CHARACTERIZATION OF THE NATIVE AND IRON-REPLACED DNA-BINDING DOMAIN OF THE RETINOIC ACID RECEPTOR

By

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A thesis submitted in conformity with the requirements for the degree of Master's of Science
Graduate Department of Biochemistry
University of Toronto

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Characterization of the Native and Iron-replaced DNA-binding Domain of the Retinoic Acid Receptor
Master's of Science (1996)
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University of Toronto

Abstract

The retinoic acid receptor DNA-binding domain (RARdbd) was shown to bind one AGGTCA site with a dissociation constant of 15.3 (± 0.5) nM. The binding affinity of the RARdbd appeared to be increased on the DR2 element, indicating potential cooperative homodimerization. The binding affinity of the RARdbd also appeared to be increased in the presence of the RXRdbd polypeptide on both the DR2 and DR5 response elements, indicating potential cooperative heterodimerization.

The second portion of this thesis demonstrated that iron was able to substitute for zinc in the retinoic acid receptor DNA-binding domain. The iron-substituted RARdbd was shown to interact with the AGGTCA consensus sequence with a dissociation constant of 15.3 (± 0.6) nM and with the same apparent DNA contacts as the native RARdbd. Most importantly, the iron-substituted RARdbd was shown to generate hydroxyl radicals, a highly reactive and oxidative species implicated in carcinogenesis through DNA strand breaks and base modifications.
**Acknowledgements**

This degree would not have been possible without the support of my committee, who helped me to direct my experiments and focus my thoughts. Dr. Peter Lewis and Dr. Reinhart Reithmeier both provided emotional support through this experience, making completion of this degree possible. I must also thank Damiano Conte for initiating the iron replacement work in the lab, and thus providing me the ideas for my second chapter as well as a great deal of feedback regarding the significance and future of the work with iron.

In looking to the future, I must also thank McMaster University, and especially Dr. Michael Rudnicki, who accepted me into the Ph.D. programme and helped me realize that I am more than capable of being a scientist. This faith and support helped me to finish my thesis and prepare me for the experience of defending my work.

Last, but definitely not least, I must thank all of those who provided love and support throughout my thesis. In particular I must thank Patricia Bronskill who helped me to develop my skills as a teacher and always provided “motherly” encouragement and support. I must also thank Nicholas Stosic, who even though he has only had to endure the final months of this experience, has provided me with stability, humour, and love. Most importantly, I must thank my parents, who have endured too many of their own hardships, but have always found enough love and strength to encourage me and help me recognize my abilities. Thank you.
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<tr>
<td>atRA</td>
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<tr>
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</tr>
<tr>
<td>ddRA</td>
<td>didehydroretinoic acid</td>
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<td>cellular retinol binding protein</td>
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Chapter One:  
Characterization of the native retinoic acid receptor  
DNA-binding domain

Introduction

Transcriptional Initiation

Proteins are important biological molecules which are involved in almost every physiological function. The nucleic acids of an organism direct the synthesis of these vital cellular products by providing a template for the transcriptional and translational machinery. The first step involves converting the stored information of the genomic DNA into messenger RNA by the transcriptional process. Translation of the message results in the synthesis of protein. The type of proteins synthesized in a cell provide that cell with its functional identity. Proteins catalyze a wide variety of reactions, provide structural support, control membrane permeability, regulate the concentration of important metabolites, effect motion and regulate the function of genes. Therefore, transcriptional and translational processes must be highly regulated so as to ensure proper embryonic development and adult homeostasis.

Transcriptional activation in eukaryotes is regulated by the formation of a multiprotein initiation complex on the promoter region of a responsive gene, as is shown in Figure 1.1. One of the primary steps in assembly of the initiation complex is binding of transcription factor IID (TFII-D) to the TATA element of the promoter DNA. This transcription factor is composed of the TATA-binding protein (TBP) and a number of associated factors. TFII-B then recruits RNA polymerase II and TFII-F to the TFII-D-DNA complex. This group of
Figure 1.1. The transcriptional initiation complex. The proteins of the basal transcriptional machinery are involved in various interactions with each other by means of a number of protein-protein interactions. This assembly of proteins is also in contact with the promoter DNA through specific interactions between TFIID and the TATA-box element. Transcriptional intermediary factors (TIFs) may then act to bridge an interaction between an activator, such as a nuclear hormone receptor, and the basal machinery so as to accelerate transcriptional initiation.
proteins represents the minimal requirements for transcriptional initiation. Association of TF<sub>H</sub> and TF<sub>E</sub> completes the formation of the basal assembly, as well as converting the initiation complex into an elongation complex (Buratowski, 1994; Heintz and Pederson, 1994).

For effective transcriptional initiation, another group of transcription factors, known as activators, must interact with the basal machinery. Activators are sequence-specific transcription factors which accelerate transcriptional initiation by binding to an upstream activating sequence (UAS) within the promoter DNA, prior to the formation of the initiation complex. Once bound to the DNA, the activator is involved in protein-protein contacts with the general transcription factors. These interactions may be direct, or mediated by transcriptional intermediary factors (TIFs). Such interactions may stabilize the initiation complex, as well as facilitate activation of the polymerase.

The first activator to be well characterized was transcription factor IIIA (TF<sub>III</sub>A), which is required for transcription of the 5S-rRNA genes by RNA polymerase III. This protein contains nine repeated domains of thirty amino acids, each of which contain conserved cysteine, histidine, and hydrophobic residues. In 1985, it was discovered that each thirty-residue repeat forms an independent DNA-binding minidomain, which tetrahedrally coordinates a zinc atom through two cysteine and two histidine residues (Miller et al., 1985). This “zinc-finger” motif has been shown to be one of the most commonly found in eukaryotic regulatory proteins. Today, the term “zinc-finger” no longer simply describes this arrangement, but also refers to a variable zinc-binding motif found in a number of proteins. To date, approximately 1% of the human genome encodes zinc-finger-containing proteins, including the classical zinc-fingers, GATA-1 proteins, LIM-domain proteins, RING-fingers, and the nuclear hormone receptors (Hoovers et al., 1992).
The Nuclear Hormone Receptors

The nuclear hormone receptors are a group of zinc finger-containing proteins which comprise a large family of ligand-inducible transcriptional activator proteins. The members of this family include receptors for molecules such as the glucocorticoids, estrogen, progesterone, aldosterone, as well as the hormonal forms of vitamin A and vitamin D, thyroid hormone, and peroxisomal activators. Hormone bound-receptors function as activators by binding to specific sequences of DNA located in the promoter region of hormone responsive genes, and effecting the activation of transcriptional initiation through protein-protein contacts. Although a number of *in vitro* studies have shown that the nuclear hormone receptors interact directly with the proteins of the basal transcriptional complex, the physiological significance of these studies is suspect. Other studies have discovered various transcriptional intermediary factors which are able to act as bridges between the nuclear receptors and the basal transcription factors. RIP140 is a potential intermediary factor which has been shown to bind to transcriptionally active forms of the estrogen receptor (Cavailles et al., 1985). A member of the RING-finger family, TIF1, has also been shown to interact with the estrogen receptor as well as the progesterone receptor, retinoic acid receptor, and the retinoid X receptor (LeDouarin et al., 1995).

One of the more interesting discoveries was that of SUG1, a yeast protein with a number of mammalian homologues, which has been shown to function as a nuclear receptor-interacting factor. This protein has also been shown to be a component of the RNA polymerase II holoenzyme (Lee et al., 1995; vomBaur et al., 1996). This suggests that SUG1, bound to a nuclear receptor, may be important in recruiting the basal transcriptional complex to the promoter region of the gene to be expressed. This further emphasizes the importance of
**Figure 1.2.** Functional domains of the nuclear hormone receptors. Domain C, the DNA-binding domain, exhibits the greatest degree of homology between the receptors while region E/F, the ligand-binding domain, is more unique. The A/B region, involved in ligand-independent transactivation, appears to display the least homology between receptors and therefore may be important in distinguishing specific hormonal pathways. Region D is known as the hinge region.
the nuclear hormone receptors in regulation and initiation of gene expression.

The nuclear hormone receptors are unified as a superfamily by structural and functional similarities, such that all receptors can be divided into common domains which exhibit discrete functions. The A/B region of the receptors appears to be involved in ligand-independent transactivation. It varies in length between the receptors and has been postulated to function in a cell- and promoter context-specific manner. The C region is the most highly conserved amongst the different receptors. This region contains an invariant pattern of cysteine residues which tetrahedrally coordinates two zinc atoms, forming a "zinc-twist", reminiscent of the zinc finger of TFIIIA (Vallee et al., 1991). It is this zinc-twist which mediates the binding of the receptor to its cognate DNA sequence. In concert with the D region, this DNA-binding domain is also involved in nuclear localization as well as the cooperative formation of homo- or heterodimers, the functional form of the receptors. Lastly, the E/F region is known as the ligand-binding domain and has been implicated in ligand-dependent transactivation. A number of the transcriptional intermediary factors which bridge the interaction with the general initiation complex have been shown to interact with a specific portion of this region. It has also been shown that heptad repeats within this region form a coiled-coil dimerization interface, aiding in the formation of functional activators or repressors.

The ligand-bound nuclear receptors transduce the hormone signal by binding as dimers to specific sequences of DNA, known as response elements, located in the promoter region of hormone responsive genes. The specific sequence of DNA to which nuclear hormone receptors bind usually consists of two core recognition motifs oriented and spaced to permit optimum protein-DNA and protein-protein contacts. The estrogen receptor (ER) and the
Figure 1.3. **Hormone response elements.** The ERE represents the DNA element recognized by the estrogen receptor. This response element is a palindromic repeat with a three base pair spacer. The glucocorticoid response element (GRE) is similar to the ERE in spacing and orientation, yet differs in the precise sequence. The RAREs represent the retinoic acid response elements. The DR2 and DR5 are direct repeats with two or five base pairs separating the half-sites, while ER8 represents an inverted repeat with an eight base pair spacer.
glucocorticoid receptor (GR) cooperatively bind to their cognate DNA sequences as ligand-bound homodimers. Both receptors bind to a palindromic repeat of a hexameric half-site spaced by three base pairs. The ER recognizes the AGGTCA half-site sequence, whereas the GR specifically binds to an AGAACA sequence. Optimization of the protein-protein contacts between the two receptors of the dimer results in cooperative binding to the DNA, increasing the affinity of the dimer for its response element.

The Retinoid Receptors

The most recently identified nuclear hormone receptors are the retinoic acid receptors (RAR) and the retinoid X receptors (RXR). In contrast to the ER and GR, which function as homodimers, RAR activates gene expression as a heterodimer with RXR. The formation of the heterodimer increases the efficiency with which the two receptors bind to the DNA. The most common retinoic acid response elements (RAREs) are direct repeats of the AGGTCA consensus half-site sequence, spaced by two (DR2) or five base pairs (DR5). An everted repeat of the same half-site with an eight base pair spacer (ER8) has also been shown to function as a response element. It has been shown that the binding of the heterodimers to the DNA response elements is ordered, such that RXR binds to the upstream half-site and RAR occupies the downstream site (Predki et al., 1994). RXR has also been shown to activate transcription as a homodimer from a direct repeat with one base separating the half-sites.

Three subtypes of the retinoic acid receptor have been identified: RARα, RARβ, and RARγ. Each subtype consists of a number of isoforms. For example, RARα and RARγ have seven identified major isoforms, while the RARβ group has four. Isoforms
are the result of each RAR gene consisting of two promoter regions. This results in alternative transcripts with varying A/B regions. The number of isoforms are further increased by RNA splice variants. A comparison of the RAR isoforms reveals a 97% similarity in amino acid sequence in the DNA-binding domain and an 80% similarity in the ligand-binding domain (Pemrick et al., 1994). In contrast, there is very little sequence similarity in the A/B and F regions of the receptors. The A/B region of the receptor is involved in ligand-independent transactivation, and has been shown to be able to act as an autonomous activator in the absence of the ligand-binding domain. The F region is part of the ligand-binding domain and may be involved in ligand-dependent transactivation. Since each isoform has a different A/B and F region, it is possible that different isoforms interact with different transcriptional intermediary factors, and therefore, activate expression of distinct genes. Thus, cell-specific expression of RAR isoforms may result in cell-specific gene expression in response to retinoic acid.

The retinoid X receptor family also consists of three subtypes, RXRα, RXRβ, and RXRγ. Each subtype potentially encompasses a number of isoforms. The retinoid X receptors exhibit a pattern of homology similar to RAR, in that there is high sequence conservation in the DNA-binding domain (93%) and the ligand-binding domain (88%). RAR and RXR display approximately 55-60% similarity in the DNA-binding domain, but only 25% in the ligand-binding domain (Pemrick et al., 1994). The differences in the ligand-binding domain can be attributed to differences in transactivation activity and ligand specificity. The retinoic acid receptor is able to bind all-trans retinoic acid, 9-cis retinoic acid, didehydroretinoic acid, and a number of other retinoic acid derivatives. However, the retinoid X receptor appears to primarily bind 9-cis retinoic acid.
In addition to serving as a partner for RAR, the retinoid X receptor is also able to heterodimerize with the thyroid hormone receptor (TR), the vitamin D₃ receptor (VDR), the peroxisome proliferator-activated receptor (PPAR), and the nerve growth factor-induced receptor (NGFI-B). The RXR-TR heterodimer binds to a direct repeat of the AGGTCAG sequence with four base pairs separating the two half-sites (DR4). In a similar fashion, the RXR-VDR heterodimer interacts with a direct repeat of the same consensus sequence, but with a three base pair spacer (DR3). RXR-PPAR heterodimers bind to a DR1 element and RXR-NGFI-B dimers interact with a DR5 response element. Therefore, the retinoids play an important role in other signalling pathways including those of vitamin D₃ and the thyroid hormone. In each case, the RXR binds to the upstream site, while the TR, VDR, PPAR, and NGFI-B occupies the downstream site. Thus, the polarity of binding may be important in dictating the type of interactions with transcriptional intermediary factors and the proteins of the basal transcriptional machinery. These interactions are likely to be responsible for recruiting the initiation complex as well as converting it to an elongation complex, thereby activating transcription.

The retinoid receptors activate gene expression in response to the various hormonal derivatives of vitamin A. Vitamin A, or retinol, is an essential nutrient derived from carotinoids in plants and retinyl esters in animal fat. Vitamin A is an indispensable molecule in vision, growth, reproduction, tissue maintenance and embryonic development. It has been shown to regulate the growth and differentiation of a number of cells, as well as possess anti-cancer activity. Various studies have shown that retinoids are capable of inhibiting the proliferation of normal cells as well as tumor cells. Therefore retinol has become an essential chemopreventive and chemotherapeutic treatment (Berard et al., 1994; Giguere, 1994).
Figure 1.4. The action of vitamin A within the cell. All-trans retinol enters the cell passively or by means of the RBP receptor (RBPR) and is metabolized to retinoic acid derivatives by means of cellular retinol binding proteins (CRBPs). Retinoic acid and its derivatives enter the nucleus and effect the expression of specific genes through the retinoid receptors (RAR and RXR). Excess retinoic acid is stored and metabolized by cellular retinoic acid binding proteins (CRABPs).
Figure 1.5. Retinoic acid derivatives. Figure 5a represents all-trans retinol, the most common form of vitamin A found in the blood. The remaining three molecules represent common active derivatives of retinoic acid: figure 5b is all-trans retinoic acid, 5c is 9-cis retinoic acid, and 5d is didehydroretinoic acid.
Physiological Significance of the Retinoid Receptors

Null mutations of RARα and RARγ have been generated in mice (i.e. all α or γ isoforms disrupted), and in both cases the observations revealed a decrease in viability as well as growth deficiency, male sterility, and congenital malformations (Kastner et al., 1995). Many of these effects have been shown to be cured or prevented by retinoic acid treatment, which may act to up-regulate the expression of the retinoid receptors. RARβ null mutants, on the other hand, did not appear to exhibit any discernible abnormalities. In contrast to these single mutants, double mutants of RAR subtypes displayed a dramatically reduced viability, and phenotypically appeared to be representative of vitamin A deficiency (VAD). Therefore, mice with single mutants may have been able to compensate through the use of other RAR subtypes, while mice containing double mutants are unable to overcome such a loss.

In another study, the effects of expressing an RARβ4-like isoform in transgenic mice was investigated (Berard et al., 1994). The results of the study indicated that this protein impaired normal growth control, resulting in hyperplasia in a number of epithelial tissues, including the lung, the salivary and sebaceous glands, and the mammary glands. Observations also revealed an increase in the incidence of primary tumors affecting these tissues in a comparison with non-transgenic littermates. A concomitant study of human lung tumor lines which express RARβ revealed a consistently higher ratio of RARβ4 to RARβ2 than that found in normal tissue. Investigations involving human mammary epithelial cells and tumor cell lines revealed a repression of RARβ2 in breast cancer cell lines and an up-regulation of the same retinoid receptor during in vitro senescence of mammary epithelial cells and also of human dermal fibroblasts (Lee et
al., 1995). These results suggest that the RARβ4 isoform is able to exert positive effects on cell growth, proliferation, and tumorigenesis, while the RARβ2 isoform appears to play a negative role in such processes. Interestingly, the RARβ4 isoform has been shown to encode for a protein which is lacking the A region normally found in retinoid receptors. This region has been implicated in ligand-independent activation, and although this domain appears to be dispensable for *in vitro* activity of a number of specific retinoic acid responsive promoters, it has also been shown to be important for expression from other promoters including that of the cellular retinol binding protein II (CRBP II). Therefore, it is possible that an excess of RARβ4 may be able to compete with other isoforms of RAR for binding to RAREs in promoters such as the one for the CRBP II gene. The lack of ligand-independent activation function in the RARβ4 isoform may result in a reduction in expression of those particular genes. If these genes encode for proteins involved in controlling cell growth and proliferation, then a deficiency would result in uncontrolled growth and possibly tumorigenesis.

In the case of RARβ2, previous studies have indicated that novel protein complexes bind to the β2 RARE in breast cancer cells, resulting in a transcriptionally repressed promoter and lower expression levels of RARβ2 (Lee et al., 1995). Other investigations have shown that RARβ2 expression is either altered or absent in a number of tumor cell lines (Gebert et al., 1991; Hu et al., 1991). Together, this suggests that RARβ2 is required to activate expression of a series of genes whose products have antiproliferative activity. The absence of such proteins may result in uncontrolled growth, and possibly tumorigenesis. The presence of these proteins would allow the cell to
maintain a state of homeostasis and potentially trigger cellular senescence. Thus, retinoic acid treatment may then act to up-regulate the expression of RARβ2 and other important retinoid receptors, so as to control and inhibit proliferation.

**The DNA-binding Domain of the Retinoid Receptors**

As stated earlier, the retinoid receptors transduce the retinoic acid signal by binding as dimers to specific sequences of DNA located in the promoter region of retinoid responsive genes. These sequences consist of a repeat of the AGGTCA consensus site with various spacings and orientations. The most commonly studied retinoic acid response elements are the direct repeats with either two (DR2) or five (DR5) base pair spacers. Binding of the receptors to these DNA elements is mediated by a region known as the DNA-binding domain. Previous work with a number of receptor DNA-binding domains has demonstrated that these polypeptides exhibit DNA binding patterns which closely resemble those of the full length receptors. It has also been shown that it is the DNA-binding domain alone which dictates specific binding to DNA response elements (Hu et al., 1992; Mader et al., 1993; Nelson et al., 1993; Rastinejad et al., 1995; Schwabe et al., 1993; Schwabe et al., 1993b; Zechel et al., 1994). Therefore, in order to study the DNA-binding properties of RAR, the DNA encoding the DNA-binding domain of human RARα and mouse RXRβ were cloned into a T7 RNA polymerase-dependent bacterial expression plasmid, p2R. BL21 (DE3) *Escherichia coli* were transformed with these plasmids and expression of the DNA-binding domain polypeptides, RARdbd and RXRdbd, were induced with isopropyl-1-thio-β-galactopyranoside (IPTG). The use of *E. coli* to produce these polypeptides has a number of advantages which include
a lack of interference from endogenous nuclear receptors which are present in animal cell preparations. After partial purification of these polypeptides, DNA-binding properties were studied using a 36-base pair oligonucleotide containing either the DR2 or the DR5 element. The ability of the RAR DNA-binding domain polypeptide to mimic the properties of the whole receptor allows us to focus on the DNA-binding function of the retinoic acid receptor while eliminating the problems associated with expressing and purifying the whole receptor.
Figure 1.6. Amino acid sequence of RARdbd and RXRdbd. The upper sequence represents that of the expressed retinoic acid receptor DNA-binding domain polypeptide (RARdbd), while the lower sequence represents the retinoid X receptor DNA-binding domain polypeptide (RXRdbd). Each DNA-binding domain polypeptide tetrahedrally coordinates two zinc atoms through eight conserved cysteine residues as shown.
Methods

Expression and Purification

BL21(DE3) *E. coli* bacteria transformed with the p2R-RARdbd or p2R-RXRdbd plasmid DNA were kept as glycerol stocks at -20°C. A preliminary bacterial culture was prepared by adding 10 μl of either the p2R-RARdbd or p2R-RXRdbd transformed *E. coli* glycerol stock to 100 ml of LB media containing 25 μg/mL of kanamycin. This culture was grown overnight at 37°C. On the following day, 1 L of LB containing 25 μg/mL of kanamycin was inoculated with the 100 ml culture and grown at 37°C until the absorbance at 600 nm was between 0.8 and 1.0. Once this absorbance was reached, isopropyl-1-thio-β-galactopyranoside (IPTG) was added to a final concentration of 1 mM so as to induce expression of the polypeptides. The incubation at 37°C was continued for a further 4 hours.

The bacteria were then harvested by centrifuging the media in a Beckman J2-MI centrifuge with a JA-10.5 rotor at 10,000 rpm for 10 minutes at 4°C. The bacterial pellet was resuspended in 20 ml of buffer A (25 mM Na/K phosphate [pH 7.6], 1 mM DTT, 10% glycerol) and PMSF was added to give a final concentration of 30 μM. The bacterial suspension was then sonicated, followed by centrifugation in a Ti50.2 rotor at 40,000 rpm for 45 minutes at 4°C. The supernatant was separated from the pellet and 2 ml of 2% polyethyleneimine was added dropwise to the supernatant. This mixture was incubated on ice for 10 minutes, and then centrifuged in a Ti50.2 rotor at 40,000 rpm for 30 minutes at 4°C. The supernatant was removed and dialyzed overnight against buffer A containing 50 μM zinc acetate.

This crude bacterial extract was applied to a 10 ml BioRex-
70 column which had been pre-equilibrated to pH 7.6 with buffer A. The column was initially washed with buffer A to remove proteins unable to bind to the resin. A number of other bacterial impurities were eluted with buffer A containing 300 mM NaCl. The RARdbd/RXRdbd polypeptide was eluted with buffer A containing 500 mM NaCl. Finally, the partially purified polypeptide was dialyzed overnight against buffer A to remove the salt.

**SDS-polyacrylamide gel electrophoresis**

SDS-polyacrylamide gel electrophoresis was used to follow the purification of the two DNA-binding domain polypeptides. The separating gel consisted of 15% acrylamide in 0.375 M Tris-HCl (pH 8.8) and 1% SDS. The stacking gel was a 4% acrylamide gel, with 0.25 M Tris-HCl (pH 6.8) and 1% SDS. The acrylamide stock used for preparation of the gels consisted of a 30:1 ratio of acrylamide to bis-acrylamide. Samples, consisting of an equal volume of protein and 2X sample buffer (50 mM Tris-HCl [pH 6.8], 100 μM β-mercaptoethanol, 2% SDS, 0.1% bromphenol blue, 10% glycerol), were heated to 100°C for five minutes prior to loading on the gel. After loading of the samples, the gel was run in 1X running buffer (25 mM Tris, 250 mM glycine, [pH 8.3], 0.1% SDS) at 180 volts for 90 minutes. The gel was then stained with 1% Coomassie blue in a mixture of 10% methanol and 10% acetic acid for 2 hours followed by destaining with a 10% methanol/10% acetic acid mixture overnight.

**Polypeptide Concentration Determination**

The concentration of polypeptide was determined using the BioRad protein assay, a method based on the Bradford dye-binding
procedure, in which the colour of Coomassie brilliant blue G-250 dye changes in response to various protein concentrations. The standard curve was constructed using bovine serum albumin with concentrations ranging from 100 μg/ml to 1000 μg/ml. For the RXRdbd, a correction factor was incorporated since the purification was not as complete as that for the RARdbd. This factor represented the percent purity of the RXRdbd based on densitometry analysis of the SDS-polyacrylamide gel using the UVP Gel Scanning system and the NIH Image 1.52 program.

**Mobility Shift Assay**

The mobility shift assay was employed to test the activity of the DNA-binding domain polypeptides. Assay samples consisted of polypeptide in a solution with buffer A containing 50 mM KCl, 1 μg dIdC (Pharmacia), and oligonucleotide 5'-end-labeled with γ-32P-ATP. These samples were incubated at room temperature for 15 minutes prior to loading on a 5.4% non-denaturing polyacrylamide gel containing 2.5% glycerol and 0.5X TBE (45 mM Tris-borate, 1 mM EDTA). This gel was first pre-run in 0.5X TBE buffer at 100 volts for 30 minutes, followed by loading the samples on the gel and running the gel in 0.5X TBE buffer at 180 volts for approximately 90 minutes. The gel was dried and exposed to Kodak XAR-5 film overnight at -20°C using an intensifying screen.

**5'-end Labeling of Oligonucleotides**

Oligonucleotide probes for the mobility shift assay were synthesized as single stranded DNA molecules by the Hospital for Sick
Children Biotechnology Centre. In order to anneal the two strands, as well as radioactively label the DNA oligonucleotides, 2 μg of strand 1 was incubated with 100 μCi of γ²²P-ATP (ICN) and 5 units of polynucleotide kinase (New England BioLabs) in reaction buffer (50 mM Tris-HCl [pH 7.6], 10 mM MgCl₂, 5 mM DTT, 0.1 mM spermidine-HCl, 0.1 mM EDTA) at 37°C for 2 hours. After this 2 hour period, the kinase enzyme was deactivated by further incubating the mixture at 85°C for 20 minutes. This was followed by the addition of 2 μg of the second strand and cooling of the mixture slowly to room temperature.

The annealed and labeled oligonucleotide was purified on a 16.5% non-denaturing polyacrylamide gel run at 150 volts in 1X TBE buffer for 4 hours. Double-stranded oligonucleotides were visualized as bands by soaking the gel in ethidium bromide for 15 minutes and exposing the gel to ultraviolet light. The bands corresponding to the proper oligonucleotides were excised from the gel and cut into smaller pieces. The labeled oligonucleotides were then eluted from the acrylamide gel by incubating the gel slices in 500 μl of metal-free water at 37°C overnight.

The specific activity of the oligonucleotides were determined by first measuring the absorbance of the eluate at 260 nm and converting the value to a concentration using a molar absorption coefficient value given by the Oligo 4.0 program. The counts per minute (cpm) in 10 μl of sample was determined using a Beckman LS6000IC β counter. The specific activity was then calculated and expressed as a cpm/pmol.

Unlabeled oligonucleotides were prepared by first heating 2 μg of strand 1 in reaction buffer (see above) to 85°C followed by the
addition of 2 μg of strand 2 and cooling of the mixture slowly to room temperature. The unlabeled oligonucleotides were not purified.

The oligonucleotides synthesized had the following sequences:

1836 (5')-GCATGGATCCAGGTCAAAAAGGTCAAGTCCGAT-(3')
1837 (5')-ATGCAGATTCTGACCTTTTGACCTGGATCCATGC-(3')
1838 (5')-GCATGGATCCAGGTCAATCACAAGGTCAGAATTCGAT-(3')
1839 (5')-ATGCAGATTCTGACCTTTTGACCTGGATCCATGC-(3')
1840 (5')-GCATGGATCCAGGTCAACAGGAGGTCAAGTCCGAT-(3')
1841 (5')-ATGCAGATTCTGACCTCTGTGACCTGGATCCATGC-(3')

Annealing of 1836 and 1837 results in a double stranded oligonucleotide which contains the DR2 response element, while 1838 and 1839 contain the DR5 element and 1840 and 1841 contain the DR4 element.

**Dissociation Constant Determination**

The dissociation constant for the RARdbd oligonucleotide bound to a single consensus site was determined using a mobility shift assay method. In these experiments, a fixed amount of protein, 1.35 pmol, was titrated with an increasing amount of DNA oligonucleotide which contained only one AGGTCA site. The gel was dried and exposed to film overnight. The bands corresponding to bound and free DNA were cut out of the gel and placed in scintillation fluid. The cpm for these bands were determined using a Beckman LS6000IC β-counter.
The cpm values were converted to nM concentration units using the specific activity of the DNA oligonucleotide and the quenching correction factor described in the previous section. These values were subjected to Scatchard analysis, which involves a plot of the ratio of the concentration of bound DNA to the concentration of free DNA as a function of the concentration of bound DNA. The slope of this plot corresponds to the negative reciprocal of the dissociation constant, and the x-intercept represents the concentration of active protein in the samples.

Cooperativity Assays

The purpose of a cooperativity assay is to evaluate the extent of DNA binding as a function of increasing polypeptide concentration. In order to accomplish this, a fixed amount of DNA oligonucleotide (DR2, DR4, and DR5) was titrated with an increasing amount of polypeptide. For each sample in this experiment, the concentration of RARdbd and RXRdbd polypeptide were identical.

Assay samples consisted of polypeptide in a solution with buffer A containing 50 mM KCl, 1 µg dIdC (Pharmacia), and oligonucleotide 5'-end-labeled with γ-32P-ATP. These samples were incubated at room temperature for 15 minutes prior to loading on a 5.4% non-denaturing polyacrylamide gel containing 2.5% glycerol and 0.5X TBE (45 mM Tris-borate, 1 mM EDTA). This gel was first pre-run in 0.5X TBE buffer at 100 volts for 30 minutes, followed by loading of the samples on the gel and running of the gel in 0.5X TBE buffer at 180 volts for approximately 90 minutes. The gel was dried and exposed to Kodak XAR-5 film overnight at -20°C using an intensifying screen.

The bands for bound DNA, both monomer and dimer, were then cut out of the gel and placed in scintillation fluid. The
counts per minute were determined using a Beckman LS6000IC β-counter. The counts per minute were converted to nM concentration units using the specific activity of the DNA and a correction factor for the quenching of counts due to the acrylamide. This correction factor was determined by comparing the cpm of a specific amount of γ-32P-labeled oligonucleotide which was run on a mobility shift assay with an identical sample which was simply added to the scintillation fluid.

DNase I Footprinting

The DNaseI footprinting method employed was adapted from a method described by Benoit Leblanc and Tom Moss (Kneale, 1994). The binding reaction consisted of RARdbd and RXRdbd polypeptide with DNA oligonucleotide in a reaction buffer which consisted of 25 mM Tris-HCl (pH 7.6), 50 mM KCl, 10% glycerol, and 1 mM DTT. After 20 minutes, a cofactor solution was added to give final concentrations of 5 mM MgCl₂ and 2.5 mM CaCl₂. This was followed by the addition of 50 ng, or 7.5 units, of DNaseI enzyme. This reaction was then allowed to proceed for exactly 2 minutes at 37°C.

An equal volume of stop solution (1% SDS, 200 mM NaCl, 20 mM EDTA) was added, followed by a 1:1 phenol:chloroform extraction. The aqueous top layer was transferred to a new tube and 130 µl of 3M sodium acetate, 1 µl of 10 mg/ml tRNA, and 800 µl of 100% ethanol was added to this solution. This precipitation reaction was incubated in a dry ice/ethanol bath for 20 minutes and then centrifuged at 12,800 rpm for 15 minutes.

The pellet from this centrifugation was washed with 500 µl of 80% ethanol and incubated in a dry ice/ethanol bath for 10 minutes. This solution was then centrifuged at 12,800 rpm for 15
minutes, followed by removal of the supernatant and a examination of the pellet with a Geiger counter. This pellet was then dried in a SpeedVac for 30 minutes.

The Cerenkov counts of the dried pellets were determined using a Beckman LS6000IC β-counter and then resuspended in loading buffer (7M urea, 0.1X TBE, 0.05% xylene cyanol, and bromphenol blue) to give a concentration of 5000 cpm/µl. The samples were heated at 90°C for 5 minutes prior to loading of 4-5 µl on a 20% urea denaturing gel. This gel was initially pre-run on a sequencing apparatus at 40 watts for 2 hours, or until the gel was uniformly heated. Subsequent to loading of the samples, the gel was run at 40 watts until the bromphenol blue dye reached the bottom of the gel (approximate time of 3 hours). The gel was then dried and exposed to Kodak XAR-5 film for 48 hours at -20°C.

Sequencing of the guanine bases in the DR5 oligonucleotide was achieved by initially methylating the guanine bases of the DNA with dimethylsulfate followed by selective cleavage of these methylated residues with piperidine. Approximately 1x10⁶ cpm of DR5 oligonucleotide was incubated in reaction buffer (10 mM Tris-HCl, pH 8.0, 60 mM NaCl, 10 mM MgCl₂, 1 mM EDTA) with 1 µl of dimethylsulfate for 5 minutes at room temperature. This was followed by the addition of 50 µl of ice cold stop buffer (1 M β-mercaptoethanol, 1.5 M sodium acetate, pH 7.0), 1 µl of a 10 mg/ml solution of tRNA, and 850 µl of ice cold 100% ethanol. This mixture was then incubated in a dry ice/ethanol bath for 10 minutes, and centrifuged at 12,800 rpm for 15 minutes at 4°C.

The supernatant was removed and the pellet was resuspended in 200 µl of an ice cold solution containing 0.3 M sodium
acetate and 1 mM EDTA. This was followed by the addition of 750 µl of ice cold 100% ethanol and an incubation in a dry ice/ethanol bath for 10 minutes. This precipitation reaction was then centrifuged at 12,800 rpm for 15 minutes at 4°C. The supernatant was removed and the pellet was again resuspended in the sodium acetate/EDTA solution and the subsequent steps were repeated.

The final pellet was washed with ice cold 70% ethanol and centrifuged at 12,800 rpm for 10 minutes at 4°C. The supernatant was removed and the pellet was dried in the SpeedVac for 15-30 minutes.

The pellet was resuspended in 50 µl of a 1:10 dilution of piperidine. This was further incubated at 95°C for 30 minutes, followed by a 5 minute incubation in a dry ice/ethanol bath. The solution was lyophilized in the SpeedVac until no liquid remained. This was followed by the addition of 100 µl of sterile water, and a second lyophilization step. This last step was repeated twice.

The tube containing the dried methylated DNA was placed in a scintillation vial and the Cerenkov counts were determined using a Beckman LS6000IC β-counter. The pellet was then resuspended in a volume of loading buffer (7M urea, 0.1X TBE, 0.05% xylene cyanol, and bromphenol blue) to give a concentration of approximately 5000 cpm/µl. The samples were heated at 95°C prior to loading of 3 µl on the 20% urea denaturing gel.
Results and Discussion

Expression and Purification of the RARdbd and RXRdbd

In order to study the properties of a bacterially expressed protein or polypeptide, it is often necessary to at least partially purify the molecule using a column of resin so as to eliminate interference from contaminating proteins. BioRex 70, a weak cation exchange resin, consists of carboxylic acid groups attached to a macroreticular acrylic polymer lattice. Within a particular range of pH, the BioRex-70 resin is negatively charged and will therefore interact with positively charged basic residues on proteins. Approximately 25% of the residues of the RAR and RXR DNA-binding domain polypeptides are positively charged, while less than 10% are negatively charged and the remaining amino acids are uncharged. This indicates that the polypeptides will bear a net positive charge, and will therefore interact with the BioRex-70 resin.

Figure 1.7 illustrates the purification of the RAR DNA-binding domain polypeptide on a BioRex-70 column at pH 7.6. Lane B represents the proteins found in the soluble fraction of the bacterial extract after sonication. A heavy band at approximately 14.4 kDa represents the RARdbd polypeptide. The third lane, lane C, illustrates the bacterial proteins which are unable to bind to the BioRex-70 resin. Lane D represents the bacterial proteins which interact with the resin, but are eluted from the column with buffer A containing 300 mM sodium chloride. The RARdbd polypeptide is eluted with buffer A containing 500 mM sodium chloride, as shown in lanes E and F.

Figure 1.8 illustrates the purification of the RXR DNA-binding domain polypeptide. The purification method is the same as that for the RARdbd and the results are similar. The major difference
**Figure 1.7.** SDS-polyacrylamide gel electrophoresis of RARdbd purification. The first lane, Lane A, represents the marker proteins which range in size from 6.0 kiloDaltons (kDa) to 200 kDa. The second lane, lane B, is the crude bacterial extract, and lane C represents the bacterial proteins which do not bind to the BioRex-70 resin. The proteins shown in lane D are those eluted with buffer A containing 300 mM NaCl. Lanes E and F both represent the proteins eluted with buffer A containing 500 mM NaCl. The RARdbd is eluted with this wash and appears to be approximately 14.4 kDa in size.
Figure 1.8. SDS-polyacrylamide gel electrophoresis of RXRdbd purification. The first lane, Lane A, represents the marker proteins which range in size from 6.0 kiloDaltons (kDa) to 200 kDa. The second lane, lane B, is the crude bacterial extract, and lane C represents the bacterial proteins which do not bind to the BioRex-70 resin. The proteins shown in lane D are those eluted with buffer A containing 300 mM NaCl. Lanes E and F both represent the proteins eluted with buffer A containing 500 mM NaCl. The RXRdbd is eluted with this wash and also appears to be approximately 14.4 kDa in size.
involves the degree of purity of the polypeptide. There appears to be more contaminating bacterial proteins found in the 500 mM NaCl eluate of the RXRdbd. This may be due to less RXRdbd expression relative to the amount of RARdbd expression. If there is a greater amount of strongly interacting polypeptide present, there will be less negative charges on the resin available for the contaminating bacterial proteins. Thus, with less RXRdbd polypeptide being expressed by the E. coli, there are more negative charges on the column available to the contaminating proteins.

**Binding of the RARdbd to the DR2- and DR5-containing oligonucleotides**

Figure 1.9 illustrates the binding of the RARdbd polypeptide to the double-stranded oligonucleotide containing the DR5 response element. This mobility shift assay represents a titration of 0.82 pmol of the DNA with an increasing amount of the polypeptide. As is expected, the amount of DNA which is bound appears to increase as the amount of RARdbd polypeptide increases. It also appears that the polypeptide binds to each AGGTCA site individually and that cooperativity is not involved in the binding of the RARdbd alone to the DR5 oligonucleotide. Figure 1.10 illustrates that the binding of the RARdbd to the DR5 response element is specific since an excess of unlabelled oligonucleotide effectively competes with the labelled oligonucleotide for the binding of the polypeptide. Specificity is further demonstrated by the fact that 1 µg of unlabelled non-specific DNA (dIdC), included in each sample, does not compete for the binding of the polypeptide.

Since each oligonucleotide contains two consensus half-sites, each of which will bind one polypeptide molecule, it would initially appear that 0.82 pmol of DNA should bind 1.64 pmol of
Therefore, when 1.53 pmol of RARdbd is used, approximately all of the DNA should be bound. Figure 1.9 indicates that when 1.53 pmol of RARdbd polypeptide is incubated with 0.82 pmol of oligonucleotide, less than half of the DNA is bound by the polypeptide. This illustrates how the binding of the protein to the DNA also depends on the affinity of the polypeptide for the AGGTCA consensus site, such that only a fraction of the RARdbd polypeptide is involved in interactions with the DR5-containing oligonucleotide. This is further illustrated in the last lane of the mobility shift assay titration in which an approximate 100-fold excess of protein over DNA is required to bind all of the oligonucleotide.

Table 1.1 represents a summary of the values obtained from the Scatchard plots shown in Figure 1.11. These plots were derived from mobility shift assays in which 1.35 pmol of RARdbd was titrated with an increasing amount of DNA oligonucleotide which contained one AGGTCA consensus site. The results indicated that the dissociation constant of RARdbd bound to one site was 15.3 nM (± 0.5 nM). This corresponds with a result of 16nM (± 0.72 nM) for the estrogen receptor DNA-binding domain (Schwabe et al., 1993b). According to this dissociation constant, incubation of an equal concentration of polypeptide and single-site oligonucleotide will result in the binding of approximately one-third of the DNA (see Appendix A for calculations). Therefore, in the mobility shift assay described above, if binding of the RARdbd to the DR5 is non-cooperative, then 1.53 pmol of active RARdbd will interact with 53% of the 0.82 pmol of DR5 oligonucleotide. If 15.3 pmol of active RARdbd is incubated with the same concentration of DR5 oligonucleotide, 96% of the DNA should be bound. The mobility shift assay shown in Figure 1.9 appears to confirm these calculations. If the binding of the RARdbd to the DR5 was cooperative, the affinity of the polypeptide for the DNA would increase
Figure 1.9. Mobility shift assay titration of the DR5 oligonucleotide with RARdbd polypeptide. Each lane represents 0.82 pmol of 5'-end labeled DR5 oligonucleotide incubated with various amounts of RARdbd polypeptide ranging from 0.76 pmol to 76 pmol.
Figure 1.10. Cold competition mobility shift assay of RARdbd binding to DR5 oligonucleotide. Lanes A, B, and C each contain 50 pmol of RARdbd polypeptide with 1.6 pmol of 5'-end labeled DR5 oligonucleotide. Lane B includes the addition of a 20-fold excess (32 pmol) of cold DR5 oligonucleotide, while lane C contains a 100-fold excess (160 pmol) of cold DR5 oligonucleotide. Lane D represents 1.6 pmol of free 5'-end labeled DR5 oligonucleotide.
Figure 1.11. Scatchard Plots for determining the dissociation constant of the RARdbd bound to a single AGGTCA site. Each line represents a single trial in determining the RARdbd dissociation constant (Series 1, 2, and 3). The concentration of bound and free DNA was calculated by converting the counts per minute of the corresponding bands from a mobility shift assay to concentration units using the specific activity of the labeled DNA and the quenching correction factor. The slope of this graph represents the negative reciprocal of the dissociation constant and the x-intercept represents the total active polypeptide concentration.
### Table 1.1. Summary Table of RARdbd dissociation constants.
The values in this table were obtained from the graphs illustrated in Figure 1.11. The dissociation constant, $K_d$, and the total protein concentration, $[P]_o$, are both in nM concentrations.

<table>
<thead>
<tr>
<th>Trat</th>
<th>slope</th>
<th>y-intercept</th>
<th>x-intercept</th>
<th>$K_d$</th>
<th>$[P]_o$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>-0.1054</td>
<td>0.39</td>
<td>3.68</td>
<td>14.81</td>
<td>229.7</td>
</tr>
<tr>
<td>2</td>
<td>-0.1035</td>
<td>0.39</td>
<td>3.79</td>
<td>15.08</td>
<td>226.6</td>
</tr>
<tr>
<td>3</td>
<td>-0.0984</td>
<td>0.97</td>
<td>9.84</td>
<td>15.86</td>
<td>230.3</td>
</tr>
<tr>
<td>Average</td>
<td>-----</td>
<td>-----</td>
<td>-----</td>
<td>15.25 ± 0.54</td>
<td>232.2 ± 3.8</td>
</tr>
</tbody>
</table>
as would the percentage of DNA which is bound.

Figure 1.12 represents the mobility shift assay titration of 0.82 pmol of DR2-containing oligonucleotide with increasing amounts of RARdbd polypeptide. The fraction of DNA bound appears to increase as the amount of polypeptide increases, as is the case with the DR5 oligonucleotide, but the relationship appears to differ from that for the DR5. It is also evident that the RARdbd has a propensity for forming a dimer on the DR2 oligonucleotide, a characteristic not seen with the DR5 oligonucleotide until high polypeptide concentrations are reached (results not shown). This indicates that the binding of the RARdbd polypeptide to the DR2 response element may be cooperative. The cold competition assay shown in Figure 1.13, as well as the fact that 1 µg of dIdC is included in each sample, demonstrates that this binding of the RARdbd polypeptide to the DR2 oligonucleotide is specific.

The possible cooperative binding of the RARdbd homodimer to the DR2 response element may have a number of physiological consequences. It is possible that the homodimer of RAR may be able to activate transcription from a DR2 response element, and thus, cooperative binding of the homodimer will increase the affinity of the RAR for its cognate DNA. The major limitation to such a proposal lies in the fact that a number of the studies completed to date indicate that it is the heterodimer of RAR and RXR which is the biologically important activator (Bugge et al., 1992; Kliewar et al., 1992; Leid et al., 1992; Valcarcel et al., 1994; Xiao et al., 1995; Yu et al., 1992; Zhang et al., 1992).

Figures 1.14 and 1.15 illustrate how the heterodimer of the RARdbd and RXRdbd has a greater propensity for forming the dimer on the DR2 and DR5 oligonucleotides than the RARdbd alone. Previous mobility shift assays, which are not shown here, as well as other studies, have indicated strongly that the RXRdbd has a much
Figure 1.12. Mobility shift assay titration of the DR2 oligonucleotide with RARdbd polypeptide. Each sample contains 0.82 pmol of 5'-end labeled DR2 oligonucleotide incubated with various amounts of RARdbd polypeptide ranging from 1.0 pmol to 76.5 pmol.
Figure 1.13. Cold competition mobility shift assay of RARdbd binding to DR2 oligonucleotide. Lanes A, B, and C each contain 50 pmol of RARdbd polypeptide with 3.0 pmol of 5'-end labeled DR2 oligonucleotide. Lane B includes the addition of a 20-fold excess (60 pmol) of cold DR2 oligonucleotide, while lane C contains a 100-fold excess (300 pmol) of cold DR2 oligonucleotide. Lane D represents 3.0 pmol of free 5'-end labeled DR2 oligonucleotide.
lower affinity for the AGGTCA half site sequence than the RARdbd, and that there is no formation of RXRdbd homodimer on either the DR2 or DR5 response element with comparable polypeptide concentrations (Mader et al., 1993). This indicates that the heterodimer has a greater affinity for the DR2 and DR5 response elements than either the RARdbd or RXRdbd homodimer. Thus, if RXR is present in sufficient quantity, the transcriptionally active heterodimer will be preferentially formed rather than the homodimer. This leads to the postulation that the RAR homodimer may function as a repressor rather than an activator. If RXR is not present to form the active heterodimer, the repressive homodimer may function to prevent activation of transcription of genes with a retinoic acid response element in the promoter region. Thus, homodimerization may represent a form of regulation of transcriptional initiation or the result of a limited concentration of RXR due to interactions with other nuclear receptors.

A recent study of the thyroid hormone receptor (TR) has revealed that the homodimer may function as a ligand-sensitive repressor when bound to specific sequences of DNA (Piekafita et al., 1995). Normally in order for the TR to bind to its DNA response elements with high affinity, and enhance transcriptional activation, the receptor must heterodimerize with RXR. It is possible that in the absence of ligand an inactive homodimer may compete with the active heterodimer for binding to the cognate DNA sequence.

Experiments have shown that nuclear receptors, such as the thyroid hormone receptor and the retinoic acid receptor, do not form complexes with heat shock protein 90 (hsp90) (Dalman et al., 1991). Other nuclear receptors, including the estrogen receptor (ER) and the glucocorticoid receptor (GR), interact with hsp90 in the absence of ligand. Upon interaction of the ligand with its receptor, hsp90 is believed to dissociate, allowing the ligand-bound receptor to interact
Figure 1.14. Mobility shift assay of RARdbd and RXRdbd binding to the DR5 oligonucleotide. Each lane contains 0.82 pmol of 5'-end labeled DR5 oligonucleotide. Lane A illustrates the binding of 1.6 pmol of RARdbd to the DR5 response element. Lane B represents the binding of the same amount of protein as lane A, but this sample contained both 0.8 pmol RARdbd and 0.8 pmol RXRdbd. Lane C represents free DR5 oligonucleotide.
Figure 1.15. Mobility shift assay of RARdbd and RXRdbd binding to the DR2 oligonucleotide. Each lane contains 1.0 pmol of 5'-end labeled DR2 oligonucleotide. Lane A contains 1.6 pmol of RARdbd, whereas lane B contains 0.8 pmol RARdbd and 0.8 pmol RXRdbd together. Lane C represents free DR2 oligonucleotide.
with its DNA response element, thereby activating the initiation of transcription.

In contrast with the ER and GR, the TR and RAR have been shown to be tightly bound to chromatin in the absence of ligand (Ribeiro et al., 1992). Therefore, the ligand encounters its receptor once it is already bound to the DNA. It can then be postulated that binding of the ligand may result in a change in the receptor which will activate transcriptional regulatory properties. Studies have shown that binding of the thyroid hormone ligand alters the properties of the TR, such that the affinity of the homodimer is weakened, while the affinity of the monomer and the RXR-TR heterodimer is enhanced (Piedrafita et al., 1995; Ribeiro et al., 1992). Thus, it is possible that the homodimer initially interacts with the chromatin, until the T₃ hormone ligand alters the binding properties such that binding of the heterodimer is preferred. Since the RAR is similar to the TR in structure and in function, it can be reasonably postulated that RAR homodimer may also function as a ligand-sensitive repressor.

Although most thyroid hormone response elements (TREs) are able to bind TR homodimers weakly, one study has shown that TR homodimers will bind efficiently and cooperatively to an inverted palindromic repeat of the AGGTCA sequence with a four, five, or six base pair spacer (IP4, IP5, and IP6) (Yen et al., 1992). However, the binding of this homodimer was shown to be prevented by the presence of the T₃ hormone ligand (Piedrafita et al., 1995). Experiments by Piedrafita et al. demonstrated that thyroid hormone decreased the binding stability of the homodimer, but not the RXR-TR heterodimer. This study further revealed that exposure to T₃ hormone was able to dramatically increase chloramphenicol acetyl transferase (CAT) activity in cells cotransfected with a TRβ expression vector and a CAT vector containing the IP6 response element. These results suggest
that the overexpressed TRβ will competitively bind to the IP6 element as a homodimer unless the thyroid hormone ligand is present. The T₃ ligand causes the homodimer to dissociate from the DNA, allowing the transcriptionally active heterodimer to form on the promoter. Thus, the IP6 thyroid response element appears to function as both an activator and repressor sequence. It can be concluded that this type of TRE is a versatile element that allows for repressive homodimer and activating heterodimer action, depending on the availability of RXR and/or ligand.

The ligand-binding domain of the nuclear receptors is currently implicated in ligand-dependent transactivation and dimerization. One study has also indicated that RAR and TR are able to repress transcription in the absence of ligand by means of a silencing function in the ligand-binding domain (Banahmad et al., 1992). Banahmad et al. postulate that positive- and negative-acting nuclear factors interact with the nuclear receptors and mediate communications with the basal transcriptional machinery. The ligand-binding domain of the TR and RAR may comprise separate binding sites for these factors, and depending on the site(s) available, transcription may be activated or repressed. Therefore, for the TR and RAR homodimers, the sites for positive-acting factors may be buried within the dimerization interface, while those for the negative-acting factors are accessible. Binding of ligand destabilizes the homodimer, and induces the formation of the heterodimer with RXR, potentially exposing sites for interactions with positive-acting nuclear factors. This may depend on the dimer structure, and thus, the orientation and spacing of the response element to which the receptors are bound. Therefore, ligand sensitive repression by TR or RAR homodimers may be sequence-specific, such that this form of transcriptional regulation may only occur on sequences such as the IP6 or DR2 element.
Therefore, the DNA-binding domain of these receptors is likely to be involved in dictating the binding specificity, such that homodimers and heterodimers are directed to form on select sequences. The heterodimer is the potentially transcriptionally active form, while the homodimer is unlikely to be involved in productive activation (Nagpal et al., 1993b). Thus, it is reasonable to postulate that the homodimer is involved in the repression of transcriptional initiation, whereas the heterodimer may function as an activator or a repressor. The binding of the ligand is important in transforming the bound receptors from a dormant state to a transcriptionally active conformation.

These studies therefore support the postulation that RAR may form homodimers which preferentially bind to DR2 response elements and repress transcription. Formation of this homodimer repressor may be dependent on a number of factors. First, the DR2 may be one of the only sequences to which binding of the homodimer is efficient and cooperative. Thus, the DR2 response element may be one of the only DNA sequences for which this hypothesis is valid.

Secondly, RXR concentration must be low in order for this occur, since the RXRdbd-RARdbd heterodimer has a greater affinity for the DR2 than the RARdbd homodimer, as shown in Figure 1.15. If the concentration of free RXR is comparable to the concentration of RAR, the heterodimer will preferentially be formed, thereby effecting transcriptional initiation.

Formation of the homodimer would also depend on the concentration of other nuclear receptors which heterodimerize with RXR. If there is a preponderance of receptors such as the thyroid hormone receptor, the vitamin D receptor, or the peroxisome proliferator-activated receptor, RXR will be involved in competing interactions with these receptors, allowing the homodimer of RAR to form (Leid et al., 1992). It is also important to consider that RXR forms
a productive homodimer on a DR1 element in response to 9-cis retinoic acid, thereby further limiting the potential availability of RXR for heterodimer formation (Lehmann et al., 1993).

Lastly, the retinoic acid ligand must also be low in concentration for two reasons. According to the study by Piedrafita et al., the homodimer may be ligand-sensitive, resulting in loss of binding to the DNA. This may be due to a change in conformation which causes the region involved in homocooperative binding to be distorted. The ligand, retinoic acid, has also been shown to up-regulate the expression of the retinoid receptors. Thus, retinoic acid would also potentially increase the concentration of RXR, procuring the formation of the heterodimer.

**Binding of the RARdbd-RXRdbd Heterodimer to the DR2- and DR5-containing oligonucleotides**

Therefore, the titration experiments demonstrated that each polypeptide was able to bind to the oligonucleotides containing the DR2 and DR5 response elements. The concomitant cold competition experiments illustrated that the binding of the RARdbd to these sequences is specific. The titration experiments further revealed that the RARdbd homodimer has a greater propensity to form on the DR2 response element as compared to the DR5 response element. This may indicate cooperative formation of RARdbd homodimers on the DR2 element.

Lane A of both Figure 1.14 and Figure 1.15 illustrates that 1.6 pmol of RARdbd polypeptide will bind mainly as a monomer to the DR2 and DR5-containing oligonucleotides. Lane B reveals that when 0.8 pmol of both polypeptides are present simultaneously, resulting in 1.6 pmol of total polypeptide, there is a significant formation of dimer. This dimer is likely to be heterodimer since the RARdbd homodimer
does not appear to form at this polypeptide concentration, as shown in Lane A. As indicated previously, assays not shown have indicated that RXRdbd will also not be able to form homodimers at this concentration. Therefore it appears that the two DNA-binding domain polypeptides bind preferentially as a heterodimer to the DR2 and DR5 response elements, and this binding is potentially cooperative.

The intensity of the dimer bands on Figures 1.14 and 1.15 suggest that the heterodimer has a greater propensity to form on the DR2 oligonucleotide as compared with the DR5 oligonucleotide. In a study by Mader et al., a "factor of cooperativity" was defined as the ratio of the amount of heterodimer formed to the amount of homodimer formed for the same amount of protein (Mader et al., 1993). Although this experiment involved the full receptors, rather than simply the DNA-binding domains, the results are significant. A factor of cooperativity of 31 was obtained for the DR5 response element, while a factor of cooperativity of 37 resulted for the DR2 element. Therefore, the heterodimer of the full receptors appears to bind with greater cooperativity to the DR2 response element.

The potential cooperative binding of the RARdbd and RXRdbd was analyzed by titrating a fixed amount of 5'-32P-labelled oligonucleotide with an increasing concentration of polypeptide. The polypeptide consisted of a combination of both the RARdbd and RXRdbd in equal concentration. The results were initially analysed by mobility shift assay, as shown in Figures 1.16, 1.17, and 1.18. Figure 1.16 represents the titration of the DR2-containing oligonucleotide, Figure 1.17, the DR5-containing oligonucleotide and Figure 1.18, the DR4-containing oligonucleotide. The DR4 was tested so as to illustrate a negative response since this sequence is a thyroid hormone response element, and binding of the RARdbd and RXRdbd should be noncooperative.
Figure 1.16. Cooperative Mobility Shift Assay for the D2 oligonucleotide. This mobility shift assay represents the interaction of 0.1 M NaCl with an increasing concentration of an equal combination of RNKdβ and RNKβd polypeptides.
Figure 1.17. Cooperative Mobility Shift Assay for the DRS oligonucleotide. This mobility shift assay represents the titration of 0.03 mol of DRK-containing oligonucleotide with an increasing concentration of an equal combination of RARδδδδ and RXXδδδδ peptides.

Free DNA

Monomer

Mol of polypeptide 7.5 6.0 5.25 3.75 3.0 1.88 0.75
Figure 1.8  Cooperative Mobility Shift Assay for the DRA oligonucleotide. This mobility shift assay represents the titration of 0.82 pmol of DR4 oligonucleotide with an increasing concentration of an equal combination of RKRkd and RKRkd polypeptides.
Figure 1.16 demonstrates the binding of the RAR and RXR DNA-binding domain polypeptides to the DR2 response element. If both the RARdbd and RXRdbd exhibited a dissociation constant of 15 nM for each AGGTCA site, only 26% of the DNA should be bound when 0.75 pmol of polypeptide is incubated with 1.01 pmol of oligonucleotide (for calculations see Appendix A). Since the RXRdbd has a lower affinity for the consensus sequence, it would be predicted that less than 26% of the DNA should be bound. The mobility shift assay in Figure 1.16 appears to indicate a greater percentage of bound DNA, as well as illustrate the propensity for heterodimer formation at this sub-saturating concentration of polypeptide. These results suggest that binding of the RARdbd and RXRdbd to the DR2 response element is potentially cooperative. It is also evident that the DNA-binding domain of both the RAR and RXR must also contain a dimerization function which is specific for the orientation and spacing of the response element (Zechel et al., 1994b; Zechel et al., 1994).

Figure 1.17 represents the mobility shift assay titration of the DR5-containing oligonucleotide with increasing amounts of RARdbd and RXRdbd. As indicated above, if both polypeptides exhibited a dissociation constant of 15 nM, then approximately 26% of the 1.03 pmol of DNA should be bound when incubated with 0.75 pmol of polypeptide. Considering that the RXRdbd binds with a lower affinity, then the percentage of DNA bound should be less than 26%. The percent of bound DNA in the first lane of Figure 1.17 appears to be close this approximation of 26%, suggesting a potentially weak cooperative binding of the polypeptides to the DR5 oligonucleotide. An interesting note is that a greater percentage of the DR2 oligonucleotide is bound by the heterodimer when compared with the DR5 oligonucleotide.
More importantly, there is no significant formation of dimer at concentrations of polypeptide which result in significant heterodimerization on the DR2 response element. This may be due to the increase in spacing of the two AGGTCA sites, causing the polypeptides to be positioned too far apart for significant interaction (Rastinejad et al., 1995). The apparently weaker binding of the heterodimer to the DR5 may also be due in part to the extended sequence of the RARdbd and RXRdbd. The polypeptides which are expressed encompass not only the DNA-binding domain, but also include a portion of the hinge, or D region, of the receptors. These polypeptides differ from typically expressed DBDs by 14 amino acids (Rastinejad et al., 1995; Zechel et al., 1994b; Zechel et al., 1994). This additional portion of the receptor is likely be disordered in the context of the polypeptide (Rastinejad et al., 1995), and may therefore interfere with dimerization when spacing between the AGGTCA half-sites permits such a freedom in structure of this RARdbd extension. In contrast with the DR5, binding to the DR2 response element brings the two polypeptides into closer proximity and may therefore result in favourable interactions between the two polypeptides, causing the extension to be forced outside of the dimerization interface. Binding to the DR5 does not bring the two polypeptides close enough to promote strong or favourable interactions, and may allow the extension of the RARdbd to be positioned within the interface. The structure of the whole receptor may allow for a more significant interaction between the DNA-binding domains of RAR and RXR on the DR5 response element.
DNaseI Footprinting of the RXRdbd-RARdbd Heterodimer on
the DR5-containing oligonucleotide

Structural determination of the ERdbd bound to its
cognate DNA, as well as of the RXRdbd-TRdbd heterodimer on the
DR4 has revealed that the each of the two DBD polypeptides interact
with adjacent major grooves of the DNA by means of an α-helix
(Rastinejad et al., 1995; Schwabe et al., 1993; Schwabe et al., 1993b). This
recognition helix is part of an overall basic structure common to all
nuclear receptors in which two amphipathic α-helices fold together
perpendicularly with the help of hydrophobic amino acids. This
structure permits optimum protein-DNA interactions between the
nuclear receptor and its cognate DNA sequence. In order to determine
the sites of specific interactions between the DBD polypeptides and
DNA, a method known as DNaseI footprinting was employed.

DNaseI is a 30-kDa endonuclease which contains a basic
pentapeptide loop that binds within the minor groove of the DNA.
The DNase enzyme also contains other lysine and arginine residues
which form salt bridges with the phosphate groups of both strands
flanking the minor groove. Thus, the enzyme interacts with an
extensive region of the DNA which includes approximately one turn
of the DNA helix. DNaseI is then able to cleave phosphodiester bonds
by a mechanism involving an activated water molecule, reminiscent of
chymotrypsin (Stryer, 1988). The interaction of the DNA with the
enzyme will be disrupted by the binding of the DBD polypeptides,
decreasing the extent of cleavage by DNaseI at specific sites of
interaction.

The last experiment of this chapter involved footprinting
of the interaction between the RARdbd and RXRdbd heterodimer and
the DR5-containing oligonucleotide, as illustrated in Figure 1.19.
Experiments have shown that RXR specifically interacts with the upstream AGGTCA site and RAR occupies the downstream site (Predki et al., 1994). It has also been established that RXRdbd has a lower affinity for the AGGTCA site than the RARdbd (Mader et al., 1993). This is augmented by the observation that the RXRdbd has fewer contacts with the DNA than the DNA-binding domain of the TR, and potentially fewer than the RARdbd (Rastinejad et al., 1995).

The X-ray crystallographic structure of the RXRdbd-TRdbd heterodimer bound to the DR4 reveals that the RXRdbd makes major groove contacts with the CA base pairs of AGGTCA through the arginine 33 residue of the polypeptide. The RXRdbd also makes phosphate backbone contacts with the first adenine base of the AGGTCA sequence. The footprint in Figure 1.19 demonstrates a decrease in DNaseI sensitivity at these bases, corresponding with the expected sites of interaction.

It can be assumed that the structure of the RARdbd is very similar to that of the TRdbd, since there is a 52% identity between the amino acid residues of the TRβdbd and the RARαdbd. According to the structural determination of the RXRdbd-TRdbd bound to the DR4, the tyrosine 16 and 18 residues of the RARdbd are expected to contact the nnAGGTCA bases through minor groove interactions with the phosphate backbone (Rastinejad et al., 1995). The lysine 27 residue of RARdbd will conceivably contact the AGGTCA residue through the major groove, while arginine 31 will contact the AGGTCA. The contacts with nnAGGTCA are illustrated in the footprint as decreases in DNaseI sensitivity. There is an increase in sensitivity at the AGGTCA base where the lysine 27 residue of the RARdbd is expected to make a contact. Other sites within the oligonucleotide also become more sensitive to cleavage after binding of the polypeptides. These sites are five nucleotides upstream and downstream of the AGGTCA of
Figure 1.19. The DNaseI footprint of the RARdbd and RXRdbd bound to the DR5 oligonucleotide. Lane A represents the sequencing of the guanine bases in the DR5 DNA oligonucleotide. Lane B is the result of DNaseI action on 0.65 pmol of free DR5 DNA oligonucleotide. Lanes C, D, E, and F represent the cleavage of 0.65 pmol of DR5 oligonucleotide which has been pre-incubated with increasing amounts of RARdbd and RXRdbd polypeptides. Lane C represents the footprint resulting from the binding of 0.76 pmol of RARdbd and 0.97 pmol of RXRdbd. Lane D represents 1.52 pmol of RARdbd and 1.94 pmol of RXRdbd, E represents 2.28 pmol of RARdbd and 2.91 pmol of RXRdbd, and F represents 3.04 pmol RARdbd and 3.88 pmol of RXRdbd.
the RARdbd site. This five nucleotide spacing represents approximately one half of a turn of the DNA helix since B-DNA has 10.4 base pairs per turn of the helix (Stryer, 1988). It is possible that interactions with the DNA-binding domain polypeptides may distort the DNA such that the phosphodiester bonds of the minor groove become more accessible to the DNase, facilitating the attack by the endonuclease (Scott et al., 1995). Previous experiments have shown that binding of the nuclear receptor causes DNA to bend, as well as resulting in an increase in the flexibility of the DNA and disrupting base stacking interactions. These consequences may result in a change in conformation which allows DNaseI greater access, and thus increases the potential for cleavage by the enzyme.

**Future Experiments**

Further experiments concerning the possible importance of RAR homodimerization in transcriptional repression include binding analyses as well as transcriptional assays of both the homo- and heterodimer. Initially in vitro work with the whole receptor would be necessary in order to determine (1) the dissociation constant of RAR binding to an AGGTCA half site, (2) the dissociation constant of RAR binding to the DR2, (3) the cooperativity constant for RAR binding to the DR2, (4) the stability of the homodimer bound to the DR2, (5) the dissociation constant for RXR-RAR binding to the DR2, (6) the cooperativity constant for RXR-RAR binding to the DR2, and (7) the stability of the heterodimer bound to the DR2. These experiments would allow one to assess the importance of the homodimer binding to the DR2 response element in relation to the transcriptionally active heterodimer.

The next series of experiments would involve testing the effects of ligand binding on the above values. According to the
previous experiments with the TR, it would be expected that the affinity and stability of the homodimer would decrease and the affinity and stability of the heterodimer would increase in the presence of ligand. This, in addition to transcriptional assays with the homodimer, would augment the hypothesis that in the absence of ligand, homodimers occupy the retinoic acid response elements preventing transcriptional initiation. The binding of the retinoic acid ligand would decrease the stability and affinity of the homodimer and promote the binding of the transcriptionally active heterodimer. This could also be shown through transcriptional assays.
Chapter Two
Characterization of the iron-replaced retinoic acid receptor
DNA-binding domain

Introduction

The Element Zinc and its Physiology

Zinc is the second most abundant metal in living organisms. It is a flexible and adaptable metal in that it can adopt four-, five-, and six-coordinate geometries. It is virtually non-toxic as it does not appear to undergo reduction or oxidation under physiological conditions. It has also been shown to interact in a stable manner with a variety of macromolecules, including a number of important enzymes. It is essential in cell division, development and differentiation. A deficiency in zinc results in a failure of cells to divide and differentiate, with a consequent impairment of growth.

The primary dietary sources of zinc include red meat, seafood, and cereal. Dietary zinc is absorbed in the jejunum and ileum and it is not known precisely how zinc is transferred to or across the mucosal surface of the intestine. Once inside the mucosal cell, zinc becomes associated with metallothionein and other macromolecules. It is then transported in the serum by means of transferrin and albumin. Inside the cell, approximately 30 to 40% of the total cellular zinc is located in the nucleus, and approximately 50% is found in the cytoplasm and its organelles. The remainder of the zinc is found in the cell membrane and/or the cell wall. Virtually all of the zinc found in these compartments is bound to macromolecules such as proteins/enzymes or nucleotides.
Zinc in Proteins and Enzymes

Zinc is the most widely employed metal in proteins and enzymes. It is the only metal found in each of the six classes of enzymes: oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. Zinc can adopt one of three roles in zinc-containing enzymes: a catalytic, coactive, or structural role. Structural sites generally involve the tetrahedral coordination of zinc through four cysteine residues, to the exclusion of water. A structural zinc atom is found within alcohol dehydrogenase, as well as aspartate transcarbamylase and protein kinase C. The binding of zinc in protein kinase C differs from that of alcohol dehydrogenase and aspartate transcarbamylase in that one of the cysteine residues is replaced by a histidine residue.

Zinc also plays a structural role in nucleic acid polymerases and transcription factors. The first transcription factor shown to bind zinc was transcription factor IIIA (TF_{III}A), which is required for the activation of transcription of the 5S rRNA gene. The TF_{III}A protein tetrahedrally coordinates each of its nine zinc atoms through two histidine and two cysteine residues (Miller et al., 1985). This “zinc-finger” motif has been shown to be one of the most commonly found in eukaryotic regulatory proteins. Today, the term “zinc-finger” no longer simply describes this arrangement, but also refers to a variable zinc-binding motif found in a number of proteins.

For example, the members of the nuclear hormone receptor superfamily also require zinc for structural stability, forming a “zinc-twist”, reminiscent of the “zinc-finger” of TF_{III}A. In contrast with the zinc-finger, the zinc-twist involves the tetrahedral coordination of two zinc atoms, each by means of four cysteine residues. The two zinc-binding motifs of the nuclear hormone receptors are folded together to form a single structural unit consisting of a pair of amphipathic α-
helices packed at right angles to each other. A zinc-binding pocket motif lies at the N-terminus of each of the helices of the DNA binding domain. Binding of the zinc atom to the first motif stabilizes the α-helix directly involved in DNA recognition, as well as providing a scaffolding for the formation of the single structural domain required for activity.

The thyroid hormone receptor (TR), a member of the nuclear hormone receptor superfamily, has been shown to lose DNA-binding activity when expressed in E. coli and solubilized in the presence of EDTA and in the absence of zinc (Miyamoto et al., 1991). DNA-binding activity of the receptor was restored with the addition of zinc, cadmium, manganese, or ferrous iron. Studies with the estrogen receptor DNA-binding domain, have shown that zinc can be removed from the polypeptide, resulting in a loss of DNA-binding activity. This activity is restored upon reconstitution of the polypeptide with cadmium, cobalt, and ferrous iron (Conte et al., 1996; Predki and Sarkar, 1992). Therefore, the structure of the DNA-binding domain may be stabilized by divalent metals other than zinc.

The Element Iron and its Physiology

Iron is the most abundant metal in living organisms. It is an essential element involved in important processes such as DNA synthesis, energy production, and oxygen transport. The average person contains 40 to 50 mg of iron per kilogram of body weight. The majority of this iron, 30 mg/kg, is bound by hemoglobin in the red blood cells. The remaining 10-12 mg Fe/kg in men, and 5 mg Fe/kg in women, is stored in the liver, spleen, bone marrow, and muscle as ferritin and hemosiderin. A tiny fraction of the iron in the body is in
transit, in the plasma, as a complex with transferrin or a small molecular weight carrier such as citrate or ATP.

The function of transferrin is to transport and deliver iron to cells by means of receptor-mediated endocytosis. This is achieved by means of the transferrin receptor located on the surface of iron-requiring cells. The current model for iron dissociation and mobilization within the cell following receptor-mediated endocytosis involves four major steps: (1) acidification of the endosome by means of an ATP-dependent proton pump; this facilitates the release of Fe(III) from transferrin, (2) reduction of Fe(III) to Fe(II), (3) translocation of Fe(II) to the opposite side of the vesicular membrane, and (4) mobilization of the Fe(II) by cytosolic low molecular weight carriers.

The identity of these low molecular weight carriers has been the subject of much discussion. Robert Crichton compares this low-molecular-weight iron pool with the Loch Ness monster, in that it disappears from view before its presence, or its nature, can be confirmed (Crichton, 1991). Many recent papers have identified this pool as Fe-ATP, but conclusive evidence of its characterization is clouded by the effects of cell disruption and purification required to isolate the low molecular weight carrier.

**Iron Storage in the Cell**

The cell stores excess iron in the form of ferrihydrite (5Fe₂O₃·9H₂O) within the core of the ferritin protein. Ferritin is a large protein aggregate which consists of 24 peptide chains of approximately 175 amino acids each. These peptide chains pack together to form a hollow sphere, approximately 100 Å in diameter, with hydrophobic and hydrophilic channels leading to the core. Ferrous iron enters the core ferritin by means of the hydrophilic channels, where it is oxidized to ferric iron and formation of the hydrated ferric oxide core is
initiated. These initiation complexes are augmented by the hydrolytic polymerization of Fe(II) and concomitant oxidation to Fe(III):

$$12\text{Fe}^{2+} + 3\text{O}_2 + 12\text{H}_2\text{O} \rightarrow 6\text{Fe}_2\text{O}_3 + 24\text{H}^+$$  \(1\)

Within this ferritin sphere, as many as 4500 atoms of iron may be stored, thus achieving a cellular iron concentration equivalent to 0.25 M. Therefore, ferritin functions to facilitate the solubilization of iron, as well as store the metal and prevent the potential toxic effects of this highly reactive atom.

Animal cells can also store iron in the form of hemosiderin, a derivative of ferritin. This iron-storage molecule is an insoluble complex of ferrihydrite and protein which is located in membranous bodies termed siderosomes. It is present in relatively small amounts in normal tissue, but has been found to be the predominant storage molecule for the excess iron found in patients with clinical iron-overloading syndromes, such as hemochromatosis and thalassemia.

The expression of ferritin and transferrin receptor protein is regulated by the concentration of iron within the cell. This regulation is mediated by an iron response element (IRE) located in the untranslated regions of the mRNA for each of these proteins. The transferrin receptor mRNA contains an IRE in the 3'-untranslated region, whereas the IRE of the ferritin mRNA is located in the 5'-untranslated region. These IRE sequences form stem-loop structures, and act as binding sites for the IRE-binding protein (IRE-BP). The affinity of the IRE-BP for the IRE is dependent upon the binding of iron to the protein. When iron concentrations are low, the conformation of the IRE-BP allows for the interaction of the protein with the mRNA. Binding of the IRE-BP to the ferritin mRNA prevents translation of the message, whereas binding of the IRE-BP to the transferrin receptor mRNA inhibits degradation of the RNA and facilitates translation.
Therefore, when the concentration of iron is high, the cell is directed to store the iron and prevent further uptake of the metal. When the concentration of iron is low, the cell is directed to stimulate uptake and prevent further storage of iron.

**The Importance of Iron in the Cell**

Iron is essential to each organism because of its role as an important constituent of a number of proteins, including hemoproteins, iron-sulfur proteins, and proteins containing oxo-bridged di-iron centres. Hemoproteins involve the binding of iron to four planar tetrapyrrole ring nitrogen atoms of the heme group and to one or two ligands from the protein. There are three main types of hemoproteins: (1) oxygen carriers, (2) oxygen activators, and (3) electron transfer proteins. Hemoglobin and myoglobin are examples of oxygen carrier proteins. Activators of molecular oxygen include cytochrome oxidase, peroxidases, catalases, and the cytochrome P450s. The electron transport proteins include the cytochromes which are essential in respiration and energy production for the cell.

The iron-sulfur proteins contain iron bound to sulfide, forming a cluster linked to the polypeptide chain by the thiol groups of cysteine residues. Iron-sulfur proteins, such as the ferredoxins and rubredoxins, are involved in a number of processes including electron transport. This family of iron-binding proteins also include enzymes with redox and non-redox functions, such as succinate dehydrogenase, nitrogenase, and aconitase.

Ribonucleotide reductase is an important enzyme responsible for the reduction of ribonucleotides to deoxyribonucleotides, the first step in DNA biosynthesis. This enzyme is an example of an iron protein which contains an oxo-bridged di-iron centre. Catalysis of the redox reaction requires a tyrosyl radical cation.
which is stabilized by an iron centre consisting of two Fe$^{3+}$ ions connected by an oxygen atom (Stryer, 1988).

**Iron Overload and its Toxic Consequences**

Although iron is essential for a number of important cellular processes including DNA synthesis and respiration, it is also a potentially toxic element when found in excess within the body. There are three mechanisms by which iron is accumulated within the body: (1) excessive dietary iron intake, (2) an inherited genetic defect which alters iron homeostasis, and (3) thalassemia.

Excessive dietary iron intake is prevalent in the sub-Saharan African endogenous populations which home brew acidic beer in steel drums. This increased availability of dietary iron acts in concert with an inborn error of metabolism distinct from that of HLA-linked hemochromatosis. The pattern of iron accumulation and deposition is also distinct from that of hereditary hemochromatosis, such that iron accumulates in macrophages and hepatic parenchymal cells.

HLA-linked or genetic hemochromatosis is an autosomal recessive inherited disease characterized by iron overloading in the liver, pancreas, bone marrow, and the heart. Iron overloading is the result of a defect in the membrane iron transport system in duodenal mucosal cells, resulting in an increased absorption of the metal from the gut. Clinical consequences include liver disease (cirrhosis), cardiac manifestations (cardiomyopathy with congestive heart failure, arrhythmias), endocrine dysfunction (diabetes mellitus, hypogonadism, and hypothyroidism), arthropathy, and skin pigmentation. Early identification of the disease and the initiation of phlebotomy is an effective means of preventing such consequences.
A transferrinemia is another example of an inherited disorder which alters the concentration of iron in the plasma. This disorder is characterized by the absence of circulating transferrin, resulting in hypochromic anemia and severe iron overload in the liver, heart, and pancreas in a manner similar to HLA-linked hemochromatosis.

An excess of iron also accumulates in patients treated for thalassemia. The thalassemias are a group of inherited disorders of globin-chain synthesis which result in ineffective erythropoiesis and an increase in iron absorption. Iron also accumulates as a result of blood transfusions which are part of the treatment of the disease for many patients. Anemias other than thalassemia also result in an increase in iron absorption. These anemias include sideroblastic anemia, congenital dyserythropoietic anemias, and a number of anemias associated with blocks in the incorporation of iron into hemoglobin.

Although iron is an important component of a number of proteins and enzymes, an overload is potentially toxic. Iron is incorporated into a number of enzymes because of its unique ability to vary oxidation state, redox potential, and electronic spin configuration in response to different ligand environments. These critical characteristics also render the metal capable of generating toxic oxygen species by means of the Fenton/Haber-Weiss reactions:

\[\text{O}_2 \cdot + \text{Fe(III)} \leftrightarrow \text{O}_2 + \text{Fe(II)} \quad (2)\]
\[2\text{O}_2 \cdot + 2\text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2 \quad (3)\]
\[\text{Fe(II)} + \text{H}_2\text{O}_2 \rightarrow \text{Fe(III)} + \text{OH}^- + \cdot\text{OH} \quad (4)\]

The hydroxyl radical (•OH), generated by reaction (4), is then able to cause oxidative damage to lipids, proteins, and DNA. Hydroxyl-radical mediated attack of nucleic acids induces strand breaks and base
modifications which eventually effect potentially carcinogenic mutations.

The hydroxyl radical reacts at diffusion-controlled rates, and within a short diffusion distance of approximately 2 nm (Zastawny et al., 1995). Therefore, in order to damage DNA, the hydroxyl radical must be produced proximate to the nucleic acid strand. This may be possible through low molecular weight complexes of iron which exist in the nucleus, and are capable of interacting with the DNA. A number of researchers have postulated that the low-molecular weight complexes may include molecules such as Fe-ATP and Fe-citrate, which interact non-specifically with the DNA and provide the potential for the generation of toxic free radicals. This interaction with low molecular weight iron complexes is non-specific, and may be hindered by the presence of histones and other DNA-binding proteins.

In this study, it is postulated that iron may also interact with the DNA by means of zinc-containing DNA-binding proteins. Under certain conditions, such as iron overload and/or zinc deficiency, the zinc may be replaced with iron, therefore allowing the iron-catalyzed production of free radicals to occur proximate to the DNA. Therefore, the zinc of the native RARdbd was removed and the apopolypeptide was reconstituted with iron and zinc. This is of particular interest since the retinoic acid receptor is able to interact with chromatin in the absence of ligand, and therefore an iron-substituted receptor will be in close proximity to its cognate DNA without the requirement of ligand activation. The further experiments in this chapter involve the characterization of the iron- and zinc-replaced RARdbd polypeptides, including a demonstration of hydroxyl radical production by the iron-replaced polypeptide.
**Methods**

**Metal Replacement**

In order to remove the naturally occurring zinc of the RARdbd, 250 µl of the polypeptide (approximately 10 µmoles) was added to 750 µl of solution I (13.3 mM 1,10-phenanthroline, 4 M acetic acid, 6.7 mM DTT) in a metal-free eppendorf tube and incubated on a waver at 4°C for 4 hours. This was followed by dialysis in metal-free dialysis tubing against metal-free buffer C (25 mM Tris-HCl, pH 6.8, 50 mM NaCl, 5 mM DTT, 12% glycerol) which had been pre-treated with argon for 30 minutes to remove oxygen. After 3 hours, buffer C was replaced. This was repeated once more to ensure that the ‘apoRARdbd’ was free of zinc and the components of solution I, as well as raising the pH to a physiological level.

Reconstitution of the ‘apoRARdbd’ was achieved by dialysis of the zinc-deficient polypeptide against argon-treated metal-free buffer D (25 mM Tris-HCl, pH 6.8, 50 mM NaCl, 1 mM DTT, 12% glycerol) with 50 µM zinc acetate or ferrous ammonium sulfate added after argon treatment. The ‘apoRARdbd’ was dialysed for at least 6 hours at 4°C. A control was also established in which the final dialysis against buffer D did not include added metal.

Removal of the zinc and reconstitution of the polypeptide was tested indirectly by mobility shift assay as described in Methods, Chapter One. The mobility shift assay is a test of DNA-binding activity. In the absence of metal the polypeptide should be unable to bind DNA because of a loss in tertiary structure. If reconstitution of the polypeptide with zinc acetate or ferrous ammonium sulfate results in DNA-activity being regained, this will also be illustrated through a mobility shift assay.
Dissociation Constant Determination

The protocol is described in Methods, Chapter One.

DNaseI Footprinting

The protocol is described in Methods, Chapter One.

Deoxyribose Assay

In order to test for the production of hydroxyl radicals, the native RARdbd and Fe-reconstituted RARdbd (Fe-RARdbd) were tested using the deoxyribose assay (Halliwell et al., 1988). All samples contained 20 mM potassium phosphate, pH 7.4, 20 mM 2-deoxyribose, 10 mM hydrogen peroxide, and 1 mM ascorbate, with a final volume of 1.2 mls.

Prior to addition, the native RARdbd and iron-reconstituted RARdbd (Fe-RARdbd) were dialysed at 4°C for 1 hour against metal free 25 mM Tris-HCl, pH 7.4, containing Chelex-100 resin to remove free metal. RARdbd, Fe-RARdbd, catalase (Sigma), superoxide dismutase (Sigma), and ferrous ammonium sulfate were added to triplicate samples as indicated in Table 2.2. These samples were then incubated at 37°C for 60 minutes. This was followed by the addition of 1 ml of 1% (w/v) thiobarbituric acid in 50 mM NaOH, and 1 ml of 2.8% (w/v) trichloroacetic acid. The samples were then incubated at 100°C for 20 minutes. The samples were cooled and the absorbance was read at 532 nm.
Metal Free Solutions and Labware

The naturally occurring metal was removed from double-distilled and deionized water by passage over a column of the chelating resin, Chelex-100 (BioRad). Metal free solutions were made using this Chelex-treated water and with products labeled “ultrapure”.

Glassware and plasticware, including tips and eppendorf tubes, was treated by incubating in 10% HCl for at least 24 hours, followed by rinsing with metal free water.

Dialysis tubing (SpectraPor6) was heated at 85°C in metal free water for 2 hours, followed by a change of the water and a subsequent 2 hour incubation at 85°C. This was repeated twice more to ensure that the dialysis tubing was metal free.
Results and Discussion

Iron Replacement of the RARdbd Polypeptide

In this study, the zinc atoms of the RARdbd polypeptide were removed, resulting in a loss of DNA-binding activity. It was also shown that these zinc atoms may be replaced with ferrous iron atoms in order to restore DNA-binding activity. In Figures 2.2 and 2.3, the lane designated “apo” represents the polypeptide after treatment with acetic acid and 1,10-phenanthroline, a metal chelator. As shown in the mobility shift assay, the apopolypeptide is unable to interact with the DR5-containing oligonucleotide. The lane designated “native” represents a comparable concentration of untreated or native RARdbd polypeptide incubated with the same concentration of oligonucleotide. The lanes designated “Fe” and “Zn” in both Figures 2.2 and 2.3 illustrate the reconstitution of the apopolypeptide with ferrous iron and zinc respectively. Therefore, the DNA-binding activity of the RARdbd polypeptide is able to be restored with both zinc acetate and ferrous ammonium sulfate under reducing conditions.

Iron Coordination within Zinc- and Iron-containing Proteins

Iron has also been shown to be able to reconstitute other zinc-containing proteins including the estrogen receptor DNA-binding domain (ERdbd), the thyroid hormone receptor (TR), and a peptide containing the C-terminal finger of the GATA-1 erythroid transcription factor (Conte et al., 1996; Miyamoto et al., 1991; Omichinski et al., 1993). The estrogen receptor and the thyroid hormone receptor are both members of the nuclear hormone receptor superfamily, as is the retinoic acid receptor. Proteins which are members of this superfamily are highly homologous and appear to be structurally similar. They are also related through a zinc-twist-containing region, known as the
Figure 2.1. Mobility shift assay of the reconstitution of apoRARdbd with zinc acetate and ferrous ammonium sulfate. Each lane demonstrates the results of incubating 15 pmol of polypeptide with 1.0 pmol of 32P-labeled DR5-containing oligonucleotide. The first lane represents apoRARdbd, the second is zinc-reconstituted RARdbd (Zn-RARdbd), and the third is iron-reconstituted RARdbd (Fe-RARdbd). The fourth lane represents the native RARdbd prior to removing the zinc. The fifth lane represents free DNA oligonucleotide without the addition of DNA-binding polypeptide.
Figure 2.2. Mobility shift assay of the reconstitution of apoRARdbd with zinc acetate and ferrous ammonium sulfate. Each lane represents the incubation of 38 pmol of polypeptide with 0.68 pmol of 32P-labeled DR5-containing oligonucleotide. The first lane contains the native RARdbd, while the second lane illustrates the effects of removing zinc on the DNA-binding properties of the RARdbd (apoRARdbd). The third lane represents the zinc-reconstituted RARdbd (Zn-RARdbd) and the fourth lane represents the iron-reconstituted RARdbd (Fe-RARdbd).
DNA-binding domain, which involves the tetrahedral coordination of two zinc atoms by means of eight cysteine residues.

The GATA-1 protein is not a member of this superfamily, yet it also contains two related but non-identical zinc-finger elements which are reminiscent of the zinc-twists of the nuclear hormone receptors. The peptide used in the study by Omichinski et al. contained the C-terminal module of GATA-1, which also contains a zinc atom tetrahedrally coordinated by means of four cysteine residues. Therefore, if this peptide, as well as the ERdbd and TR, are able to bind ferrous iron and maintain DNA-binding activity, it is then reasonable that the RARdbd will be able to bind ferrous iron and retain the ability to bind DNA.

The tetrahedral coordination of iron by means of four cysteine residues has a precedent in rubredoxin. This iron-sulfur protein is a bacterial electron transport protein which tetrahedrally binds iron by means of the sulfur atoms of four cysteine residues. Another zinc-binding protein, methionyl-tRNA synthetase, is homologous to iron-binding rubredoxin, extending the possible relationship between zinc and iron binding sites (Fourmy et al., 1993). Another study, using cytochrome c, revealed that zinc is able to substitute for the native iron of this protein without any significant disruption of structure (Anni et al., 1995). It was also revealed that under certain disease conditions involving a disruption of iron-metabolism, zinc is able to substitute for ferrous iron in a number of heme proteins. Therefore, it may also be possible that under certain conditions of iron overload and/or zinc deficiency, ferrous iron may be able to substitute for zinc. This appears to be a reasonable hypothesis since both iron and zinc are able to form tetrahedral complexes and both metal atoms are similar in their ionic and atomic radii (Anni et al., 1995).
The Physiological Potential for the Coordination of Iron or Zinc in Proteins

The cellular event of whether to incorporate iron or zinc may be determined by the availability of each metal. For example, if rubredoxin, an iron-sulfur protein from bacteria such as Clostridium and Desulfovibrio, is overexpressed in E. coli, the majority of the protein appears to incorporate zinc rather than iron (Lippard and Berg, 1994). It was also shown that ferrochelatase, an enzyme which catalyzes the insertion of iron into porphyrin and other molecules, will also catalyze the insertion of zinc at a rate which is essentially the same as that for iron (Lippard and Berg, 1994). Further thermodynamic studies have shown that zinc(II) binds more tightly than iron(II), but once bound, zinc is more easily exchanged than iron (Lippard and Berg, 1994). Therefore, if iron is to be incorporated into proteins such as rubredoxin, or RAR, there must be an abundance of iron and an accompanying deficiency of zinc.

As discussed in the introduction, iron accumulates in the body as the result of a number of diseases including hereditary hemochromatosis and thalassemia. It may also accumulate as the result of a dietary abundance of foods such as red meat. Iron overloading as the result of diet appears prevalent in areas such as North America where foods high in iron, such as red meat, are consumed in large quantities. The toxicity of this lesser-degree overload may be significant, such that a combination of high saturated fat and iron in the diet may significantly increase the chance of cardiovascular disease and cancer.

A study of zinc deficiency in rats demonstrated an increase in oxidative stress with concomitant chromosomal damage involving
base modifications and strand breaks (Morgan et al., 1995). Further results revealed that zinc deficiency also induces an increase in iron concentration in several tissues including the liver and the heart, as well as limits the production of erythrocytes (Bray and Bettger, 1990; Morgan et al., 1995). Therefore, a deficiency in zinc may increase the concentration of available iron, allowing this highly reactive metal to become incorporated into proteins such as the retinoic acid receptor or the estrogen receptor.

**Characteristics of the Iron-Reconstituted RARdbd**

Thus, it is possible that iron may substitute for zinc in the retinoic acid receptor, and other zinc-containing proteins, under certain conditions. The obvious question which arises following these results is one of whether the iron-replaced protein is able to effectively compete with the native protein for binding to the cognate DNA sequence. The ensuing experiments attempted to characterize the basic binding properties of the iron-replaced polypeptide in comparison to the native and zinc-replaced polypeptide. As shown in Table 2.1, the dissociation constant of the Zn-RARdbd was found to be 15.4 nM (± 0.5 nM), and that of the Fe-RARdbd was 15.3 nM (± 0.6 nM). These values are not significantly different from the value of 15.3 nM (± 0.5 nM) for the native RARdbd polypeptide. This indicates that an iron-replaced RARdbd is able to interact with its cognate DNA sequence with the same affinity as a native zinc-containing RARdbd protein. Therefore, if iron was able to replace the zinc in the "zinc-twist", the iron-replaced protein would be able to effectively compete with the native protein for DNA binding.

These results also indicate that binding of iron does not significantly distort the structure of the DNA-binding domain of the
Table 2.1. Dissociation constants for the native, zinc-reconstituted RARdbd and iron-reconstituted RARdbd. This chart illustrates the dissociation constants (K_d) for the native RARdbd, Zn-RARdbd, and the Fe-RARdbd. It also presents the total active protein concentration, ([P]_t), and an estimate of the extent of reconstitution with zinc and iron. The percent reconstitution represents a ratio of the resulting [P]_t and the expected [P]_t based on the original total protein concentration of the native RARdbd.
RARdbd. This appears reasonable since iron has an atomic radius of 1.17Å, which is slightly smaller than the approximate 1.25Å atomic radius of zinc (Anni et al., 1995). Therefore, there should be sufficient room for the incorporation of the iron atom into the zinc-binding pocket of the zinc-twist module, without any accompanying drastic change in conformation. A further study of iron replacement involving the ERdbd has revealed that the bond lengths of the metal-cysteine bond within the ERdbd is comparable with those of the Fe-cysteine bonds of rubredoxin (Conte, 1996). Thus, it appears reasonable that iron may replace the zinc of the RARdbd, maintaining the native structure and DNA-binding activity of the protein.

This preservation of structure is further confirmed through the DNaseI footprinting analysis of the native RARdbd in comparison to the Fe-RARdbd and Zn-RARdbd. Figure 2.4 demonstrates how the three polypeptides share a common footprinting pattern on the DR5-containing oligonucleotide. This pattern is solely due to the RARdbd polypeptides since RXRdbd was not included in this set of experiments. An interesting added note is that only the downstream site of the DR5 oligonucleotide appears to be occupied. This may be significant since it has been established that the RAR and RXR bind cognate DNA sequences with polarity, such that RXR is bound to the upstream site, while RAR occupies the downstream site (Predki et al., 1994).

**Consequences of Iron Replacement of the Zinc in the RARdbd**

Therefore, iron is able to replace the native zinc in the RARdbd, and this iron-replaced polypeptide binds to the cognate DNA sequence in the same manner and with a similar dissociation constant. The iron may be incorporated into the protein under conditions of iron
Figure 2.4. DNaseI footprinting analysis of native, zinc-reconstituted RARdbd and iron-reconstituted RARdbd. The first lane, labeled A, represents the chemical sequencing of the guanine and adenine bases of the DR5-containing oligonucleotide. The second lane, lane B, represents the chemical sequencing of the guanine bases alone. The third lane illustrates the effects of DNaseI action on 0.65 pmol of the free DR5-containing oligonucleotide. The fourth lane, lane D, represents the footprint of 2.25 pmol of native RARdbd on 0.65 pmol of DR5 oligonucleotide. The fifth lane, lane E, demonstrates the footprint of the same amount of Zn-RARdbd, and the sixth lane, lane F, represents the footprint of the same amount of Fe-RARdbd.
overload and/or zinc deficiency, and may be incorporated into other zinc-binding proteins such as the estrogen receptor, the thyroid hormone receptor, and the GATA-1 erythroid transcription factor. Interestingly, GATA-1 is an important protein in the iron-rich erythrocyte, thereby increasing the chances of iron incorporation. Other iron-rich cells may include the liver, heart, bone marrow, and lymphocytes.

Iron exerts its toxic effects through the Fenton/Haber-Weiss reactions, which involve metal catalyzed production of hydroxyl radicals. The production of hydroxyl radicals (OH\(^{•}\)) may be monitored using the deoxyribose assay. In this assay, the deoxyribose is oxidatively cleaved by the hydroxyl radical, releasing a substance which reacts with thiobarbituric acid and heat to produce malondialdehyde. Malondialdehyde further reacts with two molecules of thiobarbituric acid to produce a pink chromagen which absorbs light at 532 nm. Table 2.2 illustrates the results of the deoxyribose assay performed using the native RARdbd, iron-replaced RARdbd, and free ferrous ammonium sulfate.

Ferrous ammonium sulfate represents the positive control of the assay, and native RARdbd polypeptide represents the negative control. Thus, the absorbance at 532 nm for the samples containing native RARdbd should be low since there is no apparent catalyst available for the production of hydroxyl radicals. The samples containing ferrous ammonium sulfate, on the other hand, contain free \(\text{Fe}^{2+}\) which is able to catalyze the formation of hydroxyl radicals from hydrogen peroxide by means of the Haber-Weiss reaction. Therefore, samples containing ferrous ammonium sulfate should exhibit a significant absorbance at 532 nm for the deoxyribose assay. As shown in Table 2.2, the absorbance at 532 nm for the native RARdbd was 0.059 (± 0.009) and that for the ferrous ammonium sulfate was 0.834 (± 0.008).
Table 2.2. **Deoxyribose assay results.** The first column describes the additions made to the basic assay mixture which contains 20 mM potassium phosphate, pH 7.4, 20 mM 2-deoxyribose, 10 mM hydrogen peroxide, and 1 mM ascorbate. Native RARdbd and Fe-RARdbd was added to give a concentration of 0.5 μM, and the ferrous ammonium sulfate was added to provide a concentration of 1.25 μM. SOD represents superoxide dismutase.
The absorbance at 532 nm ($A_{532}$) for the Fe-RARdbd was 0.654 ($\pm$ 0.014), therefore demonstrating the presence of iron and its ability to catalyze the formation of hydroxyl radicals from hydrogen peroxide. This is reasonable since the iron bound to the Fe-RARdbd is tetrahedrally coordinated by four cysteine residues, leaving two coordination sites of the iron available to donate or accept electrons. Molecules such as desferrioxamine are hexadentate ligands and therefore occupy all six coordination sites of the iron. Thus, iron-desferrioxamine complexes are unable to catalyze the formation of hydroxyl radicals from hydrogen peroxide (Lippard and Berg, 1994). This allows molecules such as desferrioxamine to be used in the treatment of iron-overload disorders such as hemochromatosis and thalassemia.

The deoxyribose assay reaction with Fe-RARdbd was inhibited by the enzyme catalase as illustrated in Table 2.2 by the reduction of $A_{532}$ to 0.099 ($\pm$ 0.006). This demonstrates how the reaction is dependent upon hydrogen peroxide since catalase catalyzes the disproportionation of hydrogen peroxide to water and molecular oxygen:

$$2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$$ (1)

This also further demonstrates that hydroxyl radicals are the active oxygen species involved in the degradation of deoxyribose since the iron reacts with the hydrogen peroxide to form the hydroxyl radicals:

$$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{OH}^{-} + \cdot\text{OH} + \text{Fe}^{3+}$$ (2)

The disproportionation of hydrogen peroxide would remove one of the reactants of reaction (2), thus inhibiting the formation of hydroxyl radicals.

Addition of superoxide dismutase (SOD) to the reaction with Fe-RARdbd did not significantly inhibit the degradation of
deoxyribose as illustrated in Table 2.2 by the $A_{332}$ of 0.542 ($\pm$ 0.009). Superoxide dismutase catalyses the dismutation of the superoxide radical to hydrogen peroxide and water:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (3)$$

This suggests that Fe$^{2+}$ is the form of iron which is bound to the RARdbd since previous work has indicated that superoxide dismutase has no effect on the degradation of deoxyribose when Fe$^{2+}$ performs the role of the catalyst (Halliwell et al., 1988). This result also indicates that the superoxide radical is not involved in the degradation of deoxyribose since superoxide dismutase did not significantly inhibit the formation of the malondialdehyde chromagen. This further supports the theory that the Fe-RARdbd catalyzes the production of the hydroxyl radical from hydrogen peroxide.

Therefore, this study has shown that iron may be incorporated into the zinc-binding sites of the retinoic acid receptor, and this bound iron appears to be capable of participating in redox reactions, such as the Haber-Weiss reaction. A number of studies have indicated the potential for a direct correlation between bodily iron content and cancer development (Stevens et al., 1994; Stevens and Kalkwarf, 1990; Sussman, 1992; Weinberg, 1992). This relationship has been proposed to be dependent upon the role of iron as a catalyst in the production of hydroxyl radicals. These reactive oxygen radicals are able to damage important biological molecules such as lipids, protein, and DNA. Damage to DNA, involving strand breakage, deoxyribose degradation, and base modification, depends on the proximate production of the hydroxyl radical. Therefore, an iron-substituted retinoic acid receptor may be able to produce hydroxyl radicals when bound to DNA, thus damaging DNA and increasing the potential for carcinogenic mutations.
The Source of Hydrogen Peroxide within the Cell

Normal cellular metabolism involves the production of oxygen-derived species such as the superoxide radical (•O₂⁻) and hydrogen peroxide. Superoxide and hydrogen peroxide collaborate with iron in the Fenton/Haber-Weiss reactions to produce the toxic and highly reactive hydroxyl radical:

\[
\begin{align*}
    O_2^- + Fe^{3+} & \leftrightarrow O_2 + Fe^{2+} \\
    2O_2^- + 2H^+ & \rightarrow O_2 + H_2O_2 \\
    Fe^{2+} + H_2O_2 & \rightarrow Fe^{3+} + OH^- + •OH
\end{align*}
\]  

(4) (5) (6)

In normal cells, enzymes such as catalase, glutathione peroxidase and superoxide dismutase reduce oxidative stress through disproportionation of hydrogen peroxide and dismutation of the superoxide radical. This reduces the possibility of hydroxyl radical production, as well as reducing the release of iron from ferritin and other iron-containing proteins, as superoxide has been implicated in catalyzing the release of iron from storage molecules (Herbert et al., 1994; Reif, 1992). In contrast, tumor cells, such as human hepatoma cells, have been shown to exhibit a significant reduction in catalase gene expression and superoxide dismutase activity (Toyokuni et al., 1995). Therefore, the relative capacity of the cell to scavenge or prevent production of hydroxyl radicals may influence the risk of carcinogenesis.

An increase in oxidative stress through exposure to hydrogen peroxide has been shown to result in DNA strand breaks and base modifications (Zastawny et al., 1995). This study suggested that the increase in oxidative stress induced the release of iron from storage sites such as ferritin, allowing the free iron to react with the hydrogen peroxide, generating hydroxyl radicals and thus damaging DNA. This
appears reasonable since hydrogen peroxide is a non-polar molecule which is able to diffuse through membranes, such as the nuclear membrane, and react with iron, also present in the nucleus. A concurrent study using mouse fibroblasts also reported DNA strand breaks and malignant transformation of cells as a consequence of exposure to hydrogen peroxide (Halliwell and Aruoma, 1993). Therefore, an increase in oxidative stress appears to cause DNA damage which may be potentially carcinogenic.

The Source of Iron within the Cell

In order for this increase in oxidative stress to be carcinogenic, iron must be available to catalyze the formation of hydroxyl radicals. This increase in iron may be the result of a release of iron from storage. A study of normal liver cells and adjacent hepatoma cells have revealed that hepatoma cells have a significantly higher percentage of cellular iron in the metabolically active state, and thus a significantly lower percentage of this iron in the storage form of ferritin (Weinberg, 1992). Therefore, tumor cells have a greater amount of iron available for metabolic functions such as DNA replication and cell division. It may also be concluded that these cells also have a considerable amount of iron available for incorporation into proteins such as the retinoic acid receptor, increasing the possibility of hydroxyl radical production and DNA damage.

It is also possible that the iron may arise from an increase in iron uptake by the cell. A number of studies have examined the possibility of a transferrin-independent mechanism of iron uptake, and have concluded that neoplastic cells capitalize on this alternative in order to fuel their need for rapid growth and replication (Sussman, 1992). Neoplastic cells also exhibit an increase in transferrin receptor expression, augmenting the uptake of iron by the cell. This increased
uptake of iron may occur prior to neoplastic transformation, and therefore be involved in the transformation process through iron-catalyzed oxidative damage to DNA by means of iron-substituted DNA-binding proteins. It is also possible that transformation simply results in the ability of the cell to increase its uptake of iron for the purpose of cell growth and replication, the hallmark of neoplasia.

As mentioned earlier, the ability of iron to become incorporated into proteins such as the retinoic acid receptor will depend on the availability of zinc as well as iron. Thus, a deficiency in zinc may increase the possibility of iron incorporation into zinc-binding proteins such as the nuclear hormone receptors. A study of rat testes has shown that a zinc-deficient diet results in a 46% increase in the incidence of 8-hydroxy-2'-deoxyguanosine, a common base modification induced by reactive oxygen species such as the hydroxyl radical (Oteiza et al., 1995). Some hypothesize that zinc and iron may compete for similar binding sites, such that an alteration in the availability of one or both metals may affect which is incorporated into a specific protein (Bray and Bettger, 1990). Stevens et al. propose that a low concentration of physiological zinc may increase the danger that iron will catalyze hydroxyl radical production (Stevens and Kalkwarf, 1990). Therefore, it is possible that this increased risk is due to a misincorporation of iron into zinc-binding proteins, such as the nuclear hormone receptors.

The concentration of zinc in the cell may be determined by albumin, a plasma protein involved in zinc transport. A number of studies have revealed a correlation between lower levels of albumin and an increased risk of cancer and cardiovascular disease (Spatz and Bloom, 1992). It has also been demonstrated that a number of cancer patients exhibit a lower concentration of albumin in the plasma. This lower level of albumin may result in a reduced availability of zinc for
the cell, and thus an increased chance that iron will be incorporated into zinc-containing proteins such as the retinoic acid receptor.

**Cellular Transformation and the Role of Oxidative Stress**

A number of factors are involved in the transformation of a normal cell to a neoplastic cell. It is commonly believed that this transformation involves a genetic mutation, either inborn or induced by environmental factors. One of these factors may be an increased exposure to iron, a highly reactive redox metal capable of catalyzing the formation of hydroxyl radicals from hydrogen peroxide, a common cellular molecule. A concomitant defect in the activity of enzymes such as catalase and superoxide dismutase, as well as a low concentration of radical scavengers such as vitamin E, may increase the oxidative stress of a cell, thereby increasing the danger of carcinogenesis.

This study revealed that iron is able to bind to the retinoic acid receptor DNA-binding domain and provide the proper structural properties for DNA-binding activity. It was also demonstrated that the iron-reconstituted RARdbd was able to bind with the same dissociation constant as the native polypeptide, and provide the same footprinting pattern. Most importantly, it was shown that the iron-reconstituted RARdbd was able to produce highly reactive hydroxyl radicals. Since the retinoic acid receptor is a DNA-binding protein, it is reasonable to hypothesize that iron-substituted RAR, bound to its cognate DNA, may generate hydroxyl radicals, thereby causing potentially carcinogenic DNA damage.
Future Experiments

The most important aspect of the future work with iron and the RARdbd should involve *in vitro* tissue culture experiments. Although it has been established that iron is able to substitute for the zinc in the RARdbd, it must also be shown that this is a potential occurrence in the cell. Previous experiments in the lab have shown that *E. coli*, transformed with the ERdbd plasmid, and grown in minimal medium which contains iron and no other metal, will incorporate the iron into the ERdbd polypeptide (Conte et al., 1996). Although this is potentially relevant, it would be more exciting to show this result in mammalian cells. Cells, transfected with a plasmid containing the cDNA for the RAR, could be grown in iron-rich and zinc-deficient medium, and the expressed RAR could be analyzed for metal content by atomic absorption.

These experiments could also incorporate an investigation of the possible effects of the increase in iron and deficiency of zinc on the nucleotide bases and the chromosomes of the cell. It would also be interesting to look at the level of oxidative stress in the nucleus in the presence and absence of the RAR plasmid. In the presence of RAR, it is possible that a greater concentration of iron is able to enter the nucleus by way of incorporation into the RAR. This increase in iron concentration would then potentially increase the concentration of oxidative species in the cell nucleus, increasing the probability of base modifications, DNA strand breaks, and chromosomal abnormalities.
Appendix A: Calculations using the Dissociation Constant

The binding of a protein to DNA can be represented by the following equation:

\[
\text{DNA} + \text{Protein} \rightleftharpoons \text{DNA} \cdot \text{Protein}
\]

The dissociation constant (Kd) for this reaction is equal to the ratio of the association and dissociation rate constants for the process. If the DNA contains one binding site for the protein, then one mole of bound DNA is equivalent to one mole of bound protein: \[ [P]_b = [D]_b \]

\[
K_d = \frac{[P]_f [D]_f}{[DP]}
\]
\[
[P]_f = [P]_f + [P]_b
\]
\[
[P]_f = [P]_f - [P]_b
\]
\[
[D]_f = [D]_f + [D]_b
\]
\[
[D]_f = [D]_f - [D]_b
\]
\[
[D]_b = [P]_b
\]
\[
\therefore [P]_f = [P]_f - [D]_b
\]
\[
\therefore K_d = \frac{([P]_f - [D]_b)([D]_f - [D]_b)}{[D]_b}
\]

If the concentration of DNA half-site oligonucleotide is equal to the concentration of RARdbd polypeptide, there is enough DNA (D) to bind all of the polypeptide (P). The fraction of the DNA which is bound is dependent on the affinity of the polypeptide for the DNA and can therefore be calculated using the dissociation constant, which, for the RARdbd, is approximately 15 nM and the total concentration of DNA, which is 20.5 nM in this example:
\[
\text{If } [P]_t = [D]_t
\]
\[
K_d = \frac{[D]_b^2 - 2[D]_t + [D]_b}{[D]_b}
\]
\[
15 = \frac{(20.5)^2 - 2(20.5) + [D]_b^2}{[D]_b}
\]
\[
[D]_b = 6.7 \text{ nM}
\]
\[
\therefore \text{ percentage of DNA bound } = \frac{6.7}{20.5} \times 100\% = 32.6\%
\]

If the oligonucleotide contains two binding sites, then one mole of DNA is equivalent to two moles of binding sites. Therefore, if the concentration of oligonucleotide is 20.5 nM, then the concentration of binding sites ([D]t) is 41 nM. If 1.53 pmol of RARdbd polypeptide is used in a 40 µl volume, then the total polypeptide concentration ([P]t) is 38.25 nM:

\[
15 = \frac{(38.25 - [D]_b)(41 - [D]_b)}{[D]_b}
\]
\[
[D]_b = 21.6 \text{ nM}
\]
\[
\therefore 53\% \text{ of the DNA is bound}
\]

If 15.3 pmol of RARdbd polypeptide (382.5 nM) is incubated with the same concentration of binding sites:

\[
15 = \frac{(382.5 - [D]_b)(41 - [D]_b)}{[D]_b}
\]
\[
[D]_b = 39.3 \text{ nM}
\]
\[
\therefore 96\% \text{ of the DNA is bound}
\]

This will be the percentage of DNA which is bound as long as the interaction of the polypeptide with the double-site DNA is non-cooperative. A cooperative binding of two polypeptide molecules to the DNA would result in a greater percentage of DNA which is bound.
It would also result in a preferential formation of the dimer configuration.

For analysis of the RARdbd-RXRdbd heterodimer, the calculation is complicated by the fact that the RXRdbd has a lower affinity for the AGGTCA site than the RARdbd. This will result in a decrease in the predicted concentration of bound DNA. If the binding of the heterodimer is cooperative, then the experimental concentration of bound DNA will be greater than what is expected, and may therefore mimic the results that would be expected if the two polypeptides bound to the consensus site with an identical dissociation constant.
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