Figure 13 illustrates the protocol employed for reduction and reconstitution of zinc coordination of a nuclear receptor. The first frame (a) illustrates the second zinc finger of GR tetrahedrally coordinating a zinc atom. In the presence of 20 mM hydrogen peroxide, hydroxyl radicals efficiently oxidize cysteine residues in close proximity (b). Under the conditions used, the zinc atom released was rapidly chelated by the presence of 50 mM EDTA. Subsequently 1500 units of catalase were added to neutralize the

Figure 13. Schematic representation of the protocol for reduction and reconstitution of the GR zinc finger cysteines used in Figure 14.

a. A representation of the native structure of the GR zinc finger 2 (see figure 17a) before treatment with 20 mM hydrogen peroxide. b. Peroxide-formed hydroxyl radicals oxidize cysteine residues that are in close proximity facilitated by zinc atom coordination. The zinc was then sequestered by chelation with 50 mM EDTA. The peroxide was neutralized by 1500 U of catalase to produce water and oxygen. Following the peroxide treatment, receptor coordination was either: c. restored, using 0.1 M DTT and 250 μM $ZnCl_2$(to 10 % of its original activity) or d. abolished by reduction with 0.1 M DTT to produce a receptor fragment unable bind to DNA. The zinc coordination or stripping zinc from the finger structure of GR did not alter its ability to associate with Oct-1.


a.
H_2O_2
2 •OH
b.
EDTA
Zn^{2+}
4 •OH
$2H_2O + O_{2(g)}$
+DTT
+Zn^{2+}
+DTT
c. Binds to DNA
d. **Does not** Bind to DNA

hydroxyl radicals. When the apo finger was reduced with 0.1 M DTT in the presence of 250 μM $ZnCl_2$ zinc coordination could be restored with approximately 10% efficiency (c) (see Figure 14a). When finger structure was treated with 0.1 M DTT in the absence $ZnCl_2$, the cysteine residues were simply reduced (d).

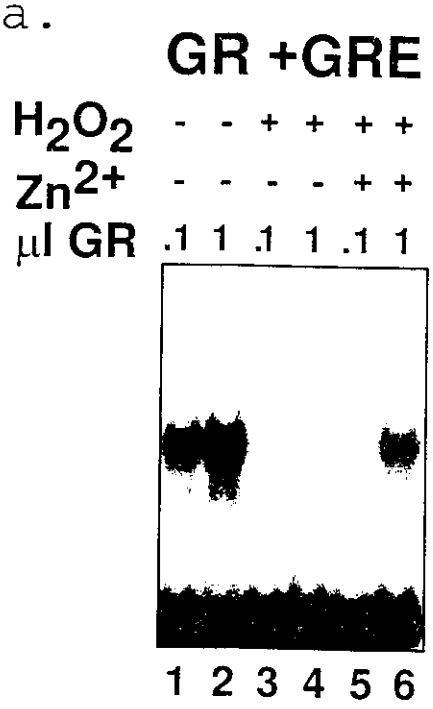
Partially purified, bacterially expressed, recombinant GR DNA binding domain (X556) was treated in a duplicate reaction along side the X616 *in vitro* translated receptor to provide a substrate to closely monitor the zinc status of the GR. These X556 receptor samples were tested for binding to a ^{32}P-labeled GRE in an EMSA. Figure 14a shows that the native form of recombinant GR (X556) was able to shift a GRE (lanes 1 and 2). However, following oxidation and reduction reactions in the absence of zinc, the ability of X566 to bind to DNA was compromised (lanes 3 and 4). The zinc coordination of reduced X556 could be restored to about 10 % of the native form (Figure 13, lanes 5 and 6) upon incubation with $ZnCl_2$.

Equal quantities of *in vitro*-translated, ^{35}S-Met labeled, GR X616 wild-type, zinc stripped- and zinc reconstituted- forms were tested in a standard binding assay with immobilized GST-Oct-1. As shown in Figure 14b, all three forms of X616 bound equally well to GST-Oct-1, independent of the receptor status with respect to zinc coordination. With these results, it appeared that the three dimensional structure of

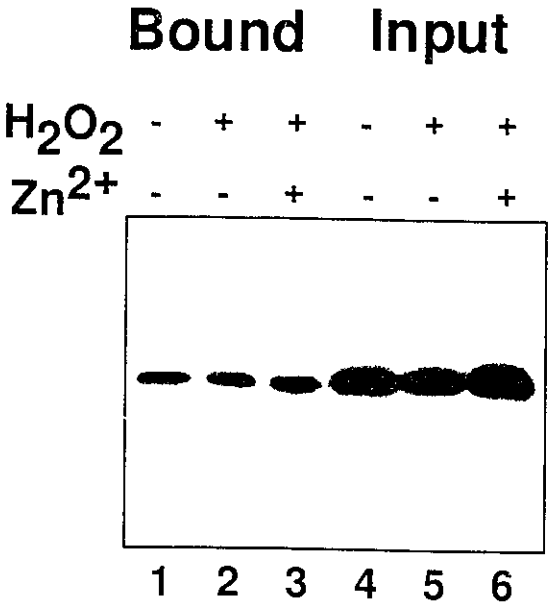
Figure 14. Zinc coordination of GR abolishes DNA binding but does not effect the GR/Oct-1 association.

a. Two volumes of recombinant partially purified GR X556 (407-556) and 20 ng ^{32}P-labeled consensus GRE were incubated in binding buffer with 1 µg of highly sheared salmon sperm DNA and 1 µg of BSA as competitor. The binding reaction was analyzed by EMSA on a 4% non-denaturing 0.5% TBE polyacrylamide gel and visualized by autoradiography. As indicated, the mock treated receptor is shown in lanes 1 and 2, while the hydrogen peroxide treated samples are shown in lanes 3-6 as described in Figure 13. Following peroxide treatment, the GR X556 was either reduced with DTT alone (lanes 3 and 4, Figure 13c) or reduced in the presence of 250 µM $ZnCl_2$ (lanes 5 and 6, Figure 13d). 0.1 µl (lanes 1, 3 and 5) and 1 µl (lanes 2, 4 and 6) represent equal relative quantities of GR X556 used in the EMSA prior to treatment, in order to precisely quantify the efficiency GR binding to a GRE. b. Equal quantities of ^{35}S-labeled, GR X616 (amino acids 407-616) receptor was incubated with GST-Oct-1 as described in the methods. The mock treated receptor is shown in lane 1 while the hydrogen peroxide treated samples are shown in lanes 2-3 as described in Figure 13. Following hydrogen peroxide treatment, the X616 fragment was either treated with DTT alone (lane 2, Figure 13c) or with of 250 µM $ZnCl_2$ (lane 3, Figure 13d). The bound proteins were resolved by 12% SDS-PAGE and visualized by fluorography. Ten percent of the input proteins are shown in lanes 4-6.

a.


GR +GRE
H_2O_2 - - + + + +
Zn^{2+} - - - - + +
µl GR .1 1 .1 1 .1 1
1 2 3 4 5 6

b.


Bound Input
H_2O_2 - + + - + +
Zn^{2+} - - + - - +
1 2 3 4 5 6

the GR DNA binding domain, stabilized by zinc atoms is not a requirement for binding to the POU domain of Oct-1, *in vitro*. Therefore, it was suspected that the region between amino acids 494 and 523 may be required for association with the POU domain of Oct-1.

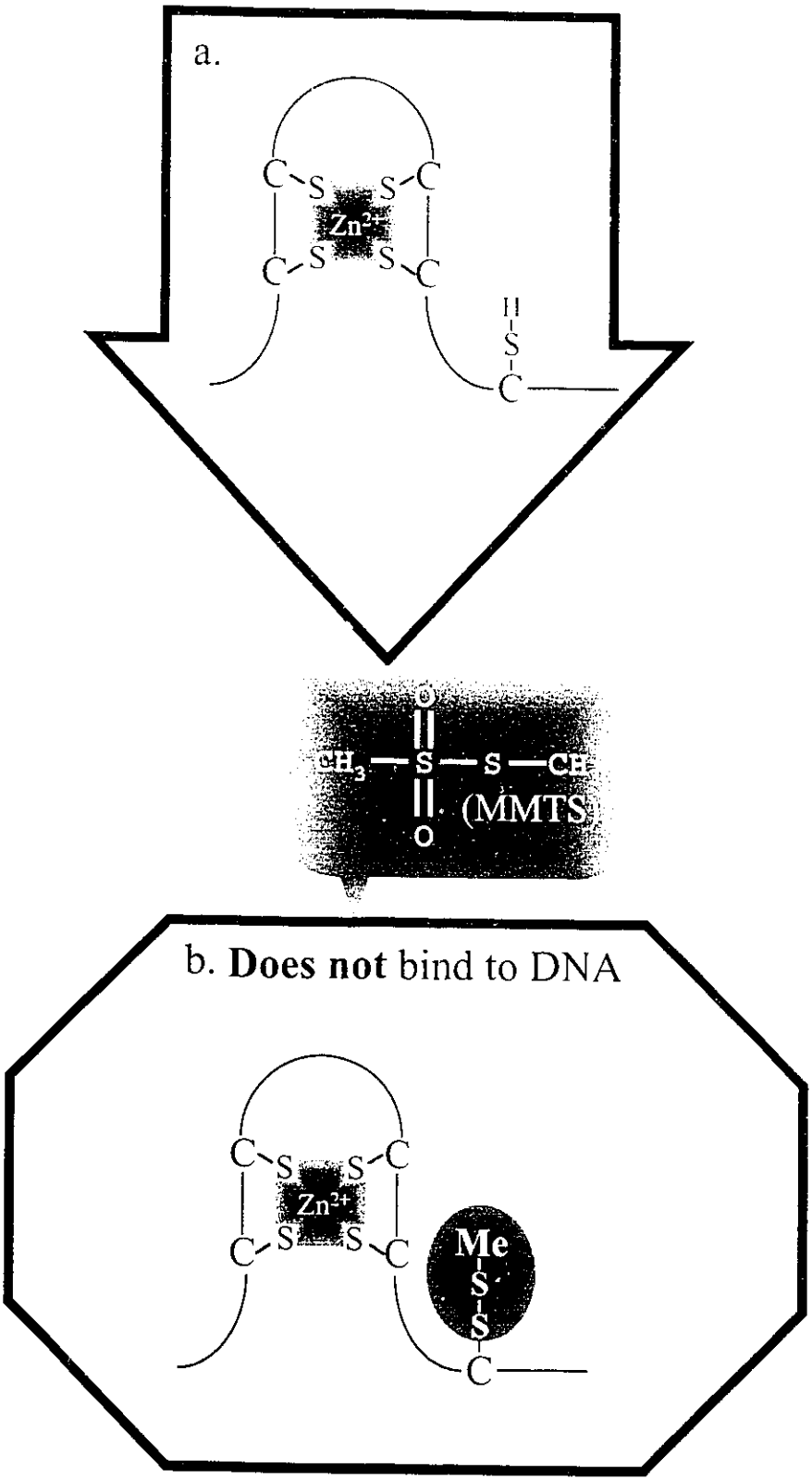
8. Modifying thiol groups in the GR DNA binding domain identifies cysteine residue at amino acid 500 of rat GR as being potentially important for binding to Oct-1.

The GR fragment encompassing amino acids 407-616 contains 10 cys residues all residing between amino acids 440-500 (see Figure 17a). Nine of the ten residues are absolutely conserved in nuclear receptors (28). Eight of these conserved residues are involved in coordinating two zinc atoms while the only other conserved residue, cys 500 is located between amino acids 494-523. MMTS, a chemical that modifies thiol groups, was used to target cysteine residues in the DNA binding domain of GR (aa 407-616). MMTS transfers a sulfyl-methyl group to reduced cysteine residues (-S-H groups) (224, 226).

Thus, in contrast to H_2O_2 treatment of zinc complexed cysteine residues, MMTS treatment also specifically targets a cysteine in the first finger that is not involved in zinc coordination. For example the schematic diagram in Figure 15b illustrates the second finger structure

Figure 15. Schematic representation of the protocol before and after MMTS treatment of GR X616 used in Figure 16.

a. Schematic representation of the second zinc finger of a nuclear receptor extending C-terminally as illustrated for rat GR. The X616 (amino acids 407-616) GR contains 10 cysteine residues all located between amino acids 440-500: 9 cysteine residues are absolutely conserved in the DNA binding domain of nuclear receptors which include a cysteine residue at amino acid 500 (see Figure 18) and 8 residues involved in zinc coordination at amino acids 440, 443, 457, 460, 476, 482, 492 and 495 (see Figure 17). b. MMTS is a thiol-specific modifying reagent which transfers a sulfyl-methyl group to thiol groups (213, 226). Illustrated is a reduced cys (aa 500 for rat GR) residue modified by a sulfyl-methyl group in the C-terminal extension of the second zinc finger following treatment with 5mM MMTS. Note the thiol groups coordinating the zinc atom may also be modified following treatment with MMTS. Modifying thiol groups in the GR DNA binding domain eliminates binding to DNA (214).


a.
Zn2+
(MMTS)
b. Does not bind to DNA
Zn2+
Me

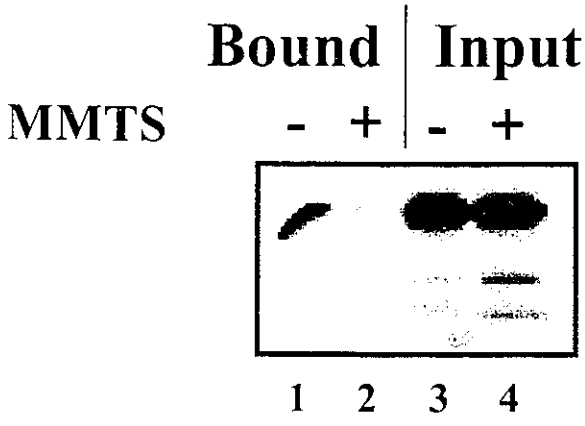
of GR with a modified cysteine residue at amino acid 500. The receptor zinc status following MMTS treatment is unknown and the cysteine residues that coordinate two zinc atoms may also be modified by MMTS. However, treatment with MMTS produces a form of GR that is unable to bind to DNA (214). Figure 16 shows the result of a standard binding assay with immobilized GST-Oct-1 using radiolabeled GR X616 treated for one hour at 4°C with or without 5 mM MMTS. In striking contrast to H_2O_2 treated GR, the MMTS treated receptor fragment was unable to bind to GST-Oct-1 (lane 2). As my previous experimental observations revealed that zinc coordination by the cysteine residues in the GR DNA binding domain was not a requirement for GR binding to GST-Oct-1 (Figure 14b), this suggested that either cys 450 and/or cys 500, the two additional cysteines in the DNA binding domain fragment used, were important for Oct-1 binding.

9. Mutations at amino acids C500 and L501 abolish GR binding to the POU domain of Oct-1.

GR DNA binding domain peptides containing individual amino acid substitutions (in the context of amino acids 407-556) were expressed to refine understanding of the determinants for GR association with Oct-1. The DNA binding mutants employed in this study were derived from a pool of saturated GR mutations that were

Figure 16. Modifying thiol groups in the GR DBD abolishes its interaction with Oct-1.

The ^{35}S labeled, X616 receptor treated, as described in Figure 16, with (lane 1) or without (lane 2) 5 mM MMTS prior to incubation with GST-Oct-1 in a standard binding assay as described in the methods. The bound proteins were resolved by 12% SDS-PAGE and visualized by fluorography. Ten percent of the input proteins are shown in lanes 3 and 4.


Bound
Input
MMTS
- + - +
1 2 3 4

either defective in DNA binding to a consensus GRE or bound DNA as monomers as opposed to the wild-type receptor which normally binds as a homodimer (37). These mutants have identified individual amino acids involved in structural and functional properties (26). Figure 17a shows a schematic representation of the zinc finger region of GR encompassing these mutations and highlights the role of individual amino acids in DNA binding and dimerization domains as determined from the X-ray crystal structure (29).

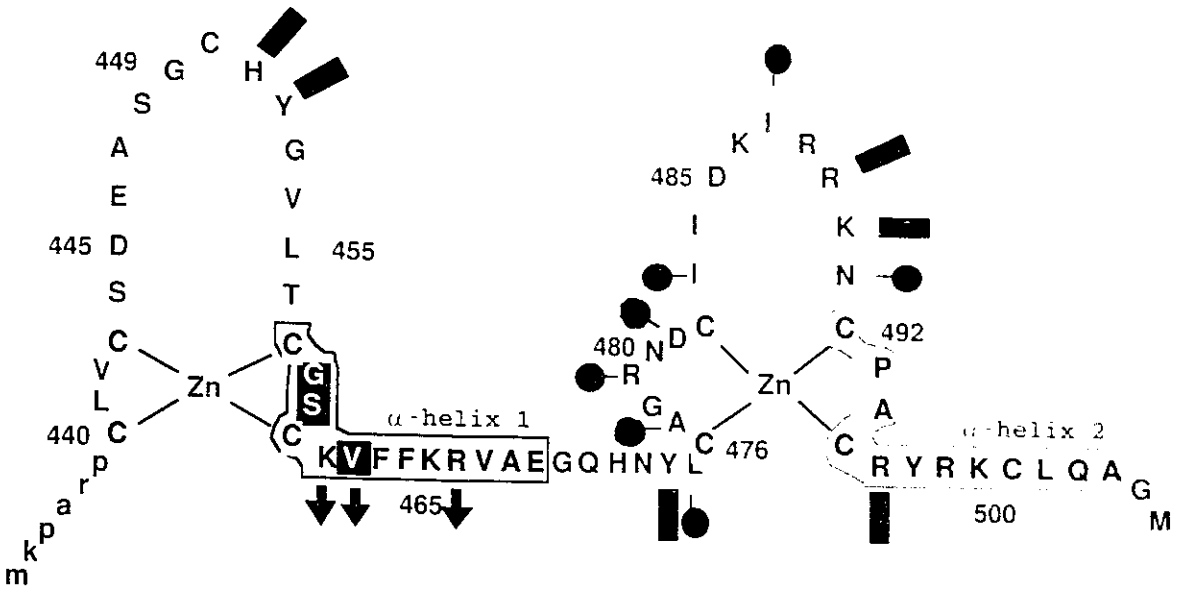
Of the 9 point mutations tested, only the amino acids that were modified at position C500Y and L501P abolished binding to Oct-1 (Figure 17, lanes 9 and 10). As the point mutations C500Y and L501P have fairly severe consequences for α-helix secondary structure, it would be imprudent to suggest that the interaction with Oct-1 occurs directly though these amino acids. However, I believe that it can be reasonably concluded that the integrity of α-helix 2 is required for the interaction of GR with Oct-1.

These binding assays with the GR point mutants support the previous observation that native zinc coordination in the GR DNA binding domain is not a requirement for interaction with Oct-1, as cysteine residues known to coordinate zinc atoms in both the first and second fingers namely, C460Y, C492Y and C495Y, produced a GR DBD

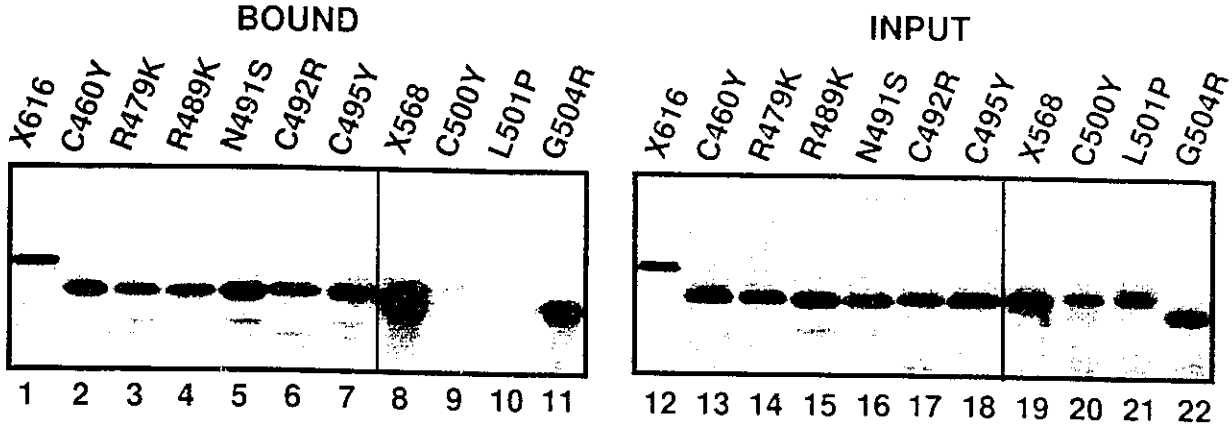
Figure 17. Mutations of amino acids at positions 500 and 501 abolish association of the GR DNA binding domain (DBD) with Oct-1.

a. Schematic representation of the GR DBD (440-505, upper case) adapted from a summary of the crystal structure of rat GR (29). The lower case letters represent 6 leader amino acids. The solid rectangles represent the amino acid residues that make phosphate 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. The boxed residues form α-helical secondary structures. The highlighted residues (G, S, V) determine the specificity of the DNA recognition sequence. b. ^{35}S-Met labeled, wild-type GR DNA binding domain (lanes 1 and 8) and point mutants (lanes 2-7, 9-11) were incubated with GST-Oct-1 in a standard binding assay as described in the methods. The bound proteins were analyzed by 12% SDS-PAGE and visualized by fluorography. The wild-type protein sequences contained amino acids 407-616 (X616, lane 1) and 407-568 (X568, lane 8). The individual point mutants indicated above contain of amino acids 407-566 with the exception of G504R which contained only amino acids 407-523 of rat GR. Ten percent in the input proteins are shown in lanes 12-22.

a.


449
445
440
485
480
476
492
500
465
455
Zn
Zn
α-helix 1
α-helix 2
KVFFKRVAEGQHNYL
RYRKCLQA

b.


BOUND
INPUT
X616
C460Y
R479K
R489K
N491S
C492R
C495Y
X568
C500Y
L501P
G504R
1 2 3 4 5 6 7 8 9 10 11
12 13 14 15 16 17 18 19 20 21 22

capable of binding to Oct-1 with wild-type affinities. The mutations that affect the dimerization properties of GR, R479K and N491S, also did not affect the GR association with Oct-1 suggesting that GR dimerization is not required to bind to immobilized GST-Oct-1 nor does Oct-1 binding occur through the GR dimerization motif in the DBD.

10. The GR interaction with Oct-1 is conserved among the nuclear receptor family.

To this point my data identified α-helix 2 in the second finger structure of GR, as a motif required for GR association with Oct-1 *in vitro*. To consider the possibility that nuclear receptors in addition to GR might bind similarly to Oct-1, the primary amino acid sequence encompassing α-helix 2 of GR was aligned with several members of the nuclear receptor family. Figure 18 shows the sequence alignment, highlighting conserved residues. This region of GR is within the most highly conserved region of nuclear receptors. Therefore, I decided to test whether it may represent a conserved Oct-1 binding motif. The first six nuclear receptors listed in Figure 17, were tested for binding to the immobilized POU domain of Oct-1 *in vitro*. When appropriately presented, all the nuclear receptors tested were able to bind to Oct-1. Ligand treated rat GR and human ER bound with similar affinities (Figure 19, lanes 1 and 2). Mouse RXR-α, RAR-α' and TR-α2 (c-erbA-

Figure 18. The interaction domain between GR and Oct-1 has been mapped to a highly conserved motif found in several members of the nuclear receptor superfamily.

Primary protein structure alignment of several members of the nuclear receptor superfamily showing a highly conserved motif in the second zinc finger. To the left are the abbreviated names of the nuclear receptors with the species origin in the second column. The third column shows the corresponding primary structures of each nuclear receptor with the amino acid numbers indicated prior to representation of the first amino acid residue illustrated. The residues encompassing α-helix 2 in the second zinc finger structure of rat GR are illustrated above the alignment. The absolutely conserved residues are highlighted. The first six nuclear receptors illustrated were tested in a standard binding assay with GST-Oct-1 (see Figure 19).

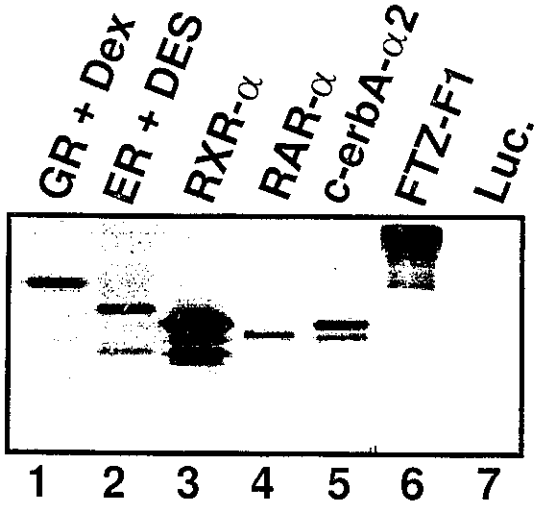
α-Helix 2

GR	rat	487-	IRRKNCPACR	YRKCLQAGMN	LEARKTKKK
RARα	mouse	135-	VTRNRCQYCR	LQKCFDVGMS	KESVRNDRK
ER	human	232-	NRRKSCQACR	LRKCYEVGMM	KGGIRKDRR
TRα2	mouse	102-	ITRNQCQLCR	FKKCIAVGMA	MDLVLDDSK
RXRα	mouse	187-	RQRNRCQYCR	YQKCLAMGMK	REAVQEERQ
FTZ-F1α	drosophila	557-	TQRKRCPYCR	FQKCLEVGMK	LEAVRADRM
RORα	human	314-	RRRNRCQFCR	FQKCLAVGMV	KEVVRTDSL
USP	drosophila	151-	RQRNRCQYCR	YQKCLTCGMK	REAVQEERQ
COUP-TFα	human	128-	HHRNQCQTCR	LKKCLKVGMR	REAVQRGRM
FXR	rat	171-	TMRRKCQDCR	LRKCREMGML	AECLLTEIQ
PPARα	human	148-	KNRNKCQYCR	FHKCLSVGMS	HNAIRFGRM
CeZK418.1	C. elegans	60-	NLILTCRQCR	YNKCLKMGMR	IVTNQYDSL
CeK06A1.4	C. elegans	148-	ELRMICRHCR	FIKCLDAGMR	RELVQARKE

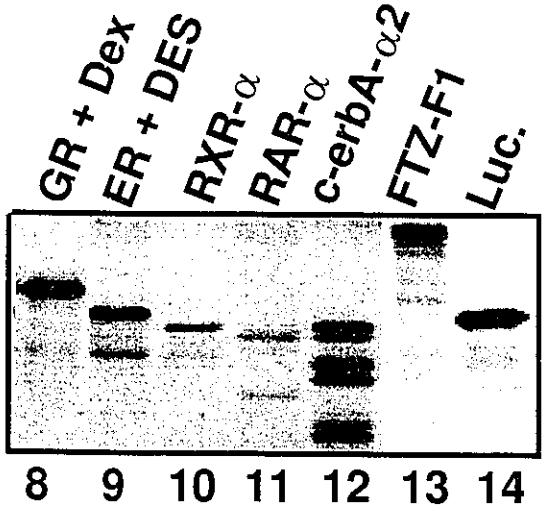
Figure 19. The interaction between GR and Oct-1 is conserved among several members of the nuclear receptor family.

^{35}S-Met labeled, ligand-treated rat GR and human ER and untreated mouse RXR-α, mouse RAR-α′, mouse c-erbA-α2 and drosophila FTZ-F1α, and firefly luciferase were incubated with GST-Oct-1 as described in the methods. The bound proteins were analyzed by 12% SDS-PAGE and visualized by fluorography. Ten percent of the input proteins are shown in lanes 8-14. GR and ER were ligand-transformed with 0.2 μM Dex and 1μM Des respectively, as described in the methods.

a.


Bound
GR + Dex
ER + DES
RXR-α
RAR-α
c-erbA-α2
FTZ-F1
Luc.
1 2 3 4 5 6 7

b.


Input
GR + Dex
ER + DES
RXR-α
RAR-α
c-erbA-α2
FTZ-F1
Luc.
8 9 10 11 12 13 14

α2) did not require ligand treatment prior to incubation with Oct-1 to maintain a stable protein-protein interaction (lanes 3-5). Furthermore, the orphan receptor Ftz-F1, that binds to DNA as a monomer (193), also associated with high affinity to the GST-Oct-1 (lane 6).

To confirm that the nature of the interaction of the nuclear receptors that bound to GST-Oct-1 was similar to that observed with GR, the binding of RAR-α' was examined more closely by C-terminal truncation. Several RAR-α' C-terminal deletion mutants were generated by *in vitro* transcription-translation of restriction digests of RAR-α' encoding plasmid DNA. These RAR-α' derivatives were then tested in a standard binding assay with immobilized GST-Oct-1. Figure 20c shows the C-terminally truncated RARα' mutants N161 and N181 bound to GST-Oct-1 with similar affinities as the full length receptor (lanes 1 -3), while further truncation into α-helix 2 (N145) abolished its interaction with Oct-1 (lane 4). This confirmed that, GR as well as

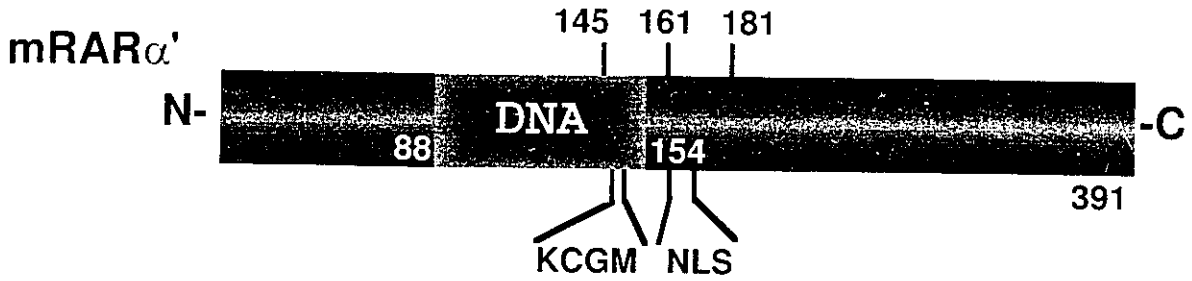
other nuclear receptors, required α-helix 2 of the second finger structure to bind Oct-1.

Therefore, it appears that multiple nuclear receptors have the potential to modify Oct-1 activity through direct protein-protein interaction.

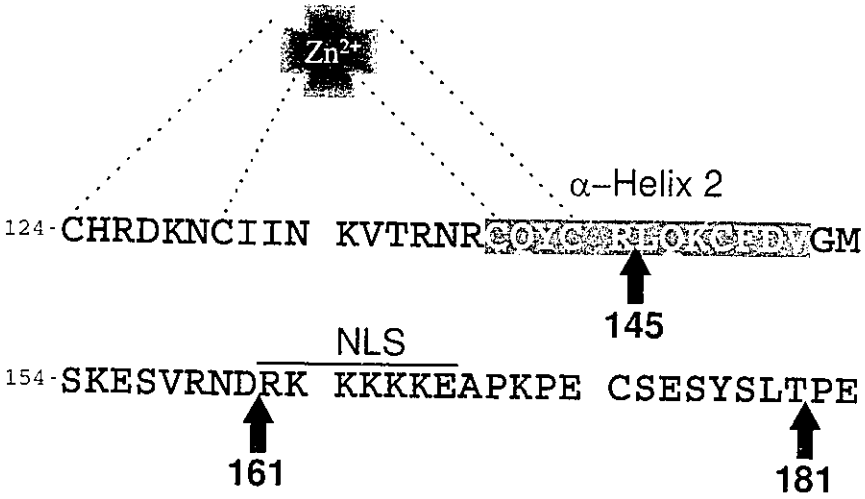
Figure 20. The interaction of RAR-α′ with Oct-1 also requires helix 2 of the second zinc finger.

a. A schematic representation of the mouse RAR-α′ with its putative DNA binding domain encompassing amino acids 88-154. The conserved region in the α-helix 2 (aligned in Figure 17) is indicated by KCGM while the region highlighted by NLS denotes a putative nuclear localization signal by homology with the amino acid sequence of the NLS of SV40 T-antigen. b. The primary and highlighted, putative secondary protein structure of the mouse RAR-α′ second zinc finger C-terminal deletions employed in c. with the solid arrows indicating the position of truncation. c. ^{35}S-Met labeled, full length (lane 1) or C-terminal truncations of RAR-α′ (lanes 2-4) were incubated with the GST-Oct-1 affinity matrix as described in the methods. The bound proteins were analyzed by 15 % SDS-PAGE and visualized by fluorography. Ten percent of the input proteins are shown in lanes 5-8. * indicates an internal control represented by the full length receptor due to incomplete DNA restriction digest prior to *in vitro* transcription-translation.

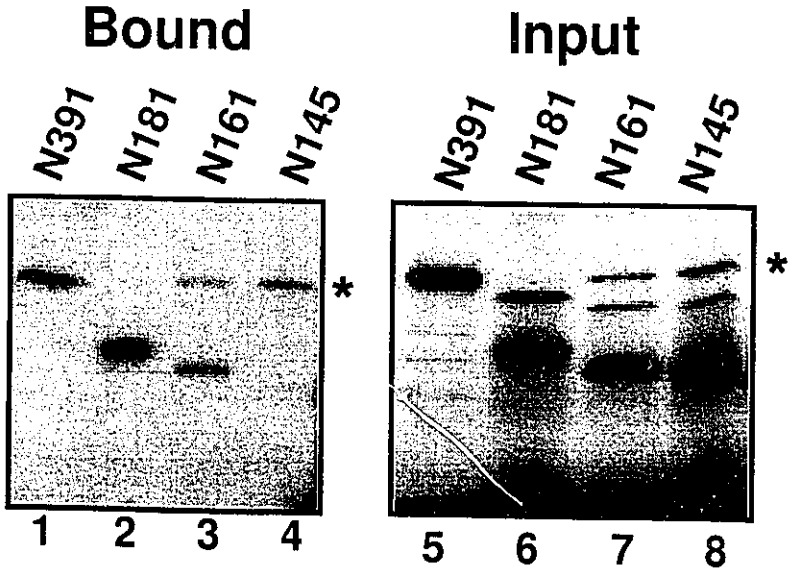
a.


mRARα'
145
161
181
N-
DNA
88
154
391
-C
KCGM
NLS

b.


Zn2+
α–Helix 2
124-CHRDKNCIIN KVTRNR
GM
145
NLS
154-SKESVRNDRK KKKKEAPKPE CSESYSLTPE
161
181

c.


Bound
Input
N391
N181
N161
N145
N391
N181
N161
N145
*
*
1
2
3
4
5
6
7
8

IV. DISCUSSION

I have investigated the interaction between GR and Oct-1 *in vitro* with the goal of providing information that will contribute to an understanding of the mechanism of functional interaction between nuclear receptor and octamer transcription factors, *in vivo*. My results demonstrate that GR and many other nuclear receptors can bind directly and specifically to the POU domain of Oct-1 in a GST pull down assay. The interaction of Oct-1 with GR was distinct from Oct-1/VP16 interaction and appears to require the dissociation of GR from heat shock proteins. For GR and RAR-α' direct binding to the POU domain of Oct-1 mapped to the zinc finger DNA binding domain region of the receptor, with point mutants C500Y and L501P in the DNA contact helix of finger 2 of GR abrogating binding. Strikingly, GR/Oct-1 binding was maintained following complete disruption of zinc coordination within the 2 zinc fingers of the DNA binding domain. These results will provide the basis for the initiation of cellular studies investigating GR/Oct binding in vivo.

1. The GR/Oct-1 interaction is specific.

The immobilized GST fusion protein system has proved to be a valuable *in vitro* tool in characterizing functionally relevant protein-

protein interactions between proteins (227-229). GR binding to Oct-1 was clearly specific with generally 10% of the input radiolabeled GR retained by the Oct-1 POU moiety following the binding procedure with GST-Oct-1. By contrast, GR failed to bind to GST alone. Similarly, firefly luciferase was not bound by Oct-1 POU.

Initially, the POU domain of Oct-1 was used because it was suspected, based on three key experiments reported elsewhere, to be required and potentially sufficient to obtain an interaction with GR. The first experiments identified a direct protein-protein interaction with purified Oct-1 and GR by coimmunoprecipitation (15). Second, disruption of the second α-helix in the POU-hd of Oct-1 disrupted coimmunoprecipitation with GR (15). The third experiment showed that Oct-2 dependent transcription could be repressed by ligand activated GR (4). As POU domains of Oct-1 and Oct-2 are highly conserved with only 9 different amino acids between their respective POU domains and that both the N- and C-terminal domains are highly unrelated, this suggested that the interaction between GR and octamer transcription factors involved the POU domain (65).

2. The DNA binding domains of GR and Oct-1 are sufficient to obtain a protein-protein interaction.

The regions encompassing the DNA binding domains of both GR and Oct-1 were found to be sufficient for binding of the two transcription factors. Interactions between DNA binding domains of transcription factors has now have been observed in several instances and seem to provide an excellent basis for combinatorial transcriptional regulation (158, 230-235). Further, most transcription factor families are characterized by the amino acid sequence homology within their DNA binding domains. Therefore, conserved interactions between transcription factor family members may in some case provide a means of generally integrating signals from separate signaling pathways. Moreover in this particular instance, extracellular hormonal signals through nuclear receptors has the potential to modify the activity of constitutive transcription factors, such as Oct-1.

3. Binding of GR to Oct-1 requires dissociation of heat shock proteins

In vitro translated GR associates with heat shock proteins into a ligand binding complex similar to that found *in vivo* (52). The only strict requirement that I was able to detect for GR binding to Oct-1 POU was the release of GR from the heat shock protein complex. This was

evident from experiments in which Dex transformed GR but not the untransformed GR bound to the GST-Oct-1 fusion protein. *In vivo,* GR is complexed with heat shock proteins in the cytoplasm and maintained in an open conformation capable of binding ligand with high affinity (53, 54). Once bound to steroid hormone, two separable events occur. The receptor is dissociated from heat shock proteins and then is rapidly translocated into the nucleus. During this process, the C-terminal domain assumes an altered conformation that is the transcriptionally active form of GR that binds to DNA regulatory elements (186).

Salt transformed receptor or Dex 21-mes transformed receptor provided strong evidence that the C-terminal altered conformation of the receptor, produced by Dex transformation of GR, was not specifically required for the binding of GR to Oct-1. This result is entirely consistent with my subsequent localization of the Oct-1 interface to the central DNA binding domain of GR. Both salt and Dex 21-mes treated GR produce a form of GR able to bind to DNA *in vivo* and *in vitro* but unable to activate transcription (54). Salt transformation simply causes the release of the heat shock proteins. Similarly, Dex 21-mes causes dissociation of the receptor from heat shock proteins by covalently modifying cys-656 of rat GR but does not

produce an altered C-terminal conformation favorable for activated transcription as observed with Dex bound GR.

In Figure 11, the salt transformed receptor bound to GST-Oct-1 with lower avidity than the ligand transformed full length GRs. About 10% of both the Dex and Dex 21-mes transformed radiolabeled full length GRs bound to GST-Oct-1, whereas less than 5% of the salt transformed receptor was retained by Oct-1 POU. For the salt transformed receptor, the *in vitro* translated GR was flash frozen for storage at -80°C and thawed prior to treatment with 0.4 NaCl. The efficiency of transformation of the receptor appeared to be influenced by the freeze-thaw procedure, which could be easily verified by equilibrium sucrose gradient centrifugation. This property was observed on numerous instances and also occurs when attempting to ligand transform full length GR following a freeze-thaw cycle. Consistently, this produced a ligand transformed receptor with decreased binding to Oct-1. However, in experiments with freshly translated GR, the salt transformed receptor is able to bind to GST-Oct-1 with similar avidity as its ligand transformed counterpart. Figure 8b shows an example following salt transformation of GR without prior to freeze-thawing, comparing the binding of GR with 10% of the input protein bound to GST-Oct-1.

The hsp90 component of the heat shock protein complex would appear to be the prime candidate in making a GR-complex unsuitable for Oct-1 association. This is because the GR truncation X616 (Figure 9b), which still has the ability to bind hsp90 (220, 221), required salt treatment to expose the Oct-1 binding interface (Figure 8b, lanes 9 and 10).

Inactive GR is anchored in the cytoplasm complexed with heat shock proteins where it is therefore theoretically unable to associate with constitutively nuclear Oct-1 protein to alter octamer factor dependent regulation of gene transcription. However upon ligand activation, GR is rapidly translocated to the nucleus and together with Oct-1 act to regulate transcriptional activation/repression of genes. Thus, my results are consistent with the proposal that ligands including both agonist and antagonist that allow for GR transformation could promote Oct-1 binding and thereby play a role in altering Oct-1 dependent gene transcription.

4. The interaction between Oct-1 and GR is distinct from the VP16/Oct-1 interaction.

Oct-1 binds with high affinity to the consensus octamer motif 5'-ATGCAAAT-3' but can also recognize the degenerate 5'-TAATGARAT-3' motif by forming a ternary protein complex with VP16 and HCF (113).

Thus, it has been established that Oct-1 can form multiprotein complexes with unrelated transcription factors. Further, an extensive study using numerous footprinting techniques have revealed the nature of the interaction of the Oct-1/VP16/HCF to a consensus octamer motif flanked 3' by a GARAT sequence (236).

The Oct-1 point mutation at amino acid 22 of the homeodomain that distinguishes binding to VP-16, did not disrupt GST-Oct-1 binding of GR. VP16 is able to bind to the POU domain of Oct-1 when a glutamate is at position 22 but is not when it is an alanine. By contrast, GR recognized the POU domain with similar avidity regardless of whether a glutamate or an alanine residue was at amino acid 22 of the homeodomain (81). GR also bound the POU domain of Oct-1 with a chimeric Oct-2 POU-hd.

5. GR binds Oct-2 as well as Oct-1.

My data supports the hypothesis that not only Oct-1, but also Oct-2 could have the ability to associate with GR. When a chimeric GST-Oct fusion protein, consisting of the Oct-1 POU domain substituted with the corresponding amino acid sequences of the Oct-2-Ho (homeodomain), was tested in a pulldown assay with ligand treated GR, the radiolabeled GR was retained at least as well as when tested with GST-Oct-1. This GST-Oct-1 Ho, chimeric protein had 2 amino acid

differences when compared to the GST-Oct-1 wild-type sequences. Two possibilities could exist. First, the two amino acid substitutions in the POU-sp domain can not abrogate the GR/Oct interaction and that Oct-2 can associate with GR. Second, the interaction between GR/Oct may be dependent on these two amino acid substitutions in the POU-sp domain. Based on results presented, neither hypothesis could be rejected. However recent experimental observations support the statement that Oct-2 can associate with GR..

Recently in our lab, using the mammalian two hybrid system with the GR DNA binding domain as the anchoring molecule and the GR L501P point mutant as a control, we have shown not only Oct-1, but also Oct-2, Pit-1 and their individual homeodomains are sufficient to associate in the nucleus of Chinese Hamster Ovary (CHO) and HeLa cells as measured by an increase in transcription from a minimal promoter (L. Pope et al. 1996, unpublished observation). These initial experiments show that GR and POU factors interact in the nucleus of mammalian cells. Moreover, it appears that this may be a conserved function of the POU factor family of transcription factors. The Oct-2 interaction supports experimental data presented by another group (4) but remains inconsistent with other data (15).

6. The avidity of GR for Oct-1 is increased in the absence of DNA.

Upon addition of ethidium bromide, transformed GR bound more avidly to Oct-1. Ethidium bromide has been used as an agent for identifying protein-protein interactions stabilized in the presence of DNA, as intercalation of ethidium bromide into DNA at the concentration employed in this work, disrupts protein/DNA binding without affecting protein/protein contacts (202). DNA can be introduced into the GST-Oct-1 binding reactions two ways. First, contamination of bacterial genomic DNA during the purification procedure of the GST-Oct-1 fusion protein may occur. Second, small quantities of either supercoiled or linear plasmid DNAs from the *in vitro* translation reaction may be carried through to the binding reaction.

When comparing the association of GR to Oct-1 in the presence of ethidium bromide, Oct-1 binding was consistently two fold higher, as determined by an increased in intensity of bands representing radiolabeled GR (Figure 11, lanes 5-8). This GR/Oct-1 binding could clearly occur in absence of DNA binding. Indeed binding of GR to DNA would appear to actually compete binding to Oct-1. Interestingly, plasmids containing EREs, as well as GREs (203) were efficient competitors for GR/Oct-1 binding. This observation may be

explainable in two ways. First, the concentration of DNA used may have been high enough to allow non-specific DNA binding by GR and Oct-1 and/or contaminating the incubation. Second, the experiment was improperly conducted. Specifically, methylation of adenosine at the N6 position in Bam HI sites have been shown to convert Bam HI sites to binding sites for GR (225). The plasmids used in this study were grown in the DH5α strain of *Escherichia coli* which contains the methylases necessary for conversion of Bam HI sites to GREs. Therefore, it will be important to repeat this experiment in the future with carefully titrated amounts of plasmid grown in methylation negative strains of *Escherichia coli* (GM48 strain). Alternatively, oligonucleotides containing GREs and EREs could be tested as competitors.

7. Zinc coordination in the DNA binding domain is not required for GR binding to Oct-1.

The requirement for zinc coordination by the GR DNA binding domain for Oct-1 binding was studied following the observation that GR N494 removed the fourth zinc coordinating cysteine residue (C495) also abolished binding to Oct-1. One possible explanation of this result was that disruption of wild-type zinc coordination of GR caused an

improper GR tertiary structure that abrogated its interaction with Oct-1.

A zinc removal strategy using hydrogen peroxide was used to evaluate the role zinc coordinated tertiary structure of the GR DNA binding domain had in binding to GST-Oct-1 (211). Previous experiments from other groups have shown that a recombinant GR DNA binding domain could reversibly coordinate two zinc atoms and that metal coordination was necessary to maintain the receptor fragment in a properly folded conformation allowing specific DNA binding (26).

Recombinant and *in vitro* translated proteins fragments containing the GR DNA binding domain were simultaneously treated with hydrogen peroxide and reduced with DTT. The reduction step was done under two conditions: 1) in the presence of $ZnCl_2$, with the intention to reconstitute zinc coordination and 2) in the absence of $ZnCl_2$, which should result in reduced cysteine residues. The radiolabeled GR tested in a GST-Oct-1 pull down assay showed that abolishing zinc coordination did not disrupt the GR/Oct-1 interaction. Subsequently, point mutations of cysteine residues that coordinate zinc atoms in both the first and second zinc binding motifs, C460Y, C492R and C495Y, did not disrupt GR binding to GST-Oct-1.

As a control for zinc removal from the GR DNA binding domain, its ability to bind to a specific DNA binding site was tested in a mobility shift assay. The *in vitro* translated radiolabeled protein product could not be used in a mobility shift assay under standard binding conditions because the unprogrammed reticulocyte lysate displayed the same shifting pattern as the lysate containing the *in vitro* translated GR (Giffin et al. 1994, unpublished observation). Therefore, the whole experiment was repeated this time using partially purified recombinant GR in a mobility shift assay. Indeed, under the conditions used, GR coordination was disrupted following peroxide treatment and the GRE binding activity could be restored to 10% of the wild-type activity following reduction in the presence of $ZnCl_2$.

The implications of these results are striking. First, total collapse of the zinc fingers of the GR DNA binding domain, and presumably therefore the finger tertiary structure, was unable to interfere with Oct-1 binding. Thus, DNA binding and Oct-1 binding are clearly separable activities of GR. Moreover, the zinc finger generated tertiary structure is also not a requirement for Oct-1 binding. Second, my results imply that the requirements for Oct-1 binding by GR are localized to specific short segments of the DNA binding domain with a zinc independent secondary structure.

8. Reduced cysteine residues in the GR DBD are required for GR/Oct-1 binding.

MMTS treatment of GR abolished its ability to bind to Oct-1. MMTS selectively modifies thiol groups on cysteine residues by adding a methylsulfyl group (226). A fragment encompassing amino acids 407-616 of radiolabeled rat GR was treated with 5 mM MMTS. The treatment has the potential to modify up to 10 cysteine residues within amino acids 440-500 of GR. As discussed above, removal of the zinc atoms from the finger structure did not affect binding to Oct-1. The two remaining cysteine residues are found at amino acid C450 in the first finger in a region of undefined secondary structure and C500 situated C-terminal to the second finger in the middle of α-helix 2. The C500 residue is absolutely conserved in nuclear receptors in contrast to C450 in which its conservation is limited to the steroid receptor subfamily. Thus, it seemed that methylation of either cysteine 450 or 500 was responsible for disrupting Oct-1 binding.

9. C500Y and L501P abolish interaction with Oct-1.

Amphipathic α-helices represent common motifs for protein-protein interactions. The crystal structure of GR bound to a

consensus GRE showed that α-helix 1 is buried in the major groove of DNA with α-helix 2 lying perpendicular across α-helix 1 (29). In solution, there is considerable exposure of the surface of helix 2 (30). Thus α-helix 2 of GR is potentially available to be a prime target for Oct-1 binding. Biochemical data presented above and C-terminal deletion analysis of GR strongly suggested that α-helix 2 may be important for Oct-1 binding as, binding was lost upon deletion into this helix. Point mutants C500Y and L501P, which abrogate GR/Oct-1 binding, confirm that helix 2 may possess determinants for interaction with Oct-1. However, X-ray/NMR indicate that C500 and L501 are normally buried towards the core of the protein, I am unable to propose at this time that either of these residues contacts Oct-1 directly. Rather, it would seem more likely that these mutations disrupt the α-helix in a manner that abrogates Oct-1 binding. Interestingly, that addition of a sulfylmethyl group at C500 with MMTS also abrogated Oct-1 binding, indicates that subtle changes to the structure of α-helix 2 could have a major impact on binding to Oct-1.

10. Oct-1 binding is a highly conserved property of nuclear receptors.

The α-helix 2 of the second zinc finger is highly conserved in nuclear receptors. To examine the possibility that POU domain binding might be a conserved property of nuclear receptors, I tested the ability of 6 nuclear receptors to bind GST-Oct-1 POU. Importantly, all of the 6 nuclear receptors tested bound Oct-1. The steroid receptors GR and ER both required transformation in order to bind to Oct-1. In contrast, the RXR-α, RAR-α' and c-erbA-α2 (TR-A-α2) bound Oct-1 in the absence of ligand. Furthermore, the distantly related *Drosophila* orphan receptor Ftz-F1α bound Oct-1 at least as well as the other receptors tested. This forms the foundation of my postulate that the DNA binding domain of nuclear receptors encode a highly conserved POU binding motif that will prove to be important for mediating functional interactions between nuclear receptors and POU factors *in vivo*. In addition, this data further reinforces my proposal that steroid receptors must dissociate from heat shock proteins prior to binding with Oct-1.

Loss of Oct-1 binding following deletion of RAR -α' C-terminally into α-helix-2 of the nuclear receptor provided evidence that the nature of the interaction between RAR-α'/Oct-1 is similar to the GR/Oct-1

interaction (compare Figure 10c to Figure 20c). Thus it can be reasonably proposed that at least the other nuclear receptors tested to date will bind Oct-1 similarly to GR.

11. A model for GR/Oct-1 binding.

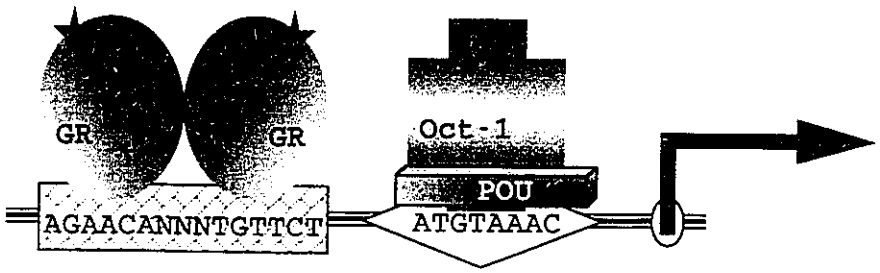
Three models can potentially explain the transcriptional modes of regulation employed by glucocorticoids to regulate gene expression through Oct-1: recruitment/co-occupancy, tethering and inhibition (Figure 21). The modes of transcriptional regulation are dependent on *cis* DNA sequences located near the promoter region of genes. The factors that bind to these specific sequences act either directly and/or with the multiprotein preinitiation complex indirectly through general or tissue specific co-factors (135, 136). These interactions are proposed to either facilitate the correct assembly of a more productive transcriptional complex that will increase active transcription or will cause a decrease in transcription by elusive mechanisms interfering with the correct assembly of the transcription initiation complex (237).

The recruitment/co-occupancy model (Figure 21a) is applicable to composite DNA regulatory elements containing a GRE and a degenerate octamer motif. In the absence of hormone, Oct-1 poorly recognizes its DNA binding octamer motif (3). In the presence of glucocorticoids, heat shock protein dissociated, active GR enters the

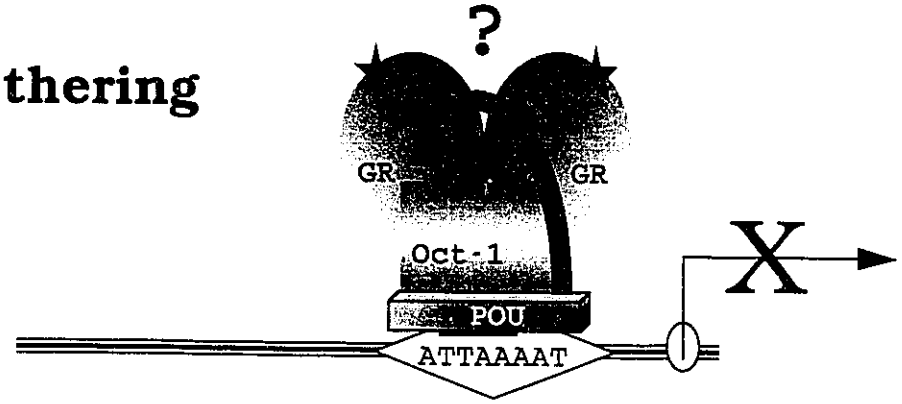
Figure 21. Mechanisms by which GR targets Oct-1 to regulate gene transcription.

a. Recruitment/co-occupancy model applies to complex response elements containing a palindromic GR in close proximity to a degenerate octamer motif as in the promoter proximal MMTV octamer motif. In the absence of active GR, Oct-1 occupancy of the promoter region is low. Once activated by ligand (star), GR may associate with Oct-1 in solution. Active GR occupies its respective responsive elements causing a local increased concentration of octamer factor which facilitates its binding to the octamer motif. This strategy may be employed by transcription factors in order to cooperatively bind to promoter regions of genes. Together the activation domains of both transcription factors act synergistically by recruiting undefined limiting co-factors to the TATA promoter region. b. Tethering may occur on select octamer motifs such as a GR negative regulatory octamer motif from the GnRH promoter as illustrated. In the absence of active GR, Oct-1 is constitutively activating transcription. Once ligand activated, GR binds to Oct-1 displaying an altered conformation based on the type of octamer motif it is bound. Neither GR nor Oct-1 can activate transcription in this configuration. The solid bar with the ? indicates co-factors may be involved in mediating these signals. The non-productive complex represses transcription from the TATA containing promoter. c. Inhibition could require a high affinity octamer motif as in the Histone H2B promoter. Constitutive Oct-1 activates transcription from a consensus octamer motif. Ligand activated GR is able to inhibit Oct-1 binding to the octamer motif in the presence or absence of stabilizing factors. In effect, transcription from the TATA promoter is down regulated.

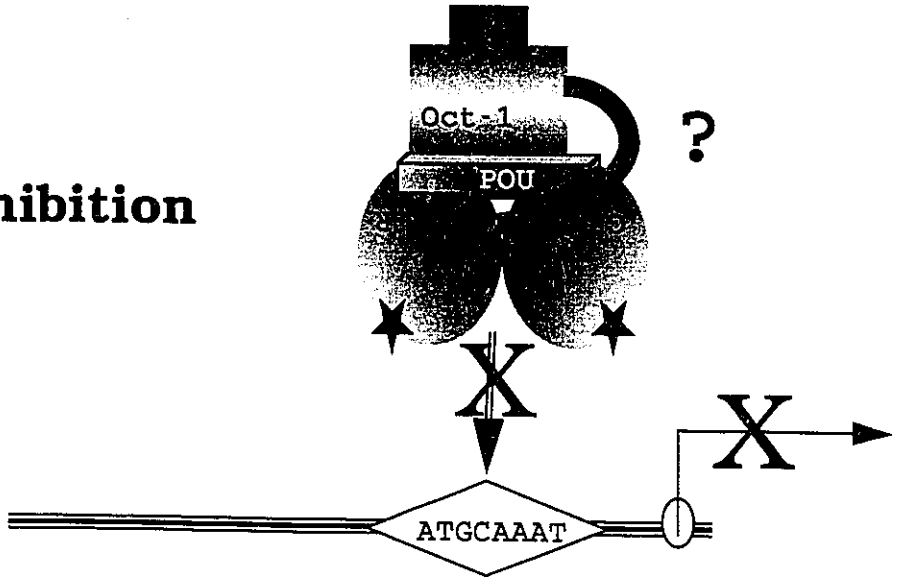
a. **Recruitment/Co-occupancy**



b. **Tethering**



c. **Inhibition**



nucleus where it can associate with Oct-1 (51). In solution, the GR/Oct-1 complex would be able to recognize a GRE with high affinity. Because DNA appears to prevent GR/Oct-1 binding I propose that the action of GR binding to its response element releases the octamer factor. In effect, the local concentration of Oct-1 is increased to facilitate its binding to juxtaposed lower affinity Oct-1 binding sites. In particular, on the MMTV promoter the binding of GR dimers has the potential to release 8 molecules of Oct-1 adjacent 2 low affinity octamer motifs. The model predicts that transcriptional activation would be synergistic at least in part due to the co-occupancy of the binding sites on a TATA containing promoter.

A recruitment/co-occupancy model could potentially explain events observed on the MMTV LTR. In the absence of hormone, ubiquitous and constitutive Oct-1 factor poorly recognizes its degenerate DNA binding sites. In the presence of glucocorticoids, GR is activated and rapidly translocated into the nucleus where it associates with Oct-1 and recruits Oct-1 to the lower affinity octamer motifs on the MMTV promoter.

The tethering model (Figure21b) requires a special type of octamer motif, a sequence similar to 5'-ATTAAAAT-3' (16), and no direct DNA binding site for GR. In the absence of hormone, Oct-1 dependent transcription is constitutively active. In response to glucocorticoids,

active GR enters the nucleus devoid of heat shock proteins and binds to Oct-1. The Oct-1/GR complex is able to recognize the octamer motif, binds to it and down regulates transcription. GR tethered to DNA through Oct-1 bound to an octamer motif and may be stabilized by additional factors to form a complex that disrupts the correct assembly of the transcription complex at the TATA promoter.

Recently, GR has been shown to be tethered by Oct-1 in a protein complex on a degenerate octamer motif to negatively regulate transcription of the GnRH promoter in response to glucocorticoids (16). The tethering model as been demonstrated on the GnRH promoter which appears to be dependent on the type of octamer response element.

The inhibition model (Figure 21c) requires a high affinity octamer motif as the Histone H2B consensus octamer motif 5'-ATGCAAAT-3'. In the absence of steroid, Oct-1 dependent transcription is constitutive. Activated, heat shock protein dissociated GR enters the nucleus where it associates with Oct-1. This association inhibits the ability of Oct-1 to bind to its octamer motif upstream of the TATA promoter to inhibit transcription.

Initially, the inhibition model has also been proposed to explain mechanisms of glucocorticoid mediated trans-repression of AP-1 dependent transcription (166, 167). Activated GR has been shown to

inhibit AP-1 recognition of its DNA response element and that the inhibition is mutual (167). More recently, GR mediated repression of AP-1 also appears involve the exhaustion of a rate limiting co-factor CBP, a common co-activator of both AP-1 and GR (155). Overexpression of GR does not appear to decrease the affinity of Oct-1 for a consensus octamer motif nor is the inhibition mutual (4). Rather, experimental observations suggest that the inhibition is due to exhaustion of a rate limiting cofactor, further supported by tissue specific transcriptional repression. The inhibition model has been proposed to explain the repression of the histone H2B gene. However, most observations to date failed to support this model (4).

12. Transcriptional synergy on the MMTV LTR

Hormonal induction of the MMTV LTR requires that steroid receptors and other transcription factors occupy their sequence specific binding sites (176). In the absence of hormone, the chromosomally integrated MMTV LTR is precisely packaged into an array of at least six nucleosomes that results a transcriptionally dormant promoter (173). Each nucleosome encompasses a region of ~200 bp. One nucleosome/core particle encompasses the hormonally responsive region including the NF-I sites and one of the two octamer motifs. The nucleosome is believed to inhibit the binding of the constitutively

active transcription factors resulting in the extremely low level of basal transcription observed (175). Following hormonal induction, GR binds to the nucleosome containing MMTV hormone responsive region which is then remodeled (174); potentially through direct interaction with SWI factors (i.e. human brahma) which increases exposure of the sequence specific binding sites for constitutively active transcription factors (i.e. NF-1 and Oct-1) (238, 239). This relieves the repression of nucleosomes on transcription and allows for transcription factor loading on the LTR. By unknown mechanisms, the basal transcriptional machinery is rapidly recruited to the promoter region and produces an increased transcription efficiency.

In contrast, when using the nucleosome-free MMTV promoter as a template for cell-free transcription experiments, basal activity is very high (178, 240). Mutating individual transcription factor binding sites has determined that the elevated basal activity can be attributed predominately to the NF-I binding sites located between the hormone response elements (HREs) and the octamer motifs (178). Thus, NF-I appears to be primarily responsible for the basal transcription on nucleosome free templates. Following hormonal induction, *in vitro* foot-printing assays have shown co-occupancy of the HREs and the octamer motifs which are attributable to the binding of steroid hormone receptors and Oct-1 to their sequence-specific binding sites (174).

Although Oct-1 is ubiquitously expressed, Oct-1 is unable to significantly activate transcription of the MMTV promoter in the absence of hormone (3). Thus, steroid receptors bound to the promoter region of the MMTV appear to facilitate the octamer motif dependent activation of MMTV transcription.

13. Understanding the tethering model.

I am proposing that GR dissociated from heat shock proteins can bind to Oct-1 in the nucleus modulating its transciptional activities. This provides mechanisms of altering transcriptional activities of ubiquitous and constitutive transcription factors. Depending on the context of the octamer motif in the promoter regions of genes and the tissue specificity transcription may be differentially regulated. On promoters containing both a GRE and an octamer motif, transcription could be synergistic. However, on promoters containing only an octamer motif and no GRE, Oct-1 dependent transcription could be repressed. The repression could be due to inhibition of Oct-1 binding or exhaustion of a rate limiting co-factor. Alternatively, GR may be tethered to the promoter through Oct-1 to trans-repress Oct-1 dependent transcription.

In order for liganded GR to activate transcription alone from a simple GRE it must be directly bound as dimer to a typical palindromic

GRE (241). GR amino acid K461 is essential for the correct interpretation of GRE signals of a DNA bound receptor (242). The lysine residue at position 461 appears to be required to arrange the transactivation domains of GR in an active conformation following binding to GREs. A GR K461A mutation produces a ligand dependent constitutively active conformation regardless of the GRE context including the plfG composite GRE described in the introduction. In short, in the presence of activated GR, GR and AP-1 (jun/fos heterodimers) repress transcription from the plfG composite element (162). By contrast, the GR K461A mutant produces a complex on this same element that is capable of activating transcription in response to hormone.

The tethering model, determined by the octamer motif context, produces an altered Oct-1 conformation that allows GR to bind to Oct-1 in a complex which may include additional factors (16). The absence of a flanking GRE does not allow GR to assume a conformation favorable for activating transcription. In effect, GR also silences transcriptional activation of Oct-1. It would be interesting to test whether the GR K461A mutation could cause transcriptional activation while being tethered to a promoter region through Oct-1

The models presented may potentially be the basis of a broadly based mechanism of transcriptional gene regulation. POU factors are

ubiquitous or tissue specific transcription factors appear to be constitutively active from simple response elements. However, their modes of transcriptional gene regulation may be altered by hormone inducible nuclear receptors on complex promoter elements.

V. CONCLUSION

This thesis provides compelling *in vitro* evidence for direct physical binding of nuclear hormone receptors to POU transcription factors in solution. Specifically, I have demonstrated that:

1) Several nuclear hormone receptors were able to bind to the POU domain of Octamer transcription factor-1 in a GST pulldown assay.

2) Binding of steroid receptors to the POU domain of Oct-1 was hormone dependent, in so much as dissociation of the steroid hormone receptors from their heat shock protein-associated complex appeared to be a prerequisite for POU binding.

3) For glucocorticoid receptor, the nuclear receptor studied most intensively, an α-helix in the second zinc finger of the DNA binding domain of the receptor appeared to contain the key determinants for Oct-1 under my experimental conditions.

4) The sufficiency of the POU domain for nuclear receptor binding, and the ability to exchange motifs within the Oct-1 POU domain with the complimentary motifs of Oct-2 without affecting binding to GR, argues that the ability to bind nuclear receptors may also be broadly conserved within the POU superfamily.

5). Results with DNA competitors suggests the intriguing possibility that nuclear receptor/octamer transcription factor binding may be exclusive of DNA binding.

6). I propose a model for testing in vivo, in which the hormone dependent binding of GR to Oct-1 leads to the recruitment and release of Oct-1 adjacent glucocorticoid receptor transcriptional regulatory sites.

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(2) Abstracts:

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