MECHANISMS OF RETINOIC ACID-MEDIATED INHIBITION OF ESTROGEN-INDUCED TRANSCRIPTION AND GROWTH IN HUMAN BREAST CARCINOMA CELLS

DAVE DEONARINE

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UNIVERSITY OF OTTAWA

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UNIVERSITY OF OTTAWA

Abstract

Retinoids have been shown to inhibit the growth of estrogen receptor-positive human breast cancer cells. In this study, it is shown that all-*trans*-retinoic acid (RA) inhibits the growth of MCF-7 cells by 60%. In these cells, we also found that RA inhibited estrogen-induced transcription. In transient transfection experiments using the vitellogenin-estrogen response element-CAT reporter construct, RA inhibited estrogen-induced transcription by 55%. Cotransfection of wildtype RARα resulted in a 75% inhibition of estrogen-induced transcription. In order to investigate the mechanisms involved in RA-mediated inhibition of transcription, deletion mutants of RARα were constructed. Point mutants of RARα were also evaluated to determine their effects on RA-mediated inhibition of estrogen-induced transcription. Cotransfection of RARα deletion mutants terminating after amino acid 414 resulted in wildtype inhibition. Using mutants terminating before amino acid 412, the inhibition was significantly reduced. RARα mutants lacking amino acids 413 and 414 also showed significantly decreased inhibition. The amino acids 413 and 414 correspond to the activating function-2 (AF-2) region in the C-terminus of the RARα. These results suggest that the RA-mediated inhibition of estrogen-induced transcription in human breast carcinoma cells is mediated by the AF-2 region of the RARα. MCF-7 cells, stably transfected with a dominant negative RARα, are growth inhibited by only 25% compared to 60% for untransfected or mock-transfected cells. Taken

together, these results suggest that RA-mediated inhibition of estrogen-induced transcription is mediated by the AF-2 region of the RARα and may play a role in RA-induced inhibition of estrogen receptor-positive human breast cancer cell growth.

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LIST OF ABBREVIATIONS

BES	N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
CRABP	cellular retinoic acid binding protein
CRBP	cellular retinol binding protein
dATP	deoxyadenosine triphosphate
DCC	dextran-coated charcoal
dCTP	deoxycytidine triphosphate
DEPC	diethyl pyrocarbonate
dGTP	deoxyguanosine triphosphate
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonuleic acid
DR	direct repeat
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
E2	estrogen
EDTA	ethylenediaminetetraacetic acid
ER	estrogen receptor
ERE	estrogen response element

FBS	fetal bovine serum
G418	geneticin
HRE	hormone response element
MOPS	(3-[N-morpholino]propanesulfonic acid])
NAD	nicotinamide adenine dinucleotide
PBS	phosphate buffered saline
RA	retinoic acid
RAR	retinoic acid receptor
RARE	retinoic acid response element
RBP	retinol binding protein
RNA	ribonucleic acid
RXR	retinoid 'X' receptor
SDS	sodium dodecyl sulfate
SSPE	saline-sodium phosphate-EDTA
TCA	trichloracetic acid
TGF	transforming growth factor
tk	thymidine kinase
TR	thyroid hormone receptor
vit	vitellogenin

CHAPTER I
INTRODUCTION

A. Retinoids

1. General Description

Retinoids are a class of fat-soluble compounds that play an important role in the proliferation and differentiation of a number of cell types. The term 'retinoids' refers to vitamin A (retinol), its natural and synthetic derivatives, and its metabolites. The most potent active metabolite of vitamin A is all-trans retinoic acid (RA), formed by the metabolism of retinol. RA exerts profound effects on vertebrate development and has been shown to inhibit the proliferation of several types of transformed cells (Sporn et al., 1994).

2. Metabolism, Storage and Transport of Retinoids

Retinoids in the body are derived from dietary retinol and β-carotene. An intestinal enzyme, β-carotene 15, 15'-oxygenase, is responsible for the cleavage of β-carotene to yield retinaldehyde (Olson, 1989). The oxidation of retinaldehyde yields retinoic acid (Fig.1). The majority of dietary retinoids are converted to retinol. Most of the body's retinoid stores are found in the liver in two different cell types: parenchymal cells and stellate cells (Batres and Olson, 1987). Other

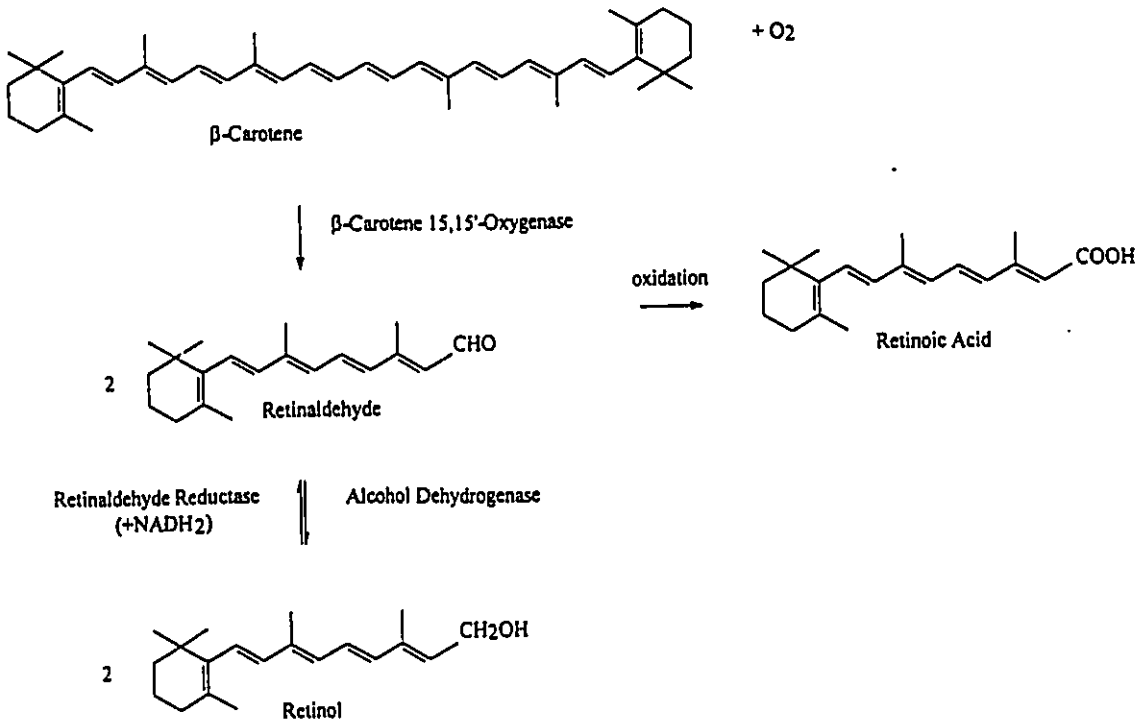


Fig.1 Schematic representation of the cleavage of β-carotene to yield retinal and conversion of retinal to retinol and retinoic acid (De Luca, 1993).

body retinoid stores include adipose tissue, eyes, testes, kidney and intestine. Only a very small portion of dietary retinoids, converted to retinoic acid, enters the circulation. The concentration of retinoic acid in the plasma is very low (4-14 nmol/L) (Eckhoff and Nau, 1990). Retinol in the circulation is bound to a specific protein: retinol binding protein (RBP) (Goodman, 1984). This protein also plays an important role in the transport of retinol from tissue stores to target tissues. The mechanisms involved in the uptake of retinol by cells from retinol bound RBP are not yet known but some groups have suggested that a specific retinol binding protein receptor may be involved (Blumhoff et al., 1988). Other studies have suggested receptor- independent uptake of retinol from retinol-RPB complexes (Hodam et al., 1991). Once retinol is delivered to the cell, it is bound by cellular retinol binding proteins (CRBPs).

3. Cellular Retinoid Binding Proteins

Cellular retinoid binding proteins are low molecular weight, cytosolic proteins. Four such proteins have been identified: CRBP I, CRBP II, CRABP I, and CRABP II (Bashor et al., 1973; Ong and Chytil, 1978; Bailey and Siu, 1988). They are members of a family of proteins consisting of carrier proteins for hydrophobic compounds and which also includes several fatty acid binding proteins. Although little is known about the function of CRABPs, it has been suggested that these proteins may be involved in the storage, transport, or metabolism of intracellular retinoids. It is thought that CRABP I and CRABP II

may function in modulating the concentration of free RA available to bind RARs in the nucleus. It has also been suggested that these proteins may sequester RA and play a role in delivering RA to the nucleus. A role for these proteins in the metabolism of RA has also been suggested (Fiorella and Napoli, 1991; Boylan and Gudas, 1992).

B. Retinoid Receptors

1. Steroid/Thyroid Hormone Receptor Superfamily

Retinoids exert their effects by interacting with two classes of nuclear receptors, the retinoic acid receptors (RARs) and the retinoid 'X' receptors (RXRs). These receptors belong to a large family of ligand inducible transcription factors which include the steroid, vitamin D and thyroid hormone receptors, peroxisome proliferator-activated receptor, insect ecdysteroid receptor and many orphan receptors whose ligands are not yet identified (Evans, 1988; Pemrick et al., 1994). These receptors bind to regulatory regions on DNA, which are referred to as hormone response elements (HREs) and are able to activate gene transcription in a ligand-dependent manner. Within this superfamily of receptors there exists two subfamilies that are functionally distinct. The type I class includes the 'classical' steroid receptors such as the glucocorticoid receptor, the progesterone receptor and the estrogen receptor. The receptors for retinoic acid, thyroid hormone, and

vitamin D, and the peroxisome proliferator-activated receptor are classified as type II receptors. These two classes of nuclear receptors differ in several ways. First, type I receptors bind their respective response elements as homodimers, whereas type II receptors require an auxiliary factor, RXR, for high affinity binding (Kliewer et al., 1992). Second, type II receptors are able to bind their response elements in the absence of ligand, but ligand is required for type I receptors to bind to DNA (De Thé et al., 1990; Allan et al., 1991). Finally, the response elements for type I receptors are composed of palindromically arranged half-sites separated by three nucleotides (Umesono and Evans, 1989). The type II receptors bind to response elements that are composed of half-sites arranged as direct repeats with variable spacing (Umesono et al., 1991). Several structural domains have been characterized, on the basis of amino acid homologies, which are responsible for the biological activity of these proteins. A conserved DNA binding domain (domain C) contains two 'zinc finger' motifs that consist of several tandemly repeated cysteine residues coordinated by zinc atoms. This region is required for the interaction of the receptors with their respective response elements (Leid et al., 1992). The two Zn-finger modules are joined primarily through interaction between the amphipathic helices formed by amino acids at the base of the two fingers. The amino acids in the stem of the first Zn-finger form an α-helix and make base contacts in the major groove of the DNA (Luisi et al., 1991). This α-helix coincides with the 'P-box' which has been implicated in the determination of specificity of binding site recognition (Mader et

al., 1989). The ligand binding domain (domain E) is located at the carboxy terminus of the receptor and is responsible not only for ligand binding but also for dimerization and transactivation (Leid et al., 1992). The region of the E domain responsible for transcriptional activation is referred to as AF-2 and was first described for the estrogen receptor. Ligand dependent transcriptional activation is modulated by the AF-2 region. It was found that deletion of amino acids 538 to 552 of the mouse ER almost completely blocked transcriptional activation while not affecting DNA or ligand binding (Danielian et al., 1992). When the ligand binding domain of any of the retinoid receptors is fused to the DNA-binding domain of GAL-4, it is capable of mediating retinoid-induced transcriptional activation of a GAL-4 response element linked to a reporter gene (Allanby et al., 1993). v-erb A, a mutant form of the thyroid hormone receptor a with oncogenic activity, has a deleted AF-2 region that accounts for the lack of transcriptional activation function of v-erbA (Damm et al., 1989). Retinoic acid receptor mutants which lack AF-2 can act as negative transcriptional regulators which block wildtype RAR function (Damm et al., 1993; Durand et al., 1994). These receptors act as dominant negative mutants in that they actively repress the basal transcription level of promoters that are normally retinoid responsive. The N-terminal region of nuclear receptors (A/B domains) contains another transactivation domain (AF-1). The AF-1 functions as a ligand- independent transactivation domain (Nagpal et al., 1993). Although the AF-2 region is highly conserved among nuclear receptors, the AF-1 region is generally not conserved.

2. Retinoid Receptor Isoforms

The family of RARs is composed of three subtypes RARα, RARβ, and RARγ (Pemrick et al., 1994). The three subtypes show strong homology in their DNA binding domains and ligand binding domains (Fig.2). Each RAR subtype has several isoforms that are generated as a result of two different promoters for each RAR gene that gives rise to alternate transcripts (Blumberg et al., 1992; Kastner et al., 1990). These transcripts generate receptor isoforms with different A domains. Alternative splicing increases the number of isoforms available in the cell (Leroy et al., 1991). A new class of retinoid receptors was identified in 1990, the RXRs, which have been shown to participate with the RARs in retinoid response (Mangelsdorf et al., 1990). The RXR family also contains three subtypes RXRα, RXRβ, and RXRγ (Pemrick et al., 1994). These subtypes also share homology in their DNA and ligand binding domains. The RARs and RXRs share ~50-60% homology in their DNA binding domains but only ~25% homology in their ligand binding domains. The RARs bind both all-trans retinoic acid and 9-cis retinoic acid with high affinity whereas the RXRs only bind 9-cis.

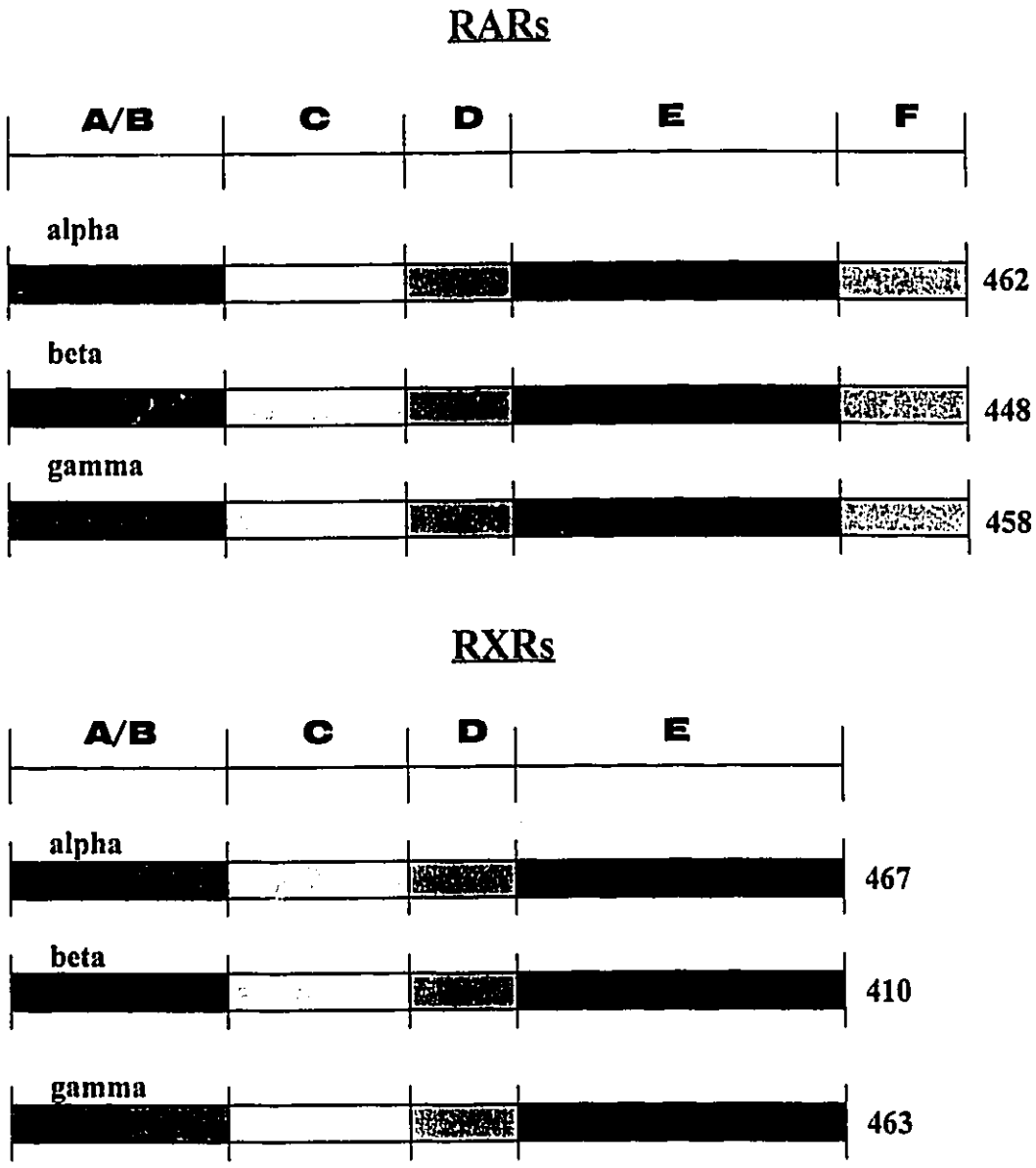


Fig.2 Illustration of two families of retinoid receptors, RARs and RXRs.
Each family contains three receptor subtypes α, β, and γ. Each receptor is composed of several domains: A-F for the RARs; A-E for the RXRs. The number of amino acids in each receptor is indicated.

C. Mechanisms of Retinoid Action

Once retinoids enter the cell, they interact with CRABPs in the cytoplasm. They then enter the nucleus where they may access their respective nuclear receptors associated with DNA. The retinoid receptors are associated with DNA as either heterodimeric complexes of RAR/RXR or homodimeric complexes of RXR/RXR. RAR/RXR heterodimers bind preferentially to retinoic acid response elements (RAREs) consisting of two direct repeats (DR) of the motif (5' - PuG(G/T)TCA - 3') separated by 1 (DR1), 2 (DR2), or 5 nucleotides (DR5) (Umesono et al., 1991; Durand et al., 1992). These heterodimers also bind to inverted repeats separated by 0 nucleotides and everted repeats separated by 8 nucleotides (for review see Mangelsdorf et al., 1995).

RXR/RXR homodimers bind preferentially to DR1 elements (Giguère, 1994). The interaction of retinoids with RAR/RXR or RXR/RXR complexes bound to the regulatory region of a retinoid responsive gene, results in the induction of expression of that gene. Although the molecular mechanisms involved in ligand-induced RAR transactivation are not well known, it has been suggested that ligand binding induces a conformational change in the ligand binding domain of the receptor that transforms the AF-2 into a transcriptionally active state (Valcárel et al., 1994; Keidel et al., 1994). It is thought that the AF-2 may be involved in

promoting or stabilizing assembly of preinitiation complexes by direct or indirect actions on components of the basal transcription machinery (Stunnenberg, 1993).

D. Physiologic Importance of Retinoids

The physiological significance of retinoids in normal growth and development was first identified in 1925 (Wolbach and Howe, 1925). It was observed that dietary deprivation of Vitamin A resulted in widespread squamous metaplasia and/or keratinizing squamous metaplasia of a wide variety of epithelia. This primarily affected the epithelial cells of the respiratory, alimentary, and genitourinary tracts and eyes and related glands. Since then, it has been determined that retinoids play a fundamental role in controlling the growth and differentiation of a number of cell types. The importance of retinoids is evident beginning from very early embryonic development where they are critical in the morphological determination of the embryo. Throughout the lifespan of the organism, retinoids continue to play an important role by affecting the processes of terminal cell differentiation and epithelial renewal by influencing the normal differentiation of epithelia (DeLuca, 1991). Null mutations of RARα, RARβ, and RARγ have been generated in order to determine the physiological function of retinoids (for a review see Chambon, 1995). In addition, isoform-specific knockouts of RARα1 (Lufkin et al., 1993), RARβ2 , RARβ4 (Mendelson et al., 1994), and RARγ2 (Lohnes et al., 1993) have been generated. RARα and RARγ

null mutants display growth deficiency, poor viability, male sterility, and congenital malformations. RARα, RARβ2, and RARγ double mutants show several congenital abnormalities.

The following abnormalities are found in one or several combinations of the double mutants :

- respiratory tract defects
- thinner "spongy" myocardium
- heart outflow tract and aortic arch derivative abnormalities
- diaphragmatic hernia
- ureter abnormalities
- male and female genital tract abnormalities
- ocular abnormalities
- kidney agenesis or hypoplasia
- skeletal abnormalities
- abnormal cartilages (thyroid, bronchial and/or tracheal rings)
- glandular abnormalities

Homeotic transformations and malformations of vertebrae are observed in RARγ and RARα single mutants. This further supports a role for RA in the patterning of the main body anteroposterior axis. RARα and RARγ mutants also display limb malformations which demonstrates a role for RA in limb morphogenesis. RXRα and RXRβ null mutants have also been generated. RXRα null mutants display a thin ventricular wall and die from cardiac failure. RXRα mutants also display

ocular abnormalities such as malformations of the anterior segment of the eye and shortening of the ventral retina. RXRβ null mutants are viable and morphologically normal but males are sterile due to abnormal spermatid maturation and release. It has also been shown that retinoids are important in immune system function (Nauss, 1986).

E. Retinoids and Cancer / Therapeutical Use of Retinoids

Retinoids are used clinically in the treatment of several diseases. They are widely used as therapeutic agents in several skin diseases such as acne, keratinizing dermatoses, and psoriasis (Peck, 1978). All-trans retinoic acid (tretinoin), 13-cis retinoic acid (isotretinoin), and 4-HPR (fenretinide) have all been used successfully in the treatment of these conditions. Since cancer is often associated with abnormal growth and a loss of differentiation, retinoids have been considered as chemopreventive agents and for the treatment of human cancers because of their role in regulating cell proliferation and differentiation. In experimental animals, retinoids have been shown to be effective in the prevention of chemical carcinogen-induced tumour formation. The effectivness of retinoids has been demonstrated in the prevention of prostate cancer, skin cancer, and urinary bladder cancer in experimental animals (Moon et al., 1994). Epidemiologic evidence demonstrates an inverse relationship between β-carotene consumption and the development of certain neoplasms. The protective effects of retinoids

have been shown against lung cancer, esophageal cancer, cervical cancer, urinary tract carcinomas, and carcinomas of the breast (Hong and Itri, 1994). Clinical data has recently suggested that 13-cis RA may be effective in the treatment of oral leukoplakia, a precancerous condition associated with the development of oral cancer (Hong et al., 1986). The use of retinoids in the treatment of acute promyelocytic leukemia (APL) has been studied extensively. Retinoids have been shown to induce complete clinical remission in patients with APL by inducing leukemic cells to differentiate into mature cells (Huang et al., 1988). Many clinical trials are currently in progress that are investigating the role of retinoids as differentiation agents, chemopreventive agents and for cancer treatment in general. There is also evidence that retinoids may inhibit the growth of human breast cancers. A link between breast cancer incidence and consumption of retinol and serum levels of retinol has been suggested based on a retrospective study (Wald et al., 1984). Carcinogen-induced (dimethylbenz(a)anthracene (DMBA)-, or N-methyl-N- nitrosourea (MNU) -induced) mammary tumour formation has been shown to be prevented by administration of retinoids in experimental animal models (Moon et al., 1977; Moon et al., 1976). Retinoids have also been shown to inhibit the growth of a number of human breast cancer cell lines (Lacroix and Lippman, 1980).

F. Breast Cancer Cells

1. General Description

Primary breast carcinomas appear to arise from the epithelial cells of the globular epithelium. They present as either intraductal or intralobular lesions. Tumours that are invasive can spread rapidly by infiltrating through tissue spaces and can invade both lymphatic and blood vessels. Many breast tumours produce a high incidence of distant metastases, mostly in the lungs, soft tissues, liver, bones and adrenals. Many human breast cancers are dependent on estrogen for growth (Lippman et al., 1988). In postmenopausal women, approximately 70% of breast tumours exhibit a hormone-responsive phenotype (Clarke et al., 1994; Sukumar et al., 1988) and it has been suggested that many breast cancers evolve into an estrogen-independent growth phenotype. This hormone- independent phenotype is often associated with a more malignant and aggressive stage of the disease. The development of a hormone-independent growth phenotype restricts the curative potential of current hormone therapies. An antiestrogen, Tamoxifen, is frequently used in the treatment of breast cancer. This drug has the advantage of a low incidence of dose limiting toxicity. The success of treatment is often limited due to the development of an anti-estrogen resistant phenotype. Many experimental models have been developed for the study of breast cancer. Among these are ER-, hormone-independent cell lines that exhibit a down-regulation of

hormone receptors (eg. MDA-MB-231). The MCF-7, ZR- 75-1, and T47D cell lines represent hormone-dependent, ER+ human breast carcinomas (Brünner et al., 1990). These cells require estrogen for growth. The MCF-7 human breast cancer cell line represents an excellent model system for the study of hormone-dependent breast cancer. As with other ER+ breast cancer cell lines, MCF-7 cells are dependent on estrogen for growth. MCF-7 cells express high levels of RARα and RARγ but undetectable levels of RARβ (Titcomb et al., 1994). RARβ is expressed in normal human mammary epithelial cells in culture but is not expressed in mammary carcinoma cell lines (Swisshelm et al., 1994).

2. Retinoids and ER+ Breast Cancer Cells

Retinoids have been shown to significantly inhibit the growth of hormone-dependent breast cancer cells in vitro and in vivo. Although retinoids inhibit the growth of ER- positive human breast carcinoma cells, they do not inhibit the growth of ER-negative cells (Fontana, 1987). Retinoids have been shown to antagonize the estrogen stimulation of MCF-7 cell growth (Fontana et al., 1990). Retinoic acid has also been shown to inhibit estrogen receptor transactivation in MCF-7 cells. RA inhibits the expression of estrogen induced genes such as pS2 and TGF-α (Fontana et al., 1992). Experiments involving transfection of MCF-7 cells with the vit-tk-CAT plasmid have demonstrated that RA can antagonize estrogen induction of expression of the CAT reporter gene (Demirpence et al.,

1994). In this reporter construct, the 5' flanking region of the Xenopus vitellogenin A2 gene (vit), which contains an estrogen response element linked to the herpes simplex virus promoter for thymidine kinase (tk), controls the transcription of the chloramphenicol acetyltransferase (CAT) gene. It has been suggested that the mechanisms by which retinoids inhibit breast cancer cell growth and estrogen-induced gene expression involves RARα-mediated pathways (Sheikh et al., 1994). Sheikh et al. demonstrated that ER- negative cell lines express lower levels of RARα than ER - positive cells. They show that retinoid-resistant ER - negative cells aquire sensitivity to growth inhibition by retinoids when transfected with RARα. Other evidence indicates that RARβ and RARγ are not involved in the growth inhibition. For example, RARγ is expressed at high levels in breast cancer cells irrespective of ER status. However, RA is unable to inhibit the growth of ER - negative cells although they express high levels of RARγ (van der Leede et al., 1995). Also, as mentioned above, breast cancer cells express no (or undetectable) RARβ (Titcomb et al., 1994). Thus, the mechanism of RA - mediated growth inhibition likely involves the RARα. Growth inhibition by retinoids is often associated with the expression of functional ER (Lacroix and Lippman, 1980; van der Burg et al., 1993). Antiestrogens such as tamoxifen, have also been shown to inhibit the growth of MCF-7 cells (Coezy et al., 1982).. It has been suggested that tamoxifen and retinoids inhibit estogen-induced gene expression and growth by different mechanisms (Rubin et al., 1994). There is no evidence that retinoids can bind the ER but it has been shown that classical

antiestrogens compete with estrogen for the ER (Rochefort et al., 1983). This further supports the idea that the mechanisms of action of antiestrogens and retinoids are different. RA and tamoxifen have been reported to have additive effects on inhibition of breast cancer cell growth (Fontana, 1987), and clinical trials are currently investigating this combination in breast cancer patients.

G. Statement of Problem

As discussed above, retinoids have been shown to inhibit the estrogen-stimulated growth of MCF-7 cells. The mechanisms involved in this growth inhibition are not known. There are several possible mechanisms which may play a role in this growth inhibition:

(i) Retinoid-mediated inhibition of estrogen-induced transcription

(ii) Induction of genes that inhibit growth

(iii) Repression of genes that promote growth

(iv) Retinoid-mediated inhibition of AP-1 activity.

Since RA inhibits the growth of ER - positive but not ER - negative cells and RAR expression is higher in ER+ cells, a role for RARα in growth inhibition has

been suggested. The mechanisms involved in the retinoid-mediated inhibition of estrogen receptor transactivation are also not known. It has been shown that the inhibition is not due to a competition between RA and estrogen for the ER (Demirpence et al., 1992). It has been suggested that the inhibition is due to RXR heterodimers bound to EREs which block ER transactivation (Segars et al., 1993). Previous work in our laboratory has shown that RAR/RXR heterodimers bind only weakly to the ERE and therefore do not compete with ER/ER homodimers for binding to EREs. It has been suggested that retinoids may be involved in downregulation of the estrogen receptor (Rubin et al., 1994). Work in our laboratory has shown, however, that RA-treatment results in no change in the levels of ER expression. It is possible that this inhibition is due to a competition between ligand bound RAR/RXR heterodimers and ER/ER homodimers for common accessory factors such as transcriptional coactivators. Since the mechanisms involved in retinoid mediated inhibition of estrogen receptor transactivation and growth inhibition of MCF-7 cells are not known, the present project was undertaken for the following purposes.

1. To investigate the mechanisms involved in retinoid-mediated inhibition of MCF-7 cell growth by:

(a) stable transfection of MCF-7 cells with a dominant negative RARα mutant (RARα' (Pratt et al., 1990)) to observe effects on retinoic acid induced growth inhibition

(b) examining the role of the RARα in retinoic acid-mediated growth inhibition of MCF-7 cells.

2. (a) To investigate the mechanisms involved in RA-mediated inhibition of estrogen receptor transactivation.

(b) To examine region(s) of the RARα involved in retinoid mediated inhibition of estrogen receptor transactivation in MCF-7 cells.

3. To examine the contributions of retinoic acid inhibition of estrogen receptor transactivation and retinoic acid regulation of retinoid responsive genes in retinoic acid mediated growth inhibition by:

(a) construction of RARα mutants which are either no longer capable of inhibiting estrogen receptor transactivation or regulating expression of retinoid responsive genes, not both.

(b) stably transfecting MCF-7 cells with such a mutant receptor and examining effects on retinoic acid induced growth inhibition.

4. To identify retinoic acid induced genes in MCF-7 cells by construction of a cDNA library made from retinoic acid treated MCF-7 cells.

CHAPTER II
MATERIALS AND METHODS

A. Cell Culture and Growth Experiments

1. Culture of MCF-7 Cells

MCF-7 cells were routinely maintained in α-MEM supplemented with non-essential amino acids (Gibco), 7% fetal bovine serum (FBS) , 0.3% glucose, and gentamicin (10μg/mL). Cells were plated at low density and the media was changed every 3 days.

2. Cell Growth Experiments

For growth experiments MCF-7 cells and RARα' -expressing MCF-7 transfectants were plated in regular media supplemented with 5% fetal bovine serum at low density in 6-well plates. After 24 hours, the cells were treated with 1mM RA (Sigma); control cells were treated with vehicle (ethanol) alone. Drug and media were changed on day three of each experiment. On the days indicated, triplicate wells were trypsinized and stained with trypan blue dye (Gibco). Viable cells were counted using a hemocytometer.

3. [^{3}H]-Thymidine Incorporation

For tritiated thymidine incorporation assays, cells plated in 24-well plates at low density in media containing 5% FBS. After 24 hours, the cells were treated with 1mM RA; control cells were treated with vehicle alone and the media was changed every 3 days. One μCi of [methyl-^{3}H]thymidine (Amersham) was added to each well on day 4 for 2 hours. The cells were lysed in 1ml 0.5%SDS. The acid-insoluble material was precipitated with 100μl of 100% trichloroacetic acid (TCA), and collected on glass microfibre filters (Whatman GF/C). After extensive washing with 5%TCA and 95% ethanol, radioactivity was measured using a liquid scintillation counter.

B. Transfections

1. Transient Transfection

For transient transfection experiments, MCF-7 cells after one passage from frozen stock were grown to 70-80% confluency in α-MEM. On Day 1, cells were washed twice with PBS and the media was changed to phenol red free DMEM (due to the estrogenic activity of phenol red) supplemented with 100 mg/L sodium pyruvate, non-essential amino acids, and 7% fetal bovine serum treated with dextran-coated charcoal as described (Dagre et al., 1983). After 48 hours (Day 3),

the cells were split and plated at low density (1:10 dilution) in 60mm dishes. On Day 4, the media was changed. On Day 5, cells were transfected using the BES (N, N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid) buffer-based calcium phosphate precipitation method (Chen and Okayama, 1987). 2.5 μg each of vit-tk-CAT, CMV-LacZ and a pcDNA3 expression plasmid (Invitrogen) for RARα or for one of the RARα mutants were mixed with 750μl of $CaCl_2$ and 750μl of 2X BES buffered saline (50mM BES, 280mM NaCl, and 1.5 mM Na_2HPO_4-$2H_2O$). 500μl of the mixture was added dropwise to each dish and the cells were transfected for 6-8 hours. After removal of the precipitate, the cells were washed with PBS and then shocked for 2 minutes at room temperature with 1.5mL of glycerol shock solution (phenol red free DMEM with 2% charcoal-stripped FBS and 20% glycerol). The cells were then washed twice with 1X PBS and incubated in phenol red free DMEM supplemented with 5% 2X-charcoal stripped FBS containing appropriate drugs (1μM RA, 1nM E2) or vehicle alone for 36-48 hours.

2. Stable Transfection

MCF-7 cells were plated at low density in regular media in 100mm dishes. After 48 hours, the media was changed and the following day, the cells were transfected as follows. 15μg of the pcDNA3-RARα' expression plasmid was mixed with 500μl of 0.25M $CaCl_2$ and 500μl 2X BES buffered saline. The mixture was then

added dropwise to the cells. After 6-8 hours, the cells were washed once with 1X PBS and then shocked for two minutes at room temperature with 2.5mL of glycerol shock solution. The cells were then washed twice with 1X PBS and incubated in regular media. After 24-48 hours, the cells were split and plated at low density. After incubation for a further 24 hours, G418 (500μg/mL) was added to the cells. After selection in media containing G418, individual colonies were isolated and placed in multiwell dishes. After expansion of the individual colonies, the cells were transferred to 60mm dishes. Northern blot analysis was then carried out in order to identify positive clones.

3. Preparation of Dextran Coated Charcoal (DCC) Stripped Serum

Fetal bovine serum was stripped of endogenous hormones by treatment with DCC (0.25% Norit A, 0.0025% dextran in 0.01M Tris/HCl pH 8.0) (Dagre et al., 1983). Serum was incubated for thirty minutes with DCC and the charcoal was removed by centrifugation for ten minutes at 4°C.

C. Chloramphenicol Acetyltransferase (CAT) Assays

Cells were washed twice with 1X PBS, the dishes were placed on ice and 200µl of 0.25M Tris (pH 7.8) was added to the cells. The cells were scraped into 1.5mL tubes and lysed by three cycles of freeze / thawing. The lysed cells were centrifuged for 10 minutes at 4°C and 40µl of the cell extract was removed for the CAT assay. The cell extracts were heated for 10 minutes at 65°C to inactivate endogenous deacetylases. 40µl of cell extract was then mixed with 160µl of CAT assay buffer (0.15M Tris (pH 7.8), 1.6mM chloramphenicol, and ^{3}H-acetyl CoA). The mixtures were then incubated at 37°C for one hour. The reactions were extracted twice with ice cold ethyl acetate and the upper phase was added to 3mL scintillation fluid and radioactivity was determined using a liquid scintillation counter.

D. β-galactosidase Assays

β-galactosidase activity in cell extracts was measured in a reaction mixture containing 800µl assay buffer (100mM $NaPO_4$ (pH7.0), 1mM $MgSO_4$, and 100mM β-mercaptoethanol), 200µl of a stock solution of o-nitrophenyl-β-D-galactopyranoside (4mg/mL) and 40µl of cell extract. Reactions were carried out

at room temperature and optical density was measured at 420nm using a spectrophotometer. Data was analyzed using a kinetics program.

E. Northern Blot Analysis

1. RNA Extraction

RNA was extracted using the LiCl/urea method (Auffray and Rougeon, 1980). Briefly, cells were washed twice with 1X PBS and 2mL of an 8M urea/ 3M LiCl solution was added to the cells. The cells were scraped into 15mL polypropylene tubes and polytroned for 2.5 minutes. The polytroned cells were incubated overnight at 4°C. The mixtures were centrifuged and the supernatant was removed. The pellets were washed with 3M LiCl using a 22 gauge syringe. The mixtures were transferred to 1.5 mL tubes and centrifuged for 10 minutes at 4°C. The supernatant was removed and the pellets were resuspended in 250µl DEPC-treated H_2O.

2. RNA Formaldehyde Gel

The RNA was separated on 1% agarose-formaldehyde gels (15mL 10X RNA gel buffer (200mM MOPS, 50mM NaOAc-$3H_2O$, and 10mM EDTA), 7.6mL formaldehyde, and 1.5g agarose in 127.4ml H_2O). RNA samples were

precipitated with ethanol and the pellets were resuspended in RNA sample buffer (50% deionized formamide, 10% formaldehyde, and 1X RNA gel buffer). Gel electrophoresis was carried out for 1-1.5 hours at 110V in a buffer containing 5% formaldehyde and 1X RNA gel buffer. The separated RNA was then transferred overnight in 20X SSC to Hybond N. After the transfer, the blot was UV crosslinked.

3. Northern Analysis

The UV crosslinked blots were incubated in hybridization solution (50% deionized formamide, 25% 20X SSPE, 1% SDS, 10% 50X Denhardt's solution, and 5% herring sperm DNA (5mg/mL) for four hours. Probe labeling was carried out using the Prime-It RmT Random Primer Labeling Kit (Stratagene). 50-100ng of template DNA was mixed with 41µl of water and the reaction mixture was heated at 95°C for five minutes. 50 µCi of [α-^{32}P]dCTP and 12 units of magenta DNA polymerase was added and the reactions were incubated for 5-10 minutes at 37°C. [^{32}P]-labeled probes were added to the hybridization solution and the hybridization was carried out overnight at 42°C. The membranes were washed 2 times for 10-20 minutes in 2X SSPE and 0.2% SDS at room temperature and then for 5-15min in 0.1X SSPE and 0.2% SDS at 65°C. The membranes were exposed to Dupont film overnight at -70°C .

F. Generation of Unidirectional Deletions

Unidirectional deletions were generated in the human RARα using ExonucleaseIII / Mung Bean nuclease digestion (Fig. 3). The full length RARα was subcloned into pTZ18R between the EcoR1 and BamH1 sites. The pTZ18R-RARα plasmid was cut with BamH1 and Sph1. Exonuclease III deletions were then carried out by incubating the cut plasmids with 500U of Exonuclease III in 1X exonuclease III buffer (66mM Tris-HCl (pH 8.0), 0.66mM MgCl2). Samples were incubated at room temperature and 25mL aliquots were removed at one minute intervals. The aliquots were added to tubes containing 1X mung bean nuclease buffer (30mM NaAC (pH 5.0), 50mM NaCl, 10mM ZnCl2, and 50% glycerol) on ice. Once all of the aliquots were removed, the tubes were heated at 68°C for 15 minutes and placed on ice. 15 U of mung bean nuclease was added to each tube and the samples were incubated at 30°C for thirty minutes. The samples were extracted with phenol / chloroform and precipitated with ethanol. The pellets were resuspended in 40mL Klenow buffer (20mM Tris-HCl (pH8.0), and 100mM MgCl2). 0.2U of Klenow DNA polymerase was added and the samples were incubated for five minutes at 37°C. After the five minute incubation, 1μl of a dNTP mix (0.125mM each of dATP, dCTP, dGTP, and dTTP) was added and the samples were incubated for an additional 15 minutes at 37°C. The DNA was then separated on a 1% agarose gel. The bands were cut out and the DNA was extracted and eluted in 30μl TE buffer. The eluted DNA was

mixed with 4µl 5X ligase buffer, and 1µl T4 DNA ligase and the reactions were incubated overnight at 15°C. DH5α competent cells were transformed with the ligated DNA and the cells were plated and incubated overnight at 37°C. Ampicillin-resistant colonies were selected and overnight cultures were inoculated. Plasmid DNA was extracted and analyzed on 1% agarose gels. Plasmids of appropriate size were further analyzed by dideoxy sequencing. Deleted mutants of desired lengths were subcloned into the EcoRV site of the pcDNA3 expression vector (Invitrogen). Each pcDNA3-RARα mutant construct was confirmed by dideoxy sequencing.

G. cDNA Library Construction

A cDNA library was constructed from retinoic acid treated MCF-7 cells using a λgt10 library kit (Amersham), as follows (Fig. 4). MCF-7 cells were treated with RA for six hours and RNA was isolated as described above.

1. Isolation of poly A+ RNA

Poly A+ RNA was isolated from total RNA using oligo(dT) cellulose columns. Total RNA was incubated with oligo(dT) cellulose for four hours at 37°C. The oligo(dT) cellulose was then loaded on a column and after several washes with 1X

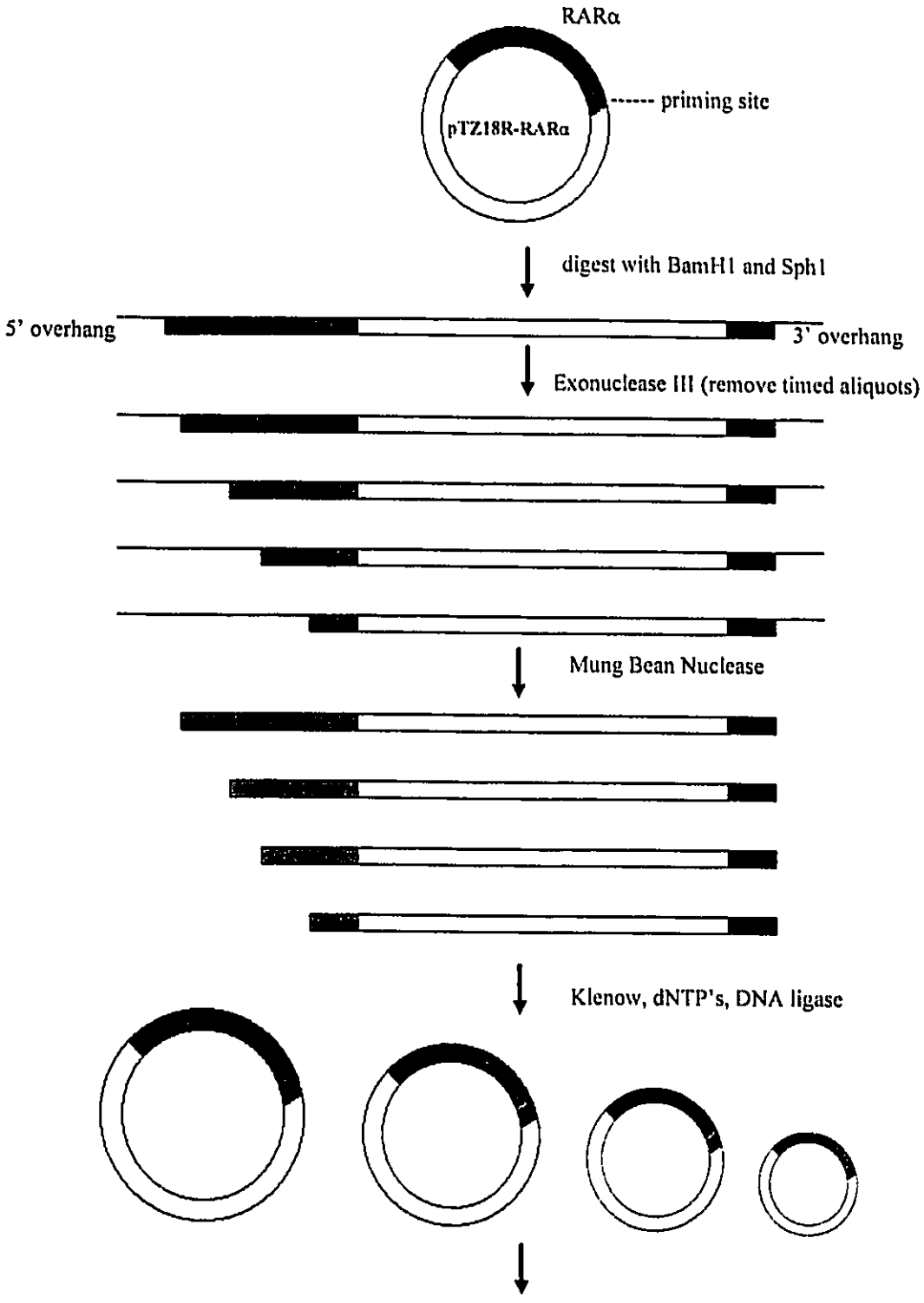


Fig. 3 Flow diagram outlining steps involved in generation of Exonuclease III / Mung Bean nuclease deletions.

binding buffer (0.5M NaCl, 20mM Tris-HCl (pH7.5), and 1mM EDTA), the poly A+ was eluted with DEPC-treated water. The RNA was precipitated with sodium acetate and ethanol, and the pellet was resuspended in DEPC water.

2. cDNA synthesis

10μg of poly A+ RNA was incubated in a reaction mixture containing 50mM Tris-HCl (pH8.3), 75mM KCl, 3mM $MgCl_2$, 10mM dithiothreitol, 500μM each of dATP, dCTP, dGTP, and dTTP, 50μg/ml oligo $(dT)_{12-18}$, and 10 000U/ml MMLV reverse transcriptase for one hour at 37°C. 40μl of the first strand reaction was added to a second strand reaction mixture (25mM Tris-HCl (pH8.3), 100mM KCl, 10mM $(NH_4)_2SO_4$, 5 mM $MgCl_2$, 250μM each of dATP, dCTP, dGTP, and dTTP, 0.15mM NAD, 5mM dithiothreitol, 250U/ml DNA polymerase I, 8.5U/mL RNaseH, 30U/mL DNA ligase). The second strand synthesis reaction was carried out for two hours at 16°C. The reaction was stopped by adding, 25μl of 0.25M Na_2EDTA (pH 7.5). After extraction with phenol and chloroform, the DNA was precipitated with ethanol and the pellet was resuspended in TE buffer.

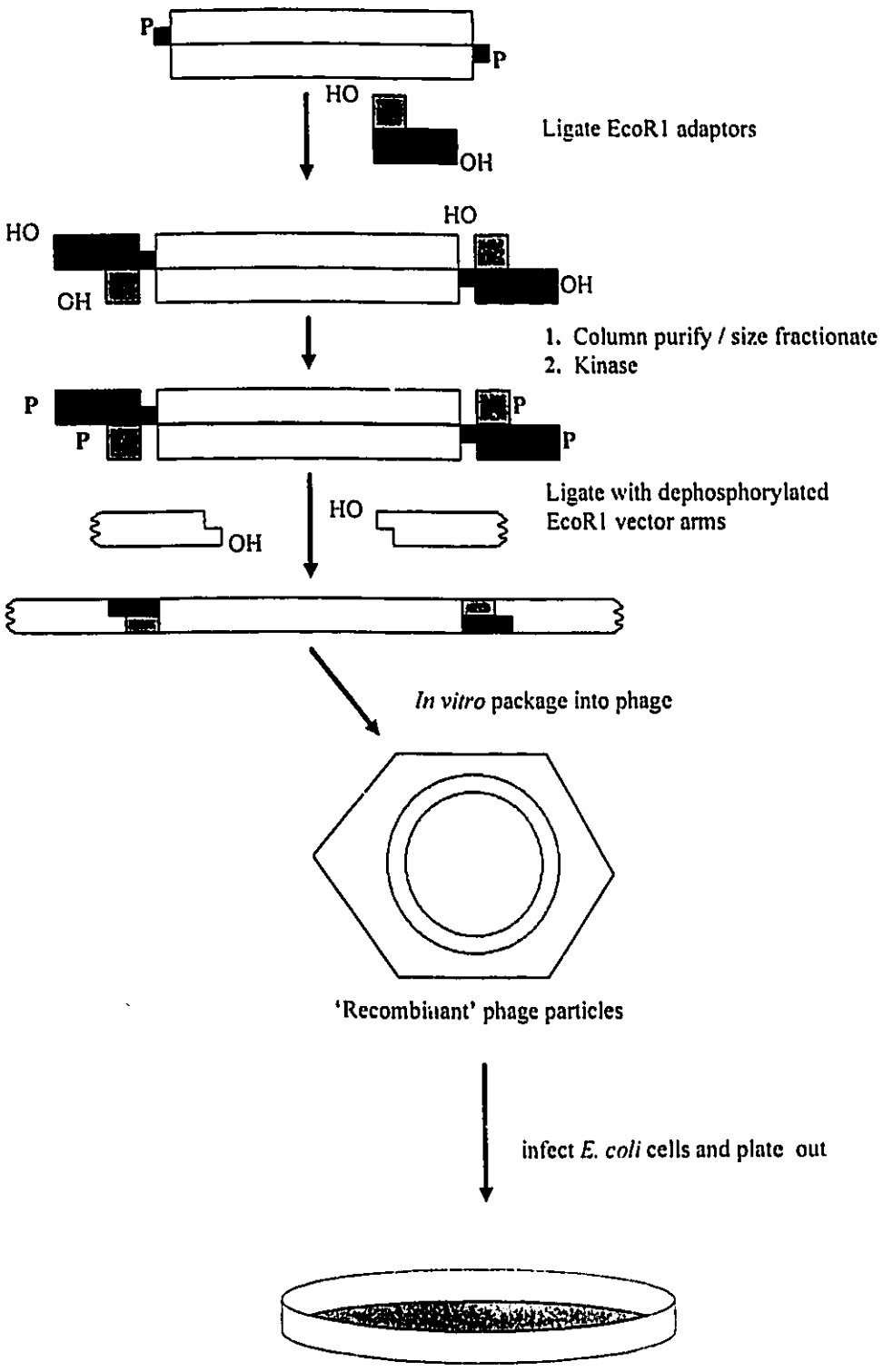


Fig. 4 Flow diagram outlining steps involved in construction of cDNA library.

3. Ligation of EcoR1 Adaptors / Kinasing

EcoR1 adaptors were ligated to the cDNA by incubating 1 μg cDNA with L/K buffer, enzyme enhancer, 12.5 pmoles/μl of EcoR1 adaptors, and 5U T4 DNA ligase for 30 minutes at 16°C. The reaction was stopped by adding 2μl of 0.25M EDTA. The 'adapted' cDNA was purified and size fractionated on a spun column. The 'adapted', purified, and size fractionated cDNA was phosphorylated by incubation with 32U T4 polynucleotide kinase in 1X L/K buffer. The kinasing reaction was carried out at 37°C for 30 minutes. The kinased cDNA was extracted with phenol / chloroform and then ethanol precipitated. The DNA pellet was resuspended in TE buffer.

4. Ligation of λgt10 Arms / *in vitro* packaging

25ng and 50ng of 'adapted' cDNA was ligated into λgt10 vector arms using 2.5μl T4 DNA ligase in 1X L-buffer. The ligation reaction was carried out at 16°C for 30 minutes. The ligated DNA was ethanol precipitated and the pellet was resuspended in TE buffer. The ligated DNA was then packaged using λ-DNA *in vitro* packaging extracts. The phage titre was determined by plating out the cDNA library on the *E.coli* host strain NM514.

H. Plasmids

The pcDNA3-RARα' construct was made by subcloning RARα' (Pratt et al., 1990) into the EcoR1 site of the pcDNA3 expression vector (Invitrogen). pcDNA3- RARα404Δ' was constructed by subcloning a 1.5-kilobase EcoR1 / BamH1 restriction fragment of the human RARα cDNA into the EcoRV site of the pcDNA3 expression vector. The pcDNA3-RARα414Δ' and pcDNA3-RARα436Δ' constructs were made as described above. The pcDNA3-RARα412Δ, pcDNA3-RARαML413,414Δ, pcDNA3-RARαE415,418A, and pcDNA3-RARα419Δ (Tate and Grippo, 1995) constructs were made by subcloning the RARα412Δ, RARαML413,414Δ, RARαE415,418A, and RARα419Δ mutants (a gift of Dr. B.F. Tate and Dr. Joe Grippo) into the EcoRV site of the pcDNA3 expression vector. All constructs were confirmed by sequencing.

I. DNA Sequencing

Five micrograms of plasmid DNA was mixed with universal primer and the mixture was heated at 95°C for five minutes. Two microiitres of 5X sequenase buffer (200mM Tris-HCl (pH 7.5), 100mM $MgCl_2$, and 250mM NaCl) was added and the reaction was heated at 37°C for 15 minutes to anneal the primer to the template. The annealed DNA mixture was mixed with 1µl 0.1M DTT, 2µl labeling mix (7.5µM dGTP, 7.5µM dCTP, 7.5µM dTTP), 6.25µCi [α-^{35}S]dATP,

and 3.25 units of Sequenase Version 2.0. The reactions were incubated for three minutes at room temperature and terminated by adding 2.5µl of termination mix (80µM dGTP, 80µM dATP, 80µM dCTP, 80µM dTTP, 50mM NaCl, and 8µM of either ddGTP, ddATP, ddCTP, or ddTTP) and incubating for five minutes at 37°C. Four microlitres of 2X stop solution (95% formamide, 20mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol FF) were added and the samples were loaded on a denaturing, polyacrylamide sequencing gel. The gels were run for 2-2.5 hours, dried and exposed overnight.

CHAPTER III
RESULTS

A. Analysis of retinoic acid effects on MCF-7 cells

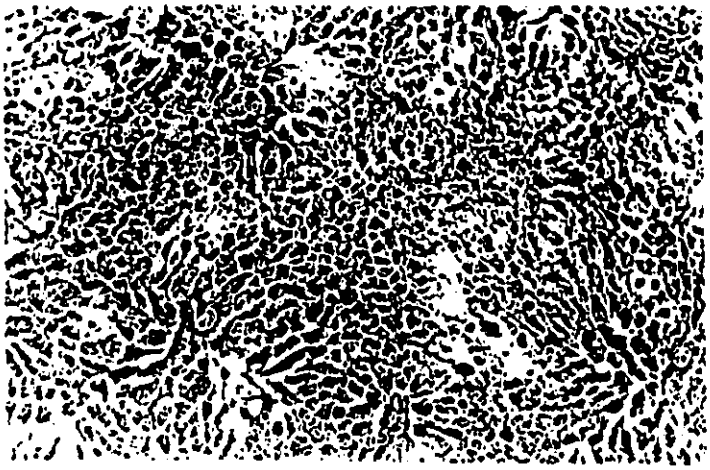
1. Effects on Morphology

Retinoic acid has several effects on the growth of MCF-7 cells. Treatment of MCF-7 cells with 1μM retinoic acid for 4 days produces changes in cell morphology (Fig.5). The cells appear longer and thinner in shape. Retinoic acid treated cells also have several processes which project from the cell.

2. Effects on Growth

Many groups have reported the growth inhibitory effects of retinoids on MCF-7 breast cancer cell lines (Rubin et al., 1994; Guilbaud et al., 1990; Fontana et al., 1990; Ueda et al., 1980; Lu et al., 1994) but the amount of inhibition varies considerably. It has been suggested that these differences in response to RA are due to clonal variations in MCF-7 cells from different laboratories (Osborne et al., 1987). In order to determine the effects of RA on the growth of MCF-7 cells from our laboratory, several growth experiments were carried out. MCF-7 cells were plated at low desity and treated with 1μM retinoic acid, control cells were treated with vehicle alone. After treatment, 90-95% of the cells remained viable as determined by trypan blue exclusion. The viable cells were counted using a

A



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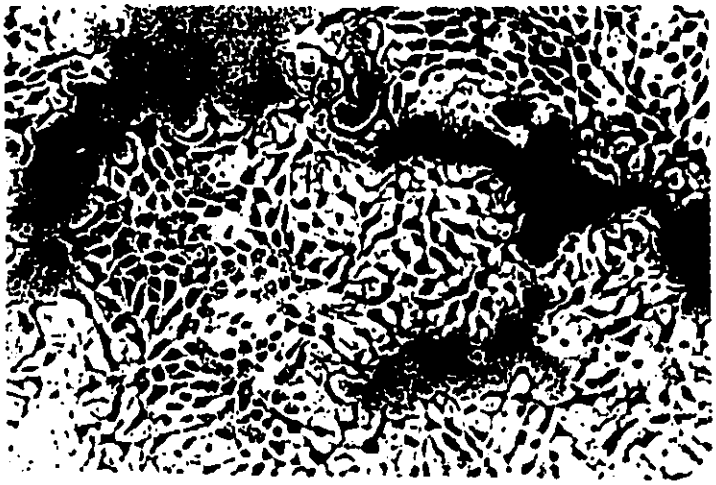


Fig. 5 **Effects of retinoic acid on MCF-7 cell morphology.** MCF-7 cells were treated with (A) vehicle alone (ethanol) or (B) 1μM RA for four days.

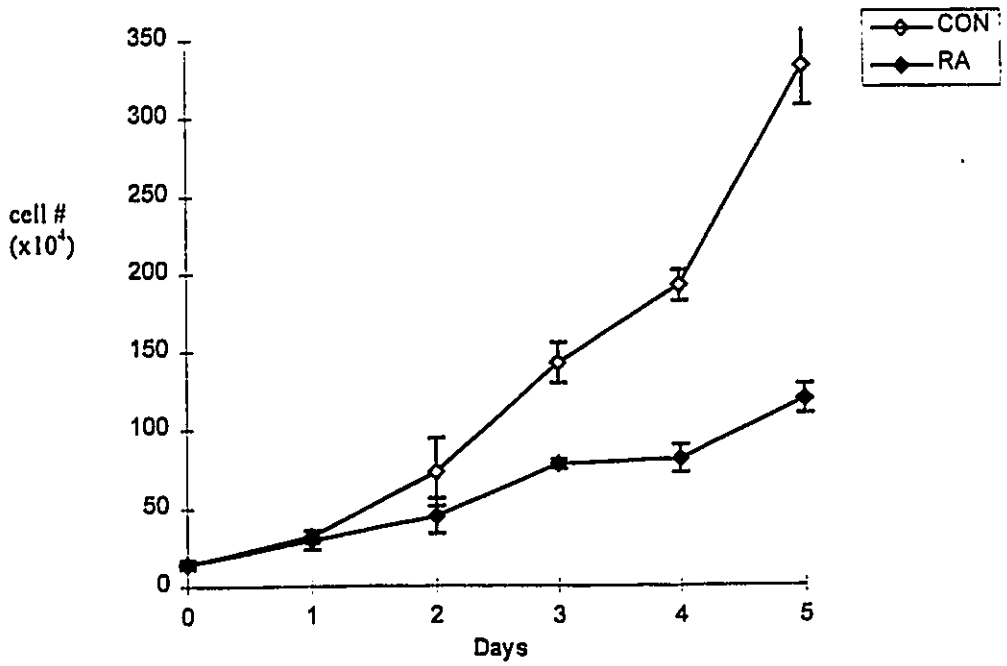


Fig. 6 Effects of retinoic acid on MCF-7 cell growth.
MCF-7 cells were treated with 1μM retinoic acid or vehicle alone. Media and drug were changed every 3 days. Values represent mean ± SEM of cell counts from triplicate experiments.

haemocytometer on the days indicated. The media was changed every three days with fresh drug. Retinoic acid treatment of MCF-7 cells results in a 64% inhibition of cell growth after 5 days of treatment (Fig.6).

3. Effects on DNA synthesis

To further demonstrate the antiproliferative effects of retinoic acid on MCF-7 cells, tritiated thymidine incorporation assays were performed as described in materials and methods. Tritiated thymidine incorporation was determined after four days of treatment with retinoic acid or vehicle alone. Retinoic acid treatment resulted in a 57% decrease in tritiated thymidine incorporation (Fig.7).

B. Analysis of Retinoic Acid Effects on Estrogen Receptor Transactivation

To examine the effects of retinoic acid on estrogen-induced transcription, MCF-7 cells were transiently transfected with the vit-ERE-CAT reporter gene. The effects of retinoic acid on estrogen-induced CAT expression was examined in cells cotransfected with the pcDNA3-RARα expression vector and in control cells (cotransfected with pcDNA3). In control cells, treatment with 10nM estrogen results in a ~6.5 fold induction of CAT activity (Fig 8). Cotreatment of 10nM estrogen and 1μM retinoic acid results in a ~57% inhibition of CAT activity. In cells cotransfected with pcDNA3-RARα, 10nM estrogen treatment results in a ~9 fold induction of CAT activity and cotreatment with 1μM retinoic acid results in a

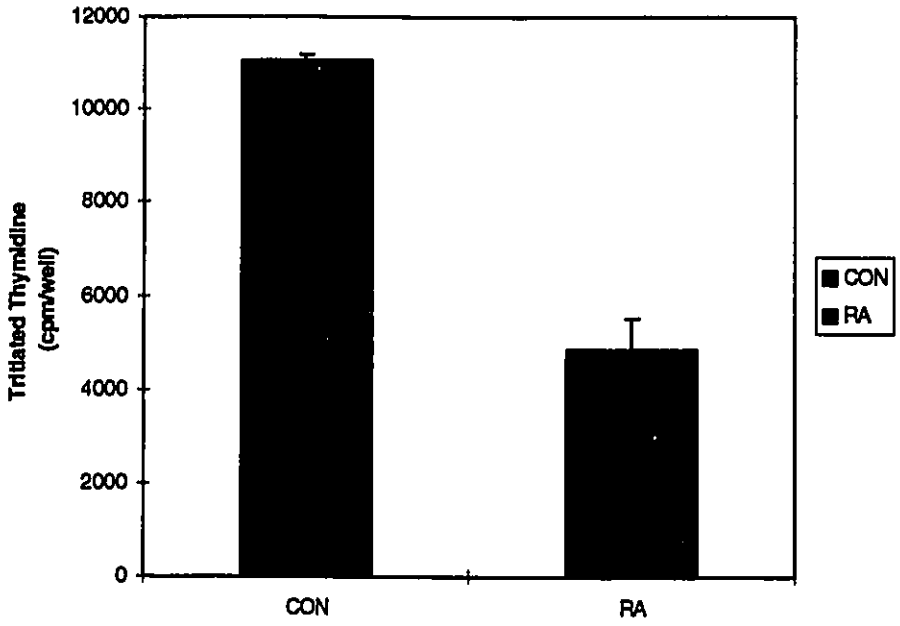


Fig. 7 Effect of retinoic acid on DNA synthesis in MCF-7 cells.
MCF-7 cells were plated in 24-well plates and incubated in the presence of 1μM retinoic acid or vehicle alone. On day four, 1μCi of [^{3}H]Thymidine was added to each well for two hours and tritiated thymidine incorporation was determined as described in materials and methods. Values represent mean±SEM of tritiated thymidine incorporation from triplicate experiments.

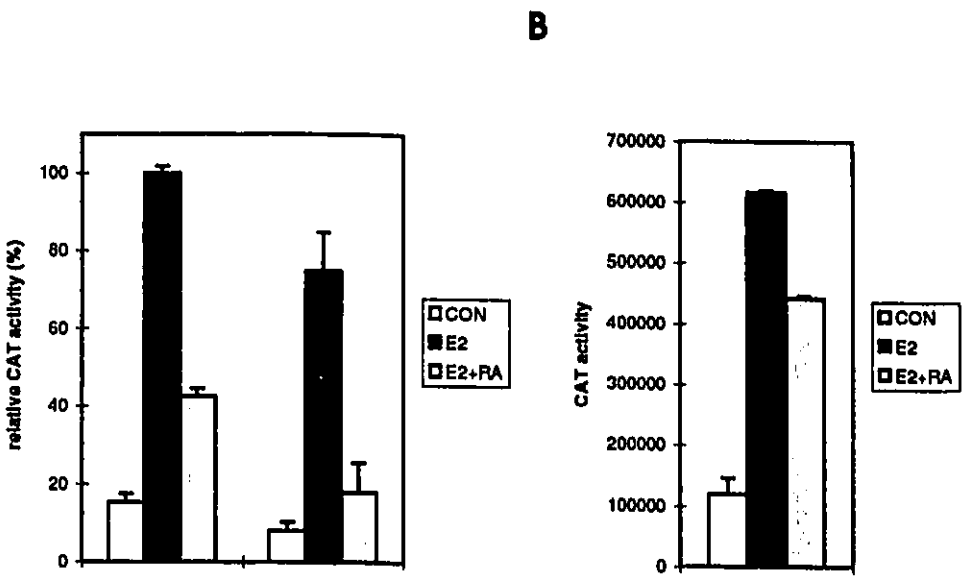


Fig 8. Effects of retinoic acid on estrogen induced CAT expression in MCF-7 cells. MCF-7 cells were transiently transfected with the vit-ERE-CAT reporter construct and either a control plasmid (pcDNA3) or expression plasmids for RARα (A) or a dominant negative mutant RARα' (B). Cells were cultured for 36-48 hours with 10nM E2, 10nM E2 + 1μM RA, or vehicle alone and then assayed for CAT activity. Values shown represent the mean CAT activity in duplicate cultures. Values are normalized with respect to β-galactosidase activity.

~76% inhibition. In cells cotransfected with a dominant negative mutant receptor, RARα', there is only 28% inhibition (Fig.8B).

C. Analysis of the Region of the RARα Involved In Inhibition of Estrogen Receptor Transactivation

1. Deletion mutants

A role for the RARα has been suggested in mediating retinoic acid inhibition of estrogen induced transcription. Since cotransfection of the RARα in the above experiment increases the retinoic acid inhibition of estrogen induced CAT expression and cotransfection of RARα' prevents this inhibition, the role of RARα was investigated more closely. Deletion of the 73 C-terminal residues (RARα') results in a mutant receptor which prevents retinoic acid inhibition of estrogen receptor. This suggested that amino acids between 391 and 462 are involved in mediating the inhibition. In order to further define the region of the RARα involved, several C-terminal deletion mutants were constructed (Fig.9). These deleted mutants were subcloned in the pcDNA3 expression vector.

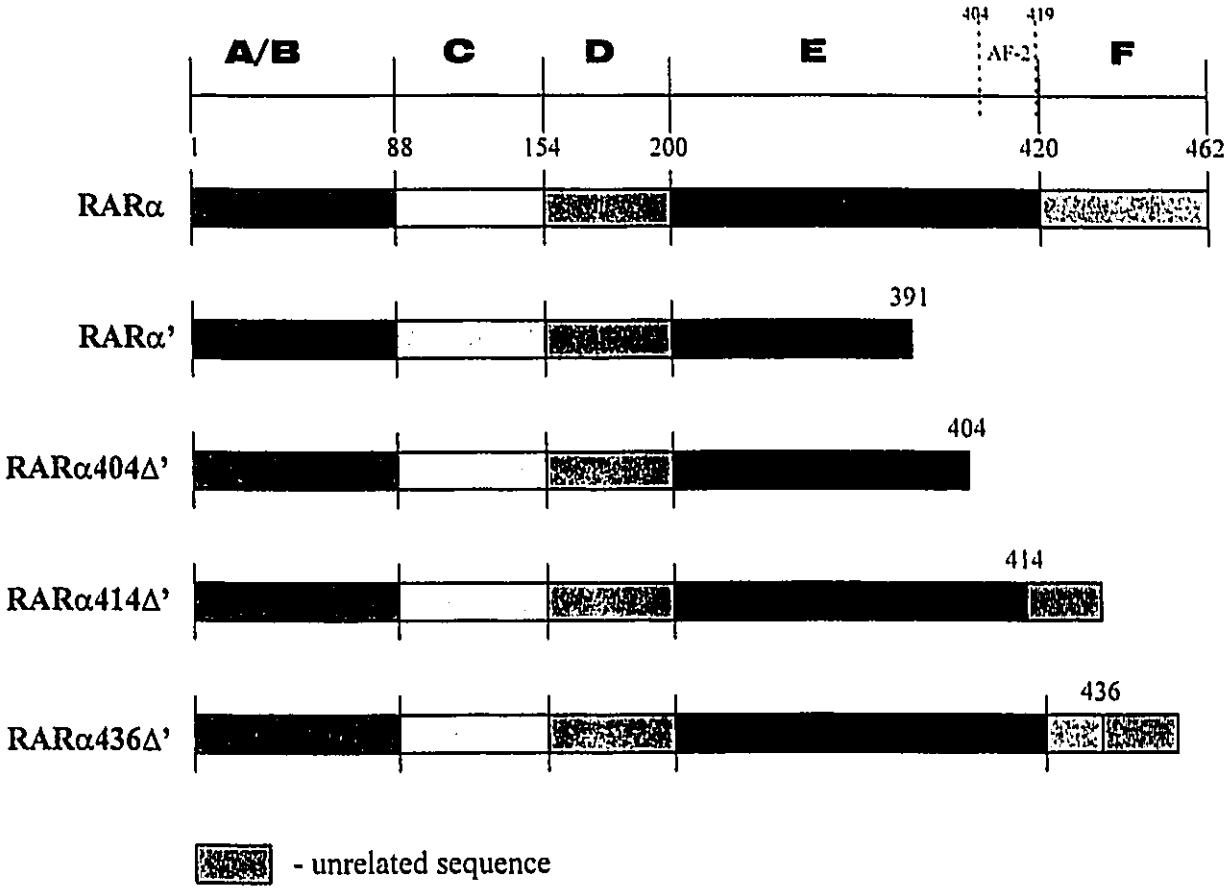


Fig. 9 Schematic representation of the truncated, mutant retinoic acid receptors. The top line represents the various domains in the retinoic acid receptor α and amino acid position. The ligand-dependent transactivation function (AF-2) region is indicated. The mutants were constructed as described in materials and methods.

Construct	% inhibition
pcDNA3(con)	51.0 ± 2.4
RARα'	18.4 ± 8.8
RARα404Δ'	29.0 ± 3.0
RARα414Δ'	61.4 ± 9.4
RARα436Δ'	59.5 ± 7.5

Table 1. Effects of transfection of indicated RARα mutants on RA-mediated inhibition of estrogen-induced CAT expression. MCF-7 cells were transiently transfected with the vit-ERE-CAT reporter construct and either a control plasmid (pcDNA3) or expression plasmids for RARα, RARα', or one of the deletion mutants. Cells were cultured for 36-48 hours with 10nM E2, 10nM E2 + 1μM RA, or vehicle alone and then assayed for CAT activity. Values shown represent the mean±SEM % inhibition of estrogen-induced CAT activity from several independent experiments. Values are normalized with respect to β-galactosidase activity.

2. Transient Transfections with Deletion Mutants

MCF-7 cells were transiently transfected with the vit-ERE-CAT reporter plasmid and either the pcDNA3-RARα404Δ', pcDNA3-RARα414Δ', or pcDNA3-RARα436Δ' constructs. Transfected cells were again treated with estrogen, estrogen + RA, or vehicle alone. In cells transfected with the control vector (pcDNA3), retinoic acid treatment inhibited estrogen induced CAT activity by ~50% (Table 1). If the cells were transfected with the pcDNA3-RARα414Δ' or pcDNA3-RARα436Δ' vectors, retinoic acid treatment resulted in approximately 60% inhibition. This inhibition was ~10% higher than the control cells. Cells transfected with the pcDNA3-RARα' or pcDNA3-RARα404Δ' constructs only showed 18% and 29% inhibition, respectively. This suggested that the region between amino acids 404 and 414 are involved in mediating the retinoic acid inhibition of estrogen induced transcription. Interestingly, this region contains the AF-2 region of the retinoic acid receptor. The AF-2 region mediates the ligand-dependent transactivation function of the RARα (Durand et al., 1994).

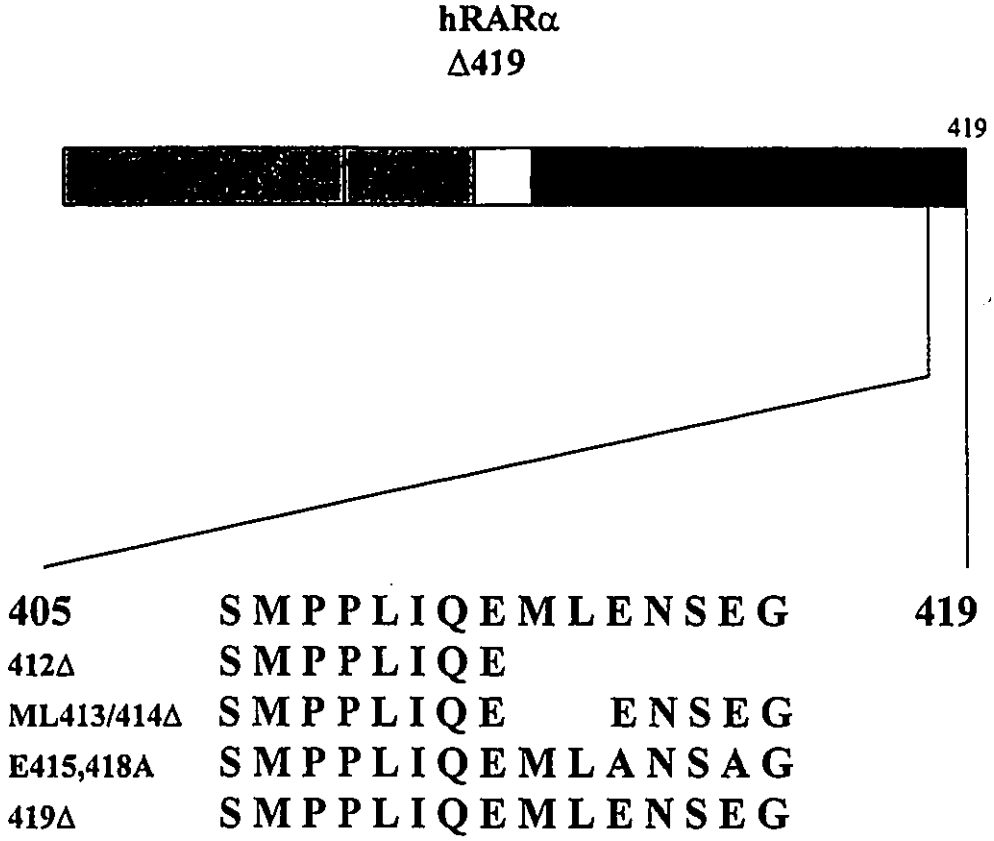


Fig. 10 Schematic representation of mutant retinoic acid receptors (Tate and Grippo, 1995). Mutant retinoic acid receptors (a gift of Dr. B.F. Tate and Dr. J. Grippo) are represented. The amino acids in the AF-2 region of the receptor are shown. The 412Δ, ML413/414Δ, and 419Δ receptors represent deletion mutants and the E415,418A receptor is an alanine substitution mutant.

D. Analysis of Effects of Specific Point Mutations of the RARα on RA-mediated Inhibition of Estrogen Receptor Transactivation

In order to determine if the specific amino acids of the AF-2 region of the RARα are involved in the retinoic acid mediated inhibition of estrogen receptor transactivation, MCF-7 cells were transfected with receptors containing point mutations between amino acids 404 and 419 (Fig.10). The 412Δ mutant is truncated at amino acid 412 and although it contains the ligand-binding and dimerization domains, it lacks the residues necessary for transactivation. The ML413, 414Δ mutant is missing amino acids 413 and 414. This mutant is also dominant negative with respect to RARα transactivation. The E415,418A mutants have substitutions of glutamic acid residues at positions 415 and 418 with alanine. This mutant is not dominant negative and maintains a wild-type AF-2 ligand-dependent transactivation function. The 419Δ mutant is truncated at amino acid 419. As with the E415,418A mutant, this receptor also maintains wild-type transactivation function (Tate and Grippo, 1995). These mutants were subcloned into the pcDNA3 expression vector. MCF-7 cells were transiently transfected as above with vit-ERE-CAT, and a control plasmid (pcDNA3), wild-type RARα, or one of the mutant constructs. In cells transfected with a control plasmid (pcDNA3), retinoic acid inhibits estrogen-induced CAT expression by ~51% (Table 2). Transfection of wild-type RARα results in approximately 65% inhibition. In cells transfected with the pcDNA3-RARα Δ412 or pcDNA3-

Construct	%inhibition
pcDNA3(con)	51.0 ± 2.4
RARα	65.4 ± 2.0
Δ412	27.3 ± 2.8
ML413,414Δ	26.8 ± 3.9
E415,418A	66.2 ± 3.6
Δ419	64.2 ± 2.4

Table 2. Effects of transfection of indicated RARα mutants on RA-mediated inhibition of estrogen-induced CAT expression. MCF-7 cells were transiently transfected with the vit-ERE-[illegible] reporter construct and either a control plasmid (pcDNA3) or expression plasmids for wtRARα, or one of the RARα mutants. Cells were cultured for 36-48 hours with 10nM E2, [illegible] E2 + 1μM RA, or vehicle alone and then assayed for CAT activity. Values shown represent the mean±SEM % inhibition of estrogen-induced CAT activity from several [illegible] experiments. Values are normalized with respect to β-galactosidase activity.

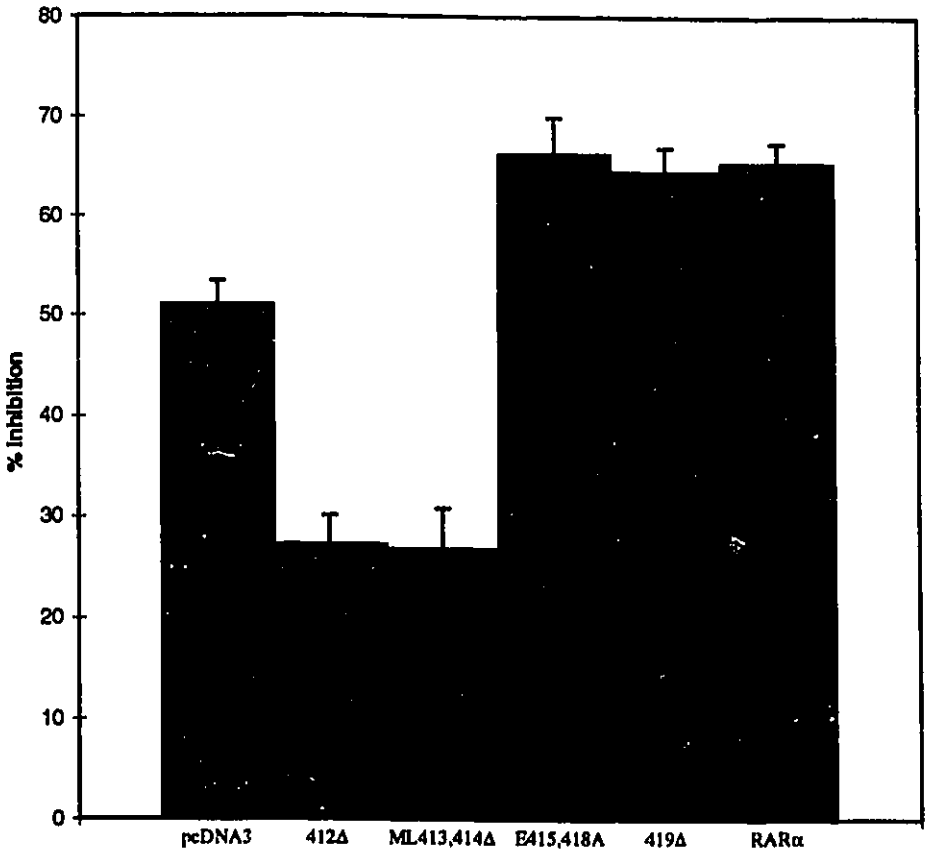


Fig.11. Effects of transfection of indicated RARα mutants on RA-mediated inhibition of estrogen-induced CAT expression. MCF-7 cells were transiently transfected with the vit-ERE-CAT reporter construct and either a control plasmid (pcDNA3) or expression plasmids for wtRARα , or one of the RARα mutants. Cells were cultured for 36-48 hours with 10nM E2, 10nM E2 + 1μM RA, or vehicle alone and then assayed for CAT activity. Values shown represent the mean % inhibition of estrogen-induced CAT activity in duplicate cultures. Values are normalized with respect to β-galactosidase activity.

RARα ML413,414Δ constructs treatment with retinoic acid results in only ~26% inhibition. Transfection of the pcDNA3-RARαE415,418A or pcDNA3-RARα419Δ constructs results in ~65% inhibition. Thus, transfection of receptors which maintain the wildtype ligand-dependent transactivation function (AF-2), results in a 65% inhibition of estrogen induced CAT expression, upon treatment with retinoic acid. This result is the same as that which occurs when the full length wildtype receptor is transfected (Fig 11). In cells transfected with receptors which no longer contain ligand-dependent transactivation function (AF-2), retinoic acid treatment results in only a 26-28% inhibition of estrogen induced CAT expression. Thus, amino acid residues which are critical for ligand-dependent transactivation (i.e., 413 and 414) are also necessary for retinoic acid-mediated inhibition of estrogen-induced gene expression. Transient transfections were carried out with the above mutants, in duplicate, in order to observe variation within one experiment and as shown in Fig.12, there is little variation.

E. Analysis of Effect of Different Amounts of Transfected Mutant Receptor

As demonstrated above, the transfection of mutant receptors lacking AF-2, results in a significant decrease in retinoic acid-inhibition of estrogen-induced gene expression. The mutant receptors also inhibit the inhibitory activity of endogenous wild type RARα receptors. In order to determine if the amount of

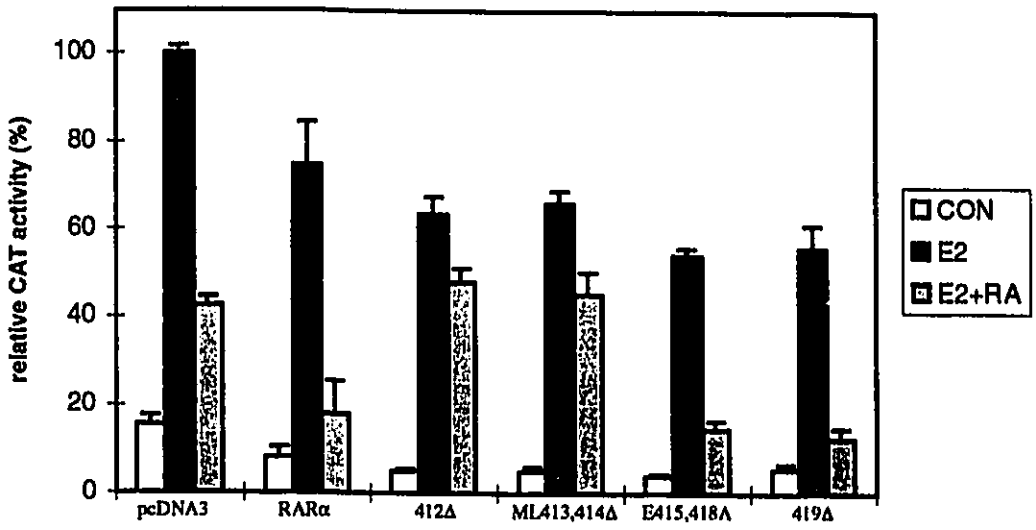


Fig. 12. Effect of retinoic acid on estrogen-induced transcription in cells cotranfected with mutant RARα's in duplicate experiments. In order to examine variation within a single experiment, duplicate dishes of MCF-7 cells were transiently transfected with the vit-ERE-CAT reporter construct and either a control plasmid (pcDNA3) or expression plasmids for wtRARα, or one of the RARα mutants. Cells were cultured for 36-48 hours with 10nM E2, 10nM E2 + 1μM RA, or vehicle alone and then assayed for CAT activity. Values shown represent the mean CAT activity in duplicate cultures. Values are normalized with respect to β-galactosidase activity.

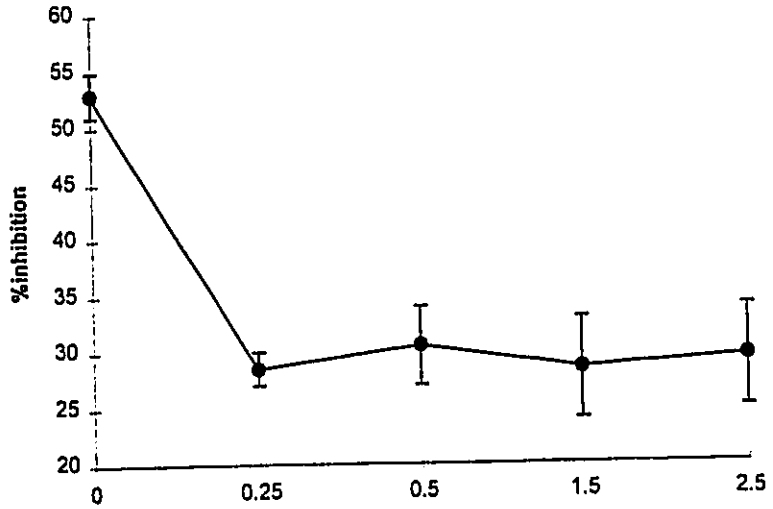


Fig. 13. Effects of increasing amounts of RARα 412Δ on retinoic-acid mediated inhibition of estrogen-induced transcription. MCF-7 cells were transiently transfected with the vit-ERE-CAT reporter construct and either a control plasmid (pcDNA3) or 0.25μg, 0.5 μg , 1.5 μg , or 2.5 μg of the pcDNA3-RARα412Δ expression plasmid. Cells were cultured for 36-48 hours with 10nM E2, 10nM E2 + 1μM RA, or vehicle alone and then assayed for CAT activity. Values shown represent the mean % inhibition of estrogen-induced CAT activity in duplicate cultures. Values are normalized with respect to β-galactosidase activity.

transfected mutant has an effect on prevention of retinoic acid mediated inhibition of estrogen induced gene expression, dose response experiments were carried out. As shown in Fig.13, even very small amounts of transfected mutant receptor (412Δ) are capable of preventing retinoic acid inhibition of estrogen receptor transactivation. In cells transfected with a control plasmid (pcDNA3), retinoic acid inhibits estrogen induced CAT expression by approximately 50%. In cells transfected with 0.25μg, 0.5μg, 1.5μg, or 2.5μg of pcDNA3-RARα412Δ, there is only 25-28% inhibition. The prevention of retinoic acid inhibition by expression of mutant receptors does not depend on the amount of transfected receptor, and the inhibition observed with different amounts of mutant receptor does not significantly differ.

F. Stable Transfection of MCF-7 Cells with a Dominant Negative Retinoic Acid Receptor (RARα')

1. Stable Transfection

As demonstrated above, retinoic acid is able to inhibit the growth of MCF-7 cells. It has been suggested that the growth inhibitory effects of retinoic acid are mediated by the RARα. In order to further examine the role of the RARα in the retinoic acid-induced growth inhibition, MCF-7 cells were stably transfected with

dominant mutant retinoic acid receptor, RARα'. This receptor lacks the ligand-binding and transactivation domains but is still capable of heterodimerization with RXRs and is also still able to bind DNA (Pratt et al., 1996). Eventhough it retains heterodimerization and DNA binding abilities this mutant receptor does not possess the ligand-dependent transactivation function (AF-2). The RARα' mutant is dominant negative and hence it is able to inhibit the transactivation function of endogenous wildtype RARα (Pratt et al., 1990). Also, as demonstrated above, the RARα' mutant prevents retinoic acid-mediated inhibition of estrogen-induced gene expression. MCF-7 cells were stably transfected with the pcDNA3-RARα' construct and clones were selected in G418. Two G418-resistant clones were found to have high levels of expresssion of the mutant receptor : RARα' (5) and RARα' (6) (Fig.14). The level of expression in the two clones was similar.

2. Analysis of Effects of Retinoic Acid on RARα' Clones

In order to determine if expression of this mutant receptor could prevent retinoic acid induced growth inhibition of MCF-7 cells, several growth experiments were conducted. Mock-transfected MCF-7 cells (ie., transfected with pcDNA3), and the two RARα' clones with high expression (RARα' (5) and RARα' (6)) were treated with retinoic acid or vehicle alone and growth assays were done. As shown in Fig.16, the growth of mock-transfected MCF-7 cells was inhibited by

Fig.14. Northern blot analysis of total RNA from G418-resistant clones of RARα'-transfected cells. Total RNA (20μg) per sample was analyzed, and the blot was probed with human RARα cDNA probe. The prehybridization, hybridization, and washing conditions were as described in "Materials and Methods". Lane (C) represents untransfected MCF-7 cells (included as the control), lane 1 represents RARα' (5) and lane 2 represents RARα' (6). Ethidium bromide staining of the gel revealed RNA integrity and comparable loading in each lane.

approximately 65%. In the RARα' clones, retinoic acid inhibited growth by only approximately 27% (Fig 16., Fig. 17). As with the mock-transfected cells, the growth of untransfected MCF-7 cells was inhibited by approximately 65%.

G. cDNA Library Construction

Since the expression of a dominant negative RARα in MCF-7 cells decreases retinoic acid inhibition of cell growth, it is possible that a component of the growth inhibition is due to the retinoic acid induction of retinoid responsive genes. It has been suggested that the retinoic acid mediated induction of genes that inhibit growth or repression of genes that promote growth is a component in retinoid-mediated growth inhibition (Fontana et al., 1990). In order to find genes that are induced by retinoic acid, a cDNA library was constructed. The library was constructed from MCF-7 cells that were treated with 1μM retinoic acid for six hours. The phage vector λgt10 was used for construction of the library. Several libraries with different titres were obtained (Table 3). The background titre (con) is low, as expected. This number reflects the reaction in which no insert was included. Since the EcoR1 arms are dephosphorylated and cannot self ligate, the titre is low. The titres for libraries 2, 4, 5, and 6 represent reactions involving 25ng of insert cDNA. The titre for library #5 represents a reaction invovling 25ng of insert cDNA. The average size of the cDNA inserts is approximately 2kb.

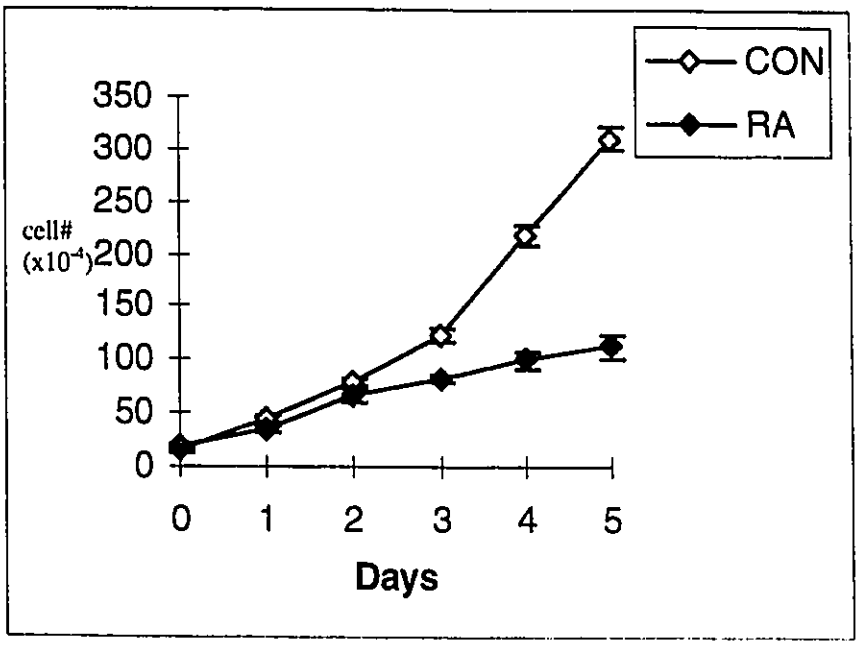


Fig 15. Effects of retinoic acid on mock-transfected MCF-7 cell growth.
MCF-7 cells were treated with 1μM retinoic acid or vehicle alone. Media and drug were changed every 3 days. Values represent mean ± SEM of cell counts from triplicate experiments.

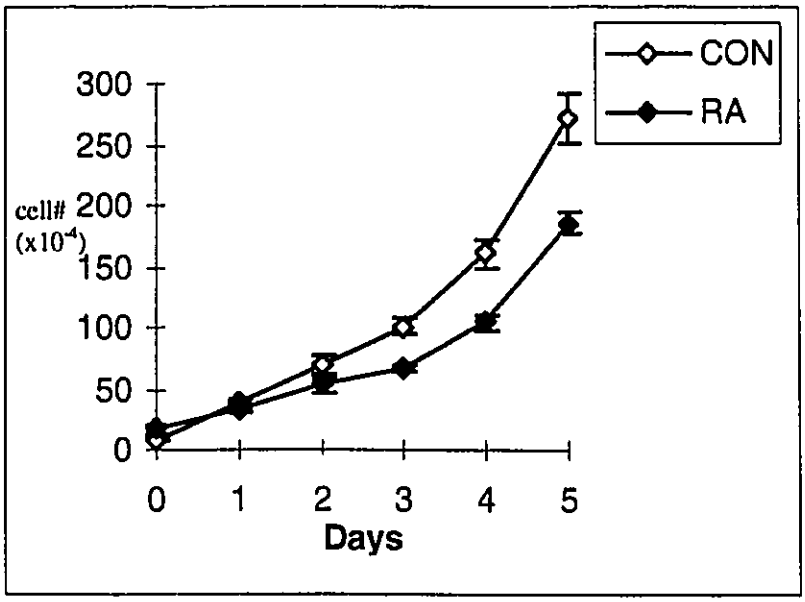


Fig 16. Effects of retinoic acid on RAR α' (5) -transfected MCF-7 cell growth.
MCF-7 cells were treated with 1μM retinoic acid or vehicle alone. Media and drug were changed every 3 days. Values represent mean ± SEM of cell counts from triplicate experiments.

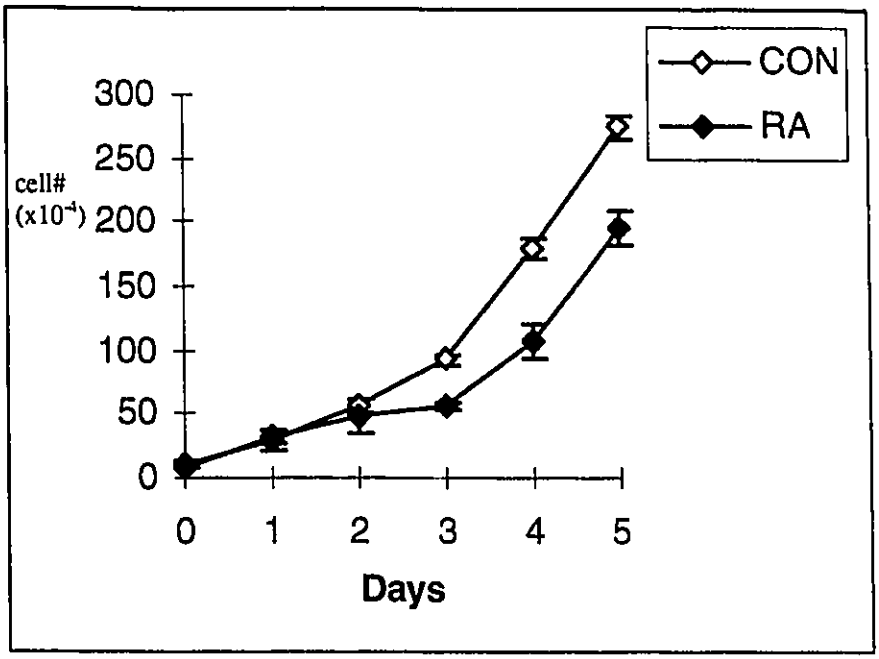


Fig 17. Effects of retinoic acid on RAR α' (6) -transfected MCF-7 cell growth.
MCF-7 cells were treated with 1μM retinoic acid or vehicle alone. Media and drug were changed every 3 days. Values represent mean ± SEM of cell counts from triplicate experiments.

library #	phage titre/ml ($x10^6$)	packaging efficiency (/μg λgt10 arms) x 10^7	cloning efficiency (/μg insert) $x10^8$
CON	0.39	0.020	---
2	23.30	1.17	4.66
3	20.40	1.02	2.04
4	8.40	0.42	0.17
5	3.63	0.18	0.072
6	7.80	0.39	0.16

Table 3. Titre, cloning efficiency, and packaging efficiency of cDNA libraries constructed. cDNA libraries were constructed from MCF-7 cells treated with retinoic acid for 6 hours. The libraries were constructed as descibed in "Materials and Methods".

CHAPTER IV
DISCUSSION

A. Effects of RA on Breast Cancer Cell Growth

The inhibitory effects of retinoic acid on breast cancer cell growth are well documented. Because the mechanisms involved in this inhibition are not known, several investigations were carried out. Retinoids have been shown to inhibit the estrogen-stimulated growth of MCF-7 cells, but the amount of inhibition reported varies from 49-80% (Rubin et al., 1994; Guilbaud et al., 1990; Fontana et al., 1990; Ueda et al., 1980; Lu et al., 1994). It has been suggested that these differences in response to RA are due to clonal variations in MCF-7 cells from different laboratories(Osborne et al., 1987). In order to determine the effects of RA on the estrogen stimulated growth of MCF-7 cells from our laboratory, growth inhibition experiments were carried out. Data presented in this study shows that exposure to RA inhibited the growth of MCF-7 cells by 64% after five days. In addition, the effect of RA on cellular proliferation was also determined by measuring thymidine incorporation in MCF-7 cells. RA inhibited thymidine incorporation by 57% after four days of treatment. Several lines of evidence have suggested a role for RARα in mediating the growth inhibitory effects of RA (Sheikh et al., 1994). Sheikh et al. demonstrated that ER-negative cell lines, which are insensitive to the growth inhibitory effects of RA, express lower levels of RARα than retinoid sensitive ER-positive cells. They also showed that retinoid-resistant ER- negative cells aquire sensitivity to growth inhibition by retinoids when stably transfected with RARα. Other evidence indicates that RARβ and RARγ are not involved in growth inhibition. For example, RARγ is expressed at

high levels in breast cancer cells irrespective of ER status. However, RA is unable to inhibit the growth of ER-negative cells although they express high levels of RARγ. Also, as mentioned above, breast cancer cells express no (or undetectable) RARβ. To further demonstrate the role of RARα in mediating the effects of RA, stable transfection experiments were carried out. MCF-7 cells were stably transfected with a dominant negative form of RARα, designated RARα', and two clones which expressed high levels of the transfected mutant receptor were isolated. The amount of growth inhibition by RA was significantly reduced in these cells which further supports the suggestion that RARα is involved in mediating the effects of RA on human breast cancer cell growth.

B. Effects of RA on Estrogen-Induced Transcription

Even though a role for RARα has been suggested, the molecular mechanisms involved are not known. Several groups have reported inhibitory effects of RA on estrogen-induced transcription. The reports of effects on estrogen-induced transcription show a significant amount of variation which may also be due to clonal variations in MCF-7 cells. Previous work in our laboratory has shown that RA inhibits estrogen-stimulated expression of the estrogen responsive gene pS2 in MCF-7 cells. In order to further demonstrate the effects of RA on estrogen-induced transcription in MCF-7 cells from our laboratory, transient transfection studies using a vit-ERE-CAT reporter construct were carried out. The results

demonstrated that RA inhibited estrogen induced transcription of CAT by 50%. When RARα was cotransfected the inhibition is increased by 10-20%. In cells cotransfected with the dominant negative RARα mutant, RARα', the inhibition is significantly reduced. This suggested that the C-terminal region of the RARα is involved in mediating the effects of RA on estrogen-induced transcription. Since the mechanisms involved in RA-mediated inhibition of estrogen-induced transcription are not known, experiments using C-terminal deletion mutants were carried out in order to determine the mechanisms by which the C-terminal region might mediate the observed RA-induced inhibition. The results of the transient transfection experiments using the C-terminal deletion mutants suggest that amino acids between 404 and 414 are involved in mediating the inhibition. In order to further define the specific region involved, RARα point mutants were used in transient transfection experiments. The results presented show that two specific amino acids, that are critical for RARα transactivation, are also required for retinoic acid induced inhibition of the estrogen response. These two amino acids 413 (M) and 414 (L) of the RARα are highly conserved among not only RARs and RXRs but also other members of the steroid/thyroid hormone receptor superfamily (Barettino et al., 1994). The conserved amino acids form a part of the AF-2 region of these receptors (Fig. 18). The AF-2 region is responsible for the ligand-dependent transactivation function. The results presented suggest that the RA mediated inhibition of ER transactivation is due to transcriptional interference or squelching. This phenomenon usually occurs between receptors which contain

related transactivation domains and possibly involves competition for cofactors. In these experiments, it has been shown that the region of the RARα that is required for transactivation is also required for retinoic acid mediated inhibition of ER transactivation.

C. Mechanisms Involved In RA-Mediated Inhibition of Growth and Estrogen Response in Human Breast Cancer Cells

The observation that amino acid 413 or 414 (or both) of the RARα, which are also conserved in the corresponding domain of the ER, are necessary for transcriptional interference suggests that this effect may be due to a competition between the two receptors for a common transcriptional coactivator. Transcriptional coactivators are thought to function by enhancing the signal of activation and transmitting effects to the polymerase in the initiation complex possibly through protein-protein interactions. Recently, several molecules that interact with the transactivation regions of nuclear receptors have been identified. These factors have also been shown to be necessary for transcriptional activation mediated by specific nuclear receptors. For example, ERAP 160 has been shown to interact with the AF-2 region of the ER and is required for transactivation (Halachmi et al., 1994). Other factors which have recently been identified are TIF-1 (Le Dourain et al., 1995), SWI2/SNF2 (Chiba et al., 1994), and SPT6 (Baniahmad et al., 1995). The current model of the mechanism of transcriptional

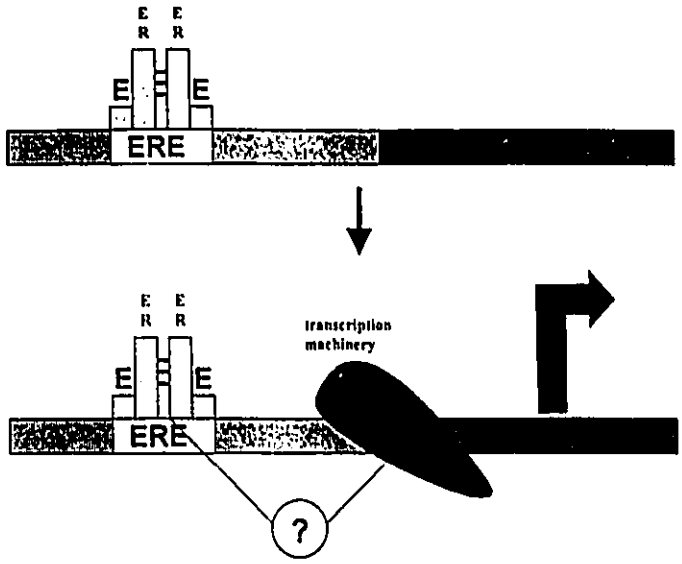
nuclear receptors is as follows. First, ligand binding induces a conformational change in the receptor which exposes the AF-2 region. The exposed AF-2 becomes accessible to transcriptional coactivators which interact with the receptor and possibly, the basal transcription machinery to induce transcription. Recently, Baur et al. have reported the isolation of a novel mouse protein, mSUG1 (Baur et al., 1996). This protein is a structural and functional homologue of the yeast protein SUG1 which is believed to be a transcriptional mediator (Swaffield et al., 1995). They show that mSUG1 interacts with the AF-2 activating domains of the RARα and the ER. They also report that mSUG1 interacts constitutively with the RARα in the absence of ligand. Mutant receptors lacking the AF-2 activation domain also interact with mSUG1 but there is a four-fold decrease in the level of constitutive interaction. Ligand binding to the wtRARα results in a 10-fold stimulation of interaction with mSUG1 but in mutants with a deleted AF-2 activation domain, ligand binding does not result in further stimulation of mSUG1 interaction. This might explain some of the observations from the transient transfection experiments carried out in this study. Cotransfection of wt RARα or mutant RARα in the absence of ligand resulted in a decrease in estrogen-stimulated transcription (Fig.12). This may due to constitutive interaction between the RARα and a transcriptional coactivator. Alternatively, this effect may be mediated by the ligand-independent transactivation domain of the RARα receptor (AF-1). Upon addition of ligand (RA), there is a further decrease in estrogen-stimulated transcription in cells transfected with wt RARα which may

P	P	L	I	Q	E	M	L	E	hRARα
P	P	L	I	Q	E	M	L	E	hRARβ
P	P	L	I	R	E	M	L	E	hRARγ
D	T	F	L	M	E	M	L	E	hRXRα
D	T	F	L	M	E	M	L	E	hRXRβ
D	S	F	L	M	E	M	L	E	hRXRγ
-	D	L	L	L	E	M	L	D	hER
E	T	L	I	R	D	M	L	L	hCOUP-TF

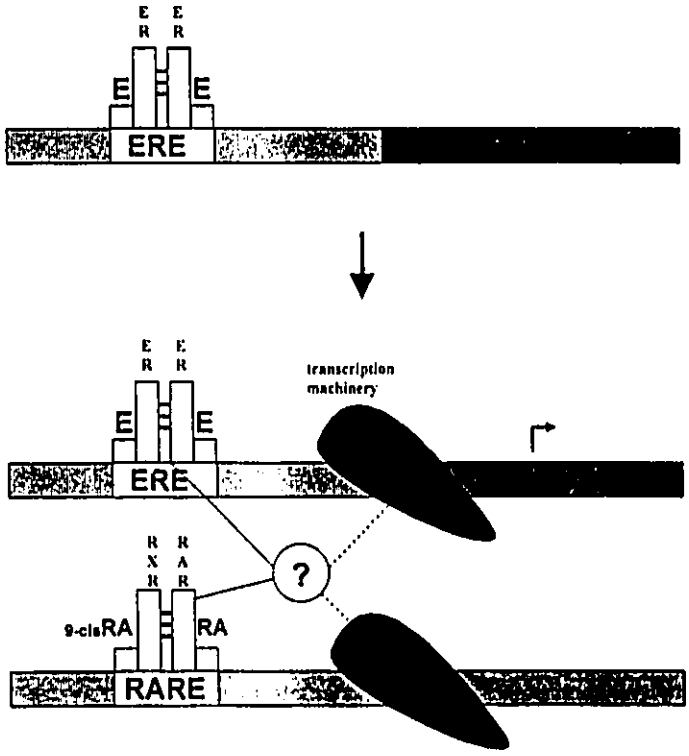
Fig. 18 Sequence alignment of AF-2 transactivation domains of various nuclear receptors. (RARα, Giguere et al., 1987; RARβ, De Thé et al., 1987; RARγ, Krust et al., 1989; RXRα, Mangelsdorf et al., 1990; RXRβ, Hamada et al., 1989; RARγ, Leid et al., 1992; hER, Green et al., 1986)

correlate with an increase in interaction between the RARα and a transcriptional coactivator (eg. mSUG1).

In cells cotransfected with mutant receptors lacking AF-2 activation domains, there was no further decrease in estrogen-induced transcription which may be due to the inability of these mutant receptors to interact with a transcriptional coactivator in a ligand dependent manner. Other evidence also supports this model for RA-mediated inhibition of estrogen-induced transcription. Barettino et al. recently reported transcriptional interference (squelching) between Type II and steroid hormone (Type I) receptors (Barettino et al., 1994). They report that $T_3R\alpha$ is capable of inhibiting RA-induced transactivation in a ligand-dependent manner. In addition, they show that the AF-2 region of the $T_3R\alpha$ is required for inhibition of RA-induced transcription. The results presented in this thesis show that the region of the RARα necessary for transactivation is identical to the region required for inhibition of estrogen-induced transcription. The ability of mutant RARα's to activate transcription in a RA-dependent manner correlates with their ability to inhibit estrogen-induced transcription. Mutations that prevent RA-induced transcription also prevent RA-mediated inhibition of estrogen-induced transcription. Taken together, the results presented in this thesis and results reported by other groups support the following model for transcriptional interference between the ER and the RAR. RAR/RXR heterodimers that are bound to RAREs bind to their respective ligands (Stunnenberg, 1993).



(a)



(b)

Fig.19 Model of molecular mechanisms involved in retinoid-mediated inhibition of estrogen induced transcription. (a) Absence of retinoids - estrogen binds to its receptor which binds, as a homodimer, to an estrogen response element in the regulatory region of an estrogen responsive gene. Ligand binding promotes an interaction between the AF-2 region of the ER and a transcriptional coactivator. This factor functions to enhance the signal of activation which results in expression of the estrogen responsive gene. (b) Presence of retinoids - the binding of the retinoids to their receptors promotes interactions between the AF-2 region of the RARα and transcriptional coactivators. The binding of the RARα to a limiting transcriptional coactivator(s) which is/are necessary for ER transactivation results in a decrease in the estrogen-induced expression of the estrogen responsive gene.

Retinoic acid binding to the RAR induces a conformational change which exposes the AF-2 region of the receptor and allows it to bind to a limiting factor(s) that is/are necessary for ER transactivation (Tate et al., 1994; Valcarcel et al., 1994; Beato et al., 1995). This competition between the RAR and the ER for a common, limiting factor results in a reduction of estrogen induced transcription of estrogen responsive genes (Fig. 19).

One of the original goals of this study was to construct a RARα mutant receptor that was no longer capable of either inhibiting estrogen receptor transactivation or regulating expression of retinoid responsive genes, not both. This would have assisted in determining whether the effects of RA on growth are due to modulation of retinoid responsive gene expression or RA-mediated inhibition of estrogen receptor transactivation. Because the region of the RARα which is necessary for transactivation is also required for RA-mediated inhibition of ER transactivation, it was not possible to construct such a mutant. It was therefore also not possible to determine which mechanism plays the more significant role in growth inhibition. Since estrogen plays a significant role in the proliferation of ER-positive breast carcinoma cells, it is possible that a component of the growth inhibitory effects of RA are due to its effects on estrogen receptor transactivation. Other compounds which have antiestrogenic acitvity such as tamoxifen and ICI 164 184 inhibit ER transactivation and MCF-7 cell growth. The antiestrogenic effects of retinoic acid differ from that of the classical antiestrogens (those which

act by competing with estrogen for ER binding) and there is an additive effect of RA and tamoxifen for inhibition of MCF-7 cell growth. The data presented in this study suggest that the antiproliferative effect of RA may be partially due to its inhibition of estrogen response. Current work in our laboratory has suggested that the antiproliferative effects of RA may also be due to other mechanisms such as regulation of genes involved in the cell cycle (unpublished results). Other studies have shown that retinoids which are selective for anti-AP-1 activity are also potent inhibitors of breast cancer cell growth (Fanjul et al., 1994). Thus it is most likely that the effects of RA on estrogen-dependent breast cancer cell growth are due to several different mechanisms. Further studies are required to determine the precise mechanisms involved and the contribution that each makes to retinoid-mediated growth inhibition of human breast cancer cells.

CHAPTER V
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