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THE ROLES OF THE RETINOIC ACID RECEPTORS IN GROWTH, DIFFERENTIATION AND DEVELOPMENT

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Abstract

In the second chapter of this thesis I have looked at the roles of the retinoic acid receptors (RARs) in differentiation by assessing their actions in the retinoic acid (RA) induced differentiation of P19 cells. I used mutant RARs to block the normal differentiation of these cells and then analyzed the possible mechanisms by which these mutants are able to exert such effects. My results indicate that overexpression of a dominant negative RAR (RARαmyc) is able to block the RA induced differentiation of P19 cells. I believe that RARαmyc acts dominantly by sterically inhibiting the binding of normal transcription complexes to the retinoic acid response element.

In the next part of my thesis, I wanted to assess the role of the RARs in tumourigenesis by overexpressing RARαmyc in the mammary gland in the mouse. Many cancers arise due to a block in the normal differentiation of an undifferentiated precursor cell and I postulated that the RARαmyc, because of its ability to block differentiation, would lead to tumourigenesis in the breast. This transgenic mouse model system has been used to assess the actions of a variety of oncogenes. Expression of my dominant negative RARα did not lead to tumourigenesis in the breast. However due to expression of the mutant RAR in the male reproductive tract these mice were infertile. My data suggests that the infertility arises due to a block in vas deferens caused by excessive production of seminal fluids.

In the last part of this thesis I analyzed a novel mechanism by which

I believe retinoids are able to integrate their growth signals with other growth signals within the cell. I found that E2F, a transcription factor which induces cell cycle progression, is able to specifically inhibit the actions of the RARs in activating transcription. My data indicates that this is due to E2F squelching of factors from RAR transcription complexes. My analysis suggests that a crucial co-activator which is required for RAR transactivation in P19 cells is being sequestered by E2F leading to the block in RAR transactivation. I believe that utilization of common co-activators by opposing growth signalling pathways is one way in which the cell is able to integrate growth signalling information.

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List of Abbreviations

AEV=avian erythroblastosis virus
AF1=activator function 1, refers to ligand independent transactivation domain
AF2=activator function 2, refers to ligand dependent transactivation domain
AP-1=activator protein-1
APL=acute promyelocytic leukaemia
AML=acute myelogenous leukaemia
CAT=chloramphenicol acetyl transferase
CDK=cyclin dependent kinase
CRBP=cellular retinol binding protein
CRABP=cellular retinoic acid binding protein
DMSO=dimethyl sulphoxide
E1A=protein product of the adenovirus early region 1, an oncogenic protein
E2F=E2 promoter binding factor (a transcription factor which activates the E2 site in the adenovirus promoter)
EC=embryonal carcinoma
ER=oestrogen receptor
F9=embryonal carcinoma cell line
GR=glucocorticoid receptor
GRE=glucocorticoid response element
HL60=acute promyelocytic leukaemia cell line
LTR=long terminal repeat, promoter of retrovirus used to replicate the virus
MMTV=mouse mammary tumour virus (often refers only to the promoter in the LTR)
NMU=N-methyl N-nitrosurea, a carcinogenic agent
NSCLC=non small cell lung cancer
P19=embryonal carcinoma cell line
PGK=phosphoglycerate kinase
POU=family of transcription factors including:Pit-1, Octamer proteins and UNC factor
PML=promyelocyte (also used to designate a transcription factor expressed highly in promyelocytes and cloned from a promyelocytic leukaemia)
PR=progesterone receptor
RA=retinoic acid
RAC65=a mutant P19 cell line which does not differentiate in response to RA
RAR=retinoic acid receptor
RARE=retinoic acid response element
RXR=retinoid X receptor
RXRE=retinoid X response element
RB=retinoblastoma protein
TBP=tata box binding protein
TAF=TBP associated factors (factors which bind TBP and cooperate in transcriptional activation)

TFIID=transcription factor II D complex, the complex containing TBP and its associated factors or TAFs.
TGFβ=transforming growth factor β
TR=thyroid hormone receptor
TRE=thyroid hormone response element
VAD=vitamin A deficient
VDR=vitamin D receptor

CHAPTER 1:

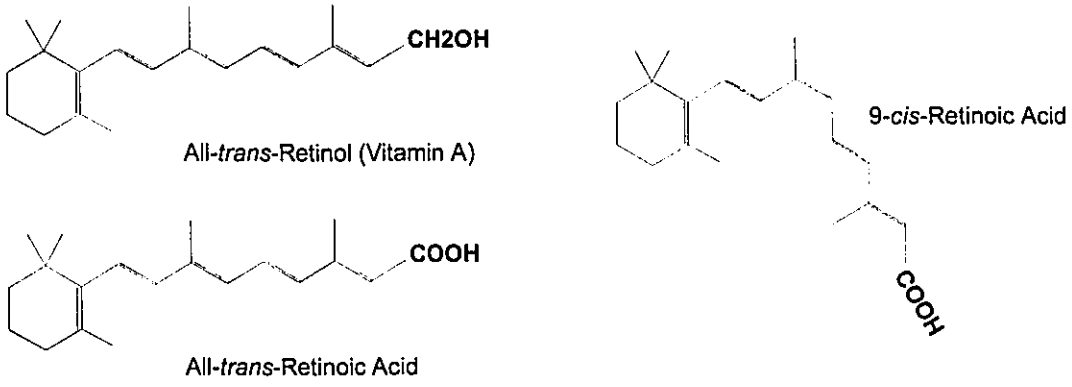
GENERAL INTRODUCTION

1) The Retinoic Acid Receptors

i. Retinoids:

Retinoids are a class of related polyisoprenoid compounds which include vitamin A (retinol) and retinoic acid (RA) (see below fig. 1.1). They are obtained from the diet as either retinol palmitate or its precursor β-carotene. Retinoids are important in normal mammalian

figure1.1



homeostasis because early studies showed that both excess and deficiency of vitamin A in the diet can lead to severe problems in vision, maintenance of epithelial tissues, reproduction, growth and mucopolysaccharide synthesis (1). All-*trans* RA is believed to be the biologically active retinoid in most cellular processes with the notable exception of vision where retinal is the active species (1). Retinoids are also potent teratogens which can induce malformations affecting all three germ lineages during development (2). Because of its striking effects on morphogenesis during development, RA has been postulated to be an endogenous morphogen which specifies body axis, cell migration and positional cues during development although the

role of RA as a morphogen is controversial (3-5). Its most important function in the adult is in the maintenance of epithelial tissues (6).

At the cellular level, retinoids have been shown to exert pleiotropic effects on many cell types and tissues. Early work demonstrated that retinoids were potent growth suppressors (1). Evidence suggests that retinoids exert this effect by shutting down the cell's responsiveness to mitogenic signals. RA is thought to effect this at least in part by the down regulation of AP-1 activity (7) and via the down regulation of the epidermal growth factor receptors present on the cell surface (8,9). Retinoids can also induce the differentiation of many tumourigenic and nontumourigenic cell lines (1) including the promyelocytic leukaemia cell line HL60 which differentiates into mature granulocytes in the presence of RA (10,11). Several embryonal carcinoma (EC) cell lines also differentiate in response to RA. F9 EC cells differentiate into endoderm (12,13) and P19 EC cells differentiate into several different cell types in response to RA; the cell type obtained depends on the dose of RA used ((14), see below).

ii. The Nuclear RARs:

Early studies demonstrated the existence of both cytoplasmic and nuclear binding sites for retinoids (1). Subsequently, cytoplasmic binding proteins for both all-*trans* retinoic acid (RA) and retinol (ROH) called the cellular RA binding proteins (CRABP) and the cellular retinol binding proteins (CRBP) were purified (15-17). The cDNAs for these proteins have

now been cloned and it has been demonstrated that two isoforms exist of each binding protein (15,16,18-20). It has been shown that the activity of various retinoids correlates well with their affinity for the nuclear RA binding sites rather than that for the cellular binding proteins (1). As well, retinoids with biological activity have been found which do not bind the cellular binding proteins (13-*cis*-RA) (21). Therefore researchers concluded that the major biological effectors for retinoids were the nuclear RA binding sites.

This led to the subsequent cloning of the nuclear retinoic acid receptors (RAR). Three isoforms of the RARs have been cloned in both humans and mice (22-28) designated RARα, RARβ and RARγ. They belong to the nuclear hormone receptor superfamily. More specifically they belong to the TR/ER subfamily of nuclear hormone receptors; this subfamily includes the retinoid receptors, the thyroid hormone receptor (TR), the vitamin D receptor (VDR) and the oestrogen receptor (ER) (29). They act as ligand inducible transcription factors. The RARs bear the greatest sequence homology with the TR and similarly they reside constitutively in the nucleus (29). Upon binding of RA they undergo a conformational change which induces DNA binding and results in transcriptional activation of target genes (fig 1.2)(30,31). The RARs have a distinct modular structure with specific regions of the protein mediating specific functions such as DNA binding, ligand binding and transcriptional activation (regions C, E and the A/B and E regions, respectively) (see below fig 1.3). These receptors have been shown to bind RA with high affinity (32,33). Upon binding of RA these proteins can activate or repress transcription (see table 1.1; and references therein).

Figure 1.2. **Mode of Action of Retinoids.**
Schematic representation of the actions of the cellular RA binding proteins and the RARs within the cell. The precise mechanism by which retinoids enter the cell is presently unclear (1).

Mode of Action of Retinoids:

1. nuclear retinoic acid receptor (RAR)
2. cellular retinoic acid binding protein (CRABP)
3. retinoic acid (RA)

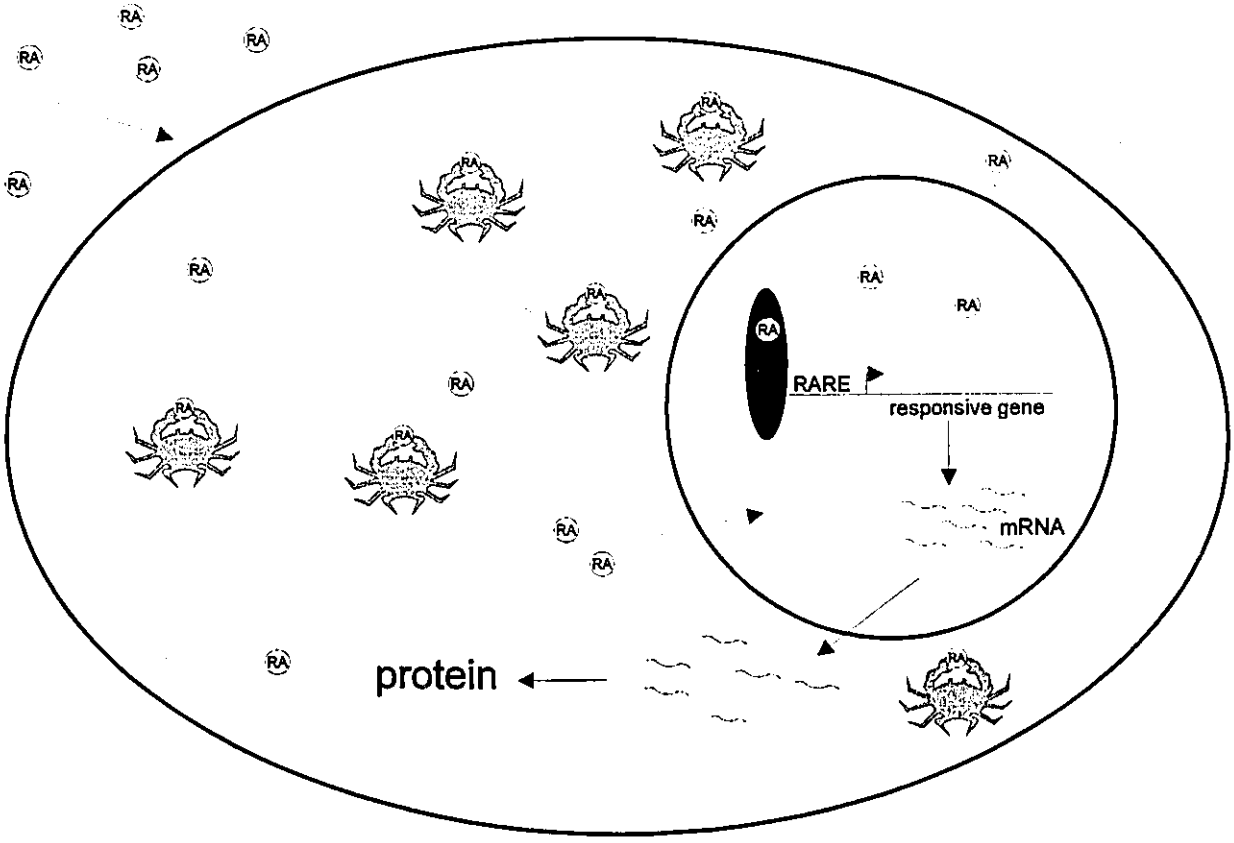
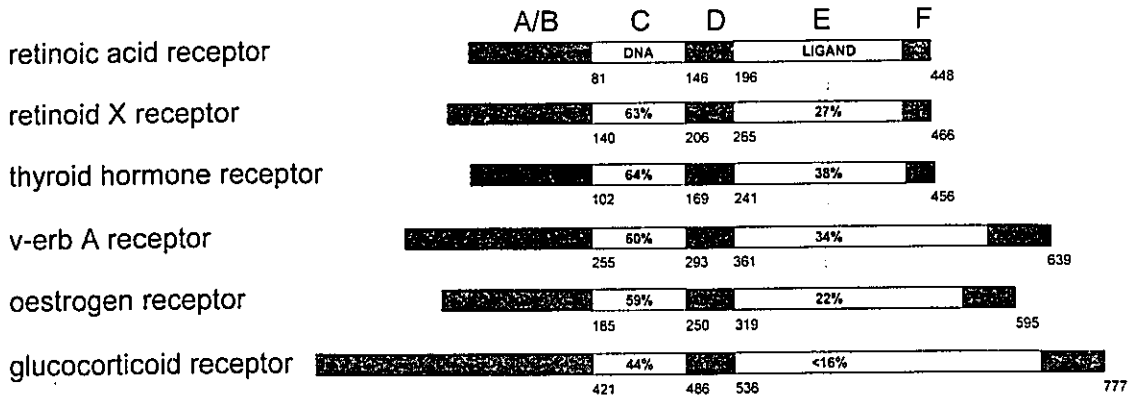


Table 1.1. **Some RA Responsive Genes and their Postulated Mode of Action.**
This represents only a selection of the RA responsive genes found and analyzed to date. This is intended to give insight into the many ways in which retinoids are able to exert such pleiotropic effects throughout the cell. DR= direct repeat (eg. DR5 is a direct repeat of core motif separated by 5 bp), IR=inverted repeat, nd=not determined.

	type of element	regulation	RAR/RXR binding	Function	References
RAR-β	DR5	up	+	development	(44)
Midkine	GGGACGTGACCG	up	+	development	(45)
CRABPII	DR5 and DR1	up	+	regulate retinoid levels	(17,46)
c-jun	4 repeats of nonconsensus site	up	-	growth / transcription	(47)
PEPCK	nonconsensus site	up	+	gluconeogenesis	(48)
Hox A1	DR5	up	+	anterior-posterior specification	(49,50)
Collagenase	AP1 site	down	inhibits binding	metalloprotease in cartilage formation	(51)
Stromelysin	AP1 site	down	inhibits binding	metalloprotease degrades proteoglycans	(52)
HIV-1	IR9	down	+	enhancer for retrovirus HIV	(53,54)
MHC Class I	DR1*	down	+	host recognition	(55,56)
Hox B1	2x DR2*	up	+	anterior-posterior specification	(57-59)
RAR-γ2	DR5	up	+	skin development	(44,60)
REX-1	octamer motif	down	-	differentiation	(61)
γF-Crystallin	everted repeats	up	+	lens / eye development	(62)
oxytocin	2xDR13	down	+	childbirth	(63,64)
LamininB1	4 repeats*	up	+	extracellular matrix component	(65,66)
Complement Factor H	palindromic repeat	up	+	host defence	(67)
EGF-receptor	1 half site	down	+	growth	(9)
N-myc	nd	down	nd	differentiation / transcription	(68)
NGF-receptor	nd	up	nd	sensory development	(69)
Hox D2	DR5	up	+	anterior-posterior specification	(70)
transglutaminase	nd	up	nd	apoptosis	(71)
Oct-3	novel element	down	nd	differentiation	(72,73)
Zif268/Krox-24/Egr-1	nd	up	nd	bone / cartilage development	(75)

Figure 1.3



A family of proteins related to the RARs has recently been characterized, the retinoid X receptors or RXRs (34,35). The RXRs were originally cloned by virtue of their high sequence similarity with the RARs (34). They do not bind all-*trans*-RA with high affinity and their natural ligand is believed to be an isomer of RA, 9-*cis* retinoic acid (9-*cis* RA) (36-38). Similar to the other steroid receptors, the RXRs have been shown to activate a specific set of 9-*cis* RA responsive genes (36,39). Interestingly, many of these genes have been shown to be involved in metabolic functions; these genes include the apolipoprotein A genes (40-42), the cellular retinol binding protein gene (16) and the medium chain acyl CoA dehydrogenase gene (43).

iii. RA Responsive Genes:

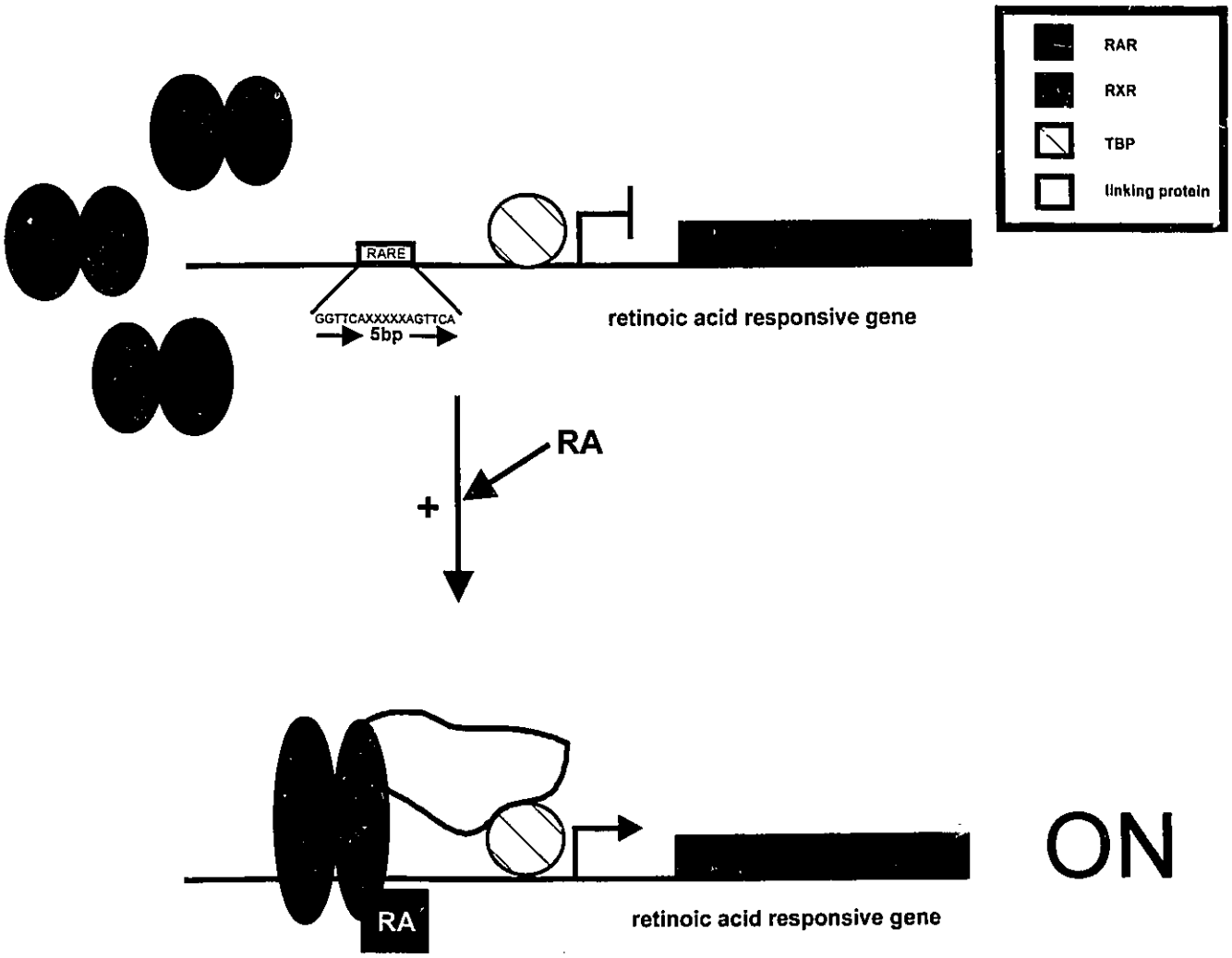
a) The Retinoic Acid Response Element

Several RA responsive genes have been characterized in many different systems. The first RA responsive gene to be characterized was the RARβ itself (76,77). In RA treated EC cells the transcript for the RARβ is rapidly induced within hours after the addition of RA (23). It was proposed to contain a specific promoter region which was activated by RA (like the other steroid hormone receptors). Subsequent cloning of the promoter region of this gene in both mouse and man delineated the functional properties which confer RA inducibility on a gene (76,77).

Like the response elements for the other steroid hormone receptors the RAR retinoic acid response element (RARE) exhibits dyad symmetry (76,77). The RARs generally recognize a specific core binding motif of 6 base pairs (G/AGT/GTC/GA) which is repeated as a direct repeat spaced by 5 nonspecific bases (figure 1.4)(76-78). This entire element is designated the RARE. Some RAREs have been found which do not exactly follow this consensus spacing of the core elements (see table 1.1). The core binding motif recognized by the RAR is identical in sequence to the core binding motif recognized by the TR, the ER, the RXR and the VDR (78,79). The major difference between these elements which allows for specific gene activation by specific receptors is the spacing and orientation of the core binding motifs (78,80). It has been postulated that the development of such a system would enable

Figure 1.4. **Mechanism of Transcriptional Activation by the RARs.**
The RARs are able to heterodimerize in solution but binding of RA allows them to bind the response element with high affinity *in vivo*. This in turn allows for the formation of an active transcription complex which uses accessory factors (also referred to as co-activators or linking proteins) to associate with TBP leading to transcriptional activation of target genes. References are sited within the text.

MECHANISM OF RARE ACTIVATION



a large amount of combinatorial diversity for gene activation within the steroid receptor subfamily. This has been shown to be true for this subfamily of steroid hormone receptors (79) because for example, the RAR has been shown to activate transcription from some palindromic TREs (81,82).

b) RA Responsive Genes

Many additional RA responsive genes have been characterized (Table 1.1). These genes encode many different types of proteins including proteases (51,52) extracellular matrix components (65), growth factors (83-85), metabolic enzymes (17,48), structural proteins (62,86) and many others. Many of the RA responsive genes characterized to date have been shown to encode transcription factors (see table and references therein). It has been postulated that RA is able to bring about such pleiotropic effects because it can alter the array of transcription factors within the cell. Thus some genes which are directly activated by RA harbour RA responsive sequences within their promoter regions. However others are regulated indirectly by RA through the activation of other transcription factors which then affect these other promoters. These indirectly responsive genes do not possess RAREs within their promoter regions. An example of such genes are the AP-1 responsive genes which have been demonstrated to be inhibited by RA through an interaction between the RARs and AP-1 (7). I will only discuss a few of the important RA responsive genes which relate to the following chapters and are known to be directly regulated by RA. Specifically, I will discuss the RA responsive genes which are expressed in P19 EC cells during neuroectodermal

differentiation.

1) The Oct-3 Transcription Factor:

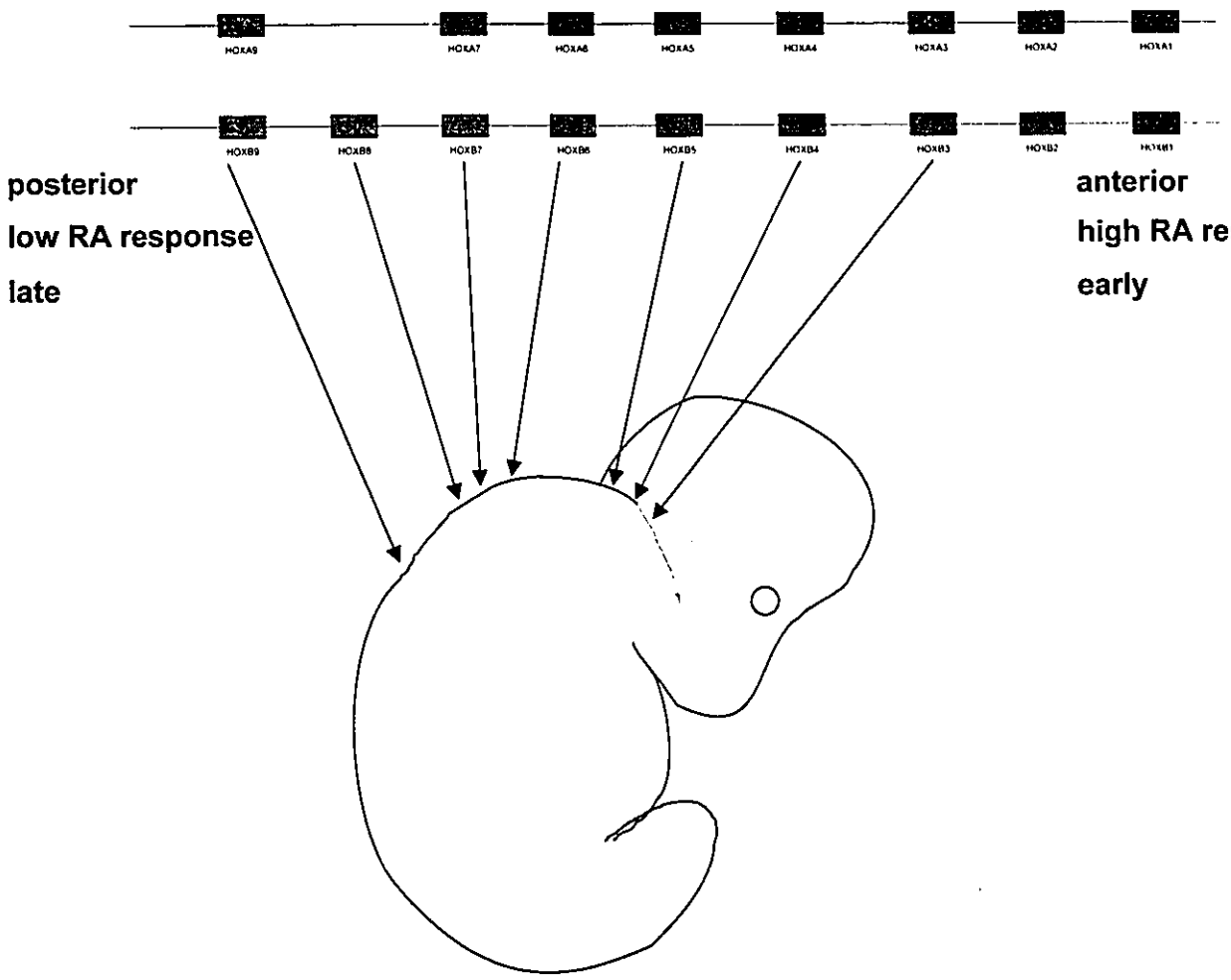
The octamer transcription factor, Oct-3 is rapidly down regulated in response to RA (72). It has been shown to harbour a RARE within its 5' promoter region (72). The Oct-3 gene encodes an octamer binding transcription factor (i.e. it recognises a specific 8 bp DNA sequence motif) which possesses a POU domain as its DNA binding domain (87-89). Oct-3 is implicated in early mammalian embryogenesis because it is highly expressed in pluripotent/totipotent cells but it is rapidly extinguished when the cells begin to differentiate (89-91). Similarly, Oct-3 is highly expressed in undifferentiated EC cells such as P19 EC cells (92). In P19 cells differentiated with RA Oct-3 expression is lost rapidly as differentiation is initiated (89,91-93). Therefore this transcription factor is an indicator of the differentiation status of the cell.

2) The Homeobox Genes:

The homeobox genes are transcription factors which all contain a conserved region of approximately 180 bp which codes for a 60 aa DNA binding motif called the homeobox (94,95). Hox genes (a subset of the homeobox genes) are expressed in a very specific spatio-temporal pattern within the anterior-posterior axis of the CNS during murine embryogenesis (94-97) (fig 1.5). Transgenic mice which have lost the expression of various Hox genes by

Figure 1.5. **Schematic Representation of the Boundaries of Hox Gene Expression Relative to Dose of RA and Position in Cluster.**
Two Hox clusters of the four Hox clusters within the genome are represented at the top and the arrows represent the most anterior boundary of their expression during development. The relative times and doses of RA which induce their expression are also indicated. This is adapted from (94).

BOUNDARY OF HOX GENE EXPRESSION RELATIVE TO POSITION IN CLUSTER



gene knock-out experiments have been shown to display various developmental defects due to deficiencies in the development of specific structures (98,99). The regions affected by each Hox knock-out correlate with to the anterior expression boundary of expression of that Hox gene in the normal embryo (98,99). This has lead researchers to suggest that Hox gene expression provides the embryo with positional cues which specify body plan during development.

The homeobox genes are clustered within the mammalian genome into four separate linkage groups (94,100)(fig 1.5). Within each cluster the individual Hox genes are present in a linear array. Studies of Hox gene expression during murine development have shown that within each group the anterior expression boundary of successive Hox genes becomes more posterior as you move through the cluster (94,100).The homeobox genes have been well characterized as RA responsive genes. Within each linear group of Hox genes their sensitivity to RA decreases with the most anteriorly expressed Hox genes being the most RA responsive of the cluster (94,101,102). The Hox genes are expressed during the differentiation of EC cells and it has been suggested that they also play a role in specifying developmental fates during EC cell differentiation (92,101,102).

3) Midkine:

The growth factor midkine (MK) is up-regulated in response to RA (45,103). The MK gene has been shown to contain a RARE within its 5' promoter region (45,103). Midkine is

a 13-15kDa cysteine rich, heparin binding, growth factor which is expressed in a restricted spatio-temporal pattern during murine development (104). It is expressed between days seven to fifteen of gestation primarily in tissues undergoing epithelial-mesenchymal inductive interactions, mesodermal tissues undergoing remodelling and neuronal tissues (104). MK has neurotropic activity (105-107).

4) The Cellular Retinoid Binding Proteins

The cDNAs for the cytoplasmic binding proteins for both all-*trans* RA and retinol called the cellular RA binding proteins (CRABP) and the cellular retinol binding proteins (CRBP) have been cloned. Two isoforms exist of each binding protein (15-18,108). One is expressed constitutively and the other (CRABP11) is transcriptionally up-regulated in response to various retinoids (15-18,108).

The cellular retinoid binding proteins are expressed during development in the same regions that are affected by dietary excess and deficiency of vitamin A (109-114). This has suggested a possible cellular function for these proteins. The CRABP expression pattern suggests that this protein serves to restrict the access of RA to the nuclear RARs in tissues which require the levels of RA to be low for normal development (111). Conversely, the expression of CRBP transcripts suggests that the function of these proteins is to store and release retinol where high levels of RA are required for specific morphogenic processes (16,112,115). Thus CRBP/CRABP regulation by retinoid may act as a cellular defence mechanism to regulate the free retinoid concentrations within the cell and maintain them

within normal physiological levels (figure 1.2).

iv. Mechanism of Transcriptional Activation by the RARs:

The RXRs have a second function in addition to activating specific 9-*cis* RA responsive genes. The RXRs also form heterodimers with the RARs and other members of TR/ER subgroup of nuclear hormone receptors (116-119). In the case of the RARs this heterodimerization was shown to be essential for the transcriptional response to RA (35,120,121). The heterodimerization of the RAR with the RXR greatly enhances the DNA binding affinity of the RAR for the RARE (35,120,121). This potentiation of DNA binding affinity does not require the 9-*cis* RA ligand (122) and in fact binding of the 9-*cis* RA ligand to the RXR is inhibited in the RAR-RXR heterodimer until RA is bound (123).

Further studies on the mechanism of RA induction of transcription have suggested that the RARs synergise with the TATA binding protein (TBP) during their transcriptional response (124). TBP is an important member of the basal transcription apparatus which has been shown to interact with many different transcription factors including: E2F (125), c-myc (126), dr1 (127), SP1 (128), E1A (126,129), p53 (130,131) and YY1 (128). Some of these interactions have been shown to require the activity of accessory transcription factors or TAFs which associate closely with TBP and together this large multiprotein complex is commonly referred to as the TFIID complex (132). In the case of the RARs the interaction with TBP is thought to require a bridging factor referred to as the cellular E1A like activity

(fig 1.4)(124).

v. Expression of the RARs:

a) In the Adult

The RARα is ubiquitously expressed throughout most tissues in the adult (23,28). The RARβ however is more restricted in its expression pattern; it has been detected in the brain, liver, kidney, heart, ovary, uterus, spleen, pituitary, testes, prostate and adrenal gland (23,27). The RARγ has been shown to be predominantly expressed in the adult skin (23,24). It has also been found at lower levels in the lung and liver (24).

b) In the Developing Embryo

The RARs have been shown to be expressed during murine development in a defined spatio-temporal pattern suggesting a role for retinoids in morphogenesis during development (110). Early embryonic expression of the RARα has been shown to be associated with regions of neural crest cell migration and the hindbrain and spinal cord within the anterior Hox gene expression boundary (108,112). Expression of this transcript (RARα) is almost ubiquitous later in development during organogenesis (111). Other groups have postulated a role for the RARα in spermatogenesis due to its restricted pattern of expression during spermatogenesis (133). The RARβ is expressed in similar regions of the hindbrain and spinal cord as the RARα during early development (108). During neurulation and organogenesis the RARβ exhibits very defined expression patterns which suggest a role for this receptor in mediating specific events during these processes (111,112). The RARγ is not expressed in almost all tissues

undergoing neurulation (112). The developmental RARγ expression pattern suggests a role for this receptor in morphogenesis, chondrogenesis and differentiation of squamous epithelia (134). The RARγ transcript is found in the developing teeth and whisker follicles (134).

c) Direct Evidence for a Role for the RARs in Development

Retinoids have been demonstrated to be present during development of the axylotl, the mouse, and the chick (96,135). The developmentally regulated expression pattern of the nuclear RARs compounded with the well documented teratogenic effects of retinoid excess (2) and developmental defects associated with dietary retinoid deficiency (111,112) suggest that they may normally play a role mediating developmental processes; however, such studies do not directly prove that the RARs normally govern such processes *in vivo*. Evidence directly linking the RARs and embryonic development has been notably scarce. In fact, knock-out experiments which have ablated any one of the RARs or the CRABPs have demonstrated that none of the RARs or CRABPs are individually essential for embryonic development (77,136-139). However, the RARα and RARγ have both been demonstrated to be essential for male reproduction (138,139). Genetic disruption of the RARγ gene, while not embryonic lethal, did produce an abnormal phenotype with defects ranging from skeletal transformations, growth deficiencies and deficiencies in the differentiation of epithelial tissues in the seminal vesicle, Harderian gland and prostate (139). The only retinoid receptor found to be singularly essential for development was the RXRβ by knock-out experiments in which it was found to be embryonic lethal at day 13.5-16.5 due to defects in the development of the chambers of the heart (140). These null mutants also exhibited defects in eye development and

growth (140). Further analysis of compound mutant mice which harbour defects in two receptor subtypes were found to exhibit much more severe defects resulting in embryonic lethality or death shortly after birth (141,142). These defects included craniofacial abnormalities, skeletal abnormalities and defects in organogenesis (141,142). The defects observed are similar to the defects seen in vitamin A deficiency (VAD) which suggests that these effects are mediated by the RARs (139-142). Some abnormalities were observed which had not previously been demonstrated in VAD (141,142). These knock-out experiments have demonstrated that a functional redundancy exists between receptor subtypes and demonstrates a role for the RARs in the ontogeny of many structures during development.

2) P19 Cell Culture System:

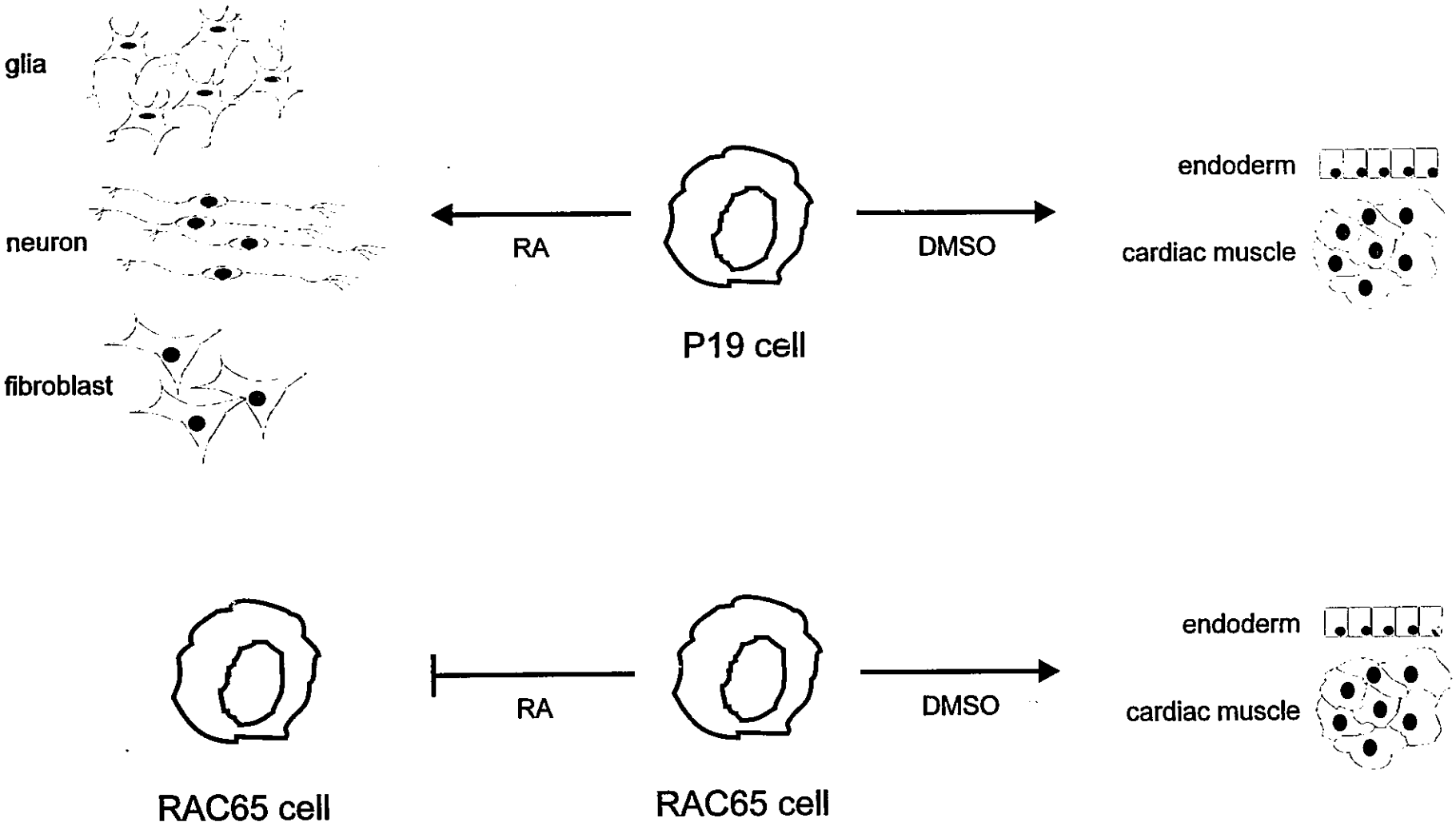
Due to the complex nature of development and differentiation it has become necessary to develop model systems to approximate and study specific areas of development. One such model system is the P19 EC cell culture system. P19 cells are derived from a teratocarcinoma in C3H/He mouse, produced by grafting a 7 day old embryo into the testis of an adult mouse (14). The resulting tumour was excised and subsequently found to be tumourigenic in that it is able to induce tumours upon reinjection into syngeneic mice (14). In tissue culture these cells proliferate and can be maintained in the undifferentiated state indefinitely (14). These cells are a good model system to study developmental processes because they possess a normal karyotype which suggests that they do not carry any gross genetic abnormalities (14). Furthermore they are pluripotent and can be induced to differentiate into derivatives of all

three germ lineages, endoderm, mesoderm and ectoderm, in response to various agents including RA and dimethyl sulphoxide (DMSO) (fig 1.6)(143-147). These cells are also ideal for such studies because they are amenable to transfection with exogenous DNAs and are able to maintain stable expression of transfected DNAs if the selective pressure is maintained (148-151).

A further advantage of this system is that it has been well characterized in its responses to various differentiation agents (14). The neuronal differentiation pathway induced by RA has been extensively studied at both the level of induction (initiation of the differentiation process) and phenotypic differentiation (expression of the characteristics of the differentiated cell type). In this process the cells are treated with $3x10^{-7}$ M RA in monolayer for two days and are then aggregated with RA overnight in non-tissue culture grade dishes. The resulting aggregates are then plated in normal medium (14). They begin to demonstrate the phenotypic characteristics of neuronal differentiation three to four days later, including cessation of proliferation and morphological characteristics such as the production of neuronal processes (14). In this differentiation model neurons, glia and fibroblasts are produced (144). Immunocytochemical analysis of these differentiating cultures demonstrated they lose expression of the embryonic stage specific antigen and acquire the expression of several markers of differentiation including glial fibrillary acidic protein and neurofilament (144), neuron specific β-III-tubulin (152) and several different neurotransmitters (153). Analysis of the regulated expression of several factors has also been carried out in attempts

Figure 1.6. **The Differentiation of P19 Cells and the Differentiation Defective Cell Line RAC65.**
P19 cells can differentiate into neurons, glia and fibroblasts upon treatment with RA and muscle and endoderm upon treatment with DMSO. RAC65 cells have lost the ability to respond to RA but maintain the ability to differentiate into muscle and endoderm upon treatment with DMSO (14).

DIFFERENTIATION OF P19 CELLS



to determine the genes governing the differentiation process. Some of these genes include the Oct-3 (92), murine wingless/wnt1 (154,155), the Hox genes (92), members of the TGFβ family of growth factors (156), the retinoblastoma protein (157), c-myc (158), c-jun (159) and the RARs (92). Many of these factors have been proposed to be important regulators of the differentiation process in both P19 cells and *in vivo* in mouse and man.

The importance of the RARs in mediating the inductive effects of RA has been elucidated in the analysis of a mutant P19 cell line which does not differentiate in response to RA, RAC65. This cell line was produced by continuous passage of P19 cells in the presence of RA and selection for clones which were resistant to the differentiating effects of RA (143). After two rounds of selection a cell line was obtained, called RAC65, which was found to be unable to differentiate into neurons or glia upon subsequent challenge with RA (143). RAC65 cells differentiate normally into muscle in the presence of DMSO (143). Further analysis of this cell line demonstrated that it harboured a mutation in the gene for the RARα (160). Cloning of this mutant receptor cDNA demonstrated that a murine early retrotransposon, ETn, was fused into the C-terminal portion of the ligand binding domain of the RARα message (160). This resulted in the generation of a premature termination codon within the protein coding region which truncated the protein at amino acid 390 (160). This removed a portion of the ligand binding domain of the RARα and generated a protein similar in structure to the v-erbA oncogene (fig 1.3). This mutant RARα protein, designated RARα', was found to act as a dominant negative mutant on the transcription of RA responsive genes within the cell (92).

3) Retinoids, Cancer and Differentiation Therapy

Many groups have studied the effects of retinoids on various cell lines. Early studies demonstrated the ability of retinoids to inhibit the growth of several transformed cell lines (1,161). As discussed previously, retinoids are believed to exert these effects at the molecular level via downregulation of the mitogenic signalling induced by AP-1(7,52-164) and the epidermal growth factor receptor pathways (9). The discovery that retinoids could act as suppressors of the malignant phenotype (165-168) led researchers to hypothesize that these effects could be utilized *in vivo* in the treatment of human cancers (169). Retinoids have since been shown to play both protective and therapeutic roles in cancer (170-175).

Retinoid deficiency has been linked to an increased risk of developing cancer (176-178). The chemopreventitive nature of retinoids is suggested by epidemiological studies which demonstrate a negative correlation between incidence of lung cancer among cigarette smokers and dietary intake of vitamin A (177,178). Another study conducted in China found that the prevalence of precancerous lesions of the oesophagus was lower in individuals receiving active treatment with vitamin A than the placebo treated group (176).

Related studies found that retinoids were also beneficial in the prevention of carcinogen induced tumours in animals. Moon, et al. (179), looked at the development of mammary tumours after treatment with the carcinogen N-methyl-N-nitrosurea (NMU). They found that both the incidence and severity of the resulting mammary carcinomas could be

significantly reduced by administering retinoids immediately after NMU treatment. Other studies have also demonstrated the prophylactic capabilities of retinoids to suppress the development of the malignant phenotype both *in vitro* (165-168) and *in vivo* in the prostate (180), bladder (181,182), intestine (183,184), skin (185) and lung (177,178). Retinoids are also beneficial in the treatment of radiation induced transformation of non-epithelial cell types (186). These studies prompted researchers to look more closely into the process of prevention of tumour development by retinoids.

Studies looking into the processes of tumour development have elucidated distinct phases in the development of the malignant phenotype. In the skin carcinogenesis model, tumourigenesis can be broken down into two distinct phases: the initiation phase (application of a subthreshold dose of a chemical carcinogen) and the promotion phase (application of a noncarcinogenic tumour promoter). Tumour promoters are not themselves carcinogens but act to promote the effects of various carcinogens. Studies looking into epidermal transformation have shown that RA exerts its anti-neoplastic effects during the promotion phase of tumourigenesis (187).

Often the anti-proliferative effects of retinoids are coupled to morphological differentiation of the cell. The HL60 human promyelocytic leukemia cell line has been shown to differentiate into granulocytes in the presence of RA (10). These differentiated cells exhibit both the morphological and phenotypic properties of mature granulocytes (10). In the presence of TPA they differentiate into monocytes (10). It has been demonstrated that these

cells express both the RARα and the RARβ proteins (188). Terminal differentiation of this cell line is believed to be mediated by the RARα protein because studies with RA-resistant subclones of HL60 cells found that in these resistant subclones that an RARα protein was being expressed with a lower molecular weight and a reduced affinity for RA (189). Further studies demonstrated that they were able to rescue the ability of these cells to differentiate by transducing a single copy of the normal RARα cDNA into these cells (189).

The effects of retinoids on the growth and differentiation of acute promyelocytic leukaemia (APL) cells such as HL60 *in vitro* has lead to a clearer understanding of the mechanisms of development of APL *in vivo*. APL in humans is characterized cytogenetically by reciprocal balanced translocation of the long arms of chromosome 17 and chromosome 15 (190,196). It appears that the rearrangement of chromosome 17 is the important feature in leukaemogenesis because patients carrying translocations from chromosomes other than chromosome 15 have been documented (192,193). APL constitutes approximately 10% of all adult nonlymphoblastic leukaemias and is characterized by a predominance of malignant promyelocytes (194). It has recently been shown that this translocation results in transposition of part of the RARα gene from chromosome 17 to a location on chromosome 15 designated PML (for ProMyelocyticLeukemia). This results in a fusion RARα-PML protein which lacks the normal RARα ligand independent transactivation (A/B) domain (AF1) and contains only regions C through E (190,195-198). The exact biochemical nature of this protein is currently under investigation (199,200).

In the early eighties, Breitman et al.(169), showed that RA could induce the differentiation *in vitro* of primary cultures of blast cells from APL patients similarly to that seen with the established APL cell line HL60. These RA treated primary cultures were found to exhibit both the phenotypic and morphological properties of terminally differentiated granulocytes (169). Other types of myelogenous leukaemia blast cells were resistant to the differentiating effects of RA (201). These *in vitro* studies coupled with the genetic evidence correlating disruption of the RARα gene with leukaemogenesis quickly led to the use of RA in the treatment regimens of selected APL patients (202-204). Warrell, et al. (203) have demonstrated the efficacy of using all-*trans*-RA in the treatment of APL in humans (also in (202,205)). Preliminary results are very encouraging because many clinical reports show complete remissions occurring in greater than 90% of patients treated with RA (203,206). Side effects have been minimal in most patients ranging from mild dryness of the lips, headaches and digestive symptoms to leucocytosis (207-209).

Warrell et al.(210) analyzed the myeloid cell population in APL patients undergoing treatment with RA and demonstrated that RA induces the maturation of APL blast cells *in vivo* (also in (211)). They looked for the expression of the abnormal RARα-PML message in the mature myeloid cell population during therapy as an indicator of the numbers of APL blasts induced to differentiate by RA treatment. They found that the clinical response (remission) was associated with an initial persistence (for 2-3 weeks) and then a reduction or elimination of the abnormal RAR transcripts by 5-8 weeks (210). Lo Coco et al. (202), have also demonstrated this response. It appears that RA acts to bring about complete remission

by inducing the differentiation of the leukaemic clone in APL patients (199). It seems however that in many cases that tumour extinction had not occurred because remissions tended to be of short duration and patients subsequently became resistant to the differentiation effects of RA (212,213). The use of RA in conjunction with other chemotherapeutic agents in maintenance therapy may prolong remission or eliminate relapses altogether (212,213).

RA is undergoing clinical trials in the treatment of other cancers and dysplastic conditions (214) as well. The term differentiation therapy has been used to discriminate between these types of therapy from other forms of conventional chemotherapy (213). Treatments like that in APL potentially serve to eliminate the tumour (leukaemia) by terminally differentiating the tumour and is referred to as differentiation extinction therapy (213). Another type of differentiation therapy called chemoprevention or adjuvant therapy, attempts to prevent the reoccurrence of tumours after surgical excision of the primary tumour (213). RA is a good candidate for such regimens because of its abilities to prevent or protect one from cancer.

This second type of differentiation therapy is also being tested *in vivo*. In these preliminary clinical trials the efficacy of RA in inducing the *in vivo* differentiation of tumour cells has been demonstrated. In one case report a patient with a refractory cutaneous lymphoma had initially received conventional chemotherapy after removal of the lesions and had relapsed several times prior to the initiation of RA treatment (21). The recurring tumours

were found to be histologically identical to the primary tumour (21). After his second relapse, combination therapy including 13-*cis*-RA was initiated (21). Upon subsequent relapse the latency was much prolonged and the histology of the tumour excised was significantly more differentiated than the original tumours (21). Thus it appears that RA has potential in affecting reoccurrence of cancer by attempting to eliminate the tumour by terminal differentiation. This type of approach has also been undertaken in the treatment of non-small cell lung (NSCLC) cancer (213). Fewer relapses, fewer metastasis and fewer new primary cancers developed in retinoid treated NSCLC patients when compared to conventionally treated controls (213). Similar studies are underway in the treatment of breast cancer (179,213), melanoma (213), cervical carcinoma (215), AML (213) and epithelial tumours (213,216).

Further studies should elucidate more clearly the mechanisms of actions of retinoids in these processes which should enable us to design more effective and longer lasting therapies for the treatment of cancer in humans. It is clear however that retinoids play an important role in the treatment of cancer in man.

4) Objectives of this Thesis Project:

I initially set out to continue our analysis of the differentiation defective P19 cell line, RAC65 in order to understand more fully how the RARα' acted to block differentiation. Previous studies in my laboratory indicated that other mutations in addition to the RARα' were required for the generation of the RAC65 cell phenotype (160). I first wanted to make dominant negative mutants with all three receptor isoforms to determine if combinations of RAR mutants could act to block RA induced differentiation in a similar way to that seen in RAC65 cells. I postulated that these mutants would also help us assess which subsets of RA responsive genes were regulated by each RAR isoform. However, C-terminal truncation of the RARβ and RARγ did not produce dominant negative mutants like the RARα'. I therefore concentrated on determining the molecular mechanism responsible for dominant negative activity in the mutant RARα protein.

Because of the pleiotropic effects of retinoids on tumour growth *in vivo* I wanted to use our three mutant RARs as probes to understand the mechanisms by which the RARs act in the prevention of tumourigenesis. The initial objective for the creation of these RAR mutants was to express them in transgenic mice to determine the roles of each of the RARs in the development of tumours *in vivo*. Initially, I attempted to generate transgenic mice which expressed these mutants ubiquitously however, all attempts to recover transgenics failed. Therefore I decided to express our mutants under control of a mammary specific promoter (MMTV) to limit expression primarily to the mammary gland.

My final objective was to determine if E2F was the cellular E1A like activity described to be required for efficient RAR transactivation. Previous studies in my laboratory indicated that the retinoblastoma protein (Rb) was regulated in a reciprocal manner to that postulated for the cellular E1A like activity. Rb's major function in the cell is as a negative regulator of the transcription factor E2F. This suggested that the cellular E1A like activity might be the activation of endogenous E2F.

CHAPTER 2:

CHARACTERIZATION OF THE ACTIVITIES OF A DOMINANT NEGATIVE RETINOIC ACID RECEPTOR IN P19 CELLS.

Introduction

P19 cells are a multipotent EC cell line which can be induced to differentiate into various cell types including neurons, muscle and endoderm in the presence various doses of RA (144,146,217). A mutant P19 cell line, RAC65, which does not differentiate in response to RA has previously been shown to harbour a mutant RAR (160). Cloning of this mutant receptor, designated RARα', demonstrated that it was a dominant repressor of a promoter carrying a RARE which suggested that this protein mediated the block to RA induced differentiation seen in RAC65 cells (92,160). Transfection of the RARα' back into P19 cells was only able to generate RA resistant clones similar to RAC65 cells at very low efficiency. Furthermore extremely high levels of the mutant transcript were present in these RA resistant clones which suggested that RAC65 cells carried another mutation in addition to the RARα' which render these cells resistant to the differentiating effects of RA.

In chicken erythroleukaemia caused by the avian erythroblastosis virus (AEV), the virus carries two transforming genes; the v-erbB oncogene which is the primary transforming agent and the v-erbA oncogene which potentiates the transformation of avian erythroblasts resulting in avian erythroleukaemia (218,219). The role of the v-erbA protein in tumourigenesis is to block the normal differentiation of the erythrocyte precursor cells resulting in an enhancement of tumourigenesis and an accumulation of undifferentiated erythrocyte precursors (218,219). At the molecular level, the v-erbA oncogene, which is a mutated form of the thyroid hormone receptor (220,221), acts as a dominant negative thyroid

hormone receptor (222,223). A strong correlation between differentiation and the induction of several thyroid and retinoid responsive genes has lead researchers to conclude that lack of induction of these genes is the cause of the block in the differentiation (224).

Comparison of the structure of v-erbA to that of the mutant RARα' indicate that they have very similar structures (compare figure 1.3 and 2.1) (225). Furthermore, both proteins act dominantly to repress the transcription of specific steroid responsive genes. Analysis of the activities of the RARα' on RA induced transcription demonstrate that it has similar activities to the v-erbA protein's activities on TH dependent transcription. In the case of the v-erbA oncoprotein, many studies have probed the role of this protein in the block to differentiation and how it is able to mediate its dominant effects.

Studies analyzing the mechanism of action of the oncoprotein v-erbA have focused on the role of heterodimerization with other auxiliary factors such as the RXRβ, in the dominant action of this protein (226-229). Current evidence suggests that DNA binding plays a key role in the ability of the v-erbA to act dominantly to inhibit the differentiation of erythrocytes by blocking the induction of TH responsive genes (225,226,230-232).

Early studies with the RARα' did not address how this mutant was able to act as a dominant repressor of RARE function. The deletion in the ligand binding domain in the RARα' suggested that it acted as a dominant repressor because it was unable to bind ligand and therefore be transcriptionally activated. However, this mutant retains most of its

heterodimerization domain, suggesting that this mutant could alternatively function *in vivo* to sequester factors, such as the RXRs, from active transcription complexes rendering them inactive.

Analysis of the actions of these types of dominant negative mutants are important because many types of cancer, including acute promyelocytic leukaemia are believed to arise via the block in the differentiation of an undifferentiated precursor cell (210). In this report, I have investigated the role of the dominant negative RARα in the differentiation of P19 cells. I have also reexamined the mode of dominant negative repression by creating a more subtle mutation in the RARα and by creating C-terminal truncations in the RARβ and RARγ cDNAs to determine if they have similar characteristics to the RARα'. I have found that overexpression of a dominant negative RAR is sufficient to block the RA induced differentiation of P19 cells. Similar to what has been shown with the v-erb A protein, the block in the differentiation correlated with the lack of induction of RA responsive genes. Analysis of the mechanism of action of our dominant negative mutant suggests that it acts by sterically inhibiting the normal receptor complexes access to the RARE.

Materials and Methods

Plasmids and Constructions

a) RAR expression plasmids

All expression plasmids were constructed using the PGK expression vectors PGKδF and PGKδR unless otherwise stated (151,233). The plasmid RAR-βCAT was a generous gift of Dr. H. Sucov (76)(La Jolla). The mutant RARαmyc was constructed by insertion of the epitope for the antibody 9E10 (234) into the BclI site of the human RARα cDNA (26) (P. Chambon, Strasbourg) as described in Maniatis and Sambrook (236). The epitope was encoded by the oligonucletides: coding 5' GATCGAGCAAAAGCTGATTTCTGAGGAG GATCTGCC 3' and noncoding 5' GATCGGCAGATCCTCCTCAGAAATCAGCTTTTG CTC 3'. The mutant RARβLacZ was constructed by fusion of the human RARβ cDNA (237)(M. Pfhal, La Jolla) from aa 1 to 319 (by digestion with StuI) in frame with the coding sequence of the N-terminus of the bacterial β-galactosidase cDNA (plasmid pSDK LacZ digested at NruI). The mutant RARγHA was constructed by insertion of the RARγ cDNA (238)(NruI-MstII fragment blunt ended) into the plasmid pTZ-STY-HA.4 (239) after liberation of its insert with BamHI and blunt ending. The PECE expression constructs were generated by lioeration of the inserts from the PGK expression constructs, blunt ending and subcloning into the PECE vector (240) digested with SmaI. The DNA binding mutant RARαmyc was constructed by PCR amplification of the DNA binding region between the 91bp and 366bp with the oligos 5' ATCGGGCCCTGGCCGCTTGGCATGGCC 3' and 5'

TAGCCTGAGGACTTGTCCTGACAGACAGACAAAGGCAGGCTTG 3'. This mutated region was then subcloned back into the construct PGK-RARαmyc. All mutant constructs were sequenced to confirm they were in frame and western blots verified expression of the epitope tagged proteins.

b) Probes for Northern Blotting

All probes were as described in Pratt and McBurney (92). The probes for the CRABPI and II were the full length cDNA (EcoRI fragments) and were a generous gift of Dr. V. Giguère (18). The MK probe was the full length PstI fragment of the cDNA clone and was a generous gift of Dr. H. Muramatsu (241). The β-III -tubulin was the C-terminal isotype defining (MscI-SmaI fragment) region and was a generous gift of Dr. A. Frankfurter (152). The plasmid mRXR-β was kindly provided by Dr. P. Chambon (Strasbourg)(35). The RXRβ probe was the full length cDNA (EcoRI-BglII fragment). The Noggin probe was the full coding region of the cDNA (SmaI fragment) and was a generous gift of Dr. R. Harland (242).

Cell Lines and Tissue Culture

P19 EC cells were maintained as described previously (14). Briefly cells were maintained in α-Modified Eagles Medium with 5% donor bovine serum (Bockneck) and 5% fetal calf serum (Cansera) with 100μg/ml gentamycin. Cells were seeded on tissue culture grade dishes (Nunclone) at a density of 2×10^5 cells per mL of supplemented medium and subcultured every 2 days. All-*trans* RA (Sigma) was dissolved in absolute ethanol (BDH) at

a stock concentration of 0.1 mM. P19 cells were induced to differentiate towards the neuronal lineage with $3x10^{-7}$ M RA as described in McBurney and Rudnicki (14).

Transient Transfection and CAT Assays

Cells were plated 12 hours prior to transfection on to 60mm diameter tissue culture dishes at a density of $2x10^5$ cells per mL. Cells were transfected with the calcium phosphate precipitation method as described by Chen and Okayama (243) for 8 hours. For each transfection a total of 12 μg of plasmid was transfected: 2 μg of the reporter RARβ-CAT was cotransfected with 2 μg of PGK-LacZ and varying amounts of the experimental plasmids (the plasmid pGEM was added to bring the amount of DNA to 12 μg as necessary). After transfection the dishes were washed with phosphate buffered saline and 5mL of growth medium was added. RA was added to appropriate dishes at a concentration of $3x10^{-7}$ M. Cells were harvested 24 hours later in 0.25M Tris pH 7.8. CAT assays were performed as described by Chen and Okayama (243) and all values were normalized to the transfection efficiencies determined by the internal standard β-galactosidase. Experiments were all performed in triplicate and each experiment was performed at least three times. Standard error calculations are for triplicate samples from a single experiment.

Stable Transfection and Production of RA Resistant Clones

For the isolation of P19 cells non-responsive to the differentiation inducing activity

of RA, 10^6 P19 cells were plated 12hrs prior to transfection on 60mm diameter tissue culture grade dishes. The cells were transfected for 8hrs by the calcium phosphate precipitation method using the following plasmids: 2μg of PGK-LacZ, 2μg of PGK-puromycin, 2μg of B17 (148) and 9μg of the RAR mutant expression plasmids. 24hrs after transfection the cells were plated at a density of $0.5x10^5$ cells/mL and 24hrs later either puromycin (2μg/mL) or puromycin plus RA ($3x10^{-7}$ M) was added to each dish. This experiment was repeated 3 times and the results are reported for each experiment where each plate was completed in duplicate.

Northern Blot Analysis

Total RNA was extracted by the LiCl urea extraction procedure of Auffray C., et al. (239). Total RNA was extracted at daily intervals after RA treatment. 10μg of total RNA was denatured and run on each lane of a 1% formaldehyde gel for 6 hours. The samples were transferred by capillary action to a nylon filter (HYbond N, Amersham) and the samples were crosslinked to the membrane with a GS Genelinker UV crosslinker (Biorad) at 125mJ. Blots were hybridized in 5X SSPE (0.75M sodium chloride/0.005M sodium phosphate/0.005M EDTA), 50% formamide, .5% SDS, 5X Denhardt's (74) and 250μg/ml salmon sperm DNA overnight at 42°C. Probes were labelled with α-^{32}P-dCTP (Dupont/NEN) with the multiprime labelling kit (Dupont). Blots were washed at high stringency (.1X SSC (0.03M sodium chloride/0.003M sodium citrate), .1% SDS) at 65°C for 30 min.

Gel Mobility Shift Analysis

Gel mobility shift analysis were performed as described in Damm et al. (223). Recombinant proteins were produced by *in vitro* translation in the presence of ^{35}S methionine (Dupont/NEN) in reticulocyte lysate using the rabbit reticulocyte lysate kit (BRL). Transcripts were obtained using the Promega transcription system using the T7/T3 bacteriophage promoter. To insure equivalent amounts of each protein were used, the relative efficiency of ^{35}S methionine incorporation for each translation was determined by running each translated protein on an SDS polyacrylamide gel to determine the relative amounts of each translation product and this value was normalized to the total number of methionines in that protein. The labelled probe was the RARE as described by Glass et al. (244). It was labelled with γ-^{32}P-dATP (Dupont/NEN) as described in Maniatis and Sambrook (245). The anti-myc antibody was the monoclonal antibody 9E10 which recognizes a 10 aa epitope of the human c-myc protein (Cambridge Research Biochemical)(234).

Western Blotting

Western blots were prepared as described in Maniatis and Sambrook (246). Proteins were transferred to Nitrocellulose (HYbond C, Amersham) and probed with the antibodies described. The RARα antibody was the polyclonal rabbit antibody #4 and was a generous gift of Dr.M.Ali (Baton Rouge) (247). The influenza haemagglutinin antibody was the monoclonal antibody 12CA5 and was a gift of Dr. J. Bell (Ottawa) (248). The β–

galactosidase antibody was the polyclonal rabbit antibody α–βGal and was purchased from Cappelle.

COS-7 Cell Culture and Transfection

COS-7 cells were maintained in α-MEM supplemented with 10% donor calf serum. Transient transfections were performed by electroporation at a capacitance of 500μF and a voltage 300Volts on a Biorad GenePulser electroporator. A total of 15 μg of each PECE expression construct DNA was electroporated into $5x10^6$ COS-7 cells. Cells were harvested in boiling SDS loading buffer (246) 48hr after transfection.

Results

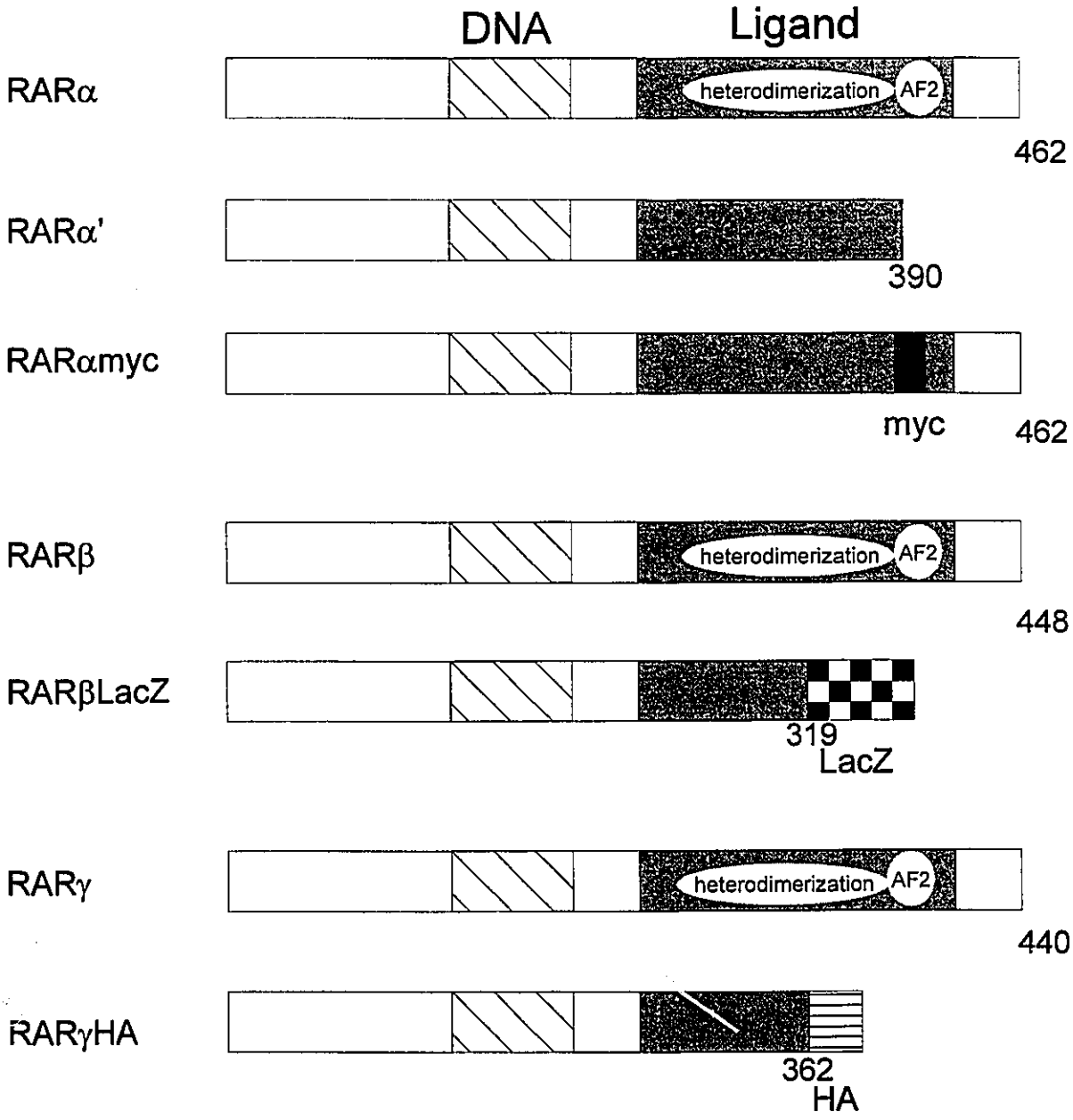
Dominant Negative Mutations in the RARs

It has previously been shown that deletion of the C-terminal 70 amino acids of the RARα results in a protein that represses transcription from promoters carrying RAREs (160).To determine if similar C-terminal deletions of RARβ and RARγ behave similarly I constructed RARβLacZ and RARγHA as shown in fig 2.1. Both proteins encode RARs truncated in the ligand binding domain and fused in frame to sequences encoding the LacZ and influenza haemaggluttinin antigen (HA) respectively.

In the mutant RARα' the C-terminal deletion removes part of the RA binding pocket as well as the ligand dependent transactivation domain (AF2) (249,250). To define more clearly the regions involved in dominant negative activity in the RARα' I inserted the 10 aa myc epitope (234) in frame into the RARα ligand dependent transactivation domain, AF2 (fig 2.1)(249). This disrupts the ligand dependent transactivation domain without affecting the region of heptad repeats which are important in heterodimerization with cofactors such as RXRβ (251).

Expression vectors encoding each of these mutant RARs were transiently transfected into P19 cells along with the reporter RARβCAT and an internal standard PGK-LacZ to determine the effect of these mutant receptors on RA dependent transcriptional activation

Figure 2.1. **Structure of RAR mutants**
Structure of mutant RARs. The mutant RARα' previously cloned from the differentiation defective cell line RAC65 is shown for comparison. The hatched regions denote the DNA binding domain and the shaded regions represent the ligand binding domain (E domain). The myc epitope is shaded black, the Lac Z is represented with the checkered pattern and the haemaggluttin epitope is represented with the horizontal stripes. Numbers denote amino acid numbers. The AF2 domain is the ligand dependent transactivation domain and is depicted with the open circle. The heterodimerization domain is depicted with the open oval and consists of a series of 9 hydrophobic heptad repeats.


DNA
Ligand
RARα
heterodimerization
AF2
462
RARα'
390
RARαmyc
myc
462
RARβ
heterodimerization
AF2
448
RARβLacZ
319
LacZ
RARγ
heterodimerization
AF2
440
RARγHA
362
HA

(fig 2.2a). Like the RARα', the RARαmyc was able to suppress the activation of the RA responsive reporter by the endogenous receptors. In contrast to the RARα' and RARαmyc, the RARβLacZ and RARγHA were unable to suppress the activation of RARβ-CAT. In fact, the RARβLacZ and the RARγHA were consistently found to produce an enhancement of transcription from this reporter relative to the endogenous levels of activation in P19 cells.

To verify that all three mutant proteins were expressed, COS-7 cells were transiently transfected with expression vectors for the three mutants and western blots were done. All three constructs produced proteins of the predicted molecular weight (figure 2.2b).

Dominant Negative Mutation of the RARα Blocks the Differentiation of P19 EC Cells.

P19 cells differentiate in response to RA. To determine whether the mutant RARs affect the response of P19 cells to RA similarly to that seen in RAC65 cells, P19 cells were stably transfected with expression vectors encoding each of the three RAR mutants. Cells were cotransfected with the selectable marker PGK-puromycin and PGK-LacZ which allowed efficient selection of clones which expressed the transfected genes by double selection for both blue colour and puromycin resistance. Selection of the cells was carried out in either puromycin or puromycin plus RA. Colonies were judged as RA resistant clones (RA^r) if they were able to grow in the presence of RA and maintain EC cell morphology (ie they don't differentiate). In cells cotransfected with empty plasmid vector , RARβLacZ or RARγHA none of the colonies were RA resistant (table 2.1). However transfection of the mutant

Figure 2.2. **Transient transfection of RAR mutants.**
Upper panel) Transient transfection of P19 cells with 8 μg of the various RAR mutants along with 2μg of RARβCAT and 2μg of PGK-LacZ (internal standard). After transfection cells were treated with RA ($3x10^{-7}$M) (black bars) or without RA (white bars)for 24 hrs and then harvested for CAT and β-galactosidase assays. Lower Panel) Western blot of COS-7 cells transiently transfected with 15 μg of the PECE expression constructs encoding the three mutant RARs. The RARαmyc (αmyc) protein was detected with the RARα antibody #4 (247) and runs between 46-68 KDa (predicted molecular weight 52 kDa). The multiple bands seen with the RARα antibody probably represent phosphorylated forms of the RAR protein which have been described for the RARs (348). The normal receptor is detected with this antibody in the untransfected COS cells. The RARβLacZ was detected with an antibody against the β-galactosidase protein (Cappelle) and runs below the 68kDa marker (predicted molecular weight 50kDa). The RARβLacZ (βLacZ) protein is detected in the transfected cells. The band in the untransfected cell lane is a cross reacting band from the β-galactosidase antibody. The RARγHA (γHA) was detected with the antibody directed against the haemagglutinin antigen, 12CA5 (248) and runs just below the 43kDa marker (predicted molecular weight 41kDa).

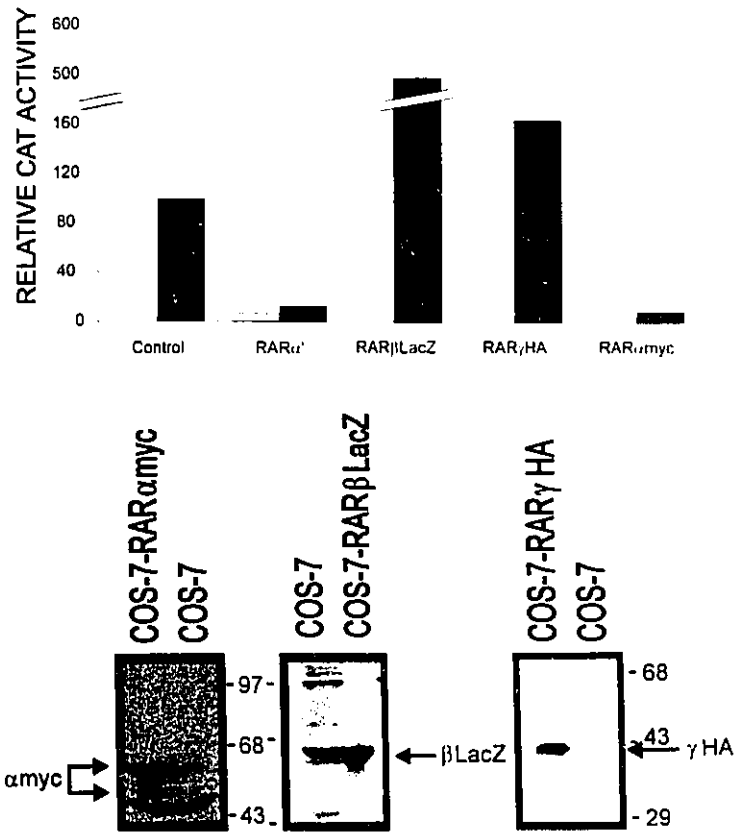

RELATIVE CAT ACTIVITY
600
500
160
120
80
40
0
Control
RARα'
RARβLacZ
RARγHA
RARαmyc
COS-7-RARαmyc
COS-7
COS-7
COS-7-RARβLacZ
COS-7-RARγHA
COS-7
97
68
43
αmyc
βLacZ
68
43
γHA
29

Table 2.1: Stable Expression of RAR Mutants and Selection of RA Resistant Clones

Experiment 1	pGEM	RARαmyc	RARβLacZ	RARγHA
Puromycin resistant colonies/$7x10^5$ cells	71	472	92	57
RA+puromycin resistant colonies (RA^r)/$7x10^5$ cells	0	39	0	0
frequency $RA^r/10^6$ $puro^r$ cells	0	56	0	0

Experiment 2	pGEM	RARαmyc	RARβLacZ	RARγHA
Puromycin resistant colonies/$1.5x10^6$ cells	80	120	30	196
RA+puromycin resistant colonies (RA^r)/$1.5x10^6$ cells	0	4	0	0
frequency $RA^r/10^6$ $puro^r$ cells	0	3	0	0

Experiment 3	pGEM	RARαmyc	RARβLacZ	RARγHA
Puromycin resistant colonies/$5x10^6$ cells	2535	2112	1619	1971
RA+puromycin resistant colonies (RA^r)/$5x10^6$ cells	0	11	0	0
frequency $RA^r/10^6$ $puro^r$ cells	0	2	0	0

Table 2.1. **Stable expression of RAR mutants and selection of RA resistant clones.** $1x10^6$ P19 cells were transfected with 9μg of the mutant expression plasmids, 2μg PGK-puromycin, 2μg PGK-LacZ and 2μg B17. 24 hrs after transfection the cells were subcloned and plated into four separate dishes. Two dishes were treated with puromycin at (2μg/ml) while the other two were treated with puromycin + RA ($3x10^{-7}$M). The puromycin was to select for transfected cells. The combination of puromycin + RA selected for transfected cells which failed to differentiate in response to RA. Failure to differentiate was determined by the ability of the cells to grow in RA and the maintenance of EC cell morphology after 8-10 days treatment. Each experiment was done in duplicate and the entire experiment was repeated three times. Three experiments are shown. The approximate frequencies for production of RA resistant clones for every 10^6 cells plated are given for comparison.

RARαmyc gave rise to RA resistant clones at very low efficiency (table 2.1).

Analysis of Expression of Several Genes in RA Treated P19 and RAC65 Cells

During the RA induced differentiation of P19 cells several genes are activated and repressed. Some of these genes are directly involved in the differentiation program while others are not. To determine which genes are important in the differentiation of P19 cells I decided to examine the expression of several genes during the differentiation of P19 cells and compare this to their expression in RAC65 cells. I also wanted to characterise the RAC65 cells more fully in order to gain insight into the mechanism by which the RARα' acted to block differentiation. RNA was isolated from both P19 and RAC65 cells for several days after treatment with RA and a northern analysis was carried out. Several genes were analyzed including RXRβ, β-III-tubulin, MK, CRABPI and II, Hox A1, A4, A5, Oct-3,and Noggin. All of the genes normally induced in response to RA in differentiating P19 cells were not induced in RAC65 cells (eg. Noggin, MK, Hox genes, CRABP genes, β-III-tubulin)(figures 2.3,4,5,6). Oct-3 is normally downregulated during the differentiation of P19 cells; however it was not down regulated in RAC65 cells indicating that they maintain EC cell character (figure 2.6). The expression of RXRβ was similar in P19 and RAC65 cells although there appeared to be a modest decrease in expression of this gene during differentiation of P19 cells whereas it increased upon RA treatment in RAC65 cells (fig 2.3). Because RAC65 cells fail to appropriately regulate most of the genes tested I cannot ascertain from this analysis which of these genes (if any) are required for the normal differentiation of P19 cells. RAC65

Figure 2.3. **Expression of RXRβ in P19 and RAC65 Cells**
Northern blot of RA treated P19 cells and RAC65 cells prepared as described in Materials and Methods. Probe was the full length mouse RXRβ cDNA (35). The blot was exposed for one day at -70°C.

18S
28S
day 10
day 9
day 8
day 7
day 6
day 5
day 4
day 2
day 1
day 0
day 0
day 2
day 4
day 5
day 6
day 7
day 8
day 9
day 10
P19
RAC65
tubulin
RXRβ

Figure 2.4. **Expression of β--III-tubulin in P19 and RAC65 Cells**
Northern blot of RA treated P19 cells and RAC65 cells prepared as described in Materials and Methods. Probe was the isotype defining region from the β-III-tubulin cDNA (152). The blot was exposed for three days.

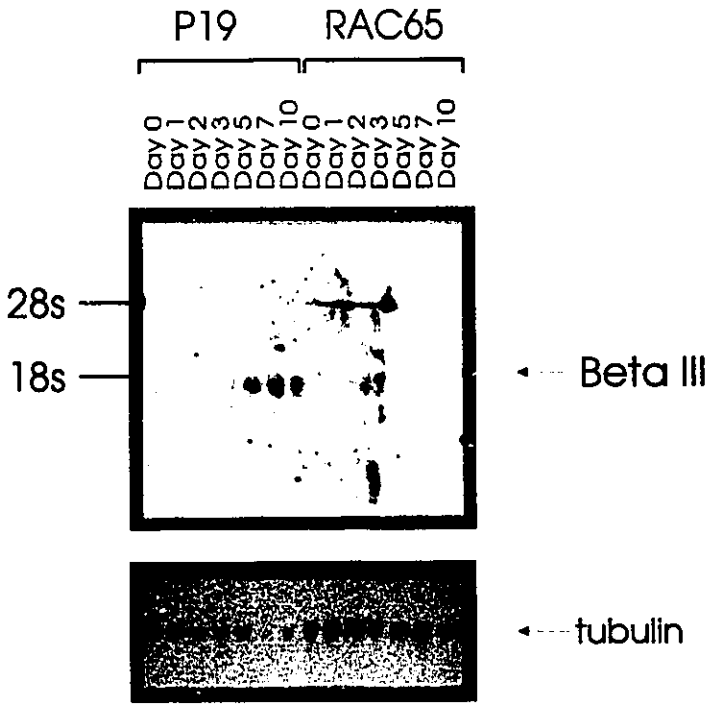

P19
RAC65
Day 0
Day 1
Day 2
Day 3
Day 5
Day 7
Day 10
Day 0
Day 1
Day 2
Day 3
Day 5
Day 7
Day 10
28s
18s
Beta III
tubulin

Figure 2.5. **Expression of Midkine in P19 and RAC65 Cells**
Northern blot of RA treated P19 cells and RAC65 cells prepared as described in Materials and Methods. Probe was the full length mouse MK cDNA (241). The blot was exposed overnight at -70°C.

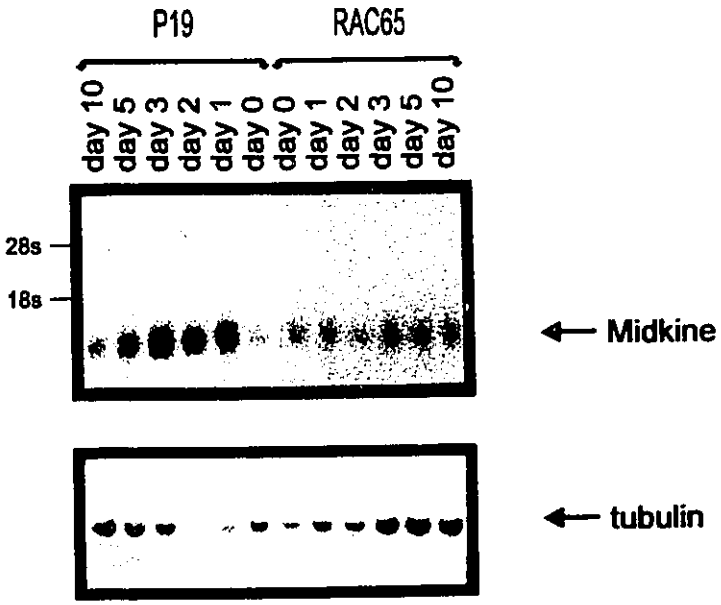
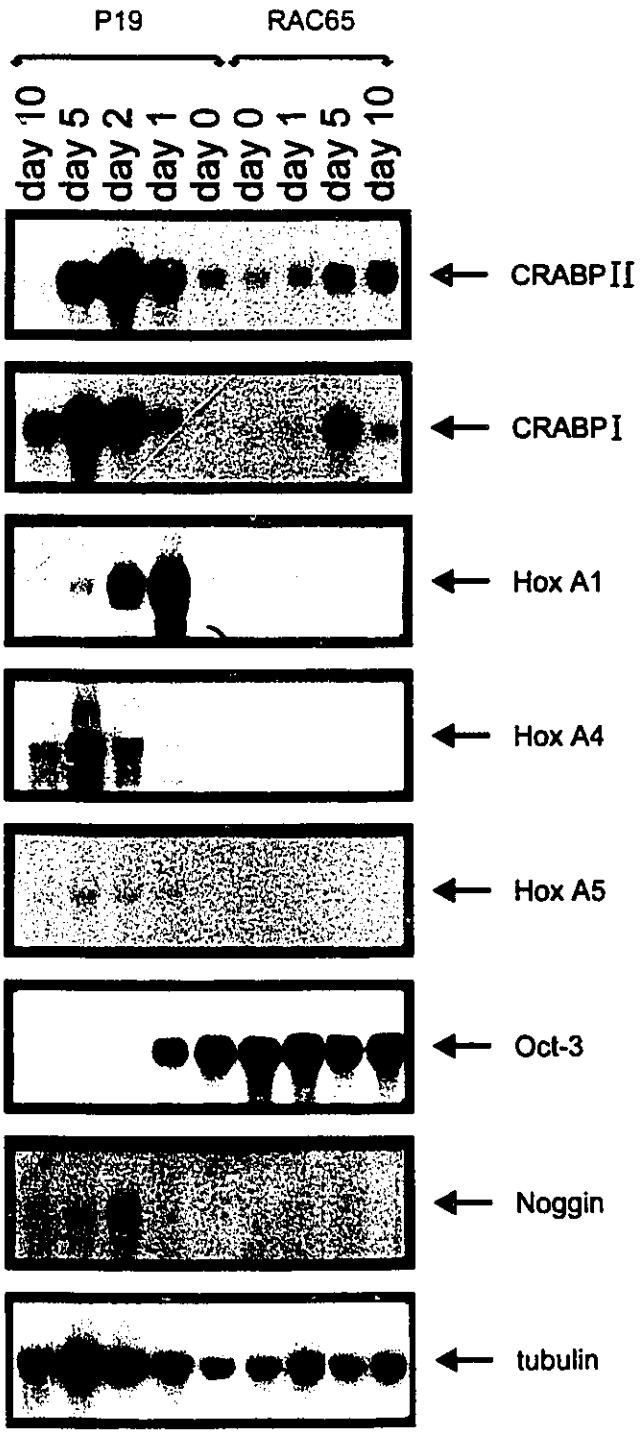

P19
RAC65
day 10
day 5
day 3
day 2
day 1
day 0
day 0
day 1
day 2
day 3
day 5
day 10
28s
18s
Midkine
tubulin

Figure 2.6. **Expression of a Series of RA Responsive Genes in P19 and RAC65 Cells** Northern blot of RA treated P19 cells and RAC65 cells prepared as described in Materials and Methods. Probes are described in Materials and Methods. Exposure times are as follows: Hox A1; 2days, Hox A4; 2days, Hox A5; 3days, Oct-3; overnight, Noggin; 5days, CRABPI and II; overnight tubulin 9 hr all at -70°C.


P19
RAC65
day 10
day 5
day 2
day 1
day 0
day 0
day 1
day 5
day 10
CRABP II
CRABP I
Hox A1
Hox A4
Hox A5
Oct-3
Noggin
tubulin

cells fail to appropriately regulate most of the RA responsive genes analyzed.

Expression of RA Responsive Genes in RARαmyc Expressing Clones.

Representative RA resistant clones produced by transfection with RARαmyc and selection in puromycin plus RA were expanded and analyzed more closely. Northern analysis of 4 of these clones revealed that they express very high levels of the RARαmyc mutant transcript (fig 2.7). The RA^r clones expressed 5 (RA.1), 11 (RA.3), 16 (RA.7) and 56 (RA.11) times higher levels of the mutant transcript than RAC65 cells (as assessed by relative intensity of bands by the phosphoimager). Since only a low percentage of transfected cells gave rise to RA^r clones, this suggests that RA resistance occurs only in cells which express very high levels of the RARαmyc. These clones differentiate normally into cardiac muscle as evidenced by the presence of beating muscle in DMSO treated cultures (data not shown) (143). Therefore, overexpression of the dominant negative mutant, RARαmyc, leads to a block in the normal RA induced differentiation of P19 cells.

In order to ascertain the effects of RARαmyc on the expression of endogenous RA responsive genes the four clones were expanded and aggregated in the presence of RA as in the normal differentiation of P19 EC cells. The expression of several RA responsive genes was analyzed by northern blotting. Expression of RARαmyc was found to alter the expression of several genes known to be RA inducible in P19 cells (92).

Figure 2.7. **Expression of RARαmyc Transcript in RA Resistant Clones.**
4 clones were picked and analyzed for expression of the mutant transcript. 10μg of RNA from each clone was assessed by northern blotting with a probe for the RARα transcript. The blot was exposed for 6 hours at -70°C with intensifying screens. The normal RARα transcript runs in the same region as the lower transcript. The high molecular weight transcripts are likely due to read through transcription from insertion of multiple copies of the plasmid or alternatively may be read through from integrations of the cotransfected plasmids. The normal transcripts expressed in P19 cells and RAC65 cells are not visible at this length of exposure time.

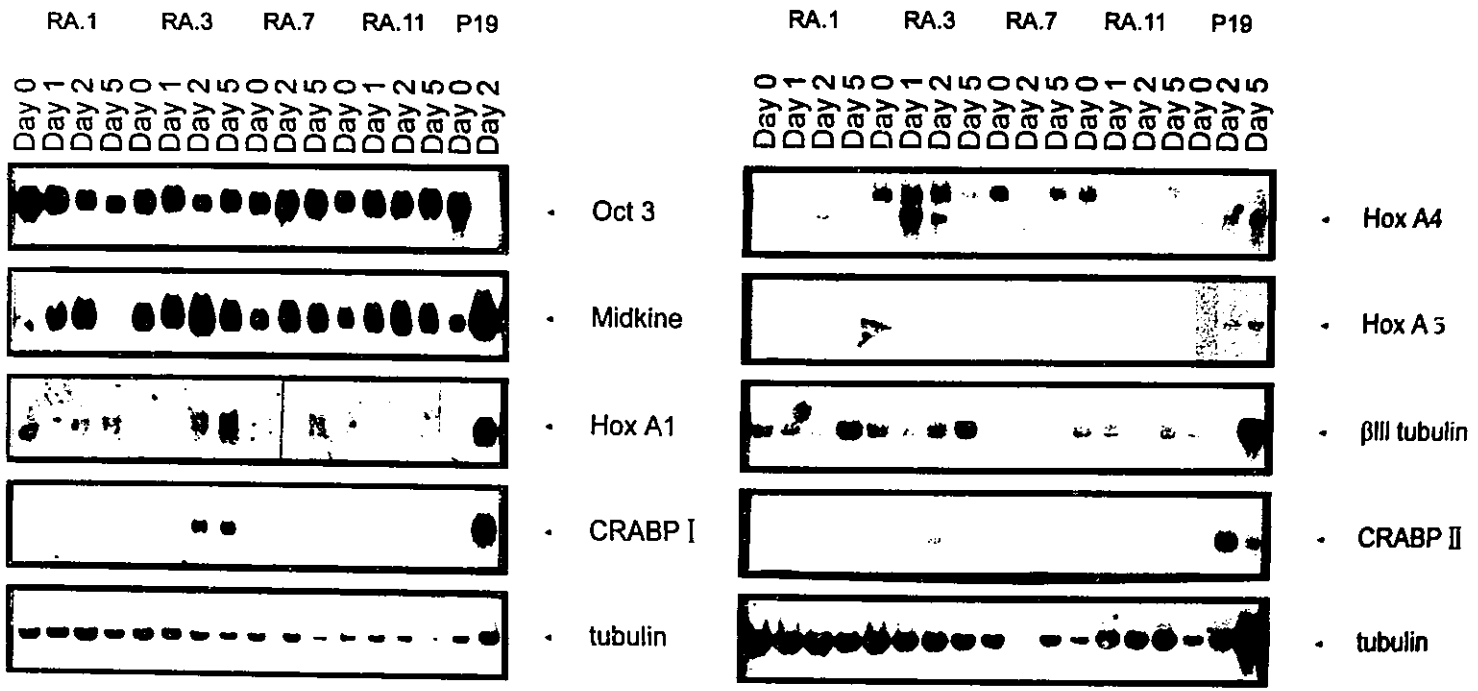
18s
28s
RAC65
RA.11
RA.7
RA.3
RA.1
P19
tubulin
RARαmyc

As expected, the 4 clones did not differentiate in response to RA. Expression of Oct-3 is indicative of early embryonic cell type and it is normally down regulated during differentiation (252). Its sustained expression demonstrates that these RA resistant cells still maintain an EC cell character (fig 2.8). Furthermore the lack of upregulation of the neuron specific β-III-tubulin (fig 2.8) demonstrates that these cells do not express differentiation markers. The induction of the Hox genes and CRABP genes is normally strong in P19 cells but in these clones their induction was much weaker or they were not induced at all (fig 2.8). I found that the later the Hox gene is induced during the normal differentiation program, the more severe the effect the RARαmyc has on its expression (fig 2.8). The expression of midkine (MK) was unaffected by overexpression of the RARαmyc. It was found to be upregulated similarly to that seen in normal differentiating P19 cells (fig 2.8)

The endogenous RA responsive genes were more severely affected in the two clones that expressed the higher levels of the mutant transcript (RA.7 and RA.11). This is consistent with the conclusion that the lack of induction of the endogenous genes is a direct result of the overexpression of the RARαmyc in these cells. These data altogether suggest that overexpression of the RARαmyc leads to a block in the normal RA induced differentiation of P19 cells by blocking the induction of RA responsive genes similarly to that seen in RAC65 cells.

This analysis demonstrates that the endogenous RA responsive genes are not induced when the RARαmyc is overexpressed even though the normal receptors are still present

Figure 2.8. **Expression of RA Responsive Genes in RA Resistant Clones.**
RA resistant clones were treated with RA as in the normal differentiation of P19 EC cells. Control P19 cells were not transfected with the expression plasmids and are shown for comparison. Probes are described in Materials and Methods. Blots were exposed at -70°C with intensifying screens overnight unless otherwise stated. The Hox A1 for the RA resistant clones (the P19 control is a 2 day exposure) was exposed for 6 days. Hox A4 is exposed for two days for the P19 control and 4.5 days for the RA resistant clones. Hox A5 P19 control is exposed for five days and the RA resistant clones are exposed for two weeks. The β-III-tubulin is exposed for 5 days. The RA resistant clones for the CRABP transcripts are exposed for three days. The tubulin standardization is a six hour exposure.


RA.1
RA.3
RA.7
RA.11
P19
Day 0
Day 1
Day 2
Day 5
Oct 3
Midkine
Hox A1
CRABP I
tubulin
Hox A4
Hox A 5
βIII tubulin
CRABP II
tubulin

within the cell. How is overexpression of the RARαmyc able to overcome the effects of the normal protein on transcription of RA responsive genes? Two possible mechanisms emerge from the current analysis to explain how the RARαmyc is able to mediate dominant effects on RA dependent transcription.

In the first model, the RARαmyc exerts such a dominant effect because it forms a complex on the RARE which prevents the active RAR containing complexes access to the promoter thereby blocking its activation. I refer to this type of mechanism as the steric hindrance mechanism.

The finding that the RARs dimerize in solution in the absence of DNA with the factors required for transcriptional activation (253) suggests a second model for dominant activity. In this model the RARαmyc because of its similarity to the normal receptor retains the ability to interact with other cellular factors required for activity of the normal RAR protein. Because the RARαmyc is overexpressed, the high levels of this protein sequester the factors required for RAR transctivation away from normal transcription complexes on the RARE. In this second model the mutant RARαmyc could be acting dominantly because it squelches the normal RARα, the RXRβ or alternatively it could be squelching other uncharacterized factors which are required for RAR transactivation. I call this model the squelching model. To distinguish between these two mechanisms of dominant inhibition of RA responsive genes, a second series of experiments was undertaken.

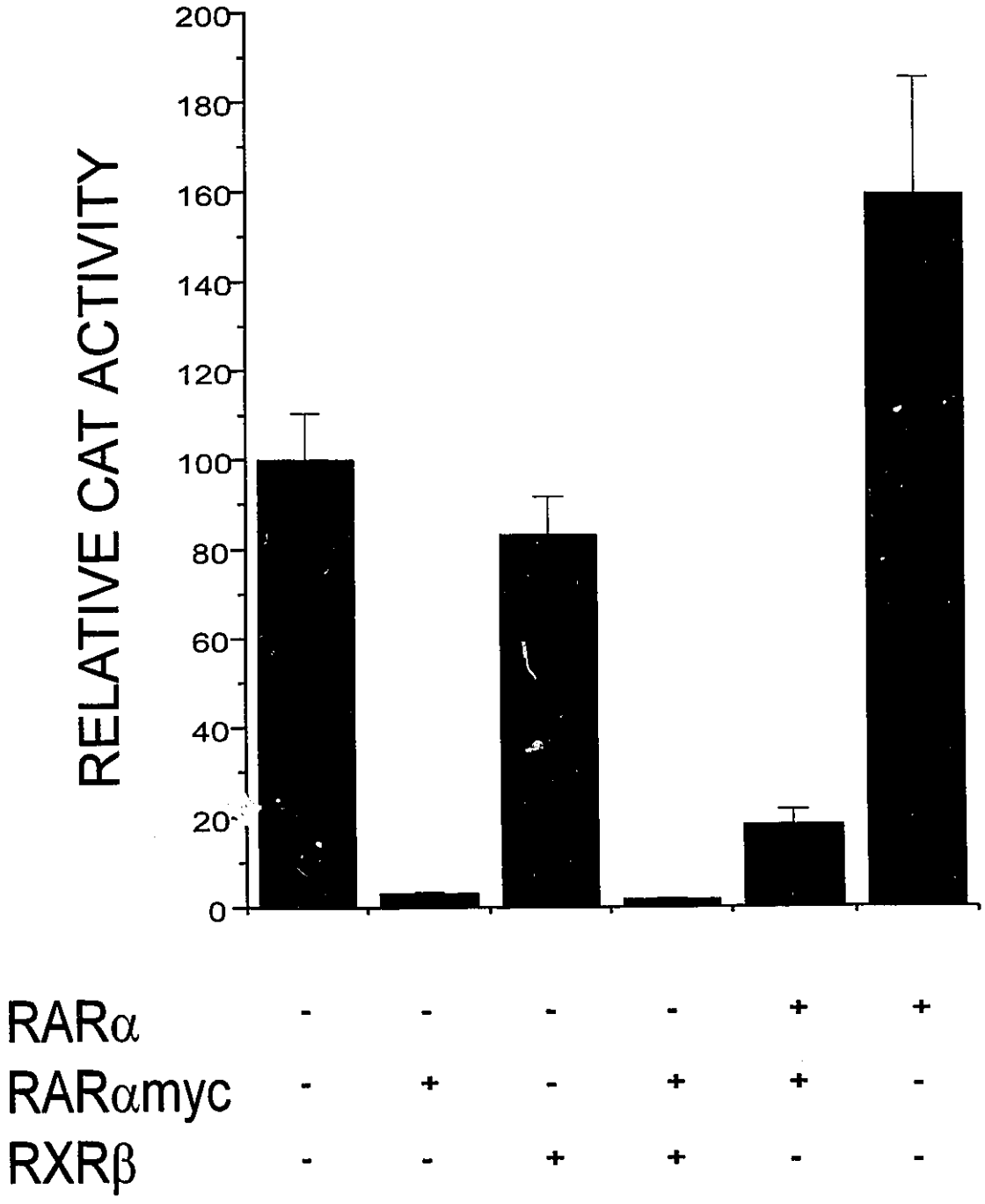
RARαmyc is Not a Repressor Because it Sequesters RARα or RXRβ

If the mutant RARαmyc was dominant because the high levels of the RARαmyc protein within the cell squelched the RARα and RXRβ away from active transcription complexes then addition of an excess of these proteins (RARα and RXRβ) should alleviate the effects of this mutant on the RARβ promoter. I performed competition experiments with the RARαmyc and the normal RARα and RXRβ to determine if this was the case (fig 2.9). The RXRβ was chosen because it is the major RXR isoform expressed in P19 cells and because heterodimerization with RXRs is essential for RAR transactivation. Cotransfection of excess RXRβ did not alleviate any of the dominant repressive effects of the RARαmyc on the RARα promoter (fig 2.9) illustrating that squelching of the RXRβ does not play a significant role in the dominant repression generated by expression of the RARαmyc. Similar to the results with RXRβ addition of excess RARα did not restore RARE activation either. Addition of excess RARα was able to alleviate a small part of the dominant repressive effect of the RARαmyc however, significant inhibition was still observed indicating that sequestration of RARα alone is unlikely to play a significant role in mediating the dominant effects of the RARαmyc protein (fig 2.9).

***In vitro* Interactions of RARαmyc**

A gel mobility shift analysis was undertaken to compare the ability of the mutant

Figure 2.9. **Addition of Excess RARα or RXRβ in the Presence of RARαmyc Does Not Restore RARE Activation.**
Competition experiment with various components of the RAR transcription complexes. 4μg of each plasmid was cotransfected with 2μg RARβCAT and 2μg of PGK-LacZ into P19 cells. After transfection cells were treated for 24 hrs with $3x10^{-7}$M RA and then harvested for CAT and β-galactosidase assays.

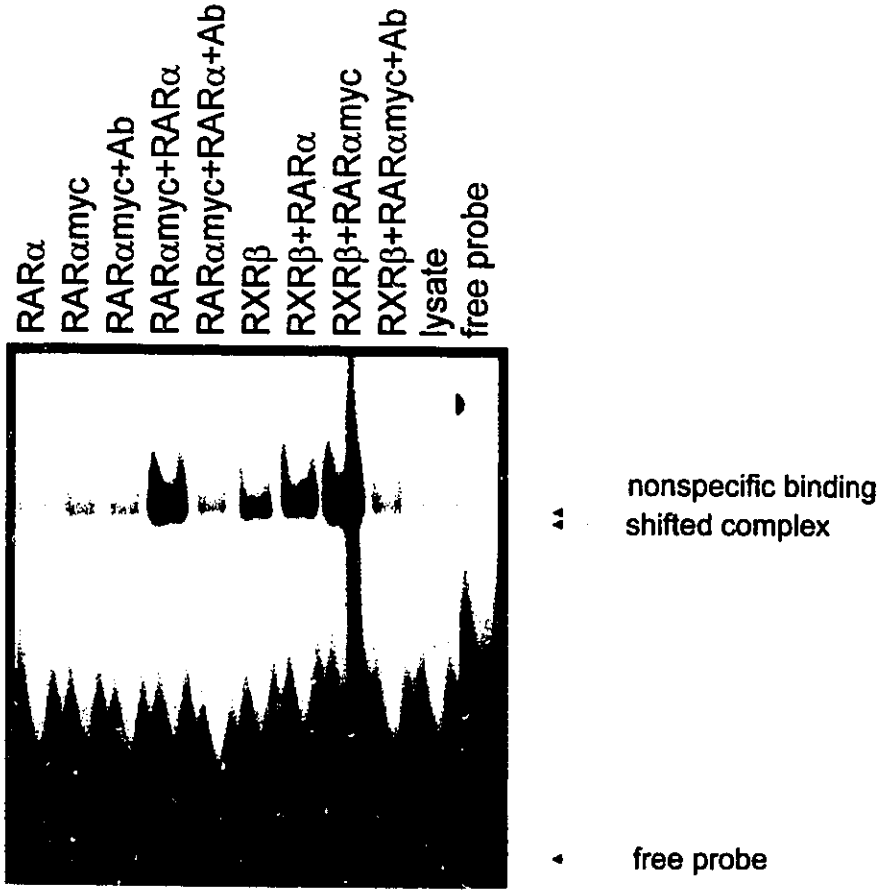

RELATIVE CAT ACTIVITY
200
180
160
140
120
100
80
60
40
20
0
RARα - - - - + +
RARαmyc - + - + + -
RXRβ - - + + - -

protein to interact with the RARE and also with known dimerization partners which are involved in transactivation. This analysis enabled us to determine if the steric hindrance model for dominant repression was a viable option because in order for this mechanism to be correct the RARαmyc has to be able to bind DNA. All proteins were translated *in vitro* in reticulocyte lysates. Both the normal RARα and the RARαmyc bound very poorly (if at all) to the RARE as homodimers but both form heterodimers with RXRβ that bound to the RARE (fig 2.10). To confirm the presence of the mutant protein in this DNA binding complex the gel shifted complex was incubated in the presence of the 9E10 antibody which recognizes the myc epitope in the RARαmyc protein. The anti-myc antibody abolished complex formation between RARαmyc and RXRβ. This is not unexpected because the binding site for the antibody is juxtaposed to the heterodimerization domain of this protein. This demonstrates that this complex contained the RARαmyc protein. The mutant RARαmyc and the normal RARα also formed heterodimers that bound the RARE. This shifted complex is also lost in the presence of the 9E10 antibody which illustrates that the mutant protein containing the myc epitope coding region was present in this complex as well (fig 2.10).

DNA Binding is Required for Dominant Negative Activity of RARαmyc

To distinguish between these two models for dominant activity of this mutant a second mutation was introduced into the RARαmyc to test the role of DNA binding in dominant activity on the RARE. This second mutation changed the first cysteine of the first zinc finger in the DNA binding domain into an alanine. This mutation has previously been shown to

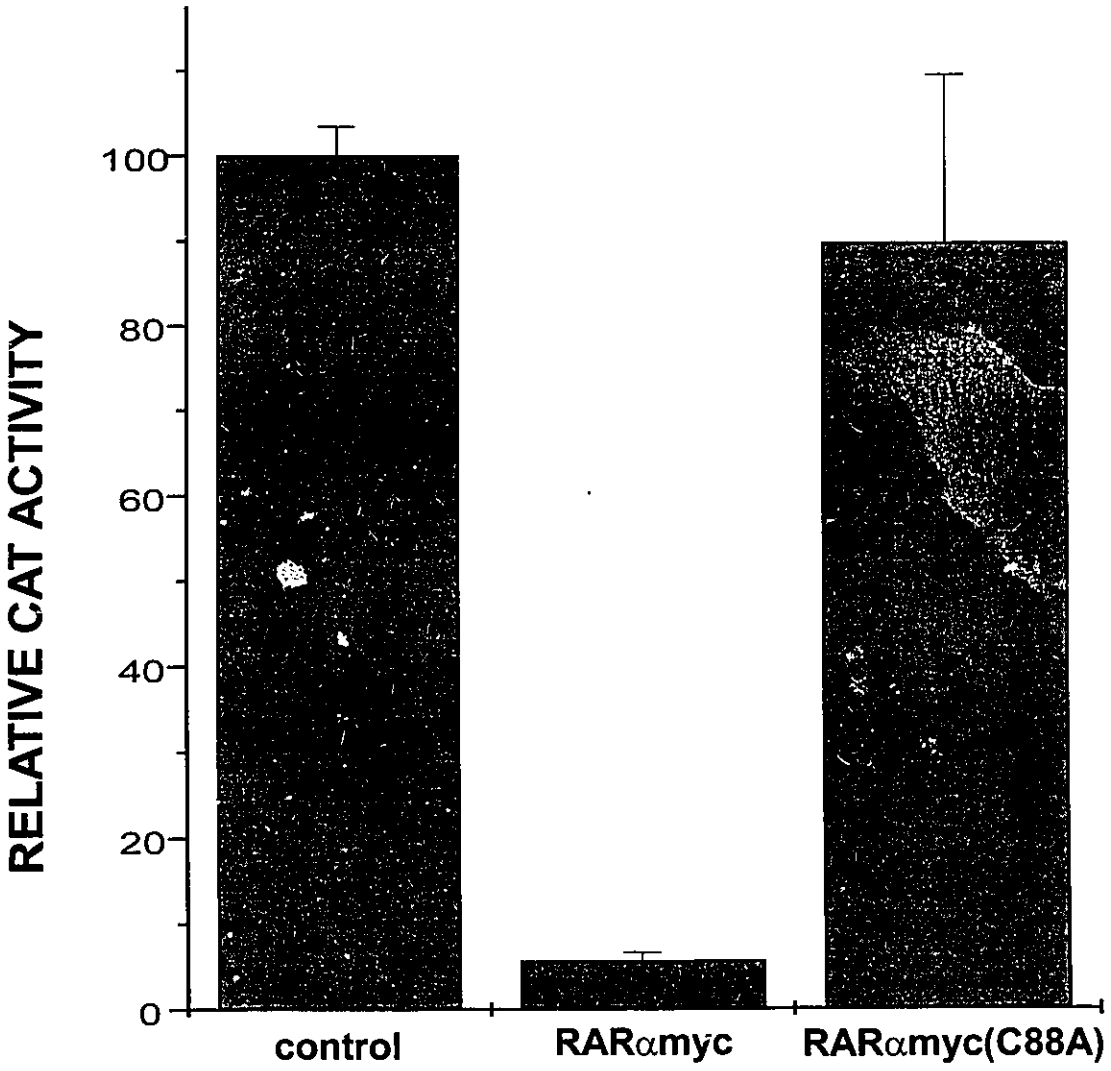
Figure 2.10. ***In vitro* binding properties of RARαmyc.**
Gel mobility shift analysis of *in vitro* translated proteins. The proteins were mixed for 15 min on ice prior to addition of the labelled probe. Antibodies were added to the proteins before the probe was added. After addition of the probe the samples were incubated at 23°C for 20 min prior to loading on the gel. Amounts of each protein were normalized by assessing relative ^{35}S methionine incorporation of parallel translations and dividing this by to the total number of methionines in each protein. The lysate lane is the probe mixed with equivalent amounts unprogrammed lysate and the free probe is the probe with the binding buffer alone.


RARα
RARαmyc
RARαmyc+Ab
RARαmyc+RARα
RARαmyc+RARα+Ab
RXRβ
RXRβ+RARα
RXRβ+RARαmyc
RXRβ+RARαmyc+Ab
lysate
free probe
nonspecific binding
shifted complex
free probe

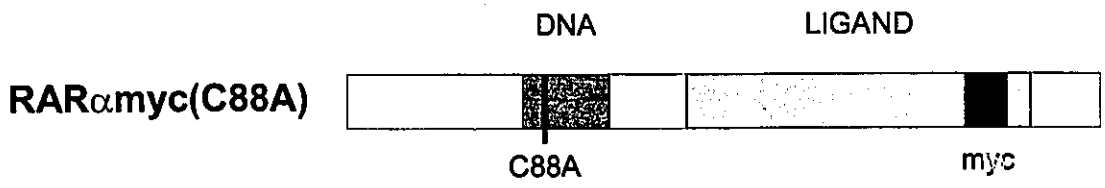
disrupt the first zinc finger in the steroid receptor DNA binding domain (254). The first zinc finger has been shown to be the region of the receptor which makes contact with the DNA and disruption of it therefore abolishes DNA binding. As is demonstrated in fig 2.11, the mutant RARαmycC88A was not a dominant repressor of the RARβCAT. This demonstrates that DNA binding is an essential component in the dominant negative activity of RARαmyc. Furthermore, this indicates that squelching of factors could not contribute significantly to the dominant negative phenotype because RARαmycC88A contains all the domains for protein-protein interactions present in RARαmyc but is unable to act dominantly (fig 2.11). The data presented is consistent with the model where the dominant effect of this mutant is mediated by the mutant inactivating or abrogating the effect of the normal RARα containing complexes by binding the DNA and preventing the normal receptor access to the RARE contain ing promoters (the steric hindrance model).

Figure 2.11. **DNA Binding is Required for Dominant Negative Activity**
a)The mutant RARαmycC88A was created by PCR mutagenesis and sequenced prior to transfection. 4μg of this plasmid or the RARαmyc expression plasmid were transiently transfected with 2μg of RARβCAT and 2μg PGK-LacZ into P19 cells. After transfection cells were treated with $3x10^{-7}$M RA and then harvested. b) Schematic representation of the mutant RARamycC88A. The point mutation generated by PCR is designated with the line.

a.


RELATIVE CAT ACTIVITY
100
80
60
40
20
0
control
RARαmyc
RARαmyc(C88A)

b.


DNA
LIGAND
RARαmyc(C88A)
C88A
myc

Discussion

In this report I demonstrate that insertion of the 10 aa myc epitope within the ligand binding domain (between aa 390 and 391) of the RARα creates a dominant negative RAR, RARαmyc which acts as a dominant repressor of RAR dependent transcriptional activation. The mutant RARαmyc can block the RA induced differentiation of P19 EC cells upon overexpression. This repression is dependent on the ability of the RARαmyc to bind DNA which implies that the mode of action is by heterodimerization and occupation of the RARE to the exclusion of normal RAR complexes.

Role of Dominant Negative RARs in the Block to Differentiation

In this report I have demonstrated a role for the dominant negative RARα, RARαmyc, in the block to RA induced differentiation of P19 cells. I demonstrate that overexpression of RARαmyc leads to a block in the normal differentiation of P19 EC cells. That overexpression of RARαmyc is required to block differentiation is demonstrated by the fact that only the cells transfected with the RARαmyc produced RA resistant clones and all RA resistant clones analyzed express high levels of this mutant transcript. High expression of RARαmyc appears to be sufficient to block the differentiation of these cells, however I cannot rule out the possibility that additional mutations are required for the development of the RA resistant phenotype because of the low frequency of recovery of these clones. I believe that

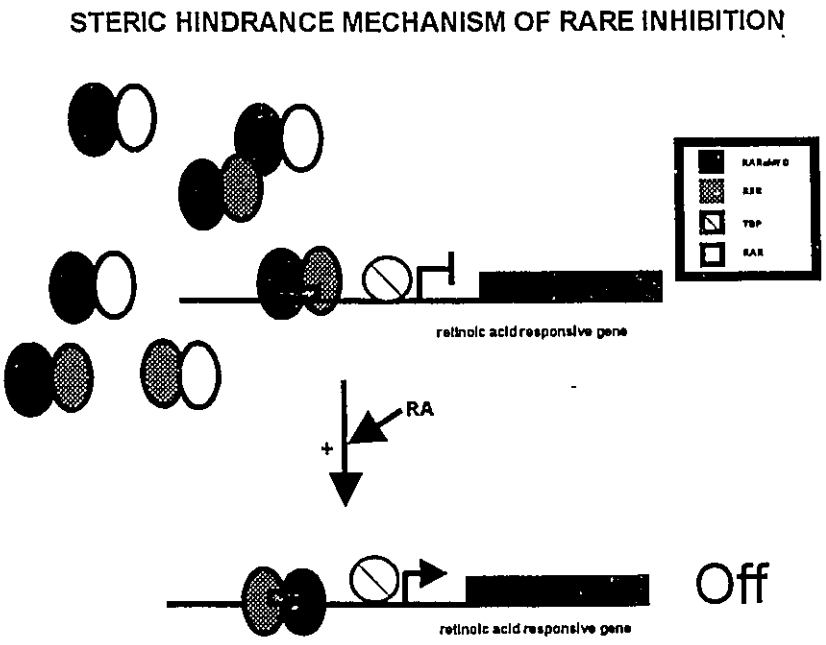
the low efficiency of recovery of these clones reflects the requirement for extremely high transcript levels in order to block differentiation. These are similar results to those reported by Espeseth et al. (255) in F9 cells. This indicates that although these cells (RA^r clones) have a similar phenotype to RAC65 cells (they do not differentiate in response to RA) the etiology of the block to differentiation seems to be different. Further evidence for this hypothesis comes from the finding that MK a gene which was not induced in RAC65 cells was regulated normally in our RA resistant clones (figure 2.8). In RAC65 cells there must be additional mutations which contribute to the RA resistant phenotype because they do not express high enough levels of the mutant transcript as I have demonstrated are required to generate the RA resistant phenotype. It is possible that in RAC65 cells there are mutations in the RAR transcriptional adaptor type proteins which have been characterized (256).

Expression of CRABPs in RAC65 Cells

It is interesting that both CRABPs are induced with RA because only CRABPII has been demonstrated to contain RA responsive sequences in its promoter (17). However, other groups have shown that CRABPI is upregulated in response to RA in F9 cells (108). The expression of the two cellular RA binding proteins was assessed to determine if their overexpression could account in part for the RA resistance of RAC65 cells. I believed that additional mutations were required for the development of the RA resistant phenotype of RAC65 cells because early attempts to isolate RA resistant clones by transfection of RARα' into P19 cells and selection in RA only produced RA resistant clones if the RARα' transfected

cells were passaged in the presence of RA (160). I thought that overexpression of the CRABPs could be the second mutation leading to this phenotype in RAC65 cells because other cell lines which are resistant to the differentiating effects of RA have previously been shown to have highly overexpressed CRABPs (257). Their expression was induced only slightly in RAC65 cells treated with RA in comparison to P19 cells. Clearly, overexpression of the CRABPs could not account for the RA resistance of RAC65 cells.

Proposed Mechanism of Dominant Negative Suppression of RAREs



It has recently been demonstrated that *in vivo* the RAR-RXR complex is not bound to the DNA in the absence of hormone (258). This is consistent with our proposed model which has RARαmyc bound to the DNA even in the absence of hormone (258). Although our work was done *in vitro*, a similar situation should exist *in vivo* because the ligand binding

domain of the RARαmyc has been compromised and it would therefore not be expected to mediate DNA binding *in vivo* as was shown for the normal RAR (258). The presence of the mutant binding to the DNA in the absence of hormone would preclude the binding of the normal receptor when hormone was added. Therefore a vast excess of the normal receptor would be required to effectively compete for the DNA binding sites. This is exactly the result that was observed.

The site occupation model for dominant negative inhibition of transactivation and differentation has been suggested for another protein; DAX-1 (259). In this report the mutant protein was postulated to block the activation of RA responsive promoters by binding to their RAREs and blocking their activation by the RARs. Furthermore, it has been demonstrated that some of the orphan receptors act to suppress RARE activation by similar mechanisms (260,261).

Comparison of the Actions of RARαmyc with the v-erbA Oncoprotein

The analysis of the mechanisms of action of the v-erbA oncogene in leukaemogenesis indicate that this mutant acts by a similar mechanism to the one proposed here for the RARαmyc. By mutational analysis it has been determined that the v-erbA protein exerts its effects on erythrocyte differentiation by blocking the induction of thyroid hormone responsive genes required for the normal TH induced differentiation program of these cells (262). The v-erbA protein also mediates this effect by acting as a dominant negative suppressor. Similar

to the results I have reported here the v-erbA oncogene is thought to block the induction of the genes involved in differentiation by binding the promoters and blocking access to other active transcription complexes (225,227,229,230,232). Similar to our results with the RARαmyc, squelching of RXRβ does not appear to play a crucial role in the dominant negative effects of v-erbA (229).

Functional Domains Required for Dominant Negative Activity

The ligand binding domain (E region) of the RARs has been subdivided into several functional domains including an all-*trans* RA binding pocket (263,264), a heterodimerization domain (a series of heptad repeats) (251,264) and a ligand dependent transactivation domain (AF2) (249). The mutation created by insertion of the c-myc epitope within the E region of the RARα does not disrupt the any of the heptad repeats which are essential in the heterodimerization of the receptor with auxiliary factors such as RXRβ (250). Correspondingly, our mutant RARαmyc was found to interact with the RXRβ efficiently. Our insertional mutation is within the AF-2, ligand dependent transactivation domain described by Barrettino et al.(249). This region was previously defined to be comprised of 35 aa immediately adjacent to the series of heptad repeats in the thyroid hormone receptor and this region is highly conserved between members of this family of hormone receptors (249). However Durand et.al.(250) have delineated a smaller portion of this 35 aa region as the AF2 function and this delineation would not include the region where our epitope is inserted as part of the AF2 domain. In that report (250) the transactivation generated by this smaller AF2

region is considerably less than that generated by the whole 35 aa region described in Barrettino et. al. (249) suggesting that this smaller region while being essential for AF2 function is not sufficient for full AF2 activity (249,250). Consistent with this hypothesis, insertion of this smaller AF2 region into the ARP-1 nuclear receptor was insufficient to confer transcriptional activity on this receptor (250). Barrettino et. al. (249) have demonstrated that there are several highly conserved amino acids which do not lie within the smaller AF2 function designated by Durand et. al. (250). Crystal structure of this entire region (35 aa) in the RXRα has indicated that it likely forms an amphipathic α-helix which sticks out from the ligand binding pocket of the receptor (265). This suggests that the entire 35 aa region functions together as the AF2 domain. It seems therefore that abrogation of this region (AF2) of the RARα is sufficient for dominant negative activity. Other groups have demonstrated that the AF-2 function and the RA binding pocket present in this region of the ligand binding domain of the RAR are separable functions (250,265). Similar to the results reported here it has been demonstrated that disruption of AF-2 function (but not necessarily ligand binding) correlates with dominant negative activity in the RARs (250).

It is not surprising that disruption of the RARα AF2 transactivation domain results in the RARαmyc being a transcriptional repressor because this domain is the major transactivation domain in the RARα (266) and disruption of it should lead to a loss in ability to transactivate as is seen in the RARαmyc. This may be because the binding site for the general transcription machinery is lost or disrupted which would make this protein unable to contact the downstream effectors which are required for transcription. Consistent with this,

hypothesis, binding of transcriptional adaptor proteins like the yeast SWI/SNF proteins has been shown to be mediated by this region of the ligand binding domain of the steroid hormone receptors (267).

C-Terminal Truncation of RARβ and RARγ Does Not Create Dominant Negative Mutants

While truncation of the ligand binding domain of the RARα can lead to repression (RARα'), in the RARβ and RARγ however truncation does not produce repressors. In contrast to our work, other groups have demonstrated in different cell lines that truncation of the C-terminus of the RARβ and RARγ can generate dominant negative mutants (268,269). I believe that this reflects differences in cell types used because Matsui and Sashihara have demonstrated cell type specific transactivation properties of a dominant negative RARγ (270). It is presently not clear what mediates such specific cell type effects however one explanation may be that some cell types harbour negative regulators which specifically regulate transactivation by the RARs. The existence of such a negative regulator has been demonstrated by Casanova et al. (261). This would also explain the finding that our two mutant proteins (RARβLacZ and RARγHA) are RA dependent transcriptional activators because their overexpression could sequester such a negative regulator of RAR function allowing for enhanced transactivation by the other receptors within the cell.

One alternative explanation for this finding that RARβ and RARγ mutants do not act

as dominant negative mutants is that they are unable to bind DNA like the RARαmyc. It is important to note that the mutant RARαmyc is unable to bind the DNA response element in the absence of heterodimerization. The addition of RXRβ or normal RAR allows the RARαmyc to bind with high affinity to the response element (fig 2.10). In our model of the mechanism of dominant repression for the RARαmyc this would mean that heterodimerization is required for repression because it allows the mutant to bind DNA. Consistent with this hypothesis, the mutations in the RARβLacZ and RARγHA while deleting the AF-2 transactivation domain have also deletions in the region of heptad repeats which should inhibit heterodimerization. This may explain why the RARβLacZ and the RARγHA are not dominant repressors because they have lost the ability to heterodimerize and therefore the ability to bind DNA. Consistent with this gel mobility shift analysis with these two proteins failed to consistently show the presence of a shifted complex (data not shown).

CHAPTER 3:

EXPRESSION OF RARαmyc IN TRANSGENIC MICE LEADS TO MALE INFERTILITY BUT NOT TUMOURIGENESIS.

Introduction

Early work in the field of retinoids demonstrated that they play both protective and therapeutic roles in cancer (170-175). How retinoids exert these effects at the molecular level has been the subject of intense study. The RARs can block oncogene induced transformation *in vitro* suggesting that the RARs may normally function *in vivo* like tumour suppressor proteins (166-168). Studies with transgenic mice expressing a mutated RARβ transgene have indicated that mutant RARs can also function dominantly to induce tumour formation (165). In APL, translocation of the RARα is the defining feature which indicates that the RARs can function dominantly to induce tumours in humans as well (192,193).

Many types of cancer, including APL, are believed to arise due to a block in the normal differentiation of an undifferentiated precursor cell. Because of this I postulated that expression of a mutant such as the RARαmyc could lead to tumourigenesis if overexpressed. Previous work has elucidated a role for the RARα in the RA induced differentiation of P19 EC cells. Overexpression of a dominant negative RARαmyc construct in P19 cells leads to a block in the RA induced differentiation of these cells. I believe that our mutant RARαmyc acts to block differentiation by heterodimerizing with the normal RARα or RXRβ and binding to the RARE thereby sterically preventing normal receptor complexes (RAR-RXR) from binding and activating the RAREs.

To determine if the nuclear retinoid signalling pathways play a direct role in

homeostasis and the development of cancer in the animal I decided to produce transgenic mice which expressed RARαmyc to determine if abrogation of RAR function *in vivo* would lead to tumourigenesis or disrupted homeostasis. The mutant RARαmyc was chosen because of its ability to block differentiation in cell culture and I postulated that it may have similar effects *in vivo*. Because of the pleiotropic effects of retinoids in many organ systems, I decided to express this mutant in a tissue specific manner to minimize the likelihood of lethal effects which could result from ubiquitous expression of such a mutant. To this end I generated transgenic mice which expressed the RARαmyc construct under the control of the MMTV promoter. This promoter drives expression primarily in the epithelial cells of the mammary glands (353). Expression of the dominant negative RARαmyc transgene in the mammary gland did not result in mice with an enhanced propensity to develop tumours. The MMTV-RARαmyc transgene was expressed in the male reproductive tract and resulted in infertility which I believe is caused by an excess of secretion product which collected in the vas deferens and epididymis causing a block to sperm progression from the testis.

Materials and Methods

DNA Constructs and Probes

Generation of the RARαmyc construct has been described previously. This mutant was subcloned into the plasmid MMTV-SV40-Bssk (271) by digestion with EcoRI and HindIII and ligation into similarly digested MMTV-SV40-Bssk. The region encompassing the promoter to the end of the polyadenylation signal was used for microinjection of fertilized zygotes. The probe for the northern and Southerns was the internal EcoRI-SacI fragment of the mouse RARα cDNA (26).

Generation of Transgenic Mice

Transgenic mice were generated in the lab of Dr. J.P. Julien by microinjection of fertilized C3H/C57Bl6 zygotes as described in Hogan et al. (272). Of the embryos injected with the RARαmyc construct five contained the transgene in their genome of which four produced transgenic lines.

Fertility Assessment and *In Vitro* Fertilization

Six to eight week old C3H females were superovulated by injection of 5 I.U. of pregnant mares' serum gonadatropin followed 48 hr later by 5 I.U. human chorionic

gonadatropin. Following the second injection the females were paired in the late evening with either transgenic or normal control mice (C3H). The following morning the females were checked for vaginal plugs indicating copulation had occurred. The females were then sacrificed and their uteri were examined for the presence of sperm. Each male was assessed with multiple females. For *in vitro* fertilization the females were injected as described above but instead of pairing the females, the following day they were sacrificed and their oviducts were removed to harvest the ovulated eggs. These eggs were mixed with the seminal fluid extracted from either control or transgenic male epididymis. Fertilization was allowed to proceed for 8 hrs and then the embryos were assessed after every 24 hrs for cleavage and progression to the blastocyst stage of development (i.e. the indication that fertilization had occurred). Analysis of the seminal fluid obtained from both the normal and transgenic epididymis was done by light microscopy.

Northern and Southern Blots

Northern blotting was done as described previously in chapter 1. For Southern blots genomic DNA was extracted from the tails of the transgenic offspring by the method described by Laird et al. (273). 10 μg of genomic DNA was digested by SacI for 16 hr and run on a 1% agarose gel overnight. The following day the gel was stained with ethidium bromide and photographed. The DNA in the gel was denatured with 0.5M NaOH/1.5M NaCl for 30 min and then neutralized for 30 min in 1M Tris pH 7.4/1.5M NaCl. The DNA was transferred to a nylon membrane (HYbond N, Amersham) by capillary action and probed as

described previously for northern blotting.

Histological Analysis

Histological analysis of tissue sections were prepared as described in Bancroft and Cook (274). Briefly tissues were fixed in 10% formalin prior to sectioning. Plastic embedded tissue cross-sections were examined with periodic acid and Schiffs-hematoxylin stain as described in Bancroft and Cook (274).

Results

Generation of Transgenic Mice Expressing RARαmyc

Transgenic mice were generated by microinjection of fertilized zygotes with the DNA construct MMTV-RARαmyc (fig 3.1a). Five heterozygous mice which harboured the RARαmyc transgene were produced. Of these, four were fertile and transmitted the transgene to their offspring, these strains were designated 1134, 1142, 1143 and 1148 (fig 3.1b). Representative Southern blots for each of these strains is depicted in figure 3.1b.

Transmission and Expression of the RARαmyc Transgene

Initially I wanted to determine if these animals were phenotypically normal. In expanding the transgenic colony I found that the RARαmyc transgene was transmitted in a normal Mendelian fashion (50% offspring transgenic) and all offspring appeared phenotypically normal (table 3.1) indicating that expression of this transgene does not cause major deleterious developmental abnormalities. Therefore expression of the RARαmyc transgene under control of the MMTV promoter does not appear to be deleterious to the development of the animal.

Analysis of expression of the RARαmyc transgene demonstrated that of the four strains only three expressed the transgene in mammary tissue, strains 1142, 1143 and 1148

Figure 3.1. **Generation of Transgenic Mice Expressing RARαmyc**
a) Schematic representation of the DNA fragment injected into fertilized zygotes for the generation of transgenic mice. The dark grey boxes depict the MMTV promoter (LTR region) and SV40 polyadenylation signals. The RARαmyc region is the open box with the DNA binding domain depicted in the medium grey and the ligand binding domain depicted in the light grey. Four strains were generated which harboured the RARαmyc construct in their genome. b) Representative Southern blots for each strain are shown. The numbers refer to the genotype of the transgenic parent and the transgenic offspring are indicated. The endogenous RAR bands are depicted with the closed arrows and the transgene specific band is depicted with the open arrow. Blots were exposed for one day at -70°C.

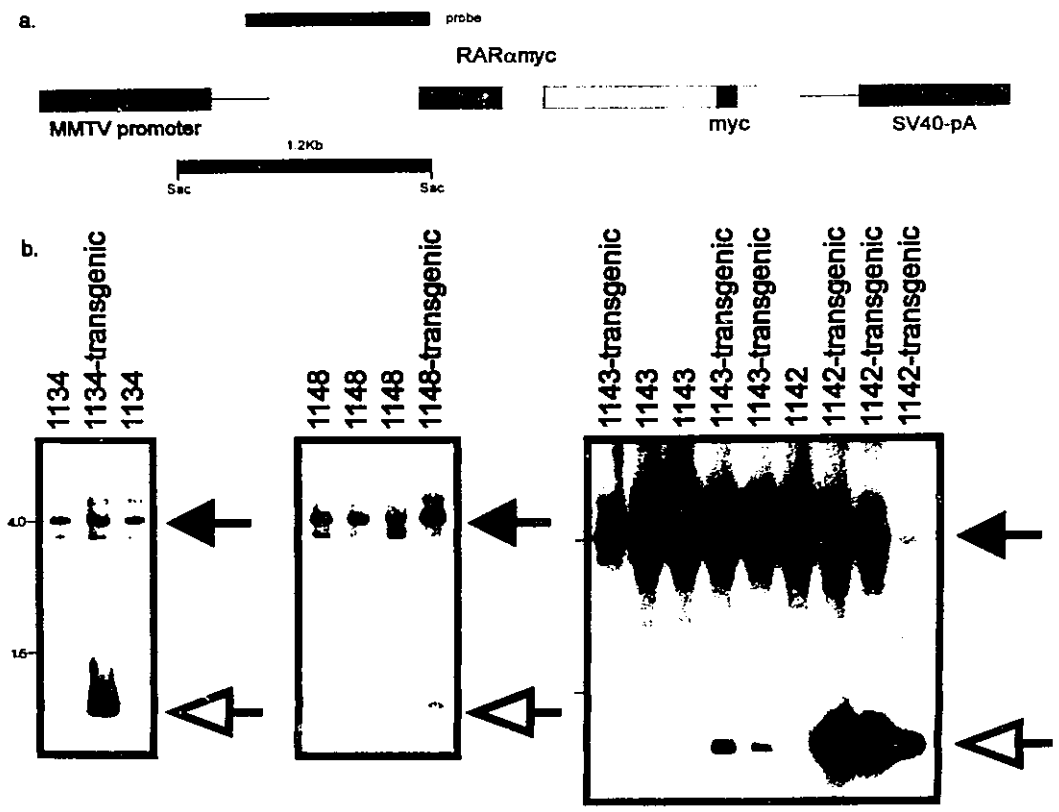

a.
probe
RARαmyc
MMTV promoter
myc
SV40-pA
1.2Kb
Sac
Sac
b.
1134
1134-transgenic
1134
1148
1148
1148
1148-transgenic
1143-transgenic
1143
1143
1143-transgenic
1143-transgenic
1142
1142-transgenic
1142-transgenic
1142-transgenic
4.0
1.6

Table 3.1. **Transmission of RARαmyc Transgene**.
All offspring of the RARαmyc founders were assayed for transmission of the RARαmyc transgene. Analysis of the genotype of these animals indicate that transmission of the transgene occurs in a normal Mendelian fashion for a heterozygous trait (50% offspring transgenic). The variations from 50% are not significant with this number of animals.

Table 3.1: Transmission of RARαmyc Transgene

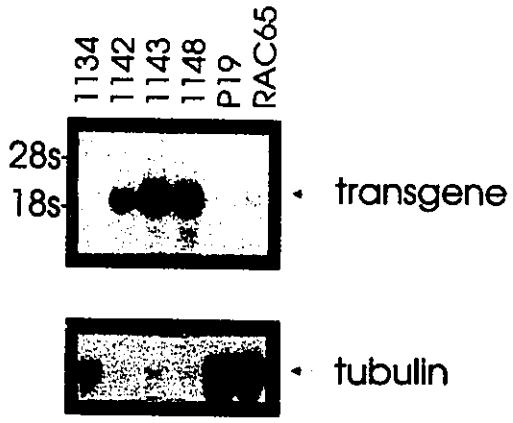
strain	total # genotyped	total # female	total # male	total # transgenic	# female transgenic	# male transgenic	transmission frequency
1134	19	8	11	9	3	6	47%
1142	45	23	22	25	15	10	56%
1143	45	21	24	20	8	12	44%
1148	54	23	31	27	12	15	50%

(fig 3.2a). The strain 1134 was not analyzed further because of its lack of expression of the transgene (fig 3.2a). Expression of the transgene was extremely high when compared to expression of the mutant RARα' in RAC65 cells. Male and female animals were taken from each strain and further analyzed for tissue specific expression of the transgene (fig 3.2b). The two upper bands on this blot represent the normal RARα transcripts which are expressed ubiquitously (fig 3.2b). Expression was found to be high in the mammary gland, lung and the salivary gland, the sites which have been demonstrated previously to have high MMTV expression (353, 271). Other tissues which expressed the transgene included the brain and kidney, two tissues which have not previously been described as tissues which express MMTV driven transgenes. The RARαmyc transcript is not found in the brain tissue of the male transgenic (fig 3.2b). In the males the seminal vesicle and the testis were found to express the transgene (fig 3.2b) .

Analysis of Mammary Gland Function and Tumourigenesis in Transgenic Mice

All animals appeared to have normal mammary gland function because they all had multiple litters and most animals survived to maturity. Transgenic animals were assessed for up to seventeen months for tumour development. One female which carried the RARαmyc transgene (the founder of the 1142 strain) developed tumours at eight months of age (table 3.2). Analysis of these tumours is presented in appendix 2. No other animals from any of the transgenic lines developed mammary tumours during the observation period. Clearly, expression of the RARαmyc transgene alone does not affect mammary gland function nor is

Figure 3.2. **Analysis of the Expression of the RARαmyc Transgene in Transgenic Mice.** a) RNA was extracted from the mammary glands from four virgin female transgenic mice (one from each line) and analyzed by northern blotting to assess expression of the RARαmyc transcript. The endogenous bands are not visible with this high stringency wash. b) RNA was extracted from various tissues of a transgenic male (1143) and female (1148) and assayed for expression of the transgene by northern blotting. The blot was washed at lower stringency (0.1X SSC and 0.1% SDS at 65°C for 5-10 min) to allow for comparison of the transgene specific bands with the endogenous RAR specific bands. The transgene specific bands are depicted with the open arrows and the endogenous bands are depicted with the closed arrows. The blots were exposed for one day at-70°C with intensifying screens. The lower panel in part b) represents the tubulin standardization and was exposed for one day. All animals were 4 months old.


1134
1142
1143
1148
P19
RAC65
28s
18s
transgene
tubulin

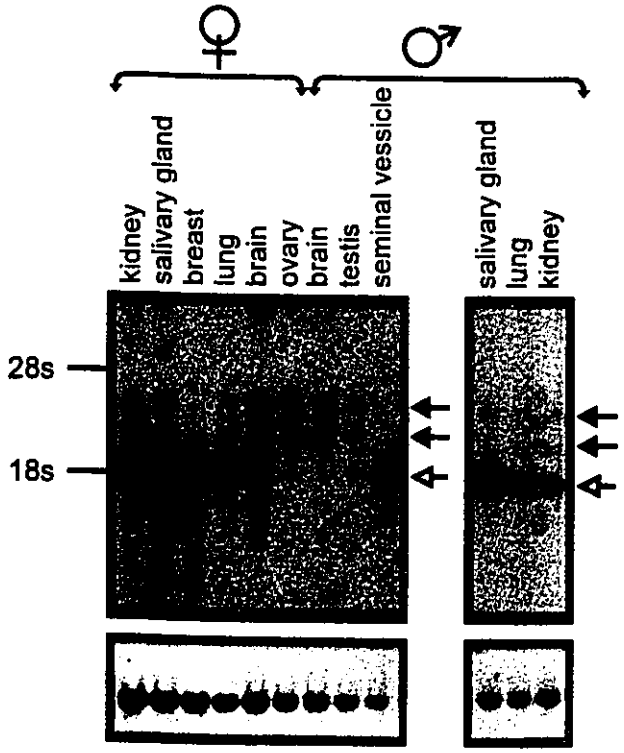

♀
♂
kidney
salivary gland
breast
lung
brain
ovary
brain
testis
seminal vessicle
salivary gland
lung
kidney
28s
18s

Table 3.2. **Tumour Development in Transgenic RARαmyc Mice.**
All female animals were examined at weekly intervals for the presence of palpable tumours. Animals were followed for up to 17 months of age. One mouse of the 1142 strain developed tumours at 8 months of age. No other animals of the same strain developed tumours. No animals of any other strains developed tumours.

Table 3.2: Tumour Development In Transgenic Mice

Tumour Incidence

transgenic strain	1142	1143	1148
# animals at 8 months	1/15	0/7	0/9
# animals at 9-17 months	0/2	0/4	0/4

sufficient to lead to tumourigenesis in the mouse.

Expression of the RARαmyc Transgene Leads to Male Infertility

In the course of developing our colony I found that the male offspring of two strains of our RARαmyc expressing mice were infertile, strains 1143 and 1148 (table 3.3). These two strains expressed the highest levels of the transgene in the mammary gland of all the transgenic strains by northern analysis (fig 3.2a). None of the males of the 1148 strain sired any offspring (table 3.3). I detected no vaginal plugs, no sperm or seminal fluid in the uteri of females caged with these males (table 3.4). There was no evidence that copulation had occurred .With the other strain, 1143, some animals were able to sire one or two litters (table 3.3). However, fertility rapidly declined because no other litters were ever produced even if superovulated females were used for the mating. I believe that copulation had occurred with these animals because vaginal plugs were present in most females however no sperm was found in the uteri of the recipient females (table 3.4). The etiology of the infertility appears to be different in these two strains.

The reproductive tracts of both normal fertile males and infertile transgenic males were dissected and fixed in 10% formalin to determine if any gross abnormalities within these tracts could account for the infertility and lack of viable sperm in the recipient females. Morphological analysis of the reproductive tracts of these transgenic animals revealed that there were no gross abnormalities, all tissues were present and of the appropriate size, within the reproductive tracts (fig 3.3a through d).

Table 3.3. **Infertility in Male Transgenic RARαmyc Mice.**
Analysis of all the male offspring of the three RARαmyc expressing strains clearly demonstrates that only two of the strains have reproductive problems. Males of the 1148 strain never sired any offspring and are completely infertile. Some of the males of the 1143 strain did produce offspring when initially paired with fertile females however after these litters no others were ever produced. I describe this strain as being only partially infertile. The etiology of the infertility appears to be different in these two strains. The 1142 strain did not demonstrate any fertility problems.

Table 3.4. **Fertility Analysis** .
Analysis of the reproductive behaviour of the infertile males. Males were analyzed for evidence of successful copulation. Copulation was assessed by the presence of a vaginal plug after pairing with fertile females for 12-18hrs. The females were then analyzed for the presence of ejaculate in their uteri which was analyzed under the light microscope for the presence of sperm. Males were 6-7.5 months. Females were 6-8 weeks old. These studies indicate that the two infertile strains exhibit differences in their reproductive behaviour.

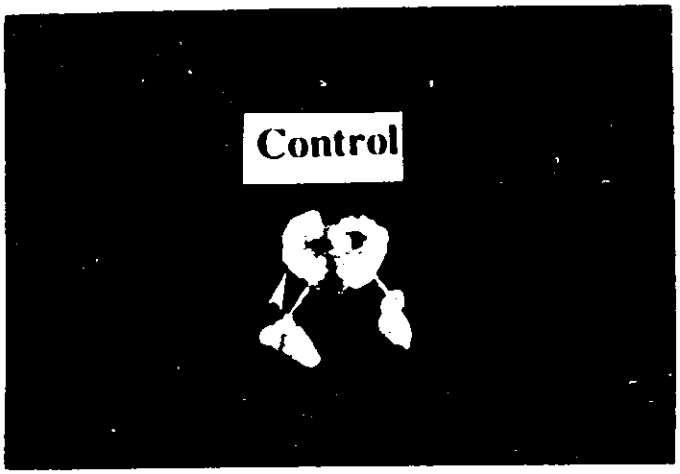
Table 3.3: Infertility in Male Transgenic RARαmyc Mice

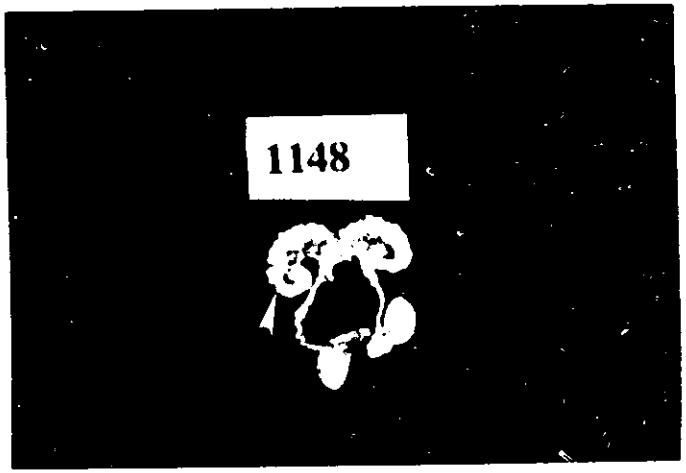
strain	total # infertile males	# males single litter	# males two litters
1142	0/9	9/9	9/9
1143	8/13	3/13	2/13
1148	10/10	0/10	0/10

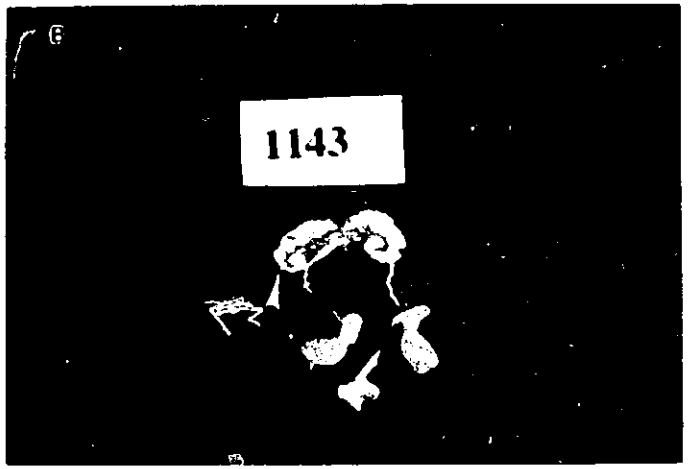
Table 3.4: Fertility Analysis of Infertile Transgenic Mice

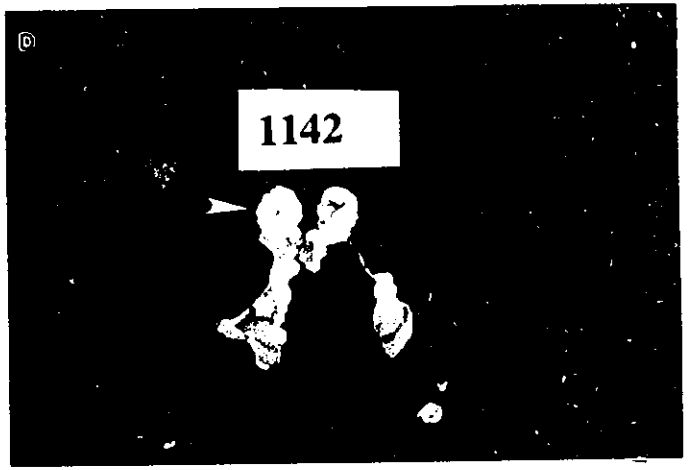
strain	vaginal plugs	sperm in uterus	ejaculate in uterus	egg development
1143	8/10	none	gelatinous	none
1148	0/10	none	none	none

Figure 3.3. **Gross Appearance of Reproductive Tracts of Infertile Males.**
a) through d) These figures depict dissections of the male reproductive tracts to assess the overall appearance of these tissues. Tissues were fixed in 10% formalin prior to photography to preserve them for further histological analysis. The arrows depict the seminal vesicles. a) normal nontransgenic b) fertile 1142 transgenic c) 1143 infertile transgenic d) 1148 infertile transgenic. All mice were 60 days old. All tissues appear normal.


Control


1148


1143


1142

Analysis of Sperm in Epididymis

The fertility analysis indicated that no sperm was present in the females used for mating after pairing. Clearly this explains why there were few or no litters sired. However I wanted to determine at what level the defect in sperm ejaculation was occurring. *In vitro* fertilization experiments were undertaken to assess if viable sperm could fertilize eggs *in vitro* to determine if the defect could be due to low sperm motility or numbers. This analysis also enabled us to determine if the transgenic sperm was capable of fertilization. *In vitro* fertilization experiments clearly demonstrate that the defect leading to infertility in both transgenic lines is due to a lack of viable sperm in the epididymis of the infertile transgenic mice (table 3.5). Analysis of the contents of the epididymis of the transgenic mice demonstrated the presence of degenerated sperm (heads and tails) in the 1143 line (fig 3.4 a and b). The epididymal contents of the 1148 transgenic did not contain any sperm (data not shown).

Spermatogenesis in Infertile Transgenics is Normal

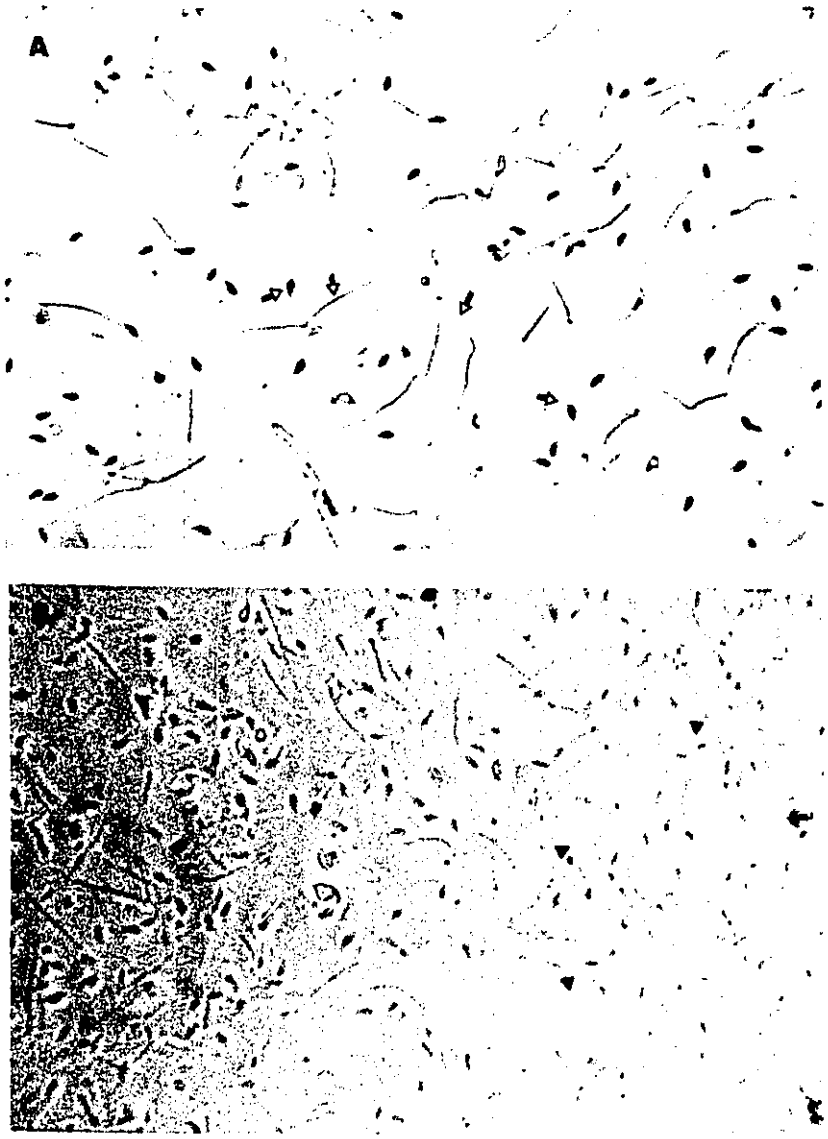
Because of the known effects of retinoids on sperm development (138) and the finding that no viable sperm were found in the epididymis of infertile transgenic males I decided to assess sperm production in the testis. This work was done in collaboration with Dr.K. Boekelheide and Mr. R.Seth at Brown University, Providence, Rhode Island. Plastic

Table 3.5. ***In vitro* Fertilization with Transgenic Sperm.**
In vitro fertilization was carried out to assess if the lack of viable sperm within the recipient females was due to a defect in sperm production or transport. Infertile males were sacrificed and the contents of their epidiymi were used for *in vitro* fertilization. No *in vitro* fertilization with transgenic males was observed due to a lack of viable sperm within the epididymal contents. Control C3H mice had normal rates of *in vitro* fertilization. Male mice were 6-7.5 months old at the time of sacrifice. The lack of viable sperm within the epididymis indicates that the defect leading to infertility occurs prior to or within the epididymis.

Table 3.5: ***In vitro*** **Fertilization with Transgenic Mice**

strain	epididymal contents	viable sperm in epididymis	egg development
1143	gelatinous	heads and tails	none
1148	gelatinous	none	none

Figure 3.4. **Sperm Isolated From Transgenic Male Epididymis.**
Transgenic mice were sacrificed and their epididymis were removed and fixed in 10% formalin to preserve them for later histological analysis. Prior to fixation these tissues were opened and their contents were removed for analysis. Analysis of the contents was carried out with live (unfixed) samples under the light microscope to assess the relative numbers of sperm and their motility. a) sperm from 1143 infertile male epididymis. The appearance of the heads separated from the tails could explain the lack of *in vitro* fertilization. Often when sperm remains in the epididymis for long periods of time (for example, due to blockage) it loses viability as it degenerates. The appearance of heads and tails is indicative of this degeneration. The open arrows depict separated heads and tails. b) normal sperm from nontransgenic mouse used as control in *in vitro* fertilization experiment. The fuzzy appearance of this photograph is due to the rapid movement of the sperm in the culture dish. The closed arrows depict the intact sperm. Animals were 6 months of age.


A

embedded testis cross-sections from formalin fixed testis were examined with periodic acid and Schiffs-hematoxylin (PAS+H) stain. Histological analysis of the testes of these animals failed to demonstrate any abnormalities in histology and appeared no different from that of normal age matched control mice (fig 3.5a,b,c).

Although testis histology appeared normal in the infertile transgenics I wanted to determine if spermatogenesis was intact in these animals. Spermatogenesis is classified into twelve stages (275) and analysis of these in our transgenic mice was done using established criteria (276). At 400x magnification, two testicular cross-sections were counted for each animal. Only round intact seminiferous tubules were staged and a minimum of 60 seminiferous tubules were coded and staged for blind evaluation of each section. This analysis demonstrated that all stages of spermatocytes were present and their distribution appeared normal when compared to age matched normal control mice as seen by normal stage distribution frequency for transgenic animals (figure 3.6a) (277,278). Furthermore diameter of the seminiferous tubules which is a sensitive indicator of anomalies in the testis (277) failed to demonstrate any significant differences between age matched normal and transgenic mice (figure 3.6b). Clearly spermatogenesis in the infertile transgenics appears intact by all criterion analyzed.

Analysis of Epididymis of Infertile Transgenic Mice

The *in vitro* fertilization (table 3.5) and light microscopic analysis of epididymal

Figure 3.5. **Histological Sections of Testis.**
Cross sections of plastic embedded testes were prepared as described in Materials and Methods. Histological analysis indicates that the infertile males have morphologically normal testes. Cross sections demonstrate no obvious histological difference between matched normal and dominant negative mice. a) age matched normal b) 1143 infertile c) 1148 infertile. Mice were 60 days old.

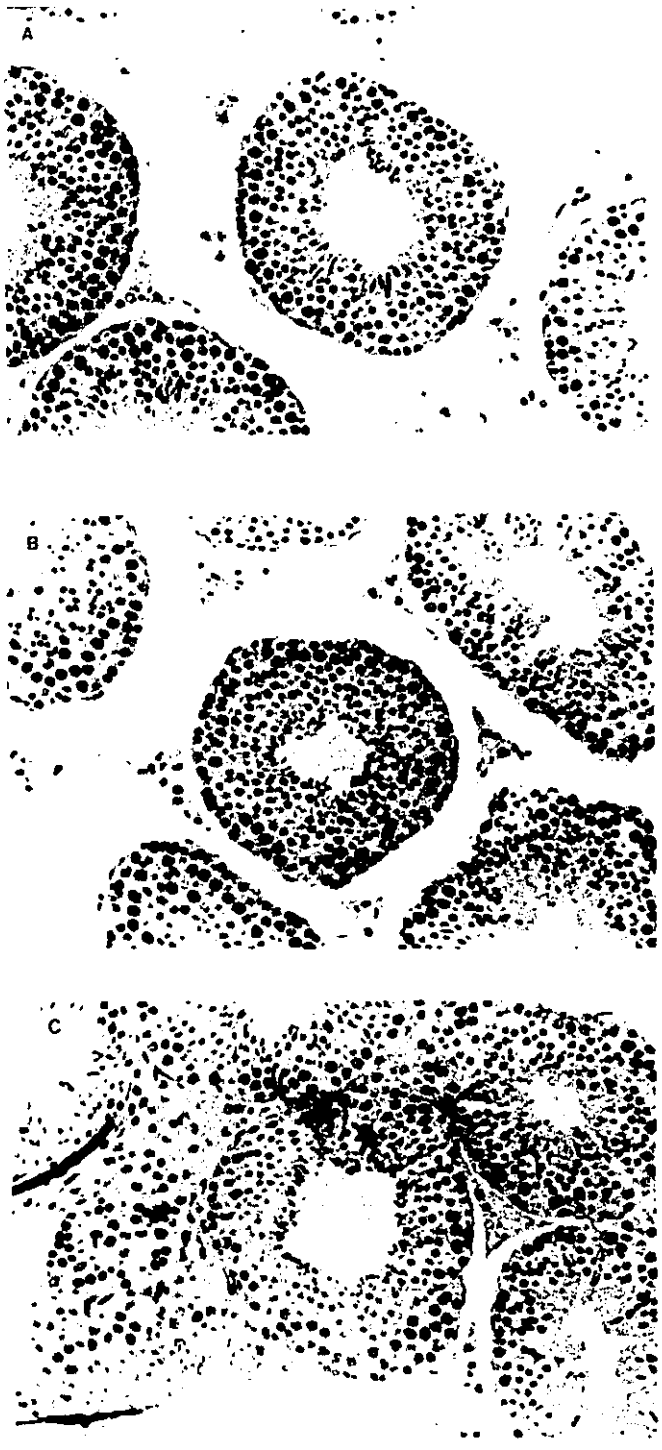
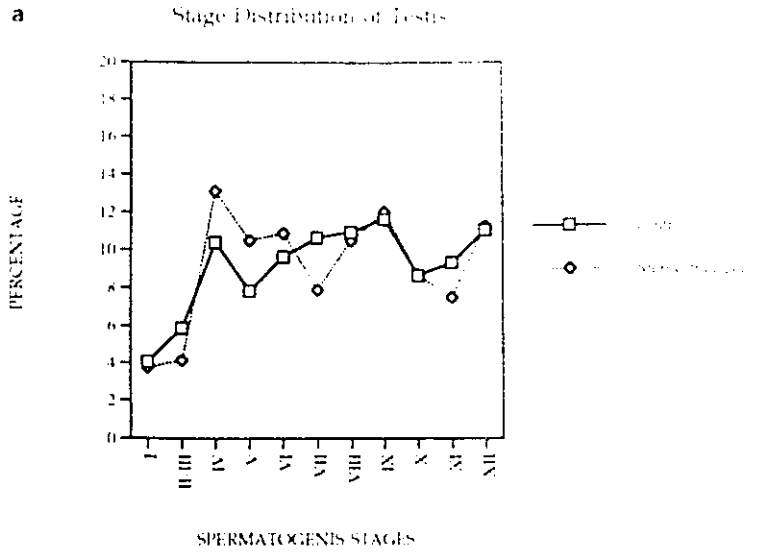
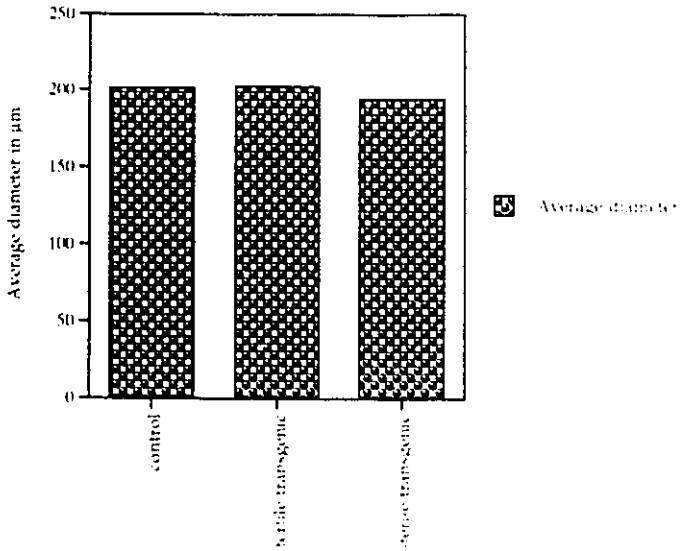

A
B
C

Figure 3.6. **Analysis of Spermatogenesis in Infertile Male Transgenics.**
a) Distribution of stages as percentages determined by counting stages for age matched normal and transgenic mice. Staging was done in a blind evaluation experiment. Parameters used for stage counting were only essentially round complete tubules at 400x magnification. 241 tubules were counted for the control and 200 tubules were counted for the transgenic. b) Tubular diameter measurements done in micrometers made by ocular micrometer at 250x magnification. The parameters used were as follows: essentially round complete tubules and the minor diameter was measured (200 tubules as control and 100 tubules for transgenics). Note there is no difference between control and age matched normal. All mice were 60 days old.

a


PERCENTAGE
SPERMATOGENIS STAGES

b


Average diameter in µm
250
200
150
100
50
0
control

contents (fig 3.4) demonstrated that the epididymis of these animals did not contain any viable sperm. To determine if the epididymis of these transgenics were histologically normal, tissue sections were prepared for histological analysis as described previously for testis cross-sections. Histological analysis of the epididymis demonstrates a difference between age matched normal and transgenic mice. These studies clearly demonstrate that both infertile strains exhibit vacuolization of the epithelial walls of the cauda epididymis which is not seen in normal control mice (fig 3.7). The effect of vacuolization can vary between transgenic lines (figure 3.7 compare 1143 and 1148). Most likely these cells represent epididymal clear cells that have been noted to enlarge and increase in number in response to toxic insults (279).

The vas deferens of the infertile transgenics contain large deposits of seminal fluid which could account for the vacuolization in the epithelial walls of the epididymis (fig 3.8a and b, closed arrows). This suggests that the infertility occurs because an excess of seminal fluid builds up within the vas deferens and the epididymis and blocks these ducts. These deposits were so great that some older animals had large protrusions which were filled with these yellow deposits along the length of the vas deferens.

Figure 3.7. **Histological Sections of Epididymis.**
Sections were prepared as described previously. Cross sections of epididymis, proxima cauda at 200x magnification. a) age matched normal b) 1143 infertile transgenic and c) 1148 infertile transgenic. Note the appearance of vacuolization of the epididymal epithelium which is absent from controls (arrows). Most likely these cells may be epididymal clear cells that have been noted to enlarge and increase in number in response to toxic insults. All mice are 60 days old.


A

Figure 3.8. **Blockages in the Vas Deferens of Infertile Transgenic Males.**
Unfixed sections of the reproductive tracts as viewed by light microscopy. The vas deferens of the infertile males were found to be enlarged and had a yellow appearance. The vas deferens were filled with large deposits of seminal fluid. These deposits filled the tubules as far up the tracts as the urethra. These deposits were not found in the fertile transgenics or control mice. a) and b) infertile male of the 1143 strain. c) and d) 1142 fertile male. The blockages are depicted with the arrows. Mice are 5.5 months.

A
C
D

Discussion

Three transgenic lines which expressed the MMTV-RARαmyc transgene were generated and these lines produced viable healthy offspring. The males of two of these lines were infertile probably due to a build up of seminal fluid within the reproductive tracts. Our data indicates that expression of dominant negative mutant RAR is not sufficient to initiate mammary tumourigenesis in the mouse but leads to infertility in the male.

Our initial analysis was to study the role of the RARs in oncogenesis in the mouse. I chose the mammary gland for several reasons. The transformation induced by MMTV driven oncogenes has been well characterized (353) and is a good model system because it allows for comparison with other oncogenes. The mammary gland is a good tissue to look at the effects of a mutant which is able to block differentiation because the mammary gland undergoes successive rounds of differentiation and regression throughout the reproductive life of the animal.

Our studies suggest that the process of differentiation of the mammary gland is not dependent on the actions of the RARs because all animals had apparently normal mammary gland function as evidenced by the normal production and maintenance of multiple litters by all transgenic mothers. This is in contrast to the work of Lee et. al. (281,282) who have demonstrated that retinoids can effect the morphogenesis, differentiation and growth of the mammary gland. However the work of Lee et al. does not present direct evidence that the

RARs direct these processes normally *in vivo* and our results suggest that the differentiation of the mammary glands is not normally governed by the actions of the RARs. The salivary gland, lung and the kidney expressed the transgenes and appeared normal. Furthermore the transgenic mice appeared healthy suggesting that RAR function is not essential to the development or homeostasis of these tissues as well.

Due to expression of MMTV driven transgenes in the reproductive system I found that expression of RARαmyc leads to male infertility in the mouse. The role of retinoids in male fertility has been well documented. One of the early effects of retinoid deficiency is infertility (283). Knock-out experiments in transgenic mice have provided useful information on the mechanisms of actions of retinoids in the male reproductive system. Ablation of RARα function was found to result in male infertility which resulted from a degeneration of the testis and a concomitant loss of spermatogenesis (138). This is clear evidence that RARα functions to maintain testis function. Knock-out experiments with the RARγ have demonstrated a different role for this receptor in male reproduction (139). In these transgenic mice, infertility resulted from a series of defects within the seminal vesicle and prostate which resulted in a keratinization of the glandular epithelium and squamous metaplasia and a concomitant lack of secretion product from these glands (139). These studies indicate that different RARs function in different regions of the male reproductive system to govern fertility.

Our analysis indicates that the defect leading to infertility is within the seminal vesicle for several reasons. The finding that no viable sperm was present within the epididymi of the

infertile transgenics indicates that either no sperm was produced or it is not transported out of the testis. I clearly show that the sperm production in the testis is normal which indicates that the problem occurs after sperm production. The presence of large deposits of seminal fluids within the reproductive tracts which would hinder the movement of the sperm from the testis lead us to the conclusion that this was the major defect leading to infertility. Our analysis suggests that there are defects in seminal fluid production. This is consistent with our northern analysis which demonstrates high expression of the transgene in the seminal vesicle. The prostate also produces some components of seminal fluid and our analysis does not address the possibility of defects in the prostate. RNA could not be isolated from the prostates of the transgenic animals to determine the levels of expression of the transgene in this gland.

Comparison of our Results with Current Work In the Field

We found that spermatogenesis in our transgenics was normal. In light of the evidence implicating the RARα in maintaining normal spermatogenesis (138), why do I not see defects in spermatogenesis? The fact that spermatogenesis is normal in our transgenics may simply reflect the low level of expression of the transgene in this tissue. Alternatively, the transgene may be expressed in a different cell type within the testis than the cell type(s) which are required for normal spermatogenesis.

Expression of the RARαmyc like the knock-out of the RARγ leads to infertility due to defects of the seminal fluid producing glands (139). However in the RARγ knock-out mice

there is an absence of seminal fluid due to the keratinization of epithelial tissues which should normally develop as secretory epithelium (139). The squamous metaplasia produced in these RARγ knock-outs is analogous to the effects produced by vitamin A deficiency where a secretory epithelium is converted to a squamous epithelium (6). Because of the lack of secretory epithelium, these glands (seminal vesicle and prostate) develop as empty glands with large areas of void space within the regions normally occupied by seminal fluids. In our transgenic mice it appears that the opposite has occurred in that the linings of these glands appear like normal glandular epithelium (data not shown, K. Boekelheide pers. comm.). The defect appears to be an excess of secretion product produced by these glands which blocks the passage of sperm from the testis into the epididymis as evidenced by the lack of viable sperm within the epididymis.

Comparison With the Actions of v-erbA in Fertility

It has been reported that expression of v-erb A in transgenic mice under control of the β-actin promoter leads to high expression of the transgene in the liver, testis and seminal vesicle (284). This was found to result in tumourigenesis in the liver and infertility in the male (284). Interestingly the phenotype observed in the infertile males was an excess of secretion product within the seminal vesicles of these mice. This resulted in infertility presumably due to blockages similar to what I saw in our transgenic mice. The phenotype observed in our transgenic mice is remarkably similar to the phenotype observed in the transgenic v-erbA mice (284). Our laboratory has previously demonstrated that the RARαmyc acts similarly to the

v-erbA protein *in vitro*. It has been suggested that the actions of v-erb A in tumourigenesis correlate more closely with its ability to block activation of RAREs than it does with its ability to activate TREs (232). It may also follow that other actions of v-erb A *in vivo* such as its ability to affect epithelial cell maturation in the seminal vesicle correlate with its ability to block RAREs. The similarity between our results and those of Barlow et al. (284) suggest that this is the case in the seminal vesicle at least.

CHAPTER 4:

E2F ANTAGONIZES THE ACTION OF THE RETINOIC ACID RECEPTOR ON THE RETINOIC ACID RESPONSE ELEMENT.

Introduction

It has recently been demonstrated that the RAR activates the RARE synergistically with TATA Binding Protein (TBP) (124). This synergism is only seen in undifferentiated EC cells (124). COS cells, a differentiated cell type, require the 13s E1A gene product for this synergism (124). This synergism is believed to be mediated by an indirect interaction between the two proteins, an interaction which requires a bridging factor which is present in undifferentiated EC cells but is absent in more differentiated cells such as COS cells (124). As a result, the transcriptional response to RA is very weak in COS cells compared to EC cells (124) and high RA responsiveness can be restored to COS cells by the addition of E1A (13s) (124). By analogy with Oct-3, whose transactivation can also be demonstrated to be 13s E1A dependent (285), the cellular bridging activity present in the EC cells was referred to as the cellular E1A like activity.

This cellular E1A like activity was first characterized as a cellular activity which enabled the replication of an E1A deficient adenovirus mutant in embryonal carcinoma cells but was absent from differentiated cells (286,287). The cellular E1A like activity present in the undifferentiated EC cells complemented the absence of the E1A gene in the adenovirus genome (286,287). This cellular activity was found to be rapidly down regulated during the differentiation of F9 embryonal carcinoma cells (288-290). Further characterisation of this activity demonstrated that it bound to a site in the adenovirus early promoter (E2) and it was referred to as E2 binding factor or E2F (289). The site which it bound in the adenovirus

promoter was designated the E2F site (289).

Recently a transcription factor was cloned by virtue of its interaction with the retinoblastoma gene product (291,292). Its identity as E2F was established because it was shown to exhibit many of the properties of the E2 promoter binding activity previously characterized including: binding to the E2F site in the E2 promoter, activation of this promoter and inactivation by the retinoblastoma gene product (293-295). A related protein, DP-1, was later cloned from F9 EC cells by virtue of its similarity with E2F (296). These two proteins exhibit homology within their DNA binding and putative dimerization domains (297). Further studies have shown that these two proteins synergistically activate the E2F site as a heterodimer (298,299). At least, three isoforms of each protein exist (297,300-302) and it has been suggested that various homo or heterodimers activate transcription from different E2F site containing promoters with different efficiencies (297). E2F sites are found in the promoters of many genes involved in cell cycle progression (303-310) and activation of E2F has been demonstrated to result in cell entry into the S phase of the cell cycle (311-314).

One of the major activities of the E1A protein which leads to tumourigenesis has been demonstrated to be the sequestration of the retinoblastoma protein (315,316). This is believed to result in tumourigenesis at least in part because the retinoblastoma protein is a negative regulator of E2F function and its sequestration results in enhanced levels of the active form of E2F and concomitant cell cycle progression (315,317). Embryonal carcinoma cells have been demonstrated to contain very little of the retinoblastoma protein (157). Furthermore they

exhibit high levels of E2F binding activity (289). This suggests that the cellular E1A like activity present in EC cells could be attributed to an excess of E2F over the low levels of the retinoblastoma protein which are present in EC cells.

In this report I examine the role of E2F and DP-1 in the activation of the mouse RARβ gene to determine if E2F has the same effect as 13s E1A on elevating expression from the RARβ promoter. I found that E2F and E1A are potent inhibitors of the RA induced transcriptional response from the RARβ promoter. I believe that E2F acts to inhibit the RARβ-promoter by sequestering a critical linking factor required for transactivation.

Materials and Methods

Cell Lines and Tissue Culture

P19 embryonal carcinoma cells were maintained as described previously in chapter 2 (14).

Plasmids and Constructions

The plasmid RAR-βCAT was a generous gift of Dr. H. Sucov (76)(La Jolla). This construct contained 3.5kb of the RARβ promoter region linked to the gene chloramphenicol acetyltransferase (CAT). Deletions of this promoter were constructed by digestion at the sites indicated, blunt ending and religation as described in Maniatis and Sambrook (318). The E2F-1 cDNA was provided by Dr E.K. Flemington (292)(Boston). The construct PGK-E2F was produced by subcloning the BamH1 fragment of the E2F cDNA into the unique BamHI site in the plasmid PKJδF which contains the PGK promoter and terminator/polyadenylation signals separated by a polylinker (151,233). The internal deletions of the E2F protein were constructed by digestion at unique restriction sites within the E2F coding region and with a second enzyme within the 3' region of the polylinker these were then religated. The DP-1 cDNA was a gift of Dr. R. Girling and Dr N.B. La Thangue (London)(296). The construct PGK-DP-1 was constructed by subcloning the EcoRI-XhoI of the cDNA into EcoRI(partial)-XhoI digested PGK-E2F. The plasmid TREpal-CAT contains a deleted version of the MMTV LTR where the glucocorticoid responsive sequences have been replaced by a palindromic thyroid response element (81). This plasmid was a gift of Dr. V. Giguère (Montreal). The

plasmids mRXR-α,β,γ,GRE-CAT and RSV-GR were kindly provided by Dr. P. Chambon (Strasbourg)(26,35). The plasmid PGK-TBP was constructed by insertion of the BamHI-BglII fragment of TATA binding protein cDNA into the BamHI site of PGK-δF, the cDNA was a generous gift of Dr. J. Greenblatt (319) (Toronto). The BRG-1 expression construct was a generous gift of Dr. Gerald Crabtree (Palo Alto) and Dr. Bruce Strober (New York) (320). The E1A constructs have been described previously (321) and were a gift of Dr. S. Bayley (322)(Hamilton). The p300 cDNA was provided by Dr. R. Eckner (Boston) and was subcloned into the PGK expression vector described above with Hind III and Not I(323).

Transient Transfection and CAT Assays

Cells were plated 12 hours prior to transfection on to 60mm diameter tissue culture dishes at a density of $2x10^5$ cells per ml. Cells were transfected with the calcium phosphate precipitation method as described by Chen and Okayama (243) for 8 hours. For each transfection a total of 15 μg of plasmid was transfected: 2 μg of the reporter RARβ-CAT was cotransfected with 2 μg of PGK-LacZ and varying amounts of the experimental plasmids (the plasmid pGEM was added as required to bring the amount of DNA to 15 μg). After transfection the dishes were washed with phosphate buffered saline. 5ml of medium with 10% serum was added to each dish. RA was added to appropriate dishes at a concentration of $3x10^{-7}$ M. Thyroid hormone was added at a concentration of $1x10^{-8}$ M and dexamethasone was added at $1x10^{-6}$ M. Cells were harvested 24 hours later in .25M Tris PH7.8. CAT assays were performed as described by Chen and Okayama (243) and all values were normalized to the transfection efficiencies determined by the internal standard β-galactosidase. Experiments

were all performed in triplicate and each experiment was performed a minimum of three times. Standard error calculations are for triplicate samples from a single experiment.

Northern Blot Analysis

Total RNA was extracted by the LiCl Urea extraction procedure of Auffray C., et al. (239). Total RNA was extracted at daily intervals after RA treatment. 10 μg of total RNA was denatured and run on each lane of a 1% formaldehyde gel for 6 hours. The samples were transferred by capillary action to a nylon filter (HYbond N, Amersham) and the samples were crosslinked to the membrane with a GS Genelinker UV crosslinker (Biorad) at 125mJ. Blots were hybridized in 5X SSPE, 50% formamide, .5%SDS, 5X Denhardt's and 250μg/ml salmon sperm DNA overnight at 42°C. Probes were labelled with α-^{32}P-dCTP (Dupont/NEN) with the multiprime labelling kit (Dupont). Blots were washed at high stringency (.1X SSC, .1% SDS) for 30 min. The E2F probe was the entire cDNA fragment (BamHI fragment). The DP-1 probe was the full length cDNA fragment (EcoRI-XhoI fragment).

Results

Expression of E2F and DP-1 in Differentiating P19 EC Cells

Initially I wanted to determine the precise expression pattern of both E2F and DP-1 to determine if both proteins were expressed in our assay system. Both E2F and DP-1 are expressed in undifferentiated EC cells (fig 4.1). E2F is expressed throughout differentiation (fig 4.1a). Expression of DP-1 is lost by day 10 (fig 4.1b).

E2F is a Dose Dependent Inhibitor of the RARβ Promoter

To determine the role these proteins play in activation of RA responsive genes in P19 cells I performed cotransfection experiments with E2F, DP-1 and the RA responsive reporter RARβ-CAT. Transfection of P19 cells with the reporter RARβ-CAT results in high RA induced transcription of the CAT gene. Addition of increasing amounts of the plasmid PGK-E2F decreases the RA induced transcriptional response in a dose dependent manner (fig 4.2a). Even small amounts of the E2F plasmid results in significant inhibition of the reporter, at 1μg of expression plasmid 70% inhibition is observed. Cotransfection of 8μg of E2F expression plasmid results in complete inhibition of the RA induced transcriptional activation of this reporter.

DP-1 and E2F heterodimers synergistically activate transcription from E2F sites (297-299). Cotransfection experiments were performed with DP-1 and the reporter RARβ-

Figure 4.1. **Expression of E2F and DP-1 in Differentiating P19 Cells**
10 μg of total RNA from differentiating` P19 cells was run on a formaldehyde gel and transferred to a nylon membrane. The northern was hybridized with a probe to either a) E2F or b) DP-1. The blots were subsequently hybridized to a internal control probe, the α-tubulin cDNA, to control for the total amount of RNA transferred to the membrane. The blots were exposed overnight at -70°C.

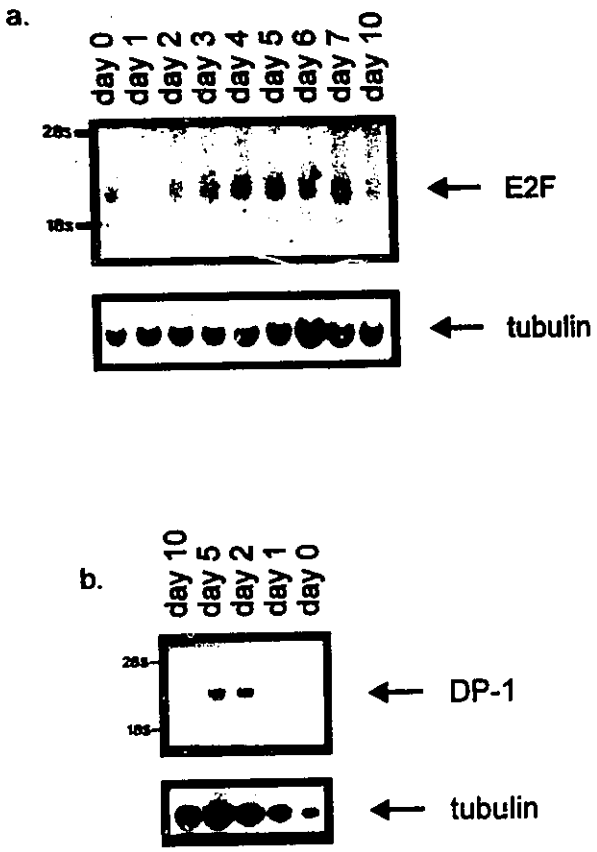
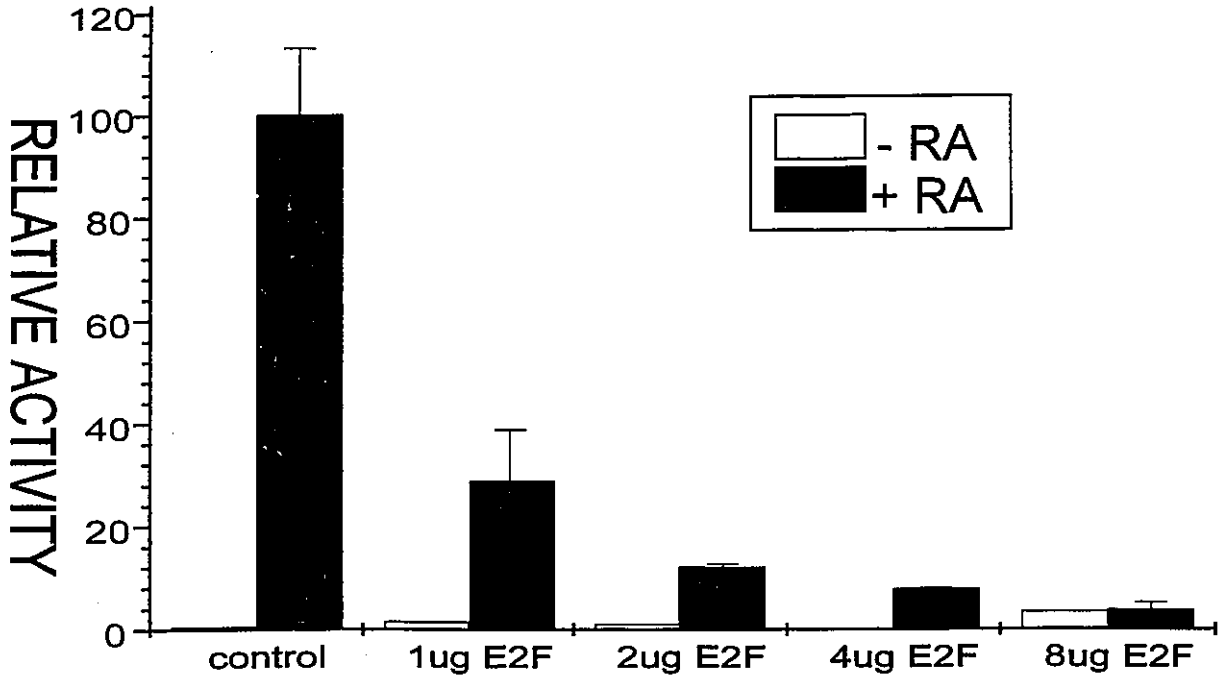
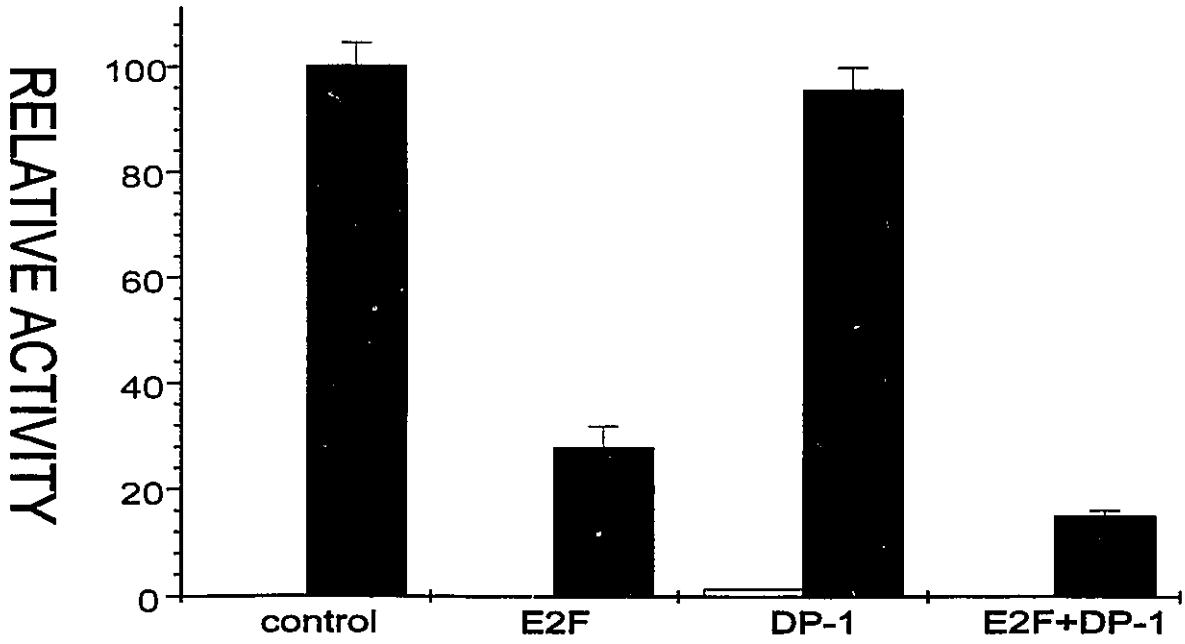

a.
day 0
day 1
day 2
day 3
day 4
day 5
day 6
day 7
day 10
28s
18s
E2F
tubulin
b.
day 10
day 5
day 2
day 1
day 0
28s
18s
DP-1
tubulin

Figure 4.2. **E2F Inhibits the RARβ promoter**
a) A total of 12 μg of DNA was transfected into P19 EC cells; An internal control plasmid PGK-lacZ (2μg) was added to each transfection, 2μg of the reporter RARβ-CAT and up to 8μg of the E2F expression plasmid PGK-E2F. For experiments where less than 8μg of the E2F plasmid was used the control plasmid pGEM was added as required to bring the total DNA transfected up to 8μg. All transfection efficiencies were normalized to total β-galactosidase activity. Results are from one representative experiment with each sample repeated in triplicate. Each experiment was repeated a minimum of three times. CAT activity was induced in the presence of $3x10^{-7}$M RA. b) The experiment was as in figure 4.2a except that a total of 4μg of the plasmids PGK-DP-1 or PGK-E2F were transfected with 4μg of pGEM to make up the total to 8μg. Where both DP-1 and E2F were transfected, 4μg of each was used and no pGEM was added.

a.


RELATIVE ACTIVITY
120
100
80
60
40
20
0
- RA
+ RA
control
1ug E2F
2ug E2F
4ug E2F
8ug E2F

b.

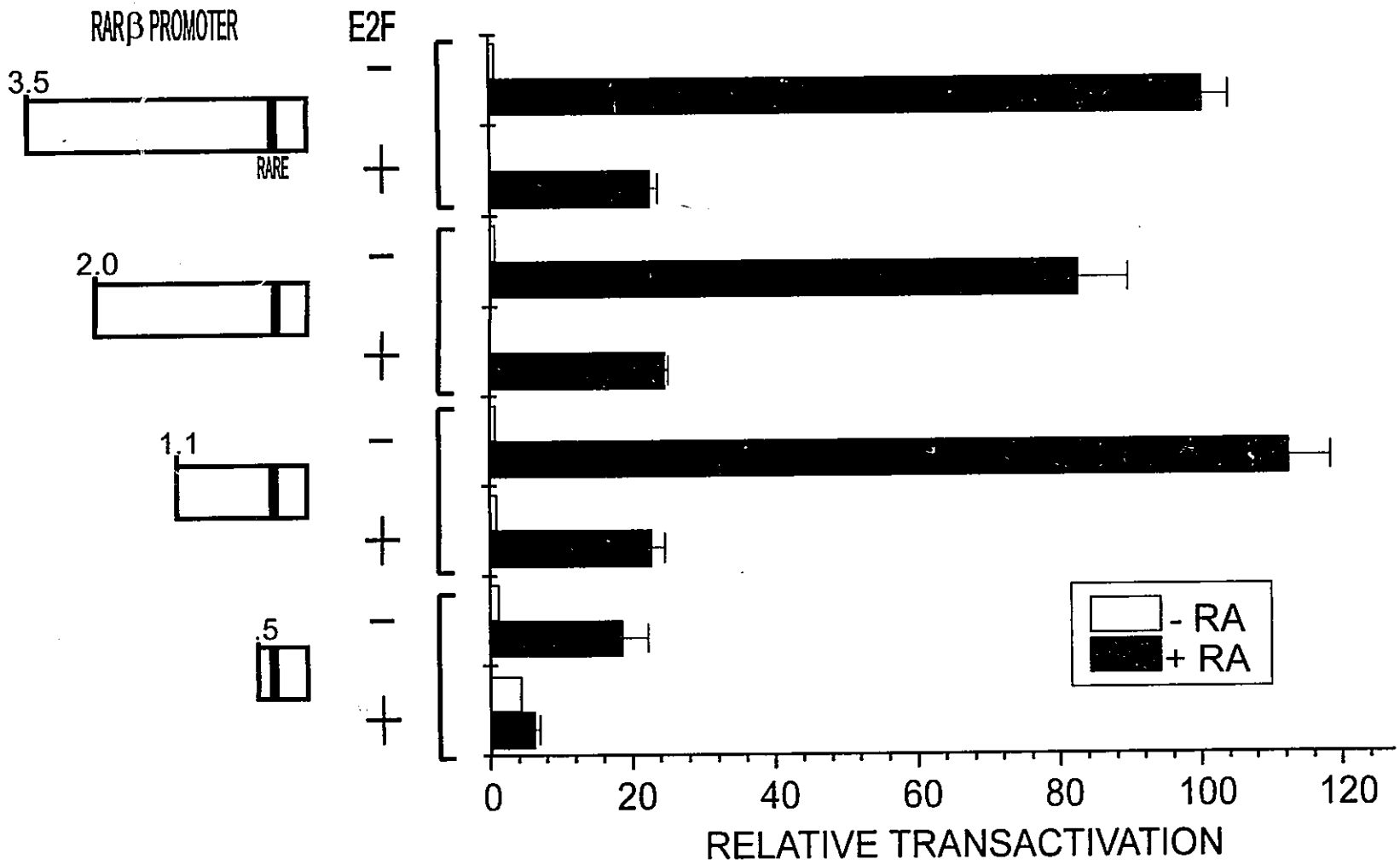

RELATIVE ACTIVITY
100
80
60
40
20
0
control
E2F
DP-1
E2F+DP-1

CAT to determine if this E2F like protein has similar effects on the RARβ promoter. DP-1 does not have a significant effect on the RARβ promoter (fig 4.2b). It is interesting that DP-1 is not an inhibitor like E2F because most studies have reported these two proteins having similar effects on E2F site dependent transcription (see fig 4.10). The contrasting effects of E2F and DP-1 on this promoter suggests that the E2F inhibition is not mediated by typical E2F sites within the RARβ promoter.

The RARβ Promoter Does Not Contain E2F sites

The reporter construct RARβCAT contains 3.5kb of promoter sequences from the RARβ gene linked to the CAT cDNA. To determine conclusively that this E2F inhibition is not mediated via E2F responsive sequences within the RARβ promoter region a deletion analysis of the promoter was carried out (fig 4.3). Sequential deletion of the promoter to within 10bp of the RARE resulted in no loss of the inhibitory effect of E2F. A significant loss of total promoter activity was observed upon deletion of the 600bp immediately upstream of the RARE however a significant inhibition by E2F was still observed (fig 4.3, last two panels). This deletion removes the putative cyclic AMP responsive sequences which have been reported to be involved in activation of this promoter (76). Analysis of the sequence of this region of the promoter failed to demonstrate the presence of any E2F site related sequences within this region (77). This demonstrates that the effect of E2F is mediated on or near the RAR/RXR transcription complex because the inhibition is seen on a minimal RARE containing promoter. Clearly, the inhibition of the RARβ promoter observed in the presence

Figure 4.3. **The Inhibition of the RARβ Promoter Is Not Dependent on E2F Sites**
The RARβ CAT plasmid was successively deleted from the 5' end to determine if E2F responsive sequences could be delineated within this promoter. 1 Kb was removed by digestion with NheI, a further 700bp was lost by digestion with PstI and finally partial digestion with SmaI removed all the 5' region of the promoter to within 10 bp of the RARE. These deleted RARβ CAT reporters were transfected with 4μg of PGK-E2F into P19 cells as described previously.


RARβ PROMOTER
E2F
3.5
RARE
2.0
1.1
.5
- RA
+ RA
0
20
40
60
80
100
120
RELATIVE TRANSACTIVATION

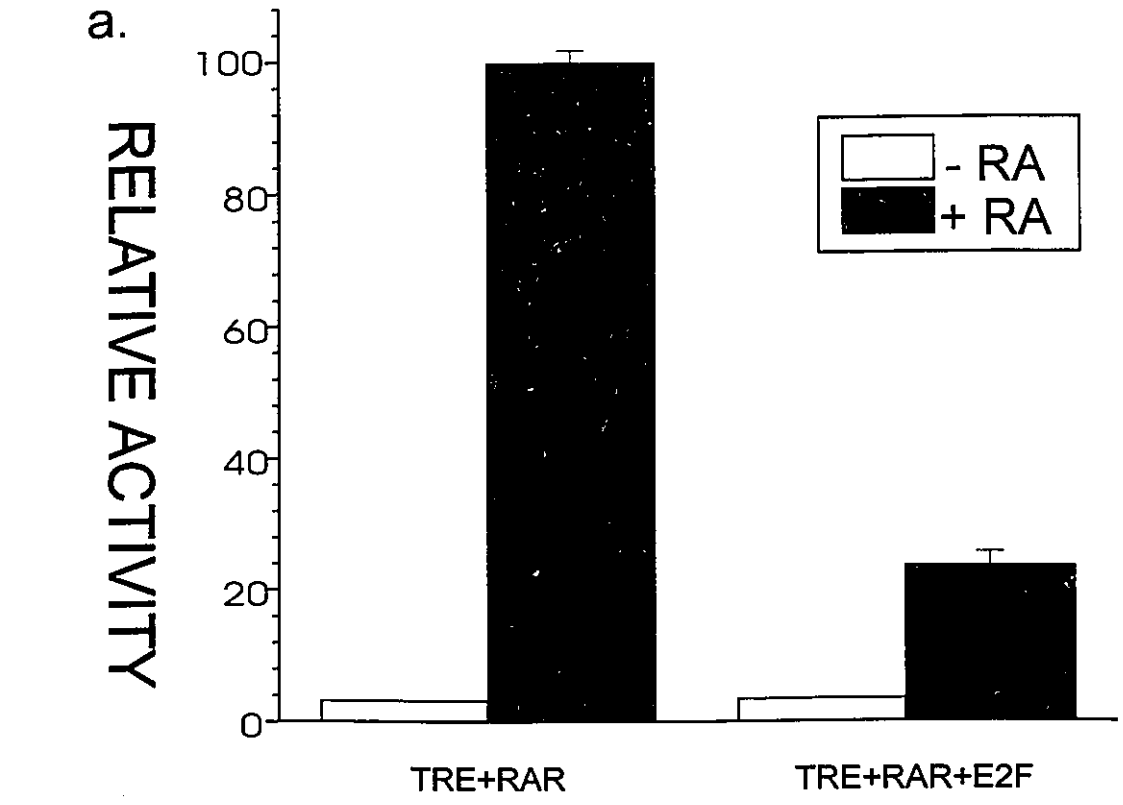
of E2F is not mediated by E2F sites within this promoter.

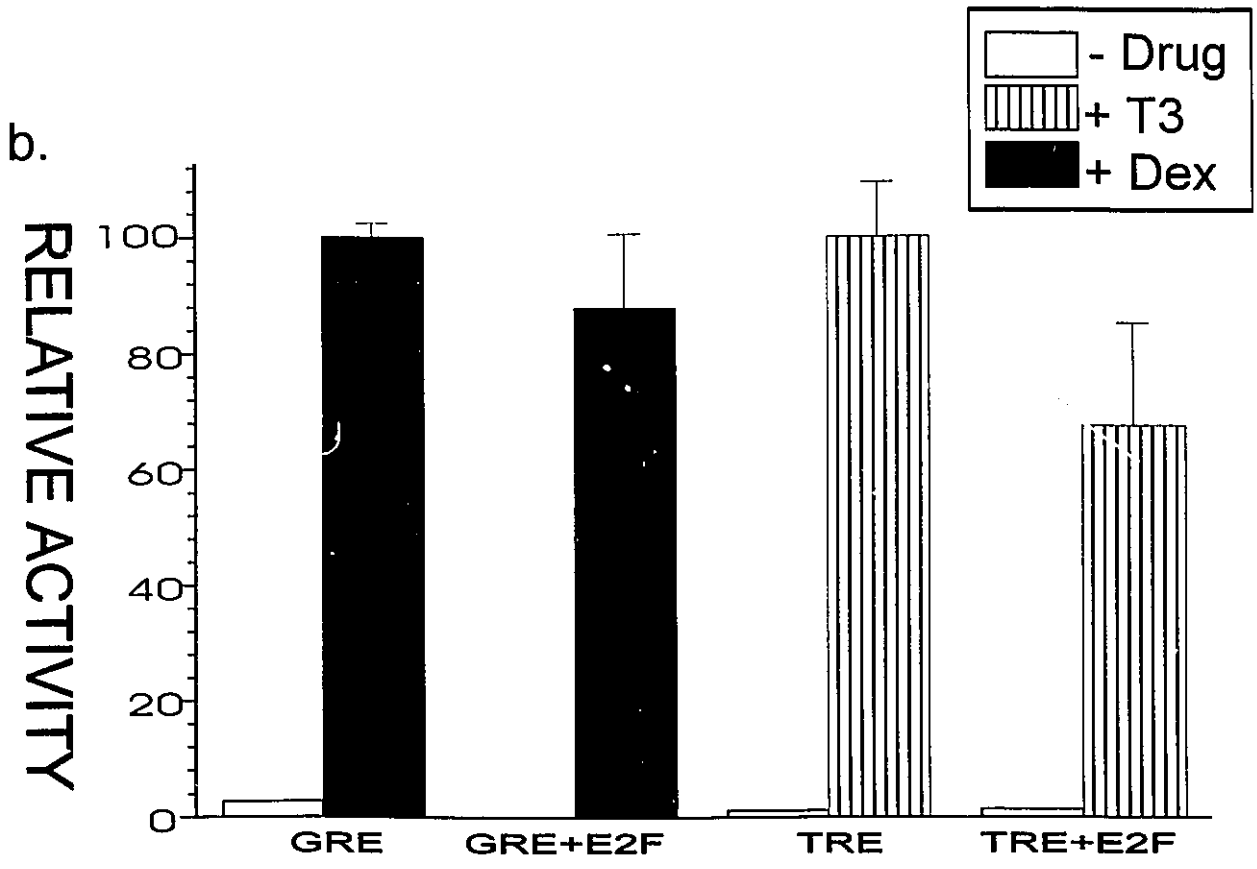
Specificity of the E2F Inhibition

To test if cotransfection of E2F inhibits transactivation by other members of the steroid hormone receptor family I carried out a similar analysis on a thyroid hormone responsive promoter, TREpal-CAT and the glucocorticoid responsive promoter, MMTV-CAT. The TREpal-CAT reporter construct is identical to the MMTV-CAT reporter except for the replacement of the GRE with a TRE (81,82). The TREpal-CAT reporter construct can be activated by either the RARs or the TRs. Cotransfection of the reporter TREpal-CAT with the RARs indicate that inhibition by E2F is observed when it is activated by the RARs (fig 4.4a). This same reporter was inhibited only slightly by E2F when the TRβ activated it in the presence of thyroid hormone (fig 4.4b).

Similar cotransfection experiments with the glucocorticoid responsive reporter MMTV-CAT and the glucocorticoid receptor clearly demonstrate that transcriptional activation by the glucocorticoid receptor is not inhibited by cotransfection of E2F (fig 4.4b). The data in figure 4.3 together with the finding that the TREpal-CAT reporter is inhibited only when activated by the RARs clearly demonstrate that the E2F inhibition is not mediated via specific E2F sites within these reporters. The finding that the inhibitory effect of E2F is limited to promoters activated by the RARs demonstrates that there is specificity to the effects of E2F between members of the steroid hormone receptor family.

Figure 4.4. **E2F inhibits RAR mediated transcription but not GR or TR mediated transcription.**
a) The reporter TREpal-CAT was cotransfected into P19 cells. A total of 4μg of the reporter was transfected with the RAR expression plasmid PGK-RARα and 4μg of the PGK-E2F plasmid (or pGEM). The internal control was 2μg of PGK-LacZ.
b) As described previously, either 4μg of the reporter TREpal-CAT plus 4μg of the thyroid hormone receptor β expression plasmid RSV-TRβ or 4μg of the reporter MMTV-CAT plus 4μg of the GR expression plasmid RSV-GR were cotransfected with and without 4μg of the plasmid PGK-E2F (or pGEM) into P19 cells. CAT activity was induced in the presence of $1x10^{-6}$M DEX (dexamethasone) or $3x10^{-8}$M T3 (triiodothyroxine).

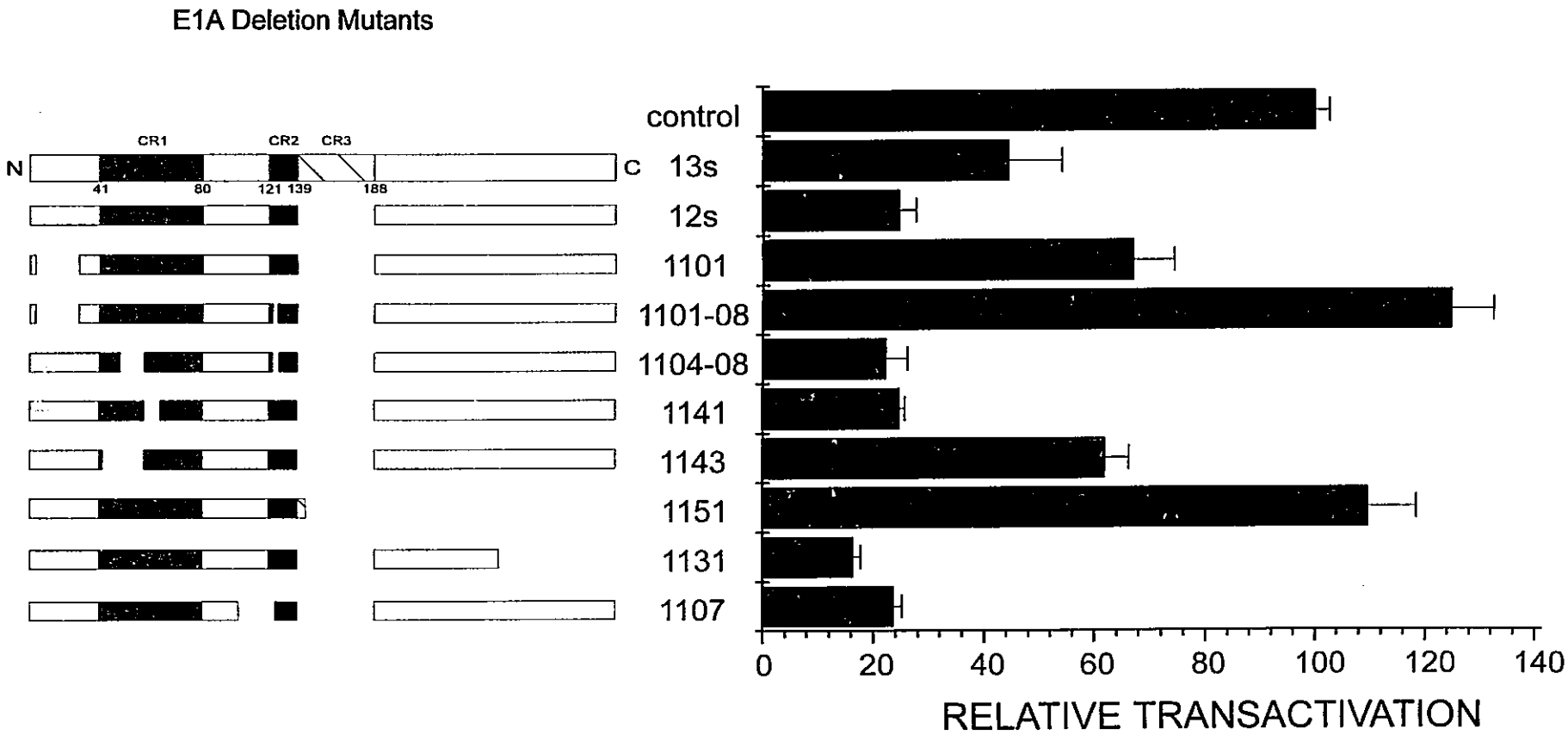

a.
RELATIVE ACTIVITY
100
80
60
40
20
0
- RA
+ RA
TRE+RAR
TRE+RAR+E2F


b.
RELATIVE ACTIVITY
100
80
60
40
20
0
- Drug
+ T3
+ Dex
GRE
GRE+E2F
TRE
TRE+E2F

E1A Also Inhibits the RARβ Promoter in P19 cells

The cellular E1A like activity was originally identified because of its ability to compensate for the absence of E1A in the adenovirus mutant Ad5 *dl*312 in embryonal carcinoma cells (287). It was characterized as an endogenous activity which activates the E2 promoter in the adenovirus genome, a function which is normally carried out by the virus encoded E1A protein (287). Because of the similarity in function of these two proteins (E1A and E2F) I wanted to determine if the functional similarity extended to their ability to inhibit the RARβ promoter. Transfection of P19 cells with E1A resulted in an inhibition of RA induced transcription from the RARβ reporter (fig 4.5). Both 13s and 12s E1A inhibited the RARE significantly. The inhibitory effect of 12s E1A was eliminated by the deletion of the N terminus (deletions 1101 and 1143) or by deletion of the N-terminal portion of exon 2 (deletions 1151 vs 1131). The N-terminus has been shown to be essential for the binding of the p300 protein (324). Both the N-terminus and the exon 2 region have been shown to be important in inhibition of other cellular promoters (322,325,326). Deletion of the CR2 domain, the portion of E1A which mediates the primary association of this protein with members of the retinoblastoma protein family (327) is not required for this inhibition. These deletion studies suggest that none of the other known E1A binding proteins (other than p300) appear to play an important role in this inhibition.

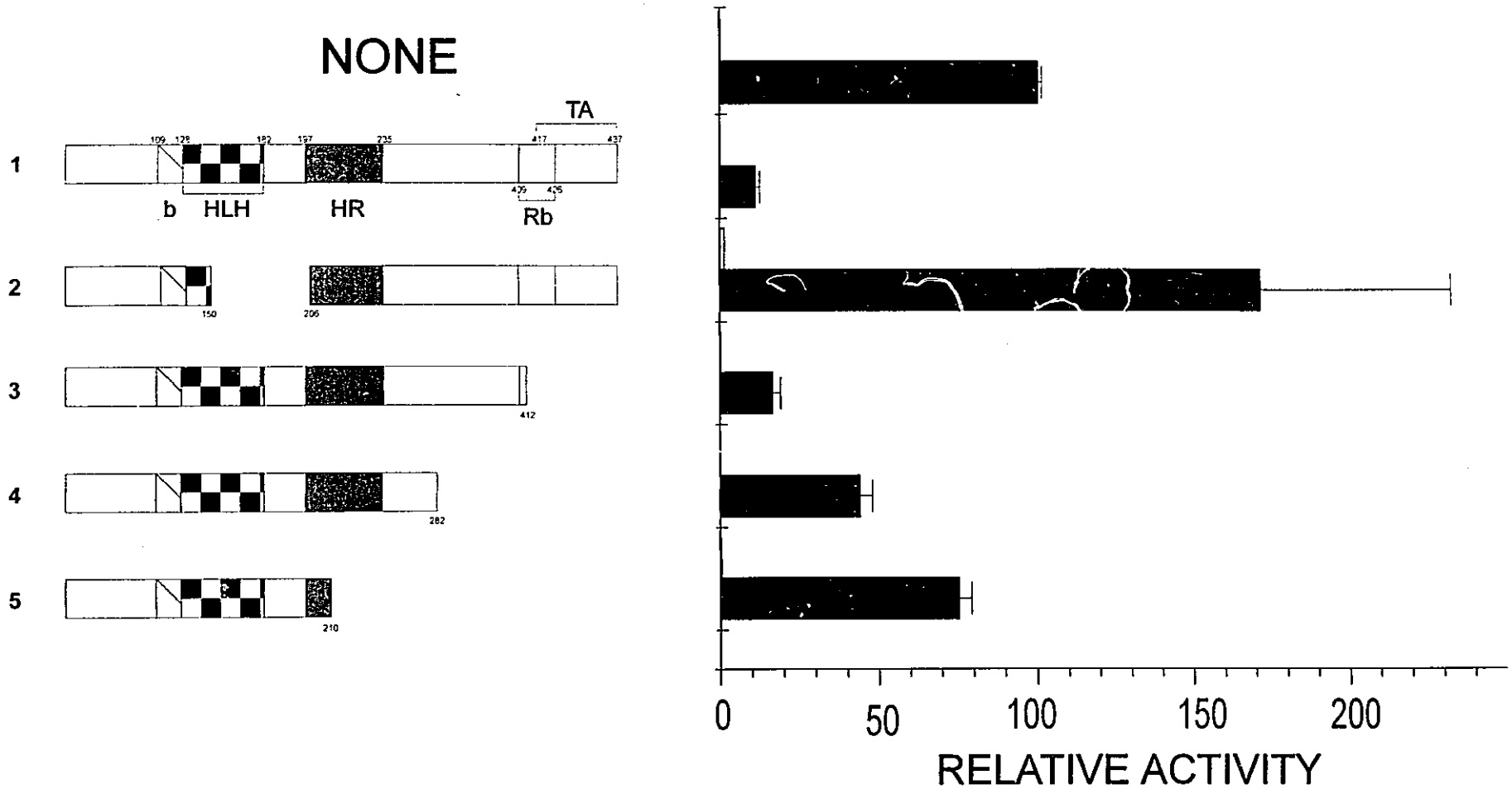
Figure 4.5. **Effect of E1A on the RARβ promoter.**
The E1A expression plasmids have been described previously (321,322) and the numbers refer to the deletions in these publications. 4μg of each of these plasmids was transfected with 2μg RARβCAT, 4μg pGEM and 2μg PGK-LacZ into P19 EC cells. Each transfection was repeated a minimum of three separate times and the results of all experiments were averaged and are reported. All dishes were treated with $3x10^{-7}$M RA after transfection.


E1A Deletion Mutants
CR1
CR2
CR3
N
C
41
80
121 139
188
control
13s
12s
1101
1101-08
1104-08
1141
1143
1151
1131
1107
0
20
40
60
80
100
120
140
RELATIVE TRANSACTIVATION

Deletion Analysis of the E2F Protein

Like E1A, E2F has been shown to interact with a number of cellular proteins including pRb and a related protein, p107, cyclins A and E, DP-1 and cyclin dependent kinases (CDK) (328-330). It is possible that the RAR transcription pathway requires one or many of these proteins to mediate its transcriptional response to RA. Thus E2F may act to inhibit RAR transactivation by sequestering these proteins. I carried out a deletion analysis of the E2F protein to ascertain which regions of E2F were required for inhibition to determine if any of these E2F binding proteins play a role in the E2F inhibition. Cotransfection of various deletion mutants with the RARβCAT illustrated the important regions for inhibition (fig 4.6). Deletion of the C-terminal 25aa of the E2F protein does not effect the inhibition indicating that the C-terminus of E2F is not necessary for inhibition (fig 4.6). The transactivation domain of E2F is contained in this 25 aa region (292) which indicates that E2F does not have to be transcriptionally active to inhibit the RARs. This indicates that E2F does not inhibit the RARβ promoter by activating the transcription of some endogenous inhibitor of RAR function. This 25 aa region has also been shown to be important in the interaction of E2F with the retinoblastoma gene product and deletion of these C-terminal 25aa eliminates most of the Rb binding domain (291). This suggests that E2F does not inhibit the RARs by directly sequestering Rb (or related proteins). This is similar to what I saw in the E1A protein deletion analysis where the Rb binding domain was not essential for the inhibition (fig 4.5).

Figure 4.6. **The Protein Interaction Domain But Not the Transactivation Domain of E2F Are Required for Inhibition of RARβ CAT.**
Several putative functional domains from the E2F protein were deleted by restriction enzyme digestion as described in Materials and Methods. 4μg of each of these E2F expression plasmids was then cotransfected into P19 cells as described previously and the resulting CAT activity was measured. The abbreviations are as follows: TA=transactivation domain, Rb= binding domain for the retinoblastoma protein, bHLH= basic helix loop helix domain (a protein interaction and DNA binding domain), HR= heptad repeats (putative protein interaction domain).


NONE
TA
1
2
3
4
5
b
HLH
HR
Rb
0
50
100
150
200
RELATIVE ACTIVITY

Further deletion of E2F into the region of the protein which contains the protein-protein interaction domains including a putative leucine zipper type motif (291,292) results in successive loss of the inhibitory effect of E2F (fig 4.6 constructs 4 and 5). These deletions do not delete the regions of E2F which are required for DNA binding (291,292). This demonstrates that the putative protein-protein interaction domain containing the series of heptad repeats plays an important role in the inhibition. An internal deletion encompassing regions required for DNA binding (construct 2) was completely devoid of any inhibitory activity suggesting that DNA binding plays an important role in inhibition. However the putative nuclear localization signal is also contained within this region so I cannot ascertain the precise role of DNA binding with this mutant. Interestingly, the regions of the E2F protein which are important for the E2F inhibition are the regions of the protein which are similar between it and DP-1 and furthermore these regions are important for its interaction with DP-1 and therefore formation of an active transcription complex (297). The protein interaction domains of E2F are necessary for this inhibition and the transactivation domain is not required for inhibition. This suggests that E2F is acting as a negative regulator of the RAR transcription pathway by squelching a protein or proteins from the RAR complexes.

The Inhibition by E2F is Not Reversed by Addition of RARs or RXRs

Because the protein interaction domains of E2F were important for inhibition I wanted to determine if either the RAR or the RXR were being bound directly and sequestered by E2F as has been shown for AP-1 and the RARs (163). If the effect of E2F is directly on the RAR

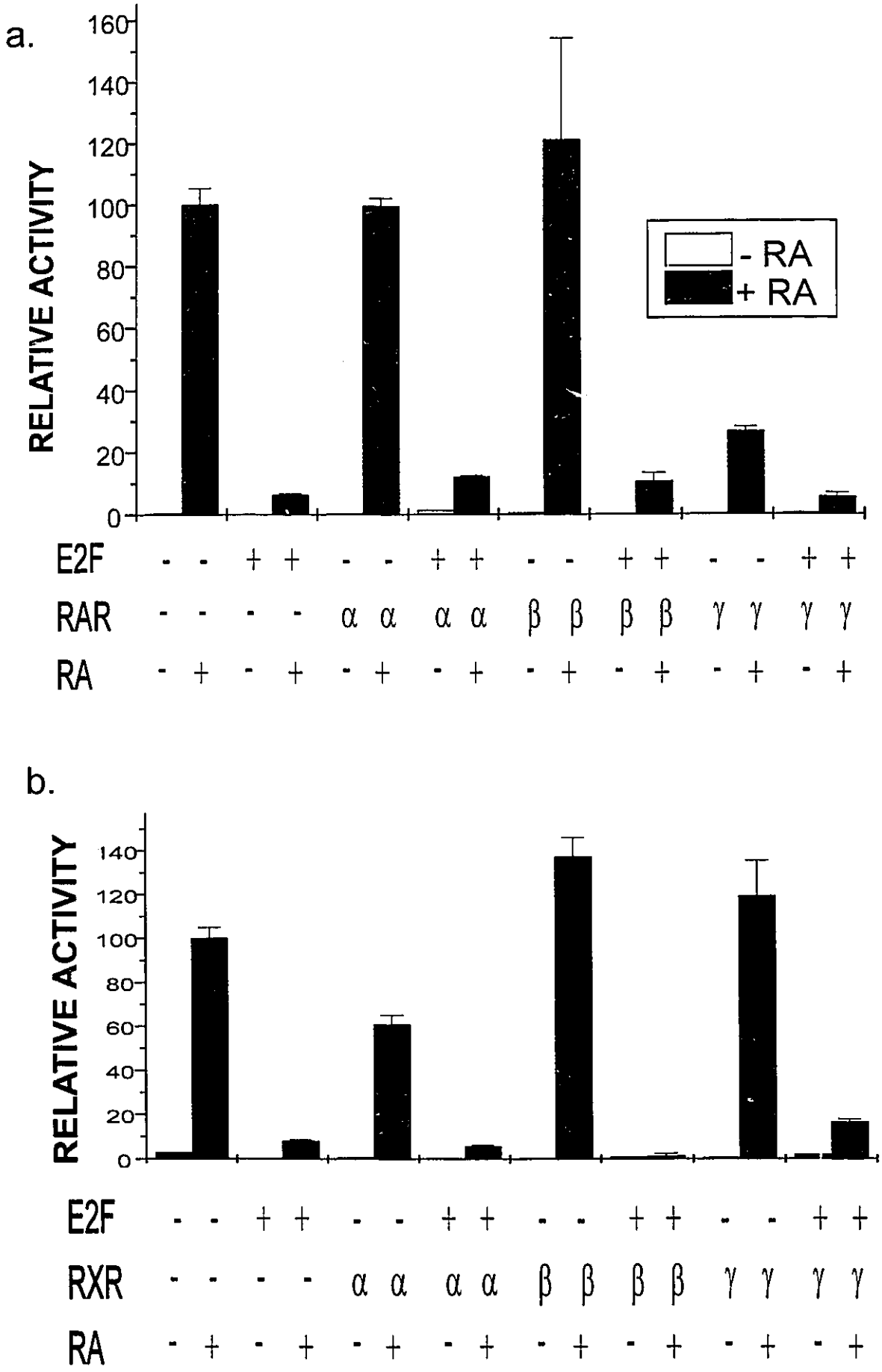
or RXR I reasoned that addition of excess of these proteins should dilute out the inhibitory effect of E2F. Thus I performed cotransfection experiments with the three RARs and three RXRs in the presence of E2F. Addition of excess RAR or RXR did not alter the inhibition and restore transactivation of the RARβ promoter suggesting that the effect of E2F is not directly on the RAR or the RXR (fig 4.7a and b).It is not clear why cotransfection of the RARγ and RXRα alone caused a decrease in transcriptional activation of this reporter (fig 4.7). This also indicates that all three receptor isoforms (both RAR and RXR) are inhibited by E2F and suggests therefore that it is a general RAR transcriptional phenomenon. Gel mobility shift analysis could not demonstrate any evidence for an interaction between these proteins (data not shown). I therefore believe the effect of E2F is indirect and is occurring downstream of the RAR/RXR heterodimer in the RAR transcriptional activation pathway .

Sequestration of Other Common Transcription Factors

Our analysis suggested that the mechanism of action of E2F might be by squelching a factor or factors from RAR transcription complexes. I reasoned therefore that if I were to add back the squelched factor I could restore activation of the RARβ promoter. To determine if any of the known elements of the RAR transcription pathway were being squelched I performed cotransfection experiments with E2F and various common components of the RAR and E2F transcription machinery.

Because transcription from both the RAR and E2F dependent promoters depends on

Figure 4.7. **The E2F Inhibition is not Reversed by Addition of RARs or RXRs**
4μg of the expression plasmids for each of (a) the RARs PGK-RARα or PGK-RARβ or PGK-RARγ or (b) the expression plasmids for the retinoid X receptors; SV40-RXRα or SV40-RXRβ or SV40-RXR-γ were transfected along with 4μg of the PGK-E2F (or pGEM as required), 2μg of PGK-LacZ and 2μg of RARβCAT into P19 cells as described previously.

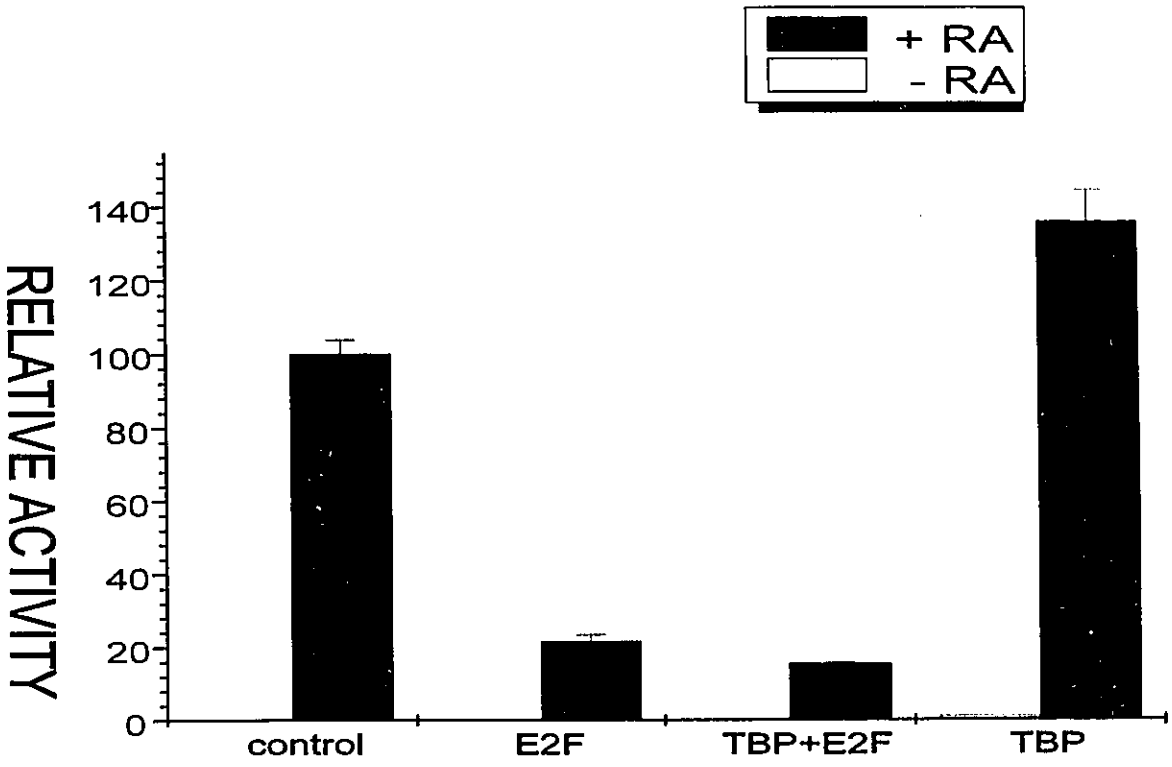

a.
RELATIVE ACTIVITY
160
140
120
100
80
60
40
20
0
- RA
+ RA
E2F - - + + - - + + - - + + - - + +
RAR - - - - α α α α β β β β γ γ γ γ
RA - + - + - + - + - + - + - + - +
b.
RELATIVE ACTIVITY
140
120
100
80
60
40
20
0
E2F - - + + - - + + - - + + - - + +
RXR - - - - α α α α β β β β γ γ γ γ
RA - + - + - + - + - + - + - + - +

association of these proteins with TBP (124,125) I wanted first to determine if under our experimental conditions I was adding enough E2F to sequester TBP from the RAR transcription complexes. Cotransfection of excess TBP in the presence of E2F was unable to relieve the repression produced by excess E2F (fig 4.8). TBP alone produced a significant activation of transcription from the RARβ promoter as has been shown by Berkenstam et al. (124) however in the presence of E2F this activation was lost and activation of the RARβ promoter was reduced to levels below that seen with E2F alone (fig 4.8). This suggests that the E2F inhibitory effect is not due simply to the sequestering of TBP.

Given the functional similarity between E1A and E2F (activation of the E2 promoter, inhibition of RARβ promoter and the fact that pRb does not play a role in RAR inhibition by either E2F or E1A) it is possible that both inhibit the RARE by similar mechanisms. The possibility exists that these proteins (E2F and E1A) share the ability to bind some common transcription factor which is present in P19 cells in limiting quantities. This common factor may also be used for transactivation by ligand activated RARs. In our model, cotransfection of E2F or E1A leads to inhibition of RAR mediated transactivation because it reduces the availability of this factor for RAR mediated transactivation. From the results of the E1A deletion analysis it is enticing to speculate that this common transcription factor is the p300 protein or a protein related to the p300 protein because of its postulated role as a transcriptional adaptor/co-activator protein (323,331).

To assess if any co-activator proteins were being sequestered by E2F thereby

Figure 4.8. **TBP Does Not Restore Activation of the RARβ Promoter.**
The plasmids PGK-E2F (4μg) and PGK-TBP (4μg) were transfected together and separately (with 4μg of pGEM) into P19 cells along with the reporter RARβCAT (2μg) and the internal standard PGK-LacZ (2μg).


+ RA
- RA
RELATIVE ACTIVITY
140
120
100
80
60
40
20
0
control
E2F
TBP+E2F
TBP

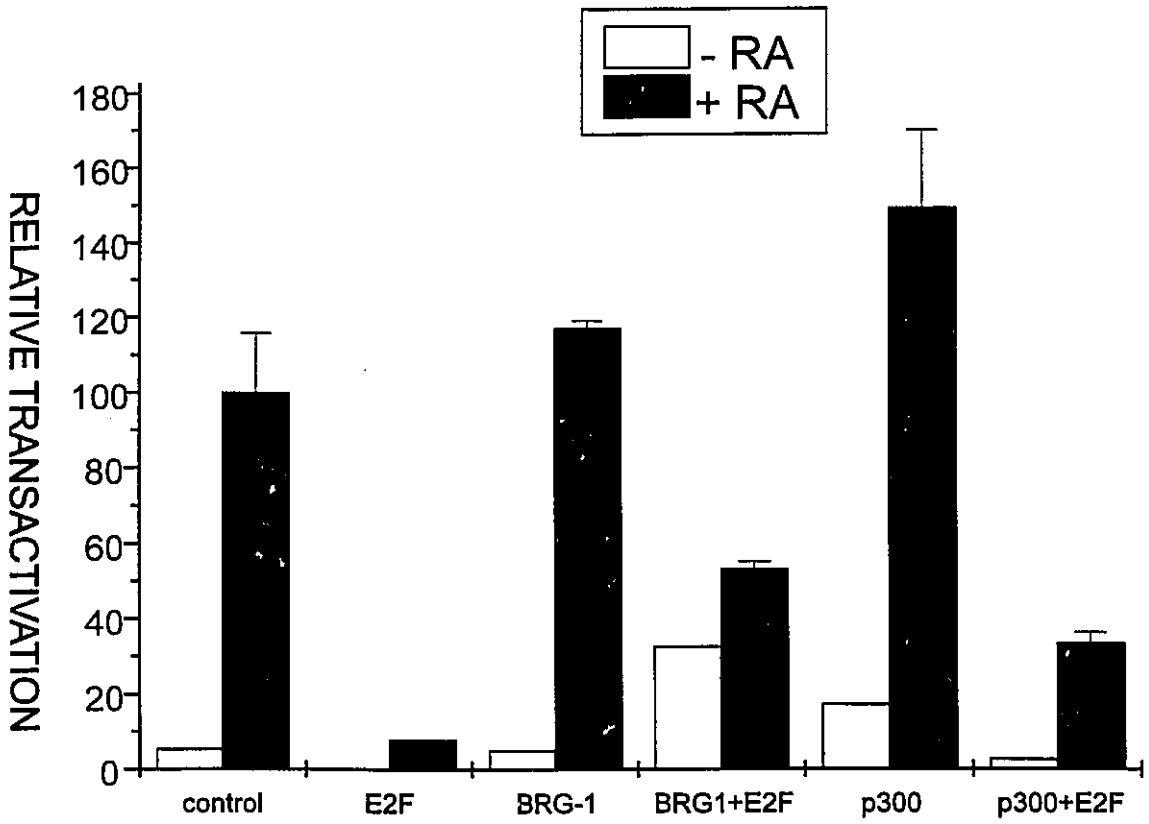
preventing activation of the RARβ promoter cotransfection experiments were undertaken. An expression construct containing the p300 cDNA was cotransfected with the reporter RARβCAT into P19 EC cells. Cotransfection of p300 only partially alleviated the inhibitory effect of E2F on this reporter (fig 4.9a). This suggests that p300 is not the factor or the only factor being squelched from RARE containing promoters by E2F. Several different types of co-activator proteins have been described and it is possible that a related bromodomain containing protein (possibly a p300 family member) or a member of the yeast SWI/SNF family of proteins is the squelched factor.

One co-activator which has been described for the RARs is the BRG-1 protein (a.k.a. SNF2β) (256). To test if a member of the yeast SWI/SNF family of proteins was the squelched factor cotransfection experiments with a BRG-1 expression construct and PGK-E2F were done. Cotransfection of BRG-1 partially reversed the inhibition of the RARβ promoter imposed by expression of E2F (fig 4.9b). In conclusion, addition of an excess of any of these co-activator type proteins alone was unable to completely restore activation of the RARE.

Effect of E2F on an E2F Site Containing Promoter

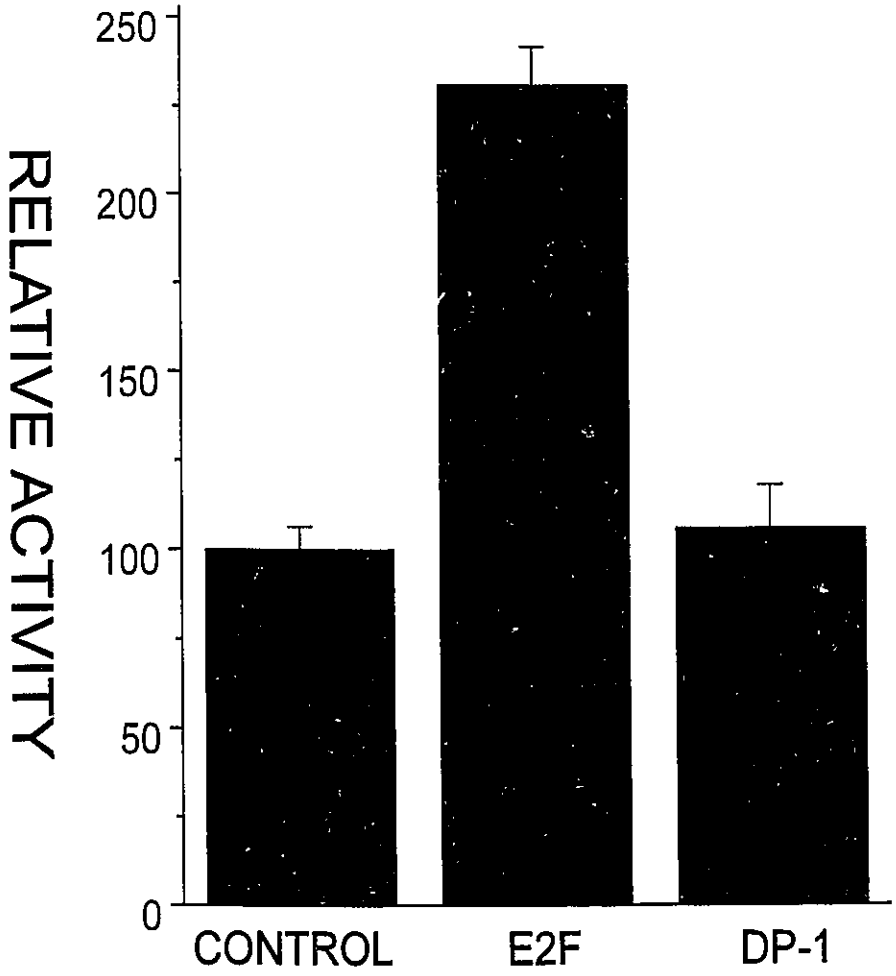
To determine if the inhibitory effect of E2F on the RARβ promoter was due to a general inhibitory effect of E2F in P19 cells or if it was a specific effect on RARE containing promoters I cotransfected E2F and DP-1 with the plasmid Rb-CAT. This promoter contains

Figure 4.9. **Two Co-activator Proteins Partially Restore Activation of the RARβ Promoter.**
An expression plasmid for the p300 protein (4μg) was transfected with and without PGK-E2F (4μg), 2μg of RARβCAT and 2μg PGK-LacZ as described previously. The mammalian expression plasmid pJB5 (320) which encodes the BRG-1 protein (SNF-2β) was cotransfected with and without E2F as above.


- RA
+ RA
RELATIVE TRANSACTIVATION
180
160
140
120
100
80
60
40
20
0
control
E2F
BRG-1
BRG1+E2F
p300
p300+E2F

an E2F site and it has been shown to be activated by E2F in transient transfections (303). E2F was found to behave normally on this promoter, activating this promoter significantly (fig 4.10). This demonstrates that this inhibition is a specific effect of E2F on the RARE containing promoters. Furthermore it indicates that addition of excess E2F by transfection increases the amount of active E2F within the cell. The finding that E2F is an activator on an E2F site containing promoter in P19 cells but an inhibitor of RARE containing promoters is interesting because E2F has not previously been demonstrated to have an inhibitory effect on non E2F site containing promoters. This experiment also indicates that I have an active E2F protein in our assays. This conclusion is supported by the finding that an independent clone of E2F was also inhibitory on the RARβ promoter (data not shown).

Figure 4.10. **E2F Is An Activator of Rb-CAT**
The E2F responsive reporter construct Rb-CAT was cotransfected with 2μg PGK-LacZ and 6μg of PGK-E2F or PGK-DP-1 or 6μg of pGEM. Experiments were as described previously.


RELATIVE ACTIVITY
250
200
150
100
50
0
CONTROL
E2F
DP-1

Discussion

We wanted to determine the role of E2F and DP-1 in the activation of RA responsive genes in P19 cells. It had been previously shown that E1A can act as a co-activator in COS cells (124) and I postulated that E2F may have a similar function because one of the major cellular effects of E1A is the activation of endogenous E2F (315-317). Transfection of P19 cells with E2F clearly demonstrated that, in contrast to the activity of E1A in COS cells, E2F is a potent inhibitor of RARβ promoter in P19 cells. Our analysis suggested that the E2F inhibition is dependent on the protein-protein interaction domain of E2F suggesting that it inhibits the RARE by squelching a protein or proteins. Studies to assess if E2F inhibited the RARβ promoter by squelching a common co-activator from the RAR transcription complex indicated that the two co-activators implicated in the actions of the RARs and E1A (p300 and BRG-1) partially restored activation of the RARβ promoter. I believe that E2F inhibits the RARβ promoter by sequestering an essential co-activator from RAR transcription complexes.

Our data indicates that the inhibitory effect of E2F depends on the regions of E2F involved in protein-protein interactions. This suggests that the mechanism by which E2F acts to inhibit the RARβ promoter is by squelching a protein or proteins from RAR transcription complexes. I believe that E2F sequesters a common transcription factor(s) such as a bridging factor/co-activator from the RAR transcription complex thus preventing transactivation by the RARs. I believe this in part because it has been demonstrated that the availability of such an accessory factor can determine the ability to activate the RARE (124) and because the co-

activators analyzed partially restored transactivation.

Co-activators and Their Role in Activation of the RARE

Co-activators are tightly associated with TBP and are required for promoter specific activators (for example the RARs, E2F, SP1, CTF and Gal4) in order to achieve stimulated levels of transcription (351). Two models have been proposed for co-activator function. One model proposes that co-activators serve as a link or bridge between the general transcription machinery and specific transcription factors and these types of co-activators are often referred to as bridging or linking proteins or mediators (351). An example of this type of co-activator is the 13s E1A in activated transcription by the Oct factors and the RARs (124,332). Another model which has been proposed for co-activator activity is that the co-activator functions by binding the general transcription machinery and inducing a change in some property (for example a conformational change) in the general machinery which causes more productive interactions which result in activated transcription (351). Many of the TBP associated factors or TAFs which have been cloned have been demonstrated to have co-activator activity (351).

Activation of RARE containing promoters has been known to require the activity of bridging factors which link the RAR/RXR complex to the general transcription machinery (124,332). Many of these bridging proteins have been cloned and found to exhibit homology to the yeast protein SWI2/SNF2 whose role in activated transcription has been well documented in yeast (333,334). Two human homologues which have recently been cloned

were found to support activated transcription by the RAR and these proteins were designated SNF2α and SNF2β (256). One of these proteins had been cloned previously (called BRG-1) and found to bind the retinoblastoma protein and enhance its role as a cell cycle regulator (335). Another co-activator, the p300 protein, was originally identified because of its interaction with the E1A protein and its molecular weight (MW 300kDa) (323). This protein was thought to act as a co-activator because it contained a domain designated the bromodomain which is found in other co-activator proteins (323). It has been recently been shown to act as a co-activator for the activation of CRE containing promoters (336).

One property which has been demonstrated *in vitro* for co-activators is the ability to be squelched (351). Early work demonstrated that overexpression of viral transactivator proteins such as VP16 leads to a decrease in activated transcription from other promoter elements (351). This inhibition was believed to be mediated by the activator proteins squelching essential co-activator proteins from these other promoter elements. Squelching of co-activators, as has been suggested to occur here with E2F and the RARs, has been demonstrated to occur in other systems (351,352).

Multiple, distinct co-activators are believed to exist (351). The specificity of the E2F inhibition for RAR mediated transcription over the other members of the steroid hormone receptor family suggests that the E2F inhibition may be mediated by the squelching of a co-activator. This is because several distinct co-activators have been cloned and shown to posess specificity for various members of the steroid receptor family (256,339).

If E2F inhibits the RARβ promoter by squelching a co-activator then why do BRG-1 and p300 not restore transactivation completely? There are two possible explanations. In the first scenario, EC cells may harbour unique co-activators which regulate the activity of both E2F and RAR dependent transcription pathways in P19 cells. In our model this EC specific factor is squelched away from RARE containing complexes by overexpression of E2F. BRG-1 and p300 may be able to support transactivation of the RARβ promoter however they are not as efficient in P19 cells as this specific P19 co-activator.

An alternative explanation for our finding that transfection of the co-activators only partially restored RARβ promoter transactivation is that other factors are required by BRG-1/p300 for these proteins to act as efficient co-activators in P19 cells. Co-activator activity has been purified from several cell types and often purifies out as a high molecular weight complex of proteins rather than as distinct proteins (351). Two candidates for such an auxiliary factor which allows the co-activators to function efficiently are the retinoblastoma protein and TBP. The retinoblastoma protein has recently been shown to bind to a co-activator similar to BRG-1 (BRM-1) and this interaction was required for efficient co-activation with the glucocorticoid receptor (339). Furthermore, recent studies have suggested that in undifferentiated EC cells Rb may act as a transcriptional co-activator with the adenovirus E4 protein (340). TBP is another candidate because it has been shown to synergise with the RARs in activation of the RARE (fig 4.8 and (124)).

E1A and Expression of the RARβ Promoter

Our results with E1A and the RARβ promoter are in contrast to experiments by Berkenstam et. al. (124) who reported that in COS cells TBP and 13s E1A synergise in activation of the RARE. Our results clearly demonstrate that 13s E1A is a potent inhibitor of the RARβ promoter in P19 cells. This difference may reflect the differences in the cell types used. It is possible that in P19 cells factors other than those which are used in more differentiated cell types such as COS cells co-operate with the RARs and TBP to activate the RARE. One other difference that may explain the inconsistencies in E1A function between our group and that of Berkenstam et. al. is a dose effect. The levels of E1A added in their experiments is less than the amounts which I used in these experiments (1μg versus 4-8μg) (H. Stunnenberg pers. comm.). Dose is a critical determinant in the effect that E1A elicits because co-transfection of 13s E1A is stimulatory at low doses but inhibitory at high doses when assessed with the transcription factor Oct-3 (285). It is likely that the levels of E1A used by Berkenstam et al. were not sufficient to squelch (124).

One further consideration is that our analysis is carried out with the 12s E1A protein primarily. Other groups have demonstrated the 12s E1A product is an inhibitor of the RARE (341). The 12s E1A protein does not contain the CR3 domain which is the domain believed to mediate this bridging function of E1A (285,342,343). Our 13s E1A transfection demonstrates that with this domain there is less inhibition of the RARβ promoter than with the 12s E1A product. It is possible that this is the result of the additive effects of the stimulatory effect of the CR3 bridging function in addition to the inhibitory effect of the N-terminus and exon 2 regions in the 12s product. It is the inhibitory function in the 12s product

which I believe to be similar in activity to E2F.

The finding that the regions of E1A which are required for this inhibition reside within the N-terminus and part of exon 2 is interesting because these regions have previously been demonstrated to be important in the inhibition of other cellular promoters (322,325,326). It has been further demonstrated that the same regions in exon 2 are required for efficient CR3 dependent transactivation of the adenovirus E4 promoter suggesting that there is interplay between the CR3 linking function and inhibition of cellular promoters by E1A exon 2. This is completely consistent with the data presented here and the published work of Berkenstam et. al. (124) and the model which I am proposing to mediate the inhibition by E2F.

Evidence for Involvement of Other Factors Regulating RARE Expression

The finding that E2F has effects on the RARE is not surprising because it has been known for some time that expression of RARE containing promoters is dependent on other factors in addition to the RAR transcription complex. For example, studies with transgenic mice using the RARE from the RARβ promoter driving the reporter β-galactosidase demonstrated that the developmental expression pattern of the RARβ could not be recapitulated by the RARE alone (344). This suggested the presence of other factors which regulate the expression of this gene. Furthermore, studies with regenerating amphibian limbs suggest that expression of the RARβ gene cannot be accounted for solely by the presence of RA (4,5,345). It is not therefore surprising to find that the regulation of the RAR

transcription pathway is dependent on the activity of another transcription pathway (E2F) as I describe here.

E2F, RARs and the Regulation of Growth

The mechanism by which I propose E2F inhibits the RARβ promoter may represent one mechanism by which the cell integrates growth signalling pathways. By having both growth stimulatory pathways (E2F induced) and growth inhibitory pathways (RA induced) utilizing common factors which are present within the cell in limiting quantities, the cell would be able turn on one pathway while at the same time turning off the other. This would not only insure that the cell produced only one growth outcome at a time it would also help to conserve energy expenditures within the cell. A similar type of coordinate regulation of growth signalling pathways is seen between RA and the AP-1 transcription complex. The AP-1 transcription complex activates growth signalling within the cell and RA has been shown to inhibit AP-1 transactivation by a direct interaction between AP-1 and the RARs (52,163). While E2F does not appear to inhibit the RARs by a direct interaction between these proteins the overall effect seems to be quite similar. This may be another way in which the cell integrates the signals produced by external stimuli to produce the greatest response with the least energy expenditures.

Conclusion

We have clearly demonstrated that overexpression of a dominant negative RAR can block the RA induced differentiation of P19 cells. Furthermore I have shown that E2F can function as a dominant inhibitor of RARE containing promoters. One question that arises in light of these observations is, can overexpression of E2F block differentiation in a similar manner to what was seen in the RA resistant clones overexpressing RARαmyc? It has recently been demonstrated in an EC cell line that overexpression of E2F can block differentiation (346). Thus I can conclude that dominant inhibition of the RARs can block the differentiation of P19 EC cells by steric hindrance (as is seen with RARamyc) or by squelching (as is seen with E2F).

It is important to understand the mechanism by which dominant negative mutants such as RARαmyc act within the cell because many types of cancer arise due to a block in differentiation of an undifferentiated precursor cell. For example the PML-RAR protein acts dominantly to inhibit the differentiation of promyelocytes (347). It is presently unclear how this mutant protein acts within the cell to exert these effects. The types of experiments as have been described here (chapter 2) could help to elucidate how this mutant protein acts within the promyelocyte to block its differentiation. Such studies may help to elucidate better treatments or cures for cancers such as APL.

Our studies with transgenic mice indicate that the differentiation of the cells of the

seminal vesicle can be affected by overexpression of RARαmyc. There appears to be an association between the defect observed (blockages in the vas deferens and epididymis) and the resulting phenotype (infertility). Further experiments need to be done to conclusively show that the blockages lead to the phenotype observed. A detailed histological analysis of the secretory epithelium within the seminal vesicle may elucidate differences between normal and transgenic mice. High doses of retinol (ROH) induce the differentiation of RAC65 cells *in vitro* (data not shown). It may follow that feeding high doses of ROH to the infertile males would reverse the infertility by eliminating the deposits of seminal fluids. Such experiments would conclusively show that the RARαmyc causes infertility by affecting the differentiation of the seminal vesicle epithelium leading to excessive seminal fluid production and blockages within the male reproductive tract.

We propose that E2F inhibits the RARs by squelching a common co-activator. This type of inhibition could be utilized much more generally to regulate opposing signalling pathways in the cell. Early work analyzing the actions of co-activators on transcription postulated that this type of regulation could occur and would be used *in vivo* to regulate diverse transcription signals (352). However this type of regulation has only been demonstrated in artificial transcription systems (for example, Gal 4 fused to various transactivators) (351,352). The model which I propose would be the first example of the regulation of two opposing signalling pathways where squelching of co-activators is demonstrated *in vivo*. Given the diversity and specificity of co-activators being discovered today, it seems likely that in the future other systems which are regulated by this type of

mechanism will be found. It is hoped that the current work will foster the identification of novel co-activator proteins that could account for the proposed squelching mechanism involving E2F inhibition of RAR mediated transactivation and furthermore initiate future experiments aimed at exploring the *in vivo* involvement of RARs in growth, differentiation and development.

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Appendix 1: **Expression of RARγHA in Transgenic Mice**

Introduction

Retinoids are potent inhibitors of tumourigenesis and have been used in the treatment and prevention of neoplastic transformation (170-175). To determine if the RARγ plays a role *in vivo* in the normal prevention of tumourigenesis I decided to generate transgenic mice which express our RARγHA mutant in the mammary gland. The RARγHA mutant was not a dominant inhibitor of the RARβ promoter in P19 cells however I chose this mutant because this type of RARγ mutant has been demonstrated to exhibit cell type specific transactivation properties (270). Specifically, this type of mutant has been demonstrated to act as a dominant negative mutant in epithelial cell types (270). The MMTV promoter which I chose to drive expression of our transgene in the mammary gland has been demonstrated to drive expression primarily in the epithelial cells of the mammary gland (353). I reasoned that this mutant RARγHA may lead to transformation of this cell type within the mammary gland.

Materials and Methods

Generation of Transgenic Mice

Embryos were injected with the RARγHA construct producing six viable transgenic lines as described previously in chapter 3.

Northern and Southern Blotting

Blots were prepared as described in chapter 3. The probe for the RARγ was the full length mouse RARγ cDNA (EcoRI fragment) and was a gift of Dr V. Giguére (238). The genomic DNA was digested with PstI.

DNA Constructs and Probes

Generation of the RARγHA construct has been described previously. This mutant was subcloned into the plasmid MMTV-SV40-Bssk (271) by digestion with EcoRI and HindIII and ligation into similarly digested MMTV-SV40-Bssk. The region encompassing the promoter to the end of the polyadenylation signal was used for microinjection of fertilized zygotes.

Results

Generation of RARγHA Expressing Transgenic Mice

Transgenic mice were generated by microinjection of fertilized zygotes with the construct MMTV-RARγHA (fig A1.1a). With the RARγHA transgene six heterozygous founder animals were generated all of which were fertile and transmitted the transgene to their offspring (see below). These strains were designated 1104, 1105, 1108, 1111, 1124 and 1126 and the representative Southern blots are depicted in figure A1.1b.

Transmission and Expression of the RARγHA Transgene

Analysis of the RARγHA transgene demonstrated the transmission of this transgene was below the expected value of 50% and varied considerably between strains (Table A1.1). Several of the RARγHA strains consistently produced significantly smaller litters than the other transgenic strains (RARαmyc, chapter 3) which suggests that some of the RARγHA transgenics may have been selectively lost during development or cannibalized by their mothers. To determine if there was a selective loss of transgenic animals, animals which were born dead or partially cannibalized by their mother were analyzed for the transgene. Of the seven animals tested from the strains which transmitted at below 50% frequency (1105,1111,1124,1126) five of the neonates were found to be transgenic (71%) which is consistent with our hypothesis.

Figure A1.1. **Generation of Transgenic Mice Expressing RARγHA**
a) Schematic representation of the DNA fragment injected into fertilized zygotes for the generation of transgenic mice. Six strains were generated which harboured the RARγHA construct in their genome. The MMTV promoter and SV40 polyadenylation signals are depicted with the dark grey boxes. The RARγ transcription unit is depicted in the open box with the DNA binding domain in the medium grey and the ligand binding domain in the light grey. The probe is the full length mouse RARγ cDNA depicted above the RARγHA fragment and the regions of the transgene which generate the transgene specific bands when the DNA is digested with PstI are shown below. b) Representative Southern blots for each RARγHA strain are shown. The genomic DNA was digested with PstI. The open arrows represent the transgene specific bands and the closed arrows depict the endogenous RARγ bands. Blots were exposed for one day at -70°C

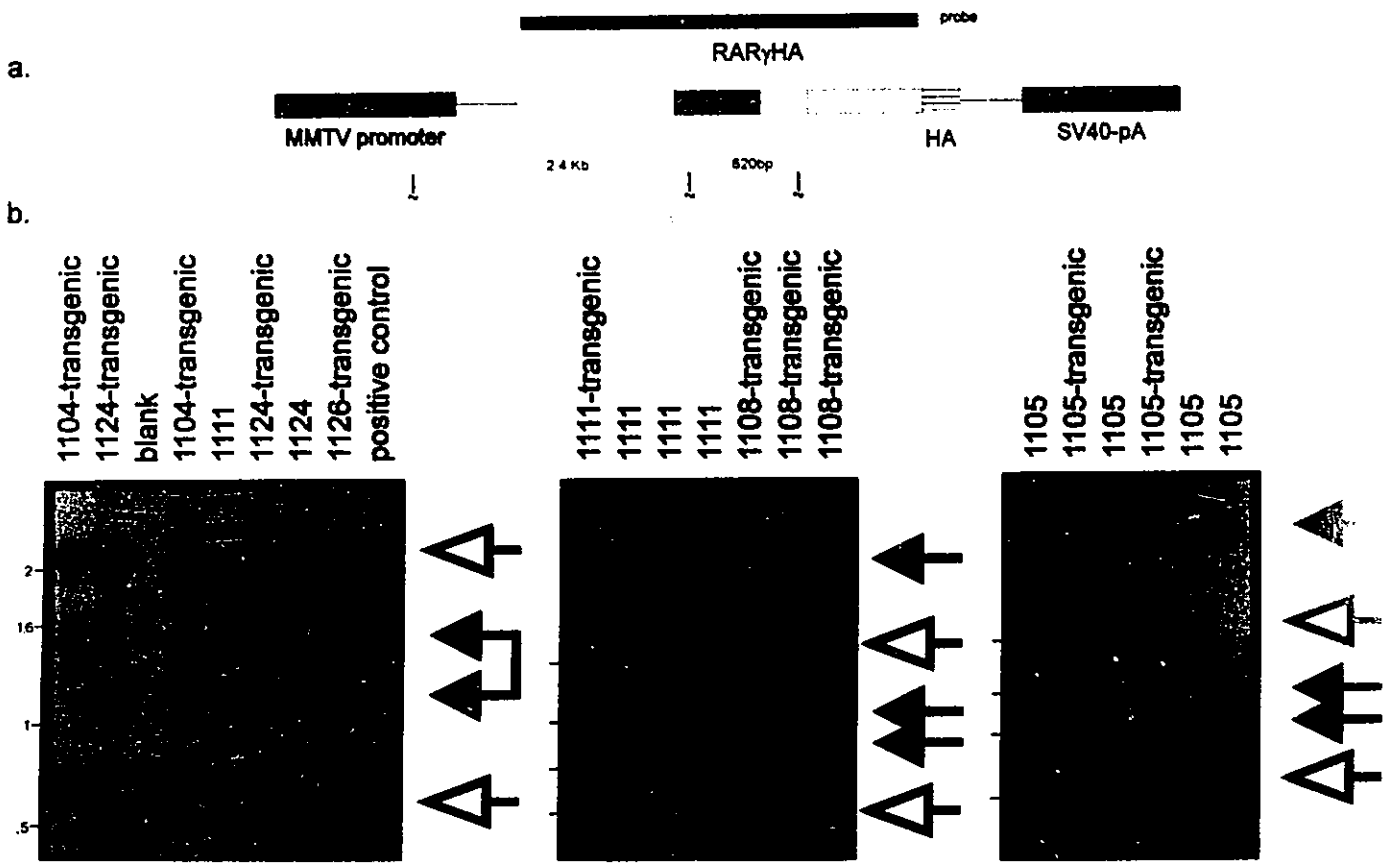

a.
probe
RARγHA
MMTV promoter
2.4 Kb
620bp
HA
SV40-pA
b.
1104-transgenic
1124-transgenic
blank
1104-transgenic
1111
1124-transgenic
1124
1126-transgenic
positive control
1111-transgenic
1111
1111
1111
1108-transgenic
1108-transgenic
1108-transgenic
1105
1105-transgenic
1105
1105-transgenic
1105
1105
2
1.6
1
.5

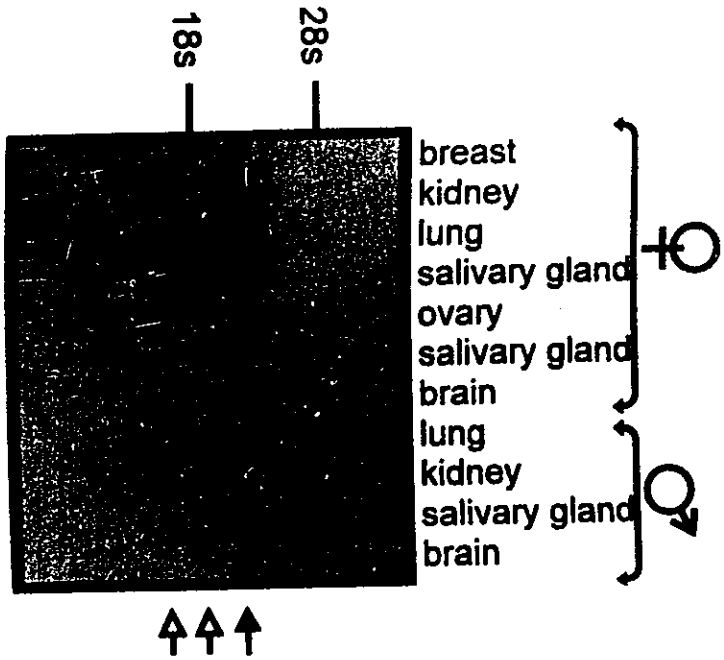
Table A1.1. **Transmission of RARγHA Transgene.**
All offspring of the RARγHA founder animals were analyzed for the presence of the RARγHA transgene in their genome by Southern analysis. Transmission of the transgene for four of the six strains occurs at a frequency below that expected for normal Mendelian inheritance of a heterozygous trait (50% offspring transgenic). The transmission for these strains was approximately 30%. Two of these transmission frequencies are significantly below the expected value (1105 and 1126). The low transmission for the 1124 strain is only marginally significant. The inheritance of 30% for the strain 1111 is not significant. More animals would need to be analyzed to determine if these latter inheritances are significant. Low transmission frequencies may be indicative of deleterious effects of expressing this transgene.

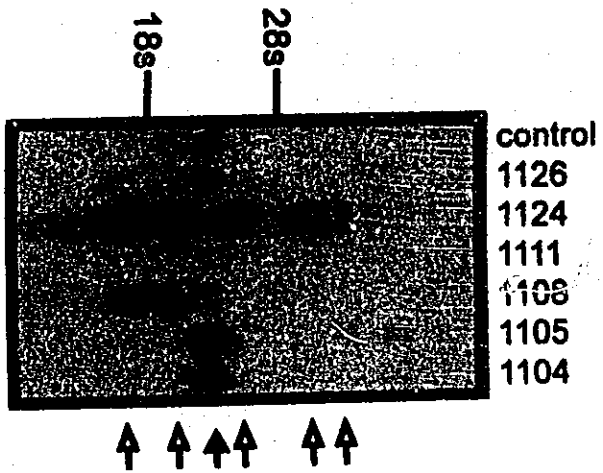
Table A1.1: Transmission of RARγHA Transgene

strain	total # genotyped	total # female	total # male	total # transgenic	# female transgenic	# male transgenic	transmission frequency	P value
1104	34	18	16	15	6	9	44%	-
1105	47	27	20	12	6	6	27%	0.025
1108	32	18	15	16	9	7	50%	-
1111	39	18	21	12	4	8	30%	0.131
1124	77	32	45	26	11	15	32%	0.061
1126	34	13	21	8	4	4	24%	0.044

To assess the levels of expression of the RARγHA transgene, one female from each strain was sacrificed and breast RNA was extracted (fig A1.2a). Only two of the lines were found to express the transgene, strains 1124 and 1108 (fig A1.2a lanes 3 and 5). The nature of the extra transcripts found in transgenic lines is currently unknown and may represent differentially processed and/or read through transcription from multiple integrations of the transgene (fig A1.2a open arrows). Upon subsequent analysis of other animals of the highest expressing transgenic line (strain 1124) none of these animals were found to express the transgene at comparable levels (fig A1.2b). Because of the lack of stable expression of the transgene none of these animals were further analyzed. This data is consistent with the hypothesis that expression of this transgene is deleterious to the development of the animal.

Figure A1.2. **Analysis of the Expression of the RARγHA Transgene in Transgenic Mice.** a) RNA was extracted from the mammary glands from six female transgenic mice (one from each line) to assess expression of the RARγHA transcript. Mice were 2-5 months of age. Expression was assessed by northern analysis. The blot was exposed for one day at -70°C with intensifying screens. The open arrows depict the transgene specific band(s) and the closed arrows represent the endogenous RARγ specific band. It is not clear why the transgene produces multiple transcripts but they may represent read through transcription from multiple integrations of the transgene or alternative splicing of the transcript. The endogenous RARγ transcript serves as a loading control for this blot. The control lane is RNA extracted from the breast of a nontransgenic (C3H) mouse. Most strains do not express the transgene. b) RNA was extracted from various tissues of a transgenic male and female of the highest expressing line 1124. Tissue specific expression was assessed by northern analysis with the RARγ probe. The endogenous transcripts are found but no transgene specific bands are seen in most tissues. The blot was exposed for six days. The expression of the transgene is considerably lower in these animals than in the first female analyzed. Expression was not detectable in the breast tissue of this female.


18s
28s
breast
kidney
lung
salivary gland
ovary
salivary gland
brain
lung
kidney
salivary gland
brain


18s
28s
control
1126
1124
1111
1108
1105
1104

Discussion

Our data suggests that expression of the RARγHA transgene is deleterious to the normal development of the mouse. Transmission of the transgene was below the expected 50% transmission for four of the six lines analyzed. For two of these strains this low transmission was significant. Analysis of embryos which failed to survive for more than a few hours after birth suggests that this may be due to a selective loss of transgenic offspring. Northern analysis demonstrated that expression of the RARγHA transgene was either absent or unstable which lends further credence to this hypothesis because it suggests that in order for animals to survive they must lose expression of the transgene. This type of transmission and early lethality has previously been demonstrated with both the RARα and RARγ knock-out mice described previously (138,139). In both these lines the mothers selectively cannibalized the homozygous knock-out offspring (138,139). In the RARα knock-out less than 10% of the homozygous offspring survived to maturity (138,139). Similar to our studies these animals which survived appeared phenotypically normal (138,139). This indicates that some animals can compensate for a loss of either RARα or RARγ. How these knock-out animals are able to compensate for this loss is currently unknown but it has been attributed to a redundancy in RAR function. Consistent with this idea, I believe that some of our RARγHA mice are able to survive and appear phenotypically normal because they have lost expression of the transgene. This hypothesis is consistent with our northern analysis which demonstrates variable expression patterns for the 1124 strain and no expression for several other strains which exhibit the low transmission frequencies (1105, 1111 and 1126).

Appendix 2: **Analysis of Tumours which Developed in 1142 Mouse**

Introduction

We wanted to determine if expression of the RARαmyc transgene in the mammary gland could lead to tumourigenesis of the breast. Our studies indicated that the expression of this transgene was insufficient to lead to tumourigenesis *in vivo*. However one mouse within our colony developed tumours. This mouse was the founder of the 1142 transgenic line. No other animals of this line or any other lines developed tumours within the observation period. This indicates that expression of the RARαmyc transgene was insufficient to lead to tumourigenesis in the mouse. However, all of the tumours analyzed by northern analysis were found to express high levels of the RARαmyc transgene which suggests that the RARαmyc transgene did play some role in the tumourigenic conversion of these tumour cells. The analysis of the tumours which arose in the 1142 founder animal are presented.

Materials and Methods

Histological Analysis

Histological assessment of the tumour tissue was carried out in the lab of Dr N. Mikhael. Formalin fixed tissues were prepared and stained with hematoxylin and eosin as described in Bancroft and Cook (274).

Northern Analysis

Northern analysis was carried out as described previously in chapter 2. The probe for the RARα was the EcoRI-SacI N-terminal probe described previously and was a gift of Dr V. Giguére (28).

Results

Analysis of Tumours Which Developed in a RARαmyc Transgenic Female

The founder of the 1142 strain developed tumours on the body wall and within the peritonium at eight months of age (fig A2.1a). Upon sacrifice eight tumours were removed for analysis (fig A2.1b). The tumours contained cells with high nuclear:cytoplasmic ratios which is characteristic of many tumour cell types. Histological analysis indicated that the tumours were metastatic and invasive (fig A2.1c and d). Some normal tissue surrounding the tumour is shown for comparison (fig A2.1c open arrow). Electron microscopy revealed however that this tumour was not mammary in origin but was a lymphoma (data not shown, analysis not carried out by the author). I believe that it originated from the lymph nodes within the salivary gland region because electron microscopy revealed that the surrounding normal tissue had the characteristics of salivary gland tissue (data not shown, analysis not carried out by the author). These tumours were found to have up regulated expression of the RARαmyc transgene significantly (fig A2.1e).

Figure A2.1. **Analysis of Tumours from 1142 Mouse.**
a) Founder of 1142 strain with the obvious appearance of tumours especially visible around the neck region. The tumours appeared at 8 months of age. b) Dissection of mouse and gross appearance of tumours on the body wall. The tumours are present where the mammary glands are normally found (where lymph nodes are present). c) Histological analysis of the tumours excised, fixed and treated with hematoxylin and eosin. The tumours' characteristics are; high nuclear to cytoplasmic ratio (nuclei stained dark), metastatic and invasive. The histology of the tumourigenic tissue is highly disorganized. Some normal adjacent tissue is shown in c and marked with the open arrow. This region appears to be more organized and has a higher proportion of cytoplasmic staining. e) Northern analysis of RNA extracted from the tumours. The normal breast RNA (labelled 1142) from a normal transgenic of the same age and strain is shown for comparison. The transgene is expressed more highly in all the tumours analyzed when compared to the normal breast tissue from the transgenic littermate. The streaky appearance of the gel is due to degradation of the RNA during processing. The blot was exposed for one day at -70°C.

B
28s
18s
1142
tumour 1
tumour 2
tumour 3
tumour 4
tumour 5
tumour 6

Discussion

Analysis of the transgenic offspring of this animal (the mouse which developed the original tumour was the founder of this strain) revealed that at up to 12 months of age none of them had developed tumours (table 3.2). This data suggests that other events in addition to expression of the RARαmyc transgene were required for the development of the tumours observed. One possibility is that extremely high levels of the transgene are required for the development of tumours and that in this mouse a mutation had occurred which caused the up regulation of the RARαmyc transgene observed (fig A2.1e). This is similar to our results in chapter two which demonstrate that extremely high levels of the RARαmyc transcript are required to block differentiation (fig 2.7).

Alternatively this tumour may have arisen due to the presence of a second random mutational event which in co-operation with expression of our RARαmyc was sufficient to lead to tumourigenesis. The random nature of the development of this tumour suggests that expression of this transgene is not sufficient to initiate tumourigenesis. This transgene may play a role in progression of tumourigenesis because of the six tumours which were assessed by northern analysis all of them had up regulated expression of the RARαmyc transgene suggesting that overexpression was necessary for tumour development (fig A2.1e). Further studies introducing our mutants into other transgenic strains which develop tumours of a known latency need to be carried out to determine this conclusively.

Another possibility is that the RARαmyc does not lead to tumourigenesis of the mammary gland but can cause transformation of lymphoid tissues as has been demonstrated in APL (210). The MMTV promoter is normally not expressed in lymphoid cells (353). However deletion of a small region of this promoter can lead to expression of an MMTV-myc transgene in the lymph nodes and concommitant tumourigenesis (354). It is possible that in this transgenic mouse a somatic mutation had occurred which lead to expression of the RARαmyc transgene in the lymphoid cells and further that expression of this transgene is sufficient to lead to tumourigenesis in the lymphoid cells. This would not be surprising because APL is believed to arise due to expression of a mutant RARα in lymphoid tissues (194-198). Expression of the RARαmyc transgene in transgenic mice under control of a lymphoid specific promoter would delineate if this was the nature of the development of these tumours.

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