

**The Retinoic Acid Response:
Modulation by Calreticulin
and
Development of a Method to Isolate
Directly Regulated Genes**

by

Mary Shago

**A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy
Graduate Department of Molecular and Medical Genetics
University of Toronto**

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Abstract

Vitamin A is a necessary nutrient for a variety of biological processes including vertebrate development, growth and reproduction. The term retinoids encompasses all naturally occurring and synthetic derivatives of vitamin A, including the active derivative retinoic acid (RA). The all-trans form of RA (atRA) serves as a ligand for retinoic acid receptors (RARs) while the 9-cis isomer of RA (9cRA) is bound by both RARs and retinoid X receptors (RXRs). As members of the nuclear receptor superfamily, RARs and RXRs function as ligand-inducible transcription factors that control a vast network of effector genes. The activity of the receptors is modulated at different levels by multiple factors. One modulator is the calcium binding protein calreticulin. I present evidence in this thesis that calreticulin can interfere with the DNA-binding activities of both RXR/RAR heterodimers and RXR homodimers, and that co-precipitation experiments suggest a direct interaction between calreticulin and RAR. Calreticulin reduces the abilities of RAR and RXR to transactivate cognate reporter genes in transient transfections. Stably over-expressed calreticulin diminishes the level of RA-induced activation of genes that are directly regulated by RA and leads to a complete inhibition of RA-induced neuronal differentiation in P19 embryonal carcinoma cells. Retinoid receptor action ultimately leads to regulated gene expression. Although a number of RA-responsive genes have been characterized, the highly pleiotropic effects of retinoids strongly suggest that there remain many more RA-regulated genes yet to be isolated. In an effort to identify RA responsive genes I have developed a method referred to as CpG-selected and amplified binding (CpG-SAAB). DNA fragments derived from CpG islands, which are genomic landmarks associated with the regulatory regions of genes, are

selected for the presence of binding sites for RAR and RXR. The transcription units associated with the fragments can then be isolated. I present in this thesis the CpG-SAAB method, the isolation of twelve novel RAR and RXR binding sites, and the characterization of a gene (NN8-4AG) associated with one of them.

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Attribution of Data

I am responsible for all of the work described in this thesis with the following exceptions. P19 EC cell lines stably over- or under-expressing calreticulin were established by Chung-Yee Leung Hagesteijn, who also performed the calreticulin/RAR coprecipitation experiments in Chapter 2. Also in Chapter 2, Kosta Milankov carried out immunofluorescence on P19 EC cells, and Greg Downey and Tom Waddel provided intracellular calcium concentrations.

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List of Abbreviations

AF, activation function
atRA, all-trans retinoic acid
BLAST, basic local alignment search tool
CAL, calreticulin
cDNA, complementary deoxyribonucleic acid
CBP, CREB binding protein
CpG-SAAB, CpG -selected and amplified binding
CRABP, cytoplasmic retinoic acid binding protein
CRBP, cellular retinol binding protein
CREB, cyclic AMP response element-binding protein
Cx, cycloheximide
DBD, DNA binding domain
DNA, deoxyribonucleic acid
DNA MTase, DNA methyltransferase
DR, direct repeat
EC, embryonal carcinoma
ECM, extracellular matrix
EMSA, electromobility gel shift assay
ER, estrogen receptor
EST, expressed sequence tag
GR, glucocorticoid receptor
LBD, ligand binding domain
LUC, firefly luciferase gene
9cRA, 9-cis retinoic acid
NN, non-nucleosomal
OR, orphan receptor
PCR, polymerase chain reaction
PPAR, peroxisome proliferator activated receptor
PR, progesterone receptor
RA, retinoic acid
RACE, rapid amplification of cDNA ends
RAR, retinoic acid receptor
RARE, retinoic acid response element
RBP, retinol binding protein

RNA, ribonucleic acid
RRL, rabbit reticulocyte lysate
RXR, retinoid X receptor
RXRE, retinoid X response element
TK, thymidine kinase minimal promoter
TR, thyroid hormone receptor
tRNA, transfer RNA
VAD, vitamin A deficiency
VDR, vitamin D receptor

Chapter 1

Introduction

General Introduction and Outline of Thesis

Retinoids are necessary for a variety of physiological processes. Retinoic acids (RA) are biologically active retinoids which function as ligands for members of the nuclear receptor superfamily. Unlike membrane-bound receptors, members of the nuclear receptor superfamily are intracellular and exert their effects directly at the level of gene expression.

In the first chapter I will introduce the signaling molecule RA and review the current state of knowledge concerning the mechanisms of action of RA and the nuclear receptors which mediate the RA cellular response. Complexity added to this system by cellular factors modulating the signaling pathway will be addressed. The calcium binding protein calreticulin will be introduced as a prelude to evidence documenting its interaction with the retinoid receptors in Chapter 2 of the thesis.

In order to decipher the molecular mechanisms of the RA signaling pathway, it is essential to isolate and characterize target genes which are regulated by the ligand/receptor complex. I will present an overview of methods which have been used to isolate directly regulated genes. Chapter 3 concerns the development of a method, termed CpG-selected and amplified binding (CpG-SAAB), to isolate genes which are directly regulated by RA and its receptors. The presence of CpG islands in the vertebrate genome constitutes a key component of CpG-SAAB and will be introduced in Chapter 1.

The experimental results will be summarized in chapter 4. Several points of interest arising from the study of the retinoid receptor/calreticulin interaction will be addressed and relevant future experiments will be proposed. Applications and limitations of CpG-SAAB, along with suggestions for improvement, will be discussed in Chapter 4. Future experiments concerning the study of genomic binding sites isolated using CpG-SAAB, as well as the novel RA responsive gene NN8-4AG, will be delineated.

1. Retinoic Acid and its mode of action

1.1 *Biological effects of retinoic acid*

In the development, growth, reproduction, and maintenance of general health of a vertebrate individual, RA plays an essential role. Retinol serves as the metabolic precursor for RA. Retinol is derived from carotenoids in plants and retinyl esters in animal fat (for review see Blomhoff et al., 1990). Carotenoids are converted to retinol in the intestinal cells, while retinyl esters are converted to retinol in the lumen of the intestine. In the intestinal cells, retinol is converted to retinyl esters and incorporated into chylomicrons, which are the main intestinal lipoproteins. The chylomicrons enter the general circulation to deliver the retinyl esters primarily to the liver where conversion to retinol and coupling with the retinol binding protein (RBP) occur. Retinol-RBP is secreted to the plasma and made available to a variety of cell types that express cell surface receptors for RBP. Inside the cells, retinol can be converted to various derivatives of the active biological metabolite RA through enzymatic processes thought to involve cellular retinol binding proteins (CRBPs). Cytoplasmic RA-binding proteins (CRABPs I and II) bind the active derivative all-trans RA (atRA) and may play roles in regulating intracellular levels of atRA by promoting the catabolism of atRA to more polar metabolites (Fiorella et al., 1993; Giguère, 1994). In addition to this major pathway a small fraction of RA, which can probably enter cells by passive diffusion, circulates in the plasma bound to albumin and provides an additional source for uptake by tissues and cells (Kurlandsky et al., 1995).

Originally, physiological functions of retinoids were deduced from vitamin A deficiency (VAD) studies in animals (for review see Means and Gudas, 1995). Deficiency studies have revealed that retinol is essential for prenatal development. Newborns of vitamin A deficient mothers exhibit a large number of congenital malformations including abnormalities of the respiratory tract, heart, ureter and genitalia,

and defects in eye development. Removal of vitamin A from the diet postnatally results in squamous metaplasia of various epithelial tissues and degeneration of seminiferous tubules and photoreceptors. Exogenous administration of RA can prevent or reverse the deleterious effects of a postnatal VAD diet except for night blindness and photoreceptor decline.

Retinoids are known to be potent teratogens for mammalian embryos when administered at pharmacological doses (Lammer et al., 1985; Rosa et al., 1986). Retinoic acid applied during gastrulation results in malformations in the hindbrain and derivatives of the neural crest (for review see Means and Gudas, 1995). At later stages, excess RA results in tail, genital and limb defects as well as spina bifida. In combination with the vitamin A deficiency studies, these observations suggest that the levels of RA within the organism are carefully controlled to facilitate normal development.

During early vertebrate development there is evidence for the involvement of retinoids in the establishment of anterior/posterior axes in the central body axis and in the limb (for review see Means and Gudas, 1995). Elevated levels of endogenous RA have been detected in regions of the embryo that possess patterning activities such as the floor plate (Wagner et al., 1990) and the posterior region of the limb bud (Thaller and Eichele, 1987).

Vitamin A was first shown to play a role in differentiation of epithelia in 1925 (Wolbach and Howe, 1925). Tissue culture systems were developed to analyze the roles of retinoids in cell differentiation and proliferation. RA was shown to be essential for normal epidermal morphogenesis at physiological concentrations (Asselineau et al., 1989). An influence of RA on differentiative processes was noted in many other cell types including neuroblastomas, melanomas and teratocarcinomas (Amos and Lotan, 1990). Treatment of the human myeloid leukemia cell line with RA leads to terminal granulocytic differentiation (Breitman et al., 1980). The embryonal carcinoma (EC) cell line F9 differentiates upon RA treatment to parietal endoderm while the P19 EC cell line

differentiates into cardiac and smooth muscle cells or neurons or glia, depending on the concentration of RA in which the cells are maintained (Edwards and McBurney, 1983; Jones-Villeneuve et al., 1982; Strickland and Mahdavi, 1978). An inhibitory effect of retinoids on tumor progression has been documented in animals and humans (Sporn and Roberts, 1983), as well as the ability to block the action of tumor promoters both *in vitro* and *in vivo* (Leder et al., 1990; Yupsa et al., 1981).

1.2 Retinoic Acid Receptors

The discovery that the all trans isomer of RA (atRA) was a high affinity ligand for a member of the nuclear receptor superfamily, the retinoic acid receptor (RAR) (Giguère et al., 1987; Petkovich et al., 1987), laid the foundation for subsequent research into the molecular mechanisms of RA and the reasons for its pleiotropic conduct in cells and in the organism. The nuclear receptor superfamily now numbers over 150 distinct members and includes the glucocorticoid receptor (GR), the estrogen receptor (ER), the vitamin D receptor (VDR) and the thyroid hormone receptor (TR) as well as many 'orphan receptors' with no known ligands (Evans, 1988; Mangelsdorf et al., 1995a). Although some of the orphan receptors may be constitutive activators or repressors, the general mode of receptor action is an allosteric change upon ligand binding which allows the receptor/ligand complex to bind to specific sites in chromatin to regulate transcription. Members of the nuclear receptor superfamily have a modular structure, the most conserved domain being the zinc finger-based DNA binding domain which determines the receptors binding site specificity (see Figure 1) (reviewed in Evans, 1988). At the C terminus of the proteins is the second highly conserved domain, the ligand binding domain (LBD). The LBD is a multifunctional domain which dictates not only ligand specificity but also encodes dimerization and ligand-dependent transactivation (termed the AF-2) functions (Fawell et al., 1990; Forman et al., 1989; Nagpal et al., 1993). The

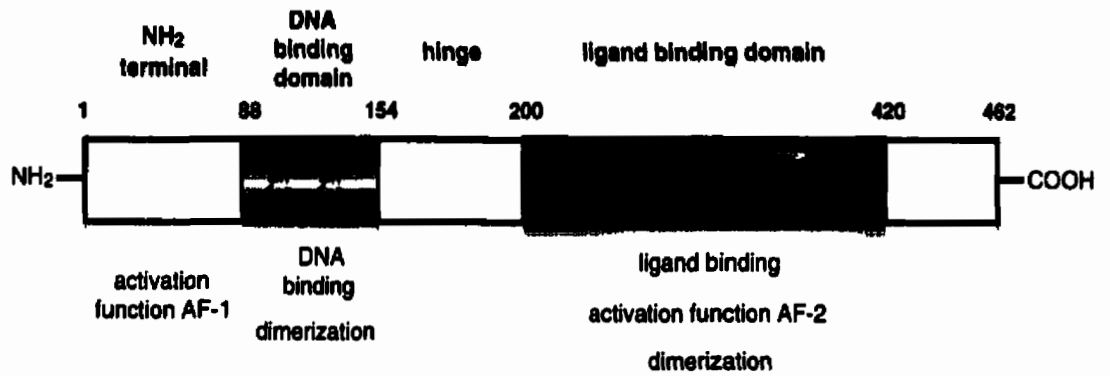


Fig. 1. Schematic representation of the functional domains of RAR α 1. Typical of the nuclear receptor superfamily, RAR α 1 contains the functional domains delineated above the diagram. Below are the activities mediated by each of these regions. Numbers represent the amino acids bordering each domain. The highly conserved DNA binding domain contains two zinc finger motifs.

receptors also feature a ligand-independent activation domain (AF-1) within the N-terminal region (Nagpal et al., 1993) and a hinge region between the DBD and the LBD which share very little amino acid similarity between members.

1.3 Retinoic Acid Response Elements and Retinoic Acid Receptor/Retinoid X Receptor Heterodimers

Three separate genes encoding RARs have been cloned, RAR α (Giguère et al., 1987; Petkovich et al., 1987), RAR β (Brand et al., 1988) and RAR γ (Krust et al., 1989; Zelent et al., 1989). Alternative splicing and differential promoter usage results in the generation of multiple isoforms of RAR α , β and γ (Giguère et al., 1990; Kastner et al., 1990; Leroy et al., 1991; Zelent et al., 1991), all with varying N termini and presumably with varying transcriptional activation capabilities (Folkers et al., 1993; Nagpal et al., 1993). Although these receptors can bind the 9-cis isomer of RA (9cRA) as well as atRA (Allenby et al., 1993), high affinity receptors which bind solely to 9cRA have also been characterized and termed retinoid X receptors (RXR α , RXR β and RXR γ) (Heyman et al., 1992; Levin et al., 1992; Mangelsdorf et al., 1992; Mangelsdorf et al., 1990). Several isoforms of RXR differing from one another in their N-terminal regions have also been identified (Liu and Linney, 1993; Nagata et al., 1994 and P. Chambon, unpublished results).

Ligand/receptor complexes control gene activity by binding to specific sites, or retinoic acid response elements (RAREs), in the regulatory regions of genes. In contrast to previously characterized members of the superfamily such as the GR and ER which bind as homodimers to DNA, RARs and RXRs form a subfamily of nuclear receptors that bind with high affinity as heterodimers to a direct repeat of the core half-site (A/G)G(G/T)TCA (Kliewer et al., 1992; Leid et al., 1992; Yu et al., 1991). It is now evident that the nuclear receptor superfamily can be divided into four groups with respect to their dimerization and DNA binding properties (Mangelsdorf et al., 1995b). The first

class includes the steroid hormone receptors that bind as homodimers to core hexamers of ACAAGA organized as inverted repeats. Members of Class II bind with highest affinity to direct repeats of the core half-site (A/G)G(G/T)TCA as heterodimers with RXR. Class III receptors bind to direct repeats as homodimers, while Class IV receptors bind to a single expanded core half-site as monomers. As members of the second class of nuclear receptors, RXR/RAR heterodimers bind with highest affinity to directly repeated hexamers with an intervening spacing of 1bp (Direct Repeat spaced by 1bp or DR1), 2bp (DR2) or 5bp (DR5) (Heery et al., 1994). Other configurations of half-sites less commonly found in RA responsive genes are half-sites positioned in a palindromic or everted fashion and spaced by varying numbers of nucleotides. Such RAREs, as well as a few characterized 'complex' RAREs which contain multiple half-sites in various configurations, are usually bound by RXR/RAR heterodimers with relatively lower affinities than direct repeats (Giguère, 1994). RXR alone in combination with 9cRA is able to bind to and activate through retinoid X response elements (RXREs) (Mangelsdorf et al., 1991), which are almost exclusively DR1s.

1.4 RXRs: Multiple Functions in Several Signaling Systems

RXR serves as a partner not only in retinoic acid signaling but also acts as a heterodimeric partner for TR, VDR, and a number of orphan receptors that include COUP-TF, ARP-1, and the peroxisome proliferator activated receptor (PPAR) (see Figure 2) (for review see Mangelsdorf and Evans, 1995a). These heterodimeric complexes also bind with highest affinity to direct repeats of core half-sites with varied spacing. For instance, RXR/VDR heterodimers bind to a DR3 while a DR4 serves as a binding site for RXR/TR heterodimers (Umesono et al., 1991).

X-ray crystallographic and nuclear magnetic resonance studies, together with previous biochemical data, have contributed to an understanding of the structure of the DNA binding domains of the nuclear receptors. The 66 amino acid DBD is highly

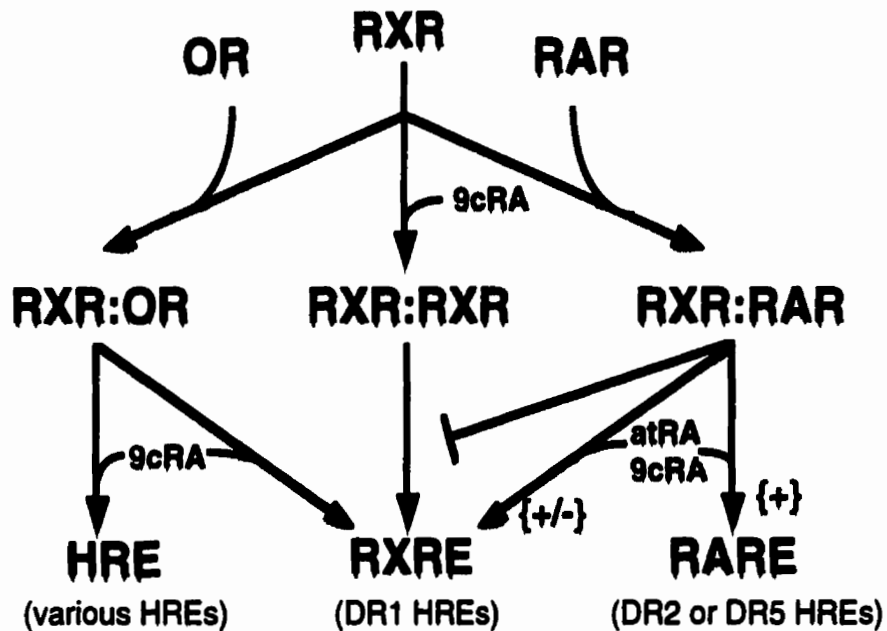


Fig. 2. RA signaling pathways. RAR activates target genes by heterodimerizing with RXR. RXR/RAR heterodimers are stimulated by atRA or 9cRA to bind and activate transcription primarily through RAREs consisting of directly oriented half-sites spaced by 2 or 5 nucleotides (DR2 or DR5 respectively). In the presence of 9cRA, RXR can homodimerize; this interaction is prevented by the presence of RAR due to the preferential formation of heterodimers. RXR homodimers confer activation through a DR1. RXR/RAR heterodimers also bind with high affinity to DR1s, leading in most cases to transcriptional repression. RXR liganded with 9cRA can also heterodimerize with certain orphan nuclear receptors (ORs). Resultant heterodimers bind to various hormone response elements (HREs) and to DR1 HREs (RXR:PPAR heterodimers).

conserved across almost all members of the receptor family and contains two α helices, one of which makes specific contacts with the DNA half-site (Hård et al., 1990; Katahira et al., 1992; Lee et al., 1993). Located in the first zinc finger, this helix contains the so-called P box which is essential in discrimination of the exact sequence of the core half-site (Lee et al., 1993; Umesono and Evans, 1989). These studies also confirmed that the receptors contain a dimerization motif within the DBD independent of the one within the carboxy-terminal LBD. Residues in the second zinc finger, termed the D box, contribute to mediation of dimeric interaction in receptors which bind as symmetric dimers to their response elements (Class I) (Glass, 1994). However, because of the head-to-tail arrangement of the RXR/RAR heterodimer on the most common type of RARE, a direct repeat, the DBD dimerization interface proffered by each partner is in a distinct region. In serving as a partner to many different members of the nuclear receptor family, RXR must possess a different interface for each partner. In the case of the RXR/TR DBDs bound to a DR4, the RXR dimerization surface is in the second zinc finger, while the TR contacts are located within and preceding the 1st zinc finger and in the 'T-box' which is C terminal to the 2nd zinc finger (Rastinejad et al., 1995). The fact that dimer interfaces can only be properly formed on the directly oriented half-sites with correct spacing leads to the preferred cognate binding sites for each RXR/partner heterodimer (Mangelsdorf and Evans, 1995a; Rastinejad et al., 1995). Heterodimerization interfaces of RAR in a RXR/RAR complex on a DR5 have been mapped to the tip of the first zinc finger (Zechel et al., 1994a). On a DR2 a different dimerization interface is formed, involving the T-box of RAR and a region of the second zinc finger of RXR excluding the D box (Zechel et al., 1994b). The dimerization function located in the LBD may act only to stabilize the heterodimeric complex while the dimerization interfaces within the DBDs actually dictate binding site specificity (Zechel et al., 1994a).

An additional binding property of the heterodimeric receptors confirmed by crystallographic studies is a distinct order in binding to direct repeats (Rastinejad et al.,

1995). In the case of the RXR/RAR heterodimer, on a DR2 or a DR5 type RARE, RXR always occupies the upstream half-site (Kurokawa et al., 1993; Predki et al., 1994), while on a DR1, the order is reversed (Kurokawa et al., 1994). Interestingly, RXR contains within the C terminal extension of its DBD an additional α helix not present in its heterodimeric partners (Lee et al., 1993). The third α helix endows RXR with a property unique to this subgroup of receptors, the ability to homodimerize on DR1 in the presence of 9cRA (see Figure 2). DR1 type binding sites thus provide high affinity binding sites for RXR homodimers as well as RXR/RAR, RXR/PPAR and RXR/ARP1 heterodimers (Kliewer et al., 1992; Nakshatri and Chambon, 1994). Studies suggest that the RXR/RAR heterodimer forms a transcriptionally inactive complex on a DR1 (Kurokawa et al., 1994), although this may not be true for all DR1s depending on promoter context and cell type (Durand et al., 1992). As mentioned above, due to the fact that RXR plus 9cRA mediates activation by a DR1, these sites are commonly called RXREs (Mangelsdorf et al., 1991).

The question of whether RXR functions as a ligand-dependent hormone receptor or a 'silent partner', particularly in the setting of a RXR/RAR heterodimer, is currently an area of interest and deliberation. In certain cases, as with RXR/PPAR, RXR/LXR or RXR/NGFI-B heterodimers on their respective half-sites, it seems clear that RXR functions as a transcriptionally active partner (Mangelsdorf and Evans, 1995a). However, contrasting results concerning dual ligand occupation in the RXR/RAR heterodimer have been obtained with receptor specific ligands. It has been reported that both RAR and RXR are liganded and transcriptionally active on a DR5 (Apfel et al., 1995). Supporting this notion are studies in which addition of either RXR- or RAR-specific ligands at concentrations which do not significantly activate endogenous RA responsive genes, leads to synergistic activation when these ligands are combined (Roy et al., 1995). On the other hand, studies have shown that RXR cannot be liganded *in vitro* and is not transcriptionally active in transient transfections unless RAR is liganded (Forman et al.,

1995), or alternatively that RXR along with RAR on a DR1 or DR5 is not able to bind ligand or transactivate regardless of whether RAR is liganded (Kurokawa et al., 1994). The expression of two endogenous genes has been demonstrated to be upregulated by an RXR specific ligand (Allegretto et al., 1995; Davis et al., 1994), however, it is unknown whether this induction is mediated by RXR homodimers or RXR with an unidentified nuclear receptor partner.

2. Physiological Roles of RARs and RXRs

2.1 Distribution of RARs and RXRs

The array of biological processes affected by RA suggests that each RAR and RXR isoform may play a unique physiological role. Detailed Northern blot analysis of receptor mRNA expression patterns in mouse and human reveals that both RAR α isoforms are expressed widely but differentially in adult tissues (Leroy et al., 1991). RAR β transcripts are found at high levels in kidney, prostate, pituitary and adrenal glands, spinal cord and cerebral cortex, with lower levels in several other tissues (Benbrook et al., 1988; de Thé et al., 1989). RAR γ transcripts are predominantly expressed in the skin and lungs (Giguère et al., 1990; Kastner et al., 1990; Zelent et al., 1989). The three RXR genes are expressed widely in adult tissues. RXR α mRNA is expressed in liver, kidney, lung, muscle and spleen (Mangelsdorf et al., 1992; Mangelsdorf et al., 1990) while RXR β is relatively abundant in many tissues but less abundant in liver, intestine and testes (Mangelsdorf et al., 1992). RXR γ transcripts are the most restricted and are expressed mainly in heart, muscle, and brain (Mangelsdorf et al., 1992). In situ hybridization analyses show spatially and temporally restricted distribution patterns of RAR and RXR mRNAs during mouse embryogenesis (Dollé et al., 1989; Dollé et al., 1990; Mangelsdorf et al., 1992; Mendelsohn et al., 1994; Ruberte et al., 1991; Ruberte et al., 1990).

2.2 Phenotypic Analysis of targeted mutations of retinoid receptors

In order to assess the function of the product of a given gene *in vivo*, loss-of-function genetic studies through gene targeting have proven indispensable. Such technology has recently allowed for the generation of mice lacking individual receptor isoforms or combinations of retinoid receptors (for review see Kastner et al., 1995). Characterization of the phenotypes seen in single receptor null mutants reveals that it may be difficult to unambiguously assign an *in vivo* role to an individual receptor using such means when it is a member of a family and moreover, when it functions in concert with a heterodimeric partner. Null mutations of RAR α , RAR β or RAR γ , as well as isoform-specific knockouts of some of the RARs have been generated. Surprisingly, mice lacking RAR β , RAR α 1, RAR β 2, or RAR γ 2 appear normal (Li et al., 1993; Lohnes et al., 1993; Lufkin et al., 1993; Luo et al., 1995; Mendelsohn et al., 1994). RAR α or RAR γ null mutants display some of the defects of the postnatal VAD syndrome including poor viability, growth deficiency and male sterility (Lohnes et al., 1993; Lohnes et al., 1994; Lufkin et al., 1993). In addition, these mutants display some congenital malformations. When RAR double null mutants are generated, almost all of the fetal VAD syndrome malformations are evident, and viability is dramatically reduced (Lohnes et al., 1994; Luo et al., 1996; Mendelsohn et al., 1994). RXR α null mutants die in utero due to heart failure (Kastner et al., 1994; Sucov et al., 1994), and although RXR β null mutants are viable and morphologically normal, males are sterile (Kastner et al., 1996). Similar to RAR double null mutants, combination mutants of RXR α plus one of the RARs display abnormalities present in VAD fetuses (Kastner et al., 1995). Conclusions from these studies so far include strong suggestions that the RAR/RXR heterodimer acts as a functional unit to transduce the RA signal *in vivo*, and that under certain circumstances, different receptors and receptor isoforms can act in a functionally redundant manner.

However, several lines of evidence persist in suggesting that the multiple effects of retinoids in the organism may reside in the control of different subsets of RA

responsive genes by various combinations of RARs and RXRs expressed in a cell specific manner. Firstly, throughout evolution there is a high degree of interspecies conservation of individual receptor isoforms, including the N terminal A region which contains a transcriptional activation function (AF-1) (Nagpal et al., 1993). Secondly, studies with F9 EC cells deficient in RAR α or RAR γ revealed a difference in their abilities to differentiate in response to RA, and a difference in the profiles of various RA responsive genes in these cells (Boylan et al., 1993; Boylan et al., 1995). RAR α and RAR β can perform some of the functions of RAR γ , but with lower efficiency (Taneja et al., 1995). The fact that double knockouts seem to be required to detect phenotypic abnormalities may indicate that the substituting receptors in the single knockout mice can still activate the lost receptors repertoire of genes enough to maintain function (Kastner et al., 1995). In order to dissect the particular functions of each individual receptor, it will be necessary to create additional RXR/RAR heterodimer knockouts, to engineer mutants in which receptors are inactivated in a spatiotemporally controlled manner, and importantly, to isolate and characterize specific subsets of RA responsive target genes.

2.3 Retinoic Acid Responsive Genes

As described above, the retinoid receptors are capable of binding to specific DNA sequences that mark particular genes as targets for regulation. Due to the multitude of effects elicited by RA, it is hypothesized that many directly regulated genes must play roles in mediating these actions. In the literature to date, many RA-responsive genes have been identified, including growth factors and their receptors, cellular enzymes, and extracellular matrix components to name a few (Sporn et al., 1994). However, it is often not known whether the response of these genes to RA is direct or indirect. Genes for which RAREs and RXREs have been characterized within regulatory regions are compiled in Table 1. As shown in Table 1, RAREs and RXREs can be divided into various classes depending on the orientation of the AGGTCA half-sites: direct repeats,

TABLE 1. Retinoic Acid Response Elements

	GENE	LOCATION	SEQUENCE^b	REFERENCE		
RARE^a	DR5	mRARβ2	-55 -> -35	AGGGTTCACCGAAAGTTCACT	Sucov et al, 1990	
		hRARβ2	-55 -> -35	AGGGTTCACCGAAAGTTCACT	de Thé et al, 1990	
		mRARα2	-57 -> -37	CGAGTTCAGCAAGAGTTCAGC	Leroy et al, 1991	
		hRARα2	-56 -> -36	CGAGTTCAGCGAGAGTTCAGC	Leroy et al, 1991	
		hRARγ2	~ -390	CCGGGTCAAGAGGAGGTGAGC	Lehmann et al, 1992	
		mCP-H	-145 -> -135	GCAGGTCACTGACAGGGCATA	Munoz-Cánoves, 1990	
		hAD3	-282 -> -302	AGGGGTCAATTAGAGTTCAGT	Duester et al, 1991	
		hGal	?	CAAGGGCAGGAGAAGGTCAAG	Costa-Giomi et al, 1992	
		hCMV-IEP	-273 -> -293	TAAGGTCACTGTACTGGGCATT	Ghazal et al, 1992	
		hMGP	-2092 -> -2112	AAGGTTCACCTTTTGTTCACC	Cancela & Price, 1992	
		mHoxa-1	3' enhancer	CAGGTTCACCGAAAGTTCAG	Langston & Gudas, 1992	
		mHoxd-4	-2523 -> -2507	TAAGGTGAATGCAGGTCA	Pöpperl & Featherstone, 1993	
		t-PA	-7319	TGGGGTCAACCTGGGGTCAAG	Bulens et al, 1995	
		DR4	TM	-1525 -> -1539	TTTGGTCACTGCAGGTTCAGT	Dittman et al, 1994
			DR2	mCRBPI	-1013 -> -996	GTAGGTCAAAAGGTCAAG
rCRBPI	?	GTAGGTCAAAAGGTCAAG		Husmann et al, 1992		
hApoAI	-197 -> -214	AGGGGTCAAGGGTTCAGT		Rottman et al, 1991; Williams et al, 1992		
mCRABPII-1	-1160 -> -1143	CCAGTTCACCAGGTTCAGG		Durand et al, 1992		
AFP	-6327 -> -6319	CATTGGAAGAGGTCA		Liu et al, 1994		
mHoxb-1	3' region	AAAGGTCAAGCAAGTTCAGC		Marshall et al, 1994		
mHoxb-1	5' region	GGAGGGCAAGAGTTCAGG		Studer et al, 1994		
ER8	myf-crystallin	-210 -> -185		AGTGACCCCTTTTAACCAAGGTTCAGT	Tini et al, 1993	
	IR8	hGH		-177 -> -162	GGGGGACATGACCCCA	Williams et al, 1992
hOST		-512 -> -497		CTAGGTGACTCACCAGG	Schüle et al, 1990	
xVitA2		-334 -> -316		TCAGGTCAAGTGCACCTGA	Forman et al, 1992	
rGH		-191 -> -164		AAAGGTAAAGATCAGGGACGTGACCCGA	Umesono et al, 1988; Williams et al, 1992	
complex		mLAMB1		-432 -> -469	GAGGTGAGCTAGGTAA(13)GGGTCAAC	Vasios et al, 1991; Williams et al, 1992
		hOXY	-76 -> -163	ATTGGTCA(14)GGGTCA(47)GGGTCAAGGTTCACC	Richard & Zingg, 1991; Lipkin et al, 1992	
hMCAD		-339 -> -309	ATTGACCTTCTCTCCGGTAAAGGTGAAG	Raisher et al, 1992		
Oct 3/4	-46 -> -27	CGGGGCCAGAGGTCAAGGCTAGA	Pikarsky et al, 1994			
RXRE^a	DR1	rCRBPII	-632 -> -602	ACAGGTCAAGGTTCACAGGTTCACAGTTCATT	Mangelsdorf et al, 1991	
		cOVAL	-87 -> -71	TGGTGTCAAAAGGTCAAA	Kliwer et al, 1992	
		rAOX	-556 -> -572	CCAGGACAAGGTTCACG	Kliwer et al, 1992	
		hApoAI	-190 -> -214	GCAGGGCAGGGGTCAAGGGTTCAGT	Rottman et al, 1991	
		mCRABPII-2	-639 -> -656	GAAGGGCAGAGGTTCACA	Durand et al, 1992	
		mMHC1	-201 -> -185	TGAGGTCAAGGGTGGGG	Nagata et al, 1992	
		rPEPCK	-437 -> -453	CACGGCCAAGGTTCATG	Lucas et al, 1991	
		hHBV	-1152 -> -1136	CGGGGTAAAGGTTCAGG	Huan et al, 1992	
		ST3	-385 -> -373	AGAGGTCAAGAGGTTCAGG	Anglard et al, 1995	

^aDR, ER and IR denote pairs of core half sites arranged as direct, everted or inverted repeats spaced by the indicated number of nucleotides; complex refers to binding sites containing multiple core half sites arranged in various orientations

^bCore half sites are underlined

palindromes and everted repeats, and complex elements with no obvious common structure. RAREs which confer the highest levels of RA-induced activation and the highest affinity binding to RXR/RAR heterodimers in vitro are DR5s; genes belonging to this class make up the largest of the groups listed in Table 1. Receptor heterodimers also bind with relatively high affinity to RAREs formatted as directly repeated half-sites spaced by 2 or 1 nucleotide(s) (DR2 or DR1). The DR1 RAREs are listed as RXREs because of their ability in transient transfection systems to confer activation upon exposure to RXR and 9cRA (Mangelsdorf et al., 1991). Other classes of RAREs generally confer a weaker RA response and are bound with lower affinity by the receptors. In theory, this heterogeneity provides a method of yielding a graded response to RA. Some of the lower affinity RAREs are promiscuous in terms of their nuclear receptor specificity, such as the response element in the mouse γ F-crystallin gene (Tini et al., 1995; Tini et al., 1993) and the palindromic response elements (Umesono et al., 1988).

The first RARE to be identified and the one that confers the highest level of RA-induced activation to date in transient transfection assays is the DR5 RARE of the RAR β 2 gene (called the β RARE) (de Thé et al., 1990; Sucov et al., 1990). In the case of the RAR β 2 gene, the β RARE likely plays a critical role in the expression of this gene, as transgenic mice harboring three copies of the β RARE driving expression of the *hsplacZ* gene (Rossant et al., 1991) show a β -galactosidase expression pattern which largely mimics the endogenous expression of the gene (Ruberte et al., 1991). Interestingly, the RAR β is only one of a number of RARE-containing genes which constitute steps of the retinoid signaling pathway (Table 1). Additional RAR genes are autoregulated, a situation which could lead to amplification of the RA signal (de Thé et al., 1990; Lehmann et al., 1992; Leroy et al., 1991; Sucov et al., 1990). Genes functioning at various levels in RA signaling contain RAREs and RXREs in their promoter regions, including cytoplasmic retinoid binding proteins (for example CRABP II, Durand et al.,

1992) and enzymes involved in vitamin A metabolism (such as alcohol dehydrogenase (ADH3) Duester et al., 1991).

Not surprisingly, several RARE-associated genes play critical roles in embryonic development. *Hox* genes are homeobox-motif-containing genes which play a role in axis formation in a variety of organisms (for review see McGinnis and Krumlauf, 1992). *Hox* genes occur in clusters in which 3' genes within the cluster are expressed more anteriorly along the developing anteroposterior axis of the embryo, while 5' located genes are expressed in posterior regions of the embryo. Additionally, 3' *Hox* genes are generally expressed earlier during development than the 5' *Hox* genes. In human embryonal carcinoma cell lines, *Hox* genes are sequentially activated by RA in a 3' to 5' direction, and activation depends on the concentration of RA (Simeone et al., 1990). Similar results were seen in mouse F9 embryonal carcinoma cells (Papaloupu et al., 1991). So far, RAREs have been identified in the regulatory regions of *Hox* genes *a-1*, *d-4* and *b-1* (Table 1).

An example of a specific role of RA in embryogenesis is provided by the homeobox gene *Hoxb-1* which has a characteristic pattern of expression in the rhombomeres of the developing mouse embryo. Initially it is expressed in both the mesoderm and neurectoderm of presomite embryos, and later becomes restricted to one particular rhombomere. Early wide expression of the gene is further stimulated by ectopically added RA (Conlon and Rossant, 1992; Marshall et al., 1992; Wood et al., 1994). A conserved DR2 RARE identified in the 3' enhancer of *Hoxb-1* is essential (along with other enhancer elements) for initiation of gene expression and confers the expanded expression seen with RA treatment (Marshall et al., 1994). Further, an additional DR-2 RARE in the 5' region of *Hoxb-1* mediates the restriction of expression to a single rhombomere following the early, unrestricted activation (Studer et al., 1994). Mutation of this sequence allows expression to leak to other rhombomeres, suggesting

that retinoids and retinoid receptors may contribute to the formation of boundaries in regions of the early embryo.

3. Modulation of the Retinoic Acid Response

3.1 Cellular Factors Modulating the Retinoic Acid Response

Although the regulation of target genes by the retinoid receptors is at first sight mechanistically simple, in reality there are multiple cellular factors which add complexity to this signaling system and contribute to the diversity of transcriptional responses within a given cellular milieu (Figure 3). Firstly, the relative availabilities of ligand within a cell will dictate not only the activity of the receptors but the type of dimer formed. Generally the formation of heterodimers is favored, and in such a configuration RXR may function as a silent, ligand-free partner (Forman et al., 1995; Kurokawa et al., 1994), although conflicting results suggest both receptors are liganded (Apfel et al., 1995). In the presence of elevated levels of RXR plus 9cRA, the formation of RXR homodimers is evident (Medin et al., 1994; Zhang et al., 1992). An additional complicating factor is that although 9cRA may be delivered directly to the cell, it can also be generated from atRA within the cell (Heyman et al., 1992). Regulation of such interconversion may vary from cell type to cell type, providing another means of receptor modulation. Regulation of expression of the cytoplasmic retinoid binding proteins (including CRBPs and CRABPs) which play a role in retinoid metabolism, contributes to the levels of various ligands available for receptor stimulation (Giguère, 1994). Several other natural retinoids to which RARs can bind have been characterized (Pijnappel et al., 1993; Thaller and Eichele, 1990), and it is possible that, as has been demonstrated with synthetic retinoids (Roy et al., 1995), each ligand results in differential regulation of responsive genes.

A cellular competition for dimeric interaction with RXR adds an additional level of complexity to gene regulation by the retinoid receptors. RXR functions as a partner

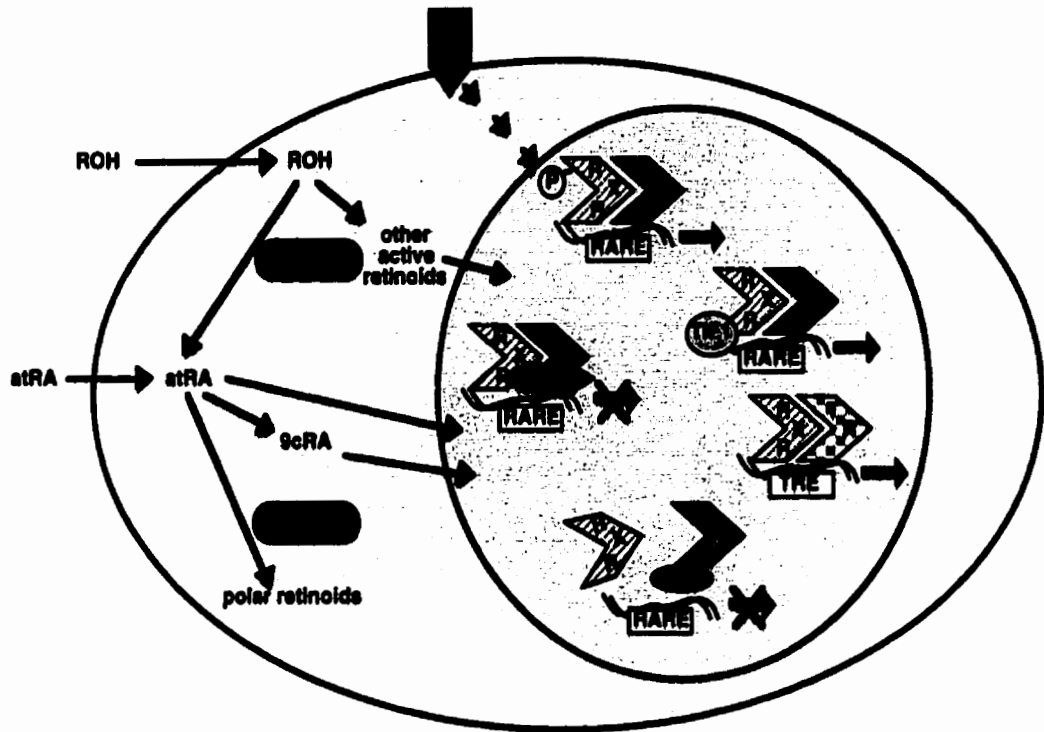


Fig. 3. Modulation of the RA response. In this model of a cell and its nucleus are presented some of the cellular factors which add complexity to RA signaling. Retinol is converted to the active metabolite atRA or to a number of other active retinoids. Cellular retinoid binding proteins (CRBPs) bind retinol with high affinity and serve as a substrate for a dehydrogenase in the metabolic cascade leading to atRA synthesis. 9cRA, a high affinity ligand for RXRs and RARs, can be isomerized from atRA in the cell. The cellular retinoic acid binding proteins (CRABPs) function as carriers for atRA in the catabolic process leading to more polar retinoids. In the nucleus, the RARs and RXRs transcriptional activity on target genes may be influenced by multiple factors including receptor phosphorylation, interaction with positive coregulators, competition with other nuclear receptors for heterodimerization with RXR, and interference with numerous corepressors or interacting factors. See text for details. ROH: retinol; EGF: epidermal growth factor; TRE: thyroid hormone response element; CAL: calreticulin.

not only for RAR but also for other members of this subfamily, including as mentioned above, TR, VDR, and PPAR (reviewed by Glass, 1994). The nature of the binding site itself confers complexity, as some RA response elements are promiscuous binding sites for several nuclear receptors (Giguère, 1994). Transduction of extracellular signals through growth-factor-induced kinase pathways may play a role in determining activities of the receptors. It has been demonstrated that growth factors can stimulate the activity of the N-terminal activation function of the ER via phosphorylation of a serine residue (Kato et al., 1995), and similar modifications may activate RAR and RXR. Another level of complexity concerns the interactions of the retinoid receptors with the transcription initiation complex, either directly or indirectly through transcriptional intermediary factors or coactivators. The C-terminal activating region of RXR interacts directly with the TATA-binding protein, both *in vivo* and *in vitro* (Schulman et al., 1995). Techniques such as far-western blotting and yeast two-hybrid screening have allowed for the elucidation of coactivators that may function as bridging factors between the retinoid receptors and the transcription initiation complex. A number of such proteins interact with the AF-2-containing LBD in the presence but not the absence of ligand and include ERAP160 (Halachmi et al., 1994), Trip1/Sug1 (Lee et al., 1995; von Baur et al., 1995), TIF1, RIP140 (Cavaillès et al., 1995) and SRC-1 (Oñate et al., 1995). Trip1/Sug1, a homologue of the yeast SUG1 gene product, is a component of the RNA polymerase II holoenzyme and interacts with RAR, TR, VDR, ER and weakly with RXR (Lee et al., 1995; von Baur et al., 1995). TIF1, which interacts with RXR γ , ER and the progesterone receptor (PR) (Le Douarin et al., 1995), belongs to a group of proteins which contain a RING domain. ERAP160 and RIP140 are novel proteins that bind the ER, RAR, and TR (Cavaillès et al., 1995; Halachmi et al., 1994).

Transcriptional repression by the retinoid receptors may occur due to competition for binding sites or competition for shared transcriptional cofactors (mentioned above), by the sequestering of the receptors into an inactive form, or by interaction of repressors

with unliganded receptors. The latter mechanism has been elucidated recently due to the identification of two proteins using the yeast two-hybrid screen, N-CoR and SMRT, which bind efficiently to unliganded RAR and TR and are released upon ligand binding (Chen and Evans, 1995; Hörlein et al., 1995; Kurokawa et al., 1995). These proteins possess novel repressor motifs and lead to a strong inhibition of basal transcriptional activity of target genes in transfected cells. Cell specific corepressor content may in part account for the varying reporter gene activity seen when equivalent levels of exogenous receptors are added to various cell types in transient transfections. An additional mechanism for the modulation of target gene activity is the sequestration of the retinoid receptors by other nuclear transcription factors. The pathways of the transcription factors AP-1 (for review see Pfahl, 1993), and Octamer (Kutoh et al., 1992; Lanigan et al., 1993) factors have been shown to 'cross-talk' with RA signaling pathways. The latter inhibition appears to be due to direct interaction between the factors leading to an inability to bind and activate target genes. Recent evidence suggests that the CREB binding protein (CBP) interacts directly with the LBD of multiple nuclear receptors including the retinoid receptors (Kamei et al., 1996). CBP is also required for activation of AP-1 and thus a competition for limiting amounts of cellular CBP may account for the inhibitory effects of RAR on AP-1 activity (Kamei et al., 1996). Also in the category of interference with transcriptional activity due to prevention of DNA binding are the proteins TRUP (Burriss et al., 1995) and the calcium binding protein calreticulin (Dedhar et al., 1994). TRUP, a novel nuclear protein, interacts with the C-terminal region of TR and RAR and prevents receptor binding. Calreticulin will be discussed in the following section.

3.2 *Calreticulin*

Calreticulin is a highly conserved, ubiquitous protein which by virtue of its KDEL signal sequence is found mainly in the lumen of the endoplasmic reticulum (Michalak et al., 1992). Originally isolated as a Ca²⁺-binding protein, it has since been implicated in

multiple cellular functions. Calreticulin contains two distinct Ca²⁺-binding sites and it is proposed that calreticulin is the predominant Ca²⁺ binding and storage protein of the endoplasmic reticulum in non-muscle cells. Calreticulin has also been detected in additional subcellular compartments including the cytoplasm and the nucleus (Opas et al., 1991; Rojiani et al., 1991), although the mechanism by which it escapes endoplasmic reticulum retention is unknown.

Upregulation of calreticulin is seen in response to a number of stimuli, including mitogenic stimulation (Burns et al., 1992), viral transfection (O'Banion et al., 1993), heat shock (Conway et al., 1995), amino acid deprivation (Plakidou-Dymock and McGivan, 1994), ionizing radiation (Ramsamooj et al., 1995), and cellular proliferation (Opas et al., 1991). Calreticulin levels in proliferating rat L6 myoblasts are downregulated upon differentiation of this cell line into myotubes (Opas et al., 1991).

Calreticulin was reisolated as a protein which interacted with the cytoplasmic domain of integrins (Rojiani et al., 1991). Integrins, cell surface receptors which mediate attachment to the extracellular matrix, may be maintained in a high affinity binding state by this interaction (Coppolino et al., 1995; Leung-Hagesteijn et al., 1994). Calreticulin binds to the conserved sequence KLGFFKR found in the α subunit of integrins. A similar sequence (KXFF(K/R)R), is present in the DBD of members of the nuclear receptor superfamily. The location of this motif between the two zinc fingers of the DBD, in a region critical for DNA binding (Giguère, 1994), suggested that interaction with calreticulin might interfere with the transcriptional activity of the receptors. Data presented in Chapter 2 of this thesis demonstrates that calreticulin can interact with the retinoid receptors via the KXFF(K/R)R motif, that such an interaction interferes with the receptors ability to bind RAREs and RXREs, and further, that expression of RA responsive genes and RA induced differentiation of P19 EC cells are downregulated when calreticulin is overexpressed. Receptor/calreticulin interactions have been documented with additional members of the nuclear receptor superfamily (Burns et al.,

1994; Dedhar et al., 1994; St-Arnaud et al., 1995; Wheeler et al., 1995; Winrow et al., 1995).

4. Isolation of target genes

4.1 Methods Used to Isolate Responsive Genes

The isolation and characterization of target genes are fundamental in the study of transcription factors. Binding sites within these genes are crucial for investigation into the mechanisms of action of transcription factors, and importantly, scrutiny of these genes offers insight into the physiological effects of the transcription factor within the organism. In addition, when studying a family of DNA binding proteins whose functions may be partially redundant, identification of target genes will aid in the elucidation of the individual roles of each family member.

Many of the genes so far characterized as retinoic acid regulated genes (Table 1) were originally noted to be upregulated upon RA treatment. These observations led to cloning of the promoter regions of the cDNAs and subsequent characterization of RAREs and RXREs within these promoters. Subtractive library screening (for example see Bouillet et al., 1995) and differential display methods (Liang and Pardee, 1992) are used extensively to isolate cDNAs of transcripts which are upregulated upon RA treatment, although very few directly regulated genes have been cloned using these protocols (LaRosa and Gudas, 1988).

A method now commonly employed to identify the consensus binding site of a given transcription factor makes use of the ability of the factor to select preferred binding sites from a pool of degenerate oligonucleotides (Giguère et al., 1994; Pollock and Treisman, 1990). Binding site preference information aids in further study of the transcriptional activity of the factor and additionally, nucleotide sequence databases can be searched for the presence of consensus binding sites in regulatory regions of

previously characterized genes. Such searches are limited by the lack of genomic sequence data within the databases but will be greatly aided by the Human Genome Project (Guyer and Collins, 1995).

The need to identify binding sites within unknown or uncharacterized genes, within the proper genomic context, led to a number of methods in which total naked genomic DNA fragments are subjected to selection using recombinant transcription factor. Fragments bound by the factor are isolated immunologically (Harrison III et al., 1990; Kinzler and Vogelstein, 1989; Sompayrac and Danna, 1990), by immobilizing the protein to a column (Oliphant et al., 1989), or by filter binding (Inoue et al., 1991), occasionally in combination with amplification by the polymerase chain reaction (PCR) (Costa-Giomi et al., 1992). However, due to the size of mammalian genomes and the typical size of a transcription factor binding site, it is inevitable that there will be a high frequency of binding sites within naked genomic DNA that play no role in gene regulation. One survey purports an average of 14 consensus binding sites for transcription factors per hundred base pairs (Prestridge and Burks, 1993). It is an enormous task to separate the binding sites playing a transcriptional role from those that do not.

Within intact cells, DNA is found in the form of chromatin, a complex of DNA and protein. The fundamental unit of chromatin is the nucleosome, consisting of two 80 bp turns of DNA around an octamer of histones H2A, H2B, H3, and H4 (Wolffe, 1992). Addition of the linker histone H1 acts to stimulate formation of higher-order or bulk chromatin. Access of transcription factors to their binding sites is made more specific by the local structure of chromatin (Felsenfeld, 1992; Wolffe, 1992). In effect the number of possible binding sites are greatly minimized by the presence of nucleosomes and other protein components of chromatin. In order to focus on endogenous binding sites for transcription factors methods have been devised in which chromatin fragments bound to

the factor are immunoprecipitated and the associated DNA cloned and analyzed (Bigler and Eisenman, 1994; Bigler and Eisenman, 1995; Gould, 1990; Tomotsune et al., 1993).

As an alternative to the latter method I have developed a technique of direct binding site isolation which utilizes the presence of unique "markers" of regulatory regions within vertebrate genomes, CpG islands (Bird, 1987).

4.2 CpG islands

A remarkable feature of the vertebrate genome is the pattern of methylation of cytosine residues. Approximately 4% of cytosines are methylated, all of these in the context of the dinucleotide CpG (Bird, 1987). Of the potentially methylatable CpG dinucleotides, 60 to 90% are methylated with the remaining unmethylated fraction concentrated in clusters referred to as CpG islands. Distributed in 45,000 regions, CpG islands constitute approximately 2% of the genome (Antequera and Bird, 1993). These regions range from 200bp to 2000bp in length and have a characteristic GC richness as well as a ratio of observed versus expected CpGs close to that statistically expected (Larsen et al., 1992). In non-CpG island regions of the genome the ratio of CpG dinucleotides is below the expected level, presumably because methylation of cytosine can lead to deamination to form thymidine (Bird, 1987). If such a change escapes the repair mechanisms of the cell, concomitant mutation to TpG and CpA results.

Methylation patterns in the vertebrate genome are maintained through subsequent cell generations by the DNA methyltransferase enzyme (DNA MTase). The importance of this activity has been demonstrated by the inability of mice targeted for disruption of the methyltransferase gene to complete development (Li et al., 1992).

CpG islands are found almost exclusively at the 5' end of genes, often coinciding with promoter regions (Bird, 1987). Surveys of vertebrate genomes indicate that these genes include all sequenced housekeeping genes and 40% of genes that show a tissue-restricted pattern of expression (Antequera and Bird, 1993; Larsen et al., 1992). The

modification of DNA by methylation plays a role in the regulation of gene expression (for review see Eden and Cedar, 1994). The questions of how methylation affects gene expression and how CpG islands maintain their unmethylated state remain to be answered definitively.

Methylation may exert a repressive effect on gene transcription directly. Treatment with 5-azacytidine, a potent inhibitor of methylation, can lead to reactivation of silent genes (Jones, 1985). Interference of methyl groups with the binding of certain transcription factors to cognate binding sites has been demonstrated *in vitro* (Bednarik et al., 1991; Watt and Molloy, 1988) although some transcription factors are not prevented from binding by a methylated target sequence (Holler et al., 1988). Alternatively, studies suggest that methylated DNA initiates a formation of chromatin which leads to transcriptional repression (Kass et al., 1993). Chromatin spreading may be initiated by proteins which bind specifically to methylated CpGs (Antequera et al., 1989; Boyes and Bird, 1991; Lewis et al., 1992; Meehan et al., 1989).

Although CpG dinucleotides are targets of DNA methyltransferases, CpG islands remain unmethylated. Evidence supports a model in which transcription factors such as Sp1 play a role in the establishment and maintenance of CpG islands. Transcription factors may preserve CpG islands by simply excluding methyltransferase or by causing formation of a chromatin state that is structured to prevent DNA MTase activity or alternatively, to attract a demethylase activity (Macleod et al., 1994). Regarding the latter alternative, it is possible that CpG islands can be methylated, but that a CpG island-specific demethylating activity removes 5-methyl-cytosine (Frank et al., 1991). Partially methylated CpG islands lose methylation when injected into embryonic cells (Frank et al., 1991).

Due to their GC rich base composition and lack of CpG deficiency, CpG islands are rich in restriction enzyme sites containing CpG dinucleotides and exclusively G and C residues. Since CpG islands frequently colocalize with gene promoters, digestion of

genomic DNA with CpG recognizing enzymes such as *EagI* (CGGCCG) and *SacII* (CCGCGG) is often used as a means to identify coding regions in genomic clones from large chromosomal regions (Cross et al., 1994; John et al., 1994).

Using enzymes which cut more frequently within CpG island DNA (such as *MspI* (CCGG) or *HinPI* (GCGC)), Tazi and Bird (Tazi and Bird, 1990) developed a method to separate CpG island chromatin from the surrounding bulk chromatin. Analysis of the CpG island chromatin revealed that it differed from bulk chromatin in that histone H1 was present in very low amounts, histones H3 and H4 were highly acetylated, and nucleosome-free regions were present. Core histone acetylation and histone H1 depletion by phosphorylation are two features of a transcriptionally active configuration of the chromatin fiber (Wolffe, 1992). Nucleosome depleted hypersensitive sites have been localized near the 5' ends of many potentially activatable genes, including those with CpG islands (Gross and Garrard, 1988; Shimada et al., 1986). By virtue of their open chromatin structure, CpG islands may be sites of interaction between transcription factors and promoters.

4.3 Introduction to CpG-SAAB, a Novel Method to Isolate Transcription Factor Binding Sites

My goal was to develop a method which could be used to isolate RA responsive genes by first isolating genomic fragments containing RAREs and RXREs. The derivation of genomic fragments exclusively from CpG island DNA would greatly diminish background binding of transcription factors to DNA playing no direct role in transcription, making the subsequent search for the associated gene less arduous. CpG island DNA is frequently associated with the 5' ends of genes, and more specifically with the promoter regions of those genes (Bird, 1987). Studies suggest that CpG island chromatin structurally resembles active or transcriptionally competent chromatin (Tazi and Bird, 1990). This relatively open configuration of CpG island chromatin as well as

its GC richness and an expected level of CpG dinucleotides make it uniquely susceptible to digestion by restriction enzymes with CpG in their recognition sites. As shown by Tazi and Bird (Tazi and Bird, 1990), fragments derived from the nucleosome-free regions of CpG island DNA can be isolated away from bulk DNA. These so-called nonnucleosomal fragments (NN fragments) are released as DNA fragments smaller than the size of a single nucleosome in digestion of intact nuclei using CpG-recognizing four basepair cutters such as *MspI* and *HinPI*.

In order to select for those NN fragments which bind RXR/RAR, fragments can be modified by the addition of linkers which will allow them to be amplified and radiolabeled using the polymerase chain reaction (PCR). Subsequently from this collection of NN fragments, those that bind are isolated in a gel mobility shift assay, amplified by PCR, repeatedly re-bound and re-amplified, and finally cloned for analysis. As the technique of reiterated binding and amplification is a modification of the protocol proposed by Blackwell and Weintraub (Blackwell and Weintraub, 1990), we chose to term the isolation method CpG-SAAB for "CpG (island fragments) -selected and amplified binding".

As starting material for the development of CpG-SAAB, we chose the murine embryonal carcinoma cell line F9. F9 and P19 EC cells are, as stated above, cell lines which differentiate upon RA treatment. Importantly, EC and embryonal stem cell lines differ from other permanent cell lines in that they maintain a methylation status in culture which is similar to in vivo tissues (Antequera et al., 1990; Frank et al., 1991; Kawai et al., 1994).

Subsequent to multiple rounds of selection using gel mobility shift assays and amplification by PCR the NN fragments are cloned for analysis. Since consensus binding sites for RAR and RXR have been extensively characterized (Giguère, 1994), it is possible to sequence the cloned NN fragments and scan for the presence of a RA response element. Functional tests, such as the ability of RXR/RAR to bind specifically

to NN fragments containing a RARE or an RXRE, as well as the ability of the fragment to confer RA responsiveness to a heterologous promoter in a transient transfection assay, can then be performed. Importantly, these fragments will constitute a set of RAREs and RXREs in a natural promoter context. These *in vivo* binding sites provide a means to investigate the possibility that the sequence of the binding site in combination with the flanking sequence may play a role in imparting differential transcriptional activity to various combinations of retinoid receptors. The possibility that cell-type-specific factors other than retinoid receptors influence receptor activity upon these genomic fragments can also be studied. Factors may exert effects by competing with the receptors for binding to these response elements, by binding to *cis* elements flanking the RAREs and RXREs, or alternatively, by interacting directly with the bound receptors.

Following functional analyses, RARE-containing NN fragments can be used as probes to isolate flanking genomic DNA. Several approaches are available for the recognition of genes within large regions of genomic DNA (for review see Gardiner and Mural, 1995). Traditional methods include the use of repeat-free genomic fragments to probe Zoo and Northern blots to detect conserved regions and transcripts respectively. Additional techniques are the *in vivo* identification of splice acceptor sites in cloned genomic DNA (exon trapping, Buckler et al., 1991; Duyk et al., 1990) and selection of cDNA sequences with genomic clones (cDNA selection, Lovett et al., 1991; Parimoo et al., 1991). It is also possible to directly sequence genomic DNA flanking the NN fragment and subject the sequence to 'software trapping' (Claverie, 1994; Legouis et al., 1991), a combination of computational analysis and database searches.

In chapter 3, I will describe the novel approach (CpG-SAAB) that has been developed to isolate RXR/RAR target genes, the functional analysis of twelve RARE- or RXRE-containing fragments isolated using CpG-SAAB, and the characterization a gene associated with one of the RXRE-containing NN fragments.

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Chapter 2

Modulation of the Retinoic Acid and Retinoid X Receptor Signaling Pathways in P19 Embryonal Carcinoma Cells by Calreticulin

This chapter is a slightly modified version of the following submitted manuscript: Modulation of the retinoic acid and retinoid X receptor signaling pathways in P19 embryonal carcinoma cells by calreticulin. Shago, M., Flock, G., Leung Hagesteijn, C.-Y., Giguère, V., and Dedhar, S. The chapter includes some data from the following publication: Inhibition of nuclear hormone receptor activity by calreticulin. 1994. Dedhar, S., Rennie, P. S. Shago, M. et al., Nature 367: 480-483.

Calreticulin is a widely expressed calcium binding protein that can bind to an amino-acid sequence motif, KXGFFKR, which is present in the cytoplasmic domain of all integrin α -subunits. Closely related sequences, KXFFKR and KXFFRR, are encoded in the DNA-binding domain of all members of the steroid/thyroid/retinoid receptor superfamily and it has recently been demonstrated that calreticulin inhibits their activity both *in vitro* and *in vivo*. Here we present novel evidence that calreticulin can interfere directly with the retinoic acid (RARs) and retinoid X (RXRs) receptor pathways. Calreticulin exhibits the ability to inhibit DNA-binding activity of both heterodimeric RXR/RAR and homodimeric RXR complexes *in vitro*. Inhibition of RXR binding to DNA is achieved with a concentration of calreticulin that is approximately four-fold lower than that required for inhibition of RXR/RAR binding to a cognate binding site. Coprecipitation experiments suggest a direct protein:protein interaction between calreticulin and retinoid receptors. In transient transfection studies, we demonstrate that calreticulin reduces RAR and RXR ability to activate retinoid-responsive reporter gene constructs. Stable over-expression of calreticulin in P19 embryonal carcinoma cells significantly decreases the activation of the endogenous RA-responsive CRABP_{II} and RAR β genes and inhibits the emergence of the RA-induced differentiated phenotype. These data demonstrate that calreticulin can interfere with the two distinct retinoid signaling pathways through a mechanism likely involving direct protein:protein interactions and that disruption of the retinoid signal alters biological processes *in vivo*.

Introduction

Control of cell proliferation and differentiation in the organism results from the precise integration of environmental cues provided by soluble mediators such as hormones and growth factors and solid substratum components of the extracellular matrix (ECM). The cell deciphers these diverse signals via the expression of intracellular and extracellular receptor systems. These regulatory proteins include nuclear receptors for steroid hormones and retinoids (Evans, 1988), protein kinase and G protein-coupled receptors for growth factors (Simon et al., 1991; Ullrich and Schlessinger, 1990) and integrins for ECM-mediated events (Hynes, 1992). Cellular activation by these signaling molecules often results in the activation and/or repression of specific gene networks whose products will ultimately dictate the decision to proliferate or differentiate. Deregulation of these and other signal transduction pathways often leads to uncontrolled cell growth and progression toward the malignant state.

Retinoids are potent regulators of cell growth and differentiation and exert profound effects in development and homeostasis (Sporn et al., 1994). Signal transduction by retinoids is mediated by two distinct families of nuclear receptors encoded by six different genes referred to as retinoic acid (RAR α , β and γ) and retinoid X (RXR α , β and γ) receptors (reviewed in Giguère, 1994). The vitamin A metabolite all-trans retinoic acid (atRA) specifically activates RARs while the 9-cis isomer (9cRA) activates both RARs and RXRs at physiological levels. RAR and RXR function as ligand-activated transcription factors that control gene expression through binding to specific DNA sequences located in the regulatory regions of target genes, known as RA response elements (RAREs) and retinoid X response elements (RXREs), respectively. While the multiple RAR isoforms bind DNA with high affinity only as heterodimers with RXR (Bugge et al., 1992; Kliewer et al., 1992; Leid et al., 1992; Marks et al., 1992; Yu et al., 1991; Zhang et al., 1992), in the presence of 9cRA, RXRs form stable homodimers that specifically recognize RXREs

(Medin et al., 1994; Zhang et al., 1992). For both heterodimeric and homodimeric retinoid receptor systems, binding to DNA is an essential step for stimulation of gene expression.

Calreticulin is a high-capacity calcium binding protein that was originally implicated to play a role in the storage of calcium in the lumen of the endoplasmic and sarcoplasmic reticulum (Michalak et al., 1992). However, its identification as a human Ro/SS-A autoantigen which is recognized by autoantibodies found in the sera of patients with Sjogren's syndrome, its localization to the nucleus and nuclear envelope (Michalak et al., 1992) and its direct association with the cytoplasmic domain of integrin (Rojiani et al., 1991) suggest that calreticulin may perform other important cellular and physiological functions. The subcellular localization of calreticulin has been shown to be affected upon inhibition of myoblast fusion by various agents, including transforming growth factor β and the tumor promoter tetradecanoyl phorbol acetate (TPA) (Michalak et al., 1991; Opas et al., 1991). In addition, the level of expression of calreticulin in B16 melanoma cells appears to correlate with their proliferating activity (Gersten et al., 1989). Taken together, these results provided evidence that calreticulin may play a significant physiological role during cell growth and proliferation.

We and others have recently shown that calreticulin can interfere with nuclear hormone receptor activity both *in vitro* and *in vivo* (Burns et al., 1994; Dedhar et al., 1994; St-Arnaud et al., 1995; Wheeler et al., 1995; Winrow et al., 1995). In particular, overexpression of calreticulin inhibited the induction of an endogenous RA-responsive gene as well as suppressing a RA-induced neuronal specific marker of differentiation in P19 embryonal carcinoma (EC) cells (Dedhar et al., 1994). The interaction of calreticulin with nuclear receptors suggested a possible role for this protein as a signal modifier between cell surface integrins and the transcription machinery.

To further increase our current understanding of the interactions between calreticulin and the two distinct retinoid signaling pathways, we investigated the effects of calreticulin on retinoid-mediated regulation of gene activation. In this study, we provide novel

evidence that calreticulin interferes with retinoid-dependent induction of gene expression by direct inhibition of the DNA-binding activity of RARs and RXRs and, as a consequence of these physical interactions, modulates the regulatory action of retinoids on cell proliferation and differentiation.

Materials and Methods

Recombinant plasmids, purified calreticulin and antibodies. Construction of the expression vectors pRShRAR α and pRShRXR α have been described elsewhere (Giguère et al., 1987; Mangelsdorf et al., 1990). The β RARETKLUC reporter plasmid consists of the human RAR β 2 gene RARE (de Thé et al., 1990) linked to sequences -105 to +51 of the TK promoter driving the firefly luciferase reporter gene. A reporter construct containing one copy of the CRBP II RXRE (RXRETKLUC) (Mangelsdorf et al., 1991) was also made in a similar manner. To construct pCMXCAL, the full length human calreticulin cDNA (McCauliffe et al., 1990) was subcloned into the pCMX expression vector (Umesono et al., 1991). pRC-CMVCAL expression vectors for the stable expression of calreticulin cDNA in the sense (CAL1) and antisense (CAL2) orientation are as described previously (Dedhar et al., 1994). p60 (calreticulin) was purified as outlined previously (Rojiani et al., 1991). Goat anti-rabbit calreticulin antiserum was a gift from Dr. Michalak (University of Alberta, Canada). Rabbit antibodies directed against a synthetic peptide specific to RAR α were a gift from Dr. A. Antalky (McGill University).

Electromobility gel shift assays (EMSAs). Plasmids used for *in vitro* transcription/translation were pCMXhRAR α (Umesono et al., 1991) and pSKmRXR β (Mangelsdorf et al., 1992), containing cDNAs for human RAR α and mouse RXR β respectively. pCMXhRAR α was linearized with *Bam*HI and transcribed with T7 RNA polymerase while pSKmRAR β was digested with *Acc*I and its mRNA synthesized with T3 RNA polymerase. Protein was generated from the resulting mRNAs using rabbit reticulocyte lysate (Promega). Probes for EMSA were radiolabeled by endfilling with Klenow and ³²P-deoxyribonucleotides. The β RARE probe is derived from the promoter of the murine RAR β 2 gene (Sucov et al., 1990) and the RXRE probe is derived from the promoter of the rat CRBP II gene (Mangelsdorf et al., 1991) (see also Table 1, Chapter 1). Approximately 1 ng of probe and a total of 6 μ l of *in vitro* transcribed/translated protein were used per reaction in the RXR/RAR heterodimer EMSAs. For the RXR homodimer

EMSA, 8 μ l of in vitro transcribed translated protein was combined with 1 ng of probe for each reaction. The reaction buffer consisted of 50 mM TrisHCl pH8, 200 mM KCl, 30% glycerol, 5 mM dithiothreitol (DTT), and 0.25% Nonidet P40 (NP40). Nonspecific competitors included 2 μ g of poly dIdC, 10 μ g of bovine serum albumin (BSA) and 0.1 μ g of denatured salmon sperm DNA in each sample. Ligands, when added to reactions, were at final concentrations of 10^{-7} M for atRA and 10^{-6} M for 9cRA. All components of the binding reaction except the probe were combined and incubated on ice for 10 min. Probe was added and the reaction was incubated an additional 10 min. at room temperature prior to loading of the entire reaction on a 4% nondenaturing polyacrylamide gel. Samples were electrophoresed for 2 hours at 150V.

Immunoprecipitation and Western blot analysis. Cells were metabolically labeled with 35 S-methionine/ 35 S-cysteine for 24 hours and RAR α was immunoprecipitated from cellular lysates as described previously (Leung Hagesteijn et al., 1994). Washes for the immunoprecipitation were performed using RIPA buffer (1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS) and 0.5% deoxycholate) with 0.5M NaCl. Immunoprecipitates were analyzed by SDS-PAGE followed by fluorography. The anti-RAR α immunoprecipitates were also analyzed by Western blot analysis for the presence of calreticulin as described previously (Leung Hagesteijn et al., 1994; Rojiani et al., 1991).

Transfection and luciferase and β -galactosidase assays. Expression vectors and reporter genes were introduced into P19 EC cell lines by calcium phosphate precipitation as described previously (Giguère et al., 1990). Five hundred nanograms of each expression vector, 2 μ g reporter plasmid, 1.5 μ g RSV β gal, and carrier plasmid to a total of 10 μ g were used per 6 cm diameter dish. The following day the cells were washed with PBS and the media (Dulbecco modified Eagle medium supplemented with 7.5% (vol/vol) fetal calf serum) with the indicated isomer and concentration of RA, was replaced. Twenty-four hours later, luciferase and β -galactosidase assays were performed as described elsewhere (Giguère et al., 1990).

Northern Blots. Total cellular RNA (15 μ g) from the indicated P19 EC cell line derivatives was hybridized to 32 P-labeled mouse CRABP II cDNA (Giguère et al., 1990) or RAR β 2 cDNA (Giguère et al., 1990) at 65°C using Rapid Hyb buffer (Amersham). After autoradiography, the filter was stripped and rehybridized with a mouse β actin cDNA probe to monitor for equal loading of RNA samples.

P19 EC cells derived subclones, RA-induced differentiation and immunofluorescence. P19 EC cell lines stably overexpressing (Cal-1) and underexpressing (Cal-2) calreticulin were described elsewhere (Dedhar et al., 1994). Cal-1 cells contain levels of calreticulin approximately five-fold greater than control cells, while Cal-2 cells express half the amount of calreticulin seen in control cells. Neuronal differentiation induced by atRA was performed as described (McBurney et al., 1988). Immunofluorescence was carried out on glass coverslips as described previously (Leung Hagesteijn et al., 1994). The cells were stained with a monoclonal antibody against class III β Tubulin (TuJ1; gift from Dr. Frankfurter).

Determination of intracellular free calcium concentration in P19 EC cells. The intracellular calcium concentration was determined by Fura-loading (Grymkiewicz et al., 1985), and fluorescence measurement utilizing a Hitachi F-2000 fluorescence spectrophotometer as described by Nasmith and Grinstein, 1987 (Nasmith and Grinstein, 1987). Equivalent numbers of P19 Neo, or P19 Cal.1(1A2 and 1D3) cells were plated onto glass coverslips and the intracellular calcium concentration was determined by utilizing the following equation:

$$[\text{Ca}^{2+}] = K_d ((F - F_{\text{min}})/(F_{\text{max}} - F))$$

where $[\text{Ca}^{2+}]$ is in nM, the K_d for Fura-2 is 224nM, F is the experimental fluorescence, F_{max} is the maximum fluorescence after exposure to ionomycin (1 μ M) in 3 mM extracellular Ca^{2+} , and F_{min} is the minimum fluorescence calculated as follows:

$$F_{\text{min}} = 1/3 (F_{\text{max}} - F_{\text{manganese}}) + F_{\text{manganese}}$$

where $F_{\text{manganese}}$ is the fluorescence after quelching with 3 mM MgCl_2 .

Results

Calreticulin interferes with RAR/RXR heterodimer DNA-binding activity. We demonstrated previously that overexpression of calreticulin in P19 EC cells reduces the ability of atRA to induce the expression of a transiently transfected RARE-driven reporter construct and of the endogenous RA-responsive CRABP-II gene (Dedhar et al., 1994 and see Figure 6A). However, these studies did not investigate the molecular mechanism of calreticulin/retinoid receptor interactions nor determine whether both RAR- and RXR-specific pathways were modulated. Since both RARs and RXRs contain within their DNA binding domain a six amino acid sequence highly related to the conserved region in integrin α subunits responsible for interacting with calreticulin (Fig. 1), we first investigated the ability of calreticulin to prevent heterodimeric RXR/RAR complexes from binding to DNA. In vitro synthesized RAR and RXR bind specifically as a heterodimer to the response element β RARE derived from the promoter of the RAR β gene (Fig. 2A, lane 2). The RXR/RAR- β RARE complexes can be efficiently competed by an excess of cold competitor (Fig. 2A, lane 3). Addition of increasing amounts of purified calreticulin, referred to as p60, resulted in a progressive reduction in the amount of RXR/RAR- β RARE complexes formed. With 200 ng of p60 present in the reaction (Fig. 2A, lane 7), heterodimeric RXR/RAR- β RARE complexes can no longer be detected. Evidence that retinoid receptors interact with calreticulin via the conserved seven amino acid region present in their respective DNA binding domain is provided by the observation that addition of an excess of the synthetic peptide KLGFFKR partially restored the ability of RXR/RAR complexes to bind to the β RARE (Fig. 2B, lane 6). Furthermore, when an antibody directed against calreticulin is included in the reaction, binding of heterodimeric RXR/RAR complexes to the β RARE is almost entirely restored (Fig. 2C, lanes 6 and 7), indicating that calreticulin and not some accompanying factor in the purified fraction is responsible for the binding interference.

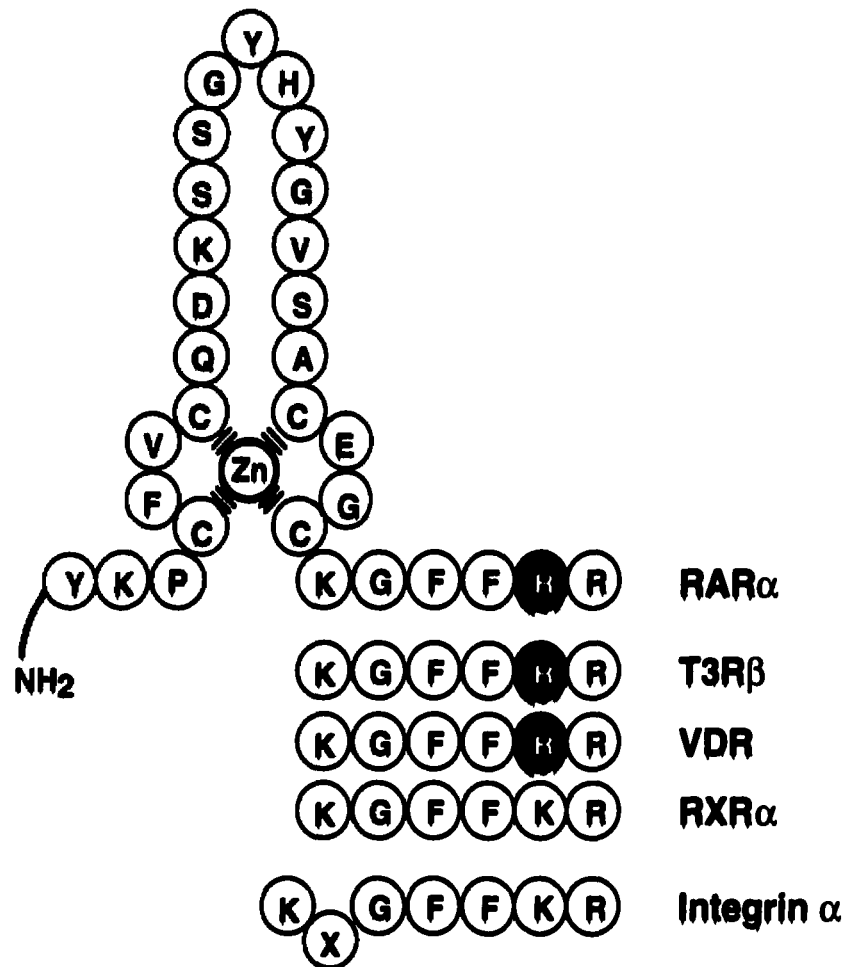
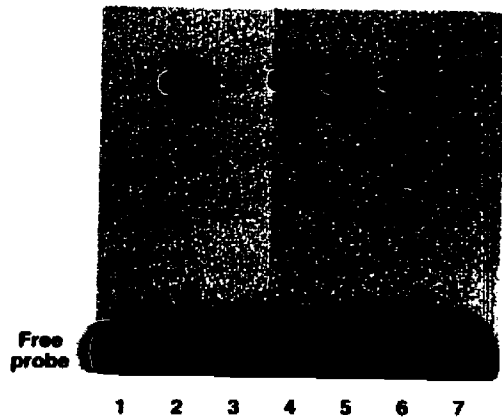


Fig. 1. Schematic representation of the first zinc finger region of the RAR α DNA binding domain including the KGFF(K/R)R sequence at the base of the zinc finger to which calreticulin is proposed to bind. Listed below the RAR α zinc finger are the putative calreticulin-interacting sequences of several other nuclear receptors and the cytoplasmic domain of the integrin α subunit.

Fig. 2. Analysis of the interaction of the RXR/RAR heterodimer with p60 (calreticulin) by EMSA. (A) Effect of the addition of increasing amounts of p60 on the RXR/RAR/ β RARE complex. *In vitro* transcribed/translated RAR and RXR were incubated with 1 ng of 32 P labeled β RARE probe. β RARE (in A, B, and C) refers to unlabeled probe. Unprogrammed lysate was included as a negative control (A, B, and C, lane 1). (B) Addition of the peptide KLGFFKR partially restores binding of RXR/RAR to the β RARE probe (lane 6). (C) Regeneration of receptor/DNA binding by inclusion of an anti-calreticulin antibody.

A

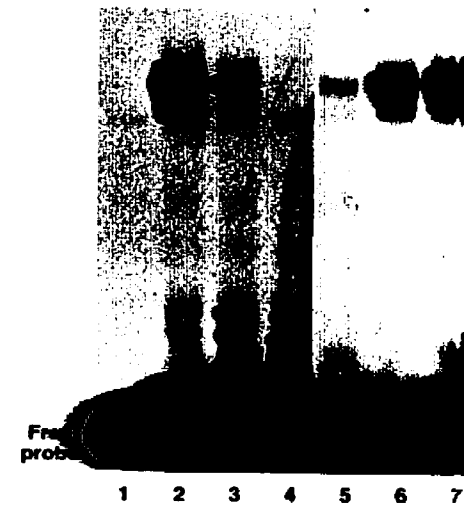
RAR/RXR	+	+	+	+	+	+
BRARE		+				
p60 (ng)			25	50	100	200

**B**

RAR/RXR	+	+	+	+	+
BRARE		+			
p60 (200 ng)			+		+
peptide (500 ng)				+	+

**C**

RAR/RXR	+	+	+	+	+	+
BRARE		+				
p60 (200 ng)			+	+	+	+
peptide (1 μg)				+		
Ab (μl)					1	2



RXR homodimers interact with calreticulin. Previous studies have demonstrated that, in the presence of 9cRA, RXR binds to its hormone response elements as a homodimer (Medin et al., 1994; Zhang et al., 1992). In addition, the putative six amino acid calreticulin interacting sequence in RXR differs from that of RAR and RXR's other heterodimeric partners (e.g. TR, VDR) by a single lysine (K) residue in place of the first arginine (R), that substitution in fact making it more similar to the calreticulin binding sequence in integrin α subunits (Fig. 1). To determine whether the inhibitory effect of calreticulin on DNA binding could be observed with RXR homodimers, we performed EMSA analysis with RXR alone and a labeled RXRE in the presence or absence of 9cRA. As shown in Fig. 3, EMSA demonstrates that homodimeric RXR-RXRE complexes were observed in significant amount only in the presence of 9cRA and that these complexes are highly susceptible to DNA binding interference by p60. Addition of increasing amounts of purified calreticulin resulted in a sharp reduction in the amount of RXR-RXRE complexes formed. Homodimeric RXR-RXRE complexes could be observed only faintly when 50 ng of p60 was included in the reaction (Fig. 3A, lane 8), and were completely abolished when the amount of p60 was increased to 100 ng (Fig. 3A, lane 9). This increased susceptibility by approximately four-fold of RXR homodimeric complexes to interference in DNA binding by p60 as compared to RXR/RAR heterodimeric complexes could be due to either the intrinsic differences in the nature of the complexes or the presence of the ligand in the RXR binding experiments, which can induce conformational changes in the receptor (Allan et al., 1992). To investigate this possibility, we next examined the effect of the inclusion of ligand on the ability of calreticulin to interfere with RXR/RAR heterodimer binding to a RARE. RXR/RAR heterodimers are responsive to both atRA and 9cRA in transient transfection assays (Allenby et al., 1993 and see Fig. 5) and show a slight change in mobility in EMSA when bound to ligand (unpublished observations). However, liganded RXR/RAR complexes appear to interact with calreticulin similarly to unliganded receptors (Fig 3B). In the presence of atRA, 9cRA or both, interference profiles are virtually

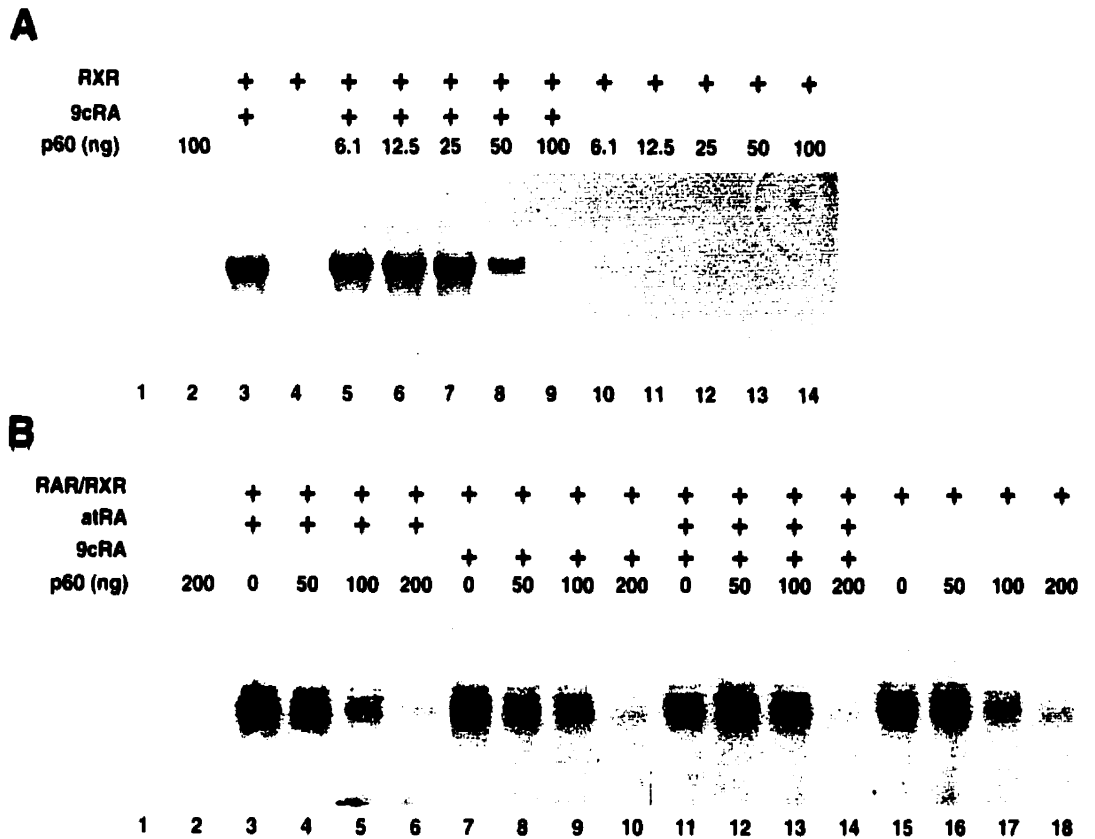


Fig. 3. (A) Interaction of p60 (calreticulin) with RXR homodimers. *In vitro* transcribed/translated RXR was combined with 1 ng of ^{32}P labeled CRBPII-RXRE in the presence (lane 3, lanes 5-9) or absence (lane 4, lanes 10 to 14) of 10^{-6}M 9cRA. Unprogrammed lysate and p60 alone (lanes 1 and 2 respectively) were included as negative controls. p60 was added to the reactions as indicated. (B) Presence of ligand does not alter the interference properties of p60. Reactions consisted of *in vitro* transcribed/translated RAR and RXR, 1 ng of ^{32}P labeled βRARE and the indicated ligand (at concentrations of 10^{-7}M for atRA and 10^{-6}M for 9cRA). As in (A), p60 was added as indicated, and unprogrammed lysate (lane 1) and p60 alone (lane 2) are included as negative controls.

identical to those of RXR/RAR alone, with binding nearly eliminated at 200 ng of p60 (Fig. 3B, lanes 6, 10, 14 and 18). This data suggests that the difference in the retinoid receptor complexes RXR/RAR and RXR/RXR to DNA binding interference by calreticulin may be due to intrinsic structural differences in their respective DNA binding domains and that the presence of bound ligand does not affect the molecular interaction between calreticulin and the retinoid receptors.

Direct calreticulin/RAR interactions. The ability of calreticulin to interfere with retinoid receptors binding to their hormone response elements, combined with the presence of the conserved six amino acid sequence known to bind calreticulin in affinity chromatography, strongly suggests that the retinoid receptors and calreticulin interact directly. Using cell lysate from ³⁵S-methionine in vivo-labeled P-19 EC cells, an antiRAR α antibody was shown to co-immunoprecipitate a protein of identical size to calreticulin (Fig. 4A). To corroborate this data, two distinct P19 EC cell clones were gently lysed and subjected to immunoprecipitation with an antiRAR α antibody. The immunoprecipitate was subjected to Western blot analysis using anti-calreticulin antibody and a band corresponding to calreticulin was consistently observed (Fig. 4B). Importantly, observation of this complex did not require the presence of protein crosslinking agents. No bands were observed when cell lysate immunoprecipitated with preimmune serum was analyzed by Western blot with anti-calreticulin antibody (data not shown). Further evidence supporting the strength of calreticulin/RAR interaction is the observation that in gel mobility shift assays, when RXR/RAR heterodimers are allowed to pre-associate with the β RARE probe, p60 (calreticulin) retains its ability to disrupt receptor/DNA complexes (data not shown).

Relative contribution of individual amino acids of the KXFF(K/R)R motif in binding to calreticulin. To discern which amino acids within the KXFF(K/R)R motif of the retinoid receptors were critical in the RXR/RAR interaction with p60 (calreticulin), synthetic peptides encoding the homologous motif from the α -subunit of

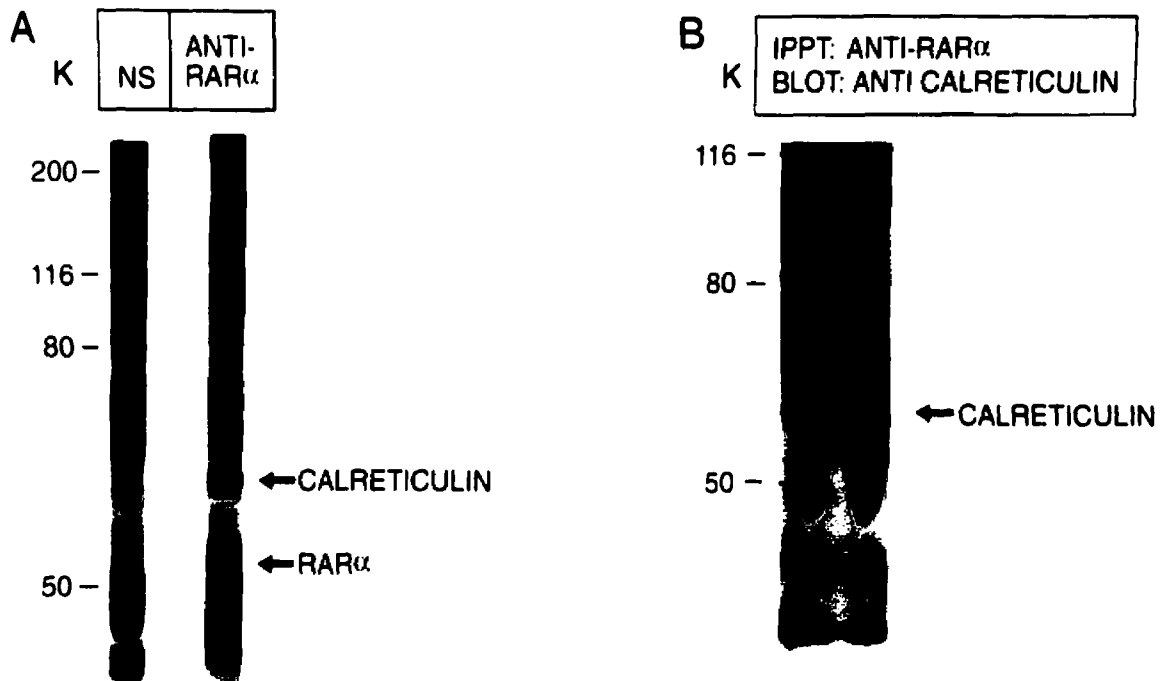


Fig. 4. Direct interaction between RAR α and calreticulin. Coimmunoprecipitation of RAR α and calreticulin. (A) P19 EC cells were metabolically labeled with ^{35}S -methionine/ ^{35}S -cysteine for 24 hours in methionine-cysteine free medium. The cells were then washed with PBS and lysed in RIPA buffer. Immunoprecipitation was carried out with anti-RAR α antiserum or normal rabbit serum (ns) as described in Materials and Methods. The immunoprecipitates were analyzed by SDS-PAGE and the proteins detected by fluorography. RAR α immunoprecipitate contains a protein with similar molecular weight to calreticulin. (B) The RAR α immunoprecipitates described in (A) were analyzed by Western blot analysis (Leung Hagesteijn et al., 1994) for the presence of calreticulin with an anti-calreticulin antibody. RAR α immunoprecipitates from two different P19 clones contain calreticulin. Pre-immune serum immunoprecipitates do not contain calreticulin (data not shown).

integrins were included in EMSA binding reactions (Table 1). As shown in Fig. 2B lane 6, addition of the peptide KLGFFKR to a RXR/RAR/ β RARE binding reaction containing p60 partially restores binding of the retinoid heterodimer to the RARE. A scrambled peptide (KLRFGFK) and peptides mutated in each position were added to similar binding reactions. Consequently, bound probe was quantitated using a Molecular Dynamics phosphoimager and calculated as a % of recovery of RXR/RAR binding (recovery of binding with the original KLGFFKR peptide was set at 100%). Average results from two experiments are shown in Table 1. Peptides mutated in any of the corresponding nuclear receptor positions fail to restore binding to an extent seen with the intact KLGFFKR peptide. Three of the mutations restore heterodimer binding to a lesser degree than the scrambled peptide; those having a mutation in either of the two phenylalanines or the final arginine of the motif. These three amino acids are absolutely conserved in each member of the nuclear hormone receptor family, as well as in integrin α -subunit cytoplasmic domains (Dedhar et al., 1994). In contrast, the first lysine, which is also conserved in all of these proteins, allows restoration of close to half of the original binding when a peptide mutated in this position is added to the EMSA binding reaction (Table 1).

Downregulation of RA response in transient transfections. When calreticulin is transiently expressed in tissue culture systems, it has been shown to interfere with the glucocorticoid and androgen receptors' activation of a reporter gene linked to an appropriate receptor binding site (Burns et al., 1994; Dedhar et al., 1994). This concentration dependent downregulation of hormone-induced gene activation is also seen with retinoid receptors in transient transfection assays (Fig. 5). In P19 EC cells exposed to atRA, 9cRA or both ligands in combination, RXR/RAR heterodimer activation through the β RARE response element, as measured by activity of a linked luciferase reporter gene, decreases gradually to about half of its maximal value with 5 μ g of transfected calreticulin expression plasmid, pCMXCAL (Fig. 5A). Strong activation of a RXRE-driven reporter gene is observed only in the presence of the ligand 9cRA and exogenously added RXR

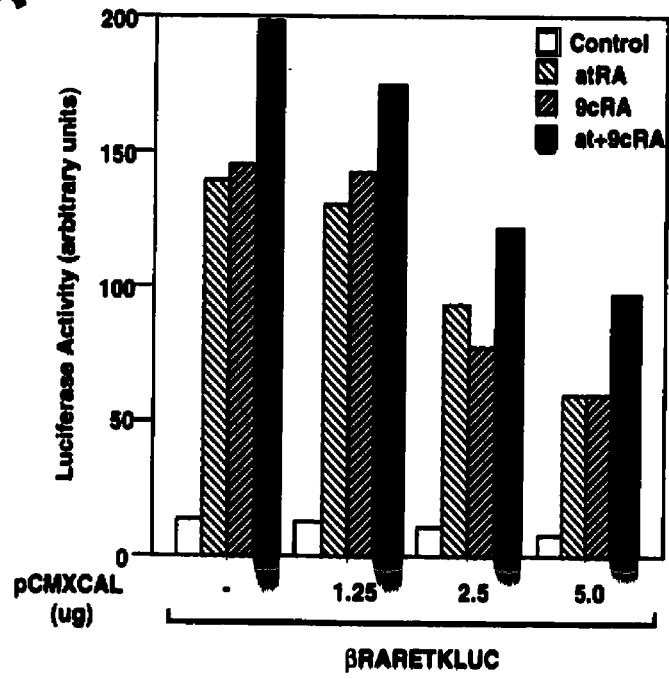
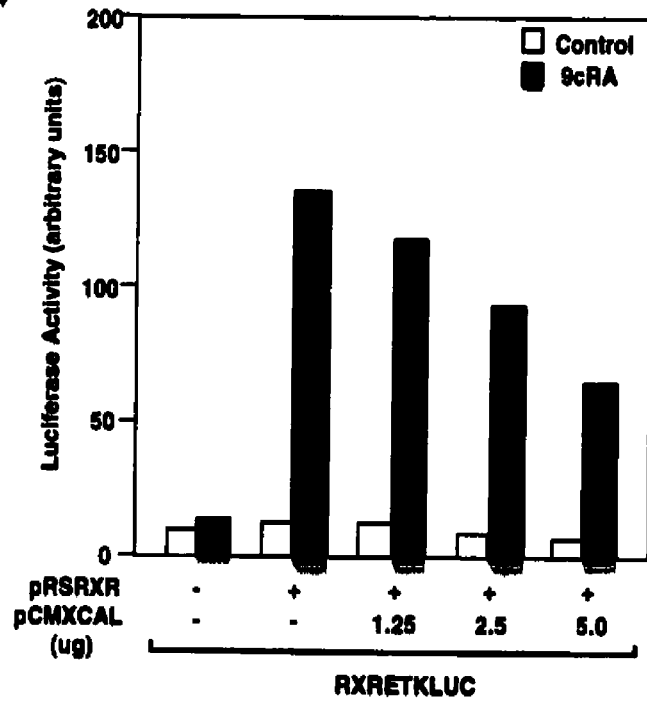
TABLE 1

Relative contribution of individual amino acids of the
KXFF[K/R]R motif in binding to calreticulin

Peptide	Recovery of RXR/RAR binding
KL <u>G</u> FFKR	100
KL <u>R</u> FGFK	34
<u>G</u> LGFFKR	45
KL <u>D</u> FFKR	63
KL <u>G</u> RFKR	24
KL <u>G</u> FRKR	20
KL <u>G</u> FF <u>G</u> R	42
KL <u>G</u> FFK <u>G</u>	22

Recovery of RXR/RAR binding is indicated as a %. Synthetic peptides were added to EMSA binding reactions in which RXR/RAR/RARE was incubated with p60 (which abolishes binding). The recovery of RXR/RAR binding to the RARE is set at 100 for the original peptide. Peptide sequence is derived from the region of the integrin α subunit that interacts with calreticulin. Underlined sequences represent amino acids that are modified from the original peptide.

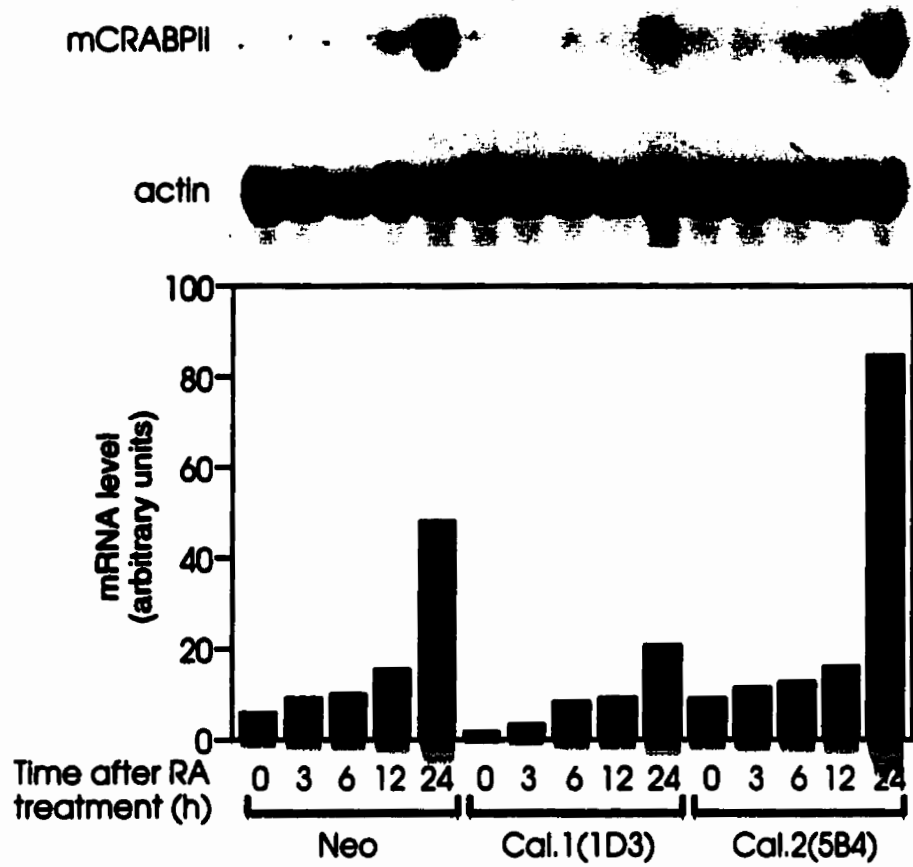
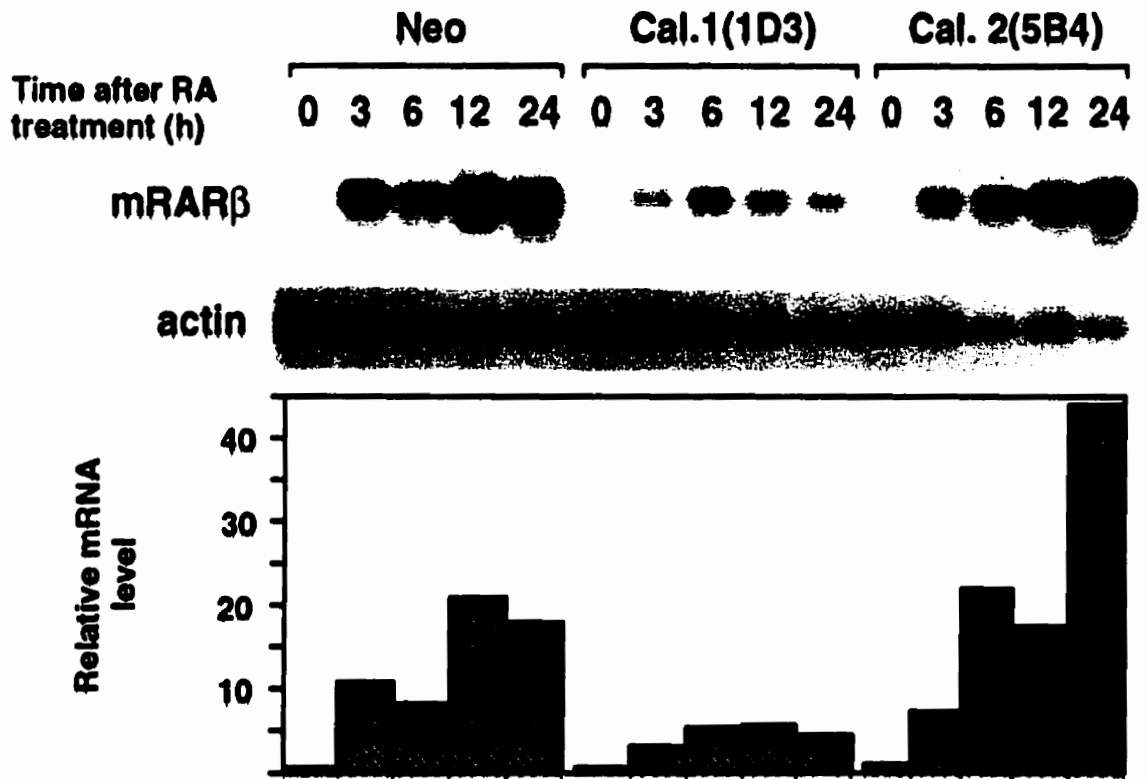
Fig. 5. Calreticulin dependent inhibition of RA induction. (A) Effect of increasing amounts of calreticulin on RAR/RXR heterodimer activation through the β RARE. P19 EC cells were transiently transfected with a TKLUC reporter with or without a single copy of the β RARE and increasing amounts of the calreticulin expression vector, pCMXCAL, as indicated. Cells were stimulated with atRA, 9cRA (both at $10^{-7}M$) or a combination of the two and cultured for a further 24 hours. Values for luciferase activity are normalized against β galactosidase activity. Control cells received no retinoid treatment. (B) Effect of increasing amounts of calreticulin on RXR homodimer activation through the CRBPII-RXRE. P19 EC cells were transfected with the indicated expression vectors and a TKLUC reporter with a single copy of the RXRE. Cells were treated with 9cRA and harvested after a further 24 hours. Luciferase values were determined as above.

A**B**

expression vector (Fig. 5B). These conditions are presumably optimal for the formation of RXR homodimers. Upon binding to RXREs, the heterodimer RAR/RXR often has a repressive effect on gene expression (Mangelsdorf et al., 1991), as seen when no exogenous receptor is added, or when both RAR and RXR expression vectors are transfected into the cells. Thus the presumptive RXR homodimer binding and activation seen in transient transfections can also be reduced to half maximal value with 5 μ g of added calreticulin expression vector (Fig. 5B). In contrast to the complete elimination of receptor/DNA interaction seen in EMSA analysis, increasing the amount of calreticulin in transient transfection assays does not completely abolish RA induced activation.

Overexpression of calreticulin decreases endogenous RA-responsive gene activation. In response to RA treatment, P19 EC cells will differentiate to a neuronal cell fate (McBurney et al., 1988). We have previously shown that overexpression or a decrease in expression of calreticulin in stably transfected P19 EC cells affects both the normal expression pattern of the RA regulated gene CRABP II (Fig. 6A) and the expression of a specific marker further downstream in the neuronal differentiation pathway (Dedhar et al., 1994). In a cell line stably overexpressing calreticulin (Cal-1), the expression of the CRABP II gene is less than half of the level observed in the parental cell line 24 hours after treatment, while in a cell line expressing antisense calreticulin (Cal-2), the amount of CRABP II transcript is approximately doubled at that same time point as compared to the parental P19 EC line (Fig. 6A). To examine how RA regulated gene expression was affected at earlier time points, the expression of the rapidly RA-induced RAR β 2 gene was examined by Northern blot analysis. The level of RAR β 2 transcript in the cell line overexpressing calreticulin is substantially decreased relative to a control cell line at 3 hours after atRA treatment (Fig. 6B) and does not increase significantly even after 6 hours of atRA treatment. In the calreticulin underexpressing Cal-2 cell line, the level of RAR β 2 transcript at 6 hours of atRA treatment is higher than that seen in control Neo cells. At the

Fig. 6. Northern blot of the (A) RA responsive CRABP_{II} or (B) early RA-responsive RAR β transcript in stably transfected P19 EC cell lines. Total RNA was isolated from control P19 EC cells (Neo), calreticulin overexpressing cells (Cal.1(1D3)), or calreticulin underexpressing cells (Cal.2(5B4)) at the indicated time points after RA treatment. Cal.1(1D3) cells express approximately five-fold greater levels of calreticulin than control cells, while Cal.2(5B4) cells contain half the amount of calreticulin relative to control cells (see Materials and Methods). A ³²P labeled mouse actin probe served as a control to check for equal loading of RNA in all lanes.

A**B**

later timepoint of 24 hours after RA treatment, the amount of RAR β 2 mRNA is more than double that of the control cell line.

Block of differentiation in P19 EC cells stably expressing calreticulin.

Results of the effect of calreticulin on RA-induced gene expression in transient transfection and stably transfected cell lines suggest that levels of calreticulin may play a crucial role in the ability of these cells to effectively differentiate in response to retinoids. A specific and early marker of neuronal differentiation, class III β tubulin, is downregulated in Cal-1 cells relative to control cells while its expression is increased in Cal-2 cell lines (Dedhar et al., 1994). Immunofluorescence studies support the proposal that levels of calreticulin present in the cell can play an important role in the ability of the cell to differentiate in response to atRA treatment. Prior to atRA treatment, while undergoing cellular proliferation, the various stably transfected cell lines, Neo (control), Cal-1 (overexpressing), and Cal-2 (underexpressing) appear similar and have a characteristic EC cell morphology (Fig. 7A, C and E). Six days after atRA treatment a number of the Neo control P19 EC cells have adopted a cell shape characteristic of neuronal differentiation (Fig. 7B). In contrast, Cal-1 cell lines maintain their EC cell morphology (Fig. 7D). A cell line expressing antisense calreticulin (Cal-2) differentiates at a more rapid rate than the Neo control cells, and all cells have a neuron-like appearance six days after atRA treatment (Fig. 7F).

Overexpression of calreticulin does not alter free intracellular calcium concentration. Calreticulin is a calcium binding protein, and therefore overexpression of this protein could result in changes in the intracellular calcium concentrations which in turn could contribute to the observed changes in gene expression and neuronal differentiation. However, the measurement of intracellular calcium concentration in the P-19 EC Neo as well as in the calreticulin overexpressing P19 EC Cal-1 clones demonstrated that the intracellular calcium concentrations in all cell lines were essentially equivalent, as shown in Table 2. Physiological activators typically increase intracellular calcium concentration by

300 to 400 nM (Nasmith and Grinstein, 1987). Therefore, the overexpression of calreticulin did not result in significant changes in intracellular Ca²⁺ concentration.

Fig. 7. Inhibition of neuronal differentiation of P19 EC cells by calreticulin. Cells were stained with anti-class III β tubulin antibody (TuJ1) followed by FITC conjugated secondary antibody as described in Material and Methods. A and B: P19 (neo) EC cells; C and D: P19 -Cal-1 EC cells; E and F: P19 Cal-2 EC cells. A, C and E; untreated cells. B, D and F: 6 days atRA (0.5 μ M) treated cells. The cells were visualized using a Zeiss Axioscop microscope under oil immersion and photographed with Kodak T-Max 400 film. Magnification 100X.



A



B

Neo



C



D

Cal-1



E



F

Cal-2

Retinoic Acid

TABLE 2Intracellular [Ca²⁺] in P19 cells

Cell line	Intracellular [Ca ²⁺] (nM)
Neo	115.9
Cal.1(1A2)	162.3
Cal.1(1D3)	137.2

Discussion

A large body of evidence supports and elucidates the existence of two distinct cellular RA response systems in which heterodimeric RXR/RAR regulates expression of downstream RARE-containing genes and RXR functions as a 9c RA-induced homodimer (for review, see Giguère, 1994). The present experiments demonstrate that calreticulin can function as a potent negative regulator of both atRA and 9cRA signaling pathways. Repression of RAR and RXR function appears to be achieved by a simple mechanism that involves direct calreticulin interaction with a conserved region in the DNA-binding domain of the receptors and consequent inhibition of their DNA binding properties. This extends the previous observations that calreticulin can also inhibit androgen, glucocorticoid, vitamin D₃ and peroxisome proliferator receptors DNA-binding activities (Burns et al., 1994; Dedhar et al., 1994; St-Arnaud et al., 1995; Wheeler et al., 1995; Winrow et al., 1995). Data presented in this study further suggests that calreticulin may have a differential or graded ability to interact with distinct members of the steroid/thyroid/retinoid receptor superfamily. Addition of increasing amounts of purified calreticulin inhibited binding of both heterodimeric RXR/RAR and homodimeric RXR complexes to their target RARE and RXRE, however, we observed a more rapid decline in DNA-binding activity of the RXR homodimer complex (Fig. 3). Thus, the data reported here suggest that the two retinoid response pathways may differ in their sensitivity to varying levels of calreticulin.

Studies have suggested that the complexing of ligand with the receptors results in conformational modification of receptors (Allan et al., 1992; Keidel et al., 1994). Changes in receptor conformation might result in an altered ability of the receptors to interact with modulator proteins such as calreticulin. However, ligand-induced conformational changes appear to center on the carboxyl terminus of the receptor (Allan et al., 1992; Keidel et al., 1994). DNA binding domain/DNA interaction, e.g. when the receptors are complexed with DNA, neither hinder or alter the conformational changes that take place upon addition of ligand (Keidel et al., 1994). We have shown that the differential ability of calreticulin to

inhibit DNA-binding of RXR/RAR and RXR/RXR complexes is not dependent on ligand/receptor interaction (Fig. 3) and may therefore be related to the difference in primary amino acid sequences between RAR and RXR in the conserved seven amino acid region that serves as the calreticulin anchoring domain (see Fig.1) or neighboring subdomains. Alternatively, differential utilization of dimerization determinants by RXR in homo- and heterodimeric complexes (Lee et al., 1993; Mangelsdorf and Evans, 1995; Zechel et al., 1994; Zechel et al., 1994) may play a role in regulating calreticulin accessibility to the six amino acid protein:protein interface present at the base of the first zinc finger of the retinoid receptors (Fig. 1).

Our *in vitro* data argue that RAR and RXR activities may be repressed by direct association with calreticulin. Firstly, calreticulin does not bind to retinoid receptor binding sites (Fig. 3A and B, lane 2) and significantly, it was possible to use RAR α -specific antibodies to immunoprecipitate cellular calreticulin (Fig. 4). These *in vitro* results do not rule out the possibility that additional accessory factors are required for stable interaction between calreticulin and retinoid receptors. However, chemical crosslinking reagents were not required for the immunoprecipitation of a RAR/calreticulin complex which suggests that this direct interaction is relatively strong.

P19 EC cells are a clonal line of mouse embryonal carcinoma cells which when treated with atRA are prompted to develop along a neuronal differentiation pathway (Jones-Villeneuve et al., 1982). The overexpression of calreticulin did not completely inhibit RA activation in the transient transfections but did result in a complete inhibition of differentiation in P19 EC cells toward the neuronal phenotype (Fig. 7). Likewise, the RA-induced endogenous class III β tubulin expression is delayed but not completely inhibited (Dedhar et al., 1994). The expression of other genes required for neurite outgrowth may therefore be specifically inhibited by calreticulin overexpression. In this regard the ECM receptors, integrins, play a significant role in the elaboration of neurites (Tomaselli et al., 1987), and the expression of α_v integrins is profoundly enhanced in RA treated P19 EC

cells (Dedhar et al., 1991). It will be interesting to determine whether calreticulin overexpression results in a complete inhibition of RA induced $\alpha_v\beta_1$ and $\alpha_v\beta_3$ expression, since the β_3 integrin promoter has recently been demonstrated to have RAREs and a vitamin D response element (VDRE) (Cao et al., 1993; Mimura et al., 1994). Inability of EC cells to differentiate upon atRA treatment has been observed when dominant negative forms of RAR or RXR are expressed in these cells (Espeseth et al., 1989; Minucci et al., 1994; Pratt et al., 1990). However, calreticulin is the first nonreceptor molecule that when inappropriately expressed in EC cells, has a similar ability to inhibit atRA-induced P19 EC cell neuronal differentiation. Taken together, these results suggest that calreticulin may provide a useful tool to dissect molecular events implicated in atRA-induced neuronal differentiation.

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Chapter 3

Isolation of a Novel Retinoic Acid-Responsive Gene by Selection of Genomic Fragments Derived from CpG-Island-Enriched DNA

This chapter is a modified version of the following manuscript: Isolation of a novel retinoic acid-responsive gene by selection of genomic fragments derived from CpG-island-enriched DNA. 1996. Shago, M. and Giguère, V. *Mol. Cell. Biol.* 16: 4337-4348.

One of the primary goals in transcription factor research is the elucidation of the genetic networks controlled by a factor or by members of a family of closely related factors. The pleiotropic effects of retinoic acid (RA) in the developing and adult animal are mediated by ligand-inducible transcription factors (RA receptors [RARs] and retinoid X receptors [RXRs]) that belong to the superfamily of nuclear receptors. Regulatory regions of RA effector genes contain RAR and RXR binding sites (RAR elements [RAREs] and RXR elements [RXREs]) that generally consist of direct or everted repeats of the core half-site motif (A/G)G(G/T)TCA. In order to identify novel genes regulated by RA, we devised a selection strategy based on the premise that regulatory regions of a large number of housekeeping and tissue-specific genes are embodied within CpG island DNA. In this method, referred to as CpG-selected and amplified binding, fragments derived from the CpG island fraction of the murine genome are selected by a gel mobility shift assay using in vitro transcribed and -translated RXR/RAR. Multiple rounds of selection coupled with amplification of the fragments by PCR enabled us to clone a population of CG-rich fragments of which approximately one-fifth contained consensus RAREs or RXREs. Twelve genomic fragments containing novel response elements are described, and the transcription unit associated with one of them, NN8-4AG, was characterized in detail. The mouse NN8-4AG transcript is upregulated by RA in F9 embryonal carcinoma cells and is homologous to an expressed sequence tag (EST41159) derived from a human infant brain cDNA library. Cloning of the murine NN8-4AG genomic sequence places the RXRE in the proximity of the transcription initiation sites of the gene. Although sequence analysis indicates that the EST41159 gene product is novel, a region of amino acid identity with sequences of a yeast polypeptide of, as yet, unknown function and the *Drosophila trithorax* protein suggest the presence of an evolutionarily and functionally conserved domain. Our study demonstrates that transcription factor binding sites and corresponding regulated genes can be identified by selecting fragments derived from the CpG island fraction of the genome.

INTRODUCTION

Vitamin A (retinol) and its derivatives are essential for embryonic development, growth, and reproduction of vertebrates, and for the regulation of the proliferation and differentiation of many cell types in the adult animal. (Sporn et al., 1994). The ability of the active retinol derivatives all-trans retinoic acid (atRA) and 9-*cis* RA (9cRA) to mediate these biological effects can be attributed to the regulation of specific target genes. Both atRA and 9cRA interact in the nucleus with ligand-inducible transcription factors that belong to the superfamily of steroid/thyroid/retinoid receptors. Two classes of retinoid receptors have been defined: the RA receptors (RAR α , β and γ) which bind both atRA and 9cRA, and the retinoid X receptors (RXR α , β and γ), which are capable of binding only 9cRA with high affinity (reviewed in Chambon, 1994; Giguère, 1994; Mangelsdorf et al., 1994). RARs and RXRs recognize short DNA elements that are present in the regulatory regions of responsive genes, termed retinoic acid response elements (RAREs) and retinoid X response elements (RXREs). These elements generally consist of direct repeats of the core consensus hexamer (A/G)G(T/G)TCA (for review see Giguère, 1994). Other sequences able to function as RAREs consist of inverted and everted repeats of the core hexamer motif as well as complex elements composed of multiple core motifs arranged in direct, inverted, or everted configurations with spacings of various lengths (Richard and Zingg, 1991; Tini et al., 1993; Umesono et al., 1988; Vasios et al., 1991).

Despite extensive knowledge of RA action at the molecular level, relatively few RA target genes have been identified (for reviews see Giguère, 1994; Gudas et al., 1994). In addition to the characterization of known genes previously shown to be regulated by RA, differential and/or subtractive hybridization strategies have been used to isolate cDNAs derived from differentially expressed mRNAs of unknown genes from untreated versus RA-treated cells (Bouillet et al., 1995; Gudas et al., 1994). The major drawbacks of this method are that only a fraction of the cloned genes will be direct targets of the retinoid

receptors and that a time- and effort-consuming step is required to identify the RAREs and RXREs associated with the transcriptional units encoding the regulated mRNAs. An alternative strategy would be to isolate RAREs and RXREs present in vertebrate genomes first and then to characterize the genes associated with these elements. However, the observation that retinoid receptor binding sites are relatively short and sometimes degenerate means that putative RAREs and RXREs are scattered throughout the genome, both in regions that play a role in transcription and in transcriptionally inert regions. In addition, to be biologically relevant, the search for retinoid receptor binding sites should concentrate not only on DNA fragments located in the regulatory region of genes but also on transcription units actively transcribed in the tissue or cells of interest. One approach directed towards the isolation of *in vivo* target genes is the immunoprecipitation of chromatin fragments with transcription factor-specific antibody (Gould et al., 1990). This approach has been used to isolate genomic targets of the thyroid hormone receptors (Bigler and Eisenman, 1994; Bigler and Eisenman, 1995).

In an effort to isolate RA target genes, we have developed a novel selection strategy that takes advantage of a unique and striking feature of the vertebrate genome: the presence of CpG islands. CpG islands are G+C rich regions that, unlike the majority of vertebrate genomic DNAs (bulk chromatin), do not show a reduced frequency of CpG dinucleotides and are not methylated at the C of these dinucleotides (Antequera and Bird, 1993; Bird, 1987). These regions are located in or near the promoter regions of genes and often include the first exon of the gene (Antequera and Bird, 1993; Bird, 1987). Moreover, Tazi and Bird (Tazi and Bird, 1990) have shown that nonnucleosomal (NN) DNA, a fraction of the genome associated with transcribed genes, is predominantly derived from the CpG island fraction of the genome. These NN fragments can be isolated away from bulk chromatin using restriction enzymes containing CpG within their recognition sequences (Tazi and Bird, 1990). Taken together, these observations suggest that CpG-island-derived NN fragments could provide an excellent source of genetic

material to scan for biologically relevant binding sites for retinoid receptors or any other transcription factors.

Using CpG-island-enriched DNA derived from nuclei of murine embryonal carcinoma (EC) cells as starting material, we utilized a combination of polymerase chain reaction (PCR) amplification and gel mobility shift assays to select NN fragments that contain RAREs and RXREs. By analyzing the genomic DNA flanking each RARE, associated transcribed regions can be identified. As this isolation method is a modification of selected and amplified binding (SAAB) (Blackwell and Weintraub, 1990), we have termed this method CpG-SAAB. In this chapter I describe CpG-SAAB, the functional characterization of twelve genomic fragments containing RAREs and RXREs, and the identification of a novel RA responsive gene associated with one of the RXRE-containing fragments. Our study demonstrates that transcription factor binding sites and corresponding regulated genes can be identified by selecting from fragments derived from the CpG island fraction of the genome.

MATERIALS AND METHODS

Nuclei isolation and chromatin digestion. Nuclei were prepared as described by Dony et al. (Dony et al., 1985) and Tazi and Bird (Tazi and Bird, 1990), with some modifications. Five 100-mm-diameter confluent plates of F9 EC cells were trypsinized, pelleted, and washed with cold phosphate-buffered saline. To gently lyse the cells, 7.5 ml of cold 0.025% Nonidet P-40 (NP40) lysis buffer was added (10 mM triethanolamine, 1.5 mM MgCl₂, 10 mM KCl, 1 mM dithiothreitol (DTT), and 0.025% NP40) and pipetted up and down 15 times. After centrifugation at 200 x g for 4 min, the pellet was resuspended in 0.025% NP40 lysis buffer, layered on a 0.8M sucrose cushion dissolved in lysis buffer then pelleted again at 200 x g for 8 min. Nuclei were resuspended gently in 500 µl of storage buffer containing 75 mM KCl, 0.25 mM MgCl₂, 1 mM EDTA, 0.5 mM (ethylene glycol-bis(β-aminoethyl ether)-N, N, N' N' -tetraacetic acid (EGTA), 0.15 mM spermine, 0.5 mM spermidine, 20 mM N-2-hydroxyethylpiperazine-N' 2-ethanesulfonic acid (HEPES) (pH7.9), 10 mM-βmercaptoethanol, 0.23 M sucrose, and 20% glycerol. An average of 4 x 10⁷ nuclei were obtained from five plates of F9 cells. Nuclei were stored at -70°C. As described by Tazi and Bird (Tazi and Bird, 1990), 500 µl aliquots of nuclei were thawed, pelleted for 10 seconds, and gently resuspended in 1X digestion buffer (50 mM Tris-HCl (pH 8.0), 10 mM MgCl₂) and 400 U (each) of *Msp*I and *Hin*PI in a total volume of 1 ml for 30 min. Digestion was followed by proteinase K treatment. Subsequent to phenol-chloroform extraction and ethanol precipitation, DNA was dissolved in 400 µl Tris-EDTA (TE).

Isolation and PCR amplification of NN fragments. Fragments for PCR amplification and gel mobility shift selection were prepared by running 10 to 30 µl of the chromatin digest on a 1.5% low melt agarose gel (Seakem). Fragments migrating to between 75 and 140 bp were endfilled with the Klenow fragment of DNA polymerase and the nucleotides dCTP and dGTP, phenol-chloroform extracted, ethanol precipitated and resuspended in 10 µl TE. The fragments were then ligated overnight at 16°C to 'catch

linkers' (catch A [5'-GAGTAGAATTCTAATATCTC-3'] and its complement, catch B) by following a procedure outlined by Kinzler and Vogelstein (Kinzler and Vogelstein, 1989). Overdigestion with *Xho*I in a total volume of 500 μ l cuts between annealed linkers and results in NN fragments with a single catch linker on either end. The digest was passed through a Qiagen tip 5 column and ethanol precipitated, and fragments of the appropriate size were reisolated from a 1.5% low melt agarose gel. The resulting NN fragments, which were quantitated by ethidium bromide agarose plate quantitation, were resuspended in 10 μ l TE, typically resulting in a concentration of 2 to 5 ng per μ l. To amplify and radiolabel the NN fragments for gel mobility shift selection, the catch A oligonucleotide was endlabeled with [γ -³²P]ATP and polynucleotide kinase (New England Biolabs). PCR reaction mixtures consisted of 15 ng NN/catch linked fragments, 250 ng (each) of labeled catch A and unlabeled catch B oligonucleotides, PCR buffer, MgCl₂ and *Taq* polymerase (according to Perkin Elmer Cetus instructions). Fifteen cycles of denaturation for 1 min 94°C, annealing for 2 min at 50°C, and extension for 2 min at 72°C, with a 4 min denaturation at 94°C prior to the first cycle, were performed. Fragments with the appropriate sizes (115-190 bp) were then isolated on a 1.5% low melt agarose gel, resuspended in 10 μ l water, and stored at -20°C.

Gel mobility shift assays. Gel mobility shift assays were performed using *in vitro*-transcribed and -translated RAR and RXR. The proteins were synthesized in rabbit reticulocyte lysate (RRL) by using the TNT-T7 kit (Promega, Madison, Wis.). Typically, 2 μ l of each RAR- and RXR-programmed RRL was combined with binding buffer (5 mM Tris-HCl [pH 8.0], 40 mM KCl, 6% glycerol, 1 mM DTT and 0.05% NP40), 2 μ g of poly(dI-dC) (Pharmacia), 0.1 μ g denatured salmon sperm DNA and 10 μ g of bovine serum albumin in a total volume of 20 μ l, and the mixture was put on ice for 10 min. One microliter of labeled NN fragments was added, and the reaction mixture was incubated for an additional ten min at room temperature. The reaction mixture was loaded on a 3.5 to 4% nondenaturing polyacrylamide gel and run at 150 V for 2 h. The gel was dried and

exposed with an intensifying screen a few hours to overnight. Control binding reactions with a known RARE (β RARE: 5'-AGCTTGAAGGGTTCACCGAAAGTTC-ACTCGCA-3' & 5'-AGCTTGCGAGTGAACCTTCGGTGAACCCTTCA-3') were run alongside to provide a marker for protein/DNA complex migration. A section of the dried gel corresponding to the migration point of bound NN fragments was cut out, minced with a razor blade, and eluted with shaking overnight at 37°C in 600 μ l of elution buffer (0.5 M ammonium acetate, 1 mM EDTA). After phenol-chloroform extraction, the selected NN fragments were precipitated with isopropanol and glycogen and resuspended in 10 μ l of water. Half of the isolated NN fragments were used in a PCR amplification as described above. Selection of NN fragments that bound to RXR/RAR consisted of eight rounds of PCR amplification followed by gel mobility shift assay isolation.

Cloning and sequencing of NN fragments. After the final PCR amplification, NN fragments were digested with *EcoRI* (which cuts within the catch linker) and ligated into pBluescriptKS (pKS; Stratagene) cut with *EcoRI* and treated with calf intestinal phosphatase (Boehringer Mannheim). Dideoxy sequencing reactions were performed with T7 DNA polymerase (Pharmacia) and the T3 and T7 primers. GC compressions were resolved with 7-deaza deoxynucleotides (Pharmacia) or by running sequencing reactions on a 40% formamide gel. Fragments were scanned for the presence of core consensus half-sites [(A/G)G(T/G)TCA] arranged in configurations (such as DR1, DR2, DR5) known to bind RXR/RAR heterodimers. Several fragments that shared weak homology with consensus RAREs or RXREs were included in the functional assays. Among them were the following sequences: NN7-22 (TGACTTTGCTGCGTGTCA), NN7-49 (CCGTCACGTGACGC), and NN7-76 (AGATCAGCAGCAGCACA). None of these non-consensus binding sites revealed specific binding or RA-induced transactivation in gel mobility shift and transient transfection assays respectively.

Genomic Southern blots and Zoo blots. Genomic DNA was isolated from R1 embryonic stem cells, human HOS cells or chicken liver and digested with *BamHI*,

EcoRI, *HindIII*, or *SacI*. Ten microgram samples were electrophoresed in 1.0% agarose gels, transferred to nylon membrane, and hybridized overnight at 42°C with the appropriate random primed NN fragment probe in a solution of 50% formamide, 1X Denhardt's solution, 5X SSPE (1X SSPE is 150 mM NaH₂PO₄ and 1 mM EDTA), 0.1% sodium dodecyl sulfate (SDS), 100 µg of denatured salmon sperm DNA per ml, and 10⁶ cpm of probe per ml. Genomic Southern blots were washed twice with 0.2X SSC (1X SSC is 150 mM NaCl plus 15 mM sodium citrate) and 0.1% SDS for 20 minutes at 60°C. Zoo blots were washed twice for 15 minutes at 55°C with 0.2X SSC and 0.1% SDS.

Recombinant plasmids. Plasmid TKLUC contains sequences -105 to + 51 of the thymidine kinase (TK) promoter linked to the firefly luciferase gene. TKLUC plasmids containing NN fragments were constructed by releasing the NN fragments from pKS with *SalI* and *BamHI*, and cloning them into the corresponding sites in TKLUC. To create NNβRARE-TKLUC, a 140 bp *MspI/HinPI* fragment from the promoter region of the murine RARβ2 gene containing the βRARE was isolated, cloned directly into pKS (Stratagene) linearized with *AccI*, and subsequently inserted into TKLUC as a *SalI/BamHI* fragment.

Transient transfection assays. P19 EC cells were cultured in α-minimal essential medium containing 7.5% fetal calf serum. Cells were transfected with 1.5 µg of RSV-β-gal and 2 µg of reporter plasmid. β-Galactosidase and luciferase assays were performed as described previously (Giguère et al., 1990).

Genomic Library Screening. A genomic clone corresponding to the promoter region of the mouse RARβ2 gene was isolated from a murine EMBL-3 SP6/T7 genomic library using a probe derived from the 5' end of the mouse RARβ2 cDNA. Clones λ7-91-2 and λ8-4-5 were isolated from a murine λ DASHII library of strain 129SV genomic DNA using a ³²P-labeled probe generated from NN fragments 7-91 and 8-4. Hybridization of duplicate nylon membranes was performed at 42°C overnight in a solution of 50% formamide, 1X Denhardt's solution, 5X SSPE, 0.1% SDS, 100 µg

denatured salmon sperm DNA per ml, and 10^6 cpm of probe per ml. Membranes were washed twice with 0.2X SSC and 0.1% SDS for 20 min at 60°C. Genomic phage clones were analyzed in detail by restriction mapping and hybridization with NN7-91 or NN8-4 and cDNA derived fragments.

Sequence determination and analysis. Homology of NN8-4 and an expressed sequence tag (EST) from the EST database (dbEST) was discovered using the BLAST search program of the National Center for Biotechnology Information (NCBI) (Altschul et al., 1990). The clone EST41159 (GenBankID R56376) was obtained through the IMAGE Consortium, Lawrence Livermore National Laboratory. EST41159 was subcloned into pKS, and both this cDNA and genomic subclones were sequenced using T7 DNA polymerase (Pharmacia). T3 and T7 primers, as well as specific primers derived from the cDNA or genomic sequences, were used to sequence both strands of the cDNA and genomic subclones. Similarity searches for putative amino acid sequences were performed with the BLASTX program (Altschul et al., 1990).

Primer Extension analysis. Fifty micrograms of total RNA isolated from F9 cells grown in the presence or absence of RA ($10^{-7}M$) for 24 hours or 10 μ g of *Escherichia coli* tRNA was hybridized to 5'- ^{32}P -end-labeled oligonucleotide 890 (5'-GGGTGGCTCGCGTCCT-CTGC-3') and extended using avian myeloblastosis virus reverse transcriptase.

S1 nuclease assay. Fifty micrograms of total RNA isolated from F9 cells grown in the presence or absence of RA ($10^{-7}M$) or 10 μ g of *E. coli* tRNA was used for S1 nuclease protection. The RNA was hybridized to a probe generated by hybridizing radiolabeled oligonucleotide 890 to a single-stranded PstI-SacI 1100bp fragment from the 5' end of the RAR β 2 gene and extending with Klenow fragment and dNTPs.

Northern (RNA) analysis. F9 EC cells were grown on 0.1% gelatin in Dulbecco modified Eagle medium supplemented with 15% fetal bovine serum which had been stripped by using charcoal/anion exchange and treated with UV light for 1 h for retinoid

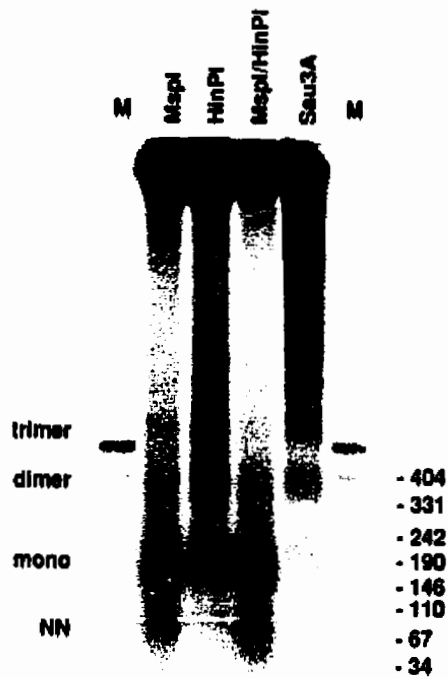
depletion. The cells were treated with vehicle alone, retinoids at a concentration of 1 μ M each, and/or cycloheximide (Cx) (final concentration, 10 μ g/ml) for various time periods. Total RNA was extracted with TRIZOL (Gibco BRL). RNA samples were electrophoresed through a 1% formaldehyde-1X morpholinepropanesulfonic acid (MOPS)-1% agarose gel, and transferred to nylon membrane in 20X SSC. RNA was UV-crosslinked to the membrane and hybridized to the appropriate random-primed probe in a solution containing 50% formamide, 5X Denhardt's solution, 5X SSPE, 0.1% SDS, and 100 μ g denatured salmon sperm DNA per ml. The membranes were washed once for 20 min at room temperature with 2X SSC-0.1% SDS and twice at 65°C in 0.2 X SSC, 0.1% SDS for 15 minutes each time. Autoradiography was carried out at -70°C with an intensifying screen. An oligonucleotide hybridizing to the 18S ribosomal RNA 5'-ACGGTATCTGATCGTCTTCGAACC-3' was used as a control probe for loading.

RACE analysis. The rapid amplification of cDNA ends (RACE) system was used according to the instructions provided by the manufacturer (Gibco BRL). The primers used as GSP1, GSP2 and GSP3 were 5'-CAGCCTCTGGGCATTCTCCTC-TGCCTTCTC-3' in exon 3, 5'-CTTCCGAATCAGCTGTGTGGCAAACAGTCCC-3' in exon 2 and 5'-CGCCACGCCACGCAGAAGGAGAACAC-3' in exon 1. First strand synthesis was performed at 50°C.

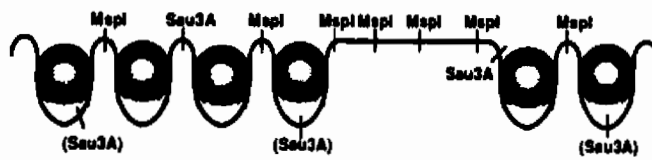
RESULTS

Isolation of NN fragments containing RAREs by CpG-SAAB. Murine EC cell lines, such as F9 and P19, constitute excellent models to study RA action (Jones-Villeneuve et al., 1982; Strickland and Mahdavi, 1978). In addition, unlike many transformed cell lines, EC cells maintain their methylation status in culture (Antequera et al., 1990). F9 EC cells were chosen as starting material for the development of the CpG-SAAB method. We first tested whether we could release CpG island DNA from F9 cell nuclei. Restriction enzymes which include a CpG in their recognition sites will preferentially digest within CpG island DNA. It has been demonstrated that chromatin digested by such CG recognizing enzymes as *MspI* and *HinPI* will release oligonucleosomes predominantly derived from the CpG island fraction of the genome (Antequera et al., 1989), as well as a population of fragments smaller than the size of DNA associated with a single nucleosome (Tazi and Bird, 1990). *MspI* and *HinPI* (the recognition sites are CCGG and GCGC, respectively) have access to CpG island chromatin not only because of the open structure of the chromatin (relative to bulk chromatin) and the characteristic GC-rich composition of this chromatin but also because of the nonmethylated status of CpG islands. Since *HinPI* is a methylation sensitive enzyme, it will not digest within methylated bulk chromatin. While *MspI* can cut methylated DNA, there appears to be a strong bias against such digestion in bulk chromatin, possibly due to protection of methylated DNA by specifically bound factors (Antequera et al., 1989; Boyes and Bird, 1991; Lewis et al., 1992). F9 cell nuclei subjected to digestion with a non-CpG-recognizing restriction enzyme such as *Sau3A* (recognition site GATC) releases a series of fragments which, after being radioactively end-labeled and electrophoresed on an agarose gel, can be seen to correspond to the DNA fragments associated with a single nucleosome, a dinucleosome, and a trinucleosome, with this pattern continuing into high-molecular-weight DNA (Fig. 1, upper panel). In contrast, digestion with *MspI* and/or *HinPI* yields DNA fragments corresponding in size

Fig. 1. Non-nucleosomal (NN) fragments are released from CpG island chromatin upon digestion with CpG recognizing enzymes. Upper panel. Intact nuclei from F9 EC cells were subjected to digestion with the restriction enzymes *MspI* (CCGG), *HinPI* (GCGC), *Sau3A* (GATC), or a combination of both *MspI* and *HinPI*. Subsequent to isolation, DNA was endlabeled and electrophoresed on a 1.5% agarose gel. An autoradiograph of the gel reveals differences in the digestion patterns caused by CpG recognizing enzymes (*MspI* and *HinPI*) and a non-CpG recognizing enzyme. Fragments corresponding to the DNA associated with a single nucleosome, dinucleosome and trinucleosome with this pattern continuing to high molecular weight DNA are seen in the *Sau3A* lane. Only fragments corresponding to smaller sized nucleosomes are visible in the *MspI* and *HinPI* lanes. A heterogeneous set of DNA fragments smaller than the size of a single nucleosome is also present in the CpG recognizing enzyme lanes. These fragments are (as illustrated in the lower panel) thought to be derived from the nucleosome-free regions within the CpG islands of activated or potentially activatable genes. The outer lanes (M) consist of endlabeled fragments of pUC19 cut with *HpaII*. The numbers represent the sizes of the marker bands in bp. Lower panel. CpG island chromatin is GC rich and contains an expected number of CpG dinucleotides, which are generally unmethylated. This type of chromatin contains an abundance of sites for restriction enzymes containing CpG in their recognition sites (such as *MspI*). Bulk chromatin has a low GC content, is deficient in CpG dinucleotides with those remaining being methylated. Very few CpG-containing recognition sites are present in bulk chromatin. Bracketed restriction enzyme sites in both types of chromatin indicate sites which are inaccessible due to blockage by nucleosomes. For details and data on the original experiments see Tazi and Bird (Tazi and Bird, 1990).



CpG island chromatin



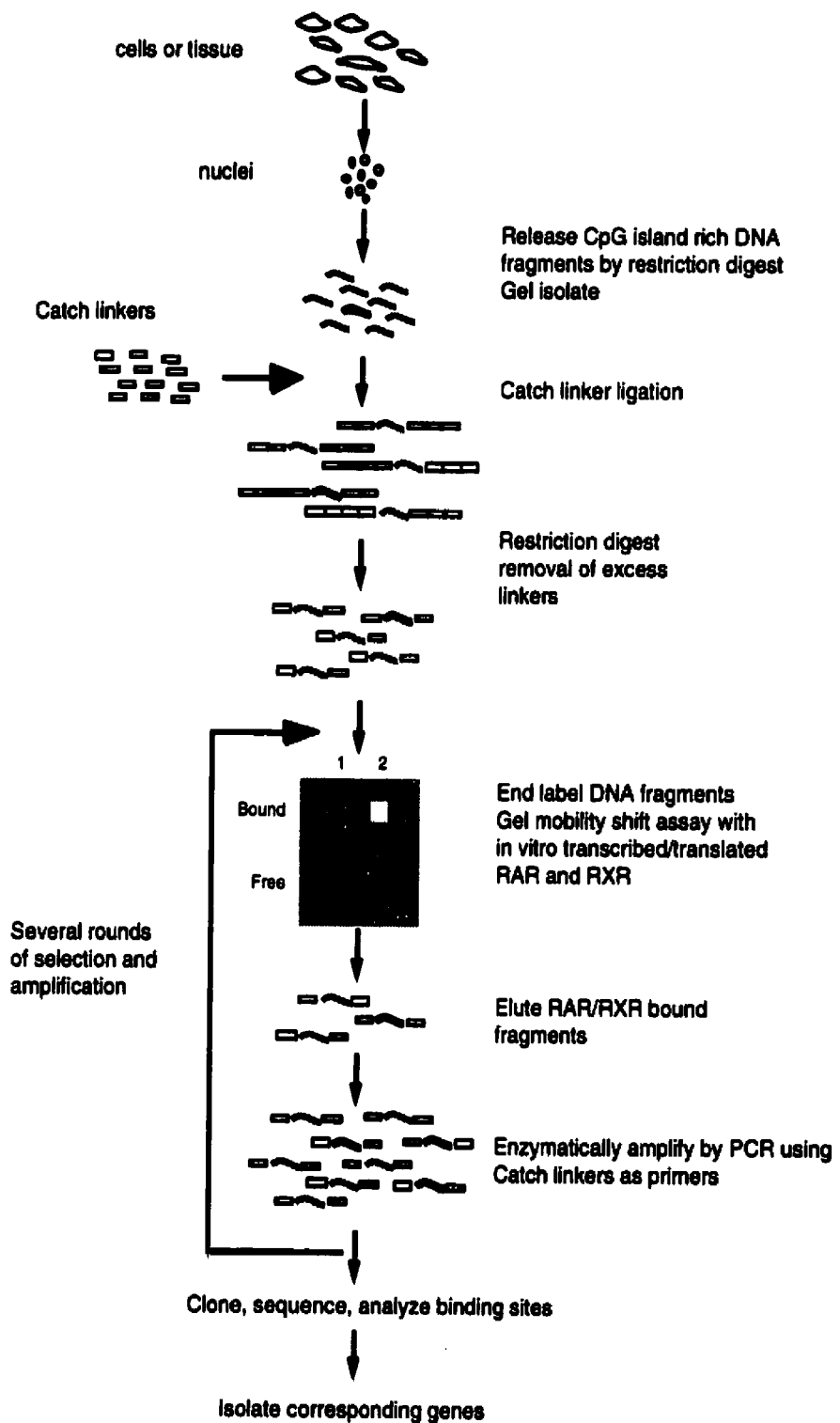
Bulk chromatin



only to one to four nucleosomes and a correspondingly greater amount of high-molecular-weight DNA (Fig. 1, upper panel). As predicted (Tazi and Bird, 1990), a heterogeneous population of fragments that are smaller than the size of a single nucleosome is released upon *MspI* and/or *HinPI* digestion. These fragments, termed NN fragments, are GC rich, are derived in large part from extended stretches of nucleosome-free DNA that are characteristic of transcribed (or potentially transcribable) genes, and are found predominantly within CpG islands (see schematic diagram in Fig. 1, lower panel) (Tazi and Bird, 1990). Thus, the CpG-island-derived NN fragments constitute an ideal fraction of the genome wherein to search for transcription factor binding sites associated with transcribed genes, in our case, RAREs and RXREs.

The strategy to isolate RARE-containing NN fragments is schematically represented in Fig. 2. Intact nuclei are first isolated, and the chromatin subjected to digestion with *MspI* and *HinPI*. The DNA is then electrophoresed on a 1.5% agarose gel and NN fragments migrating between 75 to 140 bp are extracted from it. The upper size limit was chosen to allow separation of bound fragments from unbound fragments in the gel mobility shift assay selections. NN fragments are then end-filled with the Klenow fragment of DNA polymerase I, and ligated to blunt catch linkers (Kinzler and Vogelstein, 1989). Overdigestion of the product of the ligation with *XhoI*, which cuts between ligated Catch linkers, creates a population of fragments which have a single linker on either end, facilitating PCR amplification. As was described in Materials and Methods, PCR was performed with radiolabeled Catch linkers. Binding reactions for gel mobility shift selections consisted of modified and radiolabeled NN fragments being incubated with *in vitro* -transcribed and -translated RXR/RAR. As is diagrammed in Fig. 2, parallel reactions with a known RARE are run alongside the NN fragment binding reaction as a marker for the bound fraction of fragments. A section of dried gel corresponding to the final position of the migration of bound NN fragments is cut out and the DNA is eluted. Multiple rounds of amplification by PCR (with end-labeled catch

Fig. 2. CpG-SAAB for the selection of CpG-rich genomic fragments containing RAREs. Nuclei are purified from cells or tissue samples of interest. The CpG island-rich NN fragments are released by restriction enzyme digests and gel isolated. Dark colored fragments represent those NN fragments containing a RARE or RXRE. Catch linkers are ligated to the collection of NN fragments and excess linkers are removed with a *XhoI* digest. NN fragments are then selected in a gel mobility shift assay using RAR/RXR complexes produced with an in vitro transcription-translation system. Probes represented in the schematic of the gel mobility shift assay are β RARE as a control in lane 1, and NN fragments in lane 2. NN fragments bound to the heterodimer are eluted from the gel (the cut out gel slice is represented by the white rectangle) and enzymatically amplified using the Catch linker oligonucleotides as primers. After several rounds of selection, DNA fragments are cloned into pKS and sequenced to search for the presence of a consensus RARE or RXRE. Putative RARE/RXRE-containing fragments are assessed for specific binding to RXR/RAR complexes and ability to drive reporter gene expression in transient transfection assays. Fragments showing a positive response in these assays are then used as probes to isolate associated genes.



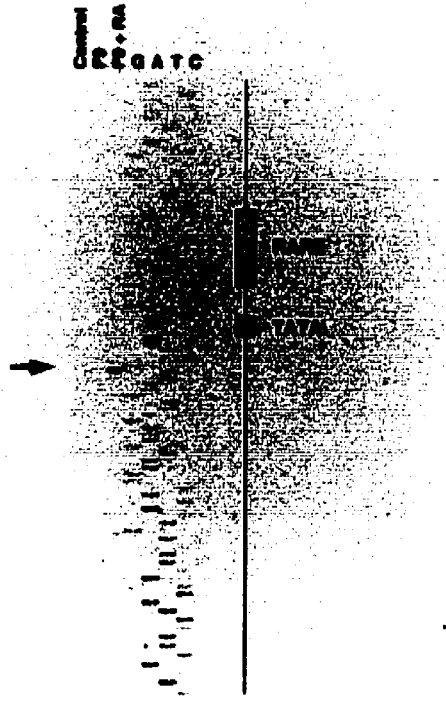
oligonucleotide) and gel mobility shift selection are then performed to enrich for NN fragments containing RAREs. By CpG-SAAB, a population of 120 RXR/RAR selected NN fragments obtained from F9 EC cell nuclei was cloned into pKS for further analysis.

Isolation of the RAR β 2 genomic clone and preliminary testing of CpG-SAAB.

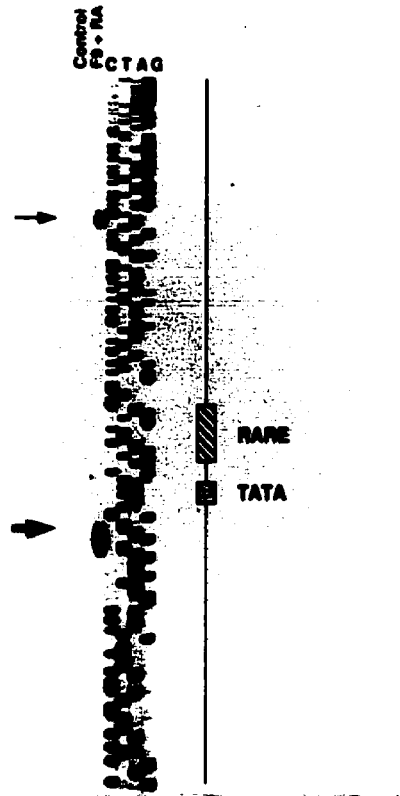
As a control for the preliminary steps of CpG-SAAB, a genomic clone corresponding to the promoter region of the RAR β 2 gene was isolated from a murine EMBL-3 SP6/T7 genomic library by using a probe derived from the 5' end of the mouse RAR β 2 cDNA. The RAR β 2 gene is highly responsive to RA and contains a DR-5 RARE (β RARE) in its promoter (de Thé et al., 1990; Sucov et al., 1990). Approximately 2500 bp surrounding the β RARE were sequenced (Fig.3A). The transcriptional start site of the murine RAR β 2 transcript was mapped using primer extension and S1 nuclease analysis (Fig. 3B & C). Surprisingly, the main CAP site is 21bp upstream of the start site mapped in the human RAR β 2 gene (de Thé et al., 1990), in spite of the high level of conservation (>90%) between the mouse and human RAR β 2 promoter sequences (our unpublished observations). An additional minor start site was identified at position -112 bp by S1 analysis (Fig 3A & C). The pattern of restriction enzyme recognition sites containing CpG dinucleotides within this region suggests that the β RARE is within a CpG island that spans approximately 1 kb (Fig. 4A). Flanking the β RARE are a *MspI* and a *HinPI* site which would enable the release of a 140bp NN fragment containing the response element. Primers internal to the *MspI* and *HinPI* sites were designed and used as PCR primers to monitor the presence of the β RARE NN fragment through the preliminary steps of CpG-SAAB. With RAR β cDNA, F9 genomic DNA, NN fragments, or fragments eluted from the gel after the first round of selection being used as template for PCR, a 140 bp fragment was visualized on an ethidium bromide stained acrylamide gel (Fig. 4B). No band was seen in the control lane without the template. The identity of this fragment was confirmed by Southern blotting with an internal probe (data not shown). These preliminary experiments established that the RARE of a known RA-responsive gene is

Fig. 3. Analysis of the promoter region of the RAR β 2 gene. (A) Nucleotide sequence of the mouse RAR β 2 5' flanking region. The major start site of transcription is indicated by an arrow; a smaller arrow denotes a possible minor upstream start site. Nucleotide sequences are numbered relative to the major CAP site. The start codon and the donor site of the first intron are underlined, and the RARE is boxed. The MspI and HinPI sites flanking the RARE are indicated. (B) Primer extension analysis. Radiolabeled primer 890 was hybridized to RNA samples and extended with AMV reverse transcriptase. RNA samples are derived from *E. coli* tRNA (control), untreated F9 cells (F9), and F9 cells treated for 24 hours with RA (F9 + RA). (C) S1 nuclease analysis. A PstI-SacI-end-labeled probe was hybridized to *E. coli* tRNA (control) or RA treated F9 cells (F9 + RA). These hybrids were digested with S1 nuclease and electrophoresed through a 6% polyacrylamide gel. No bands were seen when the probe was hybridized to untreated F9 cell RNA (data not shown). For both (B) and (C) the sequencing ladder was produced using oligonucleotide 890 as a primer. The schematic on the right indicates the position of the RARE and the putative TATA box identified in the human RAR β 2 promoter (de Thé et al, 1990; Sucov et al, 1990).

B



C



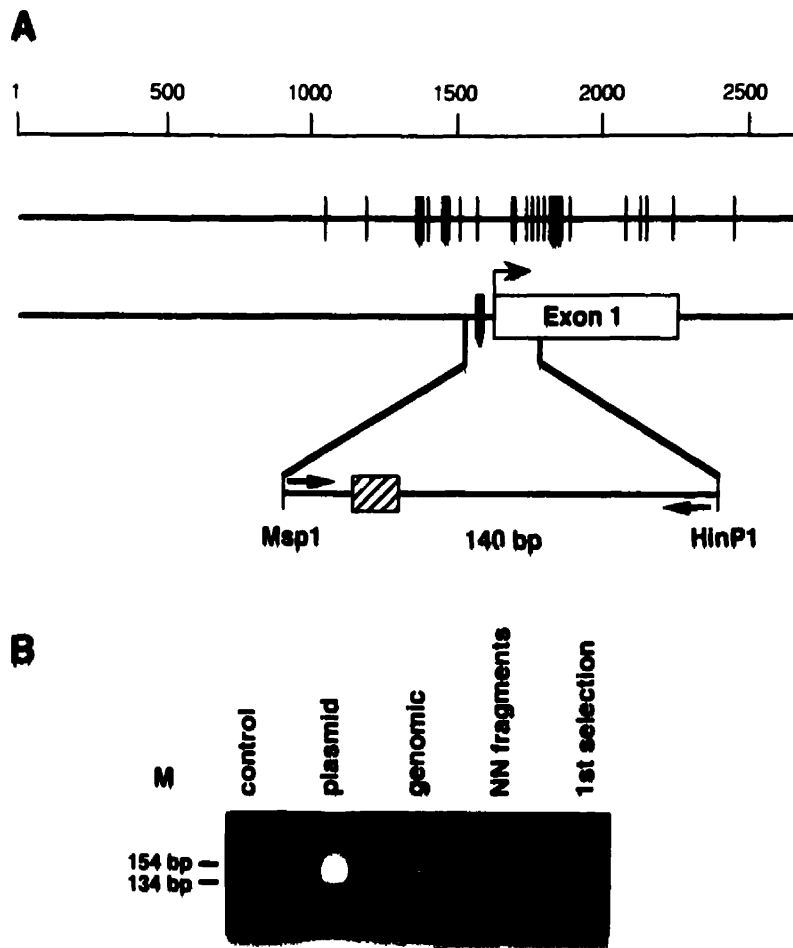


Fig. 4. A β RARE-containing fragment from the promoter of the RAR β 2 gene is within a putative CpG island, is present in the collection of NN fragments derived from F9 EC cells, and is bound by RXR/RAR in the first round of CpG-SAAB. (A) A schematic of the promoter and first exon region of the RAR β gene. The β RARE is represented by a black box, and the start of transcription by an arrow. On the line above, vertical bars represent restriction enzyme recognition sites which contain the nucleotide CpG. Their distribution pattern suggests that the β RARE is contained within a CpG island which spans approximately 1kb. Enlarged is a 140bp MspI/HinPI putative NN fragment of the RAR β transcription unit containing the β RARE. PCR primers internal to the MspI and HinPI sites were designed. (B) The PCR primers outlined in (A) were used to monitor the presence of the β RARE NN fragment through the preliminary steps of CpG-SAAB. Templates used are RAR β cDNA (plasmid), F9 EC genomic DNA (genomic), NN fragments isolated from F9 cell nuclei (NN fragments), or NN fragments eluted after the first round of selection (1st selection).

contained within a CpG island, that the element can be released as a 140 bp NN fragment upon *MspI/HinPI* digestion of intact F9 nuclei, and that this NN fragment is bound and selected by *in vitro*-transcribed and -translated RXR/RAR in the first gel mobility shift selection of CpG-SAAB.

Physical analysis of RXR/RAR selected NN fragments. The RXR/RAR selected NN fragments obtained from F9 EC cell nuclei were sequenced and scanned for the presence of consensus RAREs and RXREs. As discussed above, RARs and RXRs are members of a subfamily of the nuclear receptor superfamily that recognize direct, inverted, or everted repeats of the core consensus hexamer [(A/G)G(T/G)TCA] present in the regulatory regions of responsive genes. In general, elements containing direct repeats spaced by 1 nucleotide (DR1) function as RXREs, while DR2 and DR5 elements function as RAREs. We first performed a comparison of NN fragments prior to any gel mobility shift selection and NN fragments post-selection. The results of this comparison are outlined in Table 1. No RARE and RXRE consensus motifs of any class were present in the thirty preselection NN fragments sequenced. Among the selected NN fragment RA response elements identified were five DR1s, one DR2, seven DR5s, and 13 complex elements containing combinations of direct and everted repeats. Of the 120 selected NN fragments sequenced, approximately one in five harbored a consensus RA response element. Since some of the NN-RA response element-containing fragments were isolated more than once, in total, twelve unique RA response elements were identified. The average sizes of the genomic fragments were 91 and 98 bp for pre- and post- selection NN fragments respectively. Non CpG island bulk DNA has a G+C content of approximately 40% (Antequera and Bird, 1993). As CpG islands typically have a base composition in excess of 50% G+C (Cross et al., 1994), averages of 61% for the pre-selection NN fragments and 59.5% for the selected NN fragments indicate that the majority of the fragments are probably derived from CpG island regions of the genome.

TABLE 1. Characteristics of pre- and post-selection NN fragments

	Number	Size ^a	GC content ^b	Class of RA response elements ^c			
				DR1	DR2	DR5	complex
pre-selection	30	91	61	0	0	0	0
selected	120	98	59.5	5	1	7	13

^a Average size of the NN-fragments in bp.

^b Expressed as %.

^cDR1, DR2 and DR5 indicates NN fragments containing a single direct repeat of the core half-site motif spaced by 1, 2 or 5 nucleotides; complex refers to NN fragments containing at least one DR element plus one or more additional core half-site motifs arranged in various configurations.

To ascertain whether in general, the RA response element-containing NN fragments were derived from single-copy DNA, four of them were used to probe Southern blots of total mouse genomic DNA digested with various restriction enzymes. Three of these yielded the banding pattern expected of a single-copy probe (see example in Fig. 5), while one resulted in multiple bands of equal intensity (3 to 4 bands per digest, data not shown). These results suggest that the NN fragments examined are derived from repeat-free genomic DNA, an observation that correlates well with previous studies indicating that the frequency of repeated sequence within CpG island regions is much lower than that expected for total genomic DNA (Bird, 1986; Cross et al., 1994).

Functional analysis of RXR/RAR selected NN fragments. Although the consensus sequences for RAREs and RXREs have been well established, it is important to ascertain whether the RA response elements associated with the NN fragments fulfill certain functional requirements, namely, the abilities to specifically bind RXR/RAR complexes and to confer RA responsiveness to a heterologous minimal promoter in a transient transfection system. The RARE-containing portions of the NN fragments are outlined in Table 2, along with the class of the binding site. A 140 bp *Msp1/HinP1* genomic fragment located within a putative CpG island and containing the RARE from the promoter of the RAR β 2 gene served in the functional assays as a control (see Fig. 4A). The relative binding affinities of the fragments are shown in Table 2. A representative gel mobility shift assay is shown in Fig. 6A. All of the NN fragments except for NN7-29, which contains a DR1 with one half-site of suboptimal consensus sequence, bind RXR/RAR complexes at least as well as the genomic fragment containing the β RARE. NN fragments containing complex elements tend to bind with higher affinity to the retinoid receptors. Each of these fragments contain a perfect consensus direct repeat RARE or RXRE plus additional half sites in various configurations which presumably cooperate to increase binding affinity.



Fig. 5. NN7-71 hybridizes to a single genomic locus. 10 μ g samples of murine R1 genomic DNA were digested with the indicated restriction enzymes, electrophoresed on a 1% agarose gel, and transferred to nylon membrane. The membrane was hybridized to ³²P-labeled NN fragment 7-71.

TABLE 2. Functional analysis of non-nucleosomal retinoic acids response elements

Name	Sequence ^a	Class ^b	Binding ^c	Fold ^d Induction	Total Activity
NNB1ARE	GGGAAGGTTCA [→] CGGAAAGTTCAC [→] TCGCA	DR5	++	59	100
NN7-29	GGGTAGAGGTCAC [→] GGTGCAGTCCCG	DR1	+	4	25
NN7-51	←TGACCTTGTGAAATG(N15)TGAGCTAGTGGGTCACGGAAAGGTCA	DR2, ER4, DR5	+++	55	111
NN7-71	AGGTC [→] AAAGTTC [→] AGGTC [→] ATCC [→] AAAGGTC [→] AAAGTTA	DR2, DR0, DR5	+++	113	268
NN7-87	GGAGGTC [→] AAACTCAGGTC [→] A(N31)TGAGCC(N18)GGATCATT	DR5, 2HS	++	9	36
NN7-91	GGTTCGAGGTC [→] AGAAAGGGGTCATG	DR5	+++	49	211
NNB-3	GGAC [→] AGGGTCA [→] AGAGCAGGTC [→] ACTGTAG	DR5	+++	13	14
NNB-4	CGGACGGGTC [→] AGGGTCA [→] AGAAAGCC	DR1	++	5	75
NNB-29	GGTCA [→] CACACC [→] TGAGGTC [→] AGGATC [→] CACAGGTG GGTTC [→] CAGAGGGTCA [→] CTGGGTC [→] AGGTC [→] GGGTC [→] AGAGT [→] SACATCA	DR6, DR0, DR3, DR5	++	58	84
NNB-39	TGCCTTAGGTGA [→] AGGTC [→] AGGTC [→] ATGCCAGG	DR1	+++	5	11
NNB-44	AGTGACTCTGCAGGTC [→] GGGTC [→] ATTC [→] CAAGTTC [→] ACGG	DR5, ER8	+++	32	93
NNB-49	GGTGAAGGTC [→] AGGTC [→] ATGCCAGG	DR1	+++	4	9
NNB-66	GCCCAGAGGTC [→] ATAGGTC [→] ACCAAGA	DR2	++	6	7

^aOnly the sequence of the RA response element(s) identified within each NN fragment is displayed.

^bDR and ER denote pairs of core half-sites arranged as direct or everted repeats spaced by the indicated number of nucleotides; HS denotes individual core half-sites.

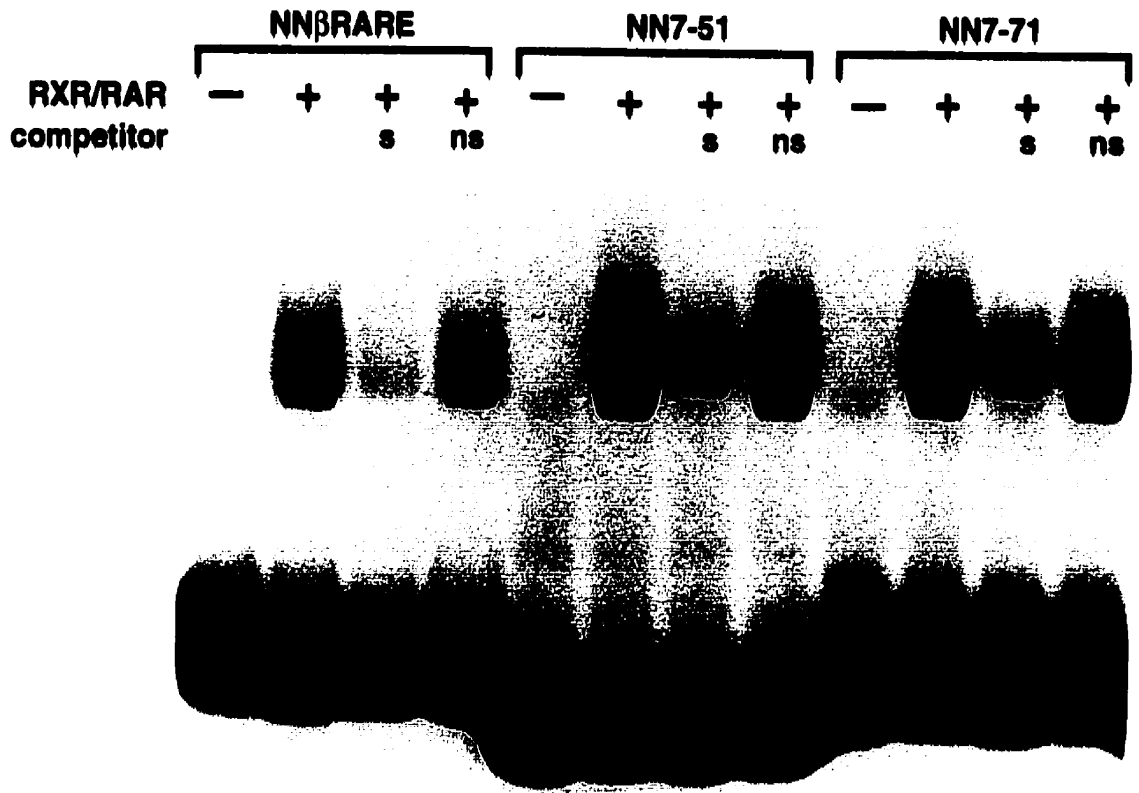
^cDifferential binding of RXR/RAR heterodimers to NN fragments in gel mobility shift assays are arbitrarily indicated as weaker (+), same (++) or stronger (+++) than NNB1ARE.

^dfold induction denotes the RA-inducibility of the RARE-containing NN fragments linked to the TK promoter.

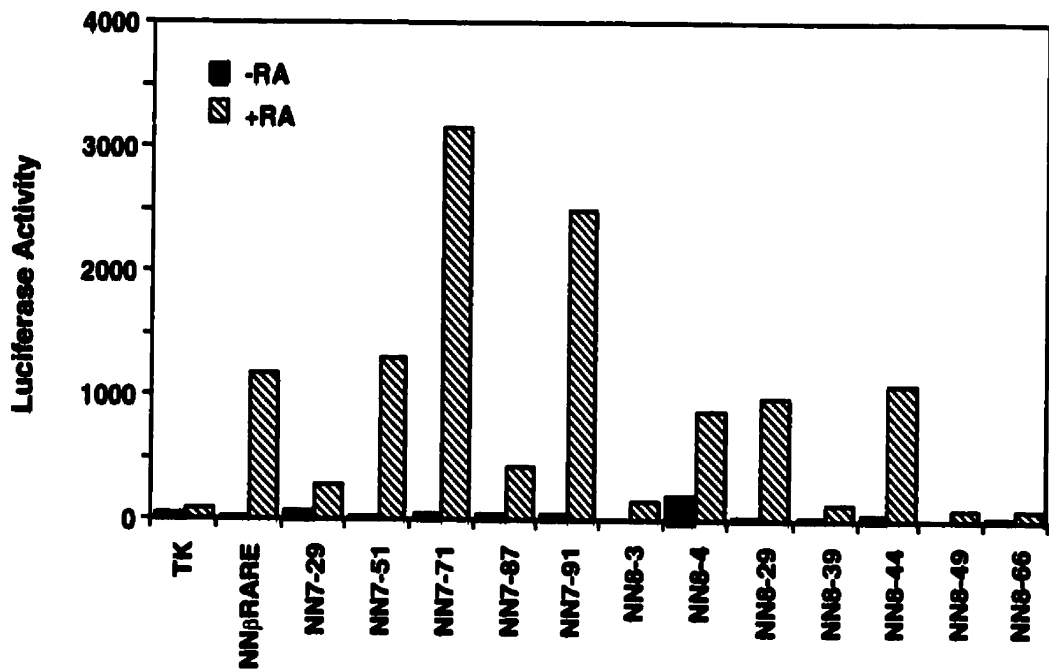
^eTotal activity refers to the RA-induced luciferase activity generated by the NN fragments normalized against the basal activity of the control TKLUC. The activity of the NNB1ARETKLUC construct was arbitrarily set at 100.

Fig. 6. Functional analysis of RXR/RAR selected NN fragments. (A) Binding of RXR/RAR to NN fragments. In this representative gel mobility shift assay, NN fragments were end-labeled and incubated with RXR/RAR complexes produced *in vitro*. A plus sign indicates the presence of *in vitro* synthesized RXR/RAR in the reaction, while a minus sign indicates the absence of the RXR/RAR heterodimer. Specificity of binding was assayed by addition of 100 fold molar excess of unlabeled specific competitor oligonucleotide (β RARE) (s) or nonspecific competitor oligonucleotide (derived from the adenovirus major late promoter) (ns). Unprogrammed lysate was used as a control. (B) RA responsiveness of RARE/NN fragments (see Table 2). NN/RARE fragments were linked to the thymidine kinase (TK) promoter and the Luciferase (LUC) reporter gene. Reporter vectors (2 μ g) were transiently transfected into P19 EC cells along with RSV β gal (1.5 μ g) as a control. No exogenous receptor was added. Cells were treated with 1 μ M RA 16 hours after transfection and harvested 24 hours after treatment. The β RARE-containing NN fragment derived from the promoter region of the murine RAR β gene served as a control in these transfection assays.

A



B



A single copy of each NN fragment was cloned upstream of the TK promoter driving the luciferase reporter gene (TKLUC). Transient transfections were performed in P19 EC cells which were treated with 1 μ M RA 16 h after transfection and harvested 24 hours after treatment (Figure 6B). Results seen with transient transfections of the constructs into F9 EC cells were qualitatively similar. Two values for each NN-RARE were calculated from this study (Table 2). Fold induction indicates the level of RA inducibility conferred to the TK promoter by each NN fragment. The total activity of the NN fragment is the luciferase activity detected upon RA treatment normalized against the basal activity of the control, TKLUC. The total activity of NN- β RARE-TKLUC was arbitrarily set at 100, and activities of the other reporters are relative to this control. Both values are calculated, because in some cases the basal activity of the fragment plus the TK promoter without RA treatment is much greater than that of TK alone. For example, although the fold induction (RA treatment versus no treatment) of NN8-4TKLUC is 5 while that of NN β RARE-TKLUC is 59, the total RA-induced luciferase activities of the reporters are relatively similar (75 units for NN8-4TKLUC, 100 units for NN- β RARE-TKLUC). The higher basal activities of some of the NN fragments suggest the presence of additional binding sites for other transcription factors that could contribute to enhancer activity.

As an additional control, these functional assays were performed on a number of selected NN fragments with no recognizable consensus RA response elements (see Materials and Methods) (data not shown). In each case these fragments did not bind specifically or at all to RRL programmed with RXR/RAR and conferred no RA-induced activation on TKLUC. Generally, the affinity of binding correlates well with the ability to confer RA induction upon a heterologous promoter, with a few notable exceptions. For example, fold induction values for DR1-containing fragments NN7-29, NN8-4, NN8-39 and NN8-49 are low. Such a result is not unexpected considering that although heterodimer RXR/RAR binds with high affinity to a DR1 configuration, repression is

thought to be the result (Mangelsdorf et al., 1991). As was mentioned above, NN8-4 (and to a lesser extent, NN7-29) is unusual in that the fragment confers a high level of total activity upon TKLUC. However, the fold induction value is low, because the basal level of transcription is greatly increased. Finally, a single copy of a DR2 element, such as that observed in NN8-66, generally confers activity more readily in transient transfections with the addition of exogenous receptor or if present in multiple copies (unpublished data).

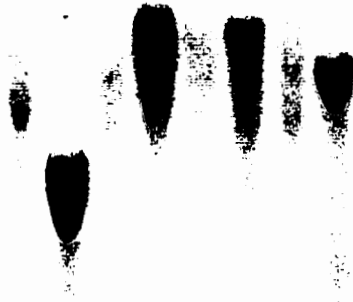
Identification of a conserved region and a putative CpG island associated with the RARE of NN7-91. Following the successful isolation of novel RARE-containing genomic fragments by CpG-SAAB, we wished to identify transcription units associated with these genomic NN fragments. Traditional methods used to identify transcribed sequences within a genomic region include the identification of conserved sequences by the screening Northern and Zoo blots. Screening of a murine λ 129 genomic library with NN fragment 7-91 yielded a number of flanking genomic clones. A 2.2kb *SpeI/Sac I* genomic fragment flanking NN fragment 7-91 was used to probe genomic Southern blots. Hybridizing bands were detected in mouse and human genomic DNA (Fig.7A), indicating the presence of a region that is conserved across species. Sequencing of approximately 3.8kb of genomic DNA surrounding the NN fragment 7-91 RARE revealed a pattern of CpG dinucleotides typical of a CpG island region (Fig. 7B).

Identification of a gene associated with the RARE of NN8-4. There exist a number of additional methods to identify transcribed sequences within a genomic region including cDNA selection, exon trapping, and direct DNA sequencing combined with use of available software and DNA databases searches, called software trapping (for review see Gardiner and Mural, 1995). The last approach is becoming increasingly powerful as more efficient computer programs and search algorithms are developed and as databases, particularly those of ESTs, are expanded. The NN fragments were first analyzed with BLAST to search the databanks of the National Center for Biotechnology Information

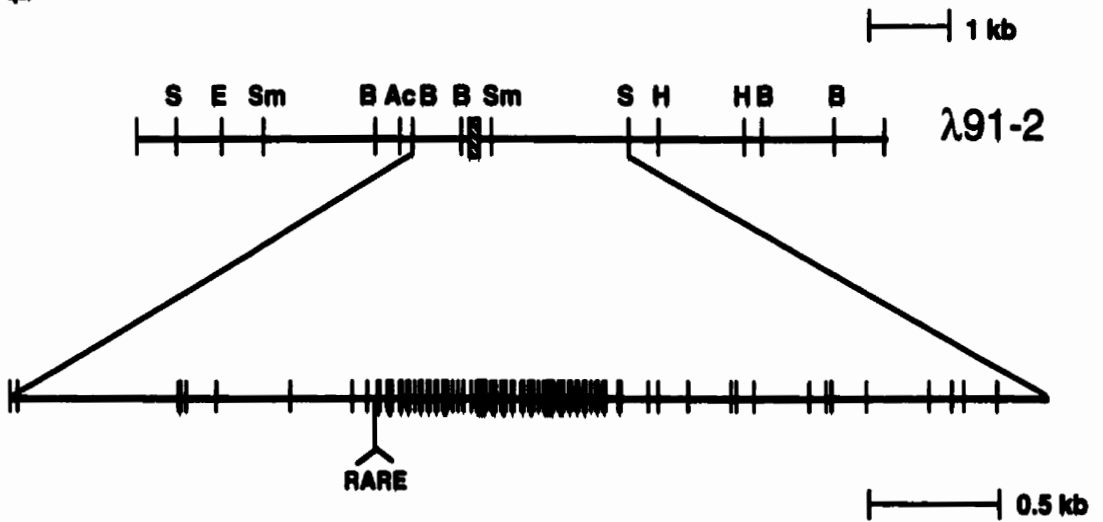
Fig. 7. Analysis of the genomic region flanking NN7-91. (A) Human/mouse sequence conservation. Genomic DNA isolated from human HOS cells or murine R1 cells were digested with the indicated restriction enzymes. 10 μ g samples were electrophoresed on a 1% agarose gel, transferred to a nylon filter, and probed with a 2.2kb radio-labeled genomic fragment flanking NN7-91. (B) The NN7-91 RARE is contained within a putative CpG island. Shown is a schematic of λ clone 91-2 which encompasses NN fragment 7-91. The position of the RARE is indicated by a stippled box. Restriction enzymes are: Ac, *AccI*; B, *BamHI*; E, *EcoRI*; H, *HindIII*; S, *SacI*; and Sm, *SmaI*. A 3.8kb region was sequenced and is enlarged. Vertical bars on the enlarged region indicate positions of CpG dinucleotides.

A

BamHI **EcoRI** **HindIII** **SacI**
H M H M H M H M



B

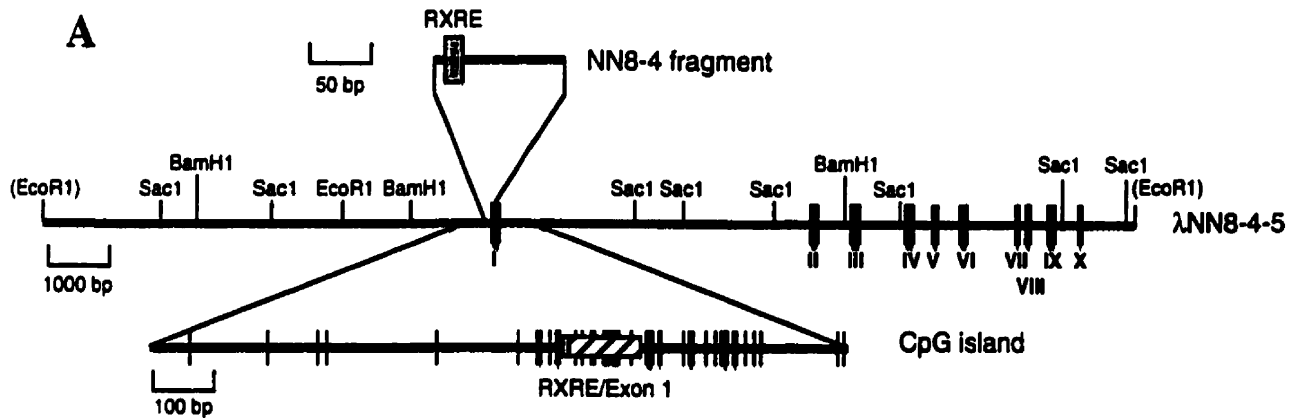


(Altschul et al., 1990). NN fragment 8-4 was found to share a 42 bp-long stretch of homology with EST41159 (Genbank accession number R56376), an EST derived from a human infant brain library (Bento Soares et al., 1994) (Fig. 8A). ESTs consist of partial sequence from randomly cloned novel cDNAs (Boguski et al., 1993). The library was constructed so that cDNAs were cloned directionally into the vector, thus allowing identification of 5' and 3' ends. As shown in Fig. 8A, alignment of EST41159 with NN8-4 places the RXRE approximately 40 bp upstream of the 5' end of the cDNA EST41159. In addition, there are only four mismatches in the 42 bp overlap between the mouse genomic NN fragment and the human cDNA, suggesting a high level of conservation within this sequence. EST41159 cDNA was obtained from the IMAGE Consortium and was sequenced in its entirety (Fig. 8B). The cDNA is 2520 nucleotides in length and includes a poly(A) tail and a consensus polyadenylation site. An open reading frame with a size of 412 amino acids commences at the extreme 5' of the cDNA, suggesting that this cDNA is not full length.

Characterization of the murine gene NN8-4AG encoding the homologue of human cDNA EST41159. To firmly establish the relationship between the murine NN8-4 genomic fragment and human EST41159 as well as to characterize the transcriptional unit associated with NN8-4 (referred to as NN8-4 Associated Gene or NN8-4AG), a murine λ 129 genomic library was screened with the NN8-4 fragment being used as a probe. Figure 9A shows a schematic of the NN8-4 fragment used as a probe to isolate λ genomic clone 8-4-5. The G+C content (67%) of NN8-4 suggested that it was derived from a CpG island. Sequence analysis of approximately 1 kb of DNA surrounding the RXRE of the NN8-4 fragment (Fig. 9B) revealed that the surrounding genomic sequence contains a putative CpG island that spans approximately 600 bp as demonstrated by the vertical bars representing CpG dinucleotides in Fig. 9A. The NN8-4AG CpG island sequence extends farther 5' of the EST41159 sequence and contains two in frame ATG codons (Fig. 9B). The homology between NN8-4AG and EST41159 continues

Fig. 8. Homology between NN8-4 and human EST41159 revealed by a BLAST search. (A) Alignment of the NN8-4 fragment with EST41159. The RXRE in NN8-4 is underlined. (B) Nucleotide and predicted amino acid sequence of EST41159. The polyadenylation signal is underlined, and the termination codon is marked with an asterisk. GenBank accession number for the complete sequence of EST41159 is U50383.

Fig. 9. Characterization of murine NN8-4 associated gene (NN8-4AG) and its putative CpG island. (A) Schematic representation of the genomic region flanking NN8-4. Above is NN fragment 8-4, used as a probe to isolate the genomic clone λ NN8-4-5. The gray box indicates the position of the RXRE. On the schematic λ NN8-4-5, exons are indicated with black boxes. Below is a representation of 1 kb surrounding NN8-4 with the RXRE and exon 1 indicated as gray and stippled boxes respectively and CpG dinucleotides represented by vertical bars. (B) Nucleotide sequence of the CpG island associated with NN8-4AG. The amino acid sequence of the first exon is indicated below the DNA sequence. The nucleotide corresponding to the furthest 5' transcriptional start site as determined by RACE analysis is indicated by an asterisk above the sequence. The RXRE and the CCAAT and GC boxes are underlined. The GenBank accession number for this sequence is U50384. (C) Comparison of the putative amino acid sequence of the first ten exons of the murine NN8-4AG gene product and the EST41159 protein. Identical amino acids are indicated by a vertical line, and similar amino acids are shown with two dots.



B

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1 TTGACCTTGAACCTCTGGTCTCCCTGCCCTCACCTCCTGAATGCAGAGGTTATAGGCAT
61 AGTCGGAAATGCTGGTTTAAAGCAGTGTGTGGCTCAAAGTCTGCTCACTCATGTOTTCAAA
121 GCAAAATCTCTCCCAACATAGAAATAAAAAATGTGCCACCAGAGGGAGGAAAAATTTTAA
181 ACCGAGTCAQCCAGCATCCCAACCAGGATGGAGGTCAAAGCAAAAAAATAGACAGTCTTC
241 TCTGGGCTAAAAAATTTGTTCCGTTGTTGGAGGTAATGACGAGGGGCTGCTTAAACAGGA
301 AGAGGGCTGCAAACTTTTGGCCAGCTGGGATACATAGTGAGACCCCTGTCTCAAACACCA
361 GACAGTGGGAAGAGCAACTATGGACACTTAAGAACACAGCTAGGATAGGCTGGAGGGTGG
421 GGGTGGGGAGTAGGGGCTCTCCCTGAGCTACTCCATCAGCCCTCATTCATGCTGTCC
481 ATCCGACATAAGCTACCGAGTCAGACACAGTCTACCAGAAGTCCAAGCAAAAAGTCAGGC
541 CAGGTCCACCCAAATCAAGCAACCAAGACACCAAAAGCCTTGAATTGCTAGCCAGTCCC
601 AGGCGGGGTAAGTGGGGAAGGGAGCGCTCGCTGCTCTGTGGCGGGCTTCCCAGGA
661 CCGGTCAGGGTCAAGAGGCAGAGGCCTGCCAAGATGGCGGCTCCATGTGGACGCTG
MetAlaAlaSerMetCysAspVal
721 TTCTCCTTCTCGGTGGGCTGGCGGACCGTGGCGGGGTTCCGTGGAAGTCCGTTATGCTG
PheSerPheCysValGlyValAlaAspArgAlaArgGlySerValGluValArgTyrVal
781 GACAGCATCAAGGTGAGGCCAGGTGGACCGGGCCCGAGAGCCACGCGGGGAACCCAC
AspSerIleLys
841 GCTCACAATCTGGAGTCCGGGCCCTCGGCCCCAGTCTGCTGCTGCTTGGGCCCGGGT
901 GCAGAGTCCGTCCTGCTGCCAGGCTCGTGGCGATATCGCGGGGGGAGGAGGAAAGC
961 CCGGTCCACTGGCGGTGGCCGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTG
1021 GACCTGGGTTGAGGATATCTGGGCCAGATTAAGCCAGGCTATCTTGGTTAGGCTG
1081 GGGCTAGTTAGCTACTATCTGGCGGTAGTGAAGGAGTACTTAGCCGAGG

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C

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NN8-8AG 1 NNAEKGVVSPCYVADRANGSEVRYVDIKRGLPATQLIRGETTIFIEELVVAAGFL
hEST41159 .....AVPFPYVAGAAKRVSEVRFVSAKAGQLPATQLIRGETTIFIEELVVAAGFL
NN8-8AG 61 NNALYTRACDNLALAEAEKAGQLTQSPQILNPEELCEVREKLNKCFKCVNYCS
hEST41159 NNALYTRACDNLALAEAEKAGQLTQSPQILNPEELCEVREKLNKCFKCVNYCS
NN8-8AG 121 AECLAAAGQTHWILCPQPSHD-PRNPLNELGEMKGVNTPETASDELNENRVAIVKGA
hEST41159 AECLAAAGQTHWILCPQPSHD-PRNPLNELGEMKGVNTPETASDELNENRVAIVKGA
NN8-8AG 181 -DKDMRRLFNKPCRTANGQATVWELLKQVFDQELLLGLFKALYEDALSLMPTPE
hEST41159 -DKDMRRLFNKPCRTANGQATVWELLKQVFDQELLLGLFKALYEDALSLMPTPE
NN8-8AG 240 GFRSLFALVGTWGGIATSELSQWVACDLDWTPQOREQLDTFTDQLYEDIEAATGDFL
hEST41159 GFRSLFALVGTWGGIATSELSQWVACDLDWTPQOREQLDTFTDQLYEDIEAATGDFL
NN8-8AG 300 NCEGSLFVLQSC...
hEST41159 NCEGSLFVLQSCM...

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downstream of the NN8-4 fragment border for an additional 12 amino acids before NN8-4AG diverges into an intronic sequence. A further nine exons of NN8-4AG were mapped onto λ NN8-4-5 with the aid of the EST41159 probe (Fig. 9A) and were subsequently sequenced for comparison with the human cDNA. Comparison of the amino acid sequence of the putative gene product coded by for the first ten exons of NN8-4AG with the EST41159 translation product reveals a level of amino acid identity of 86% (92% similarity) between the human and mouse clones (Fig. 9C). Rapid amplification of cDNA ends (RACE) was performed on mouse brain RNA using primers located in putative exons 1, 2 and 3 of the NN8-4AG transcript. A series of start sites was identified in the region of the RXRE, and the longest of these localized on the first G of the first half site, 33 bp upstream of the 5' most methionine start codon. No TATA box is evident upstream of these start sites, consistent with the presence of multiple transcriptional initiation sites (Fromm and Berg, 1983). A perfect consensus CCAAT box is located 110 bp upstream of the cluster of start sites identified by RACE analysis. In addition, two GC boxes, or potential Sp1 binding sites, are located 12 bp and 56 bp upstream of the cluster of transcriptional start sites. These results place the NN8-4 RXRE within a CpG island in the vicinity of the multiple transcriptional start sites of NN8-4AG.

NN8-4 RXRE is bound by RXR/RAR heterodimers and RXR homodimers. As can be seen from Table 2, the NN fragment 8-4 binds specifically to *in vitro*-transcribed and -translated RXR/RAR and confers RA induced activation on TKLUC in transient transfection in P19 EC cells. It is highly likely that the consensus DR1 present within NN8-4 is responsible for this activity. To verify this notion, an oligonucleotide encoding this sequence was synthesized (NN8-4 RXRE), end-labeled and analyzed in gel mobility shift assays with *in vitro*-transcribed and -translated receptors. The NN8-4 RXRE-RXR/RAR complex is effectively inhibited by competition with excess unlabeled NN8-4 RXRE, β RARE (a DR5), and CRBP II RXRE (a DR1) (Fig. 10). A similar amount of unlabeled nonspecific competitor does not interfere with binding. When RXR alone is

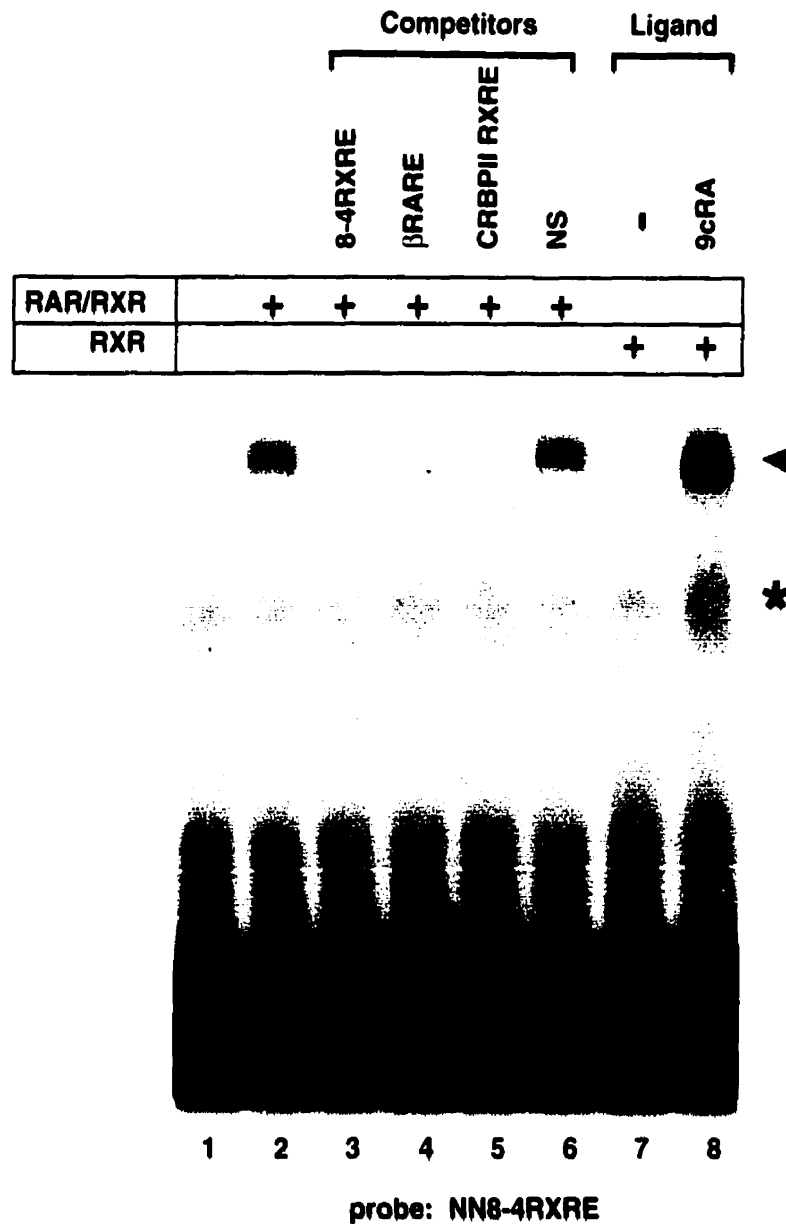


Fig. 10. Binding of RXR/RAR heterodimers and RXR homodimers to the NN8-4 RXRE. Endlabeled NN8-4RXRE was incubated with rabbit reticulocyte lysate (total 4 μ l) programmed with human RAR α 1 and/or murine RXR β mRNA. Probe was also incubated with the same amount of unprogrammed lysate as a control (lane 1). Cold competitors were used at a molar excess of 150-fold. NS indicates the addition of a nonspecific competitor oligonucleotide. 9cRA was added to the binding reaction to a final concentration of 1 μ M. The arrow indicates the specific complexes while the star indicates a nonspecific complex.

combined with labeled NN8-4 RXRE a shifted band is visible, albeit with much less intensity than that of the band seen with both RXR/RAR. With the addition of ligand 9cRA the amount of probe bound by RXR is increased to a level greater than that seen with the RXR/RAR heterodimer. This enhancement of RXR homodimer binding to a DR1 has been documented previously (Medin et al., 1994; Zhang et al., 1992). The RXRE coded for in NN8-4 interacts with receptors in a manner similar to previously characterized RXREs binding both RXR/RAR heterodimer and RXR homodimer/9cRA with high affinity (Mangelsdorf et al., 1991; Zhang et al., 1992).

The NN8-4AG transcript is directly upregulated by 9cRA in F9 cells. Our functional analyses suggest that the RXRE associated with NN8-4AG may be biologically relevant. As the human cDNA and murine NN8-4AG sequence obtained from genomic clones were highly conserved, we used EST41159 to probe RNA derived from F9 EC cells at various stages of RA treatment. A transcript of approximately 2.75 kb was present at low basal levels, and was upregulated in the presence of RA (Fig. 11). Addition of either atRA or 9cRA results in a four- to fivefold increase in the expression of NN8-4AG after 24 h of treatment (Fig. 11, lanes 4 and 8). Although the basal level of NN8-4AG mRNA varied by about 2 fold in F9 EC cells under these culture conditions, it is evident that upregulation occurred more quickly in the presence of atRA (Fig. 11, lane 2). However, it is likely that this atRA-induced gene expression is not a primary effect, as pretreatment with the protein synthesis inhibitor cycloheximide (Cx) negates the increase in expression seen at 12 h of treatment with the retinoid (Fig. 11, lane 11). No additional protein synthesis is necessary for the upregulation upon 9cRA treatment, as 12 h levels with Cx and 9cRA are similar to those with 9cRA alone (Fig. 11, lane 10). Although NN8-4AG is responsive to both atRA and 9cRA, it appears that activation with these ligands occurs via two different pathways. The Northern blot was stripped and probed with genes known to be directly regulated by RA, RAR β and CRABP II (Fig. 11). As has been demonstrated previously, RAR β is upregulated in response to both atRA and

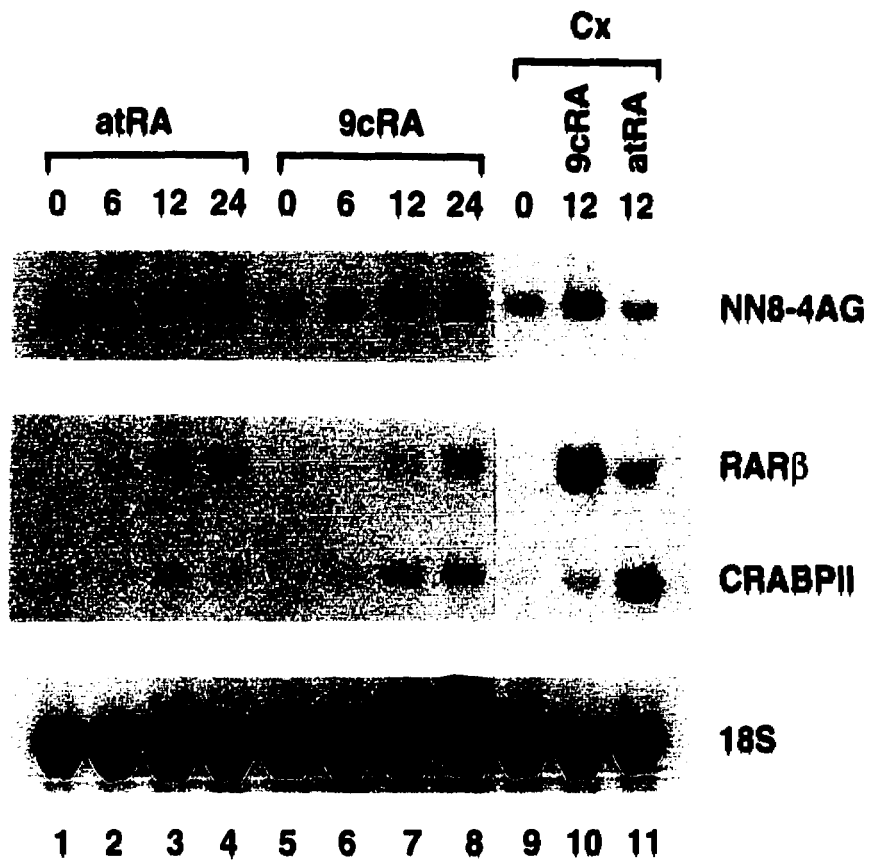


Fig. 11. Northern Analysis of NN8-4AG mRNA. EST41159 was used to probe total RNA derived from F9 EC cells which had been grown in DMEM supplemented with charcoal stripped/UV treated serum. RNA was isolated at the indicated times after RA treatment. Retinoids were added to a concentration of 1 μ M. Cycloheximide (Cx) was added to a final concentration of 10 μ g per ml, one hour prior to RA treatment. For time 0 samples, Cx was added one hour before the cells were harvested. RAR β and CRABP II, genes known to be induced by RA, were used as controls. An oligonucleotide hybridizing to the 18S ribosomal RNA was used to normalize for RNA loading.

9cRA (de Thé et al., 1989; Durand et al., 1992). 9cRA activates expression of CRABP II more effectively than atRA (Durand et al., 1992; Roy et al., 1995). Although expression levels of both mRNAs at 12 h with retinoid and Cx treatment indicate that upregulation occurs with no additional protein synthesis, RAR β is "superinduced" by a combination of 9cRA plus Cx, while CRABP II is "superinduced" only in the presence of atRA and Cx. This observation provides further evidence that the expression of these two genes is controlled by distinct retinoid signaling pathways.

Evolutionary conservation of EST41159 protein. The putative gene product of the human homologue of NN8-4AG, EST41159, was analyzed with the BLASTX program and the databases of the National Center for Biotechnology Information (Altschul et al., 1990). The predicted protein sequence of EST41159 showed significant similarity with that of a yeast (*Saccharomyces cerevisiae*) putative polypeptide of, as yet, unknown function (accession number P38890). The similarity corresponds to amino acids 308 to 381 in the human gene product and 333 to 403 in the yeast protein with a P value of 8.95e-07 (Fig. 12). This region also shares identity with the C-terminal region of the *Drosophila trithorax* protein (Mozer and Dawid, 1989), a member of a group of proteins which are thought to participate in chromatin remodeling (Orlando and Paro, 1995).

Yeast P38890	333	SFINHDCEFNAYIEQ--VEEHEELRLHARKPIKKGQIRITYVNP LHGVRLR---RRELRVNNGFLCQCDCRQNELS
EST41159	308	SCCNHSCVPNAETSF--PENNFLLHVTALEDIKPGEEICISYLDCCQERERSRHSRHKILRENYLFVCSCKLAEAD
<i>trithorax</i>	3696	RFINHCCEPNCYSKVVDILGHKHIIFAVRRIVQGEELTYDYKFPFEDEKIP-----CSCGCKRCRKVLN

Fig. 12. Comparison of the predicted amino acid sequence of human EST41159 to a yeast putative protein and to the *Drosophila trithorax* gene product. Amino acids that are identical are indicated by a vertical line (|), while conservative changes are designated by a plus sign (+). GenBank accession numbers for the putative yeast and *trithorax* proteins are P38890 and Z31725, respectively.

DISCUSSION

To be physiologically relevant, the search for transcription factor binding sites should concentrate not only on DNA fragments located in the regulatory region of genes but also on transcription units actively transcribed in the tissue or cells of interest. The previous demonstration that "active" chromatin located within CpG islands can be separated from bulk inactive chromatin and that a specific fraction of nucleosome-free DNA associated with these islands contains the promoter region of transcribed genes (Tazi and Bird, 1990), provides an elegant way to identify pertinent genomic fragments. These small DNA fragments (NN fragments) represent a highly purified fraction of the genome and can, in theory, be selected further for their ability to bind to transcription factor complexes. On the basis of these premises, we have developed a method for the isolation of RXR/RAR binding sites from CpG island DNA, isolated 12 RA response elements and characterized in detail a novel RA-responsive gene associated with one of them.

The advantages of the CpG-SAAB method are numerous for exploring the molecular mechanisms of retinoid receptors and other transcription factors. First, in contrast to such subtraction screening strategies as differential display PCR, only genes directly regulated by the receptors are cloned. Second, because NN fragments are derived from active chromatin, genes primed to respond to retinoids in the appropriate tissue or cell line will be preferentially identified. Third, the promoter and/or the regulatory region of these genes is likely to be identified first, thus providing molecular tools in the form of natural promoters and enhancers to study the action of retinoid receptors. Fourth, since this strategy targets the promoters of genes, the 5' ends of the associated transcripts, which are a region of cDNAs that is often difficult to isolate, are in many cases likely to be cloned first. Finally, CpG-SAAB can be applied to any combination of transcription factor and cell line or tissue of interest. A specific set of responsive genes is expected to be identified for each combination of transcription factor and starting chromatin.

Although not all genes are associated with CpG islands, all housekeeping and a significant fraction (40%) of tissue specific genes are (Antequera and Bird, 1993). Since CpG island DNA composes approximately 2% of the genome, the bulk of genomic DNA is excluded from the selection. In the case of the retinoid receptors at least, it may be prudent to avoid the use of naked DNA as starting material for binding site selection as members of a major *Alu* repeat subfamily and major (gamma) satellite DNA repeats contain RAREs (Rudert et al., 1995; Vansant and Reynolds, 1995). Although some of these repeats may play a role in regulating the expression of nearby genes, these elements may also interfere with the isolation of other relevant binding sites. because of their numbers and affinity for the receptors. According to preliminary analysis of a number of the RARE-containing NN fragments by genomic Southern blotting, these fragments are derived from single copy genomic DNA, a situation previously documented for the CpG island fraction of the genome (Cross et al., 1994).

We chose to use murine EC cell lines as starting material for CpG-SAAB. F9 EC cells differentiate into blastocyst-like endoderm cells upon RA treatment and provide a model system in which the genetic cascade following retinoid treatment mimics early development. CpG-SAAB enabled us to isolate a population of DNA fragments containing a variety of RAREs and RXREs. The sequencing of 120 clones resulted in the discovery of 26 that contained functional RAREs and RXREs. Fragments that did not contain any obvious consensus RA response elements were likely selected by proteins present in the RRL and, in practice, their number could be reduced by introducing an additional purification step such as immuno-precipitating with specific receptor antibodies.

The majority of the selected NN fragments containing the response elements bind the RXR/RAR heterodimer with an affinity comparable to that of the fragment containing the potent β RARE. Analysis of these fragments shows that complex elements are relatively common but also provides further evidence that the most potent RAREs are of the DR5

type (Table 2). Surprisingly, low total transcriptional activity was generated when NN8-3, a fragment containing a consensus DR5 with half sites of sequence GGGTCA and AGGTCA, was tested in the presence of RA. On the other hand, NN7-91, which contains a DR5 with the same half site sequences but in the opposite order, confers a high level of RA-induced transcriptional activity on TKLUC. A number of factors could be responsible for this difference in activities. The composition of the half sites, spacer and nucleotides 5' of the upstream half site may be critical in the determination of transcriptional activity imparted by the receptors. For example, several of the nuclear receptors which bind as monomers have been shown to require an A-T rich region extending 5' from the core half site (Giguère et al., 1994; Harding and Lazar, 1993; Wilson et al., 1991). If a monomeric receptor binding site is embodied within a RARE, monomeric nuclear receptors may interfere with the binding of RXR/RAR heterodimer complexes (Tini et al., 1995). Finally, the context of the RARE within the genomic fragment itself may be critical, as the sequences flanking the RAREs may contain binding sites for additional transcription factors which may modulate receptor activity (Nakshatri and Chambon, 1994). The cell type specific activity of the NN-RAREs may be imparted by the presence or absence of these factors within a particular cell type. The identification of a large collection of natural RAREs and RXREs by CpG-SAAB and their in-depth characterization will allow us to test these parameters in a systematic manner.

NN fragments containing functionally competent RAREs identified by CpG-SAAB can subsequently be used to isolate large flanking genomic DNA fragments containing the associated CpG island. Cross et al. (Cross et al., 1994) have demonstrated that cloned CpG islands can be used to detect transcripts of expressed genes at a relatively high frequency. We indeed observed that a genomic fragment containing NN7-91 detects both a conserved locus in mouse and human by Zoo blot analysis (Fig. 7A). In addition to these traditional methods of searching for transcripts we are also focusing on sequence

and database analysis. Particularly useful to this type of project are the EST databases. By software trapping, we have been able to quickly identify a transcript associated with one of the retinoid receptors-selected NN fragments (NN8-4). While the proximity of the RXRE contained in NN8-4 to the transcriptional start site of the associated gene (NN8-4AG) allowed for the identification of this transcribed sequence, sequencing of genomic DNA flanking the remaining selected NN fragments (1 to 2 kb in each direction) would greatly increase the probability of finding a match.

We have shown that the NN8-4AG transcript is differentially up-regulated by atRA and 9cRA (Fig. 11). Treatment of F9 EC cells with either atRA or 9cRA leads to an induction of the expression of the NN8-4AG gene, but activation by atRA seems to require the synthesis of additional factor(s). We have demonstrated that the NN8-4 fragment contains a DR1 RXRE that is recognized with high affinity by RXR/RAR heterodimers and by RXR homodimers in the presence of 9cRA and is able to confer RA-responsiveness to the TK promoter (Fig. 10 and Table 2). It can therefore be assumed that the 9cRA response is mediated via the DR1 RXRE, either through RXR homodimers (Zhang et al., 1992), RXR/RAR heterodimers, or a heterodimer complex in which RXR acts as a 9cRA-responsive partner to another member of the nuclear receptor family (for examples, see Kliewer et al., 1992; Perlmann and Jansson, 1995; Willy et al., 1995). Since 9cRA can also bind RAR it is possible that ligand occupation by both of the receptors is required for the 9cRA response. Conflicting results concerning the ability of RXR to bind ligand in the context of a RXR/RAR heterodimer have been obtained in studies with receptor-specific ligands (Apfel et al., 1995; Forman et al., 1995; Kurokawa et al., 1994; Roy et al., 1995). Further study will be required to address the nature of the dimer mediating the 9cRA response of NN8-4AG. It is not yet known whether the protein synthesis-dependent response to atRA also involves the DR1 element.

In spite of sequence analysis and database searches, the function of the novel gene product coded for by EST41159 and NN8-4AG is unknown. This result is not

unexpected, as up to 50% of ESTs from human brain are unique and do not show significant similarity to proteins with known biological functions (Adams et al., 1993). However, the observed similarity between the C-terminal domain of EST41159, a putative yeast protein, and the *Drosophila trithorax* gene product does delineate an evolutionarily conserved domain. This region of the *trithorax* gene product has been previously shown to share similarity with a number of proteins, some that are known to be associated with chromatin and others with unknown functions (Stassen et al., 1995). The region of similarity we have delineated is not as extensive as that previously defined, however; like the other homologous proteins, the motif lies at or near the C terminus of otherwise non-homologous proteins. The yeast putative protein (P38890) mentioned in this study and EST41159 share regions of homology throughout their entire sequences, suggesting they are more closely related to one another.

The CpG-SAAB method described here allowed us to isolate a subset of retinoid receptor binding sites and identify a novel RA-responsive gene with an as-yet-unknown function. The characterization of a large number of genes associated with these elements will hopefully help chart the genetic pathways implicated in the early response of EC cells to RA and improve our understanding of the mechanisms regulating cellular differentiation induced by hormonal stimuli.

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Chapter 4

General Discussion

Summary

Hormonal signals regulate the transcription of many eukaryotic genes. One class of hormones is comprised of small lipid-soluble molecules that interact directly with ligand-inducible transcription factors in the nucleus. Retinoic acids, biologically active forms of vitamin A, are members of this class of hormones. Although the physiological response to RA can be attributed to the activity of a network of target genes regulated by the retinoid receptors, it is also apparent that numerous positive and negative factors contribute to the highly controlled and graded functional response of different cells and tissues to retinoids during development, growth, or differentiation.

One of the cellular factors that opposes nuclear receptor function is calreticulin. As presented in Chapter 2, by binding to a conserved sequence in the DNA-binding domain of the retinoid receptors, calreticulin can interfere with the ability of the receptors to bind to RAREs and to induce gene transcription. Overexpression of calreticulin in P19 EC cells inhibits RA-induced neuronal differentiation, while downregulation of expression of calreticulin by antisense cDNA expression results in a more rapid progression of these cells toward the differentiated state (Dedhar et al., 1994 and Chapter 2). I will address four topics stemming from and related to these studies: (1) the effect of calreticulin on RA signaling in a calreticulin null background, (2) further investigation into the presence of calreticulin in the nucleus, (3) the possibility that calcium plays a role in the RA response in P19 cells, and (4) implications of calreticulin/receptor interactions.

In order to dissect the cellular response to RA, it will be necessary to isolate and characterize a battery of genes which are targets for RARs and RXRs. The development of a method (CpG-SAAB) to isolate RARE-containing fragments from CpG island regions of the genome constitutes a step towards this goal. In this general discussion I will make comments on and offer suggestions for the improvement of CpG-SAAB. One of the most time-consuming aspects of CpG-SAAB concerns the isolation of genes

associated with the NN-RARE fragments. An approach which employs complementary strategies will be proposed. The NN fragments described in Chapter 3 have been tested for their capacity to function as RA response elements. These genomic fragments provide useful tools to further study promoter context- and response element-dependent influences on the transcriptional activation activities of the retinoid receptors. Relevant experiments in the continuing characterization of the RA responsive gene NN8-4AG will be proposed in this chapter.

1 Modulation of the RA response by calreticulin

1.1 Effect of calreticulin on RA signaling in a calreticulin null background

There are varied reports on the effect of overexpression of calreticulin on nuclear receptor activity in transiently transfected cells. Calreticulin expression inhibits GR-, AR- and VDR-mediated transcriptional activation of a corresponding target reporter gene (Burns et al., 1994; Dedhar et al., 1994; Wheeler et al., 1995). However, little or no effect on PPAR/RXR mediated transactivation was seen with elevated levels of calreticulin (Winrow et al., 1995). The overexpression of calreticulin did not completely inhibit RA activation in the transient transfections (see Chapter 2). It is possible that such discrepancies are due to varying endogenous calreticulin content in cell lines used in these experiments. To circumvent such a problem, it would be ideal to examine the effect of various calreticulin levels on RA-induced gene expression in a calreticulin null background. Embryonic stem cells in which calreticulin has been eliminated by gene targeting are now available (S. Dedhar and R. St.-Arnaud, personal communication). In these cells, a dose response curve of calreticulin inhibition of RXR/RAR heterodimer, RXR homodimer, and other nuclear receptors abilities to transactivate through cognate reporter vectors can be established.

1.2 Further studies on the nuclear localization of calreticulin

In order to perform a physiological role in the modulation of nuclear receptor activity, calreticulin must gain access to the nucleus. Most evidence indicates that calreticulin is a resident ER protein (Michalak et al., 1992), including the presence of a signal sequence and a KDEL retention signal. Despite this, in some cells calreticulin has been shown to localize to the nucleus (Dedhar, 1994; Dedhar et al., 1994; Opas et al., 1991) and also to the cytoplasm (Leung-Hagesteijn et al., 1994; Rojiani et al., 1991). Several explanations have been proposed for the localization of calreticulin to the cytoplasm (Dedhar, 1994). Two isoforms of the protein may exist, one without ER targeting signals. Alternatively, elevated levels of calreticulin might lead to saturation of the KDEL receptor on the ER membrane with subsequent escape of calreticulin into the Golgi apparatus. Once outside the ER, the putative nuclear localization signal (NLS) (Michalak et al., 1992) may play a role in transporting calreticulin to the nucleus. Alternatively, it is possible that calreticulin could be conveyed to the nucleus as part of a complex with a protein that has a functional NLS.

The putative nuclear localization signal (PPKKIKDPD) of calreticulin is located in the central P domain of the protein (Michalak et al., 1992). Although not a perfect match, the putative NLS of calreticulin more closely resembles the NLS of SV40 T antigen (Kalderon et al., 1984) than bipartite nuclear localization signals (Dingwall and Laskey, 1991). The capacity of this sequence to serve as a NLS could be examined by addressing two questions. First, is the proposed sequence necessary for targeting of calreticulin to the nucleus, and second, is the sequence capable of directing a non-nuclear protein to the nucleus? The first question can be addressed by site-specific mutagenesis combined with immunofluorescent microscopy. In studies using the SV40 large T antigen, the minimal sequence that is critical for nuclear localization has been defined as the sequence PPKKRKV (Kalderon et al., 1984; Kalderon et al., 1984; Lanford and Butel, 1984). Particularly important in this and other proteins with defined nuclear

targeting sequences are clusters of basic residues (Dingwall and Laskey, 1986). Each of the basic residues within the putative NLS of calreticulin could be modified by site-directed mutagenesis. Comparisons between the localization of the resultant protein with intact calreticulin would best be examined in the calreticulin *-/-* embryonic stem cells. In addition, the putative calreticulin NLS can be linked to a normally cytosolic protein such as pyruvate kinase and the subsequent compartmentalization of the protein visualized by immunofluorescence (Kalderon et al., 1984).

1.3 Does the RA response in P19 EC cells involve or modify a Ca²⁺ response?

Calreticulin was first identified as a calcium binding protein. It is possible that the over- or underexpression of calreticulin in the stable P19 EC cell lines altered RA-regulated gene expression due to an effect on Ca²⁺ signaling. We have shown preliminary evidence in Chapter 2 that intracellular Ca²⁺ levels of both control and calreticulin over-expressing P19 cells are essentially the same when analyzed with the fluorescent probe Fura-2. Calreticulin is synthesized with a signal sequence and harbors a KDEL retention sequence (Michalak et al., 1992), localizing primarily to the lumen of the endoplasmic reticulum (ER). Here calreticulin has been proposed to participate in the Ca²⁺ buffering capacity of the intracellular inositol 1,4,5-trisphosphate (IP₃) sensitive, rapidly exchanging Ca²⁺ stores (Bastianutto et al., 1995; Liu et al., 1994) and may play an active role in regulation of Ca²⁺ influx into these stores (Camacho and Lechleiter, 1995; Mery et al., 1996). Ca²⁺ is released from these endoplasmic reticulum stores upon activation of IP₃ receptors (IP₃R) by IP₃, resulting in the regulation of many cellular processes (Berridge, 1993). The conversion of phosphatidylinositol (4,5)-bisphosphate to IP₃ by phospholipase C (PLC) occurs in response to ligand stimulation of various surface receptors including G protein-linked receptors and tyrosine kinase-linked receptors (Berridge, 1993). In contrast, since the retinoic acid receptors are constitutively nuclear and regulate gene expression directly, it seems unlikely that the RA receptors utilize IP₃

formation and intracellular calcium release. RA treatment does not result in generation of IP₃ or release of Ca²⁺ in human HaCaT keratinocytes (Rosenbach et al., 1993). RA-induced formation of IP₃ and release of intracellular calcium has been reported in murine keratinocytes (Tang et al., 1989), although at high doses the lipophilic retinoids could act as ionophores, resulting in an activation of PLC by an increase in intracellular Ca²⁺ (Rosenbach et al., 1993).

A rigorous study of the role, if any, of calcium signaling in the differentiation of P19 EC cells upon RA treatment is necessary. In particular, the Ca²⁺ content of control and calreticulin over-expressing cells could be analyzed both before (as in Chapter 2) and at various time points after treatment. Since calreticulin is thought to buffer IP₃ sensitive stores of Ca²⁺ in the ER, the effect of RA on PLC activity and levels of IP₃ should also be determined in these cells. If the PLC-mediated release of IP₃ is somehow triggered by RA treatment, the subsequent release of Ca²⁺ from the IP₃ sensitive ER stores may contribute to the differentiative process. The higher levels of calreticulin in the over-expressing cells allow for the sequestering of an elevated Ca²⁺ capacity in the ER (Bastianutto et al., 1995; Liu et al., 1994; Mery et al., 1996). Although the main limiting factor in the rise in Ca²⁺ concentration in the cytosol appears to be the concentration and/or modulation of IP₃ receptors and not the Ca²⁺ content of the stores (Bastianutto et al., 1995), the possibility that an increased Ca²⁺ buffering capacity of the ER somehow plays a role in RA induced P19 EC cell differentiation requires further study.

1.4 Implication of calreticulin/receptor interactions

The results presented in Chapter 2 lead to the proposal that calreticulin-retinoid receptor interactions may play a physiological role in the control of cell differentiation and proliferation. What this role might be is at present speculative but one possibility is that calreticulin may serve as a mediator in extracellular matrix-mediated change in gene expression. Calreticulin also binds to an amino acid sequence conserved in the

cytoplasmic domains of all integrin α subunits (Rojiani et al., 1991). The integrins are a family of transmembrane receptors which bind to components of the ECM, and due to interaction with the integrins and other cell surface receptors, the ECM forms a substrate for cell attachment and migration (Hynes, 1992). Cellular-ECM interactions are critical for cell attachment and migration (Hynes, 1992). Cellular-ECM interactions are critical for cellular migration and are thought to mediate signals necessary for differentiative and developmental processes (Yang et al., 1993). Retinoid-regulated pathways have previously been linked with those involving integrin action. Many components of the ECM including laminin, fibronectin, and types III and IV collagen, increase in abundance in cells treated with atRA (Chiocca et al., 1988; Horton et al., 1987; LaRosa and Gudas, 1988; LaRosa and Gudas, 1988; Wang et al., 1985). In addition, RA downregulates the expression of stromelysin and collagenase, extracellular metalloproteinases which play a role in remodeling of the ECM (Brinckerhoff et al., 1986; Nicholson et al., 1990; Saus et al., 1988). RA also concomitantly induces the expression of several integrin subunits in murine P19 EC cells (Dedhar et al., 1991) and in chicken osteoclast precursors (Cao et al., 1993). As previously mentioned, the promoter of the β_3 integrin subunit contains a RARE as well as a VDRE (Cao et al., 1993; Mimura et al., 1994). RA-induced differentiation may therefore involve interplay between retinoid receptor and ECM signaling pathways. Calreticulin, by virtue of its ability to bind specifically to components of both pathways, may perform a central regulatory role in a cell's proliferation vs. differentiation capabilities. In support of this theory, calreticulin has been localized in the nucleus and the soluble cytoplasm as well as its initial localization in endoplasmic reticulum (Dedhar et al., 1994; Rojiani et al., 1991). In proliferating muscle cells (both L6 and human skeletal muscle cells in culture) intranuclear staining of calreticulin is observed, however neither intranuclear nor intracellular expression is apparent in differentiated muscle cells (Opas et al., 1991).

It has recently been demonstrated that calreticulin expression is required for integrin-mediated cell attachment to the ECM (Coppolino et al., 1995; Leung-Hagesteijn et al.,

1994). It could therefore be argued that functional integrin activity, and therefore stronger cell-ECM interaction, should favor gene expression via nuclear hormone receptors, whereas cell rounding and release of integrin-calreticulin interaction should result in an increased availability of calreticulin for the inhibition of nuclear hormone induced gene expression. These are testable hypotheses and future work will be directed towards elucidating the role of calreticulin in ECM regulation of gene expression.

2 CpG-SAAB

2.1 Applications, limitations and suggestions for improvement

The advantages of the CpG-SAAB approach to target gene isolation were discussed in chapter 3 and include the cloning of genes that are directly regulated by the retinoid receptors and that are located in active chromatin, the isolation of genomic DNA corresponding to promoter regions and 5' ends of associated transcription units, and propensity for use with any combination of transcription factor and cell line or tissue.

CpG islands are a feature of the vertebrate genome. Invertebrate genes are generally situated in entirely nonmethylated DNA (Bird, 1987). It has been hypothesized that DNA methylation spread throughout the genome with vertebrate evolution (Antequera and Bird, 1993), possibly as a mechanism to reduce extraneous transcription (Bird, 1995). Consequently, the use of CpG-SAAB is restricted to vertebrate systems.

As mentioned above, any cell line of interest may be used as starting material for CpG-SAAB. It should be noted, however, that many permanent cell lines have a tendency to lose the nonmethylated status of CpG islands (Antequera et al., 1990). Methylation is confined to genes that are tissue-specific and non-essential in culture. Embryonal carcinoma cells such as P19 or F9 cells differ from many permanent cell lines in that their methylation patterns are similar to *in vivo* tissues (Antequera et al., 1990). CpG islands are normally free of methylation in cells of the animal, even if the associated

gene is not being transcribed (Bird, 1986), although a small fraction of loci may change their methylation profiles during development or tissue differentiation (Kawai et al., 1994). The patterns of DNA methylation are altered during tumorigenesis and such alterations may play a direct role in carcinogenesis (Balmain, 1995). To focus on transcription factor binding sites in normal development and differentiation, nuclei can be derived from EC cells such as F9 cells (see Methods in chapter 3) or from tissues (Gorski et al., 1986; Meehan et al., 1989).

Only approximately 60% of genes have CpG islands (Cross et al., 1994). Additionally, a transcription factor binding site may not be bounded by appropriately spaced CpG recognizing restriction enzyme sites (for example *MspI* and *HinPI*). Improved representation of CpG island NN fragments could be established by digesting nuclei with a number of different CpG recognizing enzymes, and then pooling the NN fragments prior to modification and selection.

CpG islands are associated with all housekeeping genes. Molecular and genetic analyses of retinoid action have shown that RXR/RAR complexes appear to play a pleiotropic role in the control of embryonic development and in homeostasis in the adult animal (Sporn et al., 1994). Retinoid receptors are therefore likely to be involved in the general maintenance of the cellular machinery and, by inference, could regulate a large number of housekeeping genes. Also amenable to isolation by CpG-SAAB are the significant fraction of genes with limited or tissue specific expression that are associated with CpG islands. Numerous examples of such genes exist, including RAR β 2 (see chapter 3), the homeobox-containing gene GBX2 (Lin et al., 1996), and many other developmental control genes (Larsen et al., 1992).

As mentioned in chapter 3, one in five selected NN fragments contained a RARE or RXRE. The remaining fragments were likely selected due to interaction with components of the rabbit reticulocyte lysate. Since the consensus binding sites for retinoid receptors are well characterized it was relatively simple to scan NN fragment

sequences for the presence of RA response elements. However, background binding might be problematic when a transcription factor binding site is degenerate or unknown. In this case it would be necessary to decrease background binding so that selected fragments can be subjected directly to functional testing. NN fragments that bind specifically to the transcription factor can be separated from nonspecific fragments by immunoprecipitation with an antibody directed against the transcription factor. Alternatively, several rounds of selection could be performed with transcription factor produced using an alternative source such as bacterial or mammalian expression systems.

2.2 Isolation of NN fragment-associated genes

Inevitably the most time-consuming component of CpG-SAAB is the identification of transcripts associated with the NN fragments. The traditional approach of identifying conserved sequences using Northern and Zoo blots, with subsequent cDNA library screening is laborious but effective. Software trapping (Gardiner and Mural, 1995 and see chapter 3), the discovery of genes using genomic sequencing and computational analysis, is increasingly proficient as a tool to identify transcribed sequences. The NN fragments can be compared with Genbank sequences using the BLAST algorithm (Altschul et al., 1990). This strategy proved successful with NN fragment 8-4, due to the proximity of the NN8-4AG transcriptional start site to the RXRE. In cases where the coding region of the associated gene may be at a greater distance from the RA response element, it is necessary to isolate a genomic clone encompassing the NN fragment for sequence analysis and software trapping. To minimize the labor necessary for obtaining sequence data, the NN fragments can be used as probes for a single round of genomic library screening. Although the resultant phage population will not contain inserts corresponding solely to the NN fragment locus, the isolated DNA should be of sufficient purity to allow for its use as template for PCR based sequencing. PCR primers can be derived from the NN fragment sequence.

Successful isolation of transcribed sequences flanking NN fragments might best be accomplished with a combination of approaches including not only software trapping but also cDNA selection (Lovett et al., 1991; Parimoo et al., 1991) or exon trapping (Buckler et al., 1991; Duyk et al., 1990). As with the identification of transcripts using Northern and Zoo blots, these techniques will require the prior isolation of flanking genomic DNA. In an effort to concentrate only on those genomic clones that contain sequences corresponding to a RA regulated transcript, we have initiated development of a preliminary nuclear run on assay in which several NN fragment-associated genomic clones can be tested simultaneously. This technique has been used extensively in the laboratory of our colleague, Dr. Alain Nepveu, who will provide protocols and technical expertise. I have previously adapted existing techniques for optimal isolation of F9 EC cell nuclei (see chapter 3). Using this technique, nuclei will be isolated from F9 EC cells grown in the presence or absence of RA and incubated with [α - 32 P]UTP. Transcripts which were initiated prior to isolation continue to be transcribed, with incorporation of the radiolabeled UTP. These transcripts can be used to probe genomic DNA clones which have been immobilized on nylon membrane. The RAR β 2 genomic clone described in chapter 3 would serve as a control. Both the genomic clones and the radiolabeled transcripts contain repetitive sequences which can hybridize and interfere with signal derived from the gene of interest. To overcome this problem, unlabeled sheared mouse genomic DNA and/or mouse cot-1 DNA can be included in the hybridization reaction. Concentrations which obscure signals due to repetitive DNA and allow visualization of target gene hybridization will be determined using the RAR β 2 genomic clone.

2.3 NN-RARE fragments as tools to study retinoid receptor action

The primary goal of the CpG-SAAB method is the isolation of target genes which are directly regulated by retinoic acid. However, the RARE/NN fragments isolated using CpG-SAAB can also provide valuable information about the mechanisms of RA

regulated transcription. CpG-SAAB not only provides a source of novel RAREs, it also allows for the isolation of RAREs within an original genomic sequence context. It seems likely that interplay between the RARE-binding retinoid receptors and unidentified factors which bind to adjacent sites plays a role in the regulation of the associated genes. These fragments thus provide a preliminary opportunity to study various aspects of retinoid receptor action. For instance, do specific combinations of RARs and RXRs preferentially activate a given RARE? What impact does the surrounding genomic sequence have on the activity imparted by the RARE, and does the RARE function similarly outside of that particular genomic context? Is the RARE bound by other nuclear receptors which contribute to its regulation? Does the RARE itself, or the RARE and surrounding genomic sequence (NN fragment) have cell-specific transcriptional activity? To date, 8 RAR isoforms and 6 RXR isoforms have been characterized (Giguère, 1994; Liu and Linney, 1993, Chambon, unpublished data; Nagata et al., 1994). Heterodimers of these receptors bind with high affinity to RAREs and RXREs. It is evident that *in vitro*, most if not all heterodimer combinations of RAR and RXR bind with similar efficiencies to these response elements (Leid et al., 1992). Targeted mutations in single receptors leads to phenotypes which are normal or less severe than expected, suggesting that there may be some functional redundancy between the receptors (for review see Kastner et al., 1995). However, several lines of evidence, including distinct expression patterns during embryogenesis as well as N-termini which are well conserved across species, point towards individual roles for each retinoid receptor (reviewed in Giguère, 1994; Hofmann and Eichele, 1994). The establishment and comparison of mice strains lacking two RAR genes revealed that certain defects are isoform specific and supports the notion that RAR isoforms perform specific functions (Lohnes et al., 1994; Luo et al., 1996; Mendelsohn et al., 1994). In addition, differences have been observed in the abilities of various RARs and RXRs to promote transcriptional activation on several RA responsive promoters. Using truncated receptors and chimeric

constructs it was determined that each RAR or RXR contains two transcriptional activation regions; the N-terminal ligand independent AF-1, and a ligand-dependent AF-2 located in the LBD (Folkers et al., 1993; Nagpal et al., 1993; Nagpal et al., 1992). Both the type of the RA response element and the promoter context seem to contribute to the differential transactivation activities imparted by the various receptors (Nagpal et al., 1992).

The RARE-NN fragments provide a 'panel' of binding sites on which to test the transactivation potentials of combinations of RARs and RXRs. The NN fragments listed in Table 2 (chapter 3) have been linked to the luciferase reporter gene driven by both a thymidine kinase minimal promoter and a basal promoter (TATA box only). P19 EC cells yield high levels of activity in assays using either of these reporters and would be ideal for this study.

A related study will examine the question of whether the RARE contained within the NN fragment functions similarly in transactivation assays when it is isolated. The transcriptional activities of the receptor on a given promoter likely reflect interactions between the AFs of the receptors and those of other transactivators (or repressors) bound to adjacent promoter regions. Studies of the transcriptional activation abilities of the retinoid receptors are often performed using transient transfections with reporter vectors consisting of an isolated response element, often multimerized to confer greater levels of activity, linked to an assayable reporter gene. However, transcriptional activity imparted by the receptors through an isolated response element has been shown to be dissimilar to the activity generated when the response element is within its promoter context (Nagpal et al., 1992). The RA responsive profile of NN8-4 in P19 EC cells is shown in Table 2 and Figure 6B, chapter 3. This fragment generates a high basal activity which is elevated 5 fold upon RA treatment to a level approaching that seen with the control, NN-RAR β . Preliminary data suggests that a different profile is seen when the RXRE alone is linked to a reporter vector, transfected and subjected to RA treatment. Under such

circumstances the NN8-4 RXRE functions much like the CRBP-II-RXRE, which is transactivated to high levels only with 9cRA treatment and cotransfected RXR expression vector (Levin et al., 1992 and our unpublished data; Mangelsdorf et al., 1991). It will be interesting to examine whether NN8-3, which contains a perfect consensus DR5 RARE and gave rise to a surprisingly low level of transcriptional activity upon RA treatment (see Table 2, chapter 3), yields greater transactivation activity when isolated away from the surrounding genomic sequence.

As mentioned in chapter 3, it is also possible that the low level of activity seen upon RA treatment of NN8-3 could be due to competition for binding by other nuclear receptors. As mentioned in chapter 3, nuclear receptors which bind as monomers have been shown to require an A-T rich region extending 5' from the core half site (Giguère et al., 1994; Harding and Lazar, 1993; Wilson et al., 1991). Preferred sites for such orphan receptors may be contained within the NN-RAREs, leading to interference with RXR/RAR binding. ROR α , an orphan nuclear receptor which functions as a monomer, can bind to and activate through a RARE in the promoter of the γ F-Crystallin gene, although RXR/RAR heterodimers repress this ROR-dependent activation by competing for occupancy of the γ F-RARE (Tini et al., 1995). Homodimers of the orphan receptors ARP-1/COUP-TFII or EAR3/COUP-TFII bind a RARE in the Oct3/4 promoter with an affinity higher than that of RXR/RAR heterodimers, and abolish RXR/RAR induced activation of the Oct-3/4 promoter completely when transiently transfected into P19 EC cells (Ben-Shushan et al., 1995). These two orphan receptors are upregulated in P19 EC cells with RA treatment (Ben-Shushan et al., 1995). The possibility that orphan receptors contribute to the regulation of NN8-3 and the other genomic fragments will be investigated using gel mobility shift assays and cotransfection assays.

The transcriptional response to RA conferred to the reporter vector by an NN-RARE may be influenced by cell-specific factors. The surprisingly low activity generated by NN8-3 in P19 EC cells may be due to the presence of a repressor which acts

on nearby genomic sequences, or directly on the receptors due to their particular conformation on NN8-3. Cell-specific activating factors may account for the high basal activity seen in transient transfections of NN8-4 TKLUC. Possible interactions will be investigated with transient transfections of various cell types, by co-transfecting NN-RARE TKLUC reporter vectors along with retinoid receptors.

2.4 Further analysis of the RA responsive gene NN8-4AG

To study the function of NN8-4AG, we first propose to examine the expression pattern of this gene in the developing embryo and in the adult mouse. Preliminary data obtained by Northern blot analysis suggests that the NN8-4AG transcript is expressed in 13.5 day old murine embryos (data not shown). In situ hybridization of both whole mount and sectioned embryos is routinely performed in our laboratory. The murine cDNA of NN8-4AG has been isolated and is currently being sequenced. RNA probes derived from this cDNA will be used to determine the temporal and spatial expression of NN8-4AG at various stages in embryonic development. In addition, possible changes in the domains of NN8-4AG expression will be assessed in embryos which have been exposed to RA (for examples see Conlon, 1995 and references therein; Lyn and Giguère, 1994). Further insight into gene function can be obtained by studying the effects of mutation or deletion of this gene. To this end, a genomic clone encoding the N terminal region of NN8-4AG (see Fig. 9A, chapter 3) will be used in the construction of a gene targeting vector for homologous recombination in embryonic stem cells.

The putative protein that is coded for by EST41159 contains a domain near its C terminal end that is homologous to the C-terminal regions of a yeast putative protein and the *Drosophila trithorax* gene product (Fig. 12, chapter 3). *Drosophila trithorax* is a chromosomal protein and a member of a group of proteins that play a role in maintaining expression patterns of homeotic genes of the Bithorax and Antennapedia complexes during development (Chinwalla et al., 1995; Kuzin et al., 1994). The C terminal region

of the *trithorax* gene product is homologous to a number of additional proteins, some that are also known to be associated with chromatin (Stassen et al., 1995). It would be of interest to determine whether NN8-4AG, the murine homolog of EST41159, is found in the nucleus. To assess the cellular localization of NN8-4AG, polyclonal antibodies can be raised against the protein for immunodetection. In an attempt to avoid cross-reactivity of the antibody with other proteins, the region of NN8-4AG selected as immunogen will be derived from a part of the protein that shows no homology with other known proteins.

As mentioned in chapter 3, the yeast putative protein (P38890) and EST41159 may be more closely related to one another than EST41159 and *Drosophila trithorax* (Fig. 1). The open reading frame of P38890 was revealed by the systematic sequencing of the *Saccharomyces cerevisiae* genome and is derived from portions of chromosome VIII contained in a cosmid numbered 9177 (Genbank accession number U00029). Since EST41159 and yeast P38890 share similar domains throughout their sequences, it is possible that the yeast putative protein is a homolog of EST41159. Functional analysis of this novel protein in yeast could be examined by disruption of the gene and examination of the resultant phenotype. A relatively simple approach is available for gene targeting in yeast, making use of a one-step PCR amplification (Baudin et al., 1993; Lorenz et al., 1995). Primers are designed which have homology to the target gene followed by sequences which will allow PCR amplification of a selectable marker such as HIS3. Target gene sequences, which are external to marker gene sequences in the PCR oligos, are of sufficient length (approximately 30 to 50 bp) to allow for integration at a homologous locus upon transformation into a recipient yeast strain. Restriction map information, derived from sequence available through Genbank databases, will enable Southern blot analysis and detection of homologous recombination events. Using this method it will be feasible to examine whether p38890 is essential for viability in yeast, or whether its deletion shows some discernable effect on phenotype.

EST41159	19	VEVRFVSSAKGKGLFATQLIRKGETIFVE
		+ + + + +
Yeast P38890	84	VEVKFIDDEHGRGLFAKRDFSKGQIILKE
EST41159	106	NCPHCQVMYCSAECRLA
		+ + ++ + +
Yeast P38890	155	DCEVCKAINCSEKCKKA
EST41159	174	KQAKDKDRWIRLFSQFCNKTANEEEEI
		+
Yeast P38890	281	KNIDDETWEKCYELFCGAFPRASEEI
EST41159	308	SCCNHSCVPNAETSPFENNFLLEHVTALEDIKPGEEICISYLDCCQRERSRHSRHK
		+ + + + + + + +
Yeast P38890	333	SFINHDCEPNAYIEQVEEHEELRLHARKPIKKGQIRITYVNPLHGVRLRRRELR
EST41159	360	RHKILRENYLFCSCPCLAE
		+ + + +
Yeast P38890	382	RRRELRVNNGFICQCDRCQNE
EST41159	383	EPNVTSEEEEEEE
		+
Yeast P38890	340	EPNAYIEQVEEHEE

FIG. 1. Extended alignment of the predicted amino acid sequence of human EST41159 to a yeast putative protein. Amino acids that are identical are indicated by a vertical line (|), while conservative changes are designated by a plus sign (+). Genbank accession numbers for EST41159 and the putative yeast protein are U50383 and P38890, respectively.

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Appendix

Appendix 1

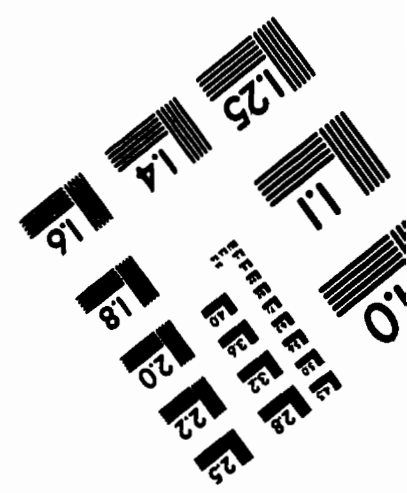
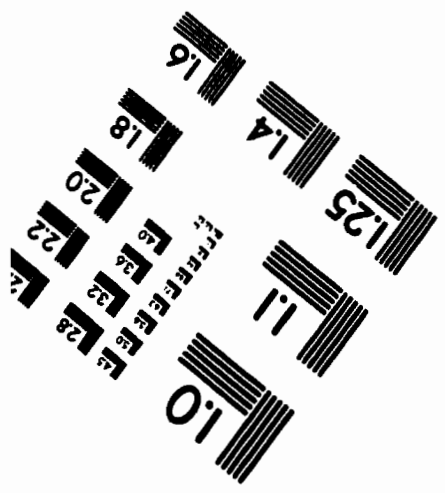
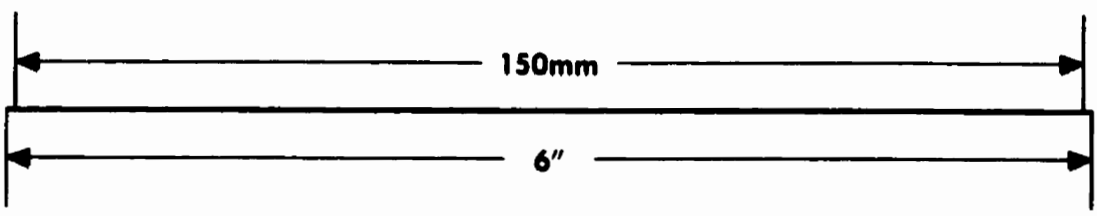
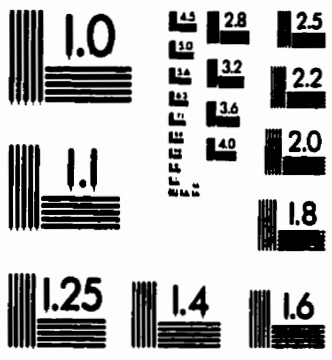
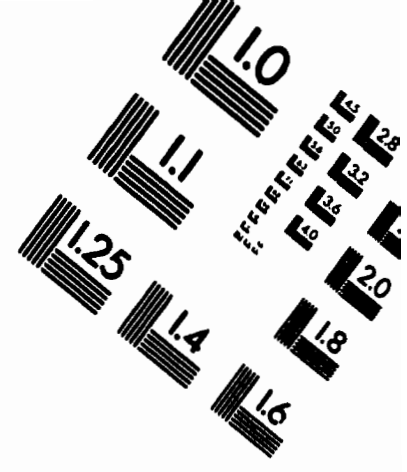
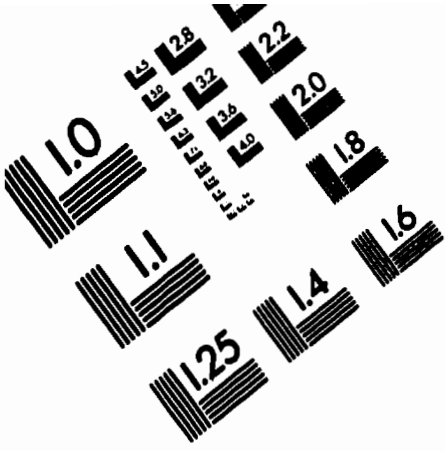
Other published papers related to this thesis in which I was involved are:

Dedhar, S., Rennie, P. S., Shago, M., Leung Hagesteijn, C.-Y., Yang, H., Filmus, J., Hawley, R. G., Bruchovsky, N., Cheng, H., Matusik, R. J., and Giguère, V. Inhibition of nuclear hormone receptor activity by calreticulin. *Nature* 367: 480-482, 1994.

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