Characterization of the DNA Binding Properties of the Thyroid Hormone Receptor

by

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We accept this dissertation as conforming to the required standard

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ABSTRACT

This thesis describes work done with the thyroid hormone receptor (TR), a nuclear protein which binds to specific DNA sequences and regulates transcription in response to thyroid hormone levels. The studies can be divided into two broad categories: structure/function studies of the TR protein, particularly with regards to DNA binding function; and, structure/function studies of the DNA sequences to which the thyroid hormone receptor binds in order to regulate gene transcription.

In order to examine the DNA binding properties of the TR an electrophoretic mobility shift assay (EMSA) was utilized. Conditions of this assay were optimized for the use of *in vitro* translated TR. Mutant forms of the β-isoform of thyroid hormone receptor were generated using a PCR-based mutagenesis protocol. Each mutant substituted a different residue of the 12 amino acid-long α-recognition helix with alanine. The mutants were analyzed for their abilities to bind to thyroid hormone response elements (TREs), and to activate transcription in transfected eukaryotic cells. The DNA binding results were consistent with a conserved α-helix structure, with conserved function for many residues, that is similar to that of the related receptors for glucocorticoids and estrogen. Only the first of the three non-conserved residues lying in the P-box (EGG), a portion of the recognition α-helix that facilitate differential binding of distinct DNA sequences, disrupted binding when substituted with alanine. The third position of the P-box, when substituted with alanine exhibits an altered ability to bind to certain natural TREs. The mutant form of TR with alanine substituted for the second P-box position displayed only a modest decrease in DNA binding affinity compared to wild-type TR (roughly 3-fold), yet was completely deficient in *trans*-activation.

The structure-function studies of TR binding sites on DNA applied a methylation interference protocol to examine the interactions of TR with direct repeats (DR) of the idealized hexameric sequence, spaced by three to five base-pairs. The interactions of both the TR/TR homodimer and the TR/RXR (9-*cis*-retinoic acid receptor) heterodimer with the DRs were examined. The methylation interference patterns for the TR/TR homodimer bound to the DR sequences are virtually identical for spacers of four and five base-pairs, but with three base-pairs, there is some evidence that at least one DNA binding domain is misaligned with the DNA to accomodate the unfavourable spacer length. The TR/RXR heterodimer methylation interference pattern is distinct on all three DRs, probably due to the fact that in the heterodimer cooperative intermolecular contacts are made between the DNA binding domains of the two receptors, but only when the spacer distance is four base-pairs. When a poorly conserved everted repeat (EvR) that overlaps the idealized DR is present,

the homodimer, but not the heterodimer, binds this cryptic EvR in competition with the DR. The binding modality of the TR homodimer and TR/RXR heterodimer to DRs was reevalutated using point mutants and EMSA. The TR homodimer and TR/RXR heterodimer both bind to idealized direct repeats with DBDs aligned appropriately for a direct repeat; however, evidence is presented that there are certain poorly conserved sequences that are intermediate between DRs and EvRs that are differentially recognized by the TR homodimer and the TR/RXR heterodimer. That is, the homodimer binds with the DBDs aligned appropriately for a EvR, and the heterodimer DBDs are aligned appropriately for a DR.

Examiners:

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A: Adenine

BSA: Bovine serum albumin

C: Cytosine

CAT: Chloramphenicol acetyl transferase

cDNA: Complementary deoxyribonucleic acid

CI: The first zinc-binding module of the nuclear receptor DNA binding domain

CII: The second zinc-binding module of the nuclear receptor DNA binding domain

COUP: Chicken ovalbumin upstream promoter

cpm: Counts per minute

dATP: deoxyadenosine triphosphate

DBD: DNA binding domain

DNA: Deoxyribonucleic acid

dNTP: deoxyribonucleotide triphosphate

DR: Direct Repeat

dsDNA: Double-stranded DNA

DTT: Dithiothreitol

EDTA: Ethylene-diaminetetraacetic acid

ER: Estrogen receptor

EvR: Everted repeat

G: Guanine

GR: Glucocorticoid receptor

GRE: Glucocorticoid response element

GRTH: Generalized resistance to thyroid hormone

GST: Glutathione S-transferase

IR: Inverted repeat

K_d: Dissociation constant

LBD: Ligand binding domain

N: Nucleotide

NMR: Nuclear magnetic resonance

PAGE: Polyacrylamide gel electrophoresis

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PPAR: Peroxisome proliferator-activated receptor

RAR: Retinoic acid receptor

rNTP: ribonucleotide triphosphate

RRL: Rabbit reticulocyte lysate

RXR: Retinoid X receptor - 9-*cis*-retinoic acid receptor

SDS: Sodium dodecyl sulphate

S.E. Standard error

ssDNA: Single-stranded DNA

T: Thymidine

T_3: Triiodothyronine

TBE: Tris, Borate, EDTA

TCA: Trichloroacetic acid

TR: Thyroid hormone receptor

TRE: Thyroid hormone response element

VDR: Vitamin D3 receptor

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DEDICATION

My Mother, Valerie Faris, passed away while I was in graduate school. I thank her for all the gifts she gave me, and my family, and dedicate this thesis to her, with love.

1.0 General Introduction

Over the last decade understanding of the activities of nuclear receptors in hormonal control of gene expression has undergone explosive growth, and thyroid hormone receptor has played a central role in characterizing this important group of proteins. Cloning of a viral oncogene homolog to the thyroid hormone receptor (TR) gene led to the identification of a variety of genes for related hormone receptors. Generally, the nuclear receptors are located in the nucleus, or translocated to the nucleus in the presence of ligand, and bind to DNA sequences called hormone response elements (HREs) as either a homodimer or a heterodimer with another nuclear receptor, although some can bind to DNA as monomers (Glass, 1994). Regulation of a gene(s) associated with an HRE depends on the presence or absence of ligand, the identity of the receptor(s) bound to the HRE, and the DNA sequence of the HRE itself. Numerous revealing studies have helped to characterize DNA binding interactions, functional domain structures and activities, regulated gene targets, ligand specificity, the ability to form homo- or heterodimers, and other fundamental properties of nuclear receptors. On the other hand, these works have also added to the complexity of the steroid/thyroid hormone receptor superfamily paradigm. It has become increasingly clear that heterodimerization plays a significant role in expanding and refining the repertoire of regulatory activities carried out by particular receptors. In a sense, our increasing understanding of receptor function has concomitantly led to an increasing number of questions with respect to physiological activity. In the end, it is the physiological questions which drive investigations in this area, for they originate from amongst such pressing areas as oncogenesis, development, genetic disorder and more basic physiologic functions.

The work described in this thesis has somewhat paralleled the course of research in the field of the steroid/thyroid receptor superfamily. Initial experiments investigating the importance of specific amino acids in the binding of TR to DNA sequences termed thyroid hormone response elements (TREs) helped elucidate the roles of these amino acids in

discriminating between related DNA sequences. Later work went on to examine differences between the interactions of TR homodimers and heterodimers with certain classes of TREs.

This introduction will provide a general overview of thyroid hormone action with particular emphasis on the DNA binding activity of the thyroid hormone nuclear receptor.

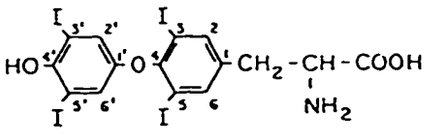
1.1 Physiology of Thyroid Hormone

1.1.1 Thyroid Hormone Production

The sole biological source of thyroid hormones (THs) is the thyroid gland of vertebrate organisms. Inside the lumen of thyroidal follicles a massive, tyrosine-rich, prohormone protein, thyroglobulin, undergoes a series of reactions which release thyroid hormones (reviewed in McNabb, 1992a). Upon entry of thyroglobulin into the lumen many tyrosine residues are iodinated at two positions of the phenolic ring. Subsequently, iodinated tyrosines are coupled and cleaved from the peptide backbone to release three related compounds: thyroxine (T_4), 3-5-3'-triiodothyronine (T_3), and 3-3'-5'-triiodothyronine (reverse T_3) (Figure 1.1). Once these reactions have occurred, thyroid hormone molecules enter the blood stream by diffusion and are distributed throughout the body.

T_4 is the most abundant species of TH in the blood stream, being produced and released in 15-fold molar excess over T_3 (Chopra, 1973). However, T_3 exhibits several-fold greater biological activity than T_4, and due to the rapid deiodination of T_4 upon entry into most cells, is the predominant form in peripheral tissues. Therefore, T_3 is considered to be the primary thyroid hormone, in that it is likely to be responsible for the majority of regulatory phenomena. Reverse T_3 is a minor form of TH which is produced from T_4 by a deiodinase activity distinct from that which produces T_3 (McNabb, 1992a). Reverse T_3 is believed to be biologically inactive, and may be produced to allow cells to internally modulate active TH levels beyond the concentrations found in the blood. THs are believed to enter cells by diffusion, so there is unlikely to be regulation at the point of hormone entry.

A)



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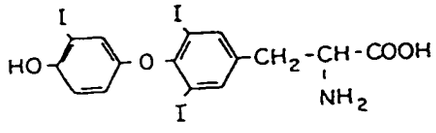

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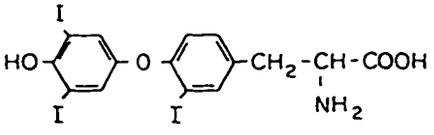


Figure 1.1: The structures of the thyroid hormones. (A) Thyroxine (T_4); (B) 3-5-3'-Triiodothyronine (T_3); and, (C) 3-3'-5-Triiodothyronine (reverse T_3).
(McNabb, 1992a)

Once in the cytosol, T_3 may be bound by several different categories of receptor. There are plasma membrane, mitochondrial, cytosolic and nuclear receptors for T_3 with varying affinities and saturability (McNabb, 1992b). The nuclear receptors are responsible for the bulk, if not all, of the gene regulation effects of T_3, and the other receptors have really only had hypothetical functions assigned to them. It is the nuclear receptors that constitute the focus of this thesis, and the many studies that will be summarized in this introduction.

1.1.2 Regulation of Thyroid Hormone Levels

Thyroid hormone levels are generally maintained by a negative feedback loop involving the hypothalamus and pituitary, termed the hypothalamic-pituitary-thyroid (HPT) axis. Basically, TH production by the thyroid gland is stimulated by thyroid stimulating hormone (TSH), or thyrotropin, released by the pituitary. TSH release is, in turn, stimulated by the hypothalamic hormone, thyrotropin releasing hormone (TRH). In order to close the regulatory loop, production of both TSH and TRH is inhibited at the transcriptional level by THs (Figure 1.2).

The HPT axis allows integration of other environmental and hormonal signals into determination of thyroid hormone levels. Examples of other factors which influence the HPT axis include such bioactive compounds as: glucocorticoids, somatostatin, serotonin, epinephrine, norepinephrine, and dopamine; as well as direct innervation of the thyroid gland by the sympathetic and parasympathetic nervous systems (McNabb, 1992c). It should, however, be emphasized that the negative feedback loop described above is the primary determinant of thyroid hormones levels in the blood of adult vertebrates.

1.1.3 General Effects of Thyroid Hormone

T_3 is important in vertebrate life from embryogenesis through growth of the individual, and as a homeostatic regulator in the adult homeotherm. In general the effects of thyroid hormone are on metabolism; THs tend to be stimulatory, increasing the metabolic activity of an organism. The influence of TH in development arises from effects on cell growth and differentiation.

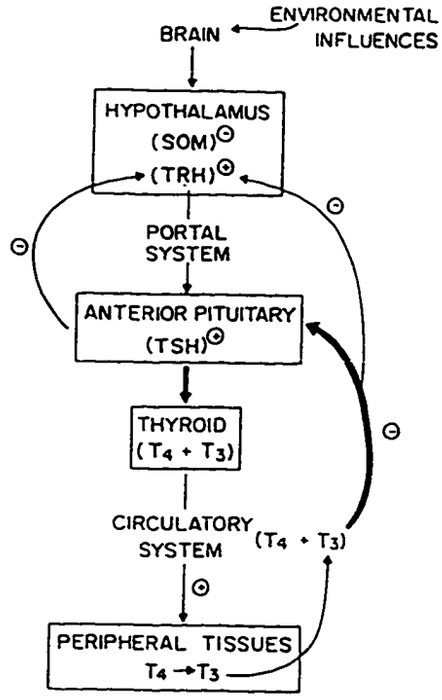


Figure 1.2: The HPT axis (hypothalamus-pituitary-thyroid) regulatory circuit that determines circulating thyroid hormone levels. Abbreviations are: SOM, somatostatin; TRH, thyrotropin releasing hormone; and, TSH, thyrotropin. - and + signs indicate inhibitory and stimulatory signals, respectively, with the dark lines indicating the most important ones.
(McNabb, 1992c)

Thyroid hormones are regulated, in adult homeotherms, to maintain the metabolism of an organism at an intrinsic "set-point" although there are several cases known in which thyroid hormone levels are altered in order to adjust the set-point in response to environmental stress. Ambient temperature, diet composition and caloric intake (starvation or over-feeding) of an individual can result in changes in serum TH levels that increase or decrease metabolism appropriately. For example, decreased caloric intake results in depressed T_3 levels and therefore lowered metabolism. Disruption of TH regulation, caused by drugs or disease, also affects metabolism. In the adult, the hyperthyroid state (elevated levels of T_3) is characterized by elevated body temperature, nervousness and weight loss. Hypothyroidism, on the other hand, is typified by lowered body temperature, lethargy, weight gain, and clinical manifestations such as slow mentation, muscle fatigue, decreased appetite, and constipation (Mooradian, 1995).

During human development, hypothyroidism results in cretinism with characteristic mental retardation, short stature, and other morphological changes (NcNabb, 1992d). Neurological defects appear to partially stem from defects in cell mobility and growth that inhibit proper spatial and temporal regulatory patterns. In lower vertebrates, thyroid hormone plays an essential role as a trigger for morphogenesis. Events in the metamorphosis of the tadpole to the adult frog such as tail resorption and leg growth arise in response to increasing levels of thyroid hormone.

1.2 Identification of the Genes for Thyroid Hormone Receptor

1.2.1 Biochemical Characterization

By the mid-1970s a nuclear receptor activity for thyroid hormone had been detected and characterization was begun. Early work with rat liver nuclear preparations suggested that there are multiple forms of the TR on the basis of chromatographically separable hormone binding activities (Latham *et al.*, 1976) and isoelectric point variants which have pIs ranging from 5.3-5.9 (MW = 49,000 kD; Ichikawa and DeGroot, 1987). The acidic

receptors were isolated from chromatin-containing fractions of nuclear extracts in the presence or absence of hormone, and maintained an affinity for double-stranded DNA (MacLeod and Baxter, 1976). The TR activity exhibits hormone binding characteristics which parallel biological hormone response. For example, the relative affinities of thyroid hormones and analogs were consistent with the compounds' biological activities. The receptor exhibited a higher affinity for T_3 than for T_4, consistent with the greater biological activity of this hormone. Furthermore, a correlation between *in vivo* receptor saturation with hormone and maximal biological effect was established (Nyborg *et al.*, 1984). Despite the increasing efficacy of protocols for receptor purification from tissue, maximum purity achieved was at best 1% in the late 1980s (Norman, *et al.* 1989). Perhaps not surprisingly, then, the route by which the TR gene came to be cloned at this time was fortuitous and involved a completely different approach.

1.2.2 Discovery of the Genes for Thyroid Hormone Receptors

The identification of the gene for TR resulted from the convergence of several diverse research projects. In particular, work on the viral oncogene v-*erbA* laid the foundation for discovery of a large number of nuclear receptors. V-*erbA* is one of two oncogenes encoded by the avian erythroblastosis virus (AEV), the other designated v-*erbB*. The transforming capacity of AEV is dependent on v-*erbB*, and enhanced by v-*erbA* which blocks differentiation when co-expressed with v-*erb*B, but which is otherwise not oncogenic (Frykberg *et al.*, 1983). V-*erbA* similarly augments the transforming capacity of other viral oncogenes including v-*src*, v-*fps*, v-*sea* and v-Ha-*ras* (Kahn *et al.*, 1986). Nucleic acid hybridization techniques were used to identify two v-*erbA* homologs each in human, c-*erbA1* and c-*erbA2* (Jansson *et al.*, 1983), and chicken chromosomes(Vennström and Bishop, 1982). Sequencing v-*erbA* revealed that it was a new class of oncogene with no homologs in contemporary protein databases (Debuire *et al.*, 1984).

Shortly thereafter, Ronald Evans' group cloned the gene for the human glucocorticoid receptor (GR) by screening an expression library with antibodies made to purified receptor

(Hollenberg *et al.*, 1985). Previous studies with purified GR had identified distinct functional domains, separable by proteolytic digest (reviewed in Weinberger *et al.*, 1985). This allowed the assignment of functions to particular regions of the GR primary sequence (Weinberger *et al.*, 1985); for example, a cysteine- and basic residue-rich region was shown to be involved in DNA binding, and the C-terminus responsible for ligand binding. The only significantly related sequence to the GR was found to be that of v-*erbA* , having 22% amino acid sequence similarity overall and greater than 40% within the 60 amino acid putative DNA binding domain. This discovery made a connection between the viral oncogene and nuclear hormone receptors.

The groups of Ron Evans and Bjorn Vennström subsequently cloned the human (Weinberger *et al.*, 1986) and chicken (Sap *et al.*, 1986) c-*erbA* genes using v-*erbA*-derived nucleic acid probes. The chicken gene encodes a 408 amino acid protein which differs from v-*erbA* by only 17 point mutations and a 9 residue deletion near the C-terminus. The human gene, located on chromosome 3, is highly homologous to its chicken counterpart, exhibiting 91% amino acid sequence similarity in the putative DNA binding domain and greater than 76% similarity C-terminal to this domain (Figure 1.3) (Goldberg *et al.*, 1989); however, the N-terminus displays less than 20% sequence similarity. Both the chicken and human c-*erbA* genes were shown to encode proteins that specifically bind T_3 and T_4 with affinities identical to those of the biochemically characterized TR, and thus the cellular homologs of the v-*erbA* gene were declared to represent genes for the TR. As it was known at this time that another human c-*erbA* gene located on chromosome 17 displayed greater homology on the basis of nucleic acid probe hybridization (Jansson *et al.*, 1983) it was suggested that the cloned gene represents not the true cellular homolog of v-*erbA* but a related, β, form of TR; thus, the gene was designated c-*erbA*β and the encoded protein, TRβ. Subsequently, the true homolog to v-*erbA* , c-*erbA*α was cloned from rat (Thompson *et al.*, 1987) and human (Benbrook and Pfahl, 1987) cDNA libraries and shown to be a TR as well. Primary sequences predicted for both TRαs display 97%

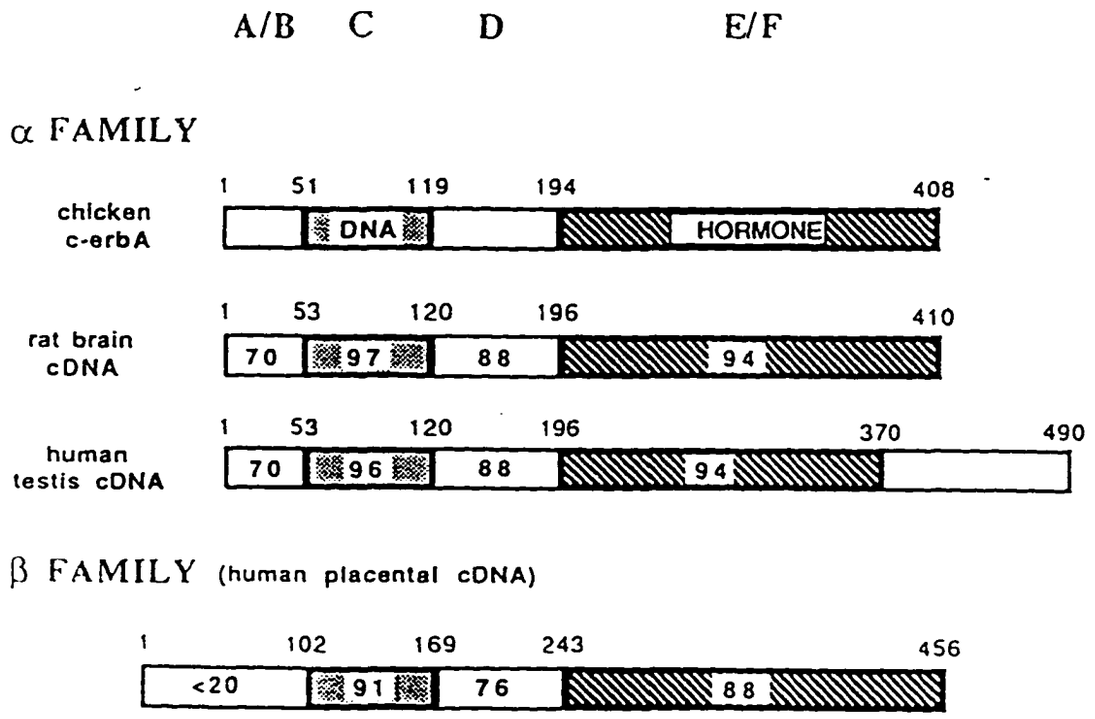


Figure 1.3: Conservation of sequence between domains of the first cloned *erbA* gene products. The amino acid position of sequence domain boundaries are indicated above each box and the domain names are indicated at the top of the figure. Amino acid similarity (%) is shown between each functional domain and the same domain in the chicken c-*erbA*α gene product. Note the high degree of similarity in the DNA binding domain (Domain C), and the ligand binding domain (Domain E/F) both between species and isoforms. The human testis cDNA is the splicing variant, TRα_2.

(Goldberg*et al.*, 1989)

sequence similarity to v-*erbA* in the putative DNA binding domain and, in contrast to TRβ, 70% similarity in the amino terminus. As predicted, the c-*erbα* gene is located on chromosome 17.

Further diversity within the c-*erbA* gene subfamily came to light as more cDNA clones became available. The first rat cDNA identified which corresponds to c-*erbAα* encodes a polypeptide of 410 amino acids (Thompson *et al.*, 1987), called TRα1. A cDNA resulting from an alternatively spliced transcript was identified which encodes a protein, TRα2, that is identical to TRα1 from the N-terminus to residue 370, just C-terminal of the DBD, but then diverges until the C-terminus, which is residue 492 (Figure 1.4) (Izumo and Mahdavi, 1988). TRα2 does not bind thyroid hormones or analogs, but is still physiologically relevant as it interferes with TRα1 function (Koenig *et al.*, 1989; Lazar *et al.*, 1989). Likewise, there are alternatively spliced forms of TRβ. The first cDNA cloned encoded TRβ1, but an alternative, pituitary-specific β2 cDNA splicing variant has been isolated from a rat cell cDNA library (Hodin *et al.*, 1989) and a chicken homolog of TRβ2 is expressed in the developing retina (Sjöberg *et al.*, 1992). Rat TRβ2 has a divergent N-terminal sequence, but is identical to TRβ1 for the final 461 amino acids, which contain the DNA and ligand binding domains. Finally, a shorter form of TRβ, TRβ0, having only 14 amino acids N-terminal to the DNA binding domain was identified from a chicken kidney cDNA library (Forrest *et al.*, 1990).

1.2.3 Thyroid Hormone Receptors Activate Transcription in Response to Thyroid Hormone

The ultimate proof that the c-*erbA* gene products mediate the biological activities of thyroid hormones was, of course, the ability of the proteins to carry out T_3-responsive transcriptional regulation of known T_3-regulated genes. This process involves, minimally, three general activities: hormone binding; sequence-specific DNA binding to sites near regulated genes; and, ligand-dependent enhancement or repression of basal transcription levels. Hormone binding had served in the initial identification of the receptors, but

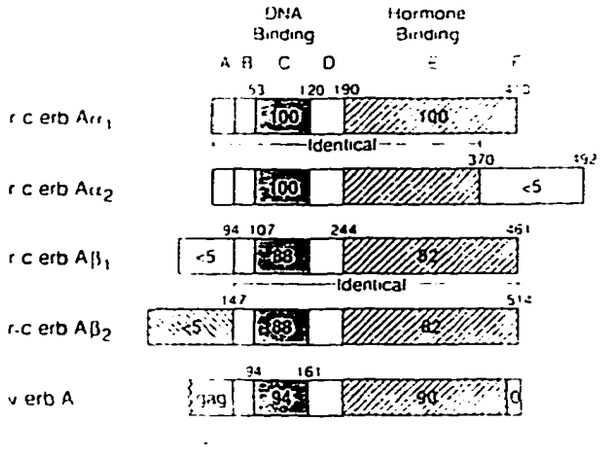


Figure 1.4: A schematic representation of the sequence divergence of TRα and TRβ splicing variants. The amino acid position of boundaries between sequence domains are indicated above each box, and the sequence similarity (%) to the equivalent domain in the rat TRα1 is indicated for the DBDs and LBDs. Alternative splicing generates TRαs with distinct C-terminal sequences, while the DBDs and N-terminal sequences are identical. Similarly, TRβ1 and TRβ2 have identical DBDs and C-terminal domains, but distinct N-termini. Included for the sake of comparison is the v-*erbA* gene product.
(Glass and Holloway, 1990)

confirmation of DNA binding and transcriptional activation required more elaborate assays.

Genes which are regulated by thyroid hormone are associated with *cis*-acting regions of DNA, known as thyroid hormone response elements (TREs). TREs were first localized using transient transfection assays. Reporter plasmids, harbouring DNA sequences of interest upstream of a promoter driving expression of the bacterial chloramphenicol acetyl transferase (CAT) gene, were transfected into cells expressing the TR. Differences in the levels of CAT activity between transfected cell cultures grown in the presence or absence of T_3 reflect whether the DNA sequence of interest contains a TRE. Typically, promoter regions of T_3-regulated genes are introduced into the reporter plasmid, and TREs are localized by deletion analysis. This assay first identified a TRE in the upstream region of the rat growth hormone gene (Flug *et al.*, 1987; Glass *et al.*, 1987) (Figure 1.6) that responds positively to T_3; that is, *trans*-activation is enhanced in the presence of T_3. The biochemically purified receptor was shown, using an electrophoretic mobility shift assay (EMSA; Fried and Crothers, 1984), to specifically bind DNA fragments containing this TRE *in vitro* (Lavin *et al.*, 1988). Shortly thereafter, the *in vitro* translation product of the human c-*erbA* gene was shown, by a different assay, to bind the same region of DNA (Glass *et al.*, 1987). Thus, the identity of the c-*erbA* gene products as TRs was confirmed on the basis of DNA binding specificity.

The electrophoretic mobility shift assay has become a standard method for analysis of nuclear receptor DNA binding activity (Figure 1.5). In this technique, a short (typically 20 to 100 base-pairs), radiolabeled DNA probe containing a TRE is incubated with TR to allow TR-TRE binding to reach equilibrium. The binding reaction is loaded directly onto a native polyacrylamide gel and electrophoresed to separate free and bound DNA, the latter migrating at a slower rate than the former. The EMSA may be analyzed visually after exposure of film to the EMSA gel, or quantitated by either direct excision of bands or densitometric analysis of the autoradiogram, and provides information that is useful in a number of ways. First, it can give a gross representation of the affinity of the interaction

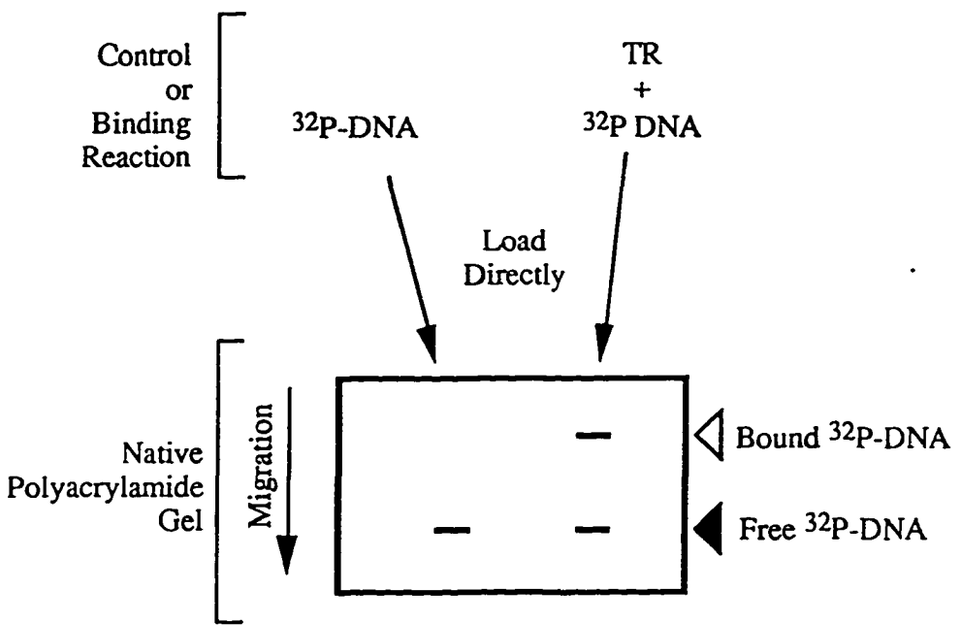


Figure 1.5: Diagrammatic representation of the electrophoretic mobility shift assay (EMSA) commonly used for studying the binding of nuclear receptors to DNA probes. Details are described in the text.

Source	Sequence	Reference
rGH -190/-167	TAAGGTAAGATCAGGGACGTGACCGCAGG	Glass *et al.*, 1987
hTSHα -22/-7	GCAGGTGAGGACTTCA	Krishna *et al.*, 1989
rMHC -124/-146	TTGGCTCTGGAGGTGACAGGAGG	Izumo *et al.*, 1989
hME -287/-257	AGGACGTTGGGGTTAGGGGAGGACAGTGGAC	Desvergne *et al.*, 1991
mMLV -351/-331	AGGACGTTGGGGTTAGGGGAGGACAGTGGAC	Sap *et al.*, 1989
cLYS -2326/-2351	TTGACCCCAGCTGAGGTCAAGTTACG	Bahiahmad *et al.*, 1990
rGH_3 1342/1388	AGGTAACTTGGGAGTCCCAGGCAGAGGTCAC	Sap *et al.*, 1990

Figure 1.6: A sample of natural TREs illustrating the diversity of half-site sequence, orientation and spacing in tandem repeats. TREs consist of core hexamers (indicated by arrows) in tandem repeats. The sequences above have been shown to confer T_3-responsiveness upon heterologous promoters, and to bind TR. The sources for the TREs are: rGH, rat growth hormone gene promoter; hTSHα, human thyrotropin α-subunit gene promoter; rMHC, rat myosin heavy chain gene promoter; hME, human malic enzyme gene promoter; mMLV, maloney murine leukemia virus promoter; cLYS, chicken lysozyme gene silencer element; and rGH_3, rat growth hormone gene, third intron.

between receptor and DNA based on the proportion of DNA bound by the receptor. Second, it allows the observation of complexes with distinct mobilities; for example, the complex of a monomer of TR bound to a TRE has a higher mobility than does that of a complex consisting of a TR dimer bound to a TRE. Third, it is possible to carry out the EMSA in the prescence of ligand, unlabeled competitor DNA, other proteins, etc. These and other modifications to the EMSA have facilitated many studies of TR DNA binding properties.

There were no *in vitro* assays for *trans*-activation by TRs in the 1980s. However, an *in vivo* assay was developed for the GR (Giguère *et al.*, 1986), which has now become the standard technique for monitoring the activation capacity of nuclear hormone receptors. The first requirement for this assay is a eukaryotic cell line that does not possess significant endogenous receptor activity. For thyroid hormone receptor assays, there are several cell lines that are commonly used: the African green monkey kidney cell line, CV1, and the SV40-transformed daughter cell line, COS; JEG-3 and HepG2. In order to assay for receptor activity the chosen cells are transfected with two plasmid vectors. One is an "expression" or *trans*-vector which constitutively expresses the gene for the receptor under investigation. The other is a "reporter" or *cis*-vector bearing a foreign gene which encodes an assayable product such as chloramphenicol acetyl transferase (CAT) or luciferase. The reporter gene is usually driven by a promoter with low basal activity, and a DNA sequence harbouring a hormone response element (HRE) may be cloned upstream. Thus, when the reporter and expression plasmids are co-transfected into the host cell line, high level expression of the reporter gene will occur only in the presence of hormone, assuming the TRE confers positive responsiveness to hormone. Cell extracts are prepared and assayed to determine reporter gene expression levels.

Application of this transcriptional assay using c-*erbA* genes cloned into an expression plasmid confirmed that c-*erbA*α and c-*erbA*β do, in fact, encode functional TRs that are capable of activating gene transcription from TREs in response to the presence of T_3

(Koenig *et al.*, 1988, Thompson and Evans, 1989). Having identified the c-*erbA* gene products and their relationship with other nuclear receptors, and developed powerful assays for DNA binding and *trans*-activation, the way was now clear to carry out detailed structural and functional studies. Contributions to our understanding of TR function have since come from such diverse areas as: studies of other receptors and the v-*erbA* gene product; examination of target gene regulation; and, structural studies of TR itself. For simplicity's sake the rest of this introduction is provided in the form of summary, rather than historical perspective.

1.3 Dissecting the Activities of the Thyroid Hormone Receptor

The thyroid hormone receptors are members of a large superfamily of receptor proteins that share a common organization of functional domains (indicated in Figure 1.3). The number of receptors identified as members of this superfamily continues to grow. In 1992 there were 32 genes identified for such receptors (Laudet *et al.*, 1992), and at present there are over 50 (Leblanc and Stunnenberg, 1995), the majority of these referred to as "orphan receptors," no specific ligand having yet been identified. The domains that characterize these receptors, and the functions they carry out, are: A/B, necessary for maximal *trans*-activation in some cases; C, the DNA binding domain (DBD); D, the hinge region, a "flexible" domain that mediates allosterism between domains C and E/F; and, E/F, the ligand binding domain (LBD), which is also involved in activation and dimerization (reviewed in Goldberg *et al.*, 1989).

While sequence conservation is generally quite low in the other domains, the DNA binding domain exhibits a high degree of similarity between receptors. Homology can be inferred not only from the conserved structure and function of this domain, but is also supported by the observation that the borders of exons encoding the DNA binding domain are consistent with gene duplication (Laudet *et al.*, 1992). The DBD consists of two zinc finger-like motifs that form an interface for both interaction with DNA, and dimerization of receptors on certain classes of TREs. In many cases, dimerization is also facilitated

through interactions of the C-terminal domain (E/F), which is also responsible for ligand binding, and contains regions important in transcriptional regulation. The hinge region (D) is so-named because it is predicted to have flexibility (Krust *et al.*, 1986). While the TR is capable of binding DNA in the presence or absence of hormone, this region may be involved in permitting independent swiveling of the DNA- and ligand binding domains. Finally, the poorly characterized domain A/B has been shown, in several cases, to be necessary for maximal *trans*-activation activity.

Before examining the details of TR structure and function, an overview of TR activity is offered. The TR is present in the nucleus, where it is presumed to exist as a monomer, homodimer, or a heterodimer formed with other members of the nuclear receptor superfamily, possibly in association with other cofactors. A particularly important heterodimer partner for TR is the receptor for 9-*cis*-retinoic acid, RXR, which readily forms heterodimers in solution that are potent activators of transcription from certain classes of TRE. The TR monomer, homodimer and various heterodimeric complexes have distinct DNA binding, ligand binding and *trans*-activation characteristics, but some general comments may be made here. The TR homodimer, bound to a TRE, represses transcription in the absence of hormone. Addition of T_3 results in dissociation of the homodimer into monomers. While it has been proposed that T_3-bound monomer is able to activate transcription, it is possible that the monomers are merely freed to form heterodimers with other receptors. In either case, the net result is usually activation of transcription, although in certain cell-types, from particular TREs, transcription is actually repressed in response to hormone. A good deal of the current work in the TR field is aimed at identifying the proteins with which TR interacts in order to regulate transcription.

1.3.1 DNA Binding

Elucidating the determinants of DNA binding specificity is critical to understanding TR regulation of gene expression. DNA binding specificity, in the case of the TR, is a function of more than just DNA sequence, as homo- and heterodimerization of the TR can

result in receptor complexes with distinct binding properties. As mentioned, the TR monomer is capable of binding to DNA. However, the sequence of DNA recognized by a single monomer is termed a half-site as these sequences are predominantly identified as tandem repeats in functional TREs. TREs are generally believed to bind dimeric receptor complexes (reviewed in Glass, 1994). Intriguingly, the functional TREs that have been characterized in the cistronic or flanking sequences of T_3-regulated genes display remarkable diversity in the configuration of half-sites (Figure 1.6). The term configuration is used throughout this work to refer to the combination of spacing (the number of base-pairs between two tandemly repeated half-sites) and relative orientation of half-sites that describes a particular tandem repeat.

Half-site sequence considerations *per se* are important for determining the affinity of binding between a given receptor DBD and a half-site and, thus, provide the basis for some differential receptor activity, as dimers with different DBD combinations may selectively bind distinct half-site combinations. The configuration of a repeat introduces another level of selectivity, as different receptor dimers have distinct preferences for the configuration of half-site repeats to which they will bind. The particular configuration of a tandem repeat may, in some cases, facilitate cooperative protein-protein interactions within a receptor dimer upon binding; interactions that are strong enough to override some sequence properties. The net result is that DNA binding by the TR is a complex function of both DNA and protein considerations, the latter subject to such physiological variables as: the availability and identity of the heterodimeric partner receptor(s), and concentrations of ligand for either receptor.

1.3.1.1 DNA Properties

TREs have been identified from a score of gene sequences from a number of organisms. It soon became clear that a TRE could not be described as a single particular sequence. Rather, TRE half-site sequences and configurations vary, presumably to facilitate different modalities and degrees of T_3 regulation. Some of the work in this thesis has been

undertaken in order to elucidate the differences in the activities of receptor complexes on TREs of different types. The background and context for this work was provided by the results of other workers who have greatly expanded our understanding of the sequence characteristics of TREs that facilitate TR binding while inhibiting the binding of inappropriate receptor complexes. Reviews which provide more details regarding the work done with other receptors, particularly those for glucocorticoid and estrogen, have been written by Glass (1990, 1994),

1.3.1.1.1 Half-site Sequence

That TREs can be tandem repeats of a half-site sequence was obvious from the first identified TRE from the rat growth hormone gene, which had two half-sites arranged head-to-head, forming an inverted repeat, or palindrome (Glass *et al.*, 1987; Glass *et al.*, 1988). Once the half-site concept was established, it was natural to generate a consensus sequence based on known TRE half-site sequences. Due to the diversity of half-site sequences that are observed in naturally occurring TREs (Figure 1.6), the hexameric consensus sequences compiled tend to be rather degenerate; some examples are: GGG(A/T)C(G/C) (Norman *et al.*, 1989), and (G/A)GG(T/a)(C/G)(a/g) (Glass, 1990), where capital letters represent nucleotides that are more conserved than those in lowercase. It is worth noting that even these degenerate consensus sequences established a basis for some differential binding of nuclear receptors; the GR, and some related receptors (for progesterones, androgens, mineralocorticoids) recognize an AGAACA half-site sequence. Point mutation and binding analysis have been used to determine which nucleotides are most favourable to TR binding at each position of the half-site. By introducing point mutations into either half-site of the inverted repeat and determining the affinity of the thyroid hormone receptor for the mutant sequence it was shown that alterations within the hexameric half-site sequence had the greatest effects on binding (Glass *et al.*, 1988). Furthermore, based on the mutations introduced the highest affinity half-site sequence was TCAGGTCA, although mutations in the two upstream 5' nucleotides did not affect binding as significantly as the other

positions. Another study, based on point mutation analysis determined AGGT(C/A)A as the favoured half-site (Brent *et al.*, 1989). Consequently, the AGGTCA hexamer was generally accepted as the preferred half-site sequence of TR for some time. Subsequent work began to expand upon this dogma. Kim *et al.* (1992) examined the influence of individual point mutations which were introduced through the length of a single half-site (ie. not part of a tandem repeat) on the binding of TRα monomers. Their results indicate that altering the nucleotide at positions F1 through F3 (see nomenclature, Figure 1.7), resulted in changes in the binding affinity of TRα. Notably, changing position F1 from a T to an A decreased binding affinity as much as any other change, including those within the core hexamer.

More recently a PCR-based binding site selection methodology has been applied to determine the highest affinity binding site(s) for TR (Figure 1.8). This technique utilizes a pool of DNA probes that have randomized DNA sequences in the center and sequences compatible with PCR primers at both ends. A binding assay is used to isolate probes with sequences which are bound with high affinity by the protein being studied, and the selected probes are amplified by PCR. The PCR-amplified probes are again subjected to binding selection. After multiple rounds of binding selection and PCR amplification, the pool of DNA probes isolated is no longer random. It is highly enriched for sequences harbouring high affinity binding sites for the protein. The resultant probes may be sequenced individually after cloning into plasmid vectors, or by directly sequencing the recovered DNA.

One application of PCR-based binding site selection used probes initially randomized at 18 positions to identify high affinity binding sites for the TRα monomer (Katz and Koenig, 1993). Bound DNA was separated from free during each round of selection by excising and eluting bands from an EMSA gel. The highest affinity site selected was TAAGGTCA, confirming both the core hexameric sequence and the significance of the two upstream flanking positions. While the two upstream flanking sequences were clearly a factor in

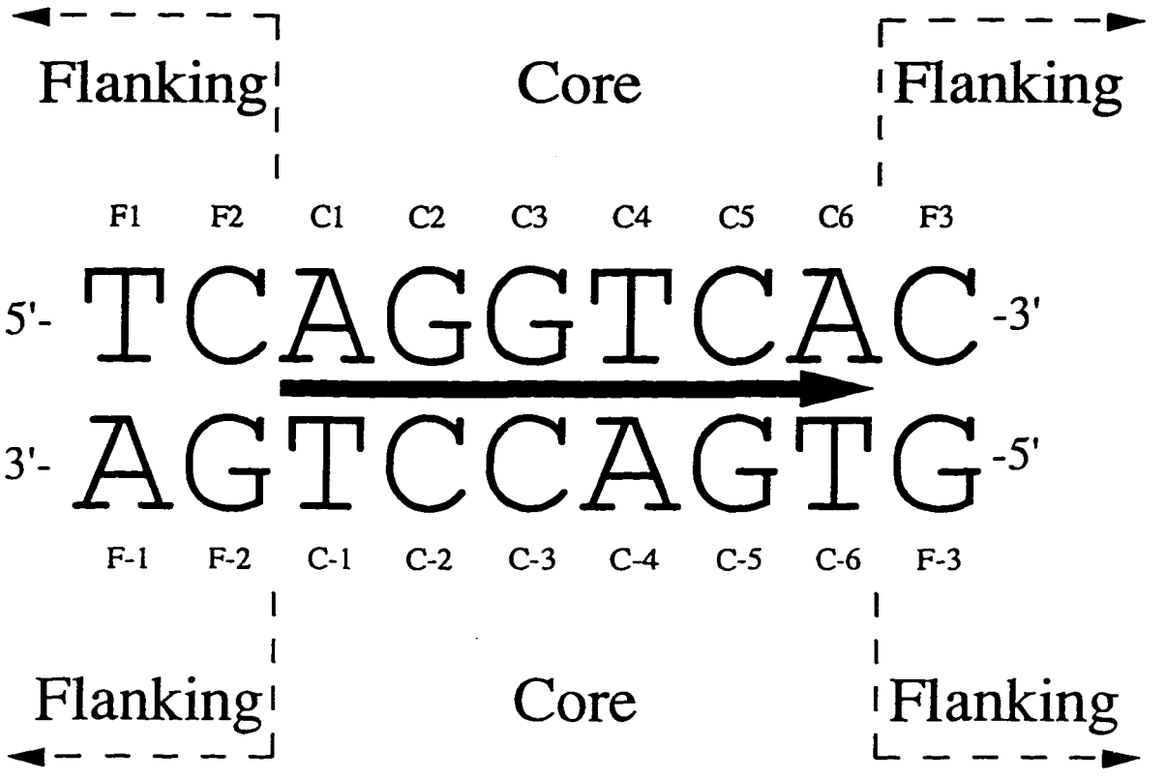


Figure 1.7: Nomenclature used in this thesis for refering to positions within a half-site sequence. The core hexamer residues are denoted C1 through C6, while the flanking sequences are F1 to F3, F3 being 3' to the half-site. The paired bases on the lower strand are indicated by negative numbers, as indicated.

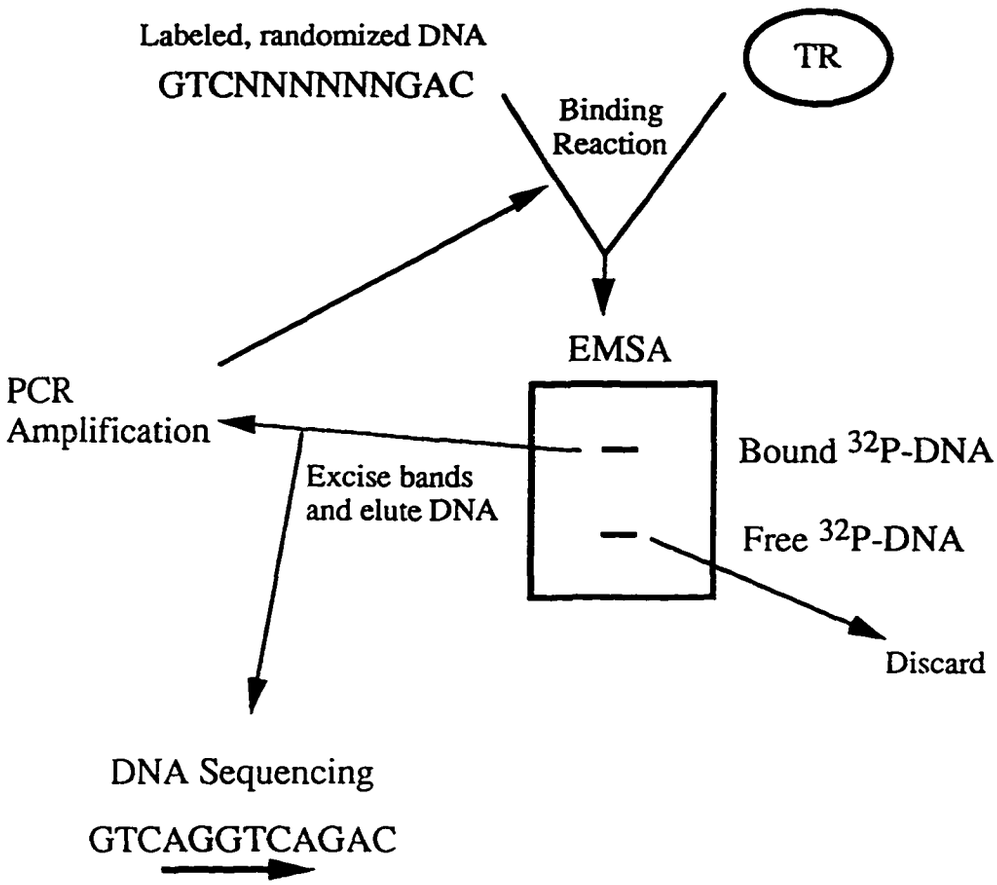


Figure 1.8: Diagrammatic representation of a PCR-based binding site selection technique. Details are provided in the text.

selection of high affinity binding sites for TR, this work did not involve the sequencing of large numbers of selected probes and as a result, the distribution and impact on binding of other bases in the flanking positions was not determined. Another study approached this problem by utilizing 16 half-site variants which consisted of the hexameric core sequence flanked by all possible combinations of bases at the two upstream positions (Schräder, *et al.* 1994a). EMSA indicated that the binding of a TRα monomer to these probes was best when position F1 was a pyrimidine, and position F2 was a purine. Unfortunately, dissociation constants (K_ds) were only determined for four of the sequences. In order of decreasing affinity the K_ds were TG (2.1 nM), CG (2.9 nM), AG (10.3 nM) and GG (15.3 nM). Thus, it is clear that the identity of the nucleotide at position F1 may influence binding affinity of the TRα monomer over a range of roughly 7-fold. Finally, a third study using PCR-based binding site selection contributed to the acceptance of the importance of flanking sequences in TR binding. This work used the TRβ/RXRα heterodimer as the selecting receptor complex and the DNA probe consisted of a single high affinity half-site upstream of an 11 base-pair stretch of randomized DNA (Kurokawa *et al.*, 1993). After two rounds of selection and amplification the heterodimer selected a half-site sequence nine base-pairs in length as revealed by direct sequencing of the selected DNA. It was clearly demonstrated in this landmark paper that the half-site selected was occupied by the TRβ component of the heterodimer (discussed below, 1.3.1.1.2.2), and not RXRα, so that the binding site selection can be accepted as having been carried out by TRβ, although the heterodimeric context must be kept in mind. The high affinity nonameric sequence selected by TRβ, as part of a heterodimer, was (T/C/g)(G/c/a)(A/g)GGTCAC. This sequence has several interesting features. First, positions F1 to C6 are essentially consistent with the other two high affinity half-sites although position F2 does not exhibit the high selection of A that is expected. It is possible that the differences between the studies result from methodological differences, the use of TRα versus TRβ, and/or the monomeric versus heterodimeric context of the TR involved. Second, the identity of a third flanking position,

F3, at the 3' end of the half-site was shown to be important for binding site selection. Third, it underscores the importance of the core hexamer in determining binding affinity. Even after only two rounds of selection, and using bulk sequencing, five out of six base-pairs of the half-site are highly selected from the initially random pool.

More recently, the crystal structure of a complex formed by the DBDs of TRβ and RXRα and a direct repeat TRE has been solved to 1.9 Å resolution (Rastinejad *et al.*, 1995). The information provided with respect to receptor structure will be discussed below, but there are some points relevant to half-site sequence that are worth making. Once again, the heterodimeric context of the interaction must be kept in mind, as the crystal structure suggests that the cooperative interactions involved in heterodimerization may influence protein-DNA interaction. Therefore, some of the interactions between the TR DBD and the half-site observed in the crystal structure may be specific for this particular combination of DNA sequence and receptor complex. Every base-pair in the core hexamer is contacted at least once by the DBD via the major groove, except for position C6 which is only contacted at the DNA backbone (Figure 1.9). Phosphate group contacts are extensive, and extend into the upstream flanking region as far as the position upstream and adjacent to F1. There are no interactions noted downstream of the half-site. Thus, there are still many questions to be answered with regards to the mechanisms by which flanking positions manifest an influence on binding affinity.

In summary, a few things are clear with respect to half-site sequence and TR binding. The core hexamer is important for binding, but flanking sequences also play a role. Position F1, in particular, is a major determinant of binding affinity. The application of different methodologies with their unique limitations confounds efforts to pool the published data to generate a comprehensive paradigm of TR-half-site interaction. In practical terms it may be sufficient to be aware of major sequence determinants of TR binding affinity, however there is the proviso that binding affinity is not intrinsically connected to biological activity. This becomes profoundly evident when the influence of

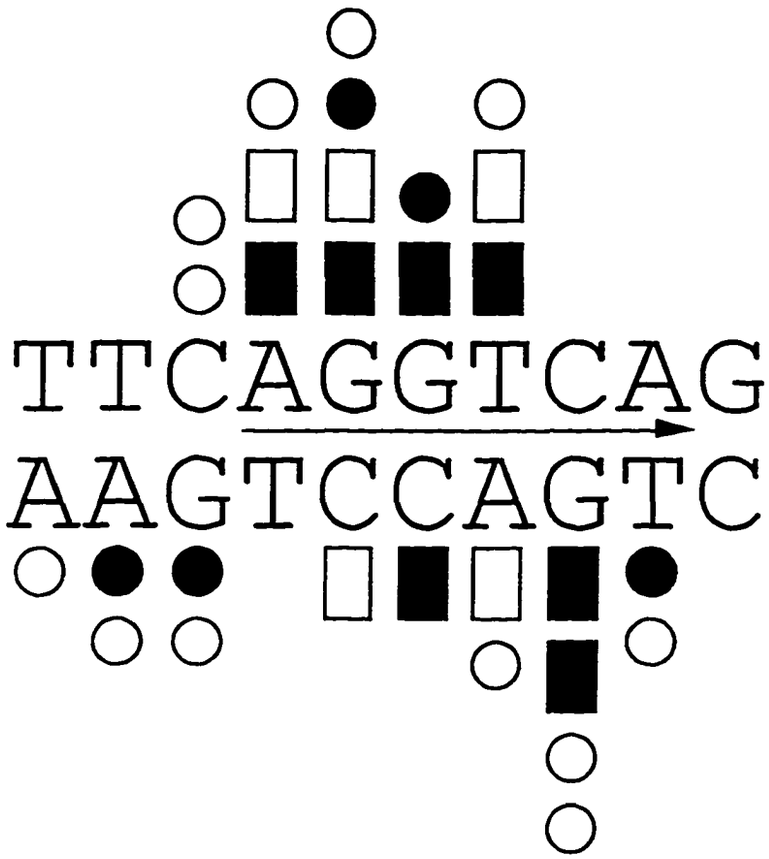


Figure 1.9: Base-specific and DNA backbone contacts made by the TR DBD in a TR/RXR DBD heterodimer bound to a DR4 sequence. The core hexamer is indicated by an arrow. Rectangles indicate direct (filled) and water-mediated (open), base-specific contacts made by the TR DBD. Circles indicate direct (filled) and water-mediated (open), DNA backbone contacts made by the TR DBD.
(Rastinejad *et al.*, 1995)

half-site configuration within tandem repeats on *trans*-activation is considered.

Given that the TR does form homo- and heterodimers in solution with other members of the nuclear receptor superfamily (Kurokawa *et al.*, 1993) a role for the monomer in *trans*-activation is not necessary to explain TR acitvity. However, several studies have claimed to provide evidence for *trans*-activation by the TR monomer. The binding of TR monomer to DNA can be readily observed by EMSA. Binding of the monomer to a single half-site served as the basis for two of the PCR-based half-site selection schemes described above, and has been reported to have a K_d as low as 2.1nM (Schräder *et al.*, 1994). Intriguingly, a natural TRE has been described which consists of only one obvious half-site, and which binds both TR isoforms as monomers (Carr and Wong, 1994; Cohen *et al.*, 1995). This latter TRE confers T_3-dependent silencing on heterologous promoters, whereas the other monomeric sites are positively T_3-responsive (Katz and Koenig, 1993; Schräder *et al.*, 1994). Unfortunately, as one can never be certain that *in vitro* binding studies reflect *in vivo* binding and activation conditions, the concept of TR monomers carrying out transcription regulation must be regarded with caution.

1.3.1.1.2 Half-site Configuration

There are essentially three possible relative orientations of non-overlapping half-sites in a tandem repeat (Figure 1.10). These three orientations result in direct repeats (DRs), inverted repeats (IRs) and everted repeats (EvRs). An alternative taxonomy that appears in the literature, but will not be used here, is to designate these orientations as direct repeat, palindrome, and inverted palindrome, respectively. Further contributing to the diversity of binding sites for TR is the fact that the number of base-pairs between half-sites may vary. For example, direct repeats may have spacer lengths of 0 base-pairs (DR0), 1 base-pairs (DR1), 2 base-pairs (DR2), etc. Spacer lengths are calculated from the boundaries of the core hexamers. As a consequence, when flanking sequences are considered, there are examples of TR binding sites in which the half-sites appear to overlap (eg. IR0).

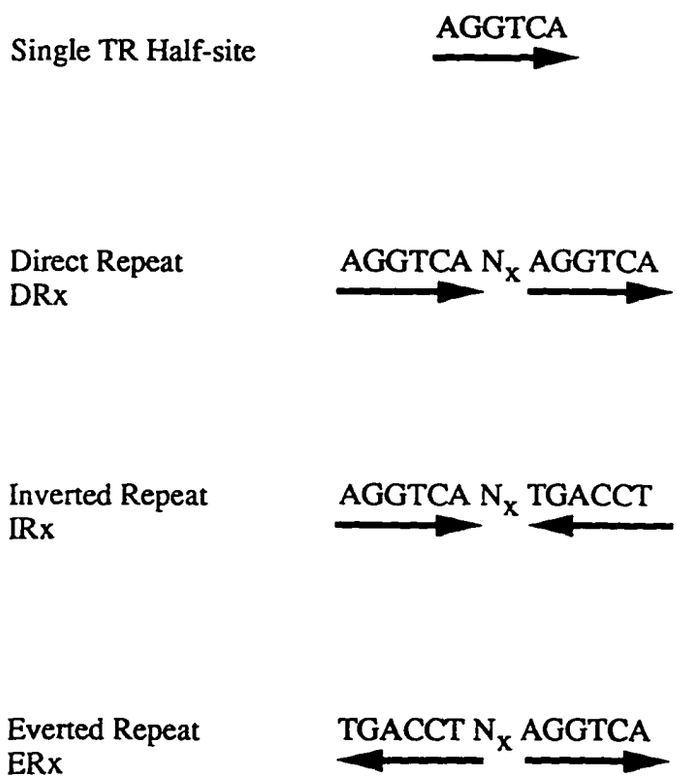


Figure 1.10: Half-site configurations in tandem repeats. The three possible orientations of half-site are illustrated. When taken in combination with the variety of spacer lengths possible, great variability is possible in half-site configuration.

1.3.1.1.2.1 Thyroid Hormone Receptor Homodimer Binding

The array of tandem repeats to which the TR homodimer binds is impressive. A study was undertaken to test binding of the TR homodimer to half-sites in all three orientations with a variety of spacer lengths (Carlberg, 1993). Unfortunately, the flanking sequences of the half-sites were not held constant between spacing variants, so it is unwise to compare binding affinities, but the results do illustrate the remarkable tolerance of the TR homodimer for different spacer distances. DR2, DR3, DR4, DR5, IR0, IR1, EvR6, EvR7, EvR8 and EvR9 were all bound by the homodimer. Various other studies have reproduced the binding of the TR homodimer to those sequences as well as to DR0, DR6, EvR3, EvR4, EvR5 (Andersson *et al.*, 1992; Kurokawa *et al.*, 1993; Näär *et al.*, 1991; Shulemovich *et al.*, 1995). While the TR homodimer has the capacity to bind many configurations of tandem repeats, the everted repeat appears to be bound with the highest affinity. When a PCR-based binding site selection protocol was applied using the TR homodimer, the selected sequences were everted repeats spaced by 4 to 7 base-pairs (Kurokawa *et al.*, 1993).

Binding of the sequences listed above by the homodimer is characterized by varying degrees of cooperativity. Cooperativity is frequently used in the thyroid receptor field to indicate that binding of the homodimer predominates over that of the monomer, usually being observed even at relatively low concentrations, thus assuming that binding of TR either occurs as a dimer, or that the binding of a monomer facilitates binding of a second monomer through positive, DNA-dependent interactions. IR0, DR3, DR4 and DR6 have been shown, using graphical methods, to exhibit cooperativity of binding to different degrees (Wahlstöm *et al.*, 1992). Likewise, another group confirmed that natural sequences of IR0, DR4 and EvR6 types are bound cooperatively by the TR homodimer, using graphical methods (Williams *et al.*, 1991; Brent *et al.*, 1992; Williams *et al.*, 1994). While cooperativity of homodimer binding to DR5 has not been directly addressed, this interaction resembles that between the homodimer and DR4 in most EMSA studies (Mader

et al., 1993; Shulemovich *et al.*, 1995), and is likely to involve a similar degree of cooperativity. An emphasis is placed on cooperativity of binding in discussing receptor binding, as it is usually concomitant with relatively high affinity of binding. When the C-terminal domain is deleted from TR, binding to many elements still takes place, but with lower affinity and a clear lack of cooperativity (Kurokawa *et al.*, 1993; Mader *et al.*, 1993).

Despite the plethora of half-site repeat configurations that the TR homodimer can bind to with significant affinity, the physiological role of the homodimer is still ill-defined. Analysis of TR homodimer activity *in situ* or *in vivo* in cells not overexpressing TR has not been published, but there is a variety of *in vitro* data that suggests the homodimer may not be the predominant active form of TR in the presence of hormone. One reason for this is that the homodimer may not be the most prevalent species of TR-containing receptor complex in most cells, as TR forms heterodimers readily with other receptors *in vitro* (discussed below). Another reason is that the presence of ligand appears to cause TRs to dissociate when bound to certain types of element, leaving only monomers bound to the DNA. Neither direct nor everted repeats are bound by the homodimer in the presence of T_3, and homodimers already bound in the absence of T_3 dissociate upon addition of the hormone (Andersson *et al.*, 1992; Yen *et al.*, 1992a; Yen *et al.*, 1992b; Yen *et al.*, 1994a; Piedrafita *et al.*, 1995). In contrast, T_3 either does not affect, or slightly enhances, binding of the homodimer to inverted repeats, and causes a subtle increase in electrophoretic mobility of the homodimeric complex that is interpreted to represent a conformational change (Andersson *et al.*, 1992; Ribeiro *et al.*, 1992). As the affinity of the TR monomer for a lone half-site is not significantly altered by T_3-binding (Schräder *et al.*, 1994a), and monomeric binding to EvRs and DRs in the presence of T_3 still occurs (Andersson *et al.*, 1992; Ribeiro *et al.*, 1992; Yen *et al.*, 1992b; Yen *et al.*, 1993), it is likely that T_3 binding induces a conformational change that selectively interferes with cooperative binding to EvRs and DRs. A possible function of this T_3 sensitivity may be to free TR monomers for association with RXR. Recently it has been shown that T_3-stimulated activation occurs at

significantly lower T_3 concentrations in the presence of RXR than in its absence (Claret *et al.* 1996). Consequently, the activity of the homodimer is likely to be one that is primarily relevant in the absence of hormone, and may be manifested most efficiently from a subset of TREs. Consistent with this idea, a recent study has shown that TR homodimers have a very slow off-rate from EvRs, but not DRs or IRs, in the absence of hormone (Piedrafita *et al.*, 1995). This is supportive of a major role for the homodimer as a repressor of transcription in the absence of hormone.

TRE configuration may serve a discriminatory role in homodimer activity at another level as well. TRα and TRβ homodimers appear to have distinct dimerization activities when binding to certain half-site configurations. TRβ exhibits a significantly greater propensity to bind several natural TREs as a dimer versus a monomer than does TRα (Darling *et al.*, 1993). Whether there is a physiological significance to this difference between TRα and TRβ activity has yet to be determined.

1.3.1.1.2.2 Thyroid Hormone Receptor Heterodimer Binding

The TR will form heterodimers with numerous other nuclear receptors. The binding of TR to TREs in association with other proteins present in nuclear extracts was recognized well before many of the heterodimeric partners had been identified (Murray and Towle, 1989; Burnside *et al.*, 1990; Lazar and Berrodin, 1990). Receptors with which TR is known to form heterodimers include: retinoic acid receptor (RAR)(Glass *et al.*, 1989); retinoid X receptor (RXR)(Yu *et al.*, 1991; Kliewer *et al.*, 1992a; Zhang *et al.*, 1992); vitamin D3 receptor (VDR)(Schräder *et al.*, 1994b); peroxisome proliferator-activated receptor (PPAR)(Bogazzi *et al.*, 1994); and, chicken ovalbumin upstream promoter transcription factor (COUP)(Berrodin *et al.*, 1992). TR heterodimers can also consist of different TR isoforms, for example; that is, TRα/TRβ (Wahlström *et al.*, 1992; Yen *et al.*, 1992; Darling *et al.*, 1993). Likewise, the oncogenic product v-*erb*A can heterodimerize with either isoform of TR (Piedrafita *et al.*, 1995; Yen *et al.*, 1994b). One important consequence of heterodimerization is that it inevitably results in novel DNA binding

characteristics. Elaborating on the list above, the heterodimers have the following preferred binding sites: TR/RAR, IR0 or DR4; TR/RXR, IR0 or DR4; TR/VDR, DR3 or DR4; TR/PPAR, DR2; and, TR/COUP, undetermined (Berrodin *et al.*, 1992; Butler and Parker, 1995).

The binding activity of heterodimeric complexes involves a number of considerations that do not apply to homodimer binding. These include: possible different half-site specificity of the two receptors, the polarity of the two receptors with respect to the asymmetric DR-type binding sites (eg. 5'-TR/RXR-3', versus 5'-RXR/TR-3'), and differential responsiveness to the two ligands, if the partner receptor binds a ligand. The first of these possibilities has not been systematically investigated, although there is some evidence for it. The polarity of receptors bound to direct repeats is believed to be spacing-dependent in some cases, and has also been shown to affect *trans*-activation. Finally, it is clear that in many cases ligand-responsiveness of heterodimers is distinct from homodimers of either receptor partner.

The configuration of half-sites in a tandem repeat overrides, to some extent, the half-site sequences in terms of determining binding affinity of heterodimers. Consequently, several of the heterodimers mentioned above preferentially bind to DR4 sequences. On the other hand, the DBDs of the heterodimeric partners may favour the recognition of sub-classes of DR4s on the basis of half-site sequence. One study used strong (S) and weak (W) TR half-sites, assigned on the basis of similarity to the consensus hexamer, arranged as either 5'-SW-3' or 5'-WS-3' DR4s. It was shown that TR/RXRγ bound SW better than WS, while TR/TRAP (a distinct nuclear factor that heterodimerizes with TR) bound WS better than SW (Force *et al.*, 1994). Such differential binding by distinct heterodimers may be sufficient to rationalize the abundance of TR-containing heterodimers for DR4 sequences.

The polarity of heterodimers bound to direct repeats is, in some cases, a function of half-site spacing. The TR/RXR heterodimer binds to DR4 sequences 5'-RXR/TR-3' (Kurokawa *et al.*, 1993; Perlmann *et al.*, 1993). While this polarity primarily results from

protein-protein interactions (discussed below), these interactions are dependent on the spacing of the DR half-sites, and thus may be described as being determined by half-site configuration. The TR/VDR heterodimer exhibits similar spacing dependent polarity with a remarkable additional phenomenon; the TR/VDR heterodimer was shown to bind 5'-VDR/TR-3' to a DR4 sequence, but 3'-TR/VDR-5' to a DR3 sequence (Schräder *et al.*, 1994c). Furthermore, the heterodimer would only *trans*-activate in response to the hormone bound by the downstream receptor. Spacing-dependent polarity is observed for other members of the receptor superfamily. RAR/RXR heterodimers are bound 5'-RAR/RXR-3' to DR1, but 5'-RXR/RAR-3' to DR5 and DR2 (Kurokawa *et al.*, 1994; Zechel *et al.*, 1994b). The heterodimer bound to DR5 mediates response to ligands for both RAR and RXR. In contrast, when the heterodimer is bound to a DR1 it does not bind either ligand (Kurokawa *et al.*, 1994). Thus, half-site configuration may determine not only polarity, but allosteric changes which influence ligand binding. It should be noted here that the heterodimer polarities described above were determined using idealized core hexamers, so it is conceivable that sequence variation within either or both half-sites of a direct repeat with the same spacing might be sufficient to reverse the heterodimer polarity. However, there are asymmetric protein-protein interactions involved in establishing polarity (discussed below) that make this unlikely.

The net result of the phenomena associated with heterodimerization, and of singular importance to the function of TR, is the effect on restricting hormonal responsiveness to appropriate DNA elements. Among all the dimerization partners with which TR interacts, RXR is believed to be the most physiologically relevant, as far as regulation of genes strictly by T_3 is concerned (reviewed in Zhang and Pfahl, 1993). RXR isoforms (α, β and γ) are ubiquitous (Mangelsdorf *et al.*, 1992), form high-affinity heterodimers with TR in solution (Zhang *et al.*, 1992; Kurokawa, 1993), and enhance the binding affinity of TR to most half-site repeat configurations. RXR also forms heterodimers with other receptors resulting in enhanced specificity of binding and activation. The most striking example of

the influence of RXR arises from examining the pattern of *trans*-activation by different receptors from direct repeats with various spacer lengths. This pattern is summarized by the "3-4-5 rule," which defines direct repeats spaced by 3, 4, and 5 base-pairs as response elements for vitamin D3, T_3, and retinoic acid, respectively (Umesono *et al.*, 1991). Implicit in this rule is a co-evolution of receptors and distinctly spaced direct repeat response elements. In generating the "3-4-5 rule", it was shown that *trans*-activation from DR3-, DR4- and DR5-linked CAT reporter plasmids was carried out exclusively in cells co-transfected with plasmids expressing VDR, TR, and RAR, respectively. Binding of the DR3, DR4 and DR5 sequences by nuclear extracts of transfected cells exhibited a parallel pattern: DR3 was bound only by nuclear protein from cells transfected with VDR, etc. When the "3-4-5 rule" was originated, the role of RXR in heterodimerization was unknown. Since that time evidence has accumulated to indicate that it is heterodimerization with RXR that generates the exclusivity of *trans*-activation observed for DR HREs.

The "3-4-5 rule" has been expanded since its inception, and renamed the "1-to-5 rule" (Mangelsdorf and Evans, 1995) with the characterization of the PPAR/RXR heterodimer which binds to and activates from DR1 (Kliewer *et al.*, 1992b). RAR/RXR heterodimers have also been shown to activate from DR2 (Durand *et al.*, 1992), although this creates some degeneracy within the "3-4-5 rule", as this heterodimer is also active from a DR5. However, at the very least, the "1-to-5 rule" is a useful paradigm for understanding the effects of heterodimerization with RXR on establishing restrictions on hormone responsiveness mediated by direct repeats of various spacings. On the other hand, there is evidence that conflicts with this increasingly simplistic model for TR activity. Adherence of *trans*-activation by TR from DR3, DR4 and DR5 elements to the "3-4-5 rule" has been shown to be dependent on half-site sequence to some extent; that is, repeats of consensus octameric half-sites (positions F1 to C6 matching the high affinity sequence) do not obey the "3-4-5 rule", but are all responsive to T_3, whereas only the DR4 is active when hexameric sites (only C1 to C6 matching the high affinity sequence) are presented (Katz *et*

al., 1995).

The formation of TR/RXR heterodimers affects the binding of half-site configurations other than direct repeats, too. The affinity of the heterodimer for IR0 sequences is higher than that of the homodimer (Zhang *et al.*, 1992; Yu *et al.*, 1991), although TR homodimers and TR/RXR heterodimers exhibit a similar preference for binding to IR0 sequences versus IRs with other spacer lengths. However, T_3-stimulated *trans*-activation occurs exclusively from IRs with no spacer base-pairs (Carlberg, 1993; Schräder and Carlberg, 1994; Wahlström *et al.*, 1992). The relative affinities of TR homodimers and TR/RXR heterodimers for everted repeats is less clear. The heterodimer does bind to everted repeats with high affinity (Baniahmad *et al.*, 1990), but the K_ds for the homodimer and heterodimer on a given EvR have not been determined. Comparison of TR/TR and TR/RXR binding to a panel of everted repeats with different spacer lengths suggests that EvRs spaced by 6-9 base-pairs bind the homodimer with high affinity, and those spaced by 4-7 base-pairs bind heterodimers (Carlberg, 1993; Schräder and Carlberg, 1994). Based on these results it is likely that the relative affinity of homo- and heterodimers for any given EvR will depend on the spacer length, aside from any half-site sequence considerations that might come into play.

The TR/RXR heterodimer exhibits ligand-responsiveness that is distinct from that of the TR homodimer, and which is key to the *trans*-activation capacity of the receptor complex. Unlike the homodimer, binding of the heterodimer to all configurations of TRE is insensitive to T_3 (Yen *et al.*, 1992a; Yen *et al.*, 1994). There appears to be no significant effect of the presence of T_3 on the affinity of the heterodimer for tandem half-site repeats of any configuration. The TR, upon binding ligand, is believed to undergo similar conformational changes whether it is in a homodimer or a heterodimer (Bendik and Pfahl, 1995). It is presumed that this conformational shift serves to dissociate the homodimer complex, and to activate transcription from the heterodimeric complex. There are conflicting data with respect to whether 9-*cis*-retinoic acid (9-*cis*-RA), the ligand of RXR,

is able to stimulate *trans*-activation by the TR/RXR heterodimer. One study has found that synergism of T_3 and 9-*cis*-RA does occur from a specific natural TRE, but not from a DR4 (Rosen *et al.*, 1992). Another study indicated no synergism on either a DR4 or an IR0 and, furthermore, that heterodimerization with TR inhibits the binding of a RXR-specific ligand to RXR (Forman *et al.*, 1995). Further research may be necessary to reconcile these data as the natural TRE used in the first study actually consists of three tandem half-sites, and the interplay of receptor complexes bound to this element is poorly understood. Regardless, it seems likely that, at least on certain types of TRE, there are allosteric interactions within the heterodimeric complex that restrict responsiveness to a particular ligand. If it turns out that this is a configuration or sequence-dependent phenomenon it would be quite consistent with the complex cross-talk that occurs between hormonal signaling pathways and further justify the diversity of TRE sequences observed.

1.3.1.2 Protein Properties

The ability of receptors to discriminate between half-site sequences and configurations is essentially a function of receptor-specific structures. The highly combinatorial, yet tightly regulated, interactions of nuclear receptors with each other, and with different configurations of HRE are manifestations of elaborate and conserved domains within the proteins. In brief, the highly conserved DNA binding domain is responsible for DNA sequence recognition, but also harbours regions involved in inter-receptor interactions that contribute to cooperative binding and spacer discrimination. The DBD harbours two zinc finger-like motifs, regions of conserved tertiary structure that provide surfaces for DNA-protein and protein-protein interaction. Several discrete regions within the zinc-binding domains have identifiable functions in these regards. The "P-box" is a set of three amino acids that discriminate between different half-site sequences. The "D-box" may be involved in protein-protein interactions contributing to cooperative binding on certain TRE configurations. Just C-terminal of the zinc-binding domain, but within the DBD, the "A/T-

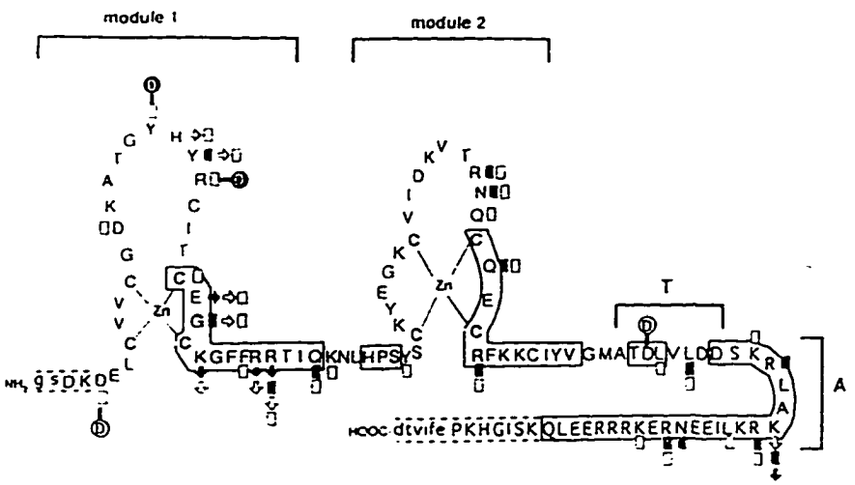
box" region is involved in DNA binding, and in dimerization as well, on certain TREs. Dimerization is also a function of interactions outside the DBD, in the LBD. The contribution of these regions to DNA recognition is considered below.

1.3.1.2.1 The Zinc-Binding Motifs and Half-Site Specificity

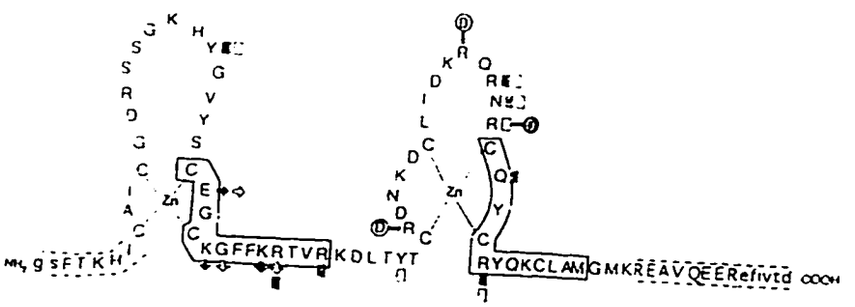
The zinc finger is a functional domain that is commonly involved in DNA recognition by eukaryotic transcription factors. There are several related types of zinc binding motif that are distinguished on the basis of the residues which they use to coordinate a single zinc atom. The zinc binding motifs of the nuclear receptor superfamily are C4-type, as they each coordinate a single zinc atom using four cysteine residues (Figure 1.11), and are referred to as class II zinc binding motifs. Tetrahedral coordination of the zinc atom stabilizes a characteristic tertiary structure that is highly conserved amongst the nuclear receptors, and which has been well characterized on the basis of crystal structures and NMR studies. NMR studies have been performed on the DBDs of GR (Härd *et al.*, 1990), ER (Schwabe *et al.*, 1990), and RXR (Lee *et al.*, 1993). X-ray crystal structures have been determined for a homodimer of GR DBDs bound to DNA at 2.9 Å (Luisi *et al.*, 1991), a similar complex with estrogen receptor (ER) DBDs at 2.4 Å (Schwabe *et al.*, 1993), and a heterodimer of TR and RXR DBDs bound to a DR4 at 1.9 Å (Rastinejad *et al.*, 1995), providing a total of four receptor DBD/DNA crystal structures. Significant similarity in overall folding is observed in the zinc binding motif region, and contacts involved in both DNA binding and protein-protein interaction are apparent from each structure.

A striking feature of the nuclear receptor DBDs, and one that distinguishes them from many zinc finger motifs, is that the two zinc binding motifs do not function autonomously with each finger recognizing a short stretch of DNA sequence, but rather form a single globular domain with a shared hydrophobic core. For this reason, the two zinc-coordinating structures (called CI and CII; Figure 1.11) are referred to as zinc modules,

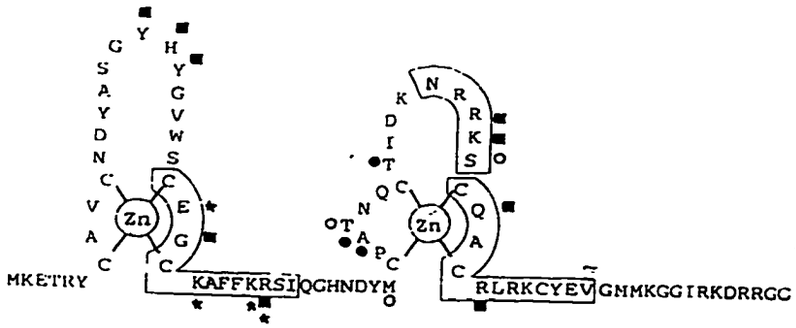
(A)



(B)



(C)



(D)

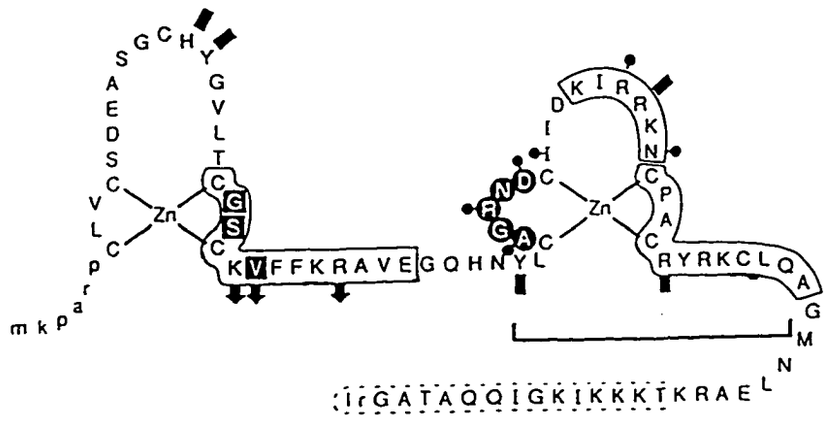


Figure 1.11: Representation of the sequence of the zinc binding motifs of four nuclear receptors. The sequences of (A) the TR, (B) the RXR, (C) the ER, and (D) the GR DBDs schematically illustrating the zinc coordinating cysteines, as well as residues involved in protein-protein or protein-DNA interactions. The boxed sequences are α-helical regions. Solid and open squares represent direct and indirect DNA backbone contacts, respectively. Solid and open arrows represent direct and indirect base contacts, respectively. In (C) direct base contacts are indicated by asterisks. Residues in the D-box of the GR are indicated by dark circles around them. The T- and A-box of TR are indicated, as well. Residues that are involved in dimerization are indicated by lollipop-like symbols for the TR, RXR and GR, and by circles for the ER.
(Luisi *et al.*, 1991; Schwabe *et al.*, 1993; Rastinejad *et al.*, 1995)

rather than fingers. Both modules have an amphipathic α-helix that starts at the third of the four coordinating cysteines and extends for 8-9 residues C-terminal (Figure 1.11). The two α-helices cross at roughly 90° angles, and between them is formed the hydrophobic core (Figure 1.12). Because the overall fold of the DBD is conserved, some regions can be identified which make contacts of a similar nature in all the X-ray crystal structures. The polar face of the CI α-helix is positioned within the major groove of the half-site and makes the bulk of base-specific contacts. In fact, the TR DBD is the only one in which residues outside this α-helix are seen to make base-specific contacts (Figure 1.11). The "tips" of both motifs make phosphate contacts which further stabilize binding. These, and other, phosphate contacts are made in conjunction with structures involved in dimerization (discussed below), hence contributing to cooperative binding. Figure 1.13 indicates the extent of base-specific interactions between the DBDs and the half-sites. Many of the contacts in the TR DBD/DNA crystal structure are indirect, being mediated by interstitial water molecules, some of which bridge to make several hydrogen bonds to protein or DNA moieties. A feature of the TR DBD which is not observed in the other crystal structures is a third major α-helix, the A-box. The A-box is indirectly involved in the asymmetric dimerization of the TR and RXR DBDs on a DR4 element, and also makes phosphate and base-specific contacts through the minor groove to positions C1 and C2.

The TR DBD makes many base-specific contacts within the half-site core hexamer, which may be divided in to two categories: those made to bases conserved amongst the nuclear receptor half-site sequences (AGAACA for the GR family, AGGTCA for the TR family), and those which provide a basis for discrimination between different classes of half-site. Contacts in the latter category are considered below in the sections on the P-box, and the A/T-box. In the first category, K_{120} (throughout this section, amino acid residues from all receptor DBDs will be numbered as if the third coordinating cysteine is position 119, as it is in TRβ) makes a direct contact and an indirect contact to the G of position C2. This nucleotide is conserved amongst HRE half-sites, the lysine residue is highly

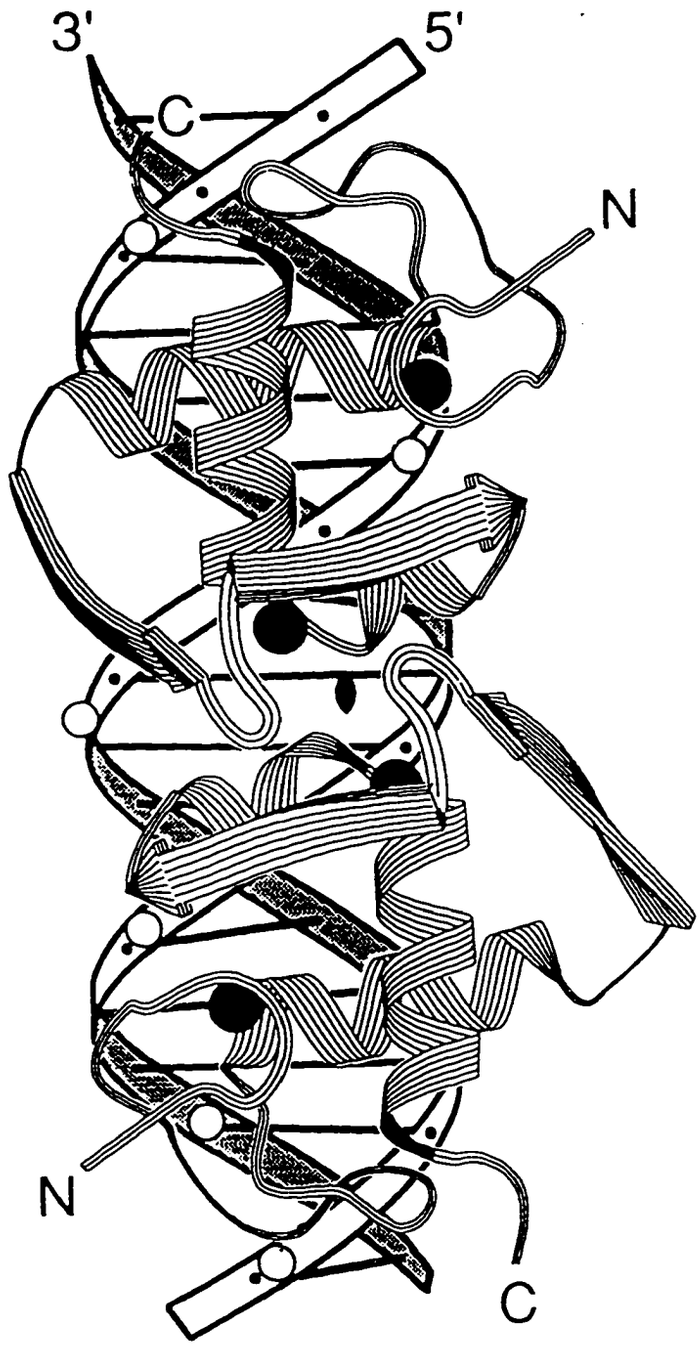
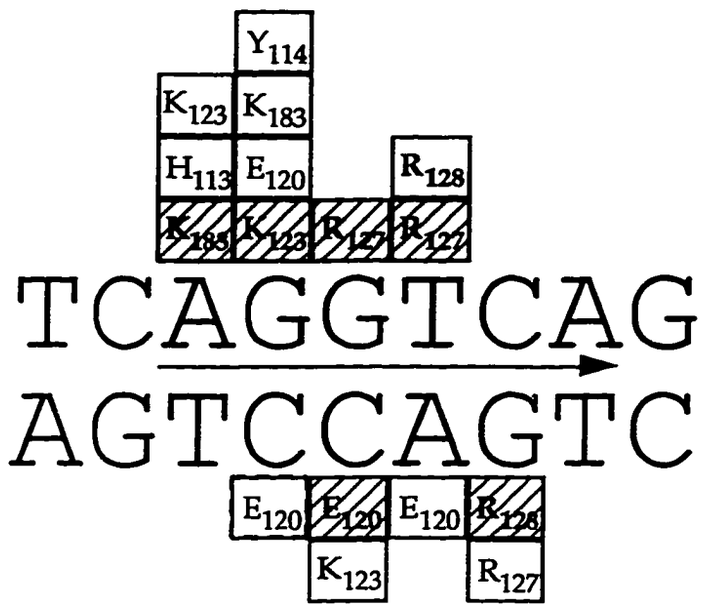
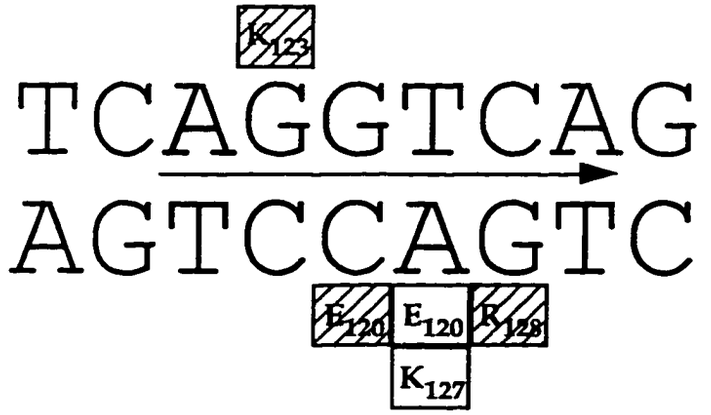


Figure 1.12: Ribbon diagram representing the crystal structure of two GR DBDs bound to a GRE-related DNA sequence. Note the two α-helices which cross at roughly 90 ° in each monomer. The N-terminal amphipathic helix lies in the major groove making base-specific contacts, while the other side contributes to the hydrophobic core of the DBD. (Luisi *et al.*, 1991)

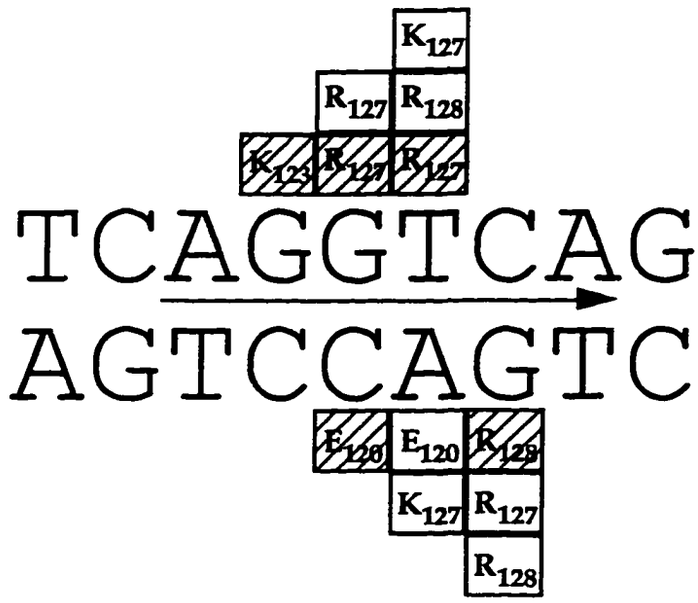
(A)


Y114
K123
K183
H113
E120
R128
K185
K123
R127
R127
TCAGGTCAG
AGTCCAGTC
E120
E120
E120
R128
K123
R127

(B)


K123
TCAGGTCAG
AGTCCAGTC
E120
E120
R128
K127

(C)



(D)

Figure 1.13: Contacts made between different receptor DBDs and bases within their DNA half-sites. The sequence of the half-sites are shown, along with the contacts made by (A) TR, (B) RXR, (C) ER, and (D) GR. The numbering system for the contacting amino acids has been transposed from TRb, so that the third cysteine residue of CI is position 119. Open squares represent direct contacts between the DBD and the indicated base, while filled boxes indicate contacts that are mediated through another atom (usually part of a water molecule). Interactions are observed that are mediated through more than one bridging atom, but these are not indicated on the figure. Each half-site has direct (solid) and indirect (open) contacts with bases (arrows) and the DNA backbone (squares) indicated adjacent to the relevant residue.
(Luisi *et al.*, 1991; Schwabe *et al.*, 1993; Rastinejad *et al.*, 1995)

conserved between receptors, and the interaction is present in all four crystal structures. The A at position C1 is also indirectly contacted by K_{120}, as well as the conserved H_{113}, in the TR crystal structure, but such an interaction is not observed for the other structures, although H_{113} makes a DNA backbone contact to this nucleotide in the GR structure and, thus, is in a similar position. The G residue of position C-5 is contacted by the conserved R_{128} residues in all DBD-half-site structures. TR and ER make a further contact with that base via R_{127} (K_{127} in ER). Despite the conservation of the A at position C6, none of the crystal structures indicate contact with this base, or that at C-6; however, backbone contacts are established which, theoretically, could be sensitive to sequence-specific structural perturbation. The remaining two positions within the core hexamer are not conserved amongst nuclear receptors and serve as the basis for differential binding. The amino acids involved in discrimination of the identify of C3 and C4 reside in the recognition α-helix and are called the P-box.

1.3.1.2.1.1 The P-box

The P-box was initially characterized as a set of amino acids involved in DNA sequence discrimination well before the crystal structures for DBDs became available. The studies took advantage of the fact that ER and GR bind IR3 sequences with distinct half-site sequence (AGAACA for GR, and AGGTCA for ER). Finger swapping between ER and GR, and DNA binding analysis with EMSA, indicated that the source of CI determined which half-site sequence is bound, the GRE-type sequence, or the ERE-type (Green *et al.*, 1988). Substitutions of ER sequence into CI of the GR further refined this result: the two amino acids between the third and fourth zinc coordinating cysteines (positions 120 and 121, Figure 1.11) were sufficient to effectively switch the specificity of the chimaeric receptor from a GRE to an ERE sequence (Danielsen *et al.*, 1989). Another study used substitutions of TR sequence into the GR DBD to find out what amino acids were involved in discriminating between a GRE (IR3) and a TRE (IR0), so that both half-site sequence and spacing were different (Umesono and Evans, 1989). This work concluded that

positions 120 and 121 were not sufficient for a complete change in specificity of half-site recognition, but that position 124 also plays a role. Thus, the sequence from positions 120 to 124 was termed the P-box (proximal box). This block of sequence includes two conserved amino acids, C_{122} and K_{123}, which, by convention, are ignored when referring to P-box sequences. Thus, the P-box of TR and ER is referred to as EGG, while the GR P-box is GSV. Substitution of a discrete sequence in CII, the D-box (distal box), was necessary to complete the conversion of the finger region so that an IR0 was recognized. That is, the D-box sequence determined whether a spacer of 0 or 3 base-pairs was tolerated between the two half-sites. The D-box is located between the first two coordinating cysteines of CII, and will be discussed under the heading of dimerization interfaces, below.

The nuclear receptors may be organized into subfamilies on the basis of P-box sequence (Umesono and Evans, 1989). In this scheme, the GR family, which includes receptors for mineralocorticoids (MR), progesterones (PR) and androgens (AR), all have GSV P-box sequences and recognize similar half-sites related to AGAACA. The ER/TR family all have a glutamate at the first P-box position and recognize a distinct sub-group of half-sites, represented by AGGTCA. A great deal of work has gone into understanding the mechanisms by which the P-box discriminates between half-site sequences, some of which comprises part of this thesis. Therefore, further discussion of P-box function will be deferred to Chapter 3.

1.3.1.2.1.2 The A-box and T-Box

Not all nuclear receptors bind to DNA with high affinity as monomers. The orphan receptor, NGFI-B was initially considered a prototypic monomeric nuclear receptor, as it binds to a single half-site with high affinity (Wilson *et al.*, 1991), and no heterodimer partners were known. Although it has more recently been shown that NGFI-B can heterodimerize with RXR and bind to a DR5 (Perlmann and Jansson, 1995) investigations of NGFI-B binding lead to the identification of a region outside the zinc binding module which is important for monomeric binding. NGFI-B has an EGG P-box sequence and, as

predicted, binds a hexameric core sequence that is the same as that bound by ER and TR, AGGTCA (Wilson *et al.*, 1991). However, as is the case for TR, two base-pairs upstream of the hexameric core also influence binding affinity, with AAAGGTCA representing the highest affinity binding site identified (Wilson *et al.*, 1992) and a functional NGFI-B response element (NBRE). RXRβ was used as the recipient receptor for construction of a series of chimaera with NGFI-B. Wild type RXRβ does not bind to the single half-site as a monomer, but after a 7 amino acid block of NGFI-B sequence was introduced C-terminal of CII, the resulting mutant bound the NBRE. The A-box, named for A-T base-pair recognition upstream of the hexamer, was thus shown to confer the ability to bind the monomeric NBRE upon RXRβ. Further swapping experiments with another orphan receptor that binds DNA as a monomer confirmed that the A-box was responsible for recognition of the base-pairs at positions F1 and F2 (Wilson *et al.*, 1993). Finally, nucleotide analogs were incorporated into oligonucleotides bearing the NBRE, and by changing features of the minor groove without influencing the major groove, it was shown that recognition of F1 and F2 by the A-box occurs through the minor groove (Wilson *et al.*, 1993). Kurokawa *et al.* (1993) used a similar approach to show that TR interaction is also dependent on the minor groove features of F1 and F2. Deleting the A-box from the TR DBD abrogates binding (Zechel *et al.*, 1994a). The A-box has subsequently been observed in NMR (Lee *et al.*, 1993) and X-ray crystallographic studies (Figure 1.11) (Rastinejad *et al.*, 1995). It comprises an α-helix that, as predicted, extends into the minor groove to make a number of stabilizing DNA backbone contacts. Thus, our understanding of half-site sequence recognition is that it is the sum of a number of activities carried out by the DBD. The recognition α-helix, including the P-box, and the A-box provide the basis for sequence recognition, while numerous DNA backbone contacts are made by other regions throughout the DBD, some of which are dependent on cooperative dimerization interfaces.

1.3.1.2.2 Dimerization

The dimerization of nuclear receptors is key to: establishing DNA binding specificity

for a specific HRE as determined by the affinity of the two associated DBDs for particular half-sites; binding affinity due to cooperative interactions between receptors; half-site configuration preference; and, ligand-responsiveness. At a more physiological level dimerization in solution can influence the available pools of particular nuclear receptors. Conversely, dimerization itself is likely to be influenced by the availability and compatibility of receptors in a cell-type dependent manner, and may also be influenced by such things as the presence of ligand, and the number of high affinity binding sites in DNA. There are two main regions that have been identified in the TR as being involved in dimerization: the LBD, and the DBD. As might be expected, the interactions of the DBD dimerization surfaces are somewhat more sensitive to half-site repeat configuration considerations, while the LBD dimerization interface is responsive to ligand binding. The functions of these two domains together contribute greatly to the complexity of TR activity and cross-talk with other receptors.

1.3.1.2.2.1 Dimerization Through the Ligand binding Domain

The ligand binding domain dimerization interface is particularly important to TR function because it facilitates the formation of both homodimer and heterodimers in solution (Kurokawa *et al.*, 1993; Zhang *et al.*, 1992). Even isolated C-terminal domains will interact in solution. This observation uncovered the phenomenon of the dominant negative effect, in which inactive receptor forms (eg. truncated forms lacking the DBD and N-terminal portion) interact with wild-type receptors to form inactive heterodimers (Forman and Samuels, 1990). This phenomenon results in an insensitivity to thyroid hormone, and may contribute to the disease, generalized resistance to thyroid hormone (GRTH) (Nagaya and Jameson, 1993).

The dimerization interaction that occurs through the LBDs is interesting for another reason: it does not appear to strongly dictate what configuration of half-sites will be bound by the dimeric complex. Interactions between LBDs stabilize the binding of TR homodimers and heterodimers to IRs, EvRs and DRs, within certain spacing limitations.

To account for the capacity to maintain LBD interactions regardless of the orientation of DBDs, a "swivel" model has been proposed, in which the LBD and DBD can rotate fairly freely with respect to one another, presumably mediated by the hinge region (Figure 1.14). This phenomenon would allow the LBD interaction to provide stability to homodimers and heterodimers without placing strict constraints on the orientation and spacing of the DBDs. Accordingly, dimerization surfaces within the DBDs cooperate to establish preferences for particular half-site configurations, at least, in isolated DBD polypeptides. For example, in the case of the TR homodimer, cooperative dimerization interactions between DBDs has only been identified for binding to IR0 (Kurokawa *et al.*, 1993), although even this result has been disputed (Mader *et al.*, 1993). Thus, in the absence of such half-site configuration-specific interactions, dimerization through the LBD affords the DBDs a certain amount of freedom to bind a variety of spacer length variants. As an illustration, when binding of full-length TR is compared to the binding of DBDs, deletion of the LBD drops dimer binding significantly, presumably due to loss of cooperative interactions, but does not result in a substantial change in half-site configuration preference (Kurokawa *et al.*, 1993; Mader *et al.*, 1993). Furthermore, when the C-terminal domains of TR and RAR are swapped, the DR spacing preference of the chimaera is the same as that of the receptor contributing the DBD, as determined by *trans*-activation (Perlmann *et al.*, 1993); that is the chimaera with the TR DBD activates transcription from a DR4, while the chimaera with the RAR DBD activates transcription from a DR5. While no cooperative interactions are believed to take place between TR DBDs in solution, there still appears to be a default or preferred orientation of the DBDs in the full-length receptor, and that is for EvRs (Kurokawa *et al.*, 1993). The role of the LBD dimerization interface in heterodimerization of TR with RXR is virtually identical to that which it plays in homodimerization of TR. Once again, isolated TR/RXR DBD heterodimers exhibit lower affinity, but similar specificity of binding to a variety of half-site configurations, than heterodimers of the full length receptors (Mader *et al.*, 1993).

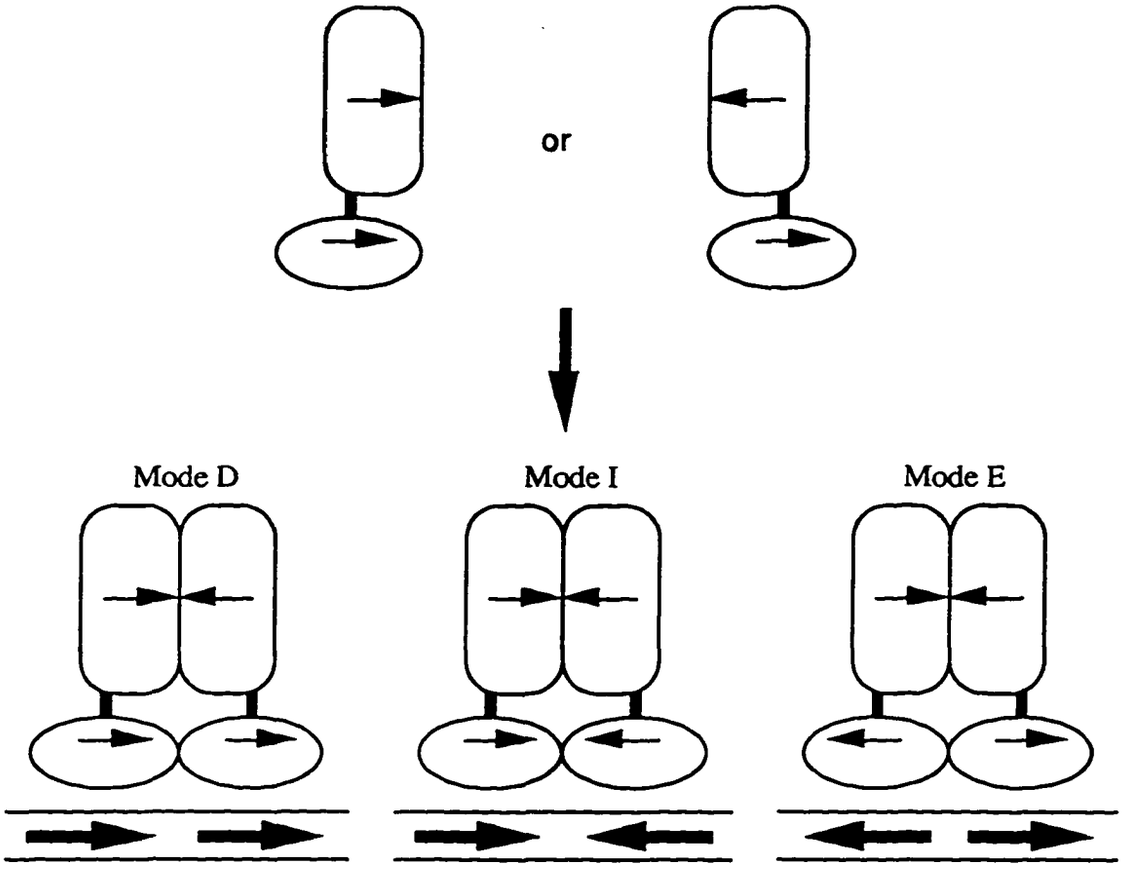


Figure 1.14: The "swivel model" of the possible relative orientations of the TR C-terminus and DBD. Conformational freedom mediated by the hinge domain allows the DBD of a TR monomomer to rotate up to 180 ° with respect to the C-terminal dimerization interface. These conformations can be combined to accomodate binding to direct, inverted and everted repeats. The receptor conformations acheived to align the DBDs appropriately for these three orientation of half-sites are referred to as Mode D, Mode I, and Mode E. (Kurokawa *et al.*, 1993)

The nature of the interaction between LBD dimerization interfaces is not well understood although recent crystal structures have certainly contributed a great deal to the picture. A series of nine heptad repeats of hydrophobic residues have been identified in the LBD sequence that were presumed to act similarly to the leucine zipper motif present in some other dimerizing transcription factors (Forman and Samuels, 1990). The basis of this interaction is that the regularly spaced hydrophobic residues create a complementary interface for interaction of two proteins. The X-ray crystal structure of the unliganded RXRα LBD (Bourguet *et al.*, 1995), which exists as a dimer in the crystal, essentially put this model to rest. It turns out that only the most C-terminal of the heptad repeats is involved in homodimerization, the others contribute to the structure of the hormone binding site. The crystal structure of the liganded TR LBD does not support a role for the first eight heptad repeats as a zipper-like motif in heterodimerization either, as they are similarly involved in ligand binding as well (Wagner *et al.*, 1995). Unfortunately, since the TR LBD is liganded, the X-ray crystal structure determined was for monomers and not homodimers. Extrapolating from the structure of the RXR LBD which has a conserved overall organization, it was suggested that the ninth heptad repeat forms a likely surface for dimerization. Solution of crystal structures for unliganded TR LBD, and for a TR/RXR LBD heterodimer will provide an interesting basis for comparison, and will likely indicate the conformational changes that occur in the LBD to disrupt the homodimer complex upon binding of hormone.

1.3.1.2.2.2 Dimerization Through the DNA Binding Domain

The configuration of half-site repeats result in DBD interactions that fall into three broad categories: sterically unfavoured, non-interacting, and positively cooperative. Presumably each of these types of interaction is important in determining what half-site repeats will constitute functional TREs, although the first two may contribute to specificity in a more passive sense, inhibiting binding to unfavoured elements. A comparison of TR DBD and full-length receptors binding to DRs with spacers of 0 to 5 base-pairs implies that two

DBDs are sterically inhibited from binding to DR0, DR1 and DR2, as only monomeric binding was observed to these half-site repeats (Mader *et al.*, 1993). No such analysis was performed for EvRs, and two DBDs bound IR0 efficiently. Other studies which have examined full-length TR homodimer binding to EvRs suggest that a spacer of 3 or fewer base-pairs is inhibitory to binding (Carlberg, 1993; Forman and Evans, 1995), but the part of the receptor which inhibits binding to this spacing is unknown. It is likely that interactions between the DBDs do not contribute substantially to determining spacing preference with respect to those EvRs that are bound (Kurokawa *et al.*, 1993), particularly since a range of spacer lengths, from 4 to 9 base-pairs can be bound efficiently (Carlberg, 1993; Forman and Evans, 1995).

Positively cooperative interactions between the DBDs on particular half-site repeat configurations imply the existence of complementary surfaces which are positioned to interact when spacing and orientation are appropriate. As would be expected due to the capacity of receptor DBDs to interact cooperatively in a variety of orientations, multiple dimerization interfaces have been characterized within the DBDs. The D-box, as described above, bestows spacer length preference on some receptors bound to IR-type sequences. In the case of the GR, the X-ray crystal structure reveals that, on an IR3, the D-boxes form a complex, symmetric interface that concomitantly results in a number of contacts with the DNA backbone, thus contributing to cooperative binding (Luisi *et al.*, 1991). Homodimers of the ER DBD bound to IR3, make qualitatively similar, interdependent protein-protein and protein-DNA contacts (Schwabe *et al.*, 1990). Substitution of the TR D-box sequence into the GR DBD resulted in a specificity for IR0 (Umesono and Evans, 1989), supporting a role for this region in spacer discrimination. However, it is unclear whether TR DBDs make cooperative interactions when bound to IR0. Mader *et al.* (1993) argue based on EMSA results that DBDs do not cooperatively interact on IRO, but Kurokawa *et al.* (1993) present contradictory data. Furthermore, when the D-box of the TR and ER are switched, only sequences with the spacing preferred by the D-box donor

are bound as homodimers of the chimaeric DBDs (Hirst *et al.*, 1992). In none of these cases was cooperativity directly tested, but it seems that the TR D-box acts permissively if not cooperatively in homodimer binding to IRs. Intriguingly, TR and RXR DBDs do display a degree of cooperativity on IR0 elements (Mader *et al.*, 1993), so there may be an as yet uncharacterized role for the D-box in heterodimerization of these two receptors, as well.

Positive cooperativity is a feature of the interaction of the TR and RXR DBDs bound as a heterodimer to DR4. This is particularly striking because it is the only case in which the binding pattern of TR and RXR DBDs to tandemly repeated half-sites is significantly different from the pattern for either homodimer (Mader *et al.*, 1993). Thus, the basis for the capacity of RXR to confer specificity for DR4 elements on TR is mediated by the DBD. Amino acid sequence swapping experiments using DBD constructs of TR and RXR gave an early picture of regions important for interaction on the DR4 element (Zechel *et al.*, 1994a). This identified the D-box of the RXR DBD, and residues just N-terminal of CI of the TR as being specifically required for heterodimerization. The X-ray crystal structure supports the involvement of these two regions in the dimerization interface (Figure 1.11), but the complete picture is more elaborate (Rastinejad *et al.*, 1995). The intrinsically asymmetric positioning of the two DBDs bound to a DR4 sequence align the CII region of RXR with both the CI and T-box of the TR DBD. Consistent with the swapping experiment results, an arginine from the RXR D-box contacts a TR T-box aspartate, and an aspartate just N-terminal of the TR CI contacts an arginine at the tip of the RXR CII. Finally, a different arginine residue in CII of RXR stacks against a tyrosine from the tip of TR CI, and also contacts a nearby TR arginine. These favourable interactions are predicted only to occur with a spacer length of four base-pairs between half-sites (Rastinejad *et al.*, 1995). Furthermore, several of these interactions are buttressed, or otherwise supported, by residues that make contacts with the DNA so that cooperativity is a function of concomitant favourable protein-protein and protein-DNA interactions.

The asymmetric interface formed between the DBDs of TR and RXR, and the cooperativity it generates, is responsible for both the specificity of this complex for the DR4 sequence, versus DRs with alternative spacer lengths, and for the observed 5'-RXR-TR-3' polarity. The X-ray crystal structure indicates that if the spacer between the half-sites is altered to 3, or fewer, base-pairs, the A-box of the TR DBD would sterically interfere with binding next to RXR (Rastinejad *et al.*, 1995). Conversely, if the spacer distance is extended to 5 base-pairs, the cooperative contacts between the two DBDs would be minimized. It is also inherent in this structure that the dimerization contacts would not exist if the polarity of the receptors were reversed. Thus, the crystal structure has provided many answers with regards to TR DBD function, including: sequence recognition and discrimination, spacing discrimination, stability of monomeric TR binding, and receptor polarity.

1.3.2 DNA Bending

The relationship between DNA bending and *trans*-activation in eukaryotes is poorly understood. In some cases a relationship between the magnitude of bending induced by a transcription factor and the level of *trans*-activation has been observed (reviewed in Nardulli and Shapiro, 1993). Furthermore, differences in the direction of DNA bending induced by *fos-fos* and *fos-jun* dimers parallels *trans*-activation levels (Kerppola and Curran, 1991). However, a mechanistic basis for these apparent relationships has yet to be elucidated. Whether DNA bending is functionally important or not, a brief summary of several studies on the DNA bending properties of TR are provided here. Two protocols for investigating bending have been used: circular permutation, and phasing analysis. Circular permutation involves performing EMSA of the protein bound to TREs located at different positions in a series of DNA probes of fixed length. If the DNA is bent by the binding of the protein, the mobility of the probes is altered more when the bend is in the middle of the probe than at the ends. The bend angle and the location of the center of bending can be calculated by graphical analysis of the probes' mobilities. An alternative strategy, phasing

analysis, uses a series of DNA probes that have an intrinsic bend at different locations with respect to the protein binding site. This methodology is less sensitive to protein conformation considerations, tends to provide more accurate estimates of bend angle, and also predicts the direction of the bend.

An early study revealed that TR bent a TRE sequence upon binding (Leidig *et al.*, 1992). Mutations which lowered the activation capacity of this TRE, and presumably the binding affinity of TR, also decreased the degree to which the DNA sequence was bent by the receptor. However, due to methodological limitations it was unclear what receptor species was binding the TRE sequence. Another work used circular permutation analysis to study bending of several TREs by a TR monomer (King *et al.*, 1993) and showed that the different sequences were bent to different extents upon binding (from 0° to 75°), and that bending magnitude was increased and the position of bend altered upon addition of a nuclear factor which heterodimerized with TR. The most recent study of DNA bending by TR has used well characterized protein and DNA components, and provides some interesting results. Shulemovich *et al.* (1995) used TRα and RXRβ, and analyzed the bending produced by the binding of TR homodimers and TR/RXR heterodimers to DR4 and DR5 elements. Circular permutation indicated bend angles from 60° to 86° for all combinations of protein and DR. However, these numbers are thought to be an overestimate, as phasing analysis indicated bend angles of 10° to 11° for DR4, and no bending at all of the DR5 sequence. Most remarkably, the direction of bending of the DR4 was opposite for the homodimer and heterodimer, the latter bending the DNA towards the minor groove. While such a dramatic difference between the bending activities of the homodimer and heterodimer may be speculated to be linked to *trans*-activation, a meaningful context for these results will require a deeper understanding of the general role of DNA bending in transcriptional regulation. It is worth noting here, that the X-ray crystal structure of the TR/RXR DBD heterodimer bound to DR4 did not indicate a bend in the DNA. This discrepancy could stem from methodological differences or reflect a true

requirement for protein features outside the DBD for DNA bending.

1.3.3 Transcriptional Regulation

TRs have multiple functions in transcriptional regulation. The classical TRE functions as a repressor of transcription in the absence of hormone, and an activator upon addition of T_3. However, there are also TREs which activate transcription in the absence of hormone, and repress in its presence. Presumably these contrasting activities result from conformational changes of the receptor upon T_3 binding, and interaction with distinct co-factors further involved in transcription. Elucidation of the mechanisms and pathways by which TR regulates transcription is currently the most dominant thrust of TR research. Several of the steps in understanding *trans*-activation by TR have already been covered. It was quickly extrapolated from GR studies, and confirmed for TR, that motifs involved in activation existed in both the N- and C-terminus, domains A/B and E/F, respectively. While efforts to refine the map of these motifs within the receptor, and to functionally characterize them, made steady progress, within the last two years a major acceleration of progress has occurred. This has largely been due to the development of the yeast two-hybrid system (Fields and Song, 1989; Le Douarin *et al.*, 1995a) and the related yeast interaction trap (Gyuris *et al.*, 1993) which facilitate the identification of unknown proteins that directly interact with a known protein. The first hybrid of the system is a fusion of the protein of interest (or fragments thereof) with a DNA binding domain such as that of GAL4 or LexA. The second hybrid results from fusing the gene for a heterologous transcriptional activation domain, for example VP16, to sequences from a cDNA library, essentially creating a library of vectors expressing VP16 fusions. When a binding site for the DBD of the first hybrid is placed upstream of a reporter gene, such as lacZ, that gene will only be expressed in cases in which the second hybrid interacts with the first to bring the activation domain to the reporter gene promoter. Thus, a library of cDNAs may be screened for proteins that interact with the protein of interest that has been fused to the DBD. Application of this technique has identified a number of proteins that specifically interact

with *trans*-regulatory domains from TR.

1.3.2.1 Refinement of Regions Involved in Transcriptional Regulation

The conservation of functional domains of nuclear receptors was dramatically illustrated by early work comparing the activities of TRβ and GR (Thompson and Evans, 1989). In this work, the receptors were divided into three segments: the N-terminus, the DBD, and the C-terminus. Determination of the *trans*-activation characteristics of these various chimaeric fusions of these domains produced many interesting results. For instance, the specificity of hormone-responsiveness was completely transferred along with the C-terminal domain, implying not only ligand binding function of this domain, but also activation domains. In contrast to the GR which has an activation domain called τ_1 in the N-terminus, the N-terminus of TRβ made no detectable contribution to *trans*-activation. More recent domain swapping experiments have shown that the TRα1 and TRβ1 isoforms differ in the function of their N-terminal domains, and that domain A/B of TRα1 does have a potent function both in T_3-dependent activation and T_3-independent silencing of transcription (Hollenberg *et al.*, 1995; Saatcioglu *et al.*, 1993). This result has been refined somewhat by Samuels' group, who have shown that the activation of TRα1 is not transferable; that is, the N-terminus of TRα1 does not activate transcription when fused to a non-receptor DBD (Hadzic *et al.*, 1995). So, the function of this domain appears to be confined to the context provided by the nuclear receptors. The domain was further defined as a 10 amino-acid region starting at position 21 of TRα (Hadzic *et al.*, 1995). Intriguingly, this region is conserved between rat and chicken TRα1 sequences, which are otherwise not well conserved in the N-terminus (Tomura *et al.*, 1995). A comparison of the *trans*-activation capacity of TRβ1 and TRβ2, which diverge only at the N-terminus (Figure 1.4) implies that there is an activation domain in the N-terminus of TRβ1, but not TRβ2 (Tomura *et al.*, 1995), in contrast to earlier results. While there is some evidence for the interaction of co-factors with the N-terminal region of TRα and TRβ1 (discussed below), the regions within TRβ1 through which these take place remain poorly defined.

Activation domains within domain E/F were the focus of more earlier investigation, due to the overlap of activation and ligand binding functions in this domain, and the interest in determining the mechanism by which ligand binding triggers *trans*-activation. A number of other transferable activation regions, τ_2-τ_4, have been identified in the C-terminus of TRβ. τ_4 is also involved in ligand-independent transcriptional silencing. The hinge region also contains sequences that are important in activation.

Recent applications of the yeast two-hybrid system have led to the identification of factors interacting with TR, and have served to unify some of the diversity of activation domains as docking sites for these factors. TFIIB, a component of the basal transcription machinery, has been shown to interact with TRβ in the N-terminal and C-terminal domains (Baniahmad *et al.*, 1993). TFIIB also interacts with the 10 amino acid sequence in the N-terminal domain of TRα described above (Hadzic *et al.*, 1995). Interestingly, unliganded TR interferes with formation of a productive preinitiation complex (PIC) on an adjacent promoter, and addition of T_3 relieves this interference (Fondell *et al.*, 1993). The interaction with TFIIB is hormone dependent; TFIIB is released upon binding of T_3 (Tong *et al.*, 1995). Thus, it is proposed that the unproductive interaction of TR with TFIIB may be a mechanism for transcriptional silencing. Likewise, TRα interacts with TATA binding protein, another component of the PIC, in a hormone sensitive fashion (Fondell *et al.*, 1996); TR may interfere with PIC formation through a number of means. However, adding to the complexity of the story is the recent identification of a "corepressor" for TR, called N-CoR (Hörlein *et al.*, 1995). This protein has the following interesting features: it is the major protein that interacts with TR, from a number of cell-lines; it interacts with the unliganded TR/RXR heterodimer, in a hormone-sensitive manner; it interacts with the C- and N-termini of TR; and, mutations in the hinge region of TR which abrogate N-CoR binding, but not TFIIB binding, eliminate transcriptional silencing. The discovery of a related co-repressor, SMRT, with similar features was published simultaneously (Chen and Evans, 1995), and both molecules are classified as a new family of thyroid-, retinoic acid-

receptor-associated co-repressors (TRAC).

Recently, a number of nuclear factors have been shown to interact with thyroid hormone receptor, some in a hormone dependent fashion. The tumour suppressor p53 binds to the DBD of TRβ and interferes with DNA binding of both the homodimer and the TR/RXRβ heterodimer (Yap *et al.*, 1996). *Trans*-activation by TRβ is also inhibited by the overexpression of p53. Another previously characterized nuclear factor, CREB-binding protein (CBP), also interacts with TR (Kamei *et al.*, 1996). In fact, CBP appears to act as an integrator of *trans*-activation signals for a variety of transcription factors including the nuclear receptors, CREB, and AP-1. For this reason, it is believed to be distinct from the specific cofactors which have also recently been shown to interact specifically with the *trans*-activation domains of TR. TIF1 (LeDouarin *et al.*, 1995b), Trip 1 (Lee *et al.*, 1995), RIP140 and RIP160 (Cavaillès *et al.*, 1995) are distinct factors which have been shown to interact with the τ_4 region in a hormone dependent fashion. These factors and their roles in TR function are poorly understood, but further studies will undoubtedly make an interesting next chapter in our understanding of TR function.

1.4 The Context of This Work

In order to completely understand the regulation of gene expression by nuclear receptors, it is necessary to understand the properties of the receptor complexes which they form, and their DNA binding properties. The studies in this thesis can be divided into two broad approaches: structure/function studies of the TR DBD, and structure/function studies of the DNA sequences to which the thyroid hormone receptor binds in order to regulate transcription. In Chapter 3.0, the construction and analysis of mutant TR receptors with alanine residues substituted individually for each residue in the recognition α-helix are described. This work confirmed the structural relatedness of the TR DBD with that of the GR, even before publication of the TR DBD X-ray crystal structure by Rastinejad *et al.* (1995). However, due to the functional analyses that were performed, this work also helped define the function of the P-box residues, in a way that purely structural studies

could not. Chapter 4.0 describes a comparison of the binding of TR homodimers and TR/RXR heterodimers to DR binding sites with a variety of spacer lengths. This analysis delineated differences in the binding site configurations that the homodimer and heterodimer bind to preferentially. A novel model for differential recognition of certain classes of half-site tandem repeats is proposed. Further investigation of this model will likely expand our understanding of the roles of the homodimer and the heterodimer in gene regulation.

CHAPTER 2.0

GENERAL MATERIALS AND METHODS

2.1 Materials

The work in this dissertation has been divided up into several projects which are described in the following chapters. Each chapter describes the specific methodology that was used to carry out the research involved, but many of the basic materials and methods are common to both. These materials and the suppliers of these materials will be summarized here to reduce repetition in the other methods sections. This summary will include not only reagents, oligonucleotide sequences, general cloning vectors, and organisms obtained from commercial vendors, but also plasmid constructs that have been generously provided by other researchers.

2.1.1 Reagents, Enzymes and Kits

General chemical reagents for the routine creation of buffer solutions were purchased from BDH. Suppliers of other specific reagents are listed in Table 2.1. Materials for bacterial cell growth were purchased from Difco (Detroit, MI). Restriction enzymes and enzymes used for recombinant DNA work such as T4 DNA ligase, Klenow fragment of DNA polymerase I and T7 RNA polymerase were purchased on the basis of price and availability from either New England Biolabs, Pharmacia, or Boehringer Manheim (Laval, Que.). Other enzymes used are listed in Table 2.2. Kits were purchased for sequencing (Sequenase - United States Biochemical, Cleveland, OH), DNA purification (Geneclean and Mermaid - Bio101 through Bio/Can Scientific, Mississauga, Ont.; and, Qiagen, Studio City, CA) and *in vitro* translation (Promega, Madison WI).

2.1.2 Oligonucleotide Synthesis, Cloning Vectors and Organisms

Oligonucleotides were synthesized on a Applied Biosystems PCR-Mate synthesizer. pUC18 or pUC19 were used for the cloning of oligonucleotides of TREs and other binding sites sequences. The sequences of oligonucleotides containing TREs are given in Table 2.2. Oligonucleotides were cloned into the site of pUC18 or pUC19 corresponding to their

Reagent/Material	Supplier
Urea (ultra pure)	Gibco BRL ICN (Montreal, Que.)
Tris(hydroxymethyl)ammonium chloride	BDH (Vancouver, B.C.)
Tris(hydroxymethyl)aminomethane	Anachemia Scientific (Richmond, B.C.)
HEPES	Sigma (Mississauga, Ont.)
Phenol (redistilled)	BDH
Acrylamide (electran)	BDH
Methylenebis-acrylamide	Pharmacia (Baie D'Urfè, Que.)
Dimethyl sulphate	BDH
Nucleotides	Pharmacia
^{32}P-dATP	New England Nuclear/DuPont (Mississauga, Ont.)
^{14}C-acetyl Co-enzyme A	Amersham (Oakville, Ont.)
Other radio-labeled compounds	DuPont (Mississauga, Ont.)
SeaPlaque Agarose	FMC Bioproducts (Rockland, ME)
NuSieve GTG agarose	FMC Bioproducts

Table 2.1: List of reagents and the supplier from whom they were purchased.

Name	Strand	Over-hangs	Sequence
TRE_{IR0}	Both	*Bam*H I	GATCCTCAGGTCATGACCTGAG
TRE_{rGH}	Top	*Bam*H I[1]	AAGGGGATCCGGTAAGATCAGGGACGTGACCGCAGG
TRE_{rGH}	Btm	*Bam*H I[1]	AGGAAGATCTCCTGCGGTCACGTCCCTGATCTTACC
TRE_{TSH}	Top	*Pst* I	GGCAGGTGAGGACTTCACTGCA
TRE_{TSH}	Btm	*Pst* I	GTGAAGTCCTCACCTGCCTGCA
TRE_{Mal}	Top	*Pst* I	GTCGTCCACTGTCCTCCCCTAACCCCAACGTCCTCTGCA
TRE_{Mal}	Btm	*Pst* I	GAGGACGTTGGGGTTAGGGGAGGACAGTGGACGACTGCA
TRE_{Lys}	Top	*Pst* I	GCTTATTGACCCCAGCTGAGGTCAAGTTACGCTGCA
TRE_{Lys}	Btm	*Pst* I	GCGTAACTTGACCTCAGCTGGGGTCAATAAGCTGCA
TRE_{rGH3}	Top	*Pst* I	GAGGCTGAGGTAACTTGGGAGTCCCAGGCAGAGGTCACTAGCTAATGCTGCA
TRE_{rGH3}	Btm	*Pst* I	GCATTAGCTAGTGACCTCTGCCTGGGACTCCCAAGTTACCTCAGCCTCTGCA
DR3	Top	*Hin*D III	AGCTTCAGGTCAAGGAGGTCAGAG
DR3	Btm	*Hin*D III	AGCTCTCTGACCTCCTTGACCTGA
DR4	Top	*Hin*D III	AGCTTCAGGTCACAGGAGGTCAGAG
DR4	Btm	*Hin*D III	AGCTCTCTGACCTCCTGTGACCTGA
DR5	Top	*Hin*D III	AGCTTCAGGTCACCAGGAGGTCAGAG
DR5	Btm	*Hin*D III	AGCTCTCTGACCTCCTGGTGACCTGA
$TRE^1/_2$	Top	*Hin*D III	AGCTTCAGGTCACTTCA
$TRE^1/_2$	Btm	*Hin*D III	AGCTTGAAGTGACCTGA
EvR4	Top	*Hin*D III	AGCTTCTGACCTCAGGAGGTCAGAG
EvR4	Btm	*Hin*D III	AGCTCTCTGACCTCCTGAGGTCAGA
DR4/EvR6	Top	*Hin*D III	AGCTTCAGCTCACAGGAGGTCAGAG
DR4/EvR6	Btm	*Hin*D III	AGCTCTCTGACCTCCTGTGAGCTGA
DR4/EvR4	Top	*Hin*D III	AGCTTCAGGCCACAGGAGGTCAGAG
DR4/EvR4	Btm	*Hin*D III	AGCTCTCTGACCTCCTGTGGCCTGA
DR3/EvR5	Top	*Hin*D III	AGCTTCAGCTCAAGGAGGTCAGAG
DR3/EvR5	Btm	*Hin*D III	AGCTCTCTGACCTCCTTGAGCTGA
PCP-2	Top	*Hin*D III	AGCTTAAAAGGCCTTCTCAGGTCAGAGA
PCP-2	Btm	*Hin*D III	AGCTTCTCTGACCTGAGAAGGCCTTTTA
PCP-2/EvR4	Top	*Hin*D III	AGCTTAAAAGACCTTCTCAGGTCAGAGA
PCP-2/EvR4	Btm	*Hin*D III	AGCTTCTCTGACCTGAGAAGGTCTTTTA

Table 2.2: Sequences of olignucleotides containing TREs. Complementary oligonucleotides (top and btm) were synthesized with overhangs compatible with the those generated by the indicated restriction enzyme. Half-sites within each sequence are underlined.

[1]See text regarding this sequence.

5'-overhangs, unless otherwise noted in the Methods and Materials sections of the experimental chapters. The oligonucleotides TRE_{rGH} were digested with *Bam*H I/*Bgl* II and cloned into the *Bam*H I site of pUC19. Due to a cloning artifact, the sequence between the two cloning sites is GATCAGGGACGTGACCGCAGGCGATC. For routine transformation and maintenance of plasmid stocks, the *Escherischia coli* strain JM109 was used.

2.1.3 Expression Vectors Provided by Other Workers

Several vectors provided by other researchers were very important to the work described in this thesis. pEA101, used for *in vitro* transcription, is pGEM-3 (Promega) with the cDNA for human TRβ1 cloned downstream of a T7 RNA polymerase promoter, into the *Eco*R I site (Weinberger *et al.*, 1986). pBS-RXRα, for *in vitro* transcription by T3 RNA polymerase, has the cDNA for human RXRα between the *Eco*R I and *Hin*D III sites of pBluescript. pCEA1 is the cDNA for chicken TRα cloned downstream of a T7 promoter in pTZ19 (Sap *et al.*, 1986). pRShTRβ is a vector for expression of the human TRβ gene in mammalian cells under the control of the long terminal repeat of the Rous sarcoma virus (Giguère *et al.*, 1986; Thompson and Evans, 1989). The cDNA is inserted between the *Kpn* I and *Bam*H I sites of the vector. MTV-CAT is a reporter plasmid for use in mammalian cells. The CAT gene is under the regulation of the mouse mammary tumour virus long terminal repeat, and 180 base pairs upstream of the transcriptional start site, there is a unique *Hin*D III site for the introduction of response elements (Hollenberg and Evans, 1988).pEA101, pRSHTRβ, and pΔMTV-CAT were kindly provided by Dr. R.M. Evans. pBS-RXRα was a gift from Dr. G. Graupner.

2.1.4 Standard Buffers

Reaction buffers for recombinant DNA work were those provided by the enzyme suppliers. Reaction buffers for experimental procedures are given in the individual chapters. The common buffer used for electrophoresis is TBE: 0.089 M Tris-borate, 0.002 M EDTA (pH 8.0).

2.2 Methods

The details of routine recombinant DNA work such as transformation, DNA preparation from bacteria, etc. will not given here as these protocols were similar, if not identical to those well-established and readily available from numerous sources. The laboratory manual which was used as a reference for protocols, buffer recipes and reaction conditions was Molecular Cloning: A Laboratory Manual (second edition) (Sambrook *et al.*, 1989). Some general comments will be made about standard procedures which are alluded to in following chapters.

2.2.1 Standard PCR Conditions

PCR was carried out on a Techne (Princeton, NJ) PHC-I thermocycler. The standard PCR reaction was 20 µl, and contained: 10 mM Tris-HCl, pH 8.3 at 20 °C; 1.5 mM $MgCl_2$; 25 mM KCl; 50 µM each dNTP; 50 µg/ml gelatin; 2 ng template DNA; 0.2 µM each primer; and 0.5 U *Taq* DNA polymerase. Samples were overlayed with 20 µl of mineral oil before the thermocycling steps. Each round of PCR consisted of a denaturation step, an annealing step, and an extension step. For the first round, the three steps were: 10 minutes at 94 °C; 1.5 minutes at 37 °C; and, 3 minutes at 72 °C. The next 24 cycles were: 1.5 minutes at 94 °C; 1.5 minutes at 37 °C; and, 3 minutes at 72 °C. The final round was 1.5 minutes at 94 °C; 1.5 minutes at 37 °C; and, 10 minutes at 72 °C. The product of the PCR was visualized by directly loading a portion of the sample on an agarose gel and electrophoresing. Before the rest of the sample was processed in any other way, a phenol:chloroform extraction and ethanol precipitation was performed.

2.2.2 Standard Recombinant DNA Techniques

Many different DNA constructs were used for this work. The recombinant DNA techniques that were used to create these constructs typically followed a single pattern: isolation of desired DNA fragments; ligation of DNA fragments; transformation of *E. coli* with ligation mixture; selection and screening of colonies transformed with plasmid vector; identification of correct DNA construct; and, preparation of DNA from bacteria.

Restriction digests of DNA were carried out per the instructions provided by the supplier of the restriction enzyme. Restriction fragments were isolated, when necessary, by running the digest out on an agarose gel. NuSieve® was used for fragments over 100 base-pairs, and SeaPlaque GTG® was used for those below 100. The desired band was excised from the gel and purified using either the Mermaid® or Geneclean® kit for short and long fragments, respectively. Synthetic oligonucleotides were sometimes used for cloning, and in these cases, the oligonucleotides were denatured at 95 °C for 5 minutes and slowly cooled in a buffer containing 50 mM Tris-HCl pH 8.3, 60 mM NaCl, and 10 mM DTT. If necessary for cloning, the overhangs of DNA fragments, and/or annealed oligonucleotides, were filled-in using the Klenow fragment of DNA polymerase I under conditions suggested by the supplier. Ligations were then carried out in ligase buffer (BRL, Burlington, Ont.) for 3 hours, at room temperature for sticky-end ligations or at 10 °C for blunt-end ligations. Ligation reactions were transformed into JM109.

When pUC19 was the cloning vector, blue/white screening was used to identify transformants in which the plasmid contained an insert. Otherwise, all colonies were treated as candidate clones. 5 ml cultures of transformants were grown overnight in LB. A cell stock with 15 % glycerol was made from each culture, and a purified DNA sample produced using a Qiagen "mini-prep" protocol. Diagnostic restriction digests were then performed to identify plasmids that appeared to bear the correct insert. These plasmids were sequenced using a Sequenase kit with ^{35}S-dATP. Electrophoresis of the sequencing gel was carried out on a 6% acrylamide gel (29:1 cross-linking ratio) in 0.5xTBE at 40W for as long as necessary to optimally resolve the bands of interest. A salt gradient system was used in which the buffer in the bottom of the apparatus contained 0.1 M Sodium Phosphate (for 1 L running buffer, 97.05 ml 1 M Na_2HPO_4 and 2.05 ml 1 M NaH_2PO_4). Autoradiography was performed overnight at room temperature with Kodak XAR or βMax film.

2.2.3 Expression of Protein *In Vitro*

For the purposes of transcription, vectors were digested with a restriction enzyme that cuts the plasmid once, downstream of the coding region of the cDNA, to allow run-off transcription from the linearized template. The restriction digest was extracted with phenol:chloroform, then chloroform, and precipitated with ethanol. The DNA was resuspended in water and used in an *in vitro* transcription reaction with the following components: 40 mM Tris-HCl, pH8.1 at 37 °C; 30 mM $MgCl_2$; 5 mM DTT; 0.1 mg/ml BSA; 0.4 mM spermidine; 5 mM rNTPs; 8% PEG8000; 0.01 % triton X-100; 0.1 µg/µl template DNA; 1.5 U/µl T7 RNA polymerase or T3 RNA polymerase; and, 0.24 U/µl RNAguard. Incubation was at 37 °C for 4-5 hours. After incubation, 1/10 volume of 0.5 M EDTA was added, the sample was extracted with phenol:chloroform, then chloroform, and precipitated with ammonium acetate and ethanol. The RNA was resuspended in water and the absence of degradation checked on a 1% agarose gel.

RNA transcribed *in vitro* was used as a template for *in vitro* translation. A rabbit reticulocyte lysate system was utilized as per the instructions. Parallel reactions were performed using ^{35}S-methionine to confirm translation and, in some cases, to equalize the amounts of translated protein used for certain purposes. TCA precipitation of the radioactive reaction was used to determine incorporation of ^{35}S-methionine, as per the instructions of the translation kit. "Unprogrammed" lysate had water added instead of RNA sample. Receptor proteins translated *in vitro* could be frozen at -70 °C indefinitely with no noticeable loss of activity.

2.2.4 Electrophoretic Mobility Shift Assay (EMSA)

While conditions for EMSA evolved throughout the course of this work, the assays in the following chapters were carried out using two similar protocols. Thus, these protocols will be given here, and any modifications noted in future references.

2.2.4.1 Standard EMSA Conditions: Set I

DNA was radiolabeled by fill-in of overhangs in the presence of ^{32}P-dATP by the Klenow fragment of DNA polymerase I. The reaction was chased briefly by addition of dATP to 500 μM before the Klenow enzyme was inactivated by incubation at 65 °C for 3 minutes. Labeling reactions were run on a 8 % acrylamide gel (29:1 cross-linking ratio), the desired band was excised, and eluted overnight in 1 mM EDTA, 0.6 M ammonium acetate, and 0.1 % SDS. The labeled DNA was precipitated and washed with ethanol, then resuspended in water.

Binding of the receptor to the radiolabeled probe was carried out in Buffer H, which, when all components had been added, contained 20 mM HEPES, pH 7.8, 50 mM KCl, 1 mM β-mercaptoethanol, 20 % glycerol, and 0.1 mg/ml poly d[I-C]. A 20 minute preincubation of 4 μl protein with 6 μl of 2x buffer on ice was performed, then 2 μl of radiolabeled DNA (diluted to 5,000 cpm/μl) was added and the incubation continued for another 20 minutes on ice. Samples were loaded onto a 5% acrylamide (29:1), 0.3 x TBE gel and run in 0.3 x TBE for 1.5 hours at 10 V/cm at 10 °C. Gels were dried onto Whatman filter paper and autoradiographed with Kodak XAR, βMAX or XK1 film at -70 °C for suitable period of time, usually overnight.

2.2.3.2 Standard EMSA Conditions: Set II

Labeling of the DNA probe was carried out as in Set I standard conditions. The binding reaction was the same except that the KCl concentration was 100 mM, and the preincubation and incubation with labeled DNA was at room temperature for 10 minutes each. Likewise, electrophoresis was carried out at room temperature. The gel buffer and running buffer were 0.5 x TBE. Gels were treated in the same fashion after EMSA.

3.0 ALANINE SUBSTITUTION MUTANTS IN THE RECOGNITION α-HELIX OF THYROID HORMONE RECEPTOR

3.1 INTRODUCTION

By 1990 the recognition α-helix of the nuclear receptor DBD was a well established model, yet the interactions of this domain with the DNA half-site were only coarsely characterized. The CI, and not the CII, region of the zinc binding motif was shown to be responsible for DNA binding specificity of the glucocorticoid and estrogen receptors through sequence swapping experiments (Green *et al.*, 1988). The identification of the role of the P-box in half-site discrimination (Umesono and Evans, 1989) further localized DNA binding features to the C-terminal region of CI. Analysis of the ten amino acid sequence following the third zinc coordinating cysteine of CI led to the prediction that this region forms an amphipathic α-helix which includes the P-box residues (Figure 3.1) (Berg, 1989). It was proposed that the hydrophobic side of the helix would form part of a hydrophobic core in conjunction with other regions of the DBD, and the polar face would be involved in DNA sequence recognition. Finally, in 1990, NMR studies confirmed the existence of the recognition helix in the GR and ER DBDs as well as the involvement of the hydrophobic face in the core of the folded zinc binding domain (Härd *et al.*, 1990; Schwabe *et al.*, 1990). It was not until the X-ray crystal structure was published that the role of individual amino acids in the recognition helix of GR became understood (Luisi *et al.*, 1990).

Prior to the publication of the TR DBD-DNA X-ray crystal structure (Rastinejad, 1995), it was of interest to contrast the known features of the GR recognition helix with that of the less well characterized TR region. The GR and TR are members of two distinct families of nuclear receptor. The two families recognize distinct half-site sequences: the former family, including receptors for glucocorticoid, progesterone, mineralocorticoid and androgens recognize AGAACA; and the latter group, including receptors for thyroid

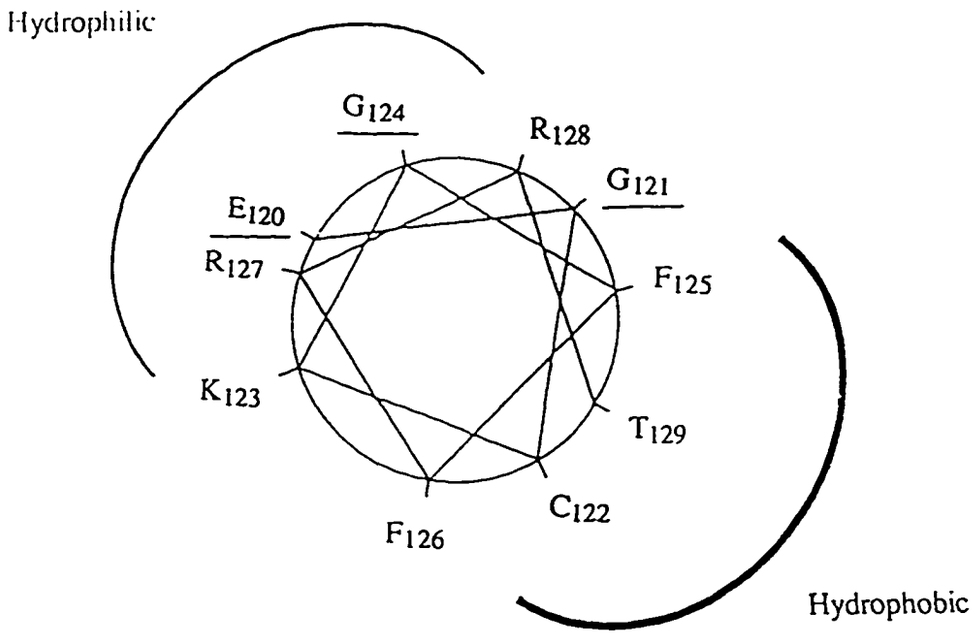


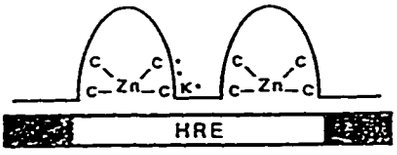
Figure 3.1: Helical wheel representation of the predicted amphipathic α-helix which follows the third coordinating cysetine (C_{119}) of CI. The P-box residues are underlined. (After Berg, 1989)

hormone, estrogen, retinoic acid and vitamin D, recognize AGGTCA (Glass, 1994). Perhaps not surprisingly, analysis of the DBD sequences of these two groups partitions the receptors similarly. One scheme based strictly on P-box sequence is shown in Figure 3.2 (Forman and Samuels, 1990). Phylogenetic analysis of the C domain from 32 nuclear receptors divides them into three subfamilies (Figure 3.3) (Laudet *et al.*, 1992). In either case, TR is grouped along with the RARs. The steroid hormone receptors, except for ER, are in a distinct group. Thus, even though conservation between the GR and TR DBDs is substantial, and a similar folding pattern for the DBDs was expected, the interactions of the TR DBD with the half-site could not be extrapolated directly from the relatively well-characterized GR and ER structures.

In order to examine the roles of amino acids within the recognition helix in TR function a series of mutant human TRβs were created, each one substituting alanine for an individual residue within the recognition α-helix, except for one position which was substituted with methionine (Figure 3.4). The mutant proteins are referred to as X-#-Y, where X is the amino acid residue in the wild-type receptor, # is the position of the mutation, and Y is the substituted amino acid, either alanine or methionine. For example, C122A is the mutant TRβ in which the fourth coordinating cysteine of CI, C_{122}, is replaced with alanine. The DNA binding affinities of the mutants were compared, as well as their abilities to mediate T_3-dependent activation. This analysis identified amino acid residues within the recognition α-helix that are important for DNA binding. Furthermore, comparison with studies of the GR P-box indicates that the TR P-box amino acids function differently, acting independently and separately at the level of *trans*-activation.

3.2 MATERIALS AND METHODS

General methods and materials for routine recombinant DNA work, electrophoresis, etc. are given in Chapter 2.0. What follows are the specific techniques used for experiments described within this chapter. Where standard conditions or procedures are referred to, they may be found in Chapter 2.0.



GROUP	I	II	III	IV
DISCRIMINATORY AMINO ACIDS	c GS ck V	c EG ck G	c EG ck S	c EG ck A
HRE consensus	AGAACA nnn TGTTCT	AGGTCA TGACCT	GTGTCA A AGGTCA	AGGTCA nnn TGACCT
RECEPTORS	GLUCOCORTICOID MINERALOCORTICOID PROGESTERONE ANDROGEN	THYROID HORMONES RETINOIDS VITAMIN D c-erbA-α2 REV-erb E75 NGFI-B	v-erbA ear-2 COUP-TF/ear-3 knirps knirps-related	ESTROGEN

Figure 3.2: A classification scheme for nuclear receptors based on P-box sequences. The P-box residues are indicated on the schematic DBD by asterisks (Forman and Samuels, 1990)

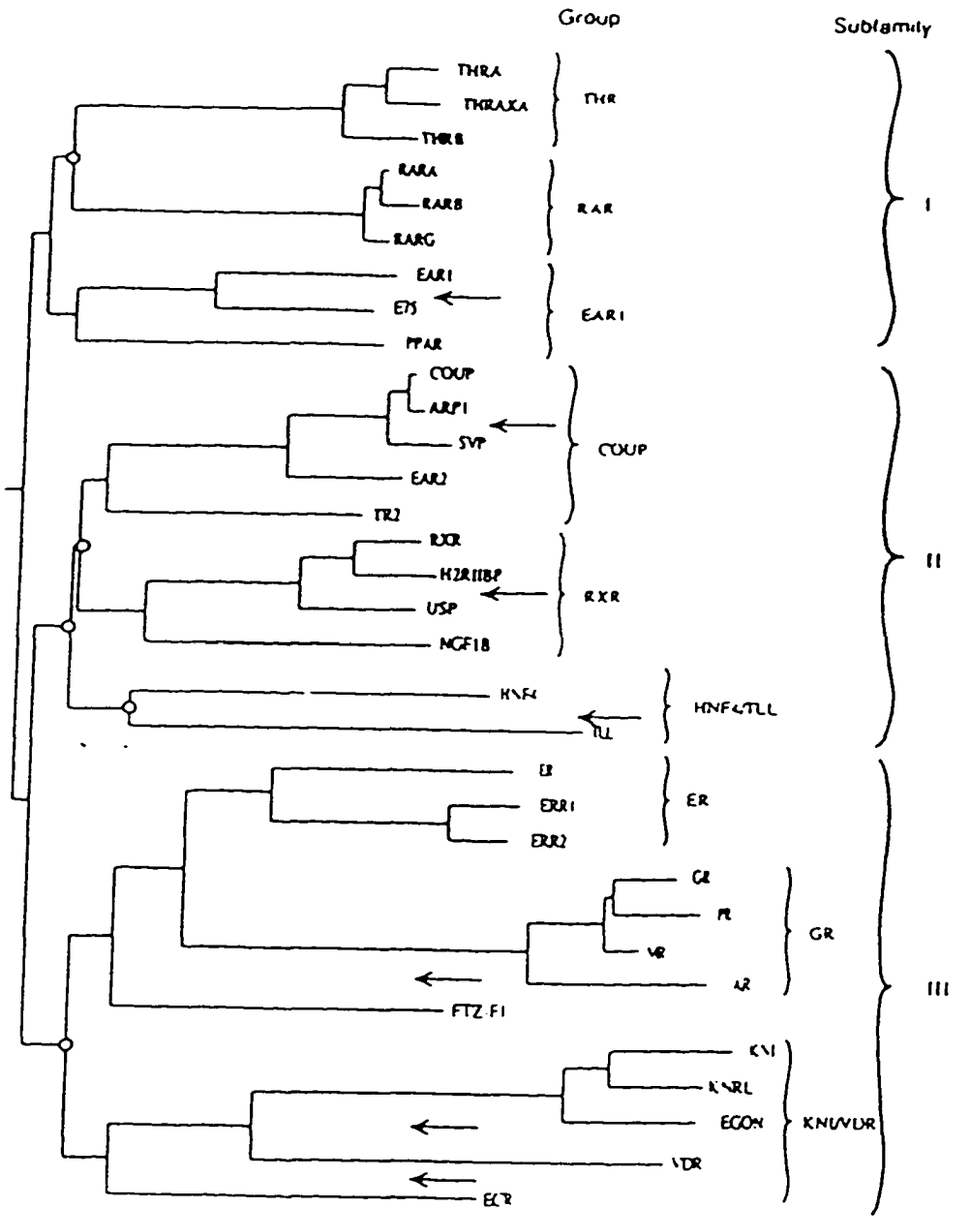


Figure 3.3: Phylogenetic analysis of 32 nuclear receptors based on the DBD sequence. Thyroid hormone receptor is abbreviated THR, and THRAXA is TRα from *Xenopus laevis*. This analysis was performed using the Fitch least square method (Fitch, 1981). (Laudet *et al.*, 1992)

```
                120         125         130
                 |           |           |
          GR C G S C K V F F K R A V E
          ER C E G C K A F F K R S I Q
      T3Rß  C E G C K G F F R R T I Q
      E120A C A G C K G F F R R T I Q
      G121A C E A C K G F F R R T I Q
      C122A C E G A K G F F R R T I Q
      K123M C E G C M G F F R R T I Q
      G124A C E G C K A F F R R T I Q
      F125A C E G C K G A F R R T I Q
      F126A C E G C K G F A R R T I Q
      R127A C E G C K G F F A R T I Q
      R128A C E G C K G F F R A T I Q
      T129A C E G C K G F F R R A I Q
      I130A C E G C K G F F R R T A Q
      Q131A C E G C K G F F R R T I A
G121A/G124A C E A C K A F F R R T I
```

Figure 3.4: Point mutations introduced into the recognition α-helix of human TRβ. The sequence of the recognitin α-helices of ER and GR are provided for comparison. (Nelson *et al.*, 1993)

3.2.1 Construction of Site-Directed Mutants of TRβ

Construction of the genes encoding the alanine substitution mutants of human TRβ was mainly performed by Dr. Colleen Nelson, with some technical assistance from myself, Mr. Stephen Hendy, Ms. Hazel Jones and Ms. Judy Wise. The introduction of codons for alanine into the coding sequence of TRβ was performed using a PCR-based mutagenesis protocol (Nelson and Long, 1989). There were essentially three major steps in the operation (Figure 3.5):

1. Introduction of mutation by PCR. PCR is carried out using mutagenic primer which introduces the mutation into the DNA sequence.
2. Extension of the mutated DNA upstream of the mutagenic primer.
3. PCR amplification specifically using the mutated strand as a template.

As indicated in Figure 3.5, the first round of PCR is carried out with a mutagenic primer, harbouring the desired DNA sequence, and a universal mutagenic primer which has two sequence components. The first is a sequence which anneals to the original template, and the second, at the 5' end, is not complementary to the original template but serves as a unique sequence for selective amplification of the mutated strand in the subsequent PCR amplification of step 3.

For the purposes of mutating the gene for TRβ, a cassette encoding both CI and CII was created from pEA101 by PCR (Figure 3.6A and B). The upstream primer used to amplify the DBD cassette introduced an *Eco*R I site, whereas the downstream primer was perfectly complementary to a region of the cDNA encompassing a unique *Pst* I site. PCR was carried out under standard PCR conditions with βL and βR as primers. The resulting DBD cassette was digested with *Eco*R I and *Pst* I, purified from an agarose gel using a tip-20 from Qiagen (Studio City, Ca), then cloned into pUC19 following standard procedures. The resulting plasmid is referred to as pUC19/TRβDBD.

For the first step of mutagenesis, PCR was performed under standard conditions with a mutagenic primer (Figure 3.6D), reverse universal mutagenic primer (RUMP), and

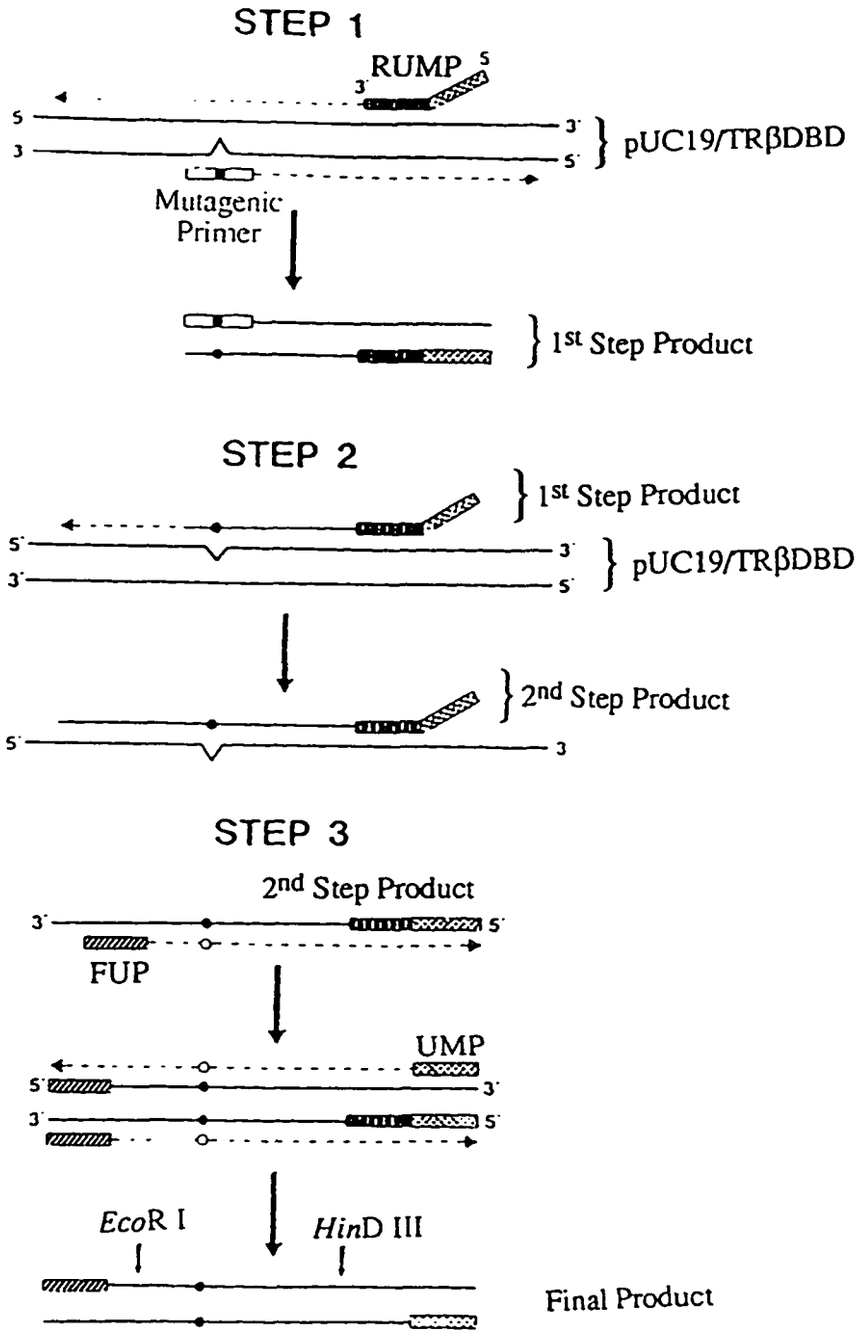
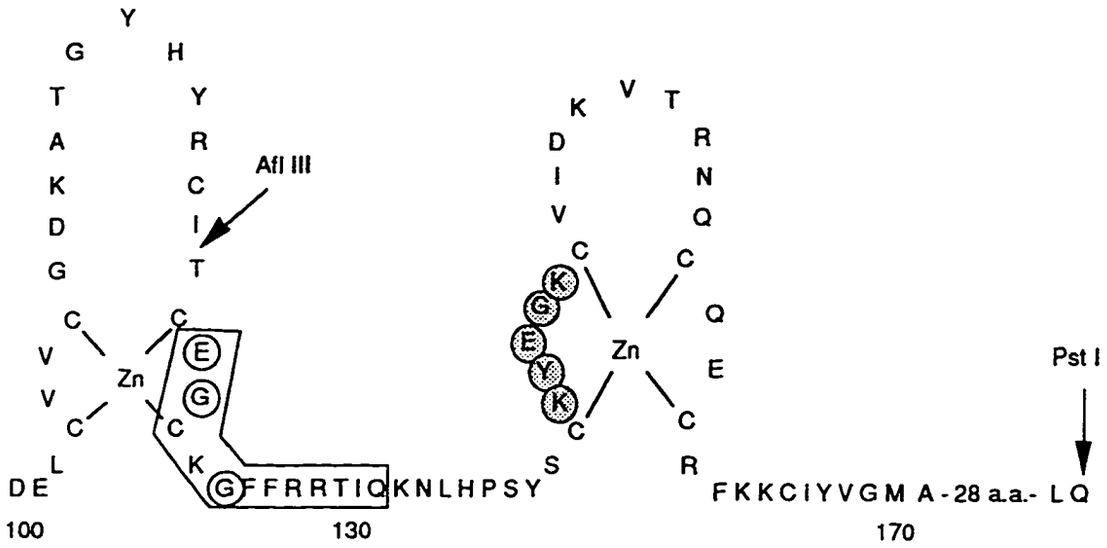


Figure 3.5: Schematic illustration of the PCR-based mutagenesis protocol used to introduce alanine substitutions into the recognition helix of TRβ. The steps are described in the text. Note that the 5' end of the universal mutagenic primer (UMP) is non-complementary in the first step, and provides the basis for specific amplification of the mutant sequence, versus the wild-type, in the third step.
(Nelson and Long, 1989)

(A)


Y
G H
T Y
A R
K C
D I
G T
Afl III
C C
V E
V Zn G
C C
L K
D E G F F R R T I Q K N L H P S Y S
100
130
V T
K R
D N
I Q
V C C
K Q
G
E E
Y Zn
K E
C C
R
Pst I
F K K C I Y V G M A - 28 a.a.- L Q
170

(B)

92
IleProSerTyrLeuAspLysAspGluLeuCysValValCysGlyAspLysAlaThrGly
ATCCCCAGTTACTTAGACAAGGACGAGCTCTGTGTAGTGTGTGGTGACAAAGCCACCGGG
βL

112
TyrHisTyrArgCysIleThrCysGluGlyCysLysGlyPhePheArgArgThrIleGln
TATCACTACCGCTGTATCACGTGTGAAGGCTGCAAGGGTTTCTTTAGAAGAACCATTCAG

132
LysAsnLeuHisProSerTyrSerCysLysTyrGluGlyLysCysValIleAspLysVal
AAAAATCTCCATCCATCCTATTCCTGTAAATATGAAGGAAAATGTGTCATAGACAAAGTC

152
ThrArgAsnGlnCysGlnGluCysArgPheLysLysCysIleTyrValGlyMetAlaThr
ACGCGAAATCAGTGCCAGGAATGTCGCTTTAAGAAATGCATCTATGTTGGCATGGCAACA

172
AspLeuValLeuAspAspSerLysArgLeuAlaLysArgLysLeuIleGluGluAsnArg
GATTTGGTGCTGGATGACAGCAAGAGGCTGGCCAAGAGGAAGCTGATAGAGGAGAACCGG

192
GluLysArgArgArgGluGluLeuGlnLysSerIleGly
GAGAAAAGACGGCGGGAAGAGCTGCAGAAGTCCATCGGG
βR

(C)

βL: ATCCCCGAATTCTTAGACAAGGACGAG
Eco RI

βR: CCCGATGGACTTCTGCAGCTCTTC
Pst I

(D)

E120A	ACGTGTGCAGGCTGCAAGGG
G121A	TGTGAAGCCTGCAAGGGTTT
C122A	GAAGGCGCCAAGGGTTTCTT
K123A	GGCTGCGCGGGTTTCTTTAG
K123M	GGCTGCATGGGTTTCTTTAG
G124A	TGCAAGGCTTTCTTTAGAAG
F125A	AAGGGTGCCTTTAGAAGAAC
F126A	GGTTTCGCTAGAAGAACCAT
R127A	TTCTTTGCAAGAACCATTCA
R128A	TTTAGAGCAACCATTCAGAA
T129A	AGAAGAGCCATTCAGAAAAA
I130A	AGAACCGCTCAGAAAAATCT
Q131A	ACCATTGCGAAAAATCTCCA
131/135-GR	TCCTATTCCTGTGCAGGTCGAAACGACTGTGTCATAGA
131/135-ER	TCCTATTCCTGTCCAGCTACAAACCAATGTGTCATAGA

RUMP GTGATCAGTACACGTGCCCAGGAAACAGCTATGAC

(E)

UMP GTGATCAGTACACGTGCC
FUP GTAAAACGACGGCCAGT

Figure 3.6: Some components of the TRβ recognition α-helix mutagenesis system. (A) The sequence of the TRβ DBD indicating the residues encoded by the *Afl* III/*Pst* I fragment which was used as a template for site directed mutagenesis. The residues that were individually substituted with alanine are boxed, starting with E_{120}. The P-box and D-box are indicated by circles. (B) The sequence of the cDNA for human TRβ, and the translated product, in the region used as a DBD cassette. Numbers refer to amino acid position. The sequences to which primers βL and βR anneal are underlined in the nucleic acid sequence. The amino acids which were substituted with alanine are underlined. (C) The primers, βL and βR, that were used to amplify the DBD cassette from the cDNA for human TRβ. Note that βL is not perfectly complementary, but includes changes which introduce an *Eco*R I site. (D) Primers used in the first step of mutagenesis. Above, the mutagenic oligos which introduce sequence changes that result in the substitution of an alanine codon for the wild-type codon. Mutagenic nucleotides are underlined. Below, RUMP. The part that is complementary to pUC19 sequence is single underlined, and the part that is complementary to UMP is double underlined. The former part is identical in sequence to the commonly used reverse universal primer (RUP). (E) Primers used in the final step of mutagenesis (Figure 3.5).

pUC19/TRβDBD as the template. Samples were electrophoresed on a 3.5% Nusieve® agarose gel, and purified with a Mermaid kit®. In the second step, PCR was performed with roughly 4 ng of the purified product from the first step as the primer. The template was, again, pUC19/TRβDBD. The reaction was carried out for 5 rounds, with the following conditions: 3 minutes at 95 °C, 1.5 minutes at 50 °C, and 1.5 minutes at 72 °C. Subsequently, universal mutagenic primer (UMP) and forward universal primer (FUP) (Figure 3.6E) were added to 0.2 μM, and 26 cycles were carried out under the standard temperature-cycling conditions. The product of the mutagenesis steps was phenol:chloroform extracted, ethanol precipitated, resuspended in water and digested with *Eco*R I and *Hin*D III. It was then cloned into pUC19 that had also been digested with *Eco*R I and *Hin*D III following standard procedures. The mutant K123A was never isolated, so an alternative mutagenic primer was made which introduced a codon for methionine in place of the wild-type lysine codon (Figure 3.6D).

In order to facilitate the shuttling of the mutant TRβ genes into vectors for expression of the protein *in vitro* and *in vivo* an intermediate vector with appropriate restriction sites had to be constructed. pEA101 was therefore modified in a number of ways to create the vector, pEAK (Figure 3.7). First, one of two *Eco*R I sites was knocked out by partially digesting pEA101 with *Eco*R I, filling in the sticky ends, and religating the vector. This vector is referred to as pMOD. An *Afl* III site was knocked out similarly. Second, a unique *Kpn* I site was introduced in the remaining *Eco*R I site using a linker oligonucleotide of the sequence AATTCGGTACCG. The oligonucleotide was self-annealed, ligated, digested with *Eco* RI, and ligated into pEA101 that had the other modifications listed above and that had been digested with *Eco*R I. Thus, as well as the unique *Kpn* I site, another *Eco*R I site was introduced into the vector. Finally, a *Pst* I site within the polylinker of the vector was removed by digestion with *Acc* I and *Hin*D III, filling in and performing a blunt-end ligation.

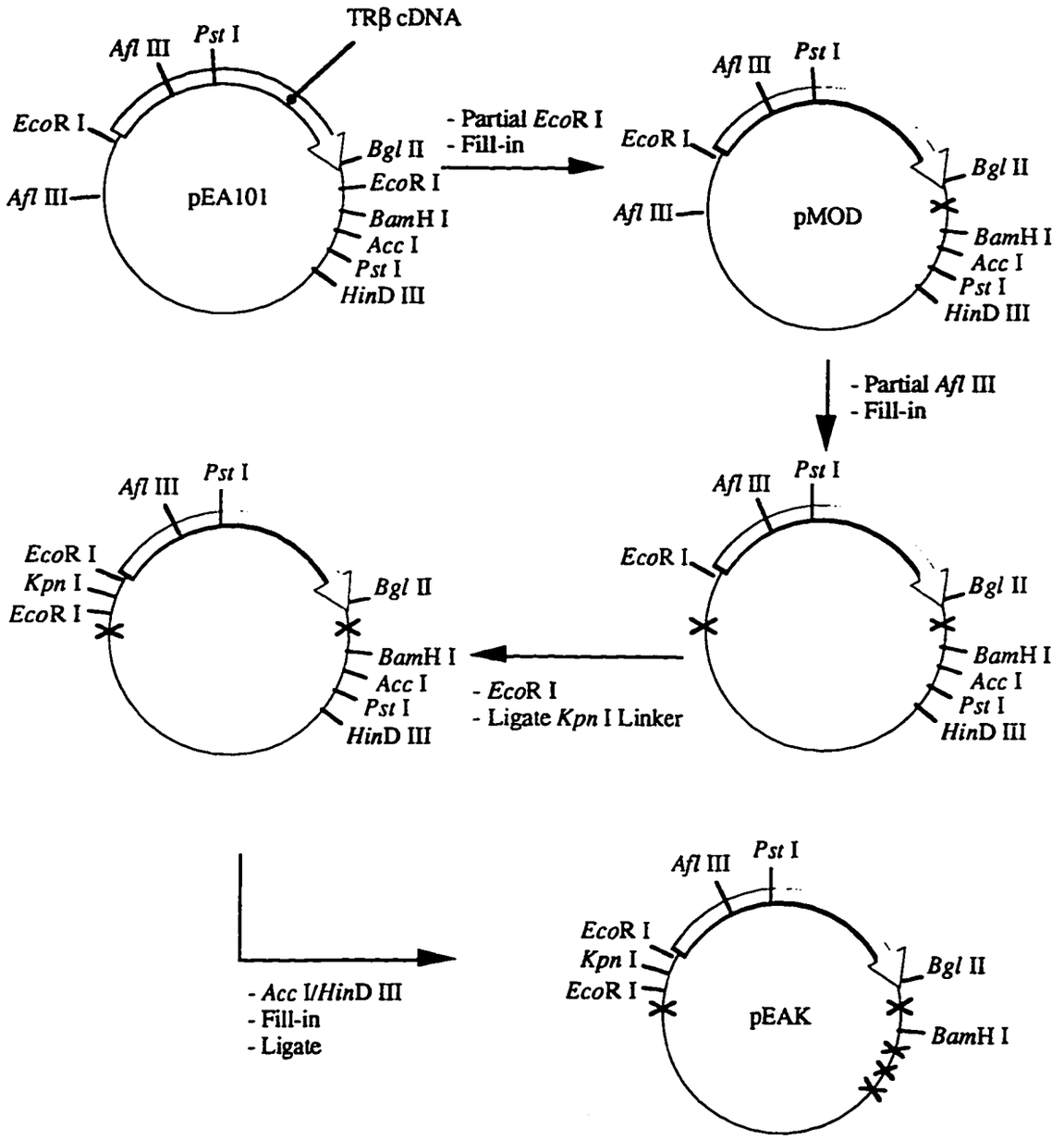


Figure 3.7: Construction of the vectors pMOD and pEAK from pEA101. A schematic illustration of the steps involved in modifying pEA101 so that the mutant TRβ genes could be shuttled into pMOD, for *in vitro* translation, and pRS for expression in mammalian cells.

The mutant DBD cassettes were transferred from pUC19 into pPEAK as *Afl* III/*Pst* I fragments. The DBD cassette fragment and pEAK digested with *Afl* III/*Pst* I were both purified from an agarose gel and ligated to yield pEAK-120, pEAK-121, etc. For transferring the mutant TRβ genes to the *in vitro* expression vector, the pEAK-mutant vectors were digested with *Eco*R I/*Bam*H I and the fragment ligated into pMOD (Figure 3.7b), yielding pMODTRβ, pMOD-120, pMOD-121, etc. To transfer the genes into the *in vivo* expression vector, the pEAK-mutant vectors were digested *Kpn* I/*Bgl* II, and the fragment ligated into the vector pRS, to yield pRShTRβ, pRSh120, pRSh121, etc.

3.2.2 Construction of pETΔNTRβ

In order to produce a form of TRβ lacking the N-terminus, the desired portion of the cDNA was extracted from pEA101 by PCR. The two primers synthesized for this purpose were: β-PET, which introduces an *Nde* I site in the sequence overlapping β-L (Figure 3.6C) and has the sequence AGTTCCGCACATATGGACGAGCTCTGTGTA; and, β-Down which anneals to the end of the cDNA, introduces a *Bam*H I site, and has the sequence GGAATCCAGGGATCCTAATCCTCGAACAC. The PCR product was digested with *Nde* I and *Bam*H I, purified from an agarose gel, and cloned into pUC19. After DNA sequencing to confirm the insert, it was shuttled into pET-3A between the *Nde* I and *Bam*H I sites. Transcription of the truncated cDNA sequence was therefore under the control of a T7 promoter which was used for *in vitro* transcription. The predicted protein product consists of residues 98-456 of human TRβ, and is referred to as TRβΔN.

3.2.3 Construction of Reporter Plasmids Containing TRE Sequences

The CAT reporter plasmid, ΔMTV-CAT, has a unique *Hin*D III site upstream of a MTV promoter which in turn directs transcription of the CAT gene. Oligonucleotide pairs with *Hin*D III sticky ends were cloned directly into this site. Oligonucleotides with *Pst* I sticky ends were ligated through the use of a *Pst* I/*Hin*D III adaptor oligonucleotide with the sequence GAAGCTTCTGCA.

3.2.4 Construction of Plasmids Bearing TRE Sequences for use in DNA Binding Assays

Oligonucleotides bearing TRE sequences were cloned into pUC for propagation and ease of single-end labeling (used for experiments in chapter 4). General cloning techniques and oligonucleotide sequences are given in Chapter 2. Unless it is explicitly stated in a figure legend, DNA probes for DNA binding assays were excised from these plasmids by digestion with *Eco*R I/*Hin*D III.

3.2.5 *In Vitro* Translation of Receptor Proteins

The expression of receptor proteins from the pMOD vectors in rabbit reticulocyte lysate (RRL) is described in Section 2.2.3.

3.2.6 Preparation of the TRE_{LYS} Affinity Column

The preparation of a TRE_{LYS} affinity column was performed as described by Kadonaga and Tjian (1986). 10 μg each of the two complementary TRE_{LYS} oligos (Table 2.2) were annealed, phosphorylated in the presence of 800 pmole (8 μCi) [γ-^{32}P]ATP and 10 μmole of unlabelled ATP, and then ligated. Hydrated, cyanogen bromide activated sepharose 4B (Pharmacia) (0.43 g)was activated in 1 mM HCl for 1 hour at room temperature, and then washed with water, and then 10 mM potassium phosphate pH 8.0 at 4 °C. The sepharose beads and oligonucleotides were incubated together for 16 hours at room temperature in the buffer solution. The beads were washed with water and ethanolamine pH 8.0, and then soaked in ethanolamine for 4 hours. The beads were finally washed with 10 ml each of 10 mM potassium phosphate pH 8.0, 1 M potassium phosphate, 1 M KCl, water, and TE/0.3 M NaCl. When running TRβ over the column, it was equilibrated with Buffer H (see Section 2.2.3.1), and 49 μl of ^{35}S-methionine containing *in vitro* translated TRβ in 300 μl of Buffer H was loaded. Washes and elutions were carried out using Buffer H with KCl concentrations increasing from 100 mM to 1 M by steps of 100 mM.

3.2.7 T_3 Binding Assay

The procedure used for determining ^{125}I-T_3 binding by TR was a filter-binding method described in Inoue *et al* (1983). From 5 to 45 fmol of ^{125}I-T_3 was incubated on ice for 18 hours with 10 µl of RRL, either unprogrammed or containing TRβ, in a total volume of 200 µl of buffer B which contains: 50 mM NaCl, 10 % glycerol, 2 mM EDTA, 5 mM β-mercaptoethanol, and 20 mM Tris-HCl, pH7.9. 1 ml of buffer B was added to each sample before it was passed through a pre-soaked nitrocellulose filter under suction. The filter was washed three times with 5 ml of buffer B, and the retained radioactivity quantified with a LKB Wallac 1282 Compugamma gamma counter.

3.2.8 Maintenance of Mammalian Cells in Culture

COS cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco) containing 10 % newborn calf serum (Gibco). HepG2 cells were maintained in MEM containing 10 % fetal calf serum (Gibco).

3.2.9 Transfection of Mammalian Cells

The transfection procedure was based on the calcium phosphate-mediated technique developed by Chen and Okayama (1988). Cells were passaged into 60 mm culture dishes and grown to roughly 20 % confluency before transfection was initiated. 1 µg of expression plasmid, plus 5 µg of reporter plasmid when CAT assays were to be performed, was mixed with 0.5 ml of 0.25 M $CaCl_2$ and 0.5 ml of 2 x BES-buffered saline (50 mM BES, 280 mM NaCl, 1.5 mM Na_2HPO_4). The mixture was incubated for 15 minutes at room temperature and then added drop-wise to each culture dish. The cultures were grown for 20 hours at 37 °C, 3 % CO_2. Then cells were washed with media, and the media replaced, containing 10^{-7} M T_3 where required for CAT assays. After another 20 hour incubation at 37 °C, 5 % CO_2 the cells were rinsed with PBS (Phosphate-buffered saline: 9.25 mM NaPO4, 0.137M NaCl, pH 7.4) and either a CAT assay was performed or nuclear extracts were prepared.

3.2.10 Preparation of Nuclear Extracts from Mammalian Cells

The protocol used for preparing nuclear extracts from mammalian cells grown in culture was published by Schreiber *et al.* (1989). Cells were grown almost to confluency on 60 mm plastic dishes, collected by scraping in 1.5 ml PBS, and pelleted by a 1 minute spin in a centrifuge at 10 °C, 3,000 x g. 400 µl of Buffer A (10 mM HEPES, pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT; 0.5 mM PMSF) was added, and the cells were incubated on ice for 15 minutes. 25 µl of 10 % nonidet NP-40 was added and the cells were immediately vortexed, centrifuged at maximum speed for 30 seconds at 10 °C and the supernatant discarded. The nuclear pellet was resuspended in 50 µl ice-cold Buffer C (20 mM HEPES, pH 7.9; 0.4 M NaCl; 1 mM EDTA; 1 mM EGTA; 1 mM DTT; 1 mM PMSF), vortexed at 10 °C for 15 minutes and then centrifuged for 5 minutes. The supernatant was frozen in aliquots for later use.

3.2.11 CAT Assays

Transfected cells were assayed for CAT activity using a protocol based on that developed by Sleigh (1986). Transfected cells were harvested and washed as was done for nuclear extract preparation. They were then resuspended in 200 µl 0.25 M Tris-HCl, pH 7.8 and subjected to 3 rounds of freezing in dry ice and thawing in a 37 °C waterbath. The lysates were heated to 65 °C for 10 minutes to inactivate deacetylase activities that might be present, and then were centrifuged for 15 minutes at 10 °C, and the supernatant collected. The OD_{280} of each sample was determined, and the quantity of sample used for the CAT assay were equalized on this basis. The CAT assay was carried out in a total of 200 µl made up with 0.25 M Tris-HCl, pH 7.5. Aside from cellular extract, each sample contained 20 µl 8 mM chloramphenicol and 20 µl ^{14}C-acetyl CoA mix (0.2 M Tris-HCl, pH 7.8, 1 mCi/L ^{14}C acetyl CoA, 0.09 mM acetyl CoA). After incubation of the samples at 37 °C for 2 hours, 200 µl of ice-cold ethyl acetate was added, the samples vortexed and centrifuged at 10 °C for 5 minutes, and 150 µl of the top layer carefully removed to a scintillation vial for counting.

3.3 RESULTS

3.3.1 Characterization of the DNA Binding Properties of TRβ

In order to analyze the binding of the TRβ mutants that were generated it was necessary to develop techniques for receptor expression, and for analysis of DNA binding activity. At the time, there was no clear consensus in the field with respect to which methods of expression were most productive or tended to produce TR in the most native form. Methods for obtaining receptor that had been used with success included: purification from liver cells (Apriletti *et al.*, 1988); over-expression in bacteria or yeast (Ribeiro *et al.*, 1992); *in vitro* translation (Glass *et al.*, 1989); and, nuclear extracts prepared from mammalian cells transfected with receptor expression plasmid, or from rat liver cells (Glass *et al.*, 1988). The latter two of these options suited the purposes of this work the best. *In vitro* translation, while not producing large quantities of receptor, or highly purified receptor, is appropriate for DNA binding studies for several reasons. It is a quick and easy technique that is easily applied to a number of different RNA transcripts simultaneously, unlike methods which require extensive purification steps. Also, it is relatively depleted of nuclear proteins, such as receptors. On the other hand, the increasing awareness that TR does interact in a physiologically relevant way with other nuclear proteins was incentive to use nuclear extracts from transfected cells as well, to ascertain whether there are gross differences between the binding patterns observed with *in vitro* translated protein and those of receptor isolated with a complement of other nuclear proteins in nuclear extracts.

At the same time, the EMSA appeared to be an optimal technique for examining DNA binding characteristics. EMSA has the unique advantage of visualizing distinct complexes of bound DNA that may form, as long as they do not fortuitously have identical migration rates on a native polyacrylamide gel. Other methods for assaying binding affinity of proteins that do not visualize bound complexes generate values for bulk bound and free DNA regardless of the number or types of proteins bound. Thus, in the case of TR, where there is an interest in determining which species of receptor complex is bound to a TRE,

such techniques are deficient. Thus, it was undertaken to develop an EMSA protocol that yielded highly resolved bands representing the high affinity complexes formed between TR and TREs.

3.3.1.1 Receptor Expression

3.3.1.1.1 *In Vitro* Translation of RNAs Encoding Receptor Proteins

The vectors pEA101, pBS-RXRα, and pETΔTRβ were linearized and used as templates for *in vitro* transcription of mRNA encoding human TRβ, human RXRα and a truncated form of TRβ lacking the N-terminus TRβΔN, respectively. Transcription with T7 or T3 RNA polymerase, as appropriate, yielded mRNA products which ran at the equivalent of about 900 base-pairs versus a dsDNA standard (Figure 3.8). *In vitro* translation from these templates in rabbit reticulocyte lysate (RRL) routinely gave from 2-4 nM receptor protein, as calculated from the amount of TCA precipitable cpm obtained from parallel translation reactions containing ^{35}S-methionine instead of unlabelled methionine. However, due the presence of smaller translation products in the RRL reaction, this must be considered a maximum value for the actual concentration of full-length receptor. Consequently, protein concentrations are not given for experiments with *in vitro* translated protein. However, when necessary the TCA precipitable counts for the ^{35}S-methionine translation reaction were used to equalize the receptor quantities in different binding reactions The TCA precipitable counts were typically around 100,000 cpm/μl. The reaction including ^{35}S was also useful for confirming that translation products were of the expected size (Figure 3.9). In fact, two major polypeptides were produced by translation of the TRβ mRNA, one of about 52 kD and one of 48 kD. The predicted molecular mass of the translation product of the pEA101 RNA is 52 kD (Weinberger *et al.*, 1986). A third strong band at 32 kD is also a product of translation of the TRβ mRNA. These three major bands are consistent in molecular weight with the sizes that would be predicted for polypeptides initiated from in-frame methionine codons downstream of the true initiation ATG. This phenomenon has been described for TRα expression, both *in vitro* and *in vivo* (Bigler and Eisenman, 1988). Translation of the other mRNAs produced major bands of

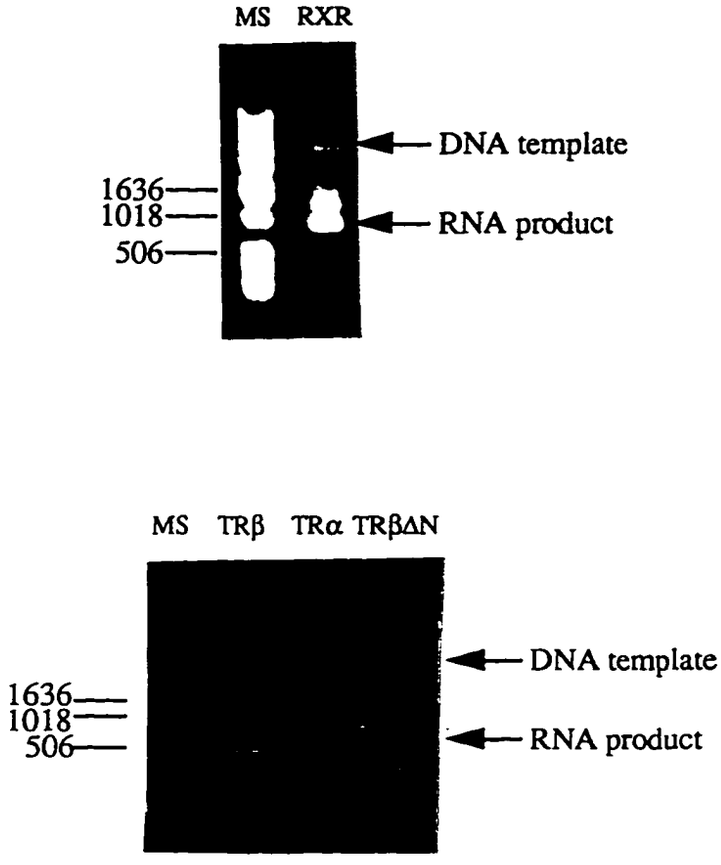


Figure 3.8: The products of *in vitro* transcription of linearized pBS-RXRα (RXRα), pEA101 (TRβ), pCEA1 (TRα), and pETΔNTRβ (TRβΔN). Roughly 1 μg of each transcription product was run on a 1% agarose gel. Gels were run short to reduce any RNA degradation that might occur during electrophoresis. The DNA template and RNA product are indicated. Numbers indicate the size, in base-pairs, of the marker bands flanking the RNA product in the Molecular Size standard lane (MS).

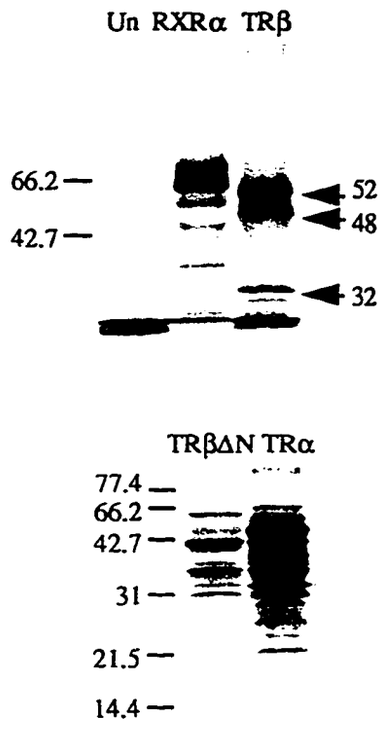


Figure 3.9: Autoradiograph of a 10% SDS-PAGE gel of ^{35}S-labeled receptor proteins. 1 µl of *in vitro* translation reaction in rabbit reticulocyte lysate was loaded in each lane. Un is an unprogrammed translation reaction in which water was added instead of RNA. The migration of non-radioactive molecular weight standards are indicated. The 32 kD band associated with translation of TRβ is indicated by the arrow.

the expected sizes, as well as numerous smaller polypeptides. The major bands for each translation reaction are: TRα, 42 kD, RXRα, 54 kD; and, ΔNTRβ, 40 kD. These values for molecular weight are consistent with those given in, or predicted from, the literature (Sap *et al.*, 1986; Mangelsdorf *et al.*, 1990; Weinberger *et al.*, 1986).

Since the *in vitro* translated TRβ was used for the majority of *in vitro* DNA binding studies performed in this work, it was undertaken to determine the involvement of the three products of translation bands in DNA binding. The codon predicted to be used for initiation of the 32 kD product lies beyond the DNA binding domain (Figure 3.10), however the ligand binding domain and the associated dimerization interface are included. Therefore, it is possible that the 32 kD fragment could dimerize with either of the two longer receptor forms. In order to determine which species of TR were active in DNA-binding, a DNA affinity column was used. Oligonucleotides containing the TRE_{LYS} sequence were ligated together and linked to sepharose beads. TRβ was translated in RRL in the presence of ^{35}S-methionine, and loaded onto the column. After washing with low salt buffer, a step-wise gradient was run and fractions collected. Samples of these were run out on a SDS-PAGE gel, and the radioactive protein visualized by autoradiography. This analysis revealed that only the two high molecular weight bands were bound to the column under low salt conditions and eluted as the salt concentration was increased to 300-400 mM (Figure 3.11). The 32 kD band, and other ^{35}S-containing material flowed through the column on loading and washing. This result indicates that the 32 kD fragment does not form DNA binding heterodimers with TRβ, at least not with high binding affinity for TRE_{LYS}. However, a possibility not addressed by this experiment is that the 32 kD polypeptide forms heterodimers with either of the two longer polypeptides in solution, and that these heterodimers are unable to bind to DNA. This would also account for the presence of some 48 and 52 kD receptor forms in the loading flow through and washes. Such a phenomenon would be similar to the "dominant-negative" model that has been proposed (Forman and Samuels, 1990) in which mutant receptor forms with disrupted

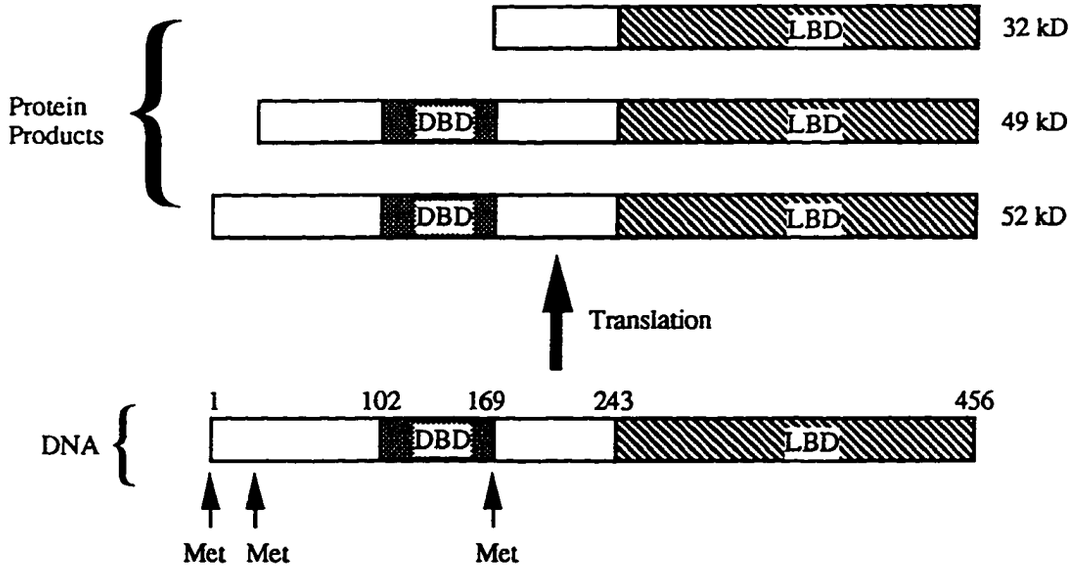


Figure 3.10: The polypeptide products that would arise from translation using the correct initation codon, and the next two in-frame methionine codons. The open reading frame of the TRβ cDNA is at the bottom with the first 3 methionine codons indicated by arrows. The three products of translation resulting from initiation at these codons are shown above. Proposed to explain the appearance of the major 52, 48 and 32 kD polypeptides produced by *in vitro* translation of the TRβ cDNA.

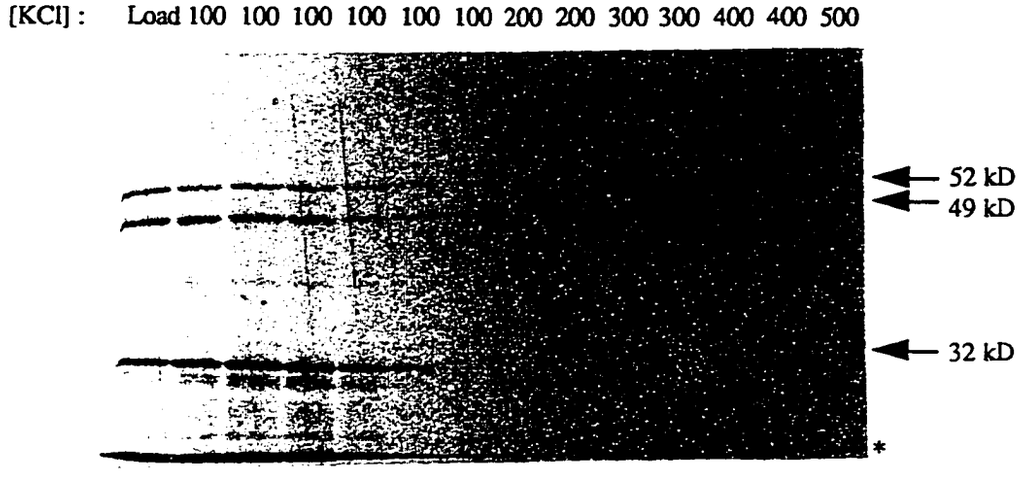


Figure 3.11: Elution profile of ^{35}S-methionine-labeled TRβ from a TRE_{LYS} affinity column. The three major polypeptide products of *in vitro* translation are indicated, as well as a band representing other ^{35}S-containing species (*), which might include free ^{35}S-methionine and charged tRNAs. The concentration of KCl in the eluting buffer is indicated below.

trans-activational capacities, or DNA or ligand binding activities, form inactive heterodimers with wild-type TRs. While the ability of the 32 kD fragment to act as a dominant negative inhibitor of binding was not examined further, other TRβ receptor forms deficient in DNA binding were studied in this regard (see section 3.3.1.3.1).

3.3.1.1.2 Production of Nuclear Extracts of Cells Transfected With TRβ Expression Plasmid

The use of nuclear extracts as a source of receptor was applied to TRβ and mutants thereof. COS and CV1 cell lines were used, as they are deficient in endogenous T_3-dependent *trans*-activation activity (Larsen *et al.*, 1986). Cells were transfected with the expression plasmid pRShTRβ or one of the variants encoding a mutant receptor, and nuclear extracts were prepared after 24 hours . The yield of receptor protein in the nuclear extract was not routinely quantified, as this protein was mainly used for qualitative studies. The presence of the receptor in nuclear extracts was most clearly demonstrated by EMSA (See below).

3.3.1.2 General Binding Considerations for TRβ

3.3.1.2.1 Source of Receptor

EMSA was used to detect the DNA binding of activities present in nuclear extracts prepared from cells transfected with pRShTRβ (Figure 3.12). Two DNA probes harbouring different HREs were used to confirm the specific DNA binding activity of the TR: TRE_{rGH}, containing a TRE from the promoter region of the rat growth hormone gene (Figure 1.6); and, GRE_{MTV}, containing an IR3 of the GR half-site sequence, AGAACA. The binding of GRE_{MTV} by proteins present in the nuclear extracts of COS cells was similar whether TRβ was present or not. On the other hand, two distinct complexes are apparent when the nuclear extract containing TRβ, but not the untransfected nuclear extract, is incubated with TRE_{rGH}. There is also a band common to both lanes in which TRE_{rGH} was the probe (lanes 3 and 5, Figure 3.12), that is not present when GRE_{MTV} is used as the probe. Whether this band represents the binding of endogenous thyroid hormone

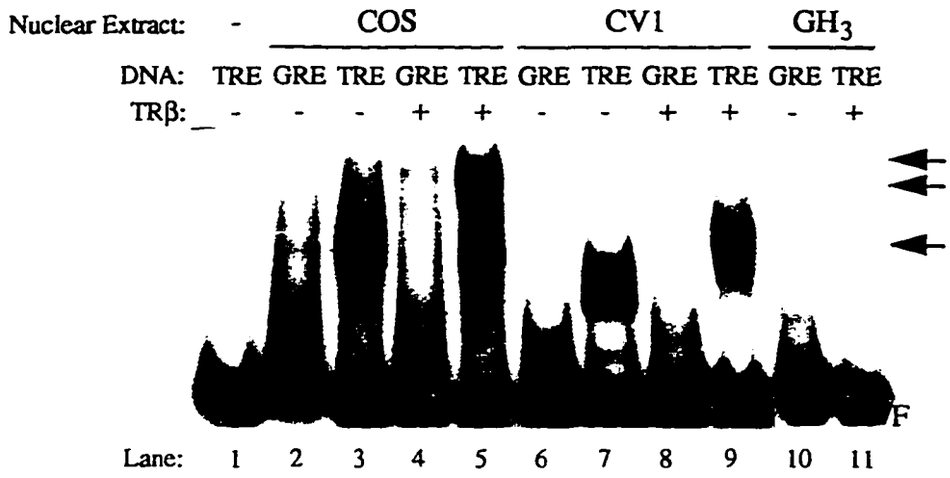


Figure 3.12: EMSA conducted with nuclear extracts and either GRE_{MTV} or TRE_{rGH}. Nuclear extracts of either COS, CV1 or GH_3 cells, either untransfected (-) or transfected (+) with plasmid expressing the TRβ cDNA were assayed for binding of radiolabeled GRE_{MTV} or TRE_{rGH}. Arrows indicate bound DNA complexes that were unique to nuclear extracts from transfected cells. The F indicates unbound, or free, DNA. EMSAs were performed under Set I standard conditions. The leftmost lane is TRE_{rGH} in the absence of nuclear extract. Nuclear extracts were prepared by Colleen Nelson.

receptor was not determined; however, subsequent *trans*-activation studies confirmed that there is little TR activity in COS cells (Section 3.3.2.2.1). It is quite possible that the TRE_{rGH} probe fortuitously harbours a sequence recognized by a distinct nuclear factor(s). Nuclear extracts of CV_1 cells also contain an endogenous factor(s) which binds TRE_{rGH} but not GRE_{MTV} (*cf* lanes 6 and 7). Intriguingly, the binding of this factor(s) is only observed in the absence of TRβ (*cf* lanes 7 and 9). Nuclear extract containing TRβ produces what appears to be a single smeary band with distinct mobility when incubated with labeled TRE_{rGH}. Finally, nuclear extract of GH_3 cells, which are not TR-deficient, was assayed with GRE_{MTV} and TRE_{rGH}. No significant bands resulted from incubation with GRE_{MTV}. Incubation with TRE_{rGH} produces two bands with mobilities similar to those of the bands resulting from the binding of TRβ in nuclear extracts of transfected COS cells.

The presence of endogenous factors in nuclear extracts of COS and CV1 cells that bind to TRE_{rGH} limits the utility of nuclear extracts as a source of TRβ for DNA binding studies. Furthermore, variables such as transfection efficiency, and yield of nuclear extraction combine to create batch-to-batch variation in the binding activities of nuclear extracts. Figure 3.13 compares the binding of TRE_{rGH} by two separate batches of nuclear extracts from COS cells transfected with pRShTRβ. The batch that was used in Figure 3.12 again produces a band with higher mobility than the two putatively TRβ-containing bands, but the second batch not only lacks the endogenous band, but has significantly greater TRβ DNA binding activity. The higher level of TRβ present in the second batch was confirmed by a second means. Nuclear extracts were incubated with ^{125}I-T_3 before use in an EMSA with unlabeled DNA probes (Figure 3.13); thus, bands result from free ^{125}I-T_3, and ^{125}I-T_3 that is bound by factors present in the nuclear extract. The additional presence of DNA bearing a TRE will increase the mobility of the ^{125}I-T_3 band that represents ligand occupied TRβ. All lanes exhibit an intense, smeared band with low mobility, which is likely to be unbound ^{125}I-T_3, since it is present in the absence of nuclear

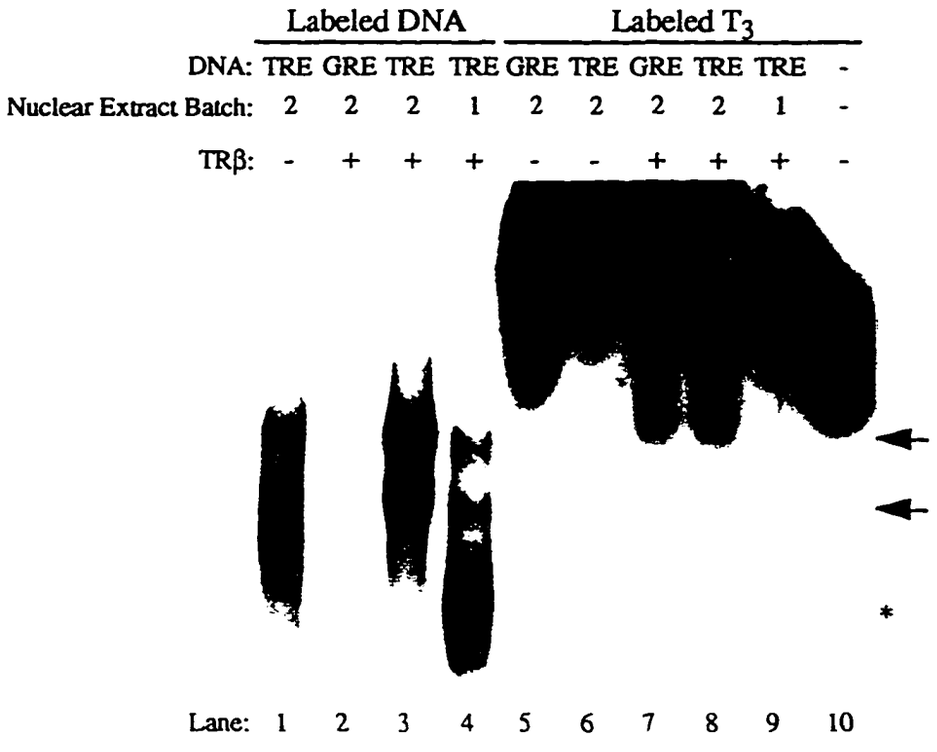


Figure 3.13: EMSA indicating batch-to-batch DNA-binding variability of nuclear extracts containing exogenous TRβ. Two batches of nuclear extract from cells transfected with pRShTRβ exhibit distinct levels of endogenous factor binding to TRE_{rGH} (indicated by the asterisk). Also, batch 2 exhibits much greater binding by the transfected receptor. Bands that are unique to nuclear extracts containing TRβ are indicated with arrows. EMSA was also performed with nuclear extracts in the presence of ^{125}I-T_3. DNA was unlabeled in these lanes. The bands that are consistent with the binding of ^{125}I-T_3 by transfected TRβ are indicated with an arrow. The free DNA was run off the bottom of the gel. EMSA was performed under Set I standard conditions. Batch 1 nuclear extract is the same as in Figure 3.12. Batch 2 was prepared by me from cells transfected by Colleen Nelson.

extract or DNA (lane 10). Note that only those lanes in which the second batch of nuclear extract was used (lanes 7 and 8) exhibit a novel band. These two lanes contain a band with higher mobility than the free ^{125}I-T_3 that is not detectable in untransfected nuclear extracts, or in the first batch of nuclear extracts containing exogenous TRβ. Thus, TRβ seems to be present at a much greater concentration in the second batch than the first batch of nuclear extract. A band is observed in the presence of both GRE_{MTV} and TRE_{rGH} implying that it represents TRβ not bound to DNA.

Two other naturally occurring TREs were also used in EMSA with nuclear extracts from transfected cells. The probe called $TRE_{TSH\beta}$ contains a sequence found in the first exon of the human thyrotropin β-subunit gene that mediates T_3-dependent inhibition of gene expression (Wondisford *et al.*, 1989). In other words, in the absence of ligand $TRE_{TSH\beta}$ enhances gene expression, but this enhancement is lost in the presence of T_3. $TRE_{TSH\beta}$ most closely resembles an IR0 sequence (Figure 1.6). The other sequence was TRE_{LYS}, an EvR6-type element from the chicken lysozyme gene that is a transcriptional silencer in the absence of T_3, and an activator in the presence of T_3 (Baniahmad *et al.*, 1990). Both these elements were bound by TRβ in nuclear extract from transfected COS cells (Figure 3.14). $TRE_{TSH\beta}$ and TRE_{rGH} form minor complexes with factors intrinsic to the untransfected COS cell nuclear extract that are similar in mobility to those in the lanes containing the transfected TRβ (*cf* lanes 1 and 2; and, 6 and 5). However, an intrinsic factor in the COS nuclear extract also bound to TRE_{LYS} with high affinity (lane 4). When nuclear extract containing TRβ is used, the band corresponding to the binding of the intrinsic factor is no longer observed although, overall, a larger proportion of TRE_{LYS} is bound (lane 3).

The concern over intrinsic factors in nuclear extract binding to TRE probes in the EMSA was eliminated by using TR translated *in vitro* in a rabbit reticulocyte lysate (RRL) system. Unprogrammed RRL (a translation reaction carried out in the absence of added RNA) produces only very faint, if any, bands when incubated with labeled TRE_{LYS} (Figure 3.15,

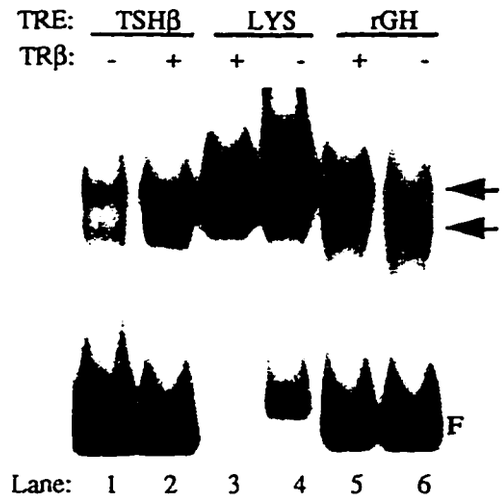


Figure 3.14: EMSA of the binding of TRβ in transfected nuclear extracts to three natural TREs. $TRE_{TSH\beta}$, TRE_{LYS}, and TRE_{rGH} were incubated with nuclear extracts of COS cells that had been transfected with pRShTRβ (+), or pGEM-3 (-). Bound DNA bands are indicated by arrows, free DNA is indicated by an F. Nuclear extracts were prepared by me from cells transfected by Colleen Nelson. EMSA was performed under Set I standard conditions.

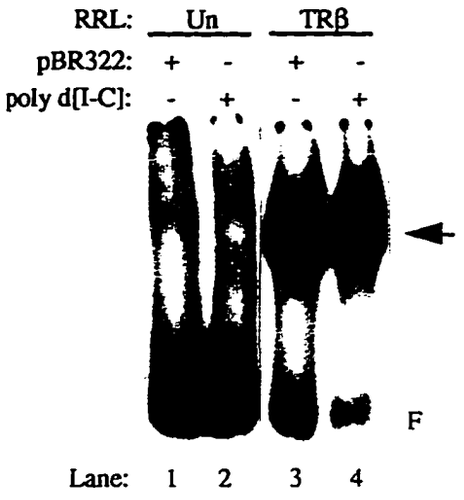


Figure 3.15: EMSA of the binding of *in vitro* translated TRβ to TRE_{LYS} in the presence of different non-specific competitor DNAs. RRL, unprogrammed (un) or containing TRβ, was incubated with either 0.5 μg poly d[I-C] or 0.45 μg pBR322 before addition of the labeled TRE_{LYS}. Bound DNA is indicated by the arrow, free by the F. EMSA was performed under Set I standard conditions.

lanes 1 and 2). On the other hand, *in vitro* translated TRβ binds to TRE_{LYS} with high affinity (lanes 3 and 4). The experiment shown in Figure 3.15 was also undertaken to find out which DNA, poly d[I-C] or pBR322, was a better choice for use as a non-specific component in the preincubation step of the EMSA. The two nucleic acids seem to allow a similar level of background "haze" when unprogrammed RRL is incubated with labeled TRE_{LYS} (Lanes 1 and 2); however, a greater proportion of the TRE_{LYS} probe is bound by TRβ when poly d[I-C] (lane 4) is used than when pBR322 is used (lane 3). This may be due to fortuitous TR binding sites within pBR322. In any case, poly d[I-C] appeared to be the more desirable non-specific competitor.

3.3.1.2.2 Salt conditions

Other tests were performed to optimize the EMSA conditions for observing TR DNA binding activity. Some DNA binding proteins require the presence of magnesium ions for binding. An EMSA was performed with TRβ binding to TRE_{rGH} in the presence of a range of magnesium ion concentrations (Figure 3.16). Binding of the two TRβ complexes with slowest mobilities is quite sensitive to magnesium, exhibiting a decline in the degree of binding in the presence of $MgCl_2$ at a concentration as low as 1 mM. Intriguingly, the decrease in amount of complex in the upper two bands is concomitant with an increase in a band with higher mobility. Whether this band represents a novel complex containing TRβ, or simply the binding of another factor which requires magnesium was not determined. The sensitivity of binding to the presence of monovalent cation was also tested. The influence of a range of KCl concentrations on TRβ binding to TRE_{IR0} and TRE_{LYS} was determined by EMSA (Figure 3.17). Binding of TRβ to these elements is observed in the presence of from 50 to 200 mM KCl, although at 200 mM the amount of DNA bound by TRβ is significantly reduced compared with that at 50 mM. The degree of binding observed in the presence of 50 or 100 mM KCl is similar. Since, higher salt is considered to create more "stringent" conditions for DNA binding proteins, in the sense of reducing

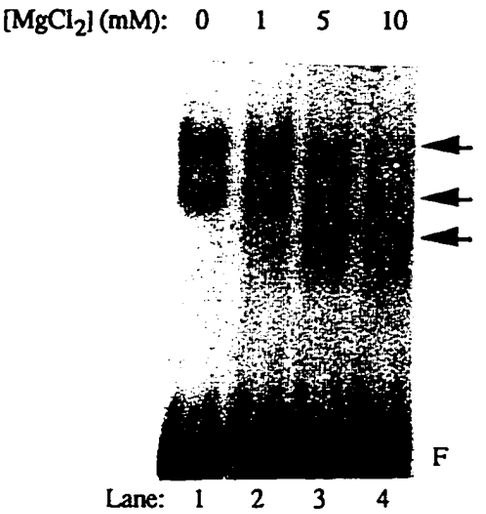
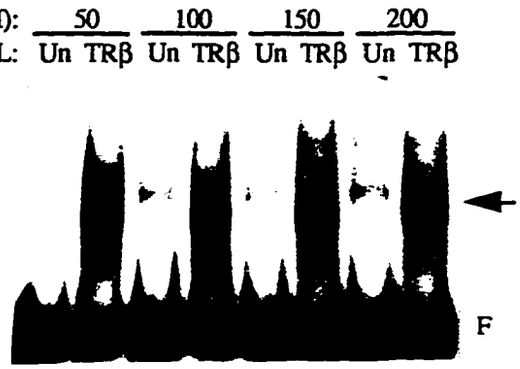


Figure 3.16: EMSA of the binding of TRβ in the presence of a range of $MgCl_2$ concentrations. Nuclear extract from COS cells tranfected with RShTRβ was incubated with labeled TRE_{rGH}. EMSA was performed under Set I conditions, with the addition of $MgCl_2$ in the binding buffer at the concentration noted. Nuclear extracts were the same as in Figure 3.14.

A)



B)

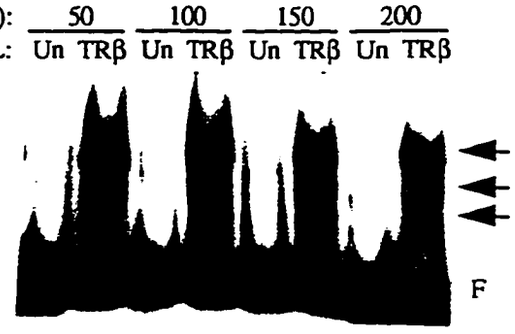


Figure 3.17: EMSA of the binding of TRβ in the presence of a range of KCl concentrations. RRL, either unprogrammed (un) or containing TRβ, was bound to (A) TRE_{IRO} or (B) TRE_{LYS} in the presence of a range of KCl concentrations. Free DNA is indicated by an F, and the arrows indicate bound DNA complexes. EMSA was performed under Set I conditions, with the addition of KCl in the binding buffer at the concentration noted.

non-specific binding, use of 100 mM KCl in the binding buffer was included in the Set II standard conditions for EMSA.

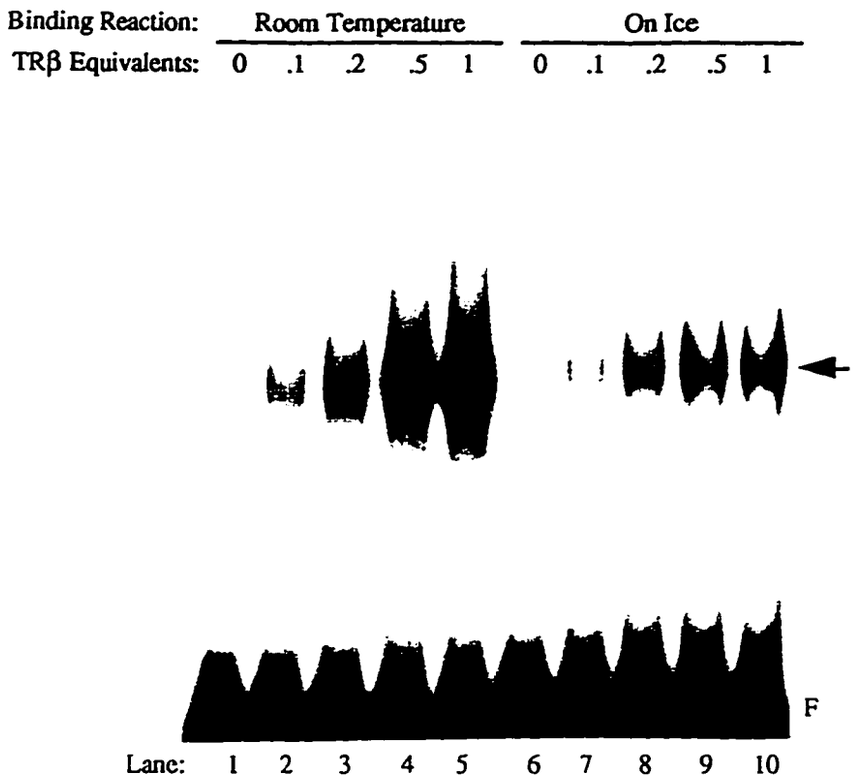
3.3.1.2.3 Temperature

Another factor which contributes to stringency of DNA binding is the temperature at which binding takes place. Higher temperature is generally considered to be more stringent. An EMSA was performed to compare the binding of TRβ to TRE_{DR4} at room temperature and 4 °C. Further, two gels were run, one at room temperature and one at 4 °C (Figure 3.18). When the gel is run at room temperature (Figure 3.18A) only one major complex is observed by EMSA, regardless of the incubation temperature of the binding reaction. However, when the gel is run at 4 °C (Figure 3.18B), two bands are apparent, regardless of the temperature at which the binding reaction was carried out, although for the reaction incubated at room temperature (lanes 1-5) there is much less DNA present in the faster migrating of the two bands. Strikingly, when the binding reaction and electrophoresis are both carried out at 4 °C (Figure 3.18B, lanes 6-10), the distribution of the bound DNA between the two bands is almost equal for most concentrations of protein tested. This experiment suggests that formation of the complex with lower mobility is more efficient at room temperature than 4 °C. Also, background smeariness and the appearance of bands in the lanes with unprogrammed lysate were reduced. Room temperature incubation and electrophoresis were incorporated into Set II standard conditions for EMSA.

3.3.1.2.4 Time Taken to Establish Binding Equilibrium

In order to ensure that the binding incubation period was long enough to permit binding to reach equilibrium, a time course experiment was performed. A series of binding reactions were preincubated for 10 minutes, after which labeled DNA was added. Samples were loaded onto a running gel at times ranging from 1 to 40.5 minutes later (Figure 3.19). This experiment indicates that equilibrium is reached in less than a minute. Hence, the

(A)


Binding Reaction:
Room Temperature
On Ice
TRβ Equivalents:
0 .1 .2 .5 1 0 .1 .2 .5 1
F
Lane:
1 2 3 4 5 6 7 8 9 10

(B)

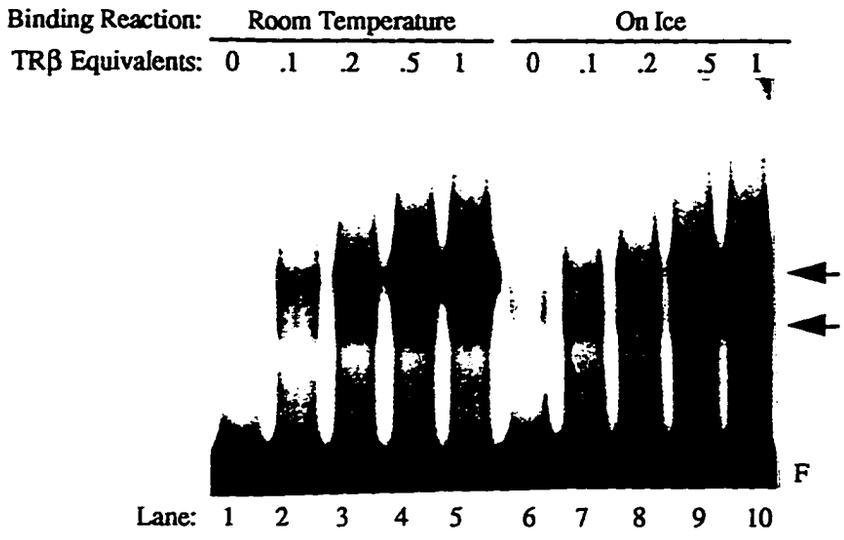


Figure 3.18: EMSA of TRβ binding to TRE_{DR4} under different temperature conditions. EMSA gels were run at (A) room temperature and (B) 10 °C. For each gel, increasing amounts of *in vitro* translated TRβ were incubated with DR4, either at room temperature or on ice. TRβ quantities are expressed as eqivalents, so that 1 is undiluted TRβ, 0.5 is a 1:2 dilution of the TRβ translation reaction with unprogrammed RRL, etc. Gels were performed under Set II standard conditions, with the modifications to temperature conditions noted above.

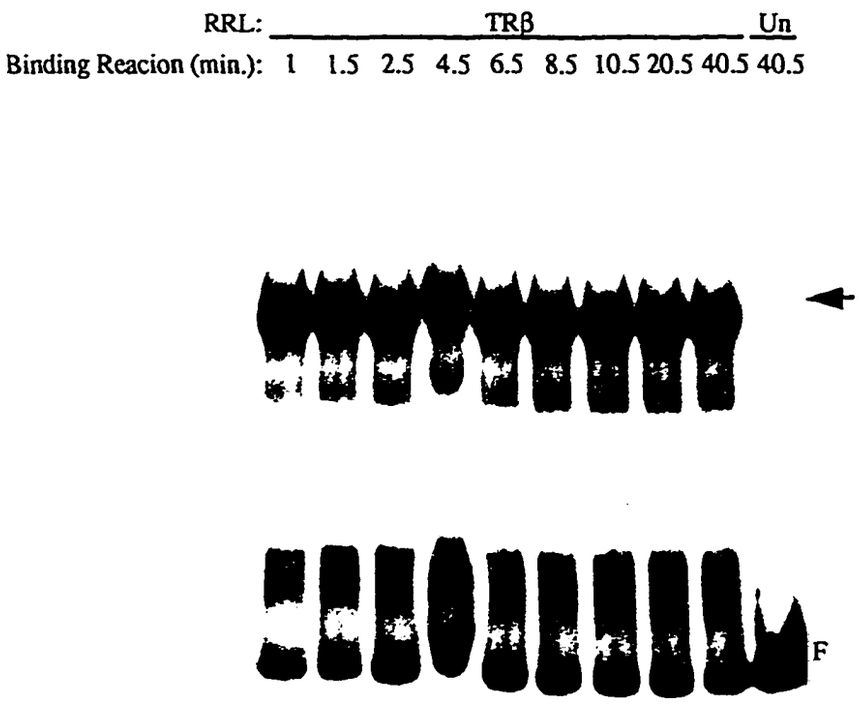


Figure 3.19: EMSA of the time-course of TRβ binding of TRE_{LYS}. Binding reactions were preincubated for 10 minutes before labeled TRE_{LYS} was added. Each binding reaction was incubated for the amount of time shown before being loaded onto a running gel. EMSA was performed under Set I conditions except that the gel buffer was 0.5 x TBE.

incubation period in Set II standard conditions is shortened, compared to Set I, to 10 minutes.

3.3.1.3 Receptor Complexes

3.3.1.3.1 Monomers, Homodimers and Heterodimers

The binding of TRβ to most of the TRE sequences described above results in two major complexes. The complex with a faster rate of migration is believed to consist of a TRβ monomer bound to the DNA probe, whereas the slower migrating complex is believed to consist of two receptor molecules bound to the probe. This assignment of complexes to bands on EMSA gels stems from two sources of data. First, there is a large body of literature which is consistent with the binding of TREs as monomer and dimer by TR in the absence of other nuclear factors (reviewed in Glass, 1994). Secondly, data such as that in Figure 3.18 clearly shows that as the TRβ:DNA ratio is increased, the proportion of DNA in the upper band increases versus that in the lower band, consistent with the increasing occupation of both half-sites of the TRE. In the case of the EMSAs performed with nuclear extracts, another possibility is that the two bands represent distinct complexes each containing two receptor molecules, the lower band representing the binding by a TRβ homodimer, and the upper one consisting of one TRβ molecule and one nuclear receptor endogenous to the nuclear extract (ie. a heterodimer). This possibility was never explored as little work was done with nuclear extracts for DNA binding analysis.

The situation for *in vitro* translated protein binding to TRE_{LYS} is less clear than for other elements, as under the conditions used here only one major band is observed (Figure 3.15). If two molecules of TRβ bind to each TRE_{LYS} sequence, the binding appears to display positive cooperativity, as virtually no monomeric binding is obvious. In order to ascertain whether this observed band represents a complex containing one or two molecules of TRβ, a mixing experiment was performed, similar to that described by Lazar *et al.* (1991). A truncated form of TRβ lacking the N-terminus, TRβΔN, and TRβ were bound to TRE_{LYS} alone or together and subjected to EMSA (Figure 3.20). TRβ (lane 1) and

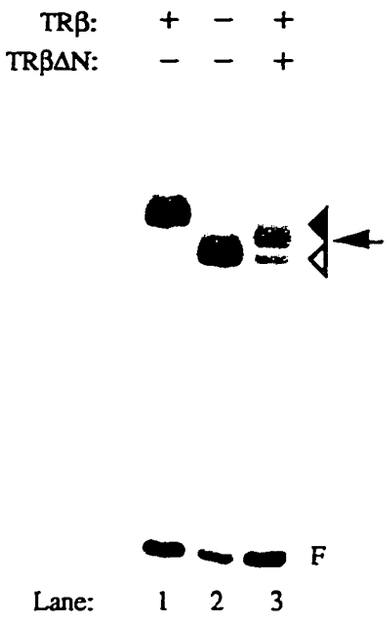


Figure 3.20: EMSA showing the binding of TRβ and TRβΔN, alone and together, to TRE_{LYS}. TRβ and TRβΔN were produced by *in vitro* translation in RRL. Complexes result from the binding of TRβ homodimers (filled triangle), TRβΔN homodimers (open triangle), and TRβ/TRβΔN heterodimers (asterisk) EMSA was performed under Set I standard conditions.

TRβΔN (lane 2) each produce single bands with distinct migrations when bound to TRE_{LYS} separately. However, when mixed (lane 3), a novel band with an intermediate rate of migration is observed. This is consistent with the slowest migrating band representing a complex containing TRβ/TRβ, the intermediate, TRβ/TRβΔN, and the fastest, TRβΔN/TRβΔN complexes.

After RXR was identified as a major heterodimerization partner for TR (Kliewer *et al.*, 1992; Zhang *et al.*, 1992), the DNA binding activity of heterodimers became of particular interest. Therefore, human RXRα was expressed *in vitro* using the same transcription/translation system as for TRβ. EMSA of TRβ and RXRα alone or together illustrates the remarkable influence of RXR on TR binding (Figure 3.21). TRE_{DR4} and TRE_{LYS} are both bound with high affinity by TRβ homodimers (lanes 2 and 6), but when RXRα is present the homodimer band disappears, and a band with a lower rate of migration is observed (lanes 3 and 7). This corresponds to the binding of one molecule of TRβ and one of RXRα to the DNA probe. RXRα has no significant affinity for either element in the absence of TRβ (lanes 4 and 8). The binding pattern illustrated in Figure 3.21 is consistent with TRβ/RXRα having a higher affinity for DR4, at least, than does the TRβ homodimer, since occupation of DR4 by the heterodimer seems to preclude the binding of the homodimer. However, that experiment was performed under conditions in which TRβ was present at a greater concentration than RXRα. In order to clearly show the higher affinity of the heterodimer for DR4, an EMSA was performed in which the concentration of TRβ was held constant in the presence of increasing levels of RXRα (Figure 3.22). Clearly, the maximum amount of binding observed by the homodimer (lane 5) is much less than that observed for the same amount of TRβ in the presence of an excess of RXRα (lane 10).

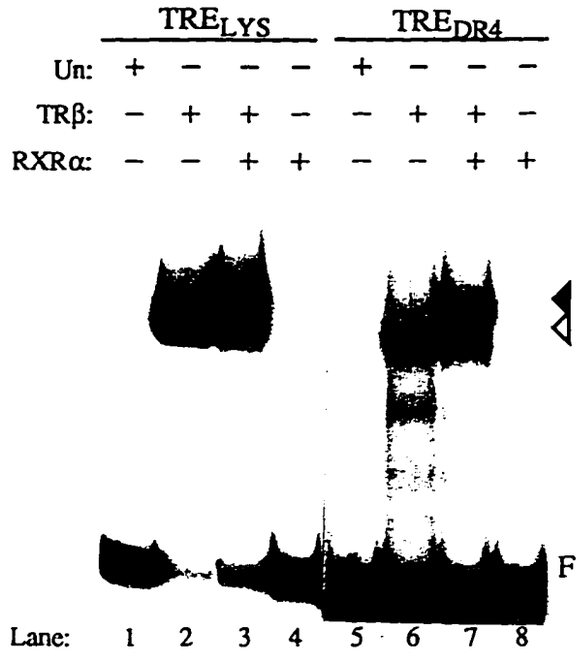


Figure 3.21: The binding of *in vitro* translated TRβ in the presence or absence of RXRα. *In vitro* translated TRβ and RXRα were present as indicated in binding reactions with either labeled TRE_{DR4} or labeled TRE_{LYS}. The TRβ homodimer is indicated by a filled triangle, and the TRβ/RXRα heterodimer by an open triangle. Free DNA is indicated by the F. EMSA was performed under Set II standard conditions.

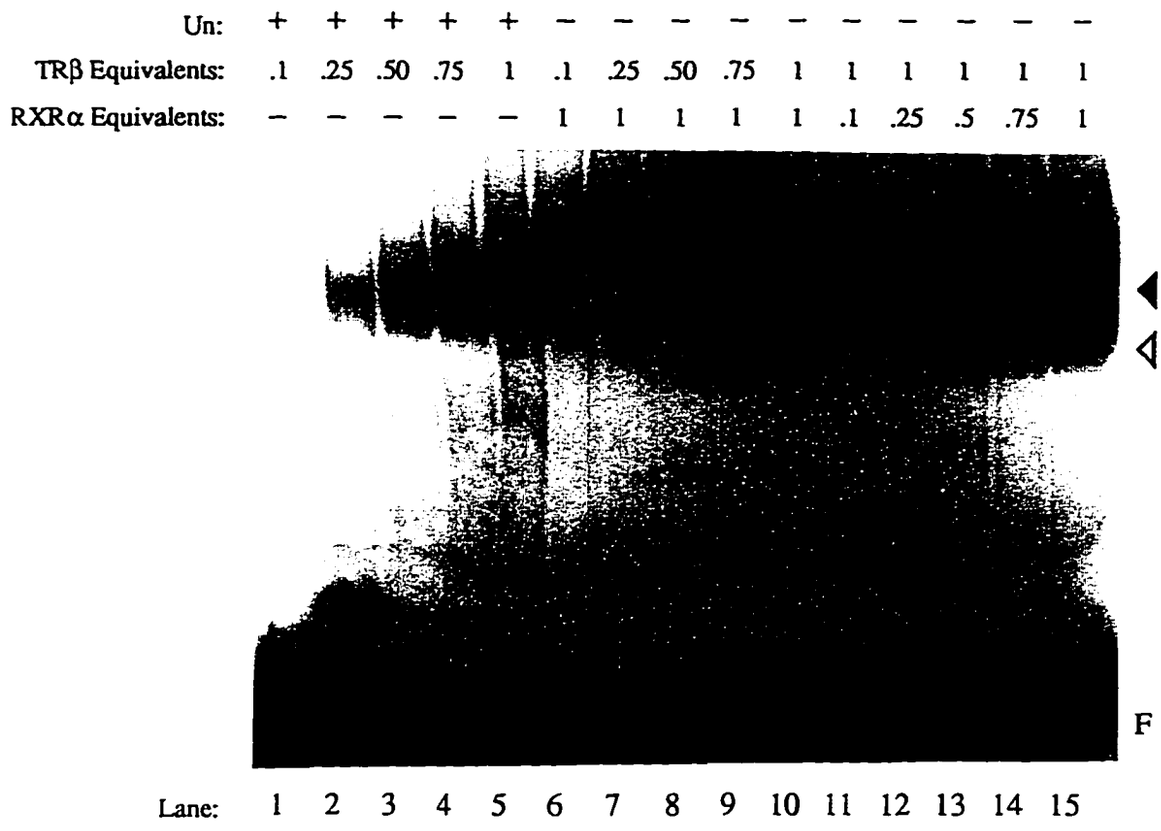


Figure 3.22: EMSA of titration experiments with *in vitro* translated TRβ and RXRα binding to TRE_{DR4}. 2 µl each of unprogrammed lysate (Un), and/or RRL containing TRβ or RXRα, were mixed as indicated. TRβ quantities are expressed as eqivalents, so that 1 is undiluted TRβ, 0.5 is a 1:2 dilution of the TRβ translation reaction with unprogrammed RRL, etc. However, 1 equivalent of TRβ does not equal 1 equivalent of RXRα. Lanes 1-5, TRβ is present in increasing concentrations. Lanes 6-10, the same increasing concentrations of TRβ in the presence of a fixed amount of RXRα. Lanes 11-15, increasing concentrations of RXRα in the presence of a fixed amount of TRβ. The band resulting from binding of TRβ homodimers to the DNA probe is indicated by an open triangle, and the band representing the binding of TRβ/RXRα heterodimer is indicated by a filled triangle. Performed under Set II standard conditions.

3.3.2 Effects of Alanine Substitutions of the Recognition α-Helix on TRβ Activity

3.3.2.1 Substitutions of Alanine in the Recognition α-Helix Have a Varying Degree of Impact on DNA Binding

A series of mutant TRβ genes were constructed by PCR-based mutagenesis. Each gene construct encodes a mutant TRβ in which one residue of the recognition α-helix (positions 120-131) has been substituted with alanine; except for K_{123}, the codon for which was refractory to mutagenesis to an alanine codon. In this case, the mutant receptor is K123M. Also, a double substitution mutant, G121A/G124A was created, as this combination converts the sequence of the P-box of TRβ to that of ER. In preparation for assaying DNA binding activity, the wild type TRβ and each alanine point mutant were transcribed *in vitro*, and the RNA was translated using rabbit reticulocyte lysate. Analysis of the mutant TRβ proteins translated *in vitro* in the presence of ^{35}S-methionine by SDS-PAGE reveals that the mutants do not exhibit any discernible differences in migration, or in the apparent relative ratios of the three major bands (Figure 3.23). This is an indication that no major disruptions to translation, or the overall structure of the TR proteins result from the altered sequences.

The alanine substitution mutants of TRβ display variations in DNA binding capacity from that of the wild-type TRβ. The DNA binding affinities of *in vitro*-translated proteins were determined by EMSA using four different DNA target sequences representing the half-site sequence and configuration variation found in naturally occurring TREs. Binding of wild-type TRβ and the 13 alanine substitution mutants was compared on TRE_{IR0}, TRE_{LYS}, TRE_{rGH3} and TRE_{MAL} (Figure 3.24). The effects of alanine substitution at each position within the recognition helix ranged from little change in binding affinity relative to wild-type (G121A, G124A, and 121/124) to no discernible binding activity (C122A, K123M, F125A, F126A, and R128A). In some intermediate cases the degree of binding varied depending on which element was used as a probe.

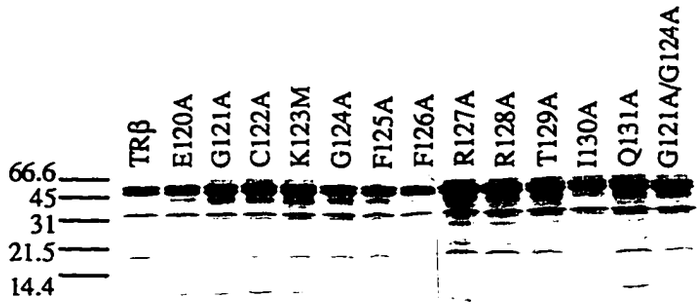
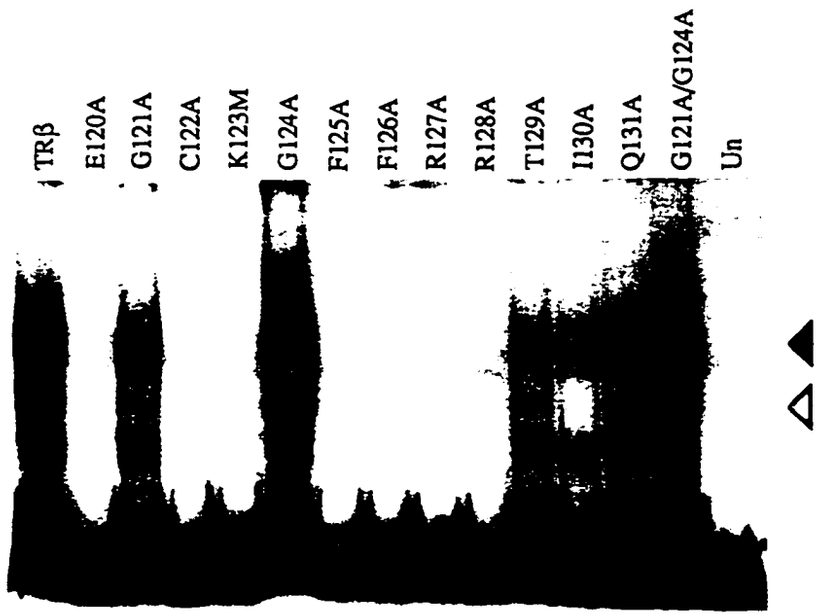
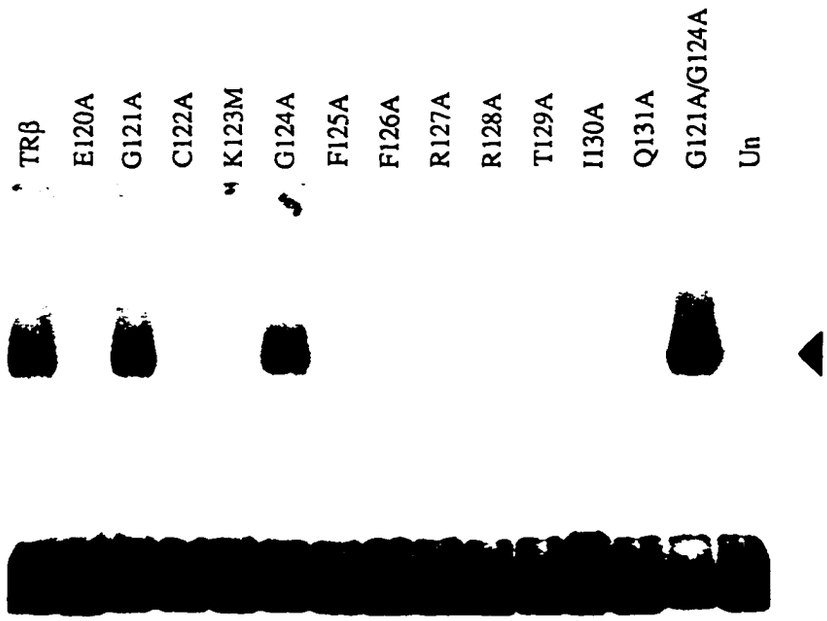


Figure 3.23: SDS-PAGE (15 % polyacrylamide) analysis of the alanine substitution mutants of TRβ, and wild-type TRβ, translated *in vitro* in the presence of ^{35}S-methionine. Molecular weight standard sizes are indicated.

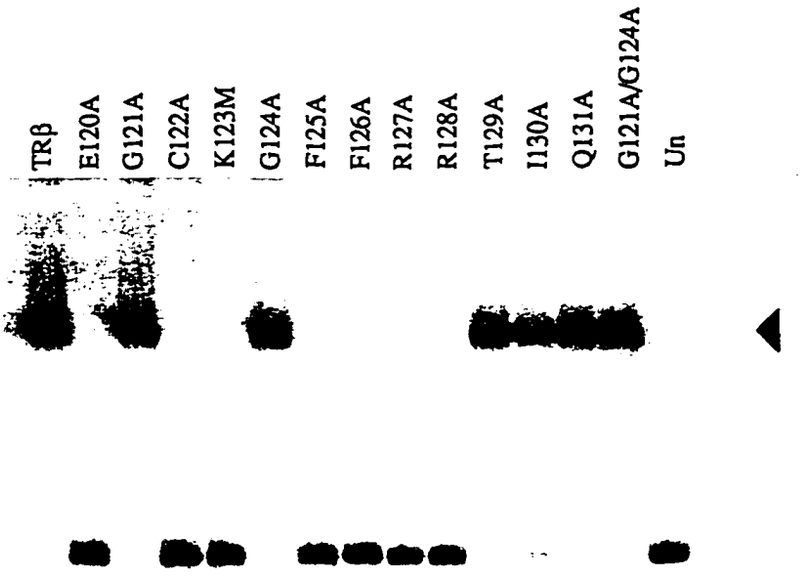
(A)


TRβ
E120A
G121A
C122A
K123M
G124A
F125A
F126A
R127A
R128A
T129A
I130A
Q131A
G121A/G124A
Un

(B)


TRβ
E120A
G121A
C122A
K123M
G124A
F125A
F126A
R127A
R128A
T129A
I130A
Q131A
G121A/G124A
Un

(C)



(D)

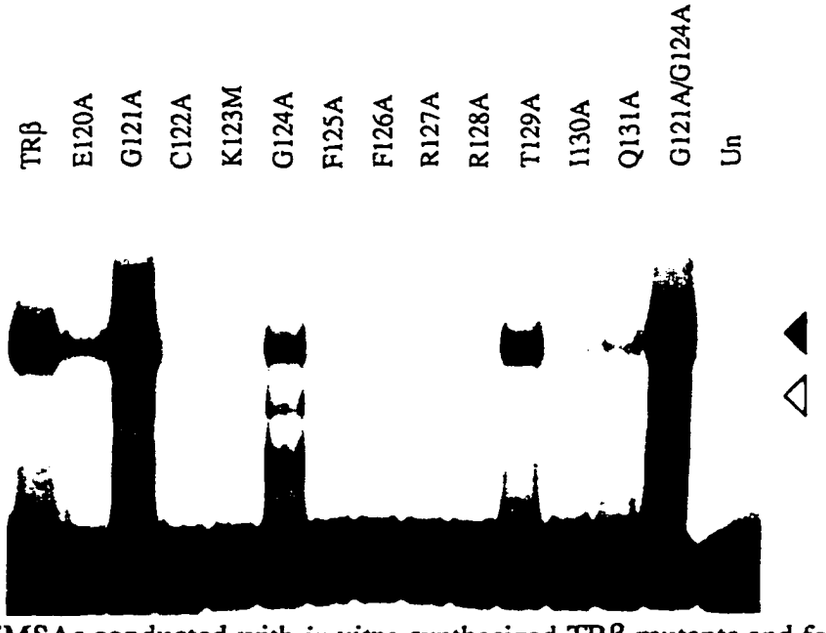


Figure 3.24: EMSAs conducted with *in vitro*-synthesized TRβ mutants and four different TREs. The TREs used were: (A) TRE_{IR0}; (B) TRE_{rGH3}; (C) TRE_{LYS}; and, (D) TRE_{MAL}. For each TRE, the indicated TRβ protein was used, and Un indicates the addition of unprogrammed rabbit reticulocyte lysate. The migration of specific receptor-DNA complexes are indicated by triangles. EMSAs were performed under Set I standard conditions.

Several of those mutants lacking DNA binding activity can be explained on the basis of what is known from DBD crystal structures (Luisi *et al.*, 1990; Schwabe *et al.*, 1993; Rastinejad *et al.*, 1995). C122A is one of the zinc coordinating cysteines of CI, and it is expected that impairment of zinc coordination would result in disruption of the zinc binding motif. Likewise, the dramatic drops in binding observed for F125A and F126A, as well as I130A, can be attributed to disruption of the folding of the zinc module. In the wild-type DBD, these positions all contribute to the hydrophobic core of the module. Substitution with alanine of distinct residues in the recognition helix that are known from the crystal structure to make base-specific contacts with the half-site DNA (E_{120}, K_{123}, R_{127}, and R_{128}) abrogates binding. On the other hand, Q131A, which makes only phosphate contacts, displays residual binding activity on some elements.

The binding affinity of those alanine substitution mutants which retained detectable activity were determined by Scatchard analysis. A DNA fragment containing the TRE_{LYS} sequence was labeled in an excess of ^{32}P-dATP to ensure complete fill-in of the sticky ends. The specific activity of the probe could thus be calculated on the basis of the specific activity of the radionucleotide and the number incorporated per molecule. A fixed amount of protein was incubated with increasing concentrations of labeled TRE_{LYS} and the bound and free DNA was separated by EMSA (example in Figure 3.25). Bands were excised from dried gels and the radioactivity determined by scintillation counting. The quantity of probe in the bound and free fractions of each binding reaction was calculated on the basis of the specific activity of the probe. A binding curve was produced (example in Figure 3.26) in order to approximate which samples lay between 20-80 % saturation of the protein. The selected samples were then used for a Scatchard analysis (Figure 3.27) to determine the K_d of the interaction of the wild-type or mutant TRβ with TRE_{LYS}. These values are given in Table 3.1 and illustrate that several of the alanine substitutions produce little disruption of TRE_{LYS} binding by TRβ. G124A, has at least as high binding affinity

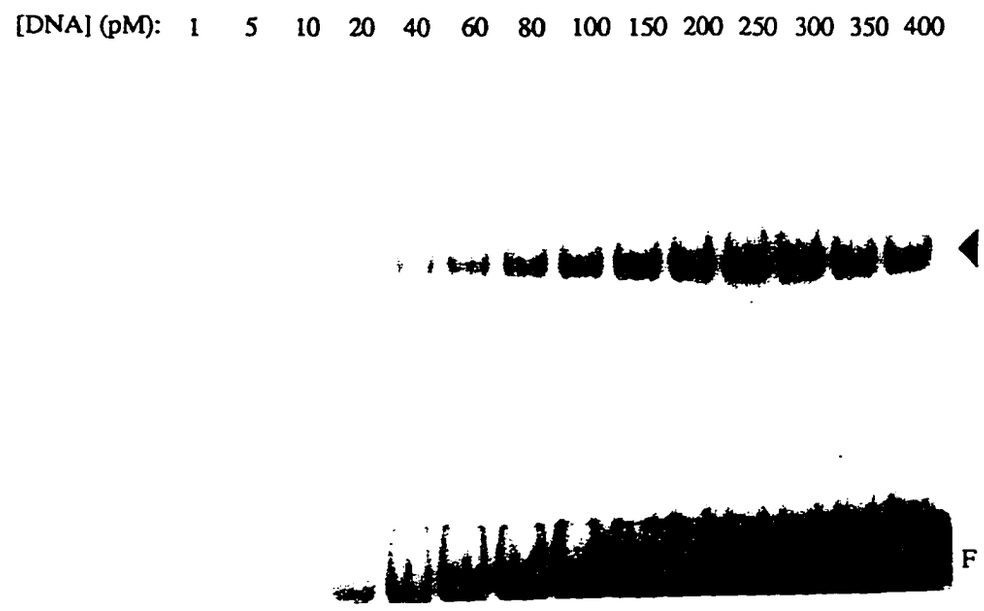


Figure 3.25: An EMSA peformed for Scatchard analysis of the DNA binding affinity of TRβ. A fixed quantity of *in vitro* translated protein was included in a binding reaction with the final concentration of TRE_{LYS} indicated. The band representing binding of TRβ homodimers to the DNA probe is indicated by the triangle. Free DNA is indicated by the F. EMSA was performed under Set I standard conditions.

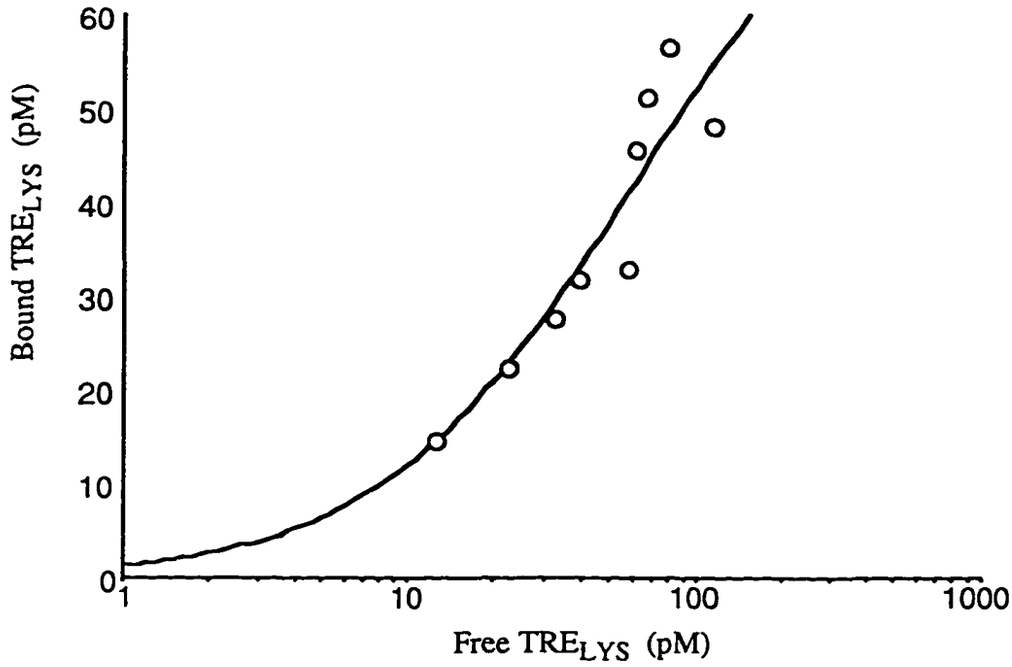


Figure 3.26: Sample binding curve for TRβ incubated with increasing concentrations of TRE_{LYS}. After scintillation counting of the radioactivity in the bands from a gel such as that illustrated in Figure 3.25, a plot of the concentration of bound TRE_{LYS} versus the concentration of free TRE_{LYS} was produced. The curve is drawn based on a non-linear least squares fitting function with the formula: m2 x m1 x m0 / (1 + m1 x m0); where m0 = ligand concentration (in this case, the concentration of ^{125}I-T_3; m1 = 10^9, initially; and m2 = 1 initially.

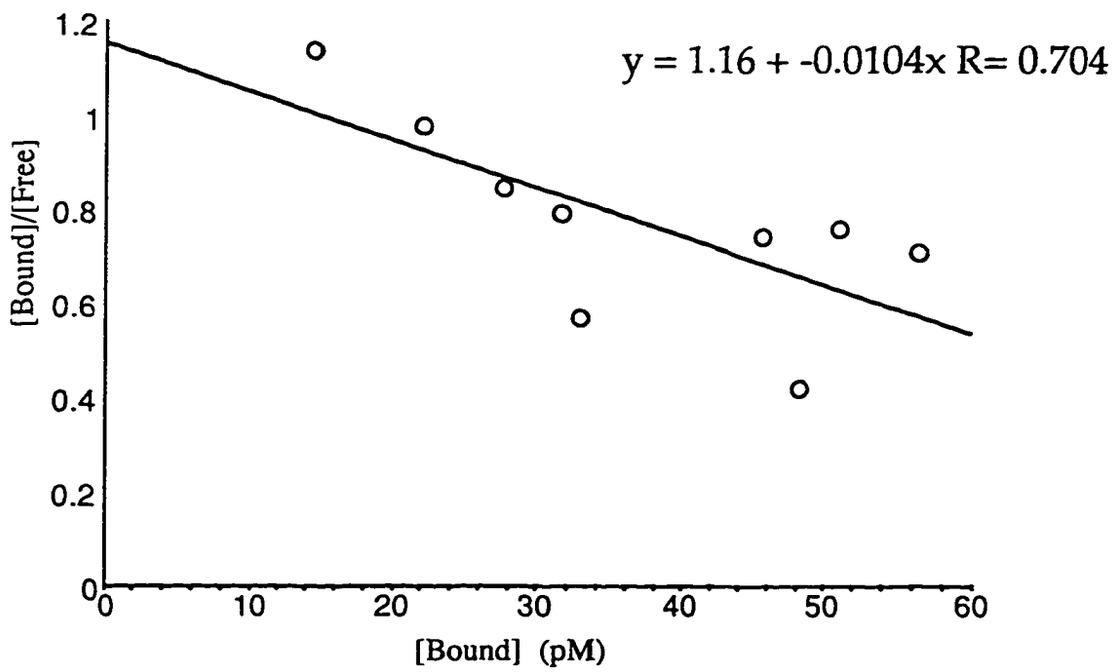


Figure 3.27: Sample Scatchard plot used to calculate the K_d of TRβ binding to TRE_{LYS}. Bands were excised from the gel used to produce the autoradiogram in Figure 3.25 and the radioactivity quantified. The specific activity of the probe was used to calculate the concentrations of bound (B) and free (F) DNA that was in each sample loaded on the gel. The equation generated for the line drawn on this graph gives a K_d value of 96 pM, calculated from the slope.

TRβ Receptor	K_d (pM) ± S.E.	Relative Affinity[a] ± S.E.
wild type	63 ± 24	1.00
E120A	>1000	<0.07
G121A	180 ± 57	0.35 ± 0.11
G121A/G124A	75 ± 35	0.86 ± 0.18
G124A	51 ± 27	1.25 ± 0.30
R127A	>1000	<0.07
T129A	76 ± 41	0.88 ± 0.16
I130A	>200[b]	<0.32[b]
Q131A	89 ± 53	0.86 ± 0.18

[a]Ratio of K_d(wild type)/K_d(mutant) measured with TRE_{LYS}. Values represent the mean of a minimum of three independent determinations. K_d values greater than 1000 pM could not be accurately determined by the assay.

[b]Although I130A had measurable DNA binding activity, saturation could not be achieved in the Scatchard analysis.

Table 3.1: Relative Binding Affinities of the TRβ Mutants for TRE_{LYS}.

for TRE_{LYS} as wild-type TRβ. G121A/G124A, T129A and Q131A are less than 2-fold defective in binding of TRE_{LYS} relative to TRβ.

3.3.2.2 Effects of Alanine Substitutions of the Recognition α-Helix on Transcriptional Activation of TRE-Linked Reporter Genes.

3.3.2.2.1 *Trans*-Activation Capacities of Mutant Receptors Reflect Their DNA Binding Affinities, With One Exception

It was of interest to see what the effects of the alanine substitutions were on *trans*-activation, since it was unknown whether the function of the P-box in differentiating half-site sequences was strictly based on DNA binding affinity, or whether some other mechanism might be responsible. All the *trans*-activation experiments were carried out by Dr. Colleen Nelson, but the data is integrally connected with the DNA binding studies I carried out, and so is included here. The ability of the alanine substitution mutants of TRβ to activate transcription in vivo was determined by cotransfection of COS cells with TRβ plasmids and TRE-linked CAT reporter plasmids in the presence or absence of hormone. As expected, TRβ mutants that were unable to bind to TRE sequences with high affinity (C_{122}, K_{123}, F_{125}, F_{126} and R_{128}) did not *trans*-activate expression of a CAT reporter gene linked to TRE_{IR0} in response to hormone (Figure 3.28).

Most of the mutant TRβ proteins displayed transcriptional activation profiles that paralleled their DNA binding abilities when tested on all four TREs in COS cells (Figure 3.29). However, transcriptional activation by mutants with intermediate DNA binding affinities, such as T129A, I130A, and Q131A was higher than expected when compared to their DNA binding affinities *in vitro*. This effect was also noted with the TRβ mutants E120A and R127A which were drastically impaired for DNA binding, but retained measurable transcriptional activation (i.e. a twenty-fold drop in DNA binding for R127A, but only a 50-80% reduction in trans-activation). The strong transcriptional activation carried out by those mutants impaired in DNA binding may result from saturating levels of

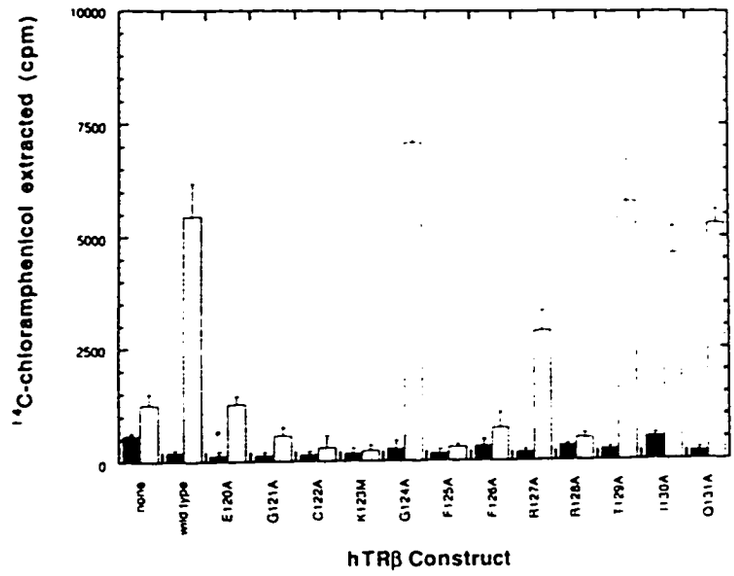


Figure 3.28: T_3-responsive transcriptional activation by wild-type and mutant TRβs of ΔMTV-CAT-TRE_{IR0}. COS cells were co-transfected with reporter plasmid (ΔMTV-CAT-TRE_{IRO}) and expression plasmid (pRSTRβ or mutant). Solid bars indicate the level of CAT activity in the absence of T_3; open bars indicate the level of CAT activity in the presence of T_3. Data represent the mean of four independent experiments with associated standard deviations. None indicates cells which were transfected with the reporter plasmid, but not the expression plasmid. These data were generated by Dr. Colleen Nelson.

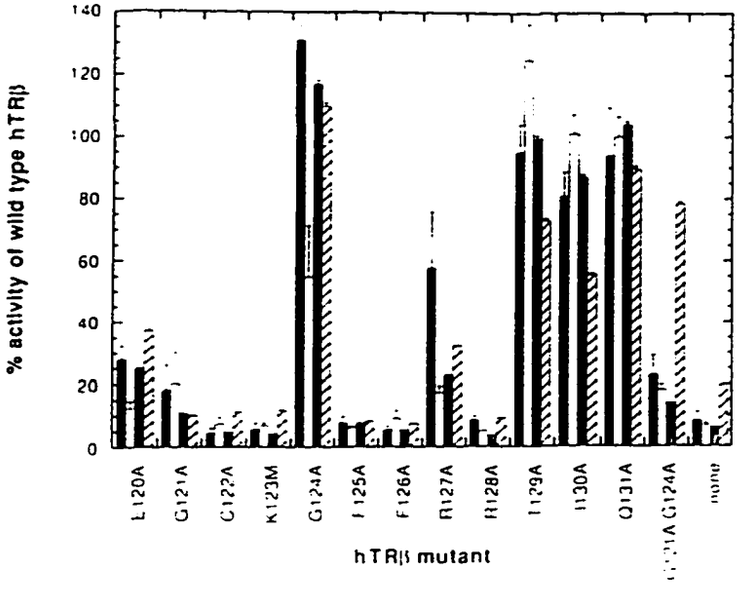


Figure 3.29: Transcriptional activation activities of mutant TRβs relative to wild-type TRβ on four different TREs. COS cells were co-transfected with reporter plasmid (ΔMTV-CAT-TRE_{IR0}) and expression plasmid (pRSTRβ or mutant) in the presence of T3. Twenty-four hours later cellular extracts were prepared and assayed for CAT activity. Data are represented as a comparison of the CAT activity induced by the wild type receptor to that induced by the indicated mutant TRβ. T_3-responsive CAT expression was measured using CAT reporter genes linked to TRE_{IR0} (solid bars), TRE_{MAL} (open bars), TRE_{LYS} (stippled bars), or TRE_{rGH3} (striped bars). Data represent the mean of at least four independent experiments with associated standard deviations. None indicates cells which were transfected with the reporter plasmid, but not the expression plasmid. These data were generated by Dr. Colleen Nelson.

receptors in transfected COS cells, which are capable of replicating the TRβ expression vector used. Therefore, identical CAT assays were repeated in HepG2 cells which lack the SV40 T-antigen and cannot replicate the receptor plasmids. In HepG2 cells the profile of *trans*-activation from the various alanine mutants was identical to that observed in transfected COS cells for all TREs tested (Figure 3.30).

To ascertain if the DNA binding deficiencies of some TRβ mutants observed *in vitro* were compensated for *in vivo* by nuclear factors, nuclear extracts from COS cells transfected with the TRβ mutants were assayed for their ability to bind to the various TREs (Figure 3.31). The mutant receptors present in nuclear extracts exhibited essentially the same pattern of binding affinities as did the *in vitro* translated receptors; however, this may be simply due to the massive overexpression of the mutant TRβs in the transfected COS cells. Considering the importance of RXRα as a heterodimerization partner for TR, and the fact that the natural levels of RXR in the COS cells may not be comparable to the overexpressed TRβ, the affinity of TR/RXR heterodimers was also examined. The influence of RXR on the DNA binding potential of the various TRβ alanine substitution mutants was assayed directly using *in vitro* synthesized TRβs in the presence or absence of *in vitro* synthesized human RXRα (Figure 3.32). At the non-saturating levels of TRβ used in this assay only the binding of TRβ/RXR heterodimers to DNA was observed. These data indicate that T129A, I130A, and Q131A mutant TRβs have a substantial DNA binding affinity on TRE_{MAL} in the presence of RXR, which correlates with the transcriptional activation shown in Figure 3.30. However, in contrast to T129A, I130A and Q131A, other alanine mutants that are defective for DNA binding, such as E120A and R127A, were not noticeably compensated by RXR. Perhaps the apparent increase of trans-activation by E120A and R127A, in comparison to their reduced DNA binding affinities, is due to the inherent levels of sensitivity of the two different assay systems.

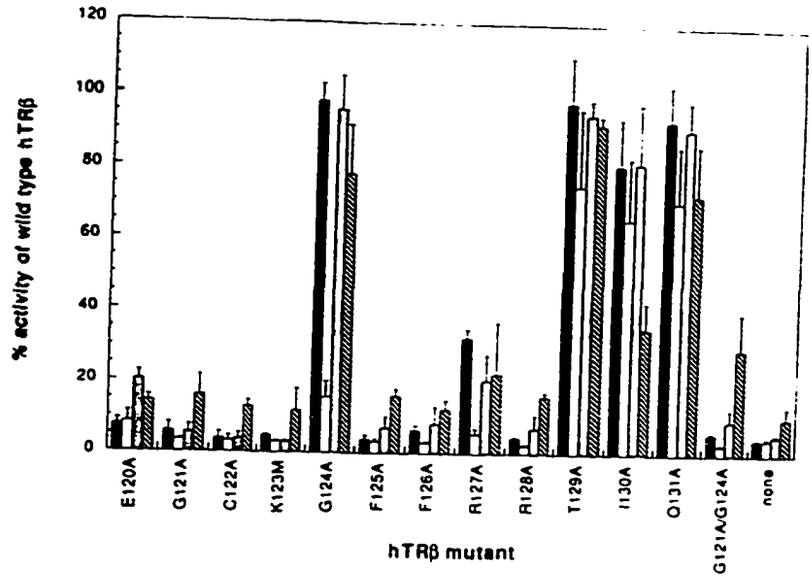


Figure 3.30: The transcriptional activity of mutant TRβs in HepG2 cells relative to wild-type TRβ on four TRE elements. HEPG2 cells were co-transfected with reporter plasmid (ΔMTV-CAT-TRE_{IR0}) and expression plasmid (pRSTRβ or mutant) in the presence of T_3. Twenty-four hours later cellular extracts were prepared and assayed for CAT activity. Data are represented as a comparison of the CAT activity induced by the wild type receptor to that induced by the indicated mutant TRβ. T_3-responsive CAT expression was measured using CAT reporter genes linked to TRE_{IR0} (solid bars), TRE_{MAL} (open bars), TRE_{LYS} (stippled bars), or TRE_{rGH3} (striped bars). Data reresent the mean of at least four independent experiments with associated standard deviations. None indicates cells which were transfected with the reporter plasmid, but not the expression plasmid. These data were generated by Dr. Colleen Nelson.

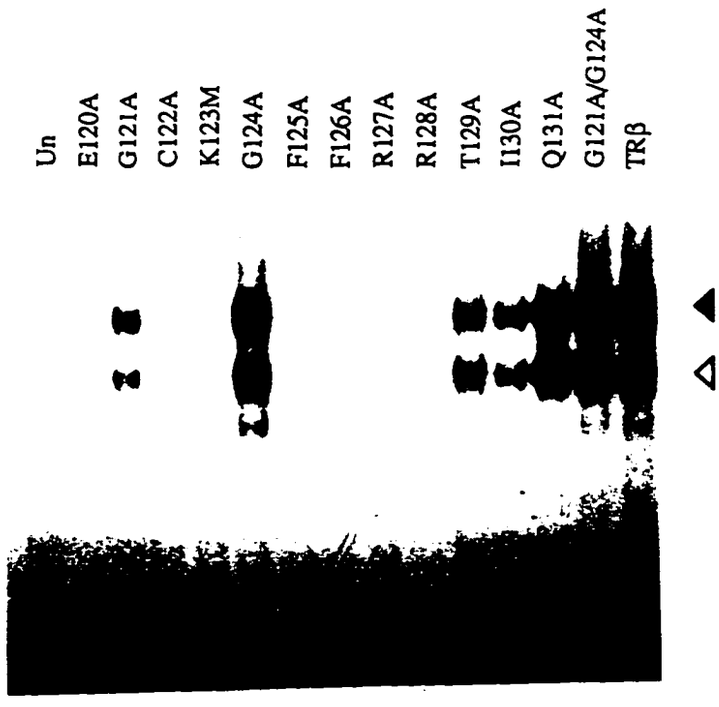


Figure 3.31: EMSA of mutant TRβs in nuclear extracts prepared from transfected COS cells. Nuclear extracts were prepared from COS cells either mock transfected or transfected with expression plasmid (pRShTRβ or mutant).The DNA probe is TRE_{IRO}. Nuclear extracts were prepared by Dr. Colleen Nelson. EMSA was performed by Stephen Hendy using Set I standard conditions. The arrowheads represent the migration of specific receptor-DNA complexes.

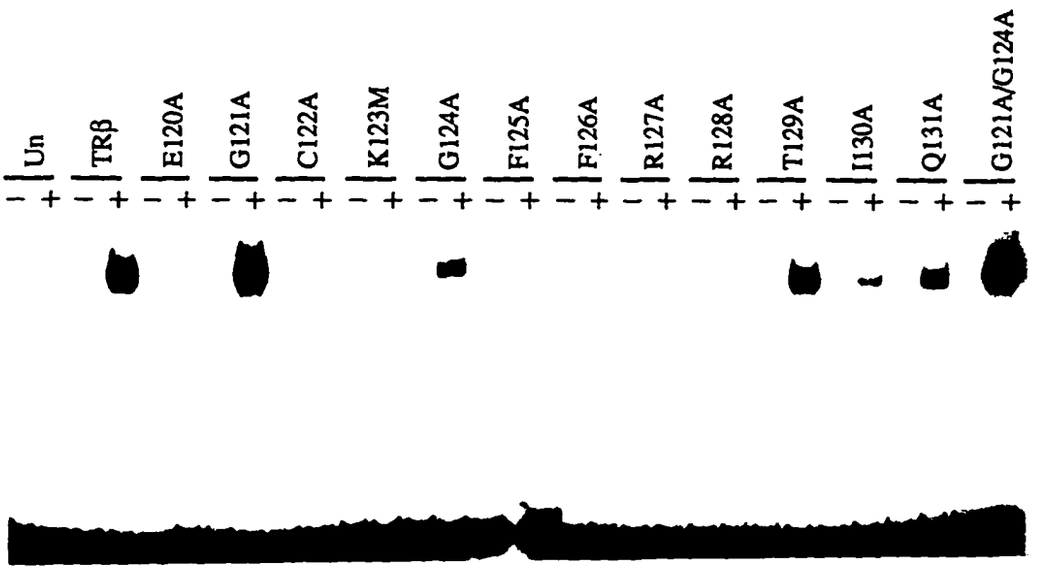


Figure 3.32: EMSA with *in vitro* synthesized TRβs on TRE_{MAL} in the presence of RXRα. Equal amounts of wild-type and mutant TRβs were subjected to EMSA in the presence or absence of *in vitro* synthesized RXRα. The EMSA was performed under Set I standard conditions. None indicates unprogrammed RRL. The arrowhead indicates migration of a complex containing TRβ and RXRα.

3.3.2.2.2 Investigation of Mechanisms by Which G121A Might Lose *Trans*-Activation Capacity While Maintaining DNA Binding Affinity

Alanine substitution of G_{121} decreased *trans*-activation of CAT reporter genes linked to all TREs in both HepG2 and COS cells to levels less than those observed for E120A (which was severely impaired for DNA binding), more closely resembling the *trans*-activation levels of mutants that disrupt zinc module structure (Figures 3.29 and 3.30). This result was unexpected: the G121A mutant is able to bind to DNA both *in vitro* and *in vivo* with affinities similar to that of the wild type receptor (Figures 3.24 and 3.31). The defect in *trans*-activation of G121A is not related to an inability to interact with RXR, since G121A binds to DNA as a heterodimer with RXR to a similar extent as the wild-type receptor (Figure 3.32). The G121A and wild-type TRβ receptors have similar T_3 binding activities, as determined by an *in vitro* T_3-binding assay (Figure 3.33); thus, the reduced ability of G121A to *trans*-activate CAT gene expression does not arise from a defect in hormone binding. Therefore, the P-box amino acid G_{121} of TRβ is required for *trans*-activation of TRE-linked genes even though substitution at this position does not affect sequence specific DNA binding.

The double substitution mutant G121A/G124A was designed, in part, to help further investigate the role of this P-box amino acid in *trans*-activation. This double mutant has wild-type DNA binding affinity *in vitro* and *in vivo* (Figures 3.24, 3.31, and 3.32). However, G121A and G121A/G124A had identical deficiencies in transcriptional activation on TRE_{IRO}, TRE_{LYS}, and TRE_{MAL} (Figures 3.29 and 3.30). In contrast, on TRE_{rGH3}, the G121A/G124A combination improved the deficiency in transcriptional activation in comparison with the G121A substitution alone.

3.3.2.2.3 The Effects of Heterodimerization with Non-Binding Mutants on the DNA binding Activity of TRβ

The availability of non-binding alanine mutants of TRβ was ideal for examining one possible mechanism of the dominant negative effect of certain non-binding TRβ receptor

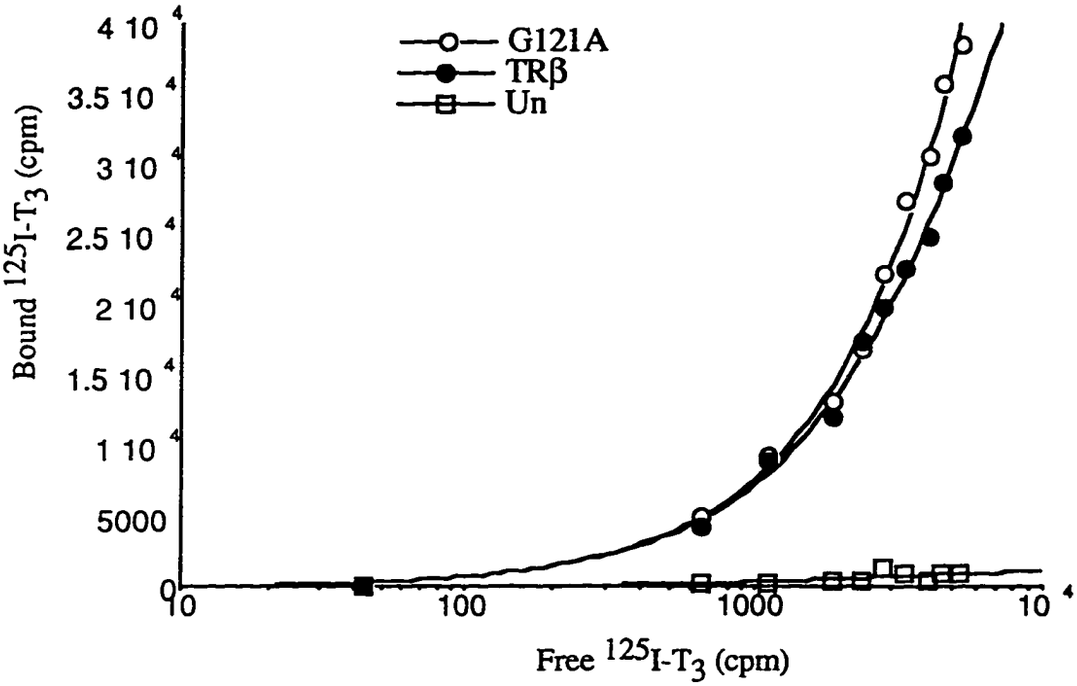


Figure 3.33: Curve showing the binding of ^{125}I-T_3 by wild-type TRβ and G121A compared with that of unprogrammed RRL. The amount of ^{125}I-T_3 retained in the filter binding assay is plotted versus the the amount of ^{125}I-T_3 in an aliquot of the binding reaction. The curve is drawn as described in Figure 2.26.

mutants: that is, the formation of heterodimers between the non-binding mutant and wild-type TRβ which bind to DNA but are not competent to carry out transcriptional activation. Another related mechanism by which non-binding TRβ mutants could inhibit the activity of TRβ is the formation of heterodimers in solution which are unable to bind DNA. The experiments presented here do not thoroughly address these possibilities; however, they do provide the basis for speculation along these lines.

The first experiment performed to see whether wild-type TRβ could heterodimerize with a non-binding mutant and still bind to DNA with high affinity was a simple mixing experiment. *In vitro* translated TRβ was mixed with a number of alanine mutant TRβs and the binding to TRE_{LYS} observed by EMSA (Figure 3.34). As expected, addition to a fixed quantity of TRβ of another mutant receptor form which retained DNA binding activity resulted in overall increased binding of the DNA probe. On the other hand, addition of the non-binding mutant C122A, or the poorly binding mutant E120A, did not enhance binding of TRE_{LYS} at all. In fact, quantification of the DNA in each bound band (Figure 3.34B) suggests that binding of the wild-type receptor is slightly reduced, which is consistent with the rather intuitive notion that a heterodimer containing a non-binding mutant TRβ will have a lower affinity for a TRE than a homodimer of wild-type TRβ. Of course, such a heterodimer might still retain some binding affinity for a TRE. This possibility was explored by using a "shift down" experiment, which is a twist on the EMSA protocol. Instead of visualizing DNA binding by the decreased mobility of labeled DNA in the presence of protein, the increased mobility of labeled protein in the presence of unlabeled DNA is detected. For this experiment T129A was used as the TRβ form with near wild-type DNA binding activity on TRE_{LYS}. EMSA of ^{35}S-labeled T129A in the presence of unlabeled TRE_{LYS} produces two distinct bands whereas C122A exhibits only a diffuse smear that likely represents the unbound protein (Figure 3.35). Mixture of labeled C122A with unlabeled T129A should reproduce the banding pattern observed for T129A alone, if C122A/T129A heterodimers are able to bind DNA. Interestingly, in this assay E120A

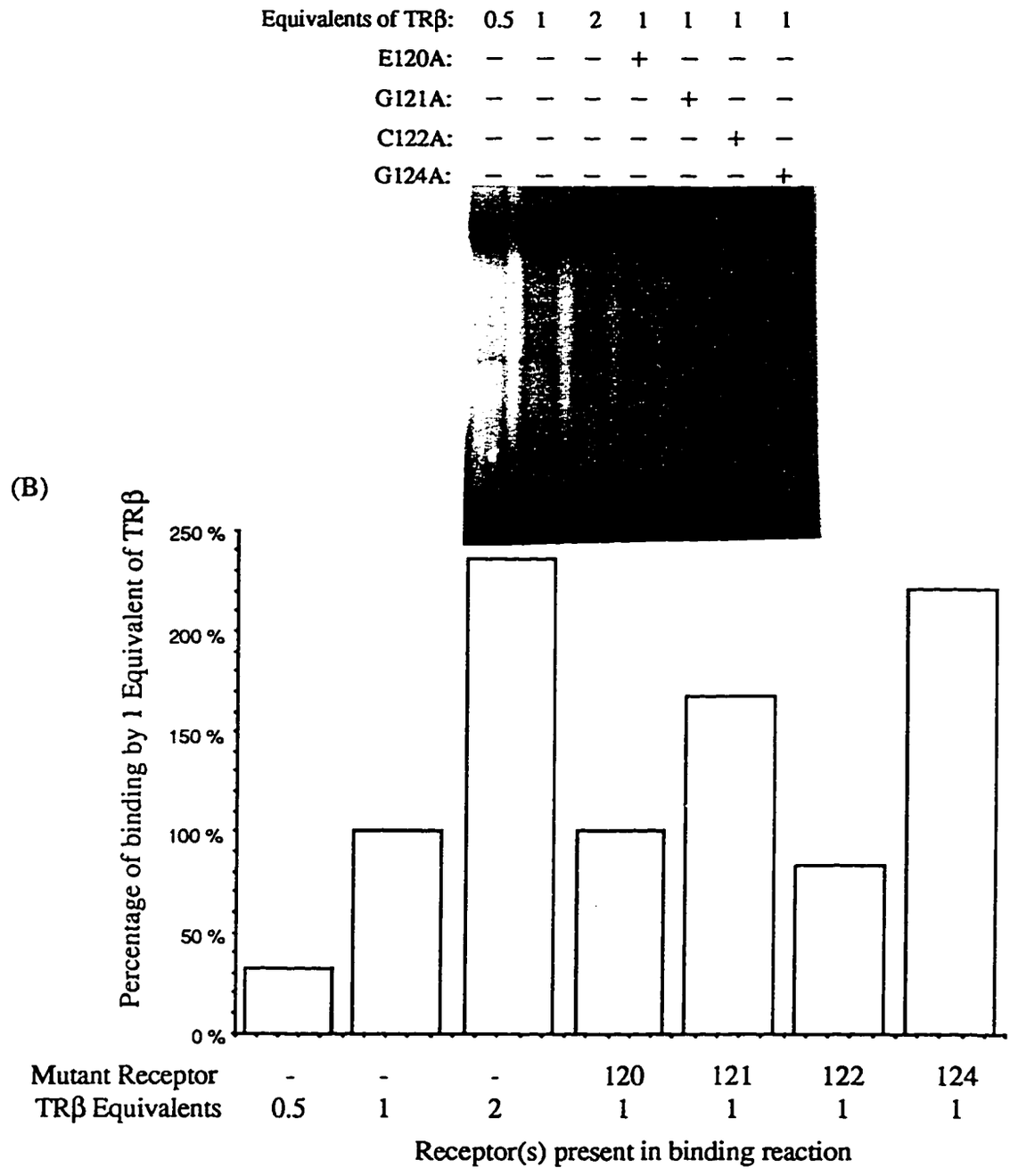


Figure 3.34: Mixing experiment comparing the effects of combining either binding or non-binding mutants of TRβ with wild-type TRβ in an EMSA. Proeins were equalized to 1 equivalent on the basis of a parallel *in virtro* translation reaction including ^{35}S-methionine. RRL, either unprogrammed or containing the indicated mutant of TRβ, was used in an EMSA with TRE_{LYS}. The EMSA was performed under Set I standard conditions.

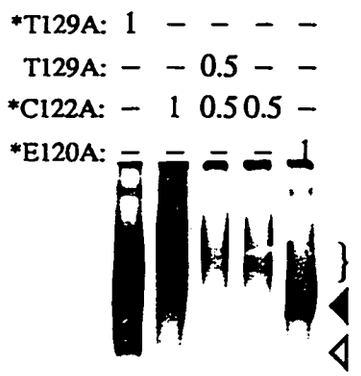


Figure 3.35: "Shift-down" EMSA examining the ability of T129A to form DNA-binding heterodimers with C122A. Unlabeled TRE_{DR4} was used in each lane in conjunction with either unlabeled or ^{35}S-methionine. A binding reaction of ^{35}S-T129A with unlabeled TRE_{DR4} produces two distinct bands (lane 1), indicated by triangles. A binding reaction of ^{35}S-C122A produces only a diffuse smear with lower mobility (lane 3), indicated by abracket. When unlabeled T129A is mixed with ^{35}S-C122A no distinct bands are observed (lane 4). The binding of ^{35}S-E120A is also observable as a single band with low mobility (lane 5). An asterisk indicates that the protein is labeled with ^{35}S. EMSA was performed under Set I standard conditions.

produces a single band, with similar migration to the slowest formed with T129A. Thus, the sensitivity of the assay is great enough to detect the binding of a TRβ mutant with relatively low DNA binding affinity. However, this assay indicates that no complex with detectable DNA binding activity is formed between T129A and C122A. Thus, T129A is unable to "rescue" the extremely low DNA binding activity of C122A, by forming a heterodimer.

3.4 DISCUSSION

3.4.1 DNA Binding Activity of Wild-Type TRβ

The EMSA for DNA binding was developed as a technique for analysis of the DNA binding properties of the thyroid hormone receptor. This assay has turned out to be the most commonly used with the thyroid hormone receptor and many workers have used variations of the technique with great success. However, when this study was initiated the optimal conditions for observing TR binding by EMSA were not well defined. In order to optimize specific binding by TRβ while limiting the binding of other nuclear factors, *in vitro* translated protein was chosen as the source of receptor. Nuclear extracts from transfected cells did provide a highly active source of TRβ which bound a number of elements (Figure 3.14). However, there were also binding activities intrinsic to the nuclear extract that bound some TRE sequences, particularly TRE_{LYS}, to a significant extent. The ability to observe the binding of intrinsic factors from mammalian cells is of obvious interest for certain problems. For example, this source of protein was used to confirm the binding of the alanine mutant TRβs in correlation with the *trans*-activation studies. However, where it is desirable to study the DNA binding properties of the TR in the absence of unidentified factors, *in vitro* translated protein proves more appropriate. Unprogrammed RRL does not produce any significant bands (Figure 3.21), whereas TRβ in RRL can be shown to bind with high affinity to a number of natural TREs by EMSA (Figure 3.24).

TRβ was shown to form two complexes with most TREs, although TRE_{LYS} was predominantly bound as the slower migrating of the two complexes. This complex is believed to represent the binding of two receptor molecules to the DNA probe. The faster migrating band is a monomer of TRβ bound to the DNA. These assignments of the two bands seen on EMSA gels are made based on several observations: as TRβ concentration is increased, the proportion of bound DNA in the upper band increases (Figure 3.18); and, mixing of TRβ and TRβΔN reveals that these two species readily form a complex with TRE_{LYS} that contains one molecule of each receptor (Figure 3.20). The literature is consistent with such assignments for the two bands, although this is a somewhat circular argument, as most workers use similar observations to make the same assignments (Lazar and Berrodin, 1990; Lazar *et al.*, 1991; references in Glass, 1994).

Wild-type TRβ can form heterodimers with the mutant TRβ lacking the N-terminus and these heterodimers bind DNA with high affinity (Figure 3.20). The ability of a TRβ mutant deficient in DNA binding activity, C122A, to form a DNA binding heterodimer with wild-type TRβ was examined. If TRβ and C122A heterodimerize, as expected, then the affinity of this complex for TRE_{LYS} must be significantly lower than that of the TRβ homodimer. The presence of C122A in a binding reaction with TRβ does not enhance the binding of TRE_{LYS} (Figure 3.34). On the contrary, a small degree of inhibition is apparent, although not enough to be consistent with the formation of stable, non-binding TRβ/C122A heterodimers in solution. There are two basic models to explain this phenomenon: 1) the formation of non-binding TRβ/C122A heterodimers in solution that are in relatively rapid exchange with monomeric receptor, so that the pool of TRβ homodimers that are competent to bind TRE_{LYS} is reduced by the presence of C122A; or, 2) the receptors heterodimerize due to DNA-dependent cooperative interactions, wherein after occupancy of one half-site of the TRE_{LYS} by TRβ, another molecule of wild-type receptor would be more likely to occupy the second half-site than a molecule of C122A. I

suggest that the first of these models is more accurate based on my results and the work of others.

The two models can essentially be broken down to the question: does TRβ dimerize in solution, or on the TRE? Evidence has been presented for the dimerization of TRβ in solution via the LBDs. Holloway *et al.* (1990) have been able to cross-link TRα, and TRβ, in solution yielding a product with a molecular weight consistent with being a dimer. Further, TRβ expressed as a fusion with GST and immobilized on microtitre wells retains ^{35}S-labeled TRβ added in the absence of DNA (Kurokawa *et al.*, 1993), although not to the extent that ^{35}S-RXRα is retained. Data presented here indicate that dimerization of T129A with C122A does not occur to a measurable extent on the DNA (Figure 3.35). Likewise, the 32 kD polypeptide produced in conjunction with the *in vitro* translated TRβ does not appear to form DNA binding heterodimers with TRβ (Figure 3.11). A significant portion of the two major products of *in vitro* translation are retained on the column, whereas the 32 kD fragment elutes completely with the low salt loading and wash volumes. Intriguingly, a significant proportion of the 48 and 52 kD polypeptides also wash off at low salt concentrations. Two possible explanations for this are that the binding sites on the column are saturated, or that the 32 kD polypeptide and the two larger polypeptides form heterodimers that do not bind to the affinity column. Since the binding sites on the column are likely to be in large excess over the number of TRβ moleucles loaded, the first option seems unlikely. Therefore, in conjunction with the data discussed above it is likely that TRβ forms binding-impaired heterodimers with mutant or non-full-length receptor forms. Bigler *et al.* (1992) have shown that expression of chicken TRα *in vivo* yields similar polypeptide products initiated from downstream, in-frame methionine codons. The form equivalent to the 32 kD polypeptide in the TRβ translation interferes with binding of full-length receptors, and has a dominant negative phenotype on *trans*-activation by wild-type TRα.

3.4.2 The Effects of Alanine Substitutions Within the Recognition α-Helix on the DNA binding Activity of TRβ

To test the involvement of specific amino acid residues in the DBD of TRβ in DNA binding and *trans*-activation, alanine was systematically substituted for those amino acids in the recognition α-helix. Since the results of this experiment were published (Nelson *et al.*, 1993) the X-ray crystal structure of the DBDs of TRβ and RXRα binding to a DR4 element has been released (Rastinejad *et al.*, 1995). Many of the effects on binding, then, can be discussed on the basis of this structure as well as the previously studied X-ray crystal structures of the GR and ER DBDs (Luisi *et al.*, 1991; Schwabe *et al.*, 1993). First, a number of the alanine substitution mutations can be grouped together as likely disrupting the zinc module structure. Because the two zinc binding domains, CI and CII, fold interdependently to created the overall structure of the DBD, disruption of the first zinc coordination site, as in C122A, or the hydrophobic core of the DBD, as in F125A, F126A or I130A eliminates the ability of the mutant receptor to bind to DNA with high affinity. I130A is not conserved in the GR which has a valine at this position, but is still involved in the hydrophobic core (Härd *et al.*, 1991). The crystal structures reveal that F_{125} and F_{126}, which are completely conserved in both the GR and ER, are centrally located in the hydrophobic core (Luisi *et al.*, 1991; Schwabe *et al.*, 1993; Rastinejad *et al.*, 1995). On the other hand, the residues corresponding to I_{130} are on the periphery, forming part of a shell around the aromatic cluster. The less integral role of I_{130} in the hydrophobic core, than those of F_{125} and F_{126}, is reflected by the fact that residual DNA binding activity is observed for I130A, but not F125A and F126A (Figure 3.24).

Other substitutions aside from the 3 discriminatory residues of the P-box also disrupted binding. A number of these residues are conserved in nature, as well as in function. For example, K_{123} is completely conserved in the four receptors for which there are crystal structures, and contacts the conserved G at position C2 of both the GR and TR/ER half-site (Luisi *et al.*, 1991; Schwabe *et al.*, 1993; Rastinejad *et al.*, 1995). Likewise, R_{128} is

conserved and contacts the conserved G at C-5. Substitution of either K_{123} or R_{128} with alanine decreases DNA binding affinity of TRβ by over two orders of magnitude, consistent with the crucial role of this residue in sequence recognition and binding of TRβ. The R127A mutant in TRβ also exhibits diminished DNA binding, however, this position is not completely conserved among the various receptors. In fact, the other three receptors have a lysine residue. Despite the lack of conservation, R_{127} of TR makes similar contacts to that of the equivalent lysine in the ER (Schwabe *et al.*, 1993; Rastinejad *et al.*, 1995). In both cases, direct and water-mediated contacts are made to the G and T residues at positions C4 and C5.

The two remaining non-P-box positions that were substituted with alanine are not conserved amongst the four receptors for which there are crystal structures. Substitution at both these positions significantly reduced binding of the mutant receptor, but not to the level of those residues previously discussed. T_{129} is identical in RXR, but in GR and ER it is replaced by an alanine and a serine, respectively. The role of this residue although obscure in the crystal structure is clearly somewhat different in the GR and TR, since TR does not maintain wild-type binding when substituted with the residue native to GR at this position. The situation for Q_{131} is somewhat different. This position is identical in ER, but is a glutamate and a arginine in GR and RXR, respectively. Despite the fact that this position is quite divergent in nature between the various receptors, substitution with alanine has a measurable effect on DNA binding affinity. In this case, however, the crystal structure indicates that Q_{131} makes direct contact with the phosphate backbone, and water-mediated contact with R_{126} (Rastinejad *et al.*, 1995), explaining the loss of DNA binding activity observed for Q131A.

The three discriminatory residues of the P-box, EGG for the TR, were of particular interest in the functional studies that were carried out. In the crystal structure of the GR DBD-DNA complex, only the third P-box residue of the GR (the valine of the GSV P-box sequence) was found to be involved in DNA binding at the level of base pair discrimination

(Luisi *et al.*, 1991). In contrast, in the ER, the first two positions of the EGA P-box make DNA contacts. E_{120} of ER contacts bases C-3 and C-4, the latter being a water-mediated contact, and G_{121} contacts the phosphate backbone (Schwabe *et al.*, 1993). Similar contacts are made within the TR DBD-DNA complex, although they are more extensive due to more elaborate water-mediated bridging. The dramatic drop in the DNA binding affinity of E120A relative to wild-type TRβ follows from these data. G_{120} makes contacts through the α-N, which may explain why substitution with alanine effects DNA binding less profoundly.

The third position of the P-box is an interesting case, because the effects of substituting alanine at this position on DNA binding affinity are dependent on the element to which the mutant is binding. Figure 3.24 indicates that while G124A binds to TRE_{IR0}, TRE_{LYS} and TRE_{rGH3} with similar affinities relative to wild-type, that TRE_{MAL} is bound with significantly lower affinity. Dr. Colleen Nelson and Mr. Stephen Hendy went on to reveal that the presence of an A residue at top-strand position of C4 (half-site sequence, AGGACA) is inhibitory to binding of TRβ mutants with alanine in the third P-box position, an effect that is largely due to the methyl group of the T residue on the bottom strand (Nelson *et al.*, 1994). In the absence of the A residue at position C4, binding of either G124A or G121A/G124A is wild-type or better (Table 3.1). Thus, in the absence of the methyl group provided by a T at C-4 no significant interference of DNA binding occurs. The third position of the P-box can be substituted with up to 10 of the amino acids, aside from glycine and alanine, and still maintain near wild-type DNA binding activity (Nelson *et al.*, 1995a) although no form of TRβ , other than wild-type, can bind an EvR composed of AGGACA half-sites with high affinity. It appears that the potential importance of the glycine residue at the third P-box position of TRβ may be in permitting the binding of TRβ to elements with this half-site, rather than in restricting the binding of TRβ to a particular sequence per se.

3.4.3 The Effects of Alanine Substitutions Within the Recognition α-Helix on Transcriptional Activation

The effects of the alanine substitutions on transcriptional activation generally paralleled their effects on binding, with the notable exception of G121A, which was defective in *trans*-activation, but not DNA binding. While the other alanine substitution mutants of TRβ that had detectable DNA binding were able to activate transcription from a TRE, G121A was unable to do so. For example, R127A has at least a 10-fold lower affinity for TRE_{LYS} than does G121A, and yet, it is able to carry out T_3-dependent *trans*-activation much more efficiently. G121A/G124A, which has a lower K_d for DNA binding than G121A (Table 3.1), is also deficient in *trans*-activation. From these comparisons it is apparent that the substitution of G_{121} with alanine differentially affects transcriptional activation far more than DNA binding.

The phenotype of the G121A mutant TRβ defines an uncoupling of DNA binding and *trans-activation* activities within the DBD. A similar phenomenon was described for the conserved lysine residue at position 123 when its counterpart in the GR was changed to a glycine (Hollenberg and Evans, 1988). In our experiments, when the equivalent conserved lysine was substituted with methionine the mutant TRβ was unable to bind DNA or *trans*-activate gene expression. This result suggests that the nature of the amino acid substitution within the different receptors may be important in uncoupling the DNA binding and *trans*-activation activities within the DBD. Other mutations in the DBD of GR that reduce transcriptional activation but not DNA binding have been described that occur in the tip of the second zinc finger (Schena *et al.*, 1989), which is spatially close to the beginning of the recognition helix in the folded GR DBD (Luisi *et al.*, 1991). Considering the recent identification of cofactors involved in active repression or activation by TR (see section 1.3.2.1), the three-dimensional clustering of these phenotypically related mutations, and the fact that none seem to be deeply buried within the zinc module structure or the DNA-

protein interface, these mutations may influence the binding of a cofactor which mediates T_3-dependent *trans*-activation by TR.

4.0 Thyroid Hormone Receptor Binding to Direct Repeat Elements Investigated Using Methylation Interference

4.1 INTRODUCTION

The role of thyroid hormone receptor in gene regulation is extremely complicated due to the relatedness of other nuclear receptors at every level, from half-site sequence and configuration to protein dimerization. While individual nuclear receptor types must restrict their activity to appropriate genes, a certain amount of cross-talk is maintained, and interaction with other non-receptor nuclear factors influences transcription. Defining the features involved in the discrimination of appropriate from non-appropriate hormone response elements for distinct receptor complexes is fundamental to describing, precisely, thyroid hormone receptor activity in gene regulation, physiology and disease.

Examples of discrimination at the level of half-site sequence and configuration have already been given in this work. The AGAACA half-site is specific for some steroid hormone receptors, while AGGTCA is a high affinity site for TR and others (Glass, 1994). A further divergence in receptor specificity occurs due to the inability of certain receptors in the latter class to bind the related sequence, AGGACA. Likewise, half-site configuration provides another level of discrimination. A central paradigm for nuclear receptor regulation of gene expression is the 3-4-5 rule, in which direct repeats spaced by 3, 4 and 5 base-pairs are HREs for vitamin D3, T_3 and retinoic acid, respectively (Umesono *et a.*, 1991). Another example involves IRs; IR0 is a TRE, whereas IR3 is an ERE (Glass *et al.*, 1988).

TR, as a homodimer, is not particularly discriminatory with respect to the binding of different half-site configurations. A wide range of spacing variation is tolerated with each orientation of half-sites. However, the EvR configuration is bound with highest affinity by TR (Kurokawa *et al.*, 1993; Williams *et al.*, 1994), and EvRs have some unique properties with respect to TR activity. Firstly, EvR6 sequences have been well characterized as potent silencers of transcription in the absence of T_3 (Baniahmad *et al.*, 1990; Piedrafita *et al.*,

1995). Secondly, the stability of the TR complex on an EvR6 is remarkably high, with a half-life for dissociation of 40 minutes (Piedrafita *et al.*, 1995). Finally, unlike DR and IR sequences, a poor correlation is observed between homodimer binding and *trans*-activation from mutated EvRs (Williams *et al.*, 1994). These observations, taken together, imply a unique, but as yet poorly understood, role for the TR homodimer in T_3 biology. In further support of a distinct role for the homodimer is the identification of natural TREs with an EvR configuration from such genes as chicken lysozyme (Baniahmad *et al.*, 1990) and human myelin basic protein (Farsetti *et al.*, 1992). Whether the homodimer also has a function in gene regulation from direct repeats is unknown, although it is certainly capable of binding to these elements with substantial affinity (Carlberg, 1993).

Discrimination of half-site configurations by TR/RXR heterodimers is better understood, particularly for direct repeats. The X-ray crystal structure of the TR and RXR DBDs bound to a DR4 element reveals a great deal of interdependent interactions that contribute to the positive cooperativity of binding of the heterodimer to this element (Rastinejad *et al.*, 1995). These interactions are predicted to be spacing dependent, and reduced if the spacer is increased or decreased or decreased by one base-pair. Molecular modeling of the vitamin D3 receptor DBD or the retinoic acid receptor DBD in a heterodimer with the RXR DBD predicts that they would make similar cooperative interactions on a DR3 and DR5, respectively (Rastinejad *et al.*, 1995). Binding studies confirm the importance of the DBD interactions; TR/RXR heterodimers have been shown to bind DR4 with higher affinity than DR3 and DR5 (Umesono *et al.*, 1991; Shulemovich *et al.*, 1995).

In order to gain further understanding of the differences and possible interplay between TR homodimer and TR/RXR heterodimer activity on DR-type elements, EMSAs and a methylation interference technique were employed to examine the interaction of both these receptor complexes with DR3, DR4 and DR5 sequences. In the process an incidental, competitive binding site for the TR homodimer was discovered that underscores differences

between the two receptor complexes. A cryptic everted repeat was identified that competed with the direct repeat for binding of the homodimer, but not the heterodimer. The ability of this non-consensus everted repeat to compete for binding with an idealized DR led to the investigation of whether direct repeats were, in fact, recognized as poor everted repeats.

4.2 MATERIALS AND METHODS

General methods and materials for routine recombinant DNA work, electrophoresis, etc. are given in Chapter 2.0. What follows are the specific techniques used for experiments described within this chapter. Where "standard" conditions or procedures are referred to, they may be found in Chapter 2.0.

4.2.1 DNA constructs and probes.

Complementary oligonucleotides with *Bam*H I sticky ends were synthesized with an Applied Biosystems (Foster City, CA) DNA synthesizer and consisted of the following top strand sequences: DR3, AGCTTCAGGTCAAGGAGGTCAGAG; DR4, AGCTTCAGGTCACAGGAGGTCAGAG; and, DR5, AGCTTCAGGTCACCAGGAGGTCAGAG. For cloning purposes, the oligonucleotides were annealed and the 5' overhangs filled-in using the Klenow fragment of DNA polymerase I from *E. coli* . The filled-in oligonucleotides were ligated into either the *Sma* I or filled-in *Bam*H I site, as described below, of pUC18 or pUC19. The resulting constructs were analyzed by DNA sequencing to confirm the sequence of the inserts, and that the orientation of each insert with respect to the pUC sequences was the same. Radioactively labeled fragments for EMSA and methylation interference were generated by cutting plasmid DNA with *Eco*R I, filling-in the resulting overhangs using [α-^{32}P]dATP in the nucleotide mixture, and then further digesting with *Hin*D III. The resulting DNA probe was purified from a native polyacrylamide gel. To label the other strand, the order of *Eco*R I and *Hin*D III digestions was reversed.

4.2.2 *In vitro* translation and EMSA.

In vitro translation of receptor proteins and EMSA were carried out as described in chapter 2.

In order to quantify the relative binding of different probes by receptor complexes, autoradiographs were used as templates to excise bound DNA bands from dried EMSA gels. Each band was then assayed with a scintillation counter.

4.2.3 Methylation Interference.

The methylation interference protocol was performed as described in Sambrook *et al.* (Sambrook, 1989), and based on Maxam and Gilbert (1980). To roughly 1 pmol of singly end-labeled DNA probe in 200 µl of DMS buffer (50 mM NaCacodylate, 1 mM EDTA, pH 8.0), 1 µl of DMS was added and incubation carried out for 5 minutes at room temperature. 50 ml of DMS Stop solution (1.5 M sodium acetate, pH 7.0, 1.0 M β-mercaptoethanol, and 100 µg/ml tRNA) was added and the DNA precipitated with 750 µl 95 % ethanol. The methylated DNA was subsequently subjected to three rounds of resuspension in 250 µl 0.3 M sodium acetate, 1 mM EDTA, and precipitation with ethanol. Finally, the DNA was washed with 70 % ethanol, dried and resuspended in water. EMSA conditions were identical to Set II standard conditions except that binding reactions were scaled up to 30 µl total volume, 8 µl of which was rabbit reticulocyte lysate, and roughly 300,000 cpm of methylated probe was added to each sample. After electrophoresis, gels were autoradiographed at 4 °C for 4 hours before bands corresponding to free and bound DNA were excised and rotated overnight in elution buffer (1 mM EDTA, 0.6 M ammonium acetate, 0.1 % sodium dodecyl sulphate). Pairs of samples corresponding to free and bound bands from the EMSA gel were equalized on the basis of cpm in preparation for loading on the denaturing gel. The samples were then heat denatured and run out on a 15% acrylamide, 8.3 M urea gel. Gels were dried and autoradiographed with preflashed film. Densities of the bands appearing on the autoradiographs were determined with a Molecular Dynamics Personal Densitometer. The sum of band densities in the bound lane was equalized arithmetically to that of the free lane, and interference values calculated for each band position. The interference value was calculated as 100*(Free-Bound)/Free, where Free is the density of the band in the lane corresponding to the free DNA from the EMSA gel and Bound is the adjusted density of the band in the lane corresponding to the bound DNA.

4.3 RESULTS

4.3.1 Binding of TRβ Homodimers and Heterodimers to Direct Repeat Sequences Varying in Spacer Length

Oligonucleotides containing direct repeats of the idealized core hexamer spaced by 3, 4 or 5 base pairs were cloned into the *Sma* I site of pUC19 and excised by *Eco*R I/*Hin*D III digestion generating 85-87 base-pair fragments (sDR3, sDR4 and sDR5; Figure 4.1) for use in DNA binding studies. The upstream and downstream half sites of the direct repeats are referred to as half sites A and B, respectively and regardless of the spacer length between them. As shown in Figure 4.1, the sequences flanking core hexamers A and B are not identical; however, they remain constant in the three DR probes used with the exception of position F3 (see Figure 1.7 for half site position reference system) of half-site A, which is an adenine in DR3, and a cytosine in DR4 and DR5 due to the distinct spacer in each probe. Thus, references to spacing-dependent differences between methylation interference patterns are made with the proviso of this one sequence difference.

Binding of the radioactively labeled sDRs by TRβ results in a major band corresponding to the binding of the homodimer under Set II standard conditions (Figure 4.2). As bands corresponding to the binding of a single monomer are not seen under the conditions of this assay the complex is presumed to involve cooperative interactions between the two TRβ monomers, and is thus referred to as a homodimeric complex. The homodimer bound sDR4 and sDR5 to a similar extent (lanes 5 and 8), while sDR3 was bound poorly (lane 2). The additional presence of a molar excess of RXRα in the binding reaction resulted in the formation of TRβ/RXRα heterodimers, which produce a complex with the DNA of lower mobility than the homodimer/DNA complex. In contrast to the homodimer, the heterodimer clearly bound sDR4 better than sDR3 or sDR5, consistent with previous results. These binding results are in general accordance with those of other workers who have examined TR binding to direct repeats (Umesono *et al.*, 1991; Shulemovich *et al.*, 1995).

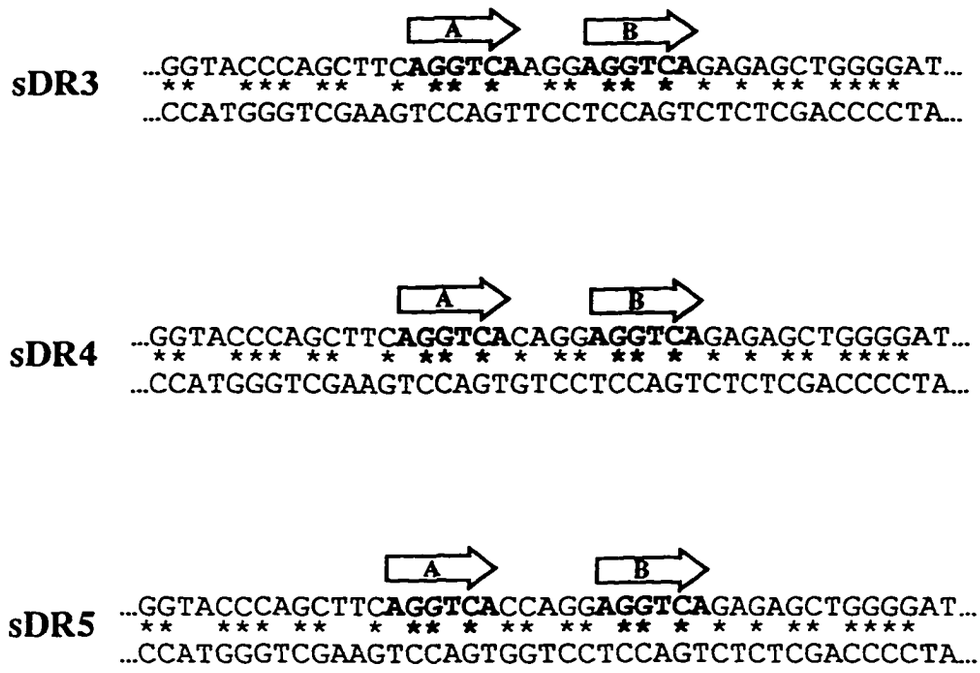


Figure 4.1: Partial sequences of the sDR probes used for EMSA and methylation interference. For sDR3, sDR4 and sDR5 the sequence of both strands are given and the position of guanine residues, on either strand, are indicated by asterisks. The half-sites, A and B, that comprise the direct repeat are in boldface and indicated by the arrows. Sequence not shown is part of the pUC19 polylinker between the *Eco*R I and *Hin*D III sites.

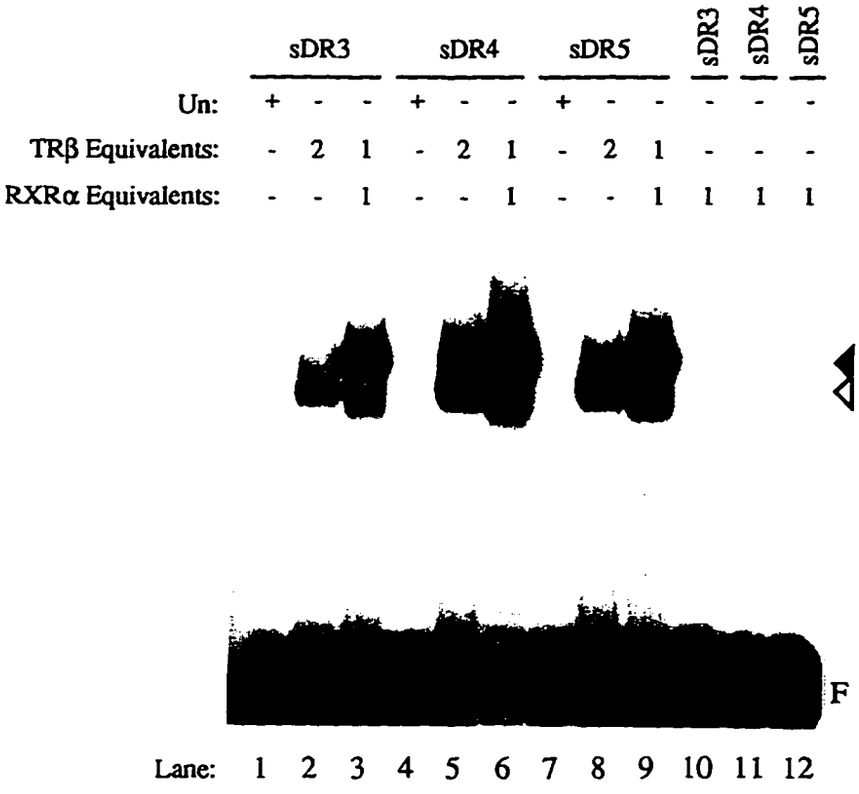


Figure 4.2: Binding of TRβ homodimers and TRβ/RXRα heterodimers to the DNA probes sDR3, sDR4 and sDR5. ^{32}P-labeled sDR3, sDR4 and sDR5 probes were incubated with TRβ and/or RXRα translated *in vitro* in RRL. Un indicates unprogrammed RRL. EMSA was performed under set II standard conditions. The autoradiogram was produced by overnight exposure of film to the EMSA gel.

4.3.2 Methylation Interference Reveals Differences Between the Binding of TRβ Homodimers and Heterodimers to the sDRs.

Methylation interference is a technique which indicates guanine residues that are involved in, or spatially close to, interactions via the major groove (Figure 4.3). The DNA sequence of interest is treated with dimethyl-sulphate, a powerful alkylating agent, such that each DNA molecule is methylated no more than once. An EMSA is performed with the alkylated DNA and the bound and free DNA collected. This step segregates DNA which has been methylated at a position such that receptor binding is inhibited from that which has been methylated at a site which does not interfere with binding. Treatment with piperidine cleaves DNA that has been methylated at the N7 position of a guanine residue. Resolution of piperidine-treated DNA from the bound and free bands of the EMSA on a denaturing gel gives bands corresponding to each G residue in the probe. However, due to the segregation of DNAs in the EMSA step, bands corresponding to G residues which inhibit binding when methylated will be underrepresented in the DNA from the bound fraction, and over-represented in the free fraction. Thus, the methylation interference procedure translates interference with binding resulting from the introduction of a novel methyl group onto the DNA into a visual pattern that reflects the involvement of G residues in DNA binding.

Methylation interference analysis was employed to identify differences between the interactions of homodimers and heterodimers with the different sDRs (Figure 4.4). For all three sDR sequences, methylation of guanine residues in either strand of half-site A strongly interfered with binding of TRβ homodimers; however, methylation of the equivalent residues within half-site B interfered with homodimer binding to a lesser extent. Homodimer binding to sDR3, in particular, showed little sensitivity to methylation of half-site B. The binding of TRβ/RXRα heterodimers to the direct repeats, on the other hand, was inhibited to a similar extent by methylation of either half-site.

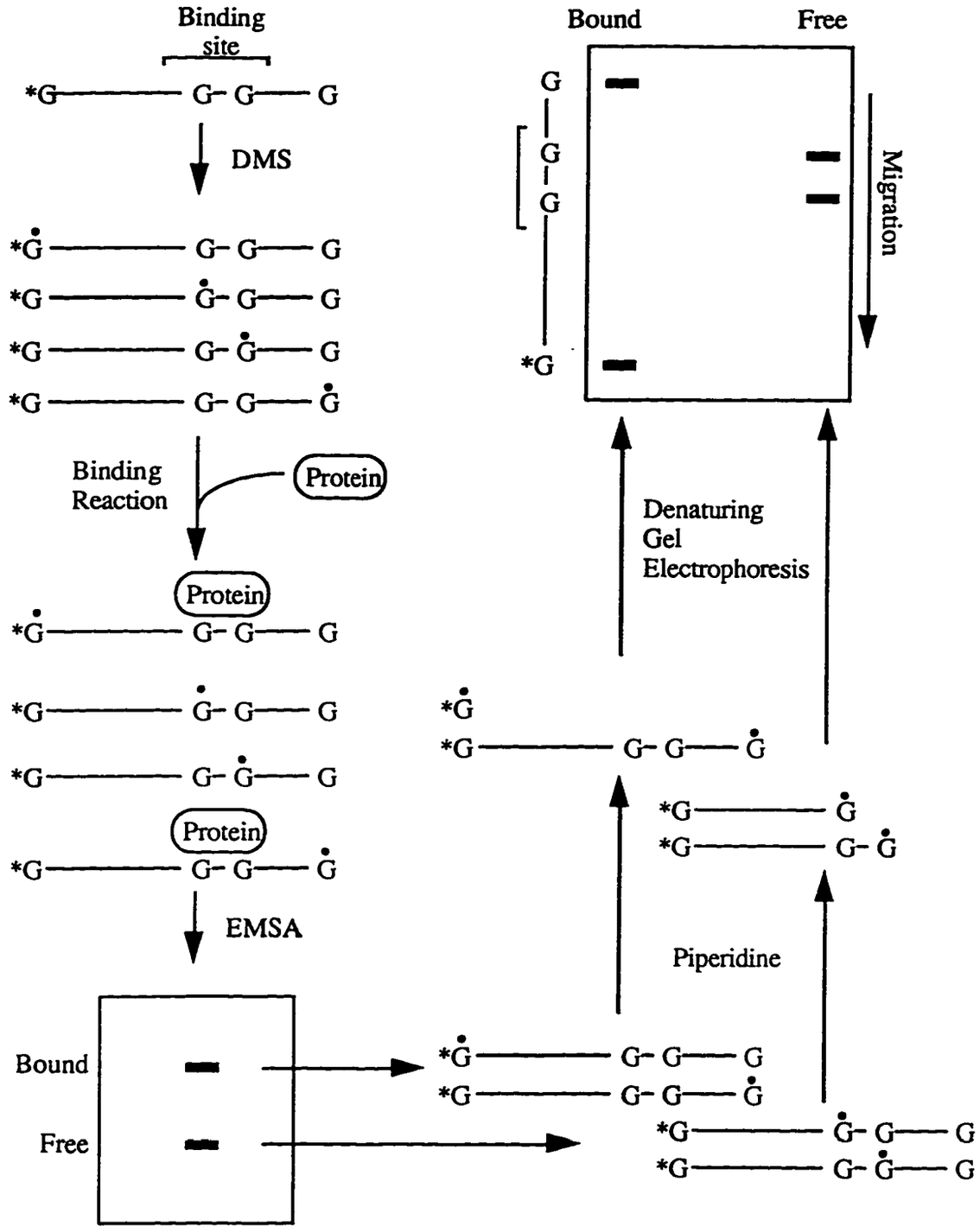


Figure 4.3: A schematic representation of the methylation interference procedure. Details are given in the text. This figure uses a generic sequence containing four G residues, two of which are within the protein binding site, as an illustration. Symbols used include: an asterisk, representing the ^{32}P-label at one end only of the DNA probe, and • which indicates methylation of a G residue.

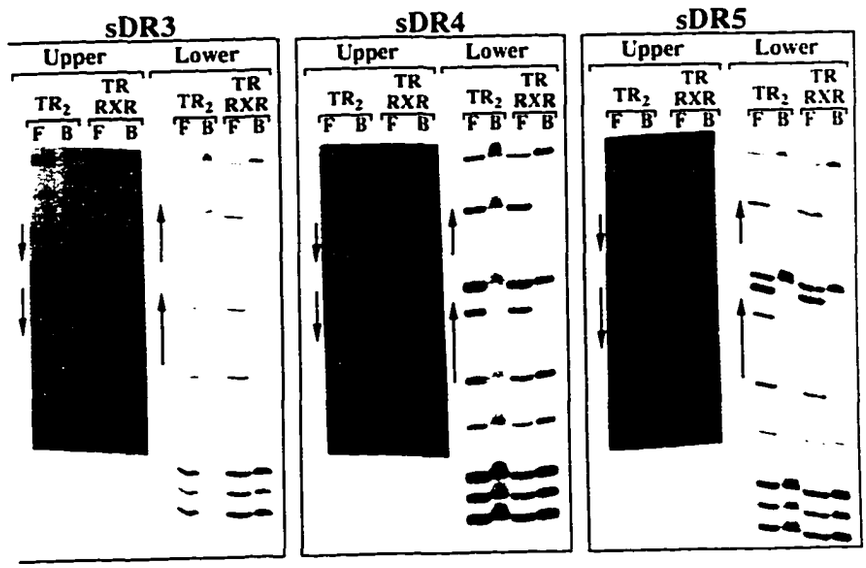
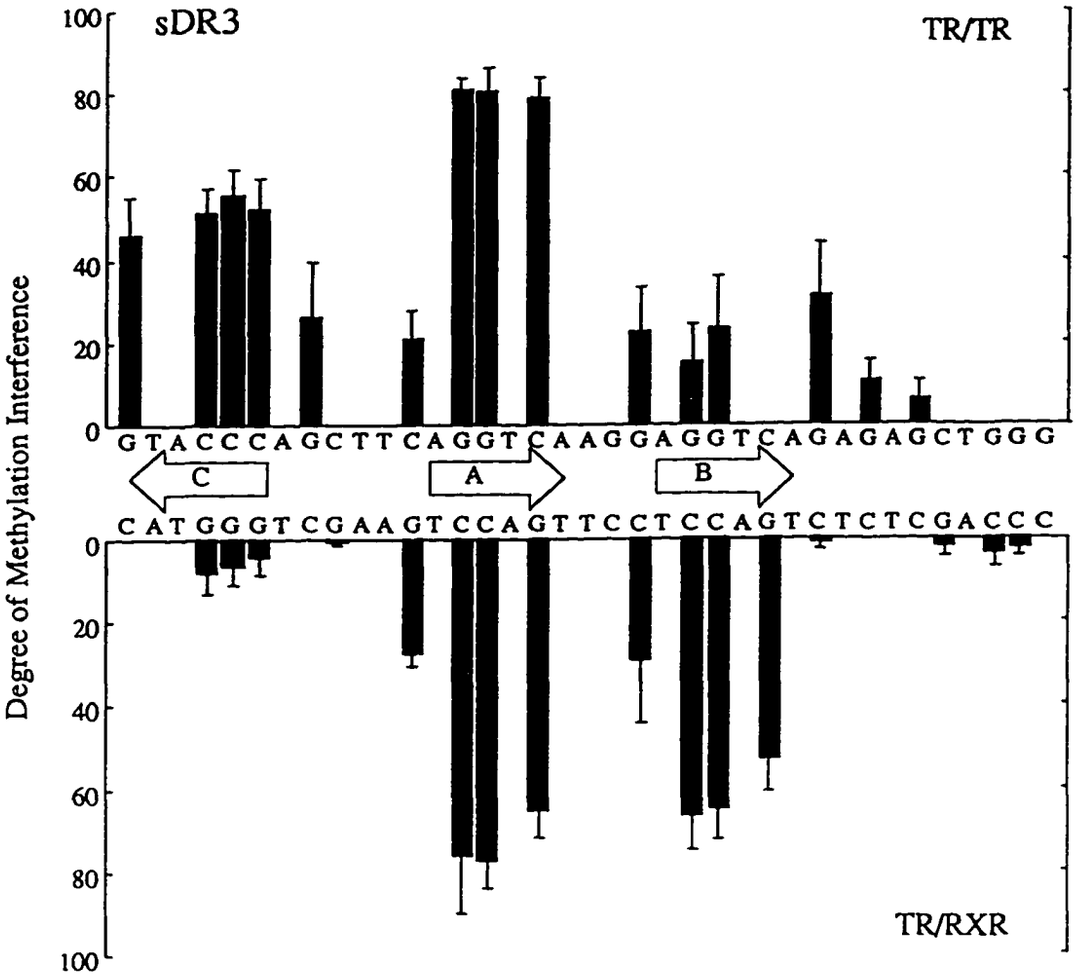


Figure 4.4: Methylation interference of the binding of TRβ homodimers and TRβ/RXRα heterodimers to sDR3, sDR4 and sDR5 sequences. Methylation interference patterns for the upper and lower strand of sDR3, sDR4 and sDR5. The arrows to the left of each section of autradiograph represent the upstream half-site A and the downstream half-site B.

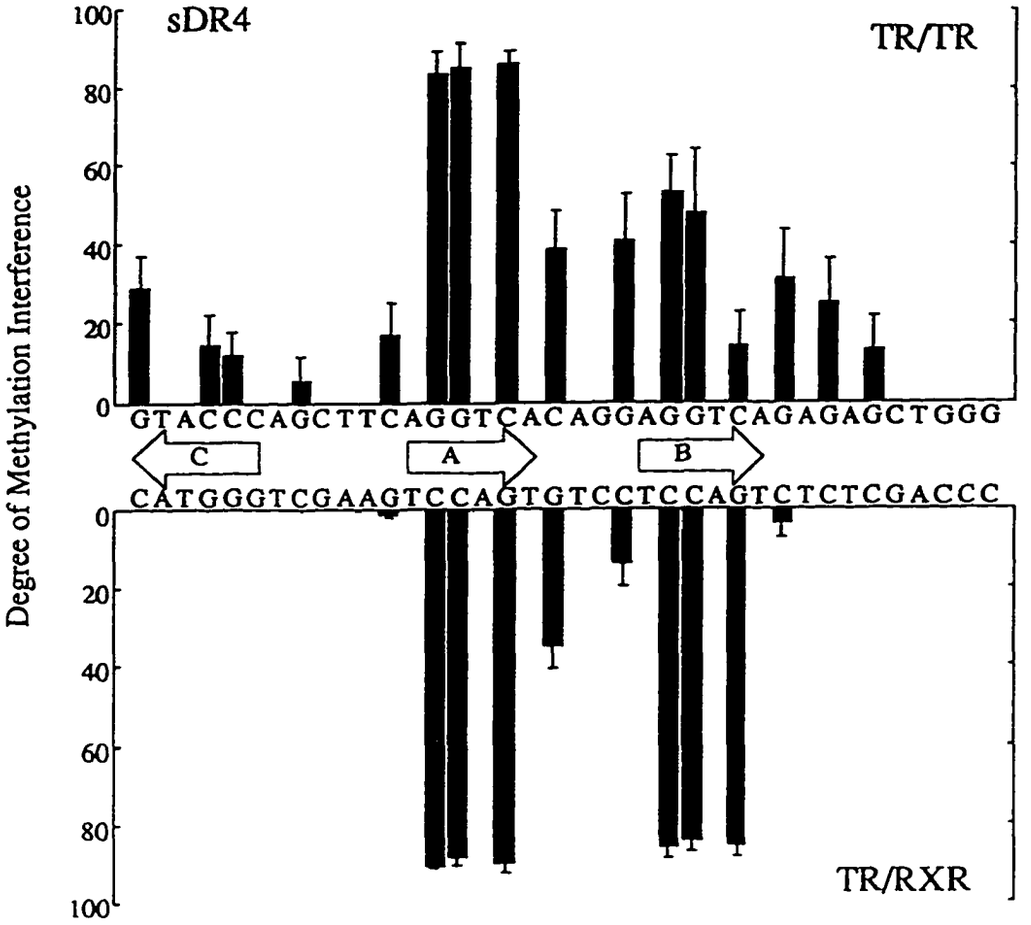
Methylation interference denaturing gel autoradiographs were scanned using a laser densitometer to allow comparison of the interference patterns for the receptor complexes on the different probes. Interference values representing the difference in intensities of bands from free and bound DNA populations collected for each binding reaction were calculated, reflecting the degree of interference caused by methylation of each residue of the sDR probes. The degree of homodimer or heterodimer binding interference produced by methylation of guanines in either strand of the sDR sequences is summarized in Figure 4.5. This analysis confirms that methylation of residues within half-site A interferes with homodimer binding significantly more than does methylation of residues within half-site B, for all three sDRs (Figure 4.5A-C). This is most strikingly the case for sDR3 (Figure 4.5A); methylation of residues within half-site B of this probe results in a minor degree of binding interference. In contrast, methylation of a number of guanines upstream of half-site A of sDR3 interferes more with homodimer binding than does methylation of residues within half-site B.

Inspection of the sequence upstream of the direct repeats reveals two potential, overlapping half-sites on the opposite strand, thus forming an everted repeat along with half-site A. The everted repeats have a spacing of 6 and 5 base-pairs (half-sites C and D, respectively; Figure 4.6), and they match the consensus hexameric sequence at 4 and 3 positions, respectively. Comparison of the methylation interference patterns obtained for homodimer and heterodimer binding to each sDR probe (Figure 4.5) indicates methylation of residues within half-sites C and/or D interferes with binding of the homodimer, but not of the heterodimer. The homodimer has previously been characterized as having high binding affinity for everted repeats with various numbers of spacing base-pairs (Carlberg, 1993; Kurokawa *et al.*, 1993). Hence, it is proposed that methylation within half-sites C/D interferes with the binding of TRβ homodimers to an everted repeat comprised of either half-sites A and C, A and D, or, mutually exclusively, to both pairs. Since it was not

(A)


sDR3
TR/TR
TR/RXR
Degree of Methylation Interference
100
80
60
40
20
0
G T A C C C A G C T T C A G G T C A A G G A G G T C A G A G A G C T G G G
C A T G G G T C G A A G T C C A G T T C C T C C A G T C T C T C G A C C C
C
A
B

(B)


sDR4
TR/TR
TR/RXR
Degree of Methylation Interference
100
80
60
40
20
0
GTACCCAGCTTCAGGTCACAGGAGGTCAGAGAGCTGGG
C
A
B
CATGGGTCGAAGTCCAGTGTCCTCCAGTCTCTCGACCC

(C)

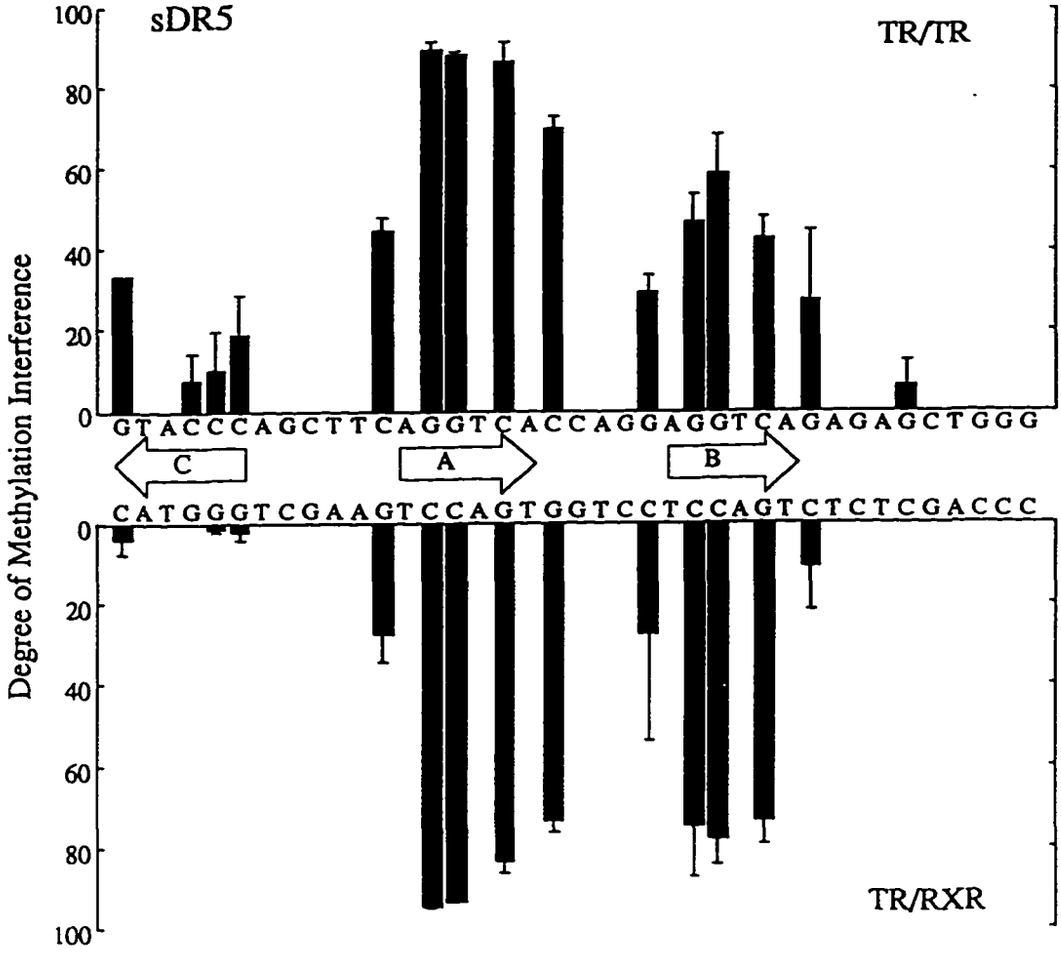
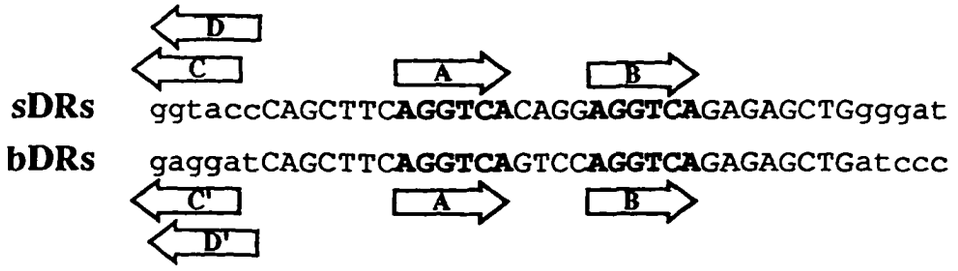


Figure 4.5: Laser densitometric analysis of the methylation interefence patterns of the binding of TRβ homodimers and TRβ/RXRα heterodimers to sDR3, sDR4 and sDR5 sequences. Interference values ranging from 0 to 100 for each guanine, regardless of strand, are shown above the sequence for the homodimer (TR/TR), and below the sequence for the heterodimer (TR/RXR), for (A) sDR3, (B) sDR4, and (C) sDR5. The higher the interference value, the greater the disruption of binding due to methylation of a particular guanine. Arrows indicate the positions of half-sites C, A, and B, from left to right.

(A)



(B)

Half-site	Sequence	Matches
	123456789	
A	TCAGGTCAC	6
B	GGAGGTCAG	6
C	CTGGGTACC	4
C'	CTGATCCTC	2
D	GCTGGGTAC	3
D'	GCTGATCCT	3
High Affinity	TGAGGTCAC	
	Ccg	
	ga	

Figure 4.6: Cryptic half-site sequences in the sDR and bDR probes. (A) The top-strand sequences of sDR4 and bDR4 are given, with putative half-sites indicated by arrows. Sequence differences between the sDR and bDR probes are in lowercase. (B) Matches between the cryptic half-sites and the high affinity core hexamer. Flanking positions are also shown for comparison.

determined whether the interaction involves half-site C, half-site D or both half-sites, this region is referred to as half-site C/D.

The methylation interference patterns for the homodimer interaction with the sDR4 and sDR5 probes indicate that half-site C/D is not occupied to the same extent as it is with the sDR3 probe (Figure 4.5). Not only is the absolute degree of interference resulting from methylation within half-site C/D less for sDR4 and sDR5 than for sDR3, but with the former probes it is also lower than the degree of interference resulting from methylation within half-site B. This phenomenon is suggestive of a mutually exclusive occupation by the homodimer of either half-sites A and C/D, or A and B.

4.3.3 The Effects of Eliminating Half-Site C/D on the Relative Degree of Methylation Interference of the Two Directly Repeated Half-Sites.

The same complementary oligonucleotides used to create the sDRs were made blunt-ended by filling in their sticky ends. They were then cloned into the filled-in *Bam*H I site of pUC19, and excised for study by *EcoRI*/*Hin*D III digestion, yielding bDR3, bDR4 and bDR5. These fragments contain the direct repeats flanked by sequences which provide less basis for an everted half-site. The upstream putatitve half-sites C' and D' of the bDR probes, which are at equivalent positions to half-sites C and D of the sDRs, match the high affinity hexameric site at only 2 and 3 out of 6 positions, respectively (Figure 4.6). The binding of these DNA sequences by TRβ and TRβ/RXRα is similar to the binding of the sDRs, as indicated by EMSA (Figure 4.7). The distinct upstream sequence present in the bDRs results in significantly different methylation interference patterns for the homodimer than those observed with the sDRs (Figure 4.8; and, compare Figure 4.5 with Figure 4.9). Methylation within half-site C'/D' does not interfere with homodimer binding to the bDRs, and the degree of interference observed due to methylation within half-sites A and B of each probe is similar. In fact, the methylation interference patterns for the bDRs with the homodimer is very similar to those with the heterodimer in terms of the relative sensitivity of binding to methylation of half-sites A and B. The methylation interference pattern

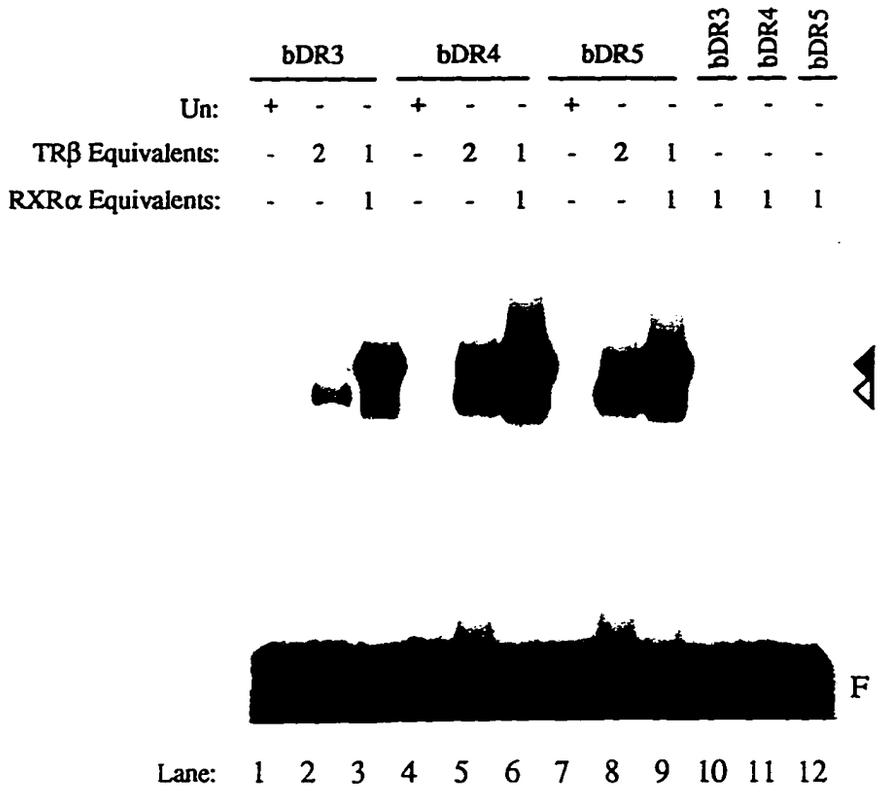


Figure 4.7: Binding of TRβ homodimers and TRβ/RXRα heterodimers to the DNA probes bDR3, bDR4 and bDR5. ^{32}P-labeled bDR3, bDR4 and bDR5 probes were incubated with TRβ and/or RXRα translated *in vitro* in RRL. Un indicates unprogrammed RRL. EMSA was performed alongside that in Figure 4.2, using identical conditions and materials, with the exception of the DNA probe. The autoradiogram was produced by overnight exposure of film to the EMSA gel.

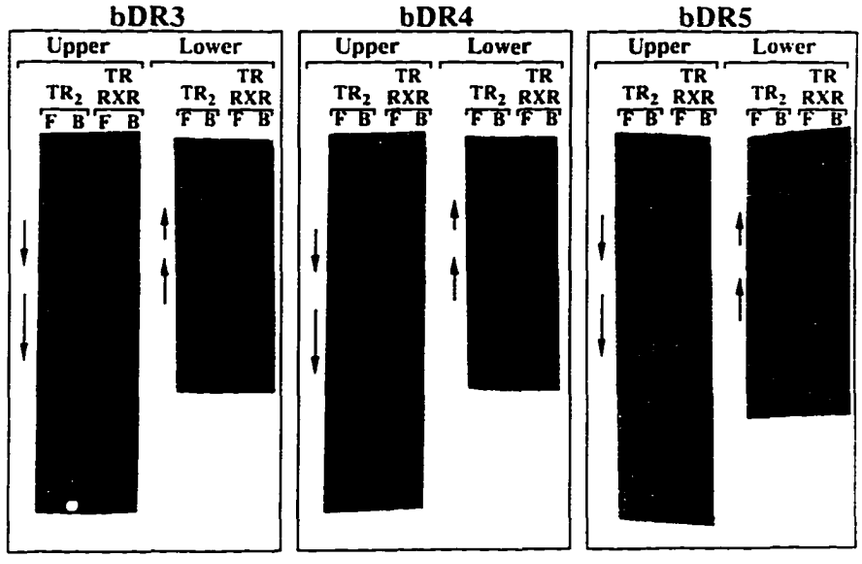
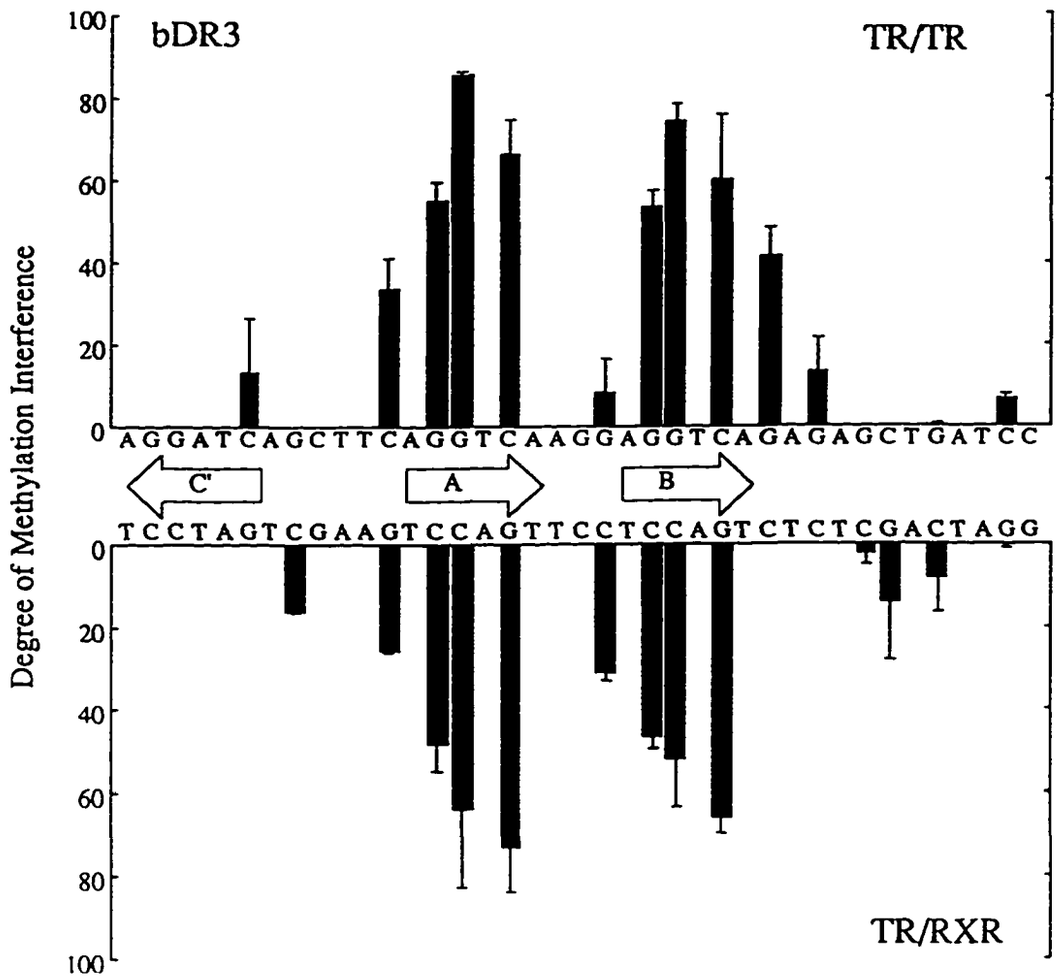
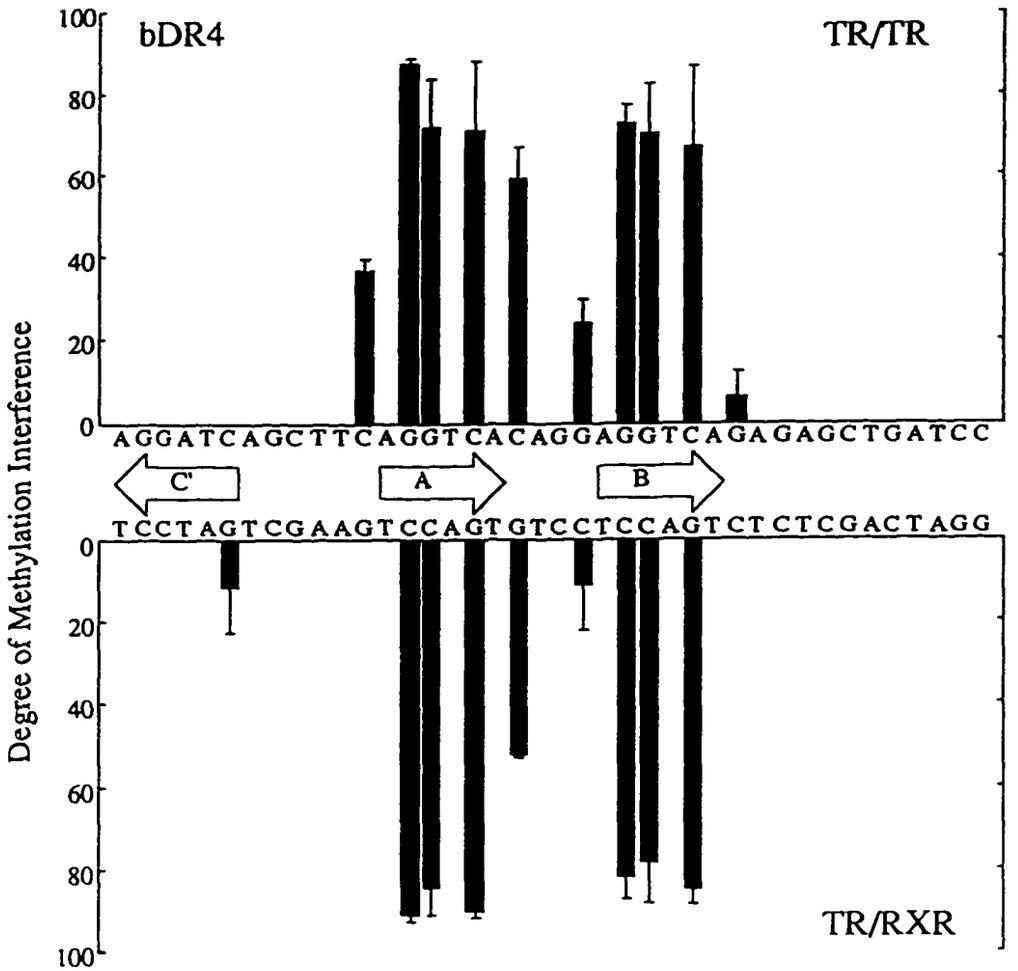


Figure 4.8: Methylation interference of the binding of TRβ homodimers and TRβ/RXRα heterodimers to bDR3, bDR4 and bDR5 sequences. Methylation interference patterns for the upper and lower strand of bDR3, bDR4 and bDR5. The arrows to the left of each section of autradiograph represent half-sites A and B.

(A)


bDR3
TR/TR
TR/RXR
Degree of Methylation Interference
100
80
60
40
20
0
AGGATCAGCTTCAGGTCAAGGAGGTCAGAGAGCTGATCC
TCCTAGTCGAAGTCCAGTTCCTCCAGTCTCTCGACTAGG
C'
A
B

(B)


bDR4
TR/TR
TR/RXR
Degree of Methylation Interference
100
80
60
40
20
0
AGGATCAGCTTCAGGTCACAGGAGGTCAGAGAGCTGATCC
C'
A
B
TCCTAGTCGAAGTCCAGTGTCCTCCAGTCTCTCGACTAGG

(C)

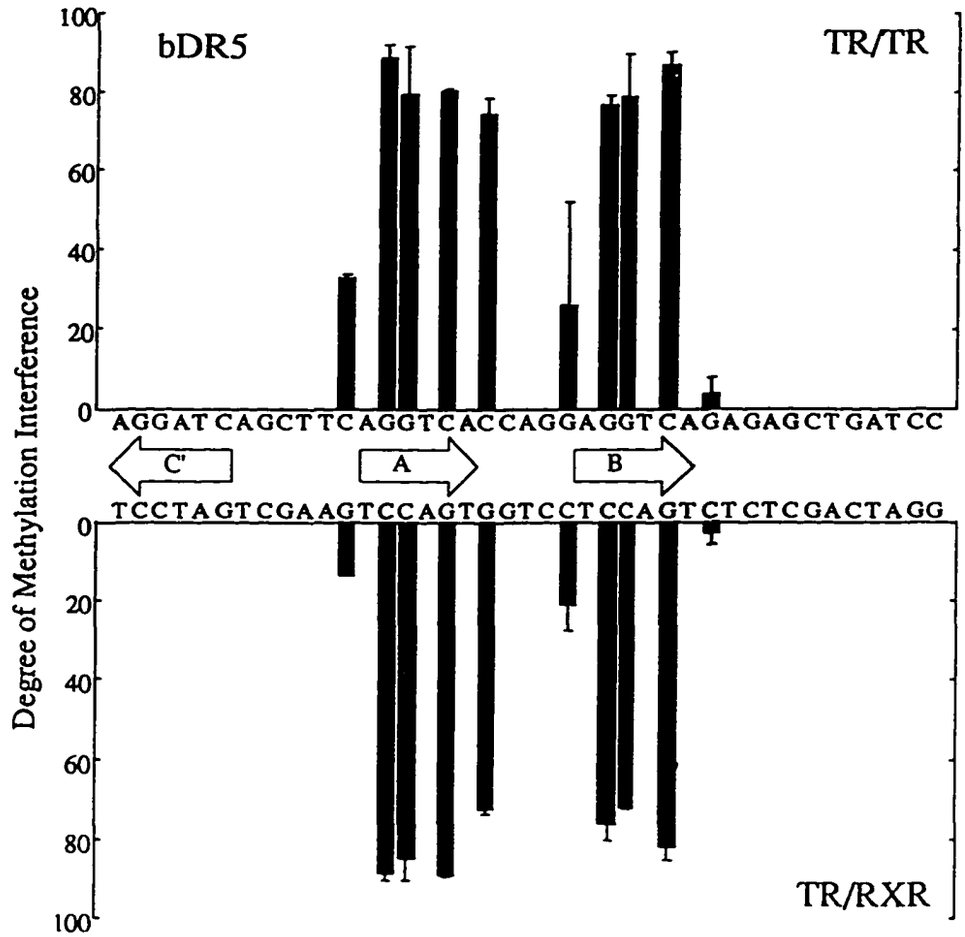


Figure 4.9: Laser densitometric analysis of the methylation interefence patterns of the binding of TRβ homodimers and TRβ/RXRα heterodimers to bDR3, bDR4 and bDR5 sequences. Interference values ranging from 0 to 100 for each guanine, regardless of strand, are shown above the sequence for the homodimer (TR/TR), and below the sequence for the heterodimer (TR/RXR), for (A) bDR3, (B) bDR4, and (C) bDR5. The higher the interference value, the greater the disruption of binding due to methylation of a particular guanine. Arrows indicate the positions of half-sites C, A, and B, from left to right.

resulting from the interaction of the heterodimer with the direct repeats is not influenced by the different upstream sequence, since the sDR and bDR patterns with the heterodimer are very similar (cf. bottoms of Figures 4.5A-C with those of Figures 4.9A-C). This is consistent with heterodimer binding occurring strictly to the direct repeat of both probes.

In order to eliminate the everted upstream half-sites altogether, the oligonucleotides (oDRs; Figure 4.1) originally cloned into pUC19 to generate the sDR and bDR probes were studied. EMSA of the oDR3, oDR4 and oDR5 was similar to that of the sDRs and bDRs (Figure 4.10). As was the case for the bDRs, the sensitivities of half-sites A and B to methylation interference were roughly equivalent for the homodimer and the heterodimer (Figure 4.11). These results further support the binding of the TRβ homodimer to the sDRs by occupying either the everted repeat (half-sites A and C/D), or the direct repeat (half-sites A and B).

4.3.4 The Relative Affinities of TRβ Homodimers and Heterodimers for DRs in the Presence or Absence of the Half-Site C/D.

The methylation interference data suggest that the TRβ homodimer has a significant affinity for the everted repeat comprised of half-sites A and C/D. On sDR3, in particular, the everted half-site appears to be occupied in preference to the direct repeat half-site B. To establish whether interaction with the everted repeat contributes substantially to the overall affinity of the sDR probes, the relative amounts of probe bound at identical receptor concentrations were determined by EMSA for all three types of probe (sDR, bDR and oDR) with all three DR spacing variants. The graph shown in Figure 4.12 indicates that the ratio of receptor complex binding to oDRs and to bDRs (oDR bound:bDR bound) is constant for homodimeric and heterodimeric complexes on the DR3, DR4 and DR5 sequences. This is as expected, since the relative affinities of homo- and heterodimers for each direct repeat sequence should be the same in either the oDR or bDR context, in which the binding affinity is determined strictly by the common direct repeat sequence. On the other hand, the ratio of sDR bound:bDR bound for the homodimer complex is substantially

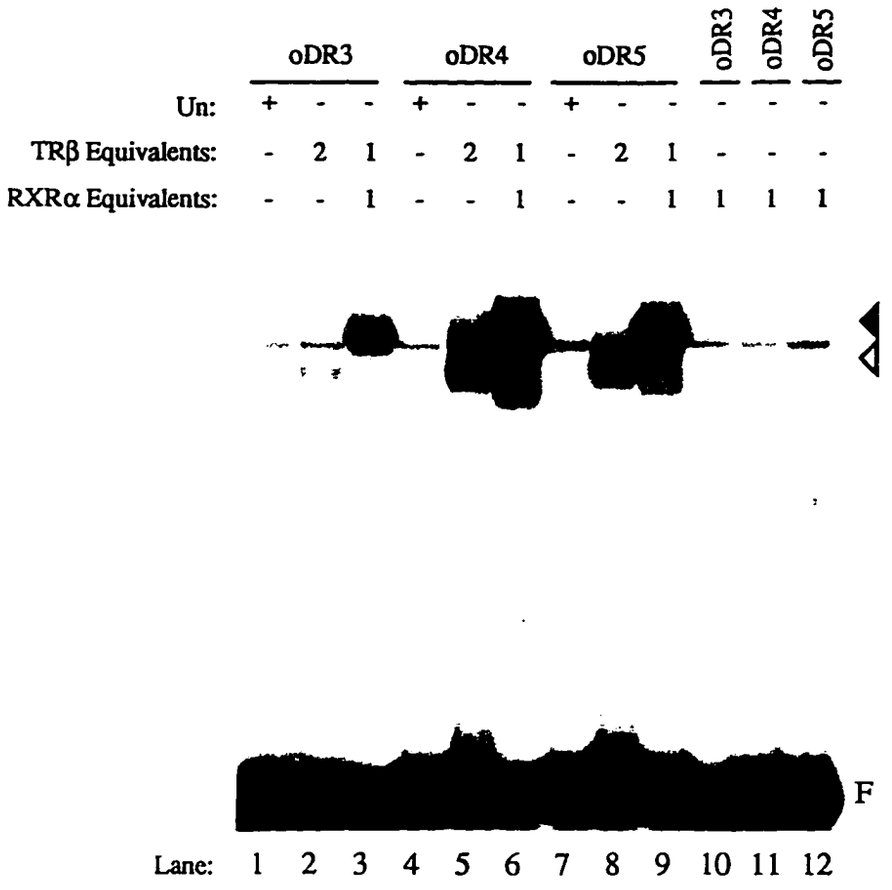
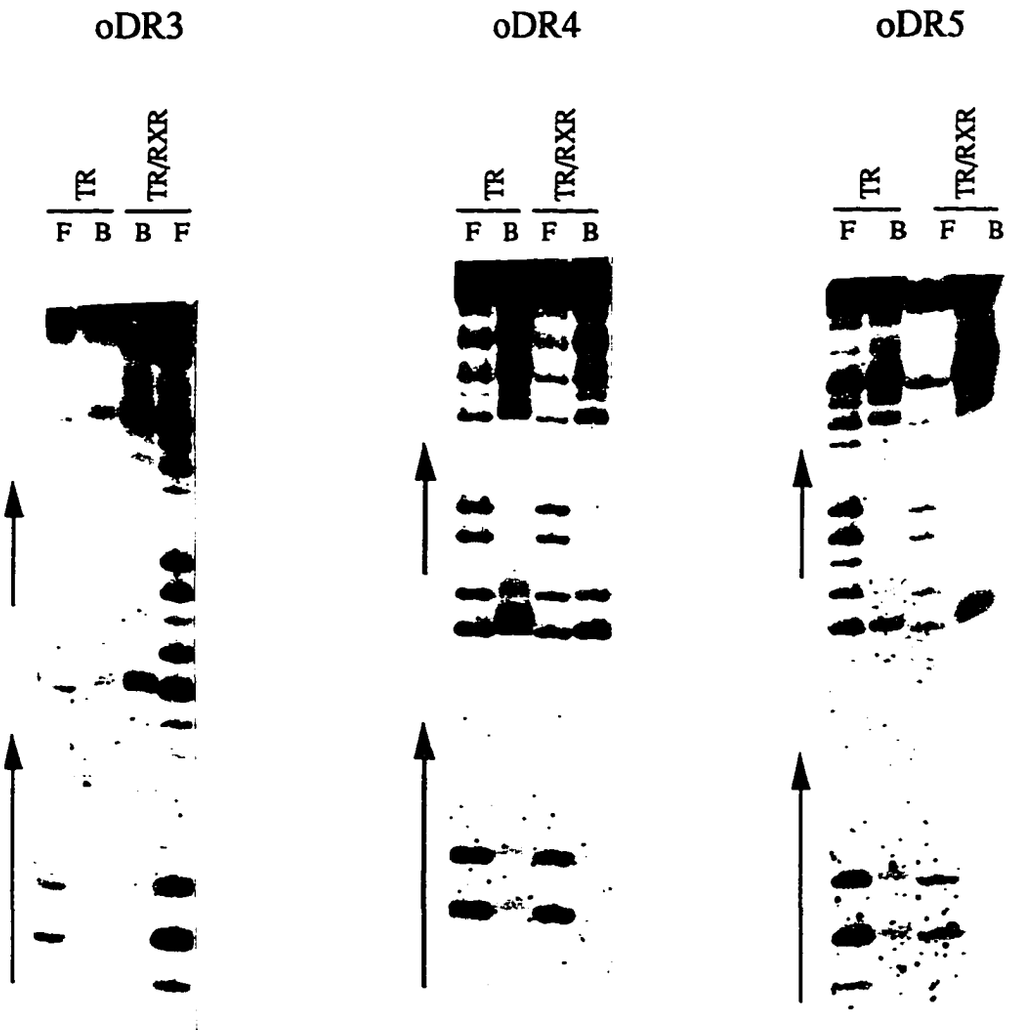


Figure 4.10: Binding of TRβ homodimers and TRβ/RXRα heterodimers to the DNA probes oDR3, oDR4 and oDR5. ^{32}P-labeled oDR3, oDR4 and oDR5 probes were incubated with TRβ and/or RXRα translated *in vitro* in RRL. Un indicates unprogrammed RRL. EMSA was performed alongside that in Figure 4.2, using identical conditions and materials, with the exception of the DNA probe. The autoradiogram was produced by overnight exposure of film to the EMSA gel.

(A)



(B)

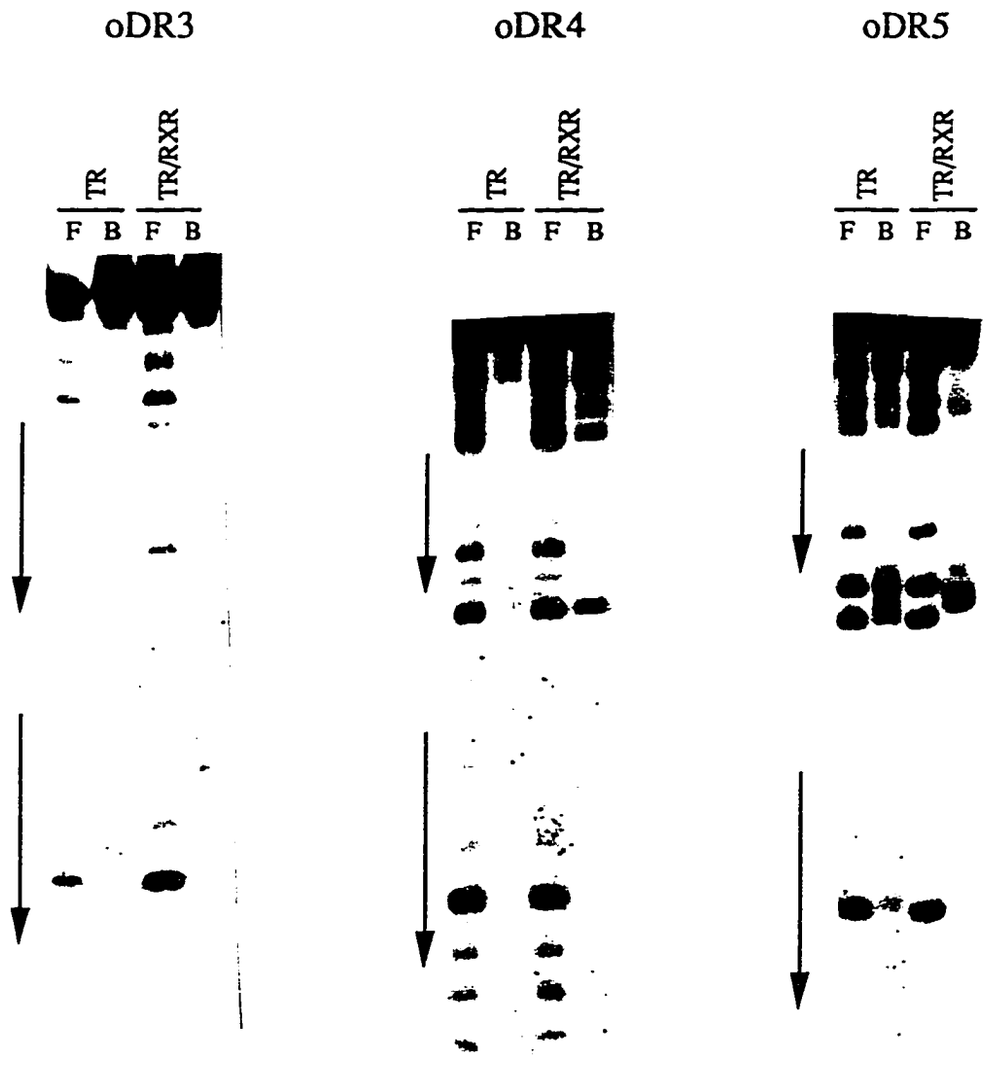


Figure 4.11: Methylation interference of the binding of TRβ homodimers and TRβ/RXRα heterodimers to oDR3, oDR4 and oDR5 sequences. Methylation interference patterns for the upper (A) and lower strand (B) of oDR3, oDR4 and oDR5. The arrows to the left of each section of autradiograph represent half-sites A and B.

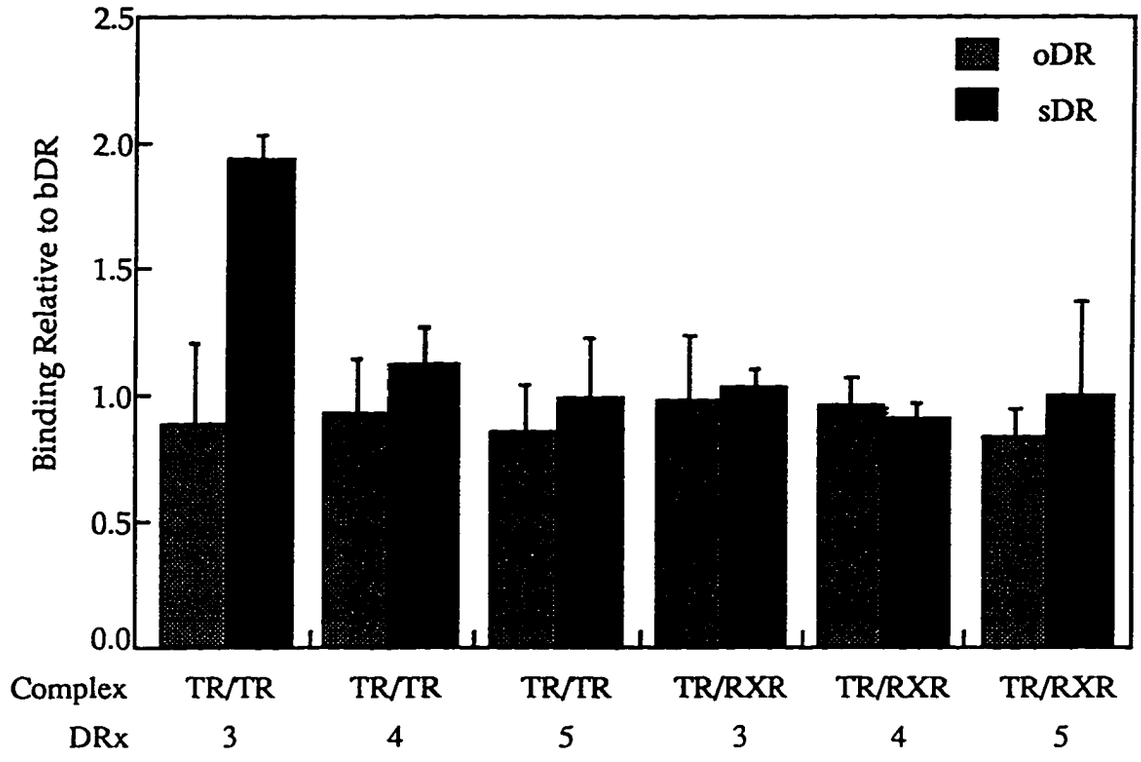


Figure 4.12: The relative binding of sDRs, bDRs and oDRs by the TRβ homodimer and TRβ/RXRα heterodimer. The ratio of oDR bound:bDR bound, or sDR bound:bDR bound was calculated for each spacing variant, and both the the TRβ homodimer and TRβ/RXRα heterodimer.The bound DNA from an EMSA was quantified by scintillation counting of excised bands. The data are the product of three independent experiments, and error bars indicate the standard error.

higher with the DR spaced by 3 than that for DRs spaced by 4 or 5 base-pairs, or the sDR bound:bDR bound ratio for heterodimer with either DR3, DR4 or DR5 sequences. Thus, the sDR3 probe, which has half-sites C and D present, is bound with higher affinity than either DR3 probe lacking these half-sites. Therefore, since occupation of the direct repeat (A and B) and the everted repeat (A and C/D) is mutually exclusive, it appears that the everted repeat composed of half-sites A and C/D is bound with greater affinity than is the direct repeat spaced by 3 base-pairs, but with less affinity than those spaced by 4 or 5 base-pairs.

4.3.5 Binding of the TRβ Homodimer to the Cryptic Everted Repeat in the Absence of the Direct Repeat.

To show that the everted repeat consisting of half-sites A and C/D is occupied by the homodimer, an oligonucleotide containing the sequence of half-site A, but not B, was cloned into pUC19 in both the *Sma* I site and the *Bam*H I site in the same fashion as were the DR oligonucleotides, to yield sTRE$^1/_2$ and bTRE$^1/_2$. Therefore, these sequences had half-site A with the same upstream sequence as the sDR and bDR probes, respectively, but lacked half-site B. EMSA with these probes reveals that sTRE$^1/_2$ was bound by the TRβ homodimer with similar affinity as was sDR3 (Figure 4.13). On the other hand, binding of bTRE$^1/_2$ by the TRβ homodimer was barely detectable. Interestingly, both TRE$^1/_2$ sequences are also bound by the TRβ/RXRα heterodimer, so it appears that in the absence of half-site B, non-ideal sequences are bound by the heterodimer.

4.3.6 Examination of the Mode in Which TRβ Homodimers Bind to Direct Repeat Elements: DBDs Aligned for Direct or Everted Repeats?

The positive cooperativity involved in a homodimeric complex binding to an everted repeat is sufficient to allow binding of an imperfect everted repeat in competition with an idealized direct repeat. In other words, favourable protein/protein interactions, facilitated by particular half-site orientation and spacing combinations can override purely half-site sequence-based considerations. This raises the possibility that direct repeats themselves

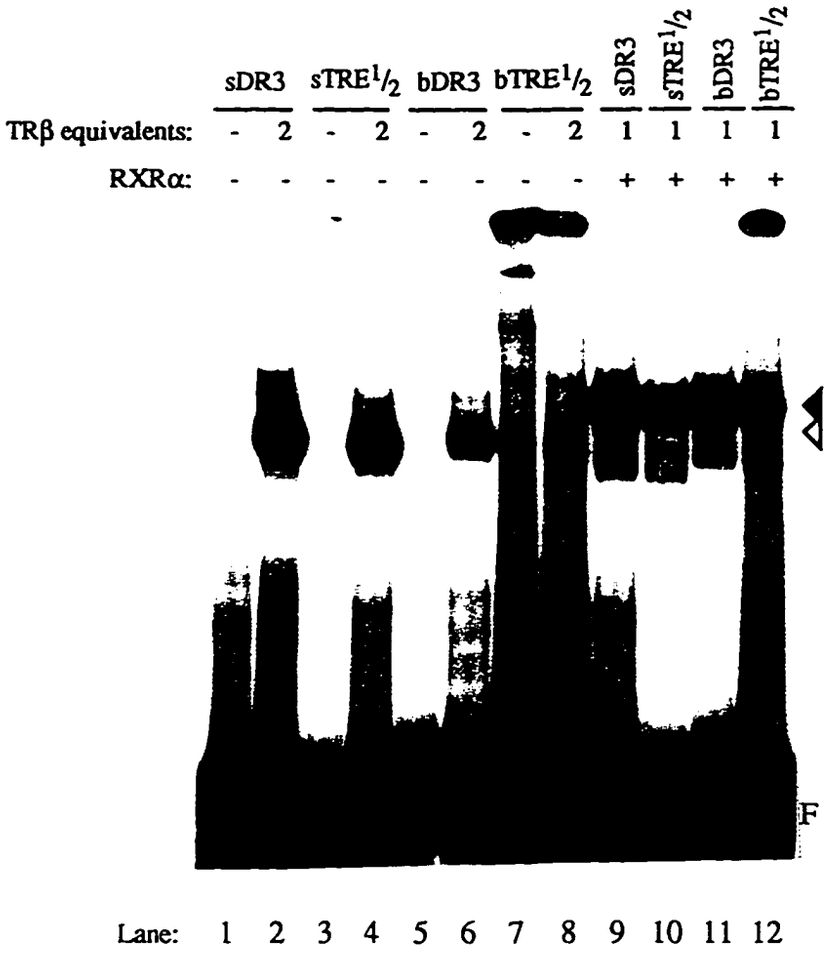


Figure 4.13: Binding of TRβ and TRβ/RXRα to the cryptic everted repeat in the absence of half-site B. EMSA was performed using *in vitro* translated TRβ and RXRα with ^{32}P-labeled DNA. TRβ and RXRα, alone or together, were bound to sDR3, sTRE$^1/_2$, bDR3, and bTRE$^1/_2$, as indicated. TRβ amounts are expressed as equivalents simply to indicate the relative amounts used in the presence and absence of RXRα. Where no receptor is indicated, unprogrammed RRL was used. EMSA was performed under Set II standard conditions, but the autoradiogram was the result of 1 week of exposure to the EMSA gel.

may be regarded as poorly conserved everted repeats; that is, direct repeats may, in fact, be bound by homodimeric complexes in the same highly cooperative conformation adopted to bind everted repeats, that is, Mode E (Figure 4.14). Inspection of the perfect direct repeat sequence used in this study reveals that, due to strand-wise redundancy of half-site A, there are two putative overlapping half-sites on the opposite strand (half-sites E and F, Figure 4.15A). In conjunction with half-site B, half-sites E and F comprise an EvR6 and an EvR4, respectively, on the DR4 element. Half-sites E and F match the consensus hexamer at 3 and 2 positions, respectively. In order to test whether the DBDs of the TRβ homodimer interact with half-site pairs A and B, E and B, or F and B, two mutant DR4 sequences were created which each reduced the matches between half-site A and the consensus hexamer sequence by one. Concomitantly, DR4/ER6 increased the number of matches between half-site E and the consensus, while DR4/ER4 improved half-site F (Figure 4.15A). The binding affinity of TRβ and TRα for DR4/ER6 and DR4/ER4 was much lower than that for the wild-type DR4 (Figure 4.15B), indicating that the idealized direct repeats are not recognized by the TR homodimers primarily as everted repeats consisting of half-sites B and E, or B and F. While these results do not rule out the possibility that the homodimer may bind to direct repeats in both modes D and E, they are consistent with the homodimer interacting with a DR4 primarily through DBDs in Mode D. If the DBDs were aligned to recognize everted repeats when binding the DR4 sequence, improvement of half-sites E and F would be expected to have increased the affinity of the homodimer for the mutant sequence relative to DR4.

In the same vein, the DR3 was tested for recognition by the homodimer in Mode E, since the less favourable spacing of this DR might shift the balance towards recognition as an everted repeat by the homodimer. The probe DR3/ER5 improves an EvR5 consisting of half-sites B and a strand-wise redundant sequence overlapping half-site A (half-site G, Figure 4.16A). Binding of DR3/ER5 by both the TRα or TRβ homodimer and heterodimer is much less than that of DR3 (Figure 4.16B). It is interesting, however, that

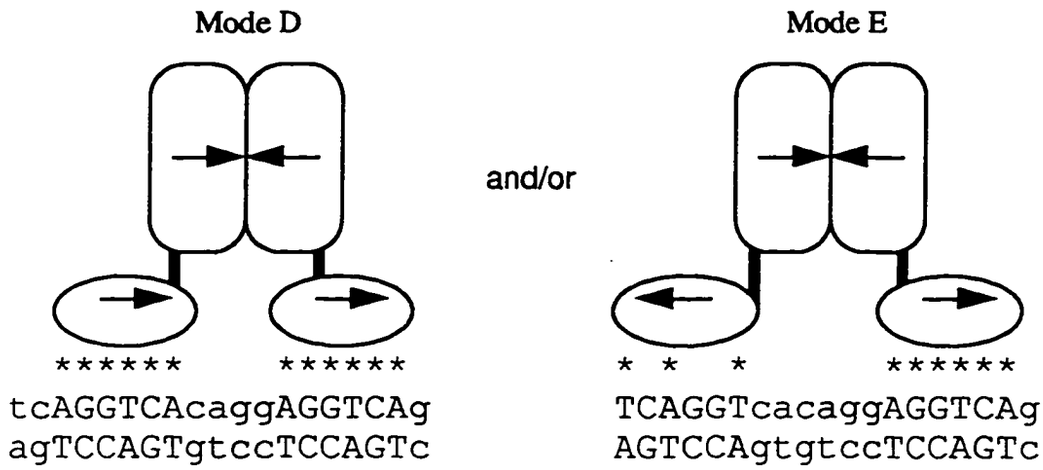
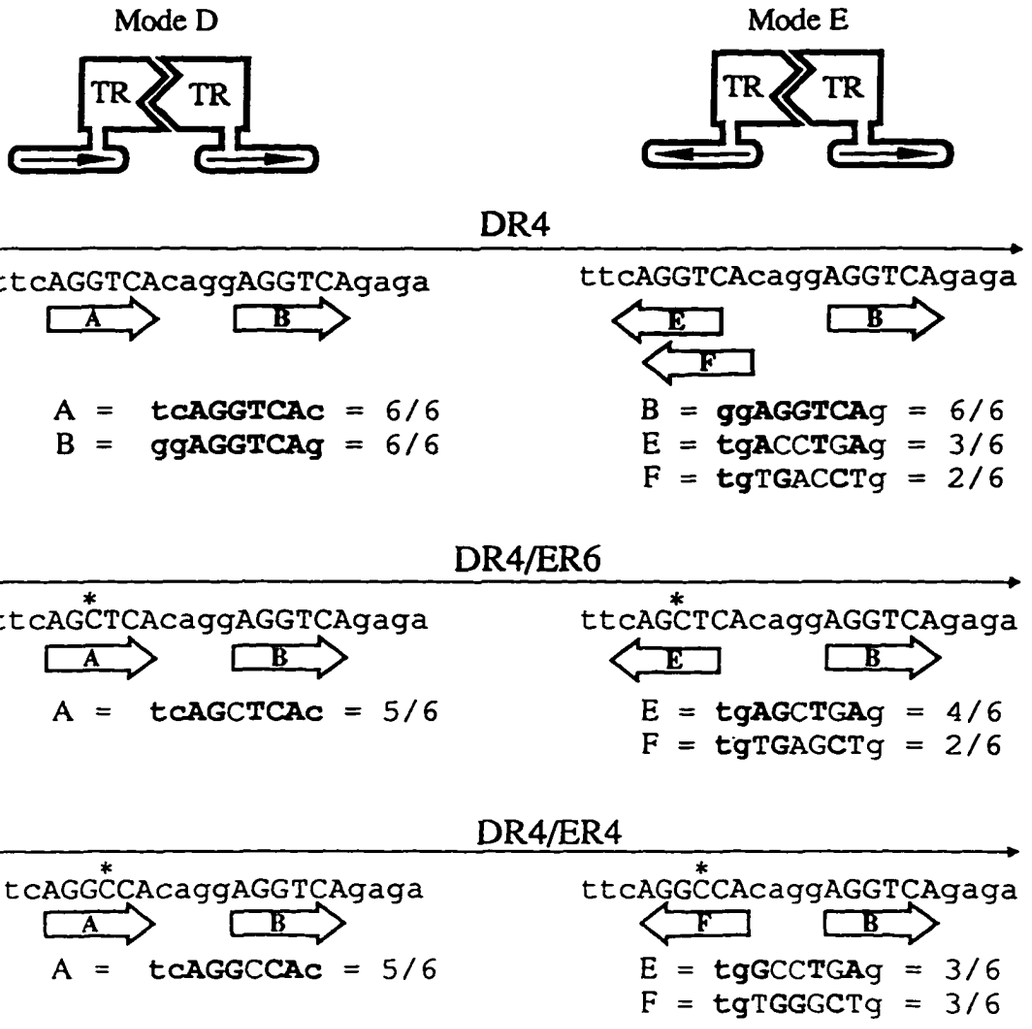


Figure 4.14: Schematic model of two possible modalities of the TRβ homodimer in which the idealized direct repeat could be recognized. The same DR4 sequence is present in both parts of the figure. On the left, the TRβ homodimer has its DBDs aligned over the two ideal core hexamers that comprise the direct repeat (in upper-case), the modality denoted as Mode D. Matches to the consensus, in this case every position, are indicated by asterisks. On the right, the homodimer binds to the same sequence, but with its DBDs aligned with a cryptic everted repeat (in upper-case), the modality denoted Mode E. There are fewer matches between the upstream half-site and the consensus sequence, but this may be compensated for by more favourable interactions between the LBDs, for example.

(A)


Mode D
TR
TR
Mode E
TR
TR
DR4
ttcAGGTCAcaggAGGTCAgaga
A
B
A = tcAGGTCAc = 6/6
B = ggAGGTCAg = 6/6
ttcAGGTCAcaggAGGTCAgaga
E
B
F
B = ggAGGTCAg = 6/6
E = tgACCTGAg = 3/6
F = tgTGACCTg = 2/6
DR4/ER6
ttcAGCTCAcaggAGGTCAgaga
A
B
A = tcAGCTCAc = 5/6
ttcAGCTCAcaggAGGTCAgaga
E
B
E = tgAGCTGAg = 4/6
F = tgTGAGCTg = 2/6
DR4/ER4
ttcAGGCCAcaggAGGTCAgaga
A
B
A = tcAGGCCAc = 5/6
ttcAGGCCAcaggAGGTCAgaga
F
B
E = tgGCCTGAg = 3/6
F = tgTGGGCTg = 3/6

(B)

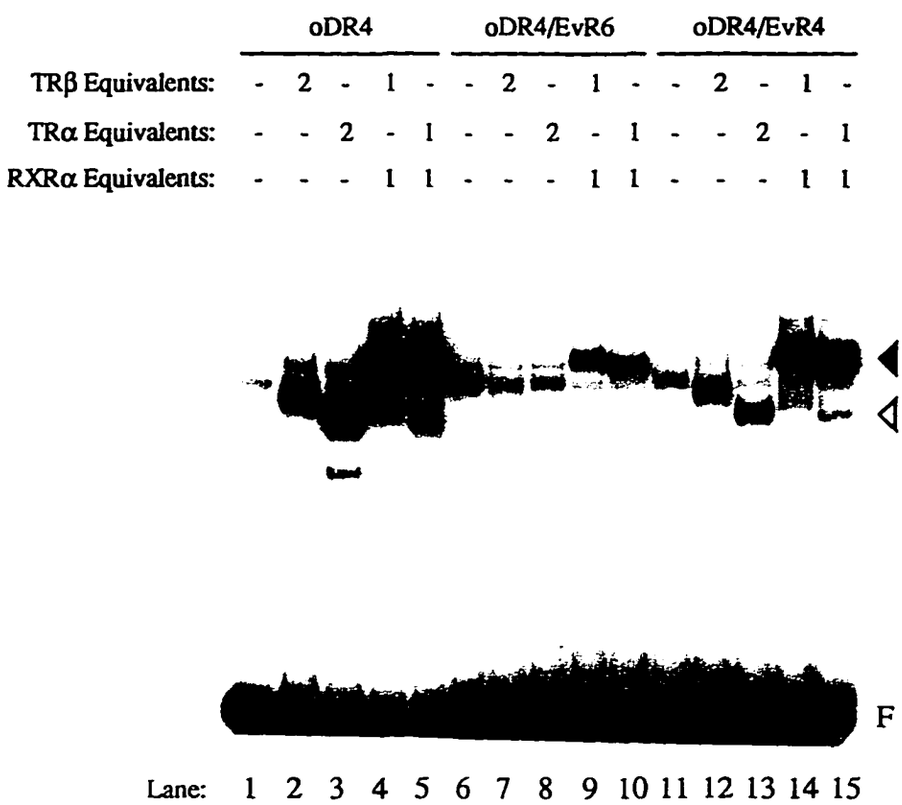


Figure 4.15: The modality of binding of the TRβ homodimer to DR4. (A) The probes DR4/EvR6 and DR4/EvR4 were designed to increase the matches of the cryptic everted repeats consisting of half-sites B and E, and B and F, respectively. The effects of the single point mutation (indicated by the asterisk) introduced into the DR4 sequence, on the sequences of half-sites A-F are shown. (B) EMSA of the TRβ or TRα homodimer and the TR/RXRα heterodimers binding to oDR4, oDR4/EvR6 and oDR4/EvR4. ^{32}P-labeled synthetic oligonucleotides were bound by *in vitro* translated receptors. Receptor quantities are given as equivalents simply to indicate that binding reactions including RXR contained half the TR receptor as those reactions in the absence of TR. An equivalent of one receptor is not necessarily equal to that of another. EMSA was performed under Set II standard conditions. The autoradiogram was produced by exposure of film to the EMSA gel for 72 hours.

(A)

DR3

ttcAGGTCAaggAGGTCAgaga
A B
A = **tcAGGTCA**a = 6/6
B = **ggAGGTCA**g = 6/6

ttcAGGTCAaggAGGTCAgaga
G B
B = **ggAGGTCA**g = 6/6
G = **tgA**CC**TGA**a = 3/6

DR3/ER5

ttcAG*CTCAaggAGGTCAgaga
A B
A = **tcAGCTCA**a = 5/6

ttcAG*CTCAaggAGGTCAgaga
G B
G = **tgAGCTGA**a = 4/6

(B)

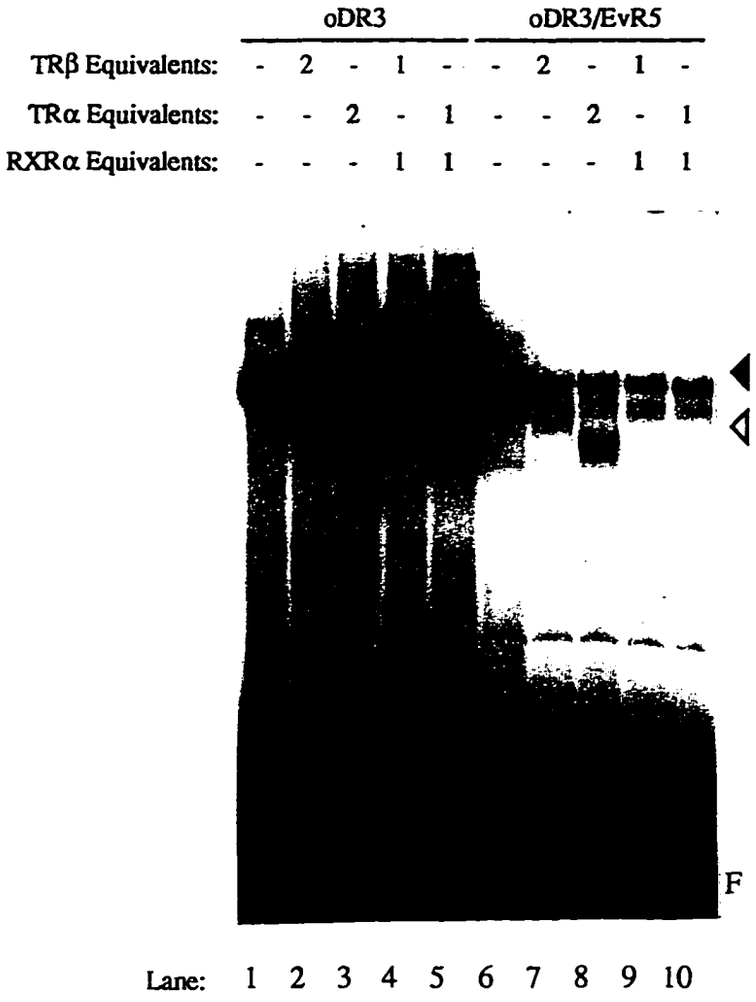


Figure 4.16: The modality of binding of the TRβ homodimer to DR3. (A) The probe DR3/EvR5 was designed to increase the matches of the cryptic everted repeats consisting of half-sites B and G. The effects of the single point mutation (indicated by the asterisk) introduced into the DR3 sequence, on the sequences of half-sites A, B and G are shown. (B) EMSA of the TRβ or TRα homodimer and the TR/RXRα heterodimers binding to oDR3, oDR3/EvR5. ^{32}P-labeled synthetic oligonucleotides were bound by *in vitro* translated receptors. Receptor quantities are given as equivalents simply to indicate that binding reactions including RXR contained half the TR receptor as those reactions in the absence of TR. An equivalent of one receptor is not necessarily equal to that of another. EMSA was performed under Set II standard conditions. The autoradiogram was produced by exposure of film to the EMSA gel for 72 hours.

on this gel, it appears that the homodimer retains some residual binding, whereas no significant binding is apparent by the heterodimer. Unfortunately, in this particular EMSA a non-specific band migrates very similarly to the heterodimer/DNA, obscuring possible binding activity by the heterodimer, although the presence of the heterodimer does not substantially increase the intensity of the non-specific band above that of the adjacent lanes. Thus, it appears that even the relatively poor affinity DR3 is recognized by the TR homodimer primarily in Mode D.

4.3.7 Differences in the Methylation Interference Patterns for the Homodimer and Heterodimer Binding to Direct Repeats are Observed in the Absence of the Cryptic Everted Repeat.

The bDR probes allow the comparison of methylation interference patterns for the binding of homodimer and heterodimer to direct repeats of the half-site sequence. Since the heterodimer only occupies half-sites A and B even in the sDR context, the sDR methylation interference patterns for the heterodimer, which are essentially identical, can be considered as well. Comparison of Figure 4.5 with Figure 4.9 reveals that the patterns of methylation interference observed within the two half-sites of the direct repeat are essentially identical for the sDRs and the bDRs bound by the heterodimer.

One constant feature of the interaction of homodimers and heterodimers with the direct repeats is that methylation of guanines within the core hexamer (in base-pairs C2, C3, and C-5) of either half-site consistently produce a greater degree of interference of binding than that seen for the flanking positions. However, methylation of the guanine in base-pair F-3 of half-site A strongly interferes with both homo- and heterodimer binding to bDR4 and bDR5. The bDR3 sequence does not have a GC base-pair at this position. While the other flanking nucleotides are subject to lesser degrees of methylation interference, there are some noteworthy differences between the patterns for the homodimer versus the heterodimer (compare top and bottom of Figure 4.9A-C), particularly with regard to the DR3 sequence. Methylation of the guanine in base-pair F3 of half-site B interferes with

binding only of the homodimer, and only in the context of DR3. There is also a slightly lower impact on binding resulting from methylation of the guanine in base-pair F2 of half-site B of bDR3 versus bDR4 or bDR5. I suggest that these two observations are consistent with the receptor occupying half-site B being out of register by one base-pair in order to maintain a four or more base-pair spacer distance from half-site A. In contrast, methylation of the guanine in base-pair F3 of half-site B of bDR3 or sDR3 does not interfere with binding of the heterodimer suggesting that no such displacement of the receptor occupying half-site B takes place in this complex.

Base-pair F1 only includes a guanine in half-site B, but in no case does methylation of this position interfere with binding of either homodimer or heterodimer. Both half-sites have a guanine at position F2 or F-2 and methylation of these guanines within either half-site interferes with both homodimer and heterodimer binding, except in the case of the heterodimer binding to DR4. Methylation of position F-2 of half-site A of bDR4 or sDR4 does not inhibit binding of the heterodimer. In summary, the homodimer interactions with DR3, DR4 and DR5 cannot be distinguished on the basis of methylation interference patterns, although they can be distinguished from the interactions of the heterodimer in some cases. Furthermore, the flanking sequences of the half-sites exhibit spacing-dependent differences in methylation interference patterns for the heterodimer.

4.3.8 Some Natural TREs May Be Recognized by the TR Homodimer and Heterodimer in Different Modalities.

The results in Section 4.3.5 show that idealized direct repeats are recognized as such; that the DBDs are aligned in the asymmetric relative orientation, Mode D, that would be expected for binding to half-sites in this orientation (Figure 4.14; left side). However, no TREs identified thus far consist of two AGGTCA sites; there is always deviation from the consensus sequence in one or both sites. Therefore, it is possible for a natural TRE to have sequence characteristics intermediate between a direct repeat and an inverted repeat. The sequence $TRE_{PCP\text{-}2}$ was characterized in the promoter region of the Purkinje cell-specific

protein gene, *Pcp-2* (Strait *et al.*, 1992; Zou *et al.*, 1994). Half-site B is a perfect match to the consensus hexamer, whereas half-site A has the internally palindromic sequence AGGCCT, so that the hexamer spaced by four base pairs from half-site B is an equally good direct or everted repeat, with respect to half site B (Figure 4.17A). Given that homodimers preferentially bind to EvRs, and heterodimers preferentially bind to DRs, the modality in which the two TRβ receptor complexes might bind to TRE_{PCP-2} was not obvious. Thus, a mutant TRE_{PCP-2} sequence was generated, PCP-2/EvR4, which improved the EvR4 at the expense of the DR4. EMSA of the binding of either TRβ or TRα alone, or in the presence of RXRα, to TRE_{PCP-2} or PCP-2/EvR4 suggests a novel phenomenon is occurring (Figure 4.17B).

The mutation in PCP-2/EvR4 slightly improves the binding of the TRβ homodimer (cf lane 2 with lane 7, and lane 3 with lane 8), while slightly decreasing binding by the TRβ/RXRα heterodimer (cf lane 4 with lane 9, and lane 5 with lane 10). Visual inspection of an EMSA of this binding interaction is not sufficient to categorically prove this phenomenon, so a binding curve was performed to compare binding of the homodimer and heterodimer to the two sequences. Saturation of the receptor with probe was not achieved under the conditions shown, but it is apparent that PCP-2/EvR4 was bound by the TRβ homodimer with greater affinity than was TRE_{PCP-2} (Figure 4.18). In contrast, the heterodimer bound to the TRE_{PCP-2} sequence with roughly 10-fold greater affinity than PCP-2/EvR4 (Figure 4.19). The consequences of the mutation present in PCP-2/EvR4 for homodimer and heterodimer binding versus TRE_{PCP-2} are interesting because they help characterize a TRE sequence that is differentially recognized by the two complexes as an EvR4 and a DR4, respectively. To confirm that an EvR4 sequence was a high affinity binding site for TRβ homodimers, oligonucleotides harbouring an idealized EvR4 sequence (oEvR4) were used in an EMSA (Figure 4.20) side-by-side with a oDR4. The binding of oEvR4 by the TRβ homodimer was at least as great as that of oDR4. Therefore, it seems that the principles discussed in section 4.3.5 are operative in the case of TRE_{PCP-2}. The

(A)

TRE_{PCP-2}

aaaAGGCCTtctcAGGTCAgag

A B

A = **aaAGGCCT**t = 4/6
B = **tcAGGTCA**g = 6/6

aaaAGGCCTtctcAGGTCAgag

H B

H = **gaAGGCCTt** = 4/6
B = **tcAGGTCA**g = 6/6

PCP-2/EvR4

*
aaaAGACCTtctcAGGTCAgag

A B

A = **aaAGGCCT**t = 4/6
B = **tcAGGTCA**g = 6/6

*
aaaAGACCTtctcAGGTCAgag

H B

H = **gaAGGTCT**t = 5/6
B = **tcAGGTCA**g = 6/6

(B)

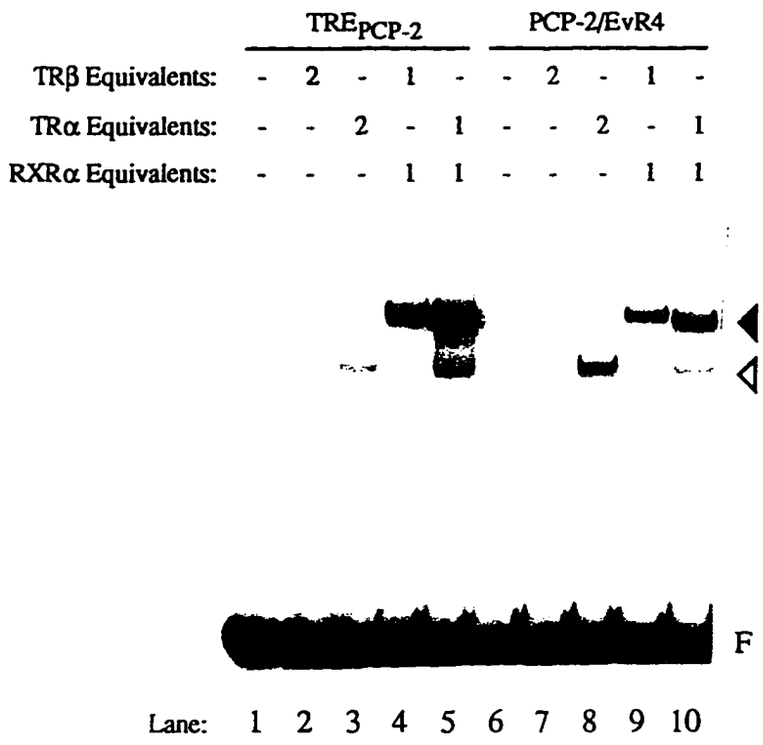


Figure 4.17: The modality of binding of the TRβ homodimer to TRE_{PCP-2}. (A) The probe oPCP-2/EvR4 was designed to increase the matches of the cryptic everted repeats consisting of half-sites B and H, compared to $oTRE_{PCP-2}$. The effects of the single point mutation (indicated by the asterisk) introduced into the DR4 sequence, on the sequences of half-sites A, B and H are shown. (B) EMSA of the TRβ or TRα homodimer and the TR/RXRα heterodimers binding to $oTRE_{PCP-2}$ and oPCP-2/EvR4. ^{32}P-labeled synthetic oligonucleotides were bound by *in vitro* translated receptors. Receptor quantities are given as equivalents simply to indicate that binding reactions including RXR contained half the TR receptor as those reactions in the absence of TR. An equivalent of one receptor is not necessarily equal to that of another. EMSA was performed under Set II standard conditions. The autoradiogram was produced by exposure of film to the EMSA gel for 72 hours.

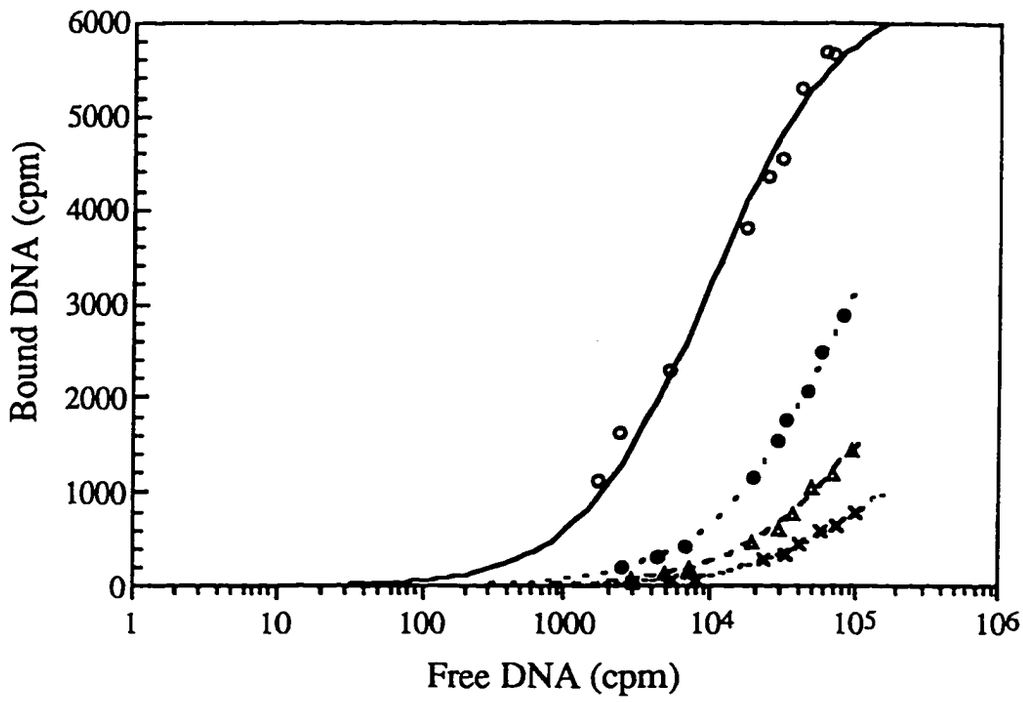


Figure 4.18: Binding curves for the binding of TRβ homodimer and TRβ/RXRα heterodimer to TRE_{PCP-2} and PCP-2/EvR4. EMSA was performed using increasing amounts of ^{32}P-labeled TRE_{PCP-2} or PCP-2/EvR4. The bound and free DNA bands were excised from the gel and the DNA quantified by scintillation counting. The amount of bound versus free DNA is plotted for each concentration. TRβ/RXRα heterodimer binding to TRE_{PCP-2} (o) and PCP-2/EvR4 (•), and homodimer binding to TRE_{PCP-2} (x) and PCP-2/EvR4 (Δ)are shown. The curve was drawn as described in Figure 3.26.

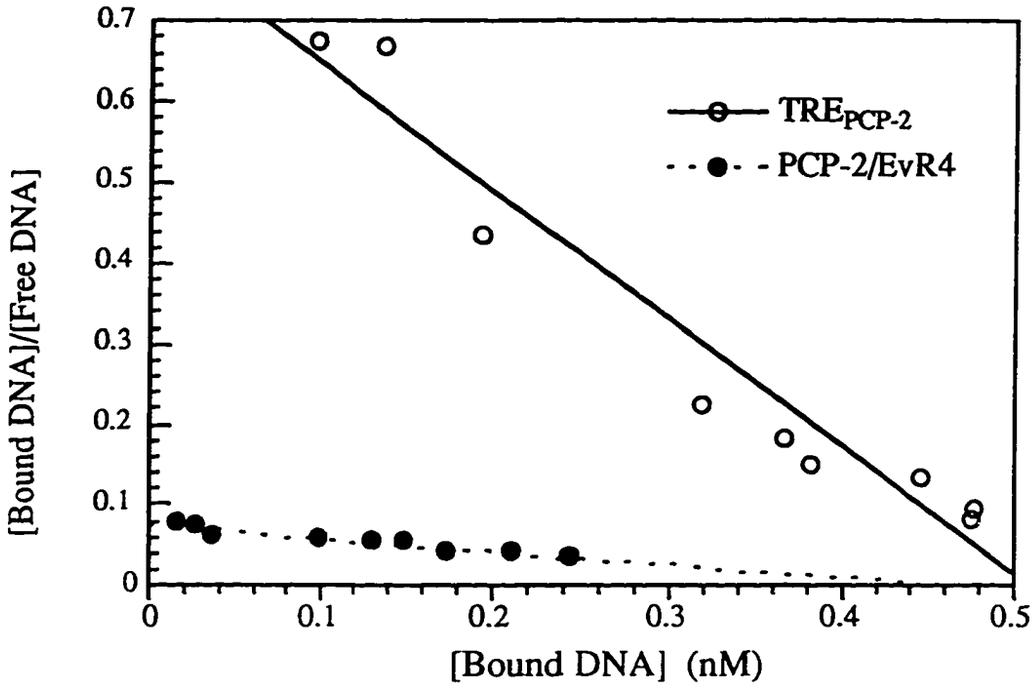


Figure 4.19: Scatchard analysis of the binding of the TRβ/RXRα heterodimer to TRE_{PCP-2} and PCP-2/EvR4. Data shown in Figure 4.18 was used to generate a Scatchard plot in order to determine the K_d for the binding of the TRβ/RXRα heterodimer to TRE_{PCP-2} and PCP-2/EvR4.

Figure 4.20: An EvR4 sequence is bound with high affinity by the TRβ homodimer. EMSA was performed with *in vitro* translated TRβ in the presence or absence of RXRα, and ^{32}P-labeled DNA. EMSA was performed under Set II standard conditions.

cooperativity of the homodimer and heterodimer binding to everted repeats and direct repeats, respectively, is great enough to override sequence considerations beyond a certain level. The internally palindromic sequence of half-site B of $TRE_{PCP\text{-}2}$ provides enough appropriate sequence on both strands to be recognized by the homodimer and heterodimer in Modes E and D, respectively.

4.4 DISCUSSION

4.4.1 General Features of TRβ Binding to Direct Repeats Characterized by EMSA and Methylation Interference.

Although both the TRβ homodimer and the TRβ/RXRα are able to bind the idealized DR3, DR4 and DR5 sequences used in this study they exhibit distinct binding patterns. The homodimer bound DR3 poorly, while DR4 and DR5 were bound with higher, and similar, affinity (Figure 4.2). In contrast, the heterodimer clearly bound the DR4 with the highest affinity, followed by the DR5, and then the DR3. Despite these differences in binding specificity, the methylation interference patterns for the binding of the homodimer and heterodimer to the different bDRs have many similarities. Methylation of guanines within the core hexamer of either half-site A or B strongly interferes with the binding of both complexes regardless of the spacer length between the half-sites. Beyond the core hexamers, methylation of guanines in either base-pairs F2 or F3 interferes with binding of the homodimer and heterodimer to all three DRs to some extent, the degree of interference varying in a spacing-dependent manner in some cases. Methylation of guanine at position F1 does not interfere with binding in any case. The identity of the nucleotide at position F1 has been shown to influence the binding of TR (Katz and Koenig, 1993; Kurokawa *et al.*, 1993; Schrader *et al.*, 1994), apparently through interaction via the minor groove (Kurokawa *et al.*, 1993; Rastinejad *et al.*, 1995). Hence, the lack of methylation interference at this position may stem from a technical limitation of the experiment in indicating minor groove interactions, and would be consistent with a lack of interaction through the major groove at this position. While the F2 position is also contacted at the phosphate groups via the minor groove (Rastinejad *et al.*, 1995), proximity to base-specific, major groove contacts with C1 may provide a basis for the observed methylation interference.

4.4.2 Differences Between the Binding of TRβ Homodimers and Heterodimers to Direct Repeats Reflected in Methylation Interference Patterns.

Comparison of the methylation interference values for the flanking sequences suggests some differences between binding of the homodimer and the heterodimer to bDR3. The data suggests that the "downstream" TR of the homodimer is misaligned with respect to half-site B, presumably assuming a position consistent with a spacing of four base-pairs from half-site A. Thus, the disposition of the receptors within a homodimer bound to the DR3 could be described as 5'-aligned-misaligned-3'. This would explain the dramatic drop in binding affinity of the homodimer for DR3 relative to DR4, as the sequence over which the downstream DBD is aligned would have few matches to the consensus half-site. Misalignment of DNA-bound nuclear receptors with respect to one half-site of an occupied binding site has been previously documented for the binding of glucocorticoid receptor to an IR4 sequence (Luisi *et al.*, 1991). Interestingly, TR DBDs non-cooperatively bind DR3 sequences with virtually identical affinity as DR4 and DR5 sequences (Mader, 1993); it is inclusion of the C-terminal dimerization domain that makes binding of the TR homodimer to a DR3, sequence less favourable than to a DR4 or DR5 sequence. There are two ways in which this could come about: binding of the homodimer to direct repeats spaced by three base-pairs could result in a conformation with decreased cooperative interactions compared to binding to DR4s; and, the cooperative interactions of the C-terminal dimerization domains could be so strong, and so predisposed to the spacer distance of four base-pairs that misalignment of one DBD results when the DR3 is bound. The results indicate that the latter case may be true to a certain extent. Due to the nature of the interference experiments it cannot be determined whether the homodimer binds to DR3 strictly in the putative misaligned state, or if it is only assumed by a sub-population of receptor complexes. It is also unclear, due to the sequence of the probe, whether the TR occupying half-site A shows evidence of misalignment as well; that is, if there might be a sub-population of

homodimers that are bound 5'-misaligned-aligned-3'. Likely, the proportion of homodimers in the two subpopulations would be determined by the relative strength of half-sites A and B. However, it is not inconceivable that this phenomenon is asymmetric (ie. occurring 5'-aligned-misaligned-3'), as the interface between the two receptors is intrinsically asymmetric itself.

If the heterodimer is subject to misalignment of one DBD when bound to a DR3 there is no evidence that the receptor on half-site B is the displaced component. Position F3 of half-site B is subject to very little methylation interference of heterodimer binding regardless of spacer length. Once again, the sequence immediately upstream of half-site A provides no guanine residues for methylation, so it is difficult to assess possible misalignment of the receptor on half-site A in that direction. If misalignment of a heterodimer DBD does occur it would likely be in this direction. In a heterodimer of TRβ and RXRα DBDs bound to a DR4 , the TR DBD makes significantly more base-specific contacts than does the RXR DBD (Rastinejad *et al.*, 1995), suggesting that if a DBD is to be misaligned it would more likely be the latter.

When the spacer between the direct repeats is four or five base-pairs, no differences in the methylation interference patterns are observed for the homodimer. This suggests that homodimer-DNA interactions are not qualitatively influenced by the spacing of the direct repeats, at least within the detection limitations of the methylation interference protocol. While it has been shown that no cooperative protein-protein interactions occur between the DBDs of the TR homodimer on DRs with spacer lengths of from 3 to 5 base-pairs (Mader *et al.*, 1993) the results presented here expand on this notion, indicating that there are no substantial qualitative differences in the protein-DNA interactions of the homodimer with a DR4 versus a DR5. This contributes to the increasing body of data that suggests that homodimer binding to direct repeats does not correspond to the 3-4-5 rule (Mader *et al.*, 1993; Shulemovich *et al.*, 1995). In fact, it seems that the affinity of homodimer binding is likely determined primarily by the affinity of the two nonameric half-site sequences, as

long as the spacer DNA is within a certain permissive range, the lower end of which would be a spacing of four base-pairs, and which is determined by the C-terminal dimerization domain. It should also be noted that, unlike the case for the DR3 sequence, it appears that no misalignment of DBDs occurs to accommodate binding to a DR5.

The heterodimer does exhibit a slight difference in methylation interference patterns between the DR4 and DR5 probes although, as with the homodimer, there is no evidence of misalignment of a DBD to maintain a spacer distance of four base-pairs when binding the DR5 sequence. The difference is manifested at position F2 of half-site A, with methylation of the guanine at this position interfering with the binding to DR5, but not DR4. This result is interesting when considered in the context of the crystallographic data that exist for the TR/RXR DBDs bound to a DR4 sequence (Rastinejad *et al.*, 1995). The crystal structure indicates that a substantial number of contacts are established between the zinc finger regions of the two receptors when the direct repeat is spaced by four base-pairs, and that these contacts serve to stabilize heterodimer binding at least partly by establishing a number of protein/DNA contacts involving the minor groove and DNA backbone. While it is predicted from this structure that many of the favourable protein-protein interactions between the DBDs would be lost when the spacing of the direct repeats is changed, it does not necessarily follow that changes in protein/DNA interaction will occur concomitantly, particularly in the major groove, or at the upstream end of the element. The data indicate that changes in protein/DNA interaction are detected by methylation interference that result from alterations in direct repeat spacing from 4 to 5 base-pairs. There are a number of possible explanations for this. The polarity of the TR and RXR on the DR5 element could be opposite to that on the DR4 element. Alternatively, subtle, spacing-dependent stereochemical differences in the interaction of the receptors with the two half-sites may exist. The crystal structure of the TR-RXR DBDs bound to a DR4 found the T box of RXR to be disordered, whereas a NMR study of free RXR (Lee *et al.*, 1993) indicated that this region would form an α-helix positioned appropriately to contact the minor groove at

the 5' end of the half-site, similarly to that of the TR T-box (Rastinejad *et al.*, 1995). Perhaps the RXR T-box can exist in different conformations on DR4 and DR5 elements that are differentially sensitive to methylation interference.

4.4.3 A Cryptic Half-Site Upstream and on the Opposite Strand From the Direct Repeat Contributes to an Everted Repeat that Competes With the Direct Repeat for TRβ Homodimer Binding. The presence of a cryptic everted half-site in the sDR probes illuminated another significant difference between the binding activities of the homodimer and the heterodimer. While half-site C/D was bound by the homodimer, it was not bound to a significant extent by the heterodimer in any case, unless half-site B was eliminated. I propose that the everted repeat (A and C/D) is bound by the homodimer in a mutually exclusive fashion with respect to the direct repeat (A and B), based on the following observations: methylation of guanine residues within the upstream half-site (C/D) interferes with binding of the homodimer to the sDR probes (Figure 4.5); half-sites A and C/D are bound in the absence of half-site B (Figure 4.13); oligomeric binding is not observed to any significant extent (Figure 4.2), ruling out the simultaneous occupation of half-sites A, B and C/D; everted repeats have been shown to have the capacity for higher affinity binding of TRβ homodimers than do direct repeats (Kurokawa *et al.*, 1993; Rastinejad *et al.*, 1995); if the everted half-site upstream of half-site A is present (sDRs), changes in spacing of the direct repeat that increase the bulk methylation interference sensitivity of half-site B are accompanied by a decrease in the bulk methylation interference sensitivity of half-site C/D, whereas the pattern on half-site A is relatively constant (compare sDR3 with sDR4 or sDR5); and, if half-site C/D is either not present (oDRs) or altered to reduce similarity to consensus (bDRs), the degree of occupation of half-site B increases, as indicated by methylation interference (Figures 4.9 and 4.11). Due to the mutually exclusive occupancy of half-sites B and C/D, the DR3 sequence, which has the lowest affinity for the homodimer of the DRs tested, exhibits the greatest occupation of the everted half-site by the homodimer. Moreover, the everted repeat has higher affinity for

the homodimer than does the direct repeat spaced by three base-pairs (Figure 4.12). Intriguingly, this phenomenon was observed in a case in which the everted repeat had significant deviation from the consensus half-site.

The observation that, for the sDRs, the everted repeat composed of half-sites A and C/D competes with the direct repeat for occupation by homodimer, but not heterodimer, highlights another form of specificity which heterodimerization with RXR confers upon TR. Not only does heterodimerization increase specificity for binding to DR4 sequences versus other DRs, it also generally confers specificity towards direct repeats versus everted repeats. This specificity is also manifested in PCR-based binding site selection assays, which yield direct repeats when performed with TR/RXR heterodimers and everted repeats with TR homodimer (Kurokawa *et al.*, 1993). The results presented here confirm this observation through direct competition of binding sites, and further delimit the phenomenon. Comparison of the methylation patterns of the homodimer and heterodimer on the sDRs reveals that half-site C/D is not occupied by the heterodimer to a significant extent compared to half-site B, regardless of spacing. However, in the absence of half-site B, heterodimer binding is still observed, so it appears that the heterodimer also finds sufficient sequence basis for binding, albeit at a lower level.

4.4.4 Investigation of the Modality of Binding of TRβ to Direct Repeats.

The cooperativity of binding of the TRβ homodimer to everted repeats is apparently sufficient to compensate to a large degree for unfavourable mutations in one half-site. Therefore, I investigated the possibility that DR sequences may simply represent an extreme example of this: the 5' half-site providing an everted half-site, with respect to half-site B, due to strand-wise redundancy in half-site B. Thus, DR4 elements might represent sites at which the homodimer has the two DBDs oriented appropriately for interaction with an everted repeat, while the heterodimer would interact with the DBDs aligned as would be expected for a direct repeat (Figure 4.14). The data provided here are not consistent with this model, since mutations which improved the everted repeat at the expense of the direct

repeat decreased binding of both homodimer and heterodimer. Even for the DR3 sequence, which is not favourably spaced, binding of the homodimer was not increased by a mutation which improved the everted repeat. Thus, despite the preferred orientation of the TR DBDs to bind EvR sequences, the conformational freedom of the homodimer is great enough to permit binding to idealized DR sequences that are suboptimally spaced.

Investigation of the natural TRE, TRE_{PCP-2}, revealed that the intrinsic, preferred orientation of DBDs of the TRβ homodimer and heterodimer can differentially overcome poor half-site sequences. The downstream half-site of TRE_{PCP-2} is a perfect match with the core hexameric sequence. The upstream hexamer, spaced by four base-pairs, has the sequence AGGCCT, which is the same on both strands. Thus, it provides an equal basis for an EvR4 and a DR4. Improvement of the EvR4, concomitantly disrupting the DR4, increases the binding affinity of the TRβ homodimer, but decreases the affinity of the heterodimer. This is consistent with differential recognition of the sequences by the homodimer and heterodimer. The results do not clearly demonstrate that both sequences are recognized by the homodimer as an EvR4 and the heterodimer as a DR4; there are a number of possible transitions in binding modality that could take place to produce this phenomena. For example, TRE_{PCP-2} could be bound as a poor DR4 by both homodimer and heterodimer, and the mutation in PCP-2/EvR4 could result in it being recognized as an EvR4 by the homodimer. Other alternatives exist, but the bottom line is that TREPCP-2 and PCP-2/EvR4 describe a novel phenomenon in TR DNA binding activity; the mutation introduced in PCP-2/EvR4 impacts homodimer and heterodimer binding in opposite directions, increasing binding by the former and decreasing binding by the latter. This is consistent with the two receptor complexes binding in distinct modalities.

The specific roles of the homodimer and the heterodimer in gene regulation are not currently clearly distinguished, so it is difficult to extrapolate these results to physiological importance. However, it may be valuable to generate a scheme of DNA recognition by homodimers and heterodimers. This scheme assumes that TR/RXR heterodimers are, in

fact, able to recognize some EvR sequences as such, rather than recognizing them as weak DR4s. This has never been clearly demonstrated, although the heterodimer has been shown to bind to idealized EvR sequences with a variety of spacer lengths (Schräder and Carlberg, 1994). However, the results presented here indicate the importance of experimentally determining binding modality despite what sequence inspection might suggest about a given binding site. Another group introduced point mutations into TRE_{LYS}, and found that mutations which disrupted homodimer binding also disrupted heterodimer binding (Williams *et al.*, 1994), so there is some evidence that heterodimers may recognize certain EvRs in Mode E. In any case, assuming that at least some idealized EvRs are bound by both homodimers and heterodimers in Mode E, a scheme is presented for recognition of sequences that range from idealized direct repeats to idealized everted repeats (Figure 4.21). In this scheme, idealized direct and everted sequence bind both the homodimer and heterodimer in Mode D and Mode E, respectively. However, in the continuum of sequences possible for the upstream half-site that are intermediate between a direct repeat and an everted repeat there are likely to be cases which are differentially recognized by the homodimer and heterodimer; in Mode E by the former, and in Mode D by the latter.

Taken together, the data presented in this chapter underscore the importance of determining the modality of binding of TR complexes to a given DNA sequence. As there are no TR binding sites known that consist of two idealized half-sites, the modality of binding may be an issue with respect to the function of natural TREs. As distinct functions are elucidated for the homodimer and heterodimer, the capacity for TREs to differentially bind the homodimer and heterodimer in distinct modalities may turn out to be functionally important in gene regulation.

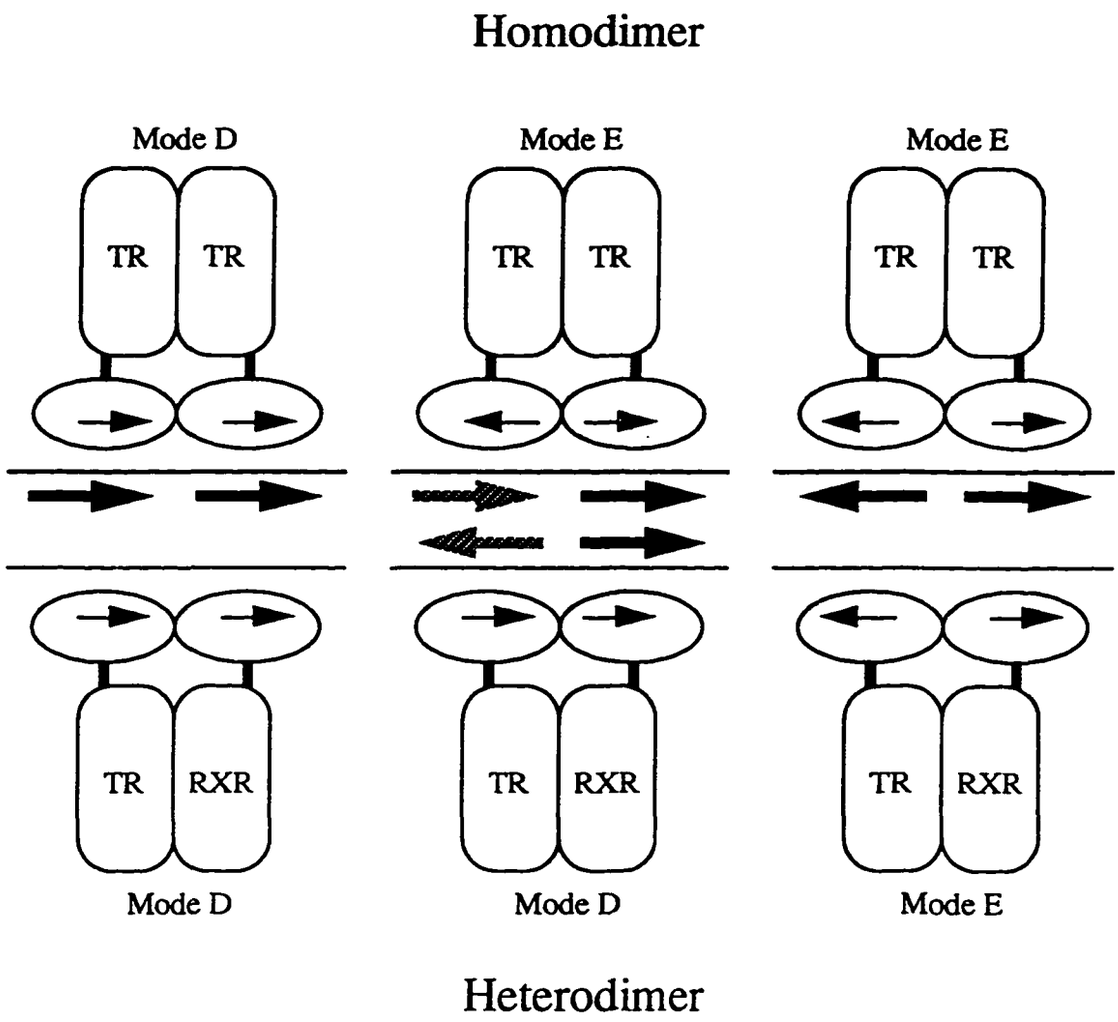


Figure 4.21: Scheme for the differential binding of TR homodimers and TR/RXR heterodimers to direct repeats, everted repeats, and sequences with intermediate sequence characteristics, referred to as "mixed-type" repeats in this work. In this model, idealized direct and everted repeats are bound in Mode D and E, respectively, by both the homodimer and heterodimer. However, sequences with non-idealized upstream half-sites (hashed arrow), may be differentially recognized by the homodimer and heterodimer, due to their predispositions to binding in Mode E and Mode D, respectively.

CHAPTER 5.0

CONCLUSIONS, PERSPECTIVES, AND SUGGESTIONS FOR FUTURE STUDIES.

5.1 INTRODUCTION

The work in this thesis has examined several phenomena in the realm of DNA binding interactions of the thyroid hormone receptor. In a sense, the work has consisted of structure-function studies of the TR protein (Chapter 3) and of the DNA sequences to which it binds (Chapter 4). In the former case, the alanine-substitution analysis of the recognition α-helix of TRβ complements the structural data that has since been obtained from the X-ray crystal structure (Rastinejad *et al.*, 1995). In the latter case, a novel paradigm for the recognition of certain classes of TRE is proposed based on the differential binding of DRs and EvRs by TRβ homodimers and TRβ/RXRα heterodimers. Taken together these results help refine current models of nuclear receptor activity, particularly with regards to the relationship between DNA binding activity of the TR and *trans*-activation.

5.2 Structure-Function Studies of the Thyroid Hormone Receptor

In applying alanine-substitution mutagenesis to the recognition α-helix, several interesting results were obtained: the importance of particular amino acids within the recognition α-helix to DNA binding was determined and analyzed in light of the available structural information; the involvement of a P-box amino acid in determining binding to a subset of half-sites sequences was recognized; and, a mutation which divorces *trans*-activation capacity from DNA binding affinity (G121A) was identified. Each of these results has contributed to the field of thyroid hormone receptor research in a timely fashion, and continues to provide a basis for interest.

The involvement of particular amino acids in the recognition α-helix of TRβ in DNA binding underscores the need for functional studies that complement strictly structural approaches. The alanine substitution mutants of TRβ confirmed the importance of certain structural components of the zinc binding module, particularly the zinc coordinating

cysteine and the residues forming part of the hydrophobic core. Likewise, substitution with alanine of several conserved residues that make base-specific contacts with the TRE disrupt binding. While many of these effects could be predicted by extrapolation from point mutagenesis experiments with the GR (Schena *et al.*, 1989), as well as the GR DBD crystal structure (Luisi *et al.*, 1991), there are enough differences to indicate that the GR DBD structural features are not completely conserved with respect to the TR DBD. For example, T_{129} in the TR is an alanine residue in the GR; however, the mutant TRβ, T129A, is quite deficient in binding to some natural TREs. Substitution of the P-box residues with alanine yielded surprising results that were significantly divergent with previous results obtained with the GR and ER.

Of the three non-conserved amino acids, EGG, that lie within the P-box of the TR DBD, only the first position, when substituted with alanine, inhibits binding of TRβ. E_{120} is now known from the TR/RXR DBD X-ray crystal structure to make base-specific contacts with the TRE half-site. Perhaps surprisingly, G_{121} also makes contacts with the DNA in the crystal structure, albeit stemming from the peptide backbone. However, the TRβ mutant, G121A, retains near wild-type DNA binding affinity. Substitution with alanine of the third discriminating P-box position results TRE binding activity that is at least as great as wild-type to all elements tested, except for TRE_{MAL}. Thus, only one out of the three P-box amino acid positions makes direct base-specific contacts, and causes general disruption of DNA binding activity when substituted with alanine. This is somewhat analogous to the situation for the GR DBD, although it is the third P-box position, V_{124}, that makes the direct DNA contact. Random mutagenesis of the DBD of the GR failed to identify mutations of the P-box residues that disrupted GRE binding (Schena *et al.*, 1989). Moreover, several studies have indicated that more than one P-box amino acid must be substituted from the ER into the GR in order to switch DNA binding specificity of the GR DBD (Umesono and Evans, 1989; Alroy and Freedman, 1992; Zilliacus *et al.*, 1992).

Thus, the alanine substitutions are consistent with this synergistic model of P-box function in the TR.

Some questions arising from the alanine substitution mutant results have been pursued further in Dr. Paul Romaniuk's laboratory; my involvement in this work has not been included in this thesis, other than some material presented in Chapter 4, as it has consisted mainly of technical assistance and advice with regards to DNA binding assays, and the work has been directed primarily by Dr. Paul Romaniuk, Dr. Colleen Nelson and Mr. Stephen Hendy. A brief description of work extending from the alanine substitution mutants follows, however, as it developed from the experiments described in Chapter 3.

The reduced capacity for the G124A mutant to bind to AGGACA half-sites has been examined (Nelson *et al.*, 1994). EGA happens to be a naturally occurring P-box sequence, being found in the ER. Other naturally occurring P-box sequences were also introduced into TRβ and assayed for DNA binding and *trans*-activation capacity. Of the related sequences, EGG, EGA, EAA, EGS, and ESG, only a subset could bind with significant affinity to AGGACA half-sites in tandem direct or everted repeats. A distinct subset could bind to AGGGCA half-sites, similarly arranged, illustrating that not only do the discriminatory amino acids of the P-box sequences function interdependently, they may endow distinct, but overlapping half-site specificity upon the DBD. The v-*erbA* protein has an EGS P-box sequence, and this difference from TRα, in conjunction with an N-terminal domain point mutation, is shown to result in a similar alteration of half-site sequence recognition by the v-*erbA* DBD to that which we observed with the EGS mutant of TRβ (Smit-McBride and Privalsky, 1994).

In an ambitious extension of this work Dr. Colleen Nelson and Mr. Stephen Hendy introduced all 19 alternative amino acids into each of the three discriminatory P-box positions of TRβ. The binding of the 57 TRβ variants to everted repeats with different half-site sequences (Nelson *et al.*, 1995a), and to TREs with distinct 5' flanking sequences (Nelson *et al.*, 1995b) was characterized. The alanine substitution mutants indicated that

the second and third positions were able to accommodate alanine residues with little to no disruption of binding. Introduction of all amino acids at these positions indicated that a surprising number of amino acids can be introduced that do not disrupt binding to distinct subsets of half-site sequence and configuration. Even the DNA-contacting E_{120} can be substituted with a number of amino acids and still bind to a subset of half-site sequences and configurations. As a further extension of this work, Miss Maya Iskandar is constructing a phage display library of TRβ DBDs that are completely randomized at all three P-box positions. Such work will undoubtedly generate mutant DBDs with novel DNA binding specificity. Screening for mutants that retain DNA binding capacity will therefore be performed using DNA sequences randomized at the central positions. The functional P-boxes thus identified will be interesting from a protein-engineering angle, as well as applying directly to understanding P-box function, possibly with respect to orphan nuclear receptors.

Heterodimerization with RXRα, like homodimerization on everted repeats, generally expands upon the repertoire of half-site sequences and configurations that are bound by a particular mutant (Nelson *et al.*, 1996), more evidence of the over-riding influence of dimerization on binding specificity. Mr. Stephen Hendy is further examining the binding of everted repeats by the random, individual P-box position mutants, as well as the *trans*-activation capacity of the mutant receptors.

The mutant receptor, G121A, was shown to retain virtually wild-type binding activity, but was unable to carry out *trans*-activation from any of the TREs assayed. Until recently it could only be vaguely posited that the alanine substitution resulted in a conformational change which was inappropriate for *trans*-activation, although it had been speculated that a mutation of the GR with a similar phenotype interfered with intramolecular communication between the DBD and the τ_1 domain (Zandi *et al.*, 1993). While that speculation may be true, recent evidence regarding the requirement of nuclear receptors for co-factors that mediate *trans*-activation suggests further experiments that could be performed to define the

mechanism by which G121A uncouples DNA binding and *trans*-activation. Assays have been developed for detecting the interaction of nuclear receptors with the positive co-factors that have been identified. It would be interesting to subject G121A to these assays, in comparison with wild-type TRβ, to see if interaction of one or more of these co-factors might be disrupted by the G121A mutation.

The phenomenon of dominant negative receptor forms has been described by many groups interested in generalized resistance to thyroid hormone (GRTH) and the activity of the v-*erbA* protein. The dominant negative forms of TR important in these cases are deficient in ligand binding and/or *trans*-activation, and thus form inactive heterodimers with wild-type TR and/or other nuclear receptors, such as RXR (Kastner *et al.*, 1995). However, TR forms lacking the DBD have also been observed to interfere, in a dominant negative fashion, with T_3-dependent *trans*-activation in transfected cells (Forman *et al.*, 1989; Bigler *et al.*, 1992). Bigler *et al.* (1992) showed that truncated forms of TRα, produced by translational initiation at internal methionine codons so that the DBD is missing, interfere with binding of full-length receptor *in vitro*. A similar phenomenon involving truncated TRβ forms is suggested by work in this thesis. Likewise, some evidence of inhibition of wild-type TRβ binding to DNA by DNA binding-deficient alanine substitution mutants of TRβ was observed. The TRβ mutants that were deficient in DNA binding as homodimers were also unable to bind DNA in the presence of RXRα. The formation of heterodimers that included DNA binding-deficient TRβ mutants was not proven, but there is no reason to believe that they would not form as normal in solution. However, an assay for receptor interaction through the LBD has been developed in which one receptor partner is expressed as a GST-fusion and immobilized on microtitre wells (Kurokawa *et al.*, 1993). The interaction of ^{35}S-labeled receptor protein with the immobilized receptor can then be easily detected. Such an assay could be applied to confirm that the DNA binding-deficient mutants do form heterodimers with RXR. To more clearly show a dominant negative effect on DNA binding, it may be necessary to express

receptor proteins at higher levels than are achieved with RRL. Such alternative expression systems as bacteria or baculovirus might be appropriate. The TRE_{LYS} affinity column could be further utilized in conjunction with the non-binding alanine substitution mutants of TRβ, since large quantities of RRL may be passed over the column. By mixing binding and non-binding receptor forms that were labeled with ^{35}S, or unlabeled, the ability of the non-binding receptors to prevent wild-type receptor from binding to the column could be assessed.

5.3 Structure-Function Studies of Thyroid Hormone Receptor Binding Sites

My most recent work regarding the recognition of direct repeats with various spacer lengths, and everted repeats, by TRβ homodimers and TRβ/RXRα heterodimers provides a basis for another level of complexity in gene regulation by TR. Half-site sequence, orientation and spacing are well established as contributing to differential binding by various receptor complexes and, consequently, restricting *trans*-activation to particular ligand signals. Strand-wise degeneracy of half-site sequences resulting in different receptor complexes binding to tandem repeats in distinct modalities (Figure 4.21), however, has not previously been described. To further define this phenomenon, a number of experiments could be performed. Since it has never been systematically shown that TR/RXR heterodimers bind to everted repeats in Mode E, it would be desirable to clarify this point. A point mutation-based approach could be applied to this problem; starting with a perfect everted repeat sequence, point mutations that improve an overlapping DR4 sequence could be analyzed for their effects on homodimer and heterodimer binding. In fact, the binding affinities of homodimers and heterodimers for EvRs with various spacer lengths have not been determined. Systematic determination of the K_ds for the interaction of homodimer and heterodimer with EvRs with spacers from 3 to 9 base-pairs in length would help predict in which modality each receptor complex was binding to binding sites with EvR characteristics. In light of the results presented here, however, it is apparent

that binding modality to each sequence would have to be determined directly through DNA binding analysis.

The importance of determining DNA binding modality, even to idealized half-site repeats, is an important consequence of the results presented here. There is a danger in making assumptions about the ability of TR complexes to bind to DNA, based solely on sequence analysis. A relevant example in the literature are two papers which present evidence for the capacity of TR monomers to carry out *trans*-activation. In one paper, a single half-site, cloned into a reporter plasmid was able to confer T_3-responsiveness on a downstream CAT gene (Schräder *et al.*, 1994). EMSA was performed, revealing that a DNA probe containing the single half-site was not bound by a TRα homodimer or a TRα/RXRα heterodimer. However, the short oligonucleotide probe used for the EMSA lacked flanking sequences present after cloning the half-site into the reporter plasmid. Thus, it is not clear that the sequences present in the reporter plasmid were bound by monomers. Results in this thesis show that a single, idealized half-site is sufficient to facilitate binding by a TRβ homodimer or TRβ/RXRα heterodimer, depending on the flanking sequence. The second paper also revealed that a single, idealized half-site cloned into a CAT reporter plasmid can mediate T_3-dependent *trans*-activation (Katz and Koenig, 1993). In this case, an EMSA was performed with DNA probes excised from the reporter plasmid, and thus contained the flanking sequences present when *trans*-activation was carried out. However, the EMSA was performed only with *in vitro* translated TRα, and thus, any heterodimers present in the nucleus that might be able to bind to the half-site and reporter plasmid flanking sequences would not have been detected. The results of my work indicate that more rigorous proof of monomer binding, *in vivo* or by factors present *in vivo*, is required to establish *trans*-activation by monomers as a legitimate phenomena.

It would be interesting to determine functional differences between elements that bind TR homodimers and heterodimers in different modalities. EvRs are known to be high affinity binding sites for TRβ homodimers, and to function as efficient transcriptional

silencers in the absence of hormone (Piedrafita *et al.*, 1995). Since it is predicted that some non-idealized tandem half-site repeats will bind the TR homodimer in Mode E regardless of the mode of heterodimer binding, mixed-type repeats may allow the repressive strength of a TRE to be modulated independently of heterodimer functionality. A possible consequence of this model is that the dynamic range of T_3-regulation from a particular TRE may be dependent on distinct characteristics of the homodimer and heterodimer, and their respective modalities of binding. For example, since the homodimer is believed to most efficiently repress transcription in the absence of T_3 from everted repeats, the affinity of the everted repeat in a mixed-type repeat may determine the bottom end of the dynamic range of T_3 regulation; that is, the level of transcription that occurs in the absence of T_3. Other characteristics, such as the affinity of the direct repeat may establish the high end of the dynamic range; the maximal amount of T_3-dependent transcription. Most studies in the field express T_3-regulation of gene expression as n-fold activation, or the ratio of expression in the presence of hormone to that in the absence of hormone. Unfortunately, such an analysis may obscure discrete effects on repression and maximal activation. An interesting example of this concept is provided by a study of TRE_{LYS}, an EvR6, published by Williams *et al.* (1994). In this paper, the absolute values of expression of the reporter gene are given for both the absence and presence of T_3, as well as the ratio of $+T_3/-T_3$ expression levels. This data is shown in Figure 5.1. Mutant 3 is of particular interest because it improves half-site C which forms a DR4 along with half-site B, while disrupting half-site A, which forms an everted repeat with half-site B. The result of this mutation is that repression in the absence of T_3 is lost, and that the maximal level of reporter gene transcription is raised by 3-fold. However, when the $+T_3/-T_3$ ratio is considered, this information is obscured, and the Mutant 3 appears to be half as effective a TRE as wild-type TRE_{LYS}. As the mutations introduced into TRE_{LYS} were not designed to address the phenomenon which I have described it is not appropriate, strictly speaking, to directly interpret them in this light. However, this study does illustrate that a complete

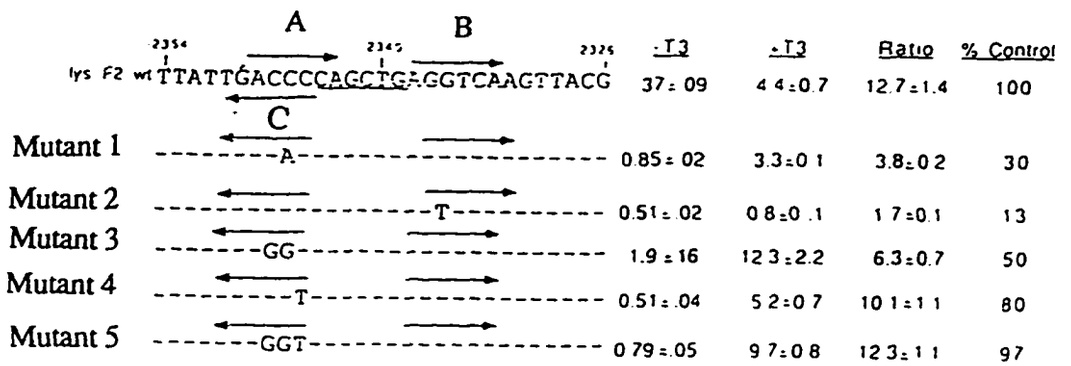


Figure 5.1: A functional analysis of TRE_{LYS}. This data is from Williams *et al.* (1994). The point mutations indicated were introduced into TRE_{LYS}, and the effects on transcriptional activation determined. Reporter plasmids bearing the wild-type or mutant TRE_{LYS} sequences upstream of the CAT gene were transfected into JEG cells. Values shown are for CAT expression in the absence of T_3 ($-T_3$), CAT expression in the presence of T_3 ($+T_3$), and the ratio of $+T_3/-T_3$, which is also expressed as a percentage of wild-type TRE_{LYS}.

analysis of TRE function requires the examination of both repression and *trans*-activation activities, and that the dynamic range of gene expression mediated by a TRE is not necessarily correlated to the level of expression in the presence or absence of T_3.

A recent model for thyroid hormone receptor function describes the receptor as being in an equilibrium between a repressive state and an activating state, an equilibrium which is shifted towards activation by the binding of T_3 (Schulman *et al.*, 1996). Analogously, I propose that mixed-type repeats may represent points on a continuum between two distinct modalities of TRE functionality. Examining this phenomenon in greater depth will not only help define the role of mixed-type repeats in gene regulation, but aid in differentiating the activities of the half-site repeats that comprise the extremes of the continuum: idealized direct repeats and idealized everted repeats, as well as dissecting the unique characteristics of the homodimer and heterodimer.

The prospects for interesting and relevant results arising from further examination of this phenomenon are good. Methodologies appropriate for approaching the problem are already well established. An assay for transcriptional repression by TRβ in the absence of T_3 has been described (Piedrafita *et al.*, 1995), which would be useful for dissecting the effects of half-site mutations on repression from the effects on activation. DNA binding assays such as those described in this thesis would be appropriate to distinguish homodimer and heterodimer binding properties to various idealized and mixed-type repeat sequences. It will be interesting to follow the developments in this regard, and I hope to make further contribution to the nuclear receptor field through further involvement in this research, and publication of these results.

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