# EFFECT OF RETINOIC ACID ON THE EXPRESSION AND FUNCTION OF AP-1 TRANSCRIPTION FACTOR IN B16 MOUSE MELANOMA CELLS : ROLE OF PROTEIN KINASE C

DISSERTATION

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**TO MY GRANDPARENTS**

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# ABSTRACT

Retinoic acid (RA) induces differentiation of B16 mouse melanoma cells. This differentiation is accompanied by an increase in protein kinase Cα (PKCα) protein level and selective enrichment in nuclear-associated PKCα. PKC is thought to regulate gene expression through the TPA response element (TRE). This element is specifically recognized by the AP-1 transcription factor composed of jun and fos family members. In this study, I have analyzed the effect of RA on the expression and function of AP-1 in B16 mouse melanoma cells. Transient transfection analysis of B16 cells using leuciferase reporter gene constructs with or without AP-1 elements indicated that RA induced a four- to fivefold increase in AP-1 transcriptional activity in a concentration-dependent manner. RA did not change the expression (mRNA and protein) of jun family members while the expression (mRNA and protein) of c-fos was decreased. In contrast, acute phorbol dibutyrate (PDB) treatment increased c-jun and c-fos expression. Analysis of the mobility shift assay by using an oligonucleotide containing the AP-1 element suggested that two of the complexes were negatively regulated by RA. There was no significant change in the binding of the other complexes by RA. Acute PDB treatment increased the binding where as chronic treatment decreased the

binding of this complex. Use of specific antibodies indicated that complexes which were decreased by RA and increased by PDB contained fos protein. Down regulation of PKCα by chronic PDB treatment inhibited both the acute PDB and the RA-induced increase in AP-1 activity. However, the potent and selective PKC inhibitor bisindolylmaleimide inhibited the PDB induced increase in AP-1 activity but had no effect on the RA-induced increase in AP-1 activity. Our results suggest that the role played by PKC in RA induced AP-1 activity is independent of its kinase activity. I also determined the role of nuclear retinoid receptors in RA-induced PKCα expression and AP-1 transcriptional activity by using receptor-specific analogs. Results suggest that RARα and RXR play an important role in RA-mediated effect on PKCα expression and AP-1 activity.

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| | |
|---|---|
| AP-1 | activator protein-1 |
| ATF2 | activator transcription factor 2 |
| cDNA | complementary deoxyribonucleic acid |
| CRABP | cellular retinoic acid binding protein |
| CRBP | cellular retinol binding protein |
| DAG | diacylglycerol |
| DMEM | Dulbecco's modified Eagle's medium |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| EDTA | ethylenediaminetetraacetic acid |
| EGTA | ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| HEPES | N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid |
| HRE | hormone response element |
| JNK | jun $NH_2$-terminal kinase |
| MAPK | mitogen activated protein kinase |
| MARCKS | myristoylated alanine rich C kinase substrate |
| PBS | phosphate buffered saline |

| | |
|---|---|
| PDB | phorbol dibutyrate |
| PKC | protein kinase C |
| PMSF | phenylmethyl sulfonyl fluoride |
| RA | retinoic acid |
| RAR | retinoic acid receptor |
| RARE | retinoic acid responsive element |
| RBP | retinol binding protein |
| RNA | ribonucleic acid |
| RXR | retinoid X receptor |
| SDS | sodium dodecyl sulfate |
| SSC | sodium chloride/sodium citrate buffer |
| TBS | tris buffered saline |
| TPA | tetradecanoylphorbol acetate |
| TRE | TPA responsive element |

# INTRODUCTION

## Vitamin A

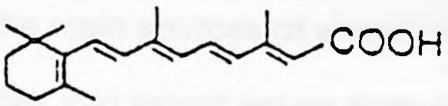
Vitamin A was discovered in 1913 as an essential component of the diet. The term "Vitamin A" is used for retinoids that exhibit the biological activity of retinol. Humans require only minute amounts of Vitamin A in their diets (400 to 1300 μg of retinol equivalents per day, depending on age and sex) (67).

It has been established that retinoids are required for normal growth, vision, maintenance of differentiation and reproduction. Widespread squamous metaplasia and keratinization of various epithelia is a hallmark of vitamin A deprivation (67). Retinoids have been shown to have a diverse range of effects on cellular growth and differentiation, vertebrate development, and homeostasis (43). Retinoids are also known to have some anti-tumor activity. Because retinoids play a very important and diverse role in overall survival, it has become a field of interest for many researchers. The structures of some natural retinoids are shown in Fig. 1.

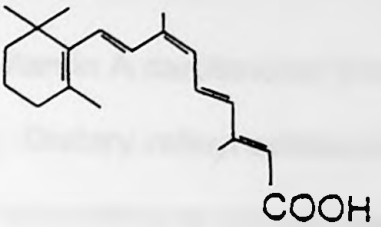
**Figure 1.** **Chemical structures of some of the natural retinoids.**

Three different forms of vitamin A are active in the body: Retinol (alcohol), retinal (an aldehyde), and retinoic (an acid). Retinol is responsible for the transport of the vitamin A. Retinal is the form active in vision and it is also an intermediate in the conversion of retinol to all-trans retinoic acid. Retinoic acid is the form of vitamin A active in cell differentiation. Didehydroretinoic acid is generated from 3,4-didehydroretinol in a metabolic pathway that parallels the synthesis of all-trans RA from retinol. The metabolic pathways leading to the synthesis of 9-cis RA and 13-cis RA are unknown but may involve a specific isomerase. Retinyl ester is a storage form of vitamin A and β-carotene is provitamin A.

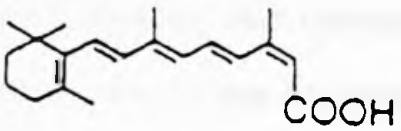
Figure 1



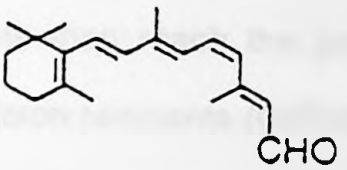

All-trans retinoic acid



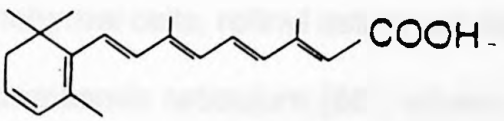

9 -*cis* -retinoic acid



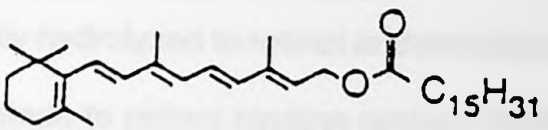

13-*cis* -retinoic acid



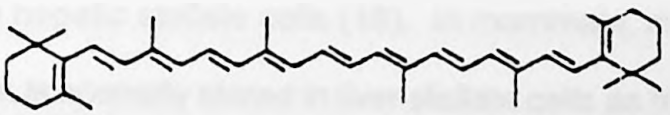

11 -*cis* - retinal




3,4-didehydro retinoic acid




retinyl palmitate



β -carotene

## Retinoid Metabolism

The main sources of vitamin A in the diet are provitamin A carotenoids from vegetables and retinyl esters from animal tissues (227). Dietary retinyl esters are hydrolyzed to retinol (ROH) in the intestinal lumen before absorption by enterocytes, while carotenoids are absorbed and then partially converted to retinol in the enterocytes (227). Within enterocytes, retinol reacts with fatty acids to form esters before incorporation into chylomicrons. Chylomicrons then reach the general circulation by way of the intestinal lymph, and chylomicron remnants (CMRs) are formed in capillaries (68). Chylomicron remnants, which contain almost all the absorbed retinol in the form of retinyl esters, are mainly cleared by the liver parenchymal cells (16) and to some extent by cells in other organs (14, 16, 66). In liver parenchymal cells, retinyl esters are rapidly hydrolyzed to retinol and transferred to the endoplasmic reticulum (86), where it binds to retinol binding protein (RBP). Binding of retinol to RBP initiates a translocation of retinol-RBP to the Golgi complex, followed by secretion of retinol-RBP from the cells (15,191). Retinol-RBP is secreted and transported to hepatic stellate cells (16). In mammals, about 50 to 80% of the total body vitamin A is normally stored in liver stellate cells as retinyl esters (12, 194). Stellate cells store retinyl esters in large cytoplasmic lipid droplets, the size and number of which depend on amounts of vitamin A present (17). Stellate cells are also found in the intestine, kidneys, heart, large blood vessels, ovaries, and testes,

and these cells also store retinyl esters when large doses of vitamin A are consumed (17). The ability of the stellate cells to control storage and mobilization of retinol ensures that the blood plasma retinol concentration is close to 2 μM in spite of normal fluctuations in daily vitamin A intake (230). Stellate cells may then secrete retinol-RBP directly into plasma. Most retinol-RBP (21 kD) in plasma is reversibly complexed with transthyretin (TTR) (55 kD), and therefore less susceptible to filtration by kidney glomeruli (230). The uncomplexed retinol-RBP is presumably taken up in a variety of cells by cell surface receptors specific for RBP. Most of the retinol taken up will then recycle to plasma, either on the "old" RBP or bound to a newly synthesized RBP (225). In addition to 2 μM retinol-RBP, there is a 5 to 10 nM plasma concentration of retinoic acid, presumably bound to albumin (227). Retinoic acid bound to albumin can be spontaneously transferred to cells and there elicit biological activity (70).

After cellular uptake, retinol can be oxidized to retinoic acid (RA) in target cells. Furthermore, RA may be synthesized from β-carotene in several tissues, and retinol and retinal are probably not metabolites in this reaction (23). Thus, in addition to retinol, β-carotene may be a source of RA in certain cells.

The concentration of RA in various cells may be regulated and mutable. The local intracellular RA concentration may be determined by the cellular uptake of retinoids and the rate of retinoid synthesis or degradation. Alternatively, it is also possible that the RA level is not regulated and most cells have an excess of RA.

Under these latter conditions, the availability of protein factors such as binding proteins and nuclear receptors, would determine the retinoid response (186).

**Role of Retinoids in Development and Cellular Growth and Differentiation**

Retinoids have been shown to regulate embryonic development and to induce or maintain the differentiated state of many cell types in culture (13, 114, 120, 213). They also protect against carcinogenesis at various sites (21). Offspring from animals deficient in vitamin A during pregnancy exhibit a number of developmental defects, indicating that retinoids are important during embryogenesis (13). The dramatic teratogenic effects of excess maternal RA administration on mammalian embryos, including human embryos of mothers treated with RA for acne, and the spectacular digit duplications observed after topical administration on vertebrate limbs during development, led to the hypothesis that RA was a morphogen (43, 67), i.e., a molecule conferring positional information during development. It may also play a critical role during organogenesis. In addition, RA has been shown to induce differentiation of various tumor cells in culture, including HL 60, a human promyelocytic-like cell line (26), F9 teratocarcinoma cells (213), and melanoma cells (114,120, 121, 122, 124). The mechanism by which retinoids exert their effects on cells is not very clear; however, several possibilities have emerged. Perhaps the most attractive among these is the modification of gene expression by a mechanism

analogous to that of steroid hormones. This hypothesis is based on the discovery that there are several retinoid-binding proteins in the cytoplasm of many normal tissues and in tumor cells (36, 123). It has been suggested that these proteins may mediate the effects of retinoids by transporting them to the cell nucleus where they may alter gene expression (36, 123). This hypothesis has been modified by the more recent discovery of the nuclear receptors for retinoic acid. These receptors, which are structurally similar to steroid hormone receptors, bind to specific DNA elements present in the regulatory region of genes whose expression is controlled by retinoic acid (10, 25, 51, 63, 104, 184).

**Mechanism of Action of Retinoids within Target Cells**

The diversity of effects generated by retinoids may be accounted for by the fact that within target cells the mechanism of retinoid action is extremely complex. There are several biologically active metabolites of retinol, several types of cytoplasmic retinoid binding proteins, and several nuclear DNA binding receptors. The intricate balance between all these factors probably results in the distinct effects seen in response to retinoid treatment in different cell types.

Once retinol is taken up by the target cells, it is metabolized to several active retinoid species. These include all-trans retinoic acid, didehydroretinoic acid, and 9-cis retinoic acid (60, 69, 78, 130). All-trans retinoic acid is produced by oxidation

of retinol (186). It is the most biologically active form of all the endogenous retinoids. The majority of 9-cis retinoic acid is believed to be the product of an isomerization reaction from all-trans retinoic acid (60, 69). In some cells, 9-cis retinoic acid may be the oxidative product of 9-cis retinol. Dehydrogenation of all-trans retinol, followed by two oxidation reactions is postulated to result in the formation of didehydroretinoic acid, with an intermediate of 3,4-didehydroretinol ( 78, 130).

The cytosol of retinoid-responsive cells may contain several retinoid binding proteins. Cellular retinol binding proteins (CRBP) I, II and III bind retinol in the cytoplasm. Cellular retinoic acid binding proteins (CRABP) I and II bind all-trans retinoic acid but not 9-cis retinoic acid (113). CRBP I and CRABP I are the predominant binding proteins in most cells. A potential function of CRBP may be to store retinol until it is needed for conversion to retinoic acid, thus providing a mechanism for controlling the intracellular retinoic acid concentration (186).

The nuclear retinoic acid receptors are members of the steroid receptor family of proteins that function as ligand-dependent transcription factors. RA exerts its effect on transcription through two classes of these nuclear receptors: the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). The classification into the RAR and RXR subfamilies is based on the differences in primary structure, sensitivity to synthetic retinoid ligands, and ability to regulate expression of different target genes (10, 63, 184, 215). Each retinoic acid receptor subfamily consists of

several receptor isoforms referred to as RARα, β, and γ (2, 25, 63, 131, 184) and RXRα, β, γ (10, 87).

The three RAR genes map on distinct chromosomes: RARα, RARβ, and RARγ genes are localized on chromosomes 11, 14, and 15 in the mouse and on 17q21.1, 3p24, and 12q13 in the human genome (234). Each RAR gene generates multiple isoforms which differ in the amino-terminal region of the proteins (92, 111, 132, 135,). Different mechanisms are used to generate the multiple RAR isoforms: differential usage of two promoters in each RAR gene, alternative splicing of exons, and initiation of translation at an internal CUG codon (110, 146, 233). The physiological significance of the existence of multiple RAR isoforms may explain in part the diversity of the biological processes controlled by all-trans RA. Comparison of the amino acid sequences of the three human receptors with the mouse receptors showed that the interspecies conservation of a member of the RAR subfamily is much higher than the conservation of all the receptors within a given species, suggesting that RARα, RARβ, and RARγ each have their own specific function. Since almost all cell types in the body express one or several of the RARs (87, 186, 233), it is likely that RA has even more pleiotropic effects than was previously thought. In some cases, lack of one isoform can be substituted by other isoforms suggesting some redundancy in the RA signaling system (115).

In 1990, Mangelsdorf et al. (130) observed that all-trans RA apparently does not bind to RXRα. This is in contrast to RARs, which bind all-trans RA at nanomolar

concentrations (131,228). It was postulated that cells convert all-trans RA to an RXR-specific ligand (132). The high divergence of the primary amino acid sequence in the ligand binding domain between RARα and RXRα indicates a potential difference in ligand specificity. Subsequently, the identification of 9-cis RA as a high affinity ligand for RXRα was described (78). 9-cis RA, generated from all-trans RA *in vivo*, is up to 40 times more potent than all-trans RA in binding to RXRα. It was also shown to bind to all three RXR subtypes with nanomolar affinity (78). Since 9-cis RA is present in mammalian tissues, it is proposed that this isomer is a natural hormone (78). However, 9-cis RA is not a unique ligand for RXRs since the retinoid can also bind and activate the three RAR subtypes in the nanomolar range (113).

The three RXR genes map to separate loci in the mouse genome; RXRα, RXRβ, and RXRγ localize to chromosome 2,17, and 1, respectively (87). To date, only the mouse RXRγ gene has been shown to generate more than one isoform (117). The RXRγ1 and RXRγ2 differ in their amino-terminal regions and are generated by alternative usage of two distinct promoters.

Although both RARs and RXRs bind the same ligand (9-cis RA) with high affinity, RARs are more closely related to the thyroid hormone receptor than to the RXRs. RXRs belong to a subgroup of nuclear receptors distinct from the RARs (106) that includes the orphan receptors (172).

The RARs must heterodimerize with RXR to bind DNA with high affinity, whereas RXRs can form a functional homodimers (99, 235). Each complex activates transcription through related response elements composed of direct repeats with different spacings (129 220).

Nuclear receptors control the expression of specific genes by binding to short DNA sequences known as the hormone response elements (HRE), generally located in the promoter of target genes. The identification of the first RA response element (RARE), located in the promoter of the RARβ2 gene, revealed a distinct organization for the RAR binding site (referred to as βRARE) (41, 214). The βRARE contains two direct repeats of the sequence PuGTTCA separated by 5 bp (DR-5). The characterization of the promoter region of a number of RA-responsive genes led to the identification of RAREs arranged in a multitude of configurations (64). Since binding of RARs to RARE is independent of RA, it is likely that RA activates gene expression by binding to RARs already attached to their DNA response element (112).

Most of the RA-responsive genes characterized to date encode proteins that participate in the transduction of the retinoid signal at various steps (64). Like most hormonal systems, retinoid signaling appears to be the subject of autoregulation by positive and negative feedback mechanisms. All the RAR genes contain a RARE in one of their two promoters (41, 109, 112, 214), and this autoinduction of RAR expression could lead to a potential amplification of the retinoid signal.

## Therapeutic Implications of RA

Retinoids have a marked effect on skin and are presently used not only for therapy of psoriasis and acne, but also to reverse the damage of sun-damaged skin. RA has also been found to antagonize some of the effects of arthritis. Retinoids have been shown to be effective in inhibiting the progression of some tumors. Acute promyelocytic leukemia (APL) has been treated with RA, resulting in remissions that last for several months or even years (35, 84, 226). It has been discovered that the chromosomal translocation characteristic of APL entails the breakage and fusion of chromosome 17 within the RAR$\alpha$ gene and chromosome 15 within a gene termed PML (promyelocytic leukemia). This translocation event results in the production of an aberrant mRNA and fusion protein (1, 22, 44). The fusion protein contains almost all of the RAR$\alpha$ and a large portion of PML. The fusion protein still functions as an RA dependent transcription factor (44, 90). Retinoic acid may induce remission of APL by regulating the activity of this aberrant fusion transcription factor.

Retinoid treatment of squamous cell carcinoma (SCC) of the upper aerodigestive tract has yielded promising results. Patients who had initial surgery for SCC of the head and neck were treated with 13-cis retinoic acid for one year. The incidence of secondary epithelial tumors was greatly reduced in patients treated

with this protocol. There are currently many trials underway to investigate whether RA can be used as therapy for additional types of cancers.

Many of the clinical studies which have been performed on retinoids and cancer utilize retinoic acid at a supra physiological concentration. This results in many toxic side effects. Researchers are studying the mechanism of action of retinoic acid using various retinoic acid analogs in order to develop agents which may lack the teratogenic and other toxic side effects of retinoic acid.

**Retinoic Acid Analogs**

Bollag and Matter (21) have used the two step carcinogenesis model to test the *in vivo* effectiveness of many synthetic retinoids. The two stage model involves an initiation step, in which a carcinogen is applied to the skin of mice. Tumor formation is then promoted by applying the phorbol ester, tetradecanoylphorbol acetate (TPA), to the skin. A series of natural and synthetic retinoids can then be tested for their ability to inhibit tumor formation. From the results of their study, Bollag and Matter developed a therapeutic index for many retinoid compounds. The index compares the effectiveness of the compound in inhibiting skin carcinogenesis versus its toxicity. Using this index, the effectiveness of various retinoids can be compared. One group of compounds contains an N-substituted carboxyl amide group in place of the terminal carboxyl group of retinoic acid. These compounds are

referred to as retinamides. They appear to maintain the same in vitro biological activity as natural retinoids but have much fewer toxic effects (142). These compounds may prove to be useful in the clinical treatment of cancers.

Recently, receptor specific retinoic acid analogs have been synthesized (39, 40, 53). These structurally restricted analogs have higher affinity for one of the RAR or RXR receptor isotypes. Structures of some of these analogs are shown in Fig. 2. These receptor specific analogs help to elucidate the mechanism of action of retinoic acid. Combination studies of these analogs will help to find synergistic and antagonistic or redundant effects among receptor isotypes.

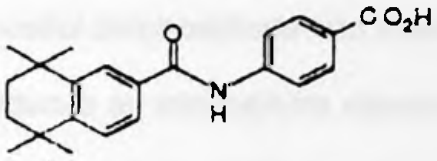
**Figure 2.** **Chemical structures of the synthetic retinoid analogs used in the present study.**

AM 580 - RARα-selective retinoid.

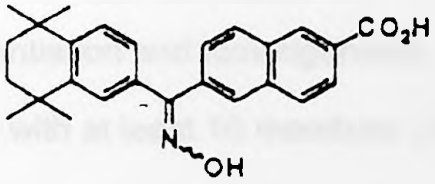
SR11254 - RARγ-selective retinoid.

SR11246 - RXR-selective retinoid.

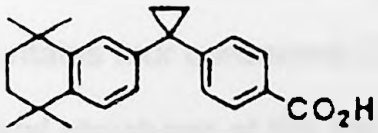
**Figure 2**




Am580




SR11254




SR11246

## PROTEIN KINASE C

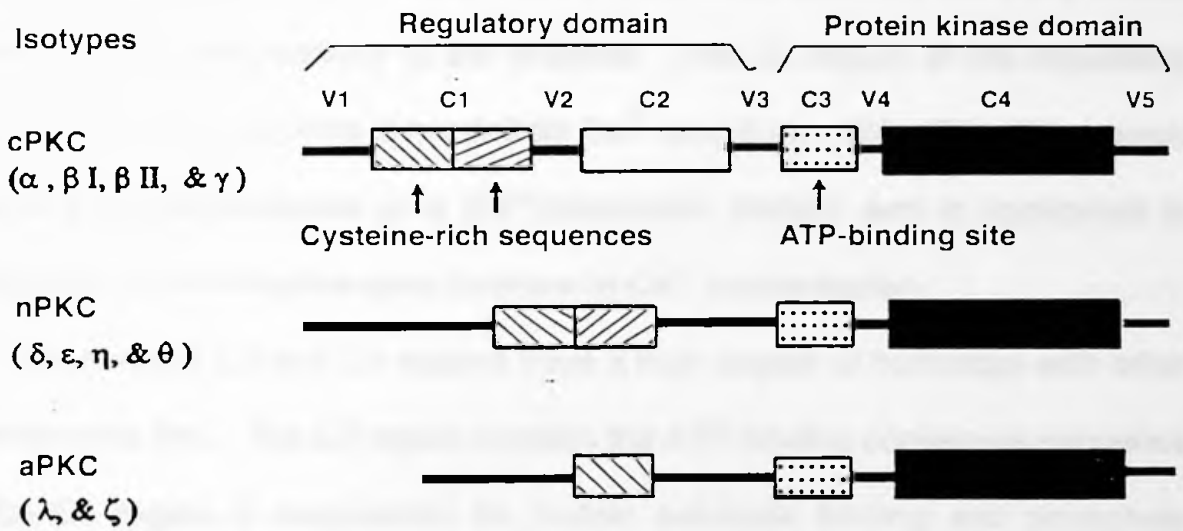
Protein kinase C (PKC) is an intracellular calcium-, phospholipid-dependent enzyme which is activated by diacylglycerol (144). It phosphorylates proteins on serine and threonine residues (159). Many hormones, growth factors, and neurotransmitters bind to cell surface receptors and activate phospholipase C, which in turn hydrolyzes phosphatidylinositol bisphosphate into inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 induces an intracellular release of calcium, whereas DAG binds and activates PKC (162, 164). PKC can also act as a receptor for phorbol ester tumor promoters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) (18, 29, 152, 189). PKC has been shown to play a role in a multitude of cellular functions such as growth, differentiation and tumorigenesis.

PKC is a multigene family with at least 10 members (159). These members can be divided into three groups. The $\alpha$, $\beta$I, $\beta$II and $\gamma$ isoforms are members of the conventional PKC family. $\beta$I and $\beta$II are alternatively spliced products of one gene. Conventional isoforms are calcium- and phospholipid-dependent (159). Each isoform is one polypeptide and contains four conserved (C1-C4) and five variable regions (V1-V5) (163). The general structures of the PKC isoforms are shown in Figure 3.

**Figure 3. General structure of the PKC family of proteins.**

The differences in the domains of the different isozymes are shown. The conserved (C1-C4) and variable (V1-V5) regions of PKC are indicated in the regulatory and catalytic domain. The cysteine-zinc motifs and ATP-binding site are indicated by arrows.

**Figure 3**


Isotypes
Regulatory domain
Protein kinase domain
V1
C1
V2
C2
V3
C3
V4
C4
V5
cPKC
(α , β I, β II, & γ )
Cysteine-rich sequences
ATP-binding site
nPKC
( δ, ε, η, & θ )
aPKC
(λ, & ζ)

The amino terminal half of the regulatory domain of cPKC isoforms contains two conserved regions, C1 and C2, that play a critical role in the regulation of enzyme activity. The carboxy terminal half of the enzyme contains two additional highly conserved regions, C3 and C4. The C1 region comprises a pseudosubstrate sequence followed by two tandem repeats of a cysteine-rich, zinc finger like motif. The zinc finger motif is responsible for DAG and phorbol ester binding (158, 170). It is suggested that the exact binding sites for DAG and phorbol ester are not the same in the C1 region (209). Thus, the two activators act at distinct sites and induce different active conformations of the enzyme. The C2 region in the regulatory domain of the cPKC isoforms is needed for $Ca^{2+}$-sensitivity (126, 170). This region interacts with phospholipids in a $Ca^{2+}$-dependent fashion and is implicated in translocation to membranes upon increase in $Ca^{2+}$ concentration.

The carboxy C3 and C4 regions have a high degree of homology with other protein kinases (94). The C3 region contains the ATP-binding consensus sequence and the C4 region is responsible for protein substrate binding and phosphate transfer. The catalytic and regulatory domains are separated by V3 which is sensitive to proteolysis by the calcium-dependent protease calpain (98, 137).

The second group of PKCs identified were the novel PKCs (nPKC). These nPKCs include the $\delta, \varepsilon, \eta$ and $\theta$ isoforms. Although these isoforms are very similar to the conventional PKCs, they are distinguishable due to the lack of C2 domain. Because of the lack of C2 domain, the nPKCs are calcium-independent (158).

The final group of PKC isoforms known as atypical PKC (aPKC) includes the ζand λ isoforms. They lack the C2 region and contain only one cysteine-rich zinc finger-like motif. These isoforms do not respond to DAG or to phorbol esters (89, 171).

The newly discovered μ isoform has an extended N-terminal region. It lacks the C2 region but does contain two cysteine-rich zinc finger-like motif separated by 73 amino acids. It does not require phorbol ester and $Ca^{2+}$ for activation.

The above observations suggests that the members of the PKC family respond differently to various combinations of lipids, and hence the patterns of activation of the PKC isoforms may vary in extent, duration and intracellular localization. In fact, various reports (7, 102, 136, 160, 168, 173, 174, 199, 224) have demonstrated that PKC isoforms show distinct tissue distribution and specific intracellular localization.

**Tissue Distribution**

Several groups have found that PKC α, βI, βII and γ are expressed in the bovine (37, 178), rat (95), rabbit (167) and human (37). From these results, it is believed that interspecies differences in the expression of each isoform are very small (95). PKC has also been found to be expressed in lower organisms such as *Drosophila (196,203), Xenopus laevis* (30), sea urchin egg (206), *Dictyostelium*

*discoideum* (88, 125) and *Saccharomyces cerevisiae* (216). PKC in yeast has a different substrate specificity than mammalian PKC (166). PKCα is widely distributed in many tissues and cell types. Most tissues, including liver, kidney, spleen and testis, additionally contain βI- and βII- subspecies in variable ratios (102). PKC βI- and βII also display differential expression in brain and other tissues including endocrine tissues such as the pituitary gland (7). The activity of βII-subspecies far exceeds that of the βI- subspecies. Cultured fibroblasts, such as the NIH 3T3 cell line, express α- but not β- subspecies (136). PKCγ is expressed solely in the brain and spinal cord and is not found in any other tissues or cell types (199). PKCδ, ε and ζ are present in brain, lung, thymus, spleen and skin (160, 168, 224). PKCε is also expressed in the lung, and PKCδ is found in several tissues including testes and ovaries (Ohno unpublished data). PKCθ is predominately found in muscle, but also to a lesser extent in lung, skin, spleen, and brain (173). PKCη is abundantly expressed in the skin and lung and less abundantly in the brain and spleen (168,174). The exact function of each isozyme is not clear; however, their unique tissue distribution leads to the belief that each has a specific role.

Studies in which a particular PKC isoform is over expressed in different cells have led to contradictory results. For example, transfection of PKCβII into rat fibrolasts resulted in an increased rate of cell growth (82). However, when the βII isoform was expressed in HT29 cells, a human colon cancer cell line, it caused a

slower growth rate and the transfected cells were less tumorigenic in nude mice (32). Overexpression of PKCα in NIH 3T3 fibroblasts resulted in a slightly transformed phenotype (59), whereas overexpression of PKCα in B16 mouse melanoma cells induced a differentiated phenotype (73). These contradictory results suggest that a particular isoform may play a role in cellular proliferation which is inhibitory or stimulatory depending on the cell type.

**Regulation of Expression of PKC Isozymes**

The variation of expression between PKC isoforms in the same tissue or the cell line sugests a distinct mechanism of regulation for each PKC gene. To date, the gene regulatory regions of only the β and γ genes have been analyzed, and they appear to be distinct. The 5' upstream promotor region of the PKCβ gene was found to contain multiple positive and negative transcriptional regulatory elements (154). Three positive (P1, P2, and PN) and two negative elements (N1 and PN) were found. Different combinations of these elements resulted in a different transcriptional response in different cell lines. Thus, the regulation of cell type-specific transcription is very complex. A CCAAT sequence which potentially binds NF1 is located at position -110 within the P1 region. One of the negative elements, N1, contains an AP-1 binding sequence with one base pair change.

The PKCγ 5' regulatory region contains an SP1 binding site but no TATA or CAAT box, similar to the promoter regions of housekeeping genes. The promoter region of the γ gene contains two AP-2 binding sites, one AP-1 binding site and one cAMP response element (30). Because AP-1 and AP-2 binding sites are known to be phorbol ester-inducible, these sites within the two genes may prove to be important in regulation by other PKC isozymes or by autoregulation.

**Activation of PKC**

Most isoforms of PKC are activated by DAG. This mechanism involves the translocation of PKC to the plasma membrane in response to growth factor receptor binding (97). The inactive PKC, which is translocated to the membrane, presumably interacts with phosphatidylserine (PS) and calcium, which are crucial cofactors in the activation of the cPKC isoforms (9). In the membrane, DAG is formed by phospholipase C cleavage of phosphatidyl inositol. DAG can also be formed by phospholipase D cleavage of phosphatidyl choline, followed by phosphatidic acid phosphatase (52, 159, 182). Once DAG binds to the PKC-calcium-PS complex, PKC is activated (85). For activation of the cPKC isoforms, an increase in intracellular $Ca^{2+}$ concentration and phospholipid degradation are intimately interrelated, and sometimes complementary to each other. At higher concentrations of Ca2+, enzyme activation requires less phospholipid degradation, while in the

presence of intense phospholipid degradation, less $Ca^{2+}$ is needed for enzyme activation (164).

In the inactive state of PKC, the pseudosubstrate region of the enzyme is bound to the substrate binding site. The pseudosubstrate is an amino acid sequence which resembles a PKC substrate and is located within the regulatory domain of the molecule. Once DAG binds, PKC undergoes a conformational change and the substrate binding site is unmasked (8). Studies which support this hypothesis include the rendering of the enzyme constitutively active by cleaving (81) or mutating (180) the pseudosubstrate, or by incubating with antibodies directed at the pseudosubstrate region (128).

More recent evidence indicates that autophosphorylation may be required for enzyme activity. The PKC molecule undergoes intrapeptide autophosphorylation at six different serine/threonine residues in both the catalytic and regulatory domains (61, 83, 140, 150). All identified PKC isoforms e.g. PKC$\alpha$ (181), $\beta$I (100), $\beta$II (61), $\gamma$ (179), $\delta$ (165), $\varepsilon$ (101), $\zeta$ (85), $\eta$ (174), and $\theta$ (173), are capable of autophosphorylation.

Proteolytic cleavage by calpain is thought to be the another mechanism by which PKC can be activated (55). Calpain cleaves the regulatory domain from the catalytic domain at physiological concentrations of calcium (98). Other evidence suggests that proteolytic cleavage may initialize the rapid PKC degradation (98). It

is still remains to be determined whether this mechanism provides the cell with a means of rapid PKC activation followed by immediate degradation (177).

Another class of PKC activators are the phorbol ester tumor promoters. Phorbol esters bind to PKC in the cysteine rich C1 region (170). Phorbol esters are more potent and are not metabolized as rapidly as DAG (9). Yeast PKC is unique in that it is activated by DAG but not by phorbol esters (166).

A recent report suggests that a novel lambda-interacting protein (LIP) binds to the zinc finger domain of the atypical protein kinase C isotype λ and stimulates its kinase activity in vitro and in vivo (48). This study raises the possibility of other novel proteins that may interact with different PKC isotypes and activate them in vivo.

Regulation of phospholipase D by protein kinase C without the involvement of its kinase activity is also reported (208). This study suggests the structure function of PKC.

**Role of PKC in Cell Growth and Differentiation**

Although, the role of PKC in signal transduction has been intensively studied, its effect on cell growth and differentiation is not well defined. Because PKC is known to be a major receptor for phorbol ester tumor promotors, several studies have been carried out to define the function of PKC in response to cell growth stimuli. When cells are stimulated by phorbol esters, there is an initial activation of

PKC. Activation is followed by down regulation and degradation of PKC. Since PKC has been shown to decrease the tyrosine kinase activity of certain growth factor receptors, such as the epidermal growth factor (EGF) receptor, phorbol esters may initiate uncontrolled cellular proliferation by inactivating the negative regulators of growth factors. When PKC is downregulated, the EGF receptor may be constitutively activated resulting in uncontrolled cell growth (118, 204, 232).

The role of PKC in cell growth or differentiation is cell type specific. Several lines of evidence suggest that PKC may play a positive role in cell growth by activating gene transcription. The expression of both c-fos and c-jun is induced by PKC (71, 145, 161, 198). These proteins, which form heterodimers bind to DNA sequences and transactivate gene expression (31, 75, 148, 191, 201, 223). The DNA sequence to which the fos-jun heterodimer specifically binds was originally called the TPA response element. Fos and jun are the components of the activator protein-1 (AP-1) and are involved in cell proliferation of many cell types. Recent studies suggest that AP-1 activity is also involved in the cell differentiation pathway (96, 119).

Several direct substrates of PKC have been identified by in vitro experimentation. MARCKS (myristoylated alanine rich C kinase substrate), and histone were the earliest substrates discovered. Phosphorylated MARCKS protein causes actin filaments to be redistributed from the cellular membrane to the cytoplasm (76). The substrates of PKC can generally be classified into categories

of proteins based on their functions within the cell (153, 161). One class includes proteins which are involved in signal transduction and PKC activation such as the EGF receptor, T cell receptor, and RAS. Proteins involved in metabolic pathways, including ion channels and active transport pumps, are another class of PKC substrates. Several transcription and translation factors are also activated by PKC (138). Nuclear proteins such as DNA topoisomerase I and lamin B have been shown to be phosphorylated by PKC (58, 80, 185). Another class of PKC substrates includes cytoskeletal and contractile proteins such as myosin light chain, troponin and caldesmon.

## AP-1 Transcription Factor

The diversity of upstream stimulatory sequences in eukaryotic genes and the variability of their position suggests that they may be recognized by different specific proteins rather than by a single polymerase. RNA polymerase II by itself can not recognize promoter sites and begin transcription. However, the addition of cell extracts restores this ability. Several specific stimulatory proteins called transcription factors have been isolated and their sites of action are being identified by footprinting experiments. Transcription factors are the proteins that recognize specific DNA sequences present upstream of specific genes. They can bind as a single moiety, or as a homodimers or heterodimers. Many proto-oncogenes are transcription

factors, such as AP-1, C-MYC, and NF-κB. These transcription factors bind to particular cis-acting elements and regulate gene transcription and thereby cell growth and differentiation.

Activator Protein-1 (AP-1) was first identified as a transcription factor that binds to an essential cis-element of the human metallothionein IIa (hMTIIa) promoter (108). Shortly thereafter the binding site for AP-1 was also recognized as the TPA response element (TRE) of several cellular and viral genes whose transcription is induced in response to treatment with phorbol ester tumor promoters (4). Comparison of several TREs led to the derivation of a palindromic consensus sequence that is recognized by AP-1 : 5'-TGAG/CTCA-3' (4, 168)

**Members of AP-1 Complex and Their Structural Analysis**

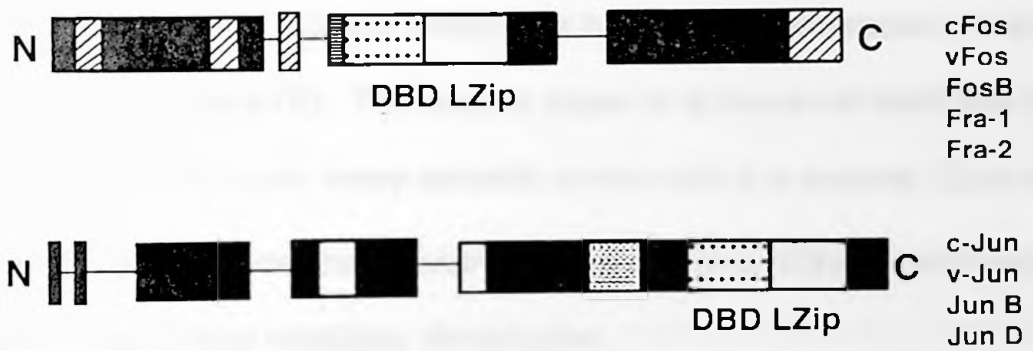
Using affinity columns constructed by coupling of synthetic oligodeoxynucleotides corresponding to the TREs of either the hMTIIa or the collagenase genes to agarose beads, AP-1 was purified from extracts of HeLa cells. Despite multiple rounds of affinity chromatography, the purified AP-1 preparations contained several distinct polypeptides. Later, it became evident that these polypeptides correspond to products of members of the jun and fos gene families (3, 19, 20, 31, 62, 193, 202). Jun and fos family proteins are proto-oncogenes.

The structures of jun and fos family members are illustrated in Fig. 4.

**Figure 4. Structural organization of the Fos and Jun proteins.**

Jun and Fos proto-oncogenes are the members of the basic leucine zipper family proteins. The hatched box represents the 'leucine-zipper' region. The cross-hatched box indicates the 'basic domain.' The solid boxes denote the transactivation regions in Jun family. The dotted boxes denote highly conserved regions in the Fos proteins of yet unknown function.

**Figure 4**


N
C
DBD LZip
cFos
vFos
FosB
Fra-1
Fra-2
N
C
DBD LZip
c-Jun
v-Jun
Jun B
Jun D

The jun family contains v-jun, c-jun, jun B, and jun D members where as the fos family contains v-fos, c-fos, fosB, fos-related antigen 1 (fra-1), and fos-related antigen 2 (fra-2) (5). The c-jun proto-oncogene is the cellular homolog of the transforming gene of avian sarcoma virus 17 v-jun. C-fos is the cellular homologue of v-fos, a viral oncogene carried by the Finkel-Biskis-Jenkins and the Finkel-Biskis-Reilly murine osteosarcoma viruses.

Both c-jun and c-fos belong to the basic leucine-zipper family proteins. The dimerization of jun and fos proteins occurs via hydrophobic interactions between their leucine zipper regions (5). The leucine zipper is a structural motif that forms an extensive $\alpha$ helix in which every seventh amino acid is a leucine. Due to this arrangement, the leucine side chains protrude from one side of the $\alpha$ helix and form a hydrophobic surface that mediates dimerization.

Measurement of the temperatures at which the dimers dissociate into their individual components indicates that the increased DNA-binding activity of the jun:fos heterodimer is due to its increased thermostability in comparison to the jun:jun homodimer (211). On the other hand, c-fos does not dimerize with another c-fos molecule. This idiosyncratic behaviour of c-fos can be explained by electrostatic repulsions between negatively-charged side chains which are abundant in its leucine zipper region. The same residues are likely to account for a net increase in the number of salt bridges between the two leucine zipper regions upon interaction with basic residues in the leucine zipper of c-jun. These additional salt

bridges are probably responsible for the increased stability of the heterodimer (134).

While the leucine zipper region mediates the dimerization of these proteins and thereby dictates the specificity of complex formation, the interaction with DNA occurs via a region immediately upstream to the leucine zipper (93, 134). Due to the abundance of positively charged residues, this area is known as the basic region (149, 222). Sequence comparisons indicate that the basic region is highly conserved among all of the jun and fos proteins (5). It is also conserved in the various cAMP responsive element binding protein (CREB) and activator transcription factor (ATF) proteins that interact with a sequence similar to the TRE (93).

The interaction between c-jun and c-fos plays an important role in cellular regulation. Transcription of both c-jun and c-fos is induced by TPA and other activators of PKC in many cell types, resulting in increased production of c-jun and c-fos proteins. In the majority of the cells analyzed so far the c-fos protein and mRNA have shorter half-lives than the c-jun protein and mRNA (5).

Therefore, the composition of AP-1 complex changes from predominantly jun homo- and heterodimers (e.g., c-jun:jun D), before induction, to mostly jun:fos heterodimers immediately after induction, followed by jun homo- and heterodimers after the fos proteins have decayed. This decrease in c-fos is likely to lead to decreased dimer formation followed by decreased occupancy of the TREs and a gradual decay of the induction response until a certain baseline, determined by the level of c-jun expression and dimerization is reached. The jun B and jun D proteins

are poor transactivators that may act to further dampen the response to c-jun, a potent transactivator. Thus, formation of the jun:fos heterodimer appears to be mostly responsible for initiating the induction response while c-jun homodimers are involved in this maintenance.

Regulation of AP-1 activity is found to be induced by many stimuli such as TPA, cytokines, T cell activators, neurotransmitters, and UV irradiation (5). Several mechanisms are involved in induction of AP-1 activity and may be classified as those that increase the abundance of AP-1 components and those that stimulate their activity.

**Transcriptional Control of c-jun Expression**

Examination of the promoter of the intronless human c-jun gene revealed the sequence 5'-GTGAC**A**TCAT-3' (6, 77). Most of the inducers of c-jun operate through this major cis element, the c-jun TRE. Apart from insertion of an A residue (in bold), this sequence is identical to a consensus TRE. Due to this subtle change it is more efficiently recognized by c-jun:ATF2 heterodimers than by conventional AP-1 complexes (6, 221). Unlike c-jun, ATF2 is a constitutively expressed protein. However, despite its inducible expression, most cell types contain some c-jun protein prior to their stimulation. The c-jun TRE is constitutively occupied in vivo (197). Following exposure to stimuli, both c-jun (47) and ATF2 (74) are rapidly

phosphorylated. The constitutive occupancy of the c-jun TRE indicates that this phosphorylation occurs while the proteins are bound to the c-jun promoter. Phosphorylation of c-jun and ATF2 stimulates their ability to activate transcription, thereby leading to c-jun induction. Thus, part of the increase in AP-1 activity is due to increased c-jun synthesis. The question arises here that is what causes the phosphorylation of c-jun and ATF2.

**Transcriptional Control of c-fos Expression**

Most of the genes that encode AP-1 components behave as immediate-early genes, i.e., genes whose transcription is rapidly induced, independent of de novo protein synthesis, following cell stimulation. Several cis elements mediate c-fos induction in response to a diverse spectrum of extracellular stimuli (5). A cAMP response element (CRE) mediates c-fos induction in response to neurotransmitters and polypeptide hormones which, by using either cAMP or $Ca^{2+}$ as second messengers, activate either protein kinase A or calmodulin-dependent protein kinases, respectively (207). A serum response element (SRE) mediates c-fos induction by growth factors, cytokines, and other stimuli that activate mitogen activated protein kinases (MAPKs) (218). Given this complexity, it is not surprising that c-fos transcription is rapidly induced in response to a variety of extracellular stimuli.

The increase in c-fos transcription results in increased synthesis of c-fos protein, which upon translocation to the nucleus combines with pre-existing jun proteins to form AP-1 dimers that are more stable than those formed by jun proteins alone (211). Increased stability results in higher levels of AP-1 DNA binding activity because it shifts the equilibrium toward dimer formation, which is essential for DNA binding.

**Posttranslational Control of AP-1 Activity**

Primary gene induction or repression in eukaryotes does not require *de novo* protein synthesis, suggesting the involvement of posttranslational modifications. Since many different types of stimuli that affect gene expression also lead to the activation of protein kinases, it is likely that transcription factor function will be directly regulated by phosphorylation. Even though other types of posttranslational modifications will undoubtedly be important in regulating transcription factor function, phosphorylation is the best studied modification system.

The activities of both pre-existing and newly synthesized AP-1 components are modulated through their phosphorylation. So far, this form of posttranslational control has been demonstrated for c-jun, c-fos, and ATF2, but it is likely that other jun and fos proteins are similarly regulated.

The biological function of c-jun as a transcription factor has been reported to

be regulated by two distinct phosphorylation systems. Phosphorylation of the $NH_2$-terminal residues serine 63 and serine 73 (human amino acid sequence) increases in response to growth factors or UV signaling, enhances the transcriptional activity of c-jun and also induces AP-1 target genes (11, 188). In contrast, phosphorylation of the COOH-terminal residues threonine 231, serine 243 and serine 249 inhibit DNA binding (24). The repression of c-jun DNA binding by COOH-terminal phosphorylation seems mechanistically straight forward, conceivably involving electrostatic repulsions between the phosphate groups on c-jun and the DNA binding site. However, the regulation of COOH-terminal phosphorylation is obscure. The existence of an activated phosphatase (91) remains hypothetical. Conversely, previous analysis has suggested that dephosphorylation can occur as a consequence of DNA binding and does not require the activation of signal transduction cascades (176). There is some evidence that phosphorylation in $NH_2$-terminal residues of c-jun stimulates the dephosphorylation of the COOH-terminal sites, and consequently increases the DNA-binding activity of the transcription factor (175).

There are many candidate protein kinases that phosphorylate c-jun *in vitro*. Pulverer et. al (187) suggested that extracellular regulated kinase (ERK1 and ERK2) phosphorylate serine 63 and serine 73. However, others have found that ERK1 and ERK2 phosphorylate COOH-terminal residues and inhibit AP-1 binding to DNA (139). Casein kinase (CKII) and glycogen synthase kinase-3 (GSK3) also

phosphorylate carboxy terminal residues (24, 116). Hibi et. al have recently proposed that c-jun $NH_2$ terminal kinase (JNK) directly binds to c-jun and phosphorylates serine 63 and serine 73 residues (79). JNK is a distant relative of mitogen activated protein kinase (MAPK) and so far two different proteins (p55 and p46) have been shown to exhibit JNK activity. Even though both of these proteins phosphorylate c-jun, they have been proposed to have different functions *in vivo* (210).

## B16 Mouse Melanoma Cells

Melanocytes are derived from the neural crest during development and share many characteristics of nerve cells; e.g., presence of nerve growth factor receptors. Fidler (57) selected a series of mouse melanoma cell lines that differed in their metastatic potential. These lines, derived from B16 melanoma, were selected for their ability to form pulmonary metastases. The cells of high metastatic potential not only formed more pulmonary metastases but were more invasive at the primary tumor site (56). Thus these tumor cell lines are useful in studying cell properties that render one line highly metastatic.

One of the major biochemical functions of melanocytes is the production of melanin. There is a correlation between the loss of regulatory controls for melanogenesis and enhanced metastatic potential. Melanocyte-stimulating hormone

(MSH) controls melanin production and cyclic AMP (cAMP) is involved in this regulation (103, 155). Tyrosinase is the key enzyme in the melanin synthesis pathway. In B16 -$F_1$ cells (low metastatic potential), MSH or cAMP greatly elevated tyrosinase activity and melanin content while inhibiting cell proliferation (155). The same parameters in B16-$F_5$ cells (intermediate metastatic potential) were altered to a much lesser degree, whereas B16-$F_{10}$ (high metastatic potential) were not significantly affected by MSH or cAMP (155). Therefore, a correlation exists between loss of hormonal regulation and increased metastatic potential.

**Effect of RA on Cell Growth and Differentiation of B16-$F_1$ Mouse Melanoma Cells**

Retinoic acid (RA), a biologically active metabolite of vitamin A, has been shown to play an essential role in maintaining normal epithelial cell differentiation (143, 212). In B16 mouse melanoma cells RA inhibits growth by blocking progression early in G1, eliminates the ability of these cells to grow in soft agar, and induces differentiation (157). In B16 cells RA induces a 5-8-fold increase in PKCα message and protein level (156). The induction of PKCα protein starts at 16 h after the RA treatment and reaches a maximum level after 48 h of treatment (195). PKCα is the major isozyme present in B16 cells. PKCβ mRNA was not detectable, and only a very small amount of PKCγ mRNA was seen at 48 h after RA treatment. B16

melanoma cells also express mRNA for PKCε, δ and ζ but not for PKCλ. None of these PKC mRNA species were induced during RA treatment of cells (Huang and Niles, unpublished data).

The effect of RA on PKCα mRNA expression is indirect, since inhibition of protein synthesis by cycloheximide abolishes the induction of PKCα mRNA. Therefore, new protein synthesis is required. It is not yet known which proteins are mediating the induction of PKCα. Lotan et. al (122) and our lab (236) have shown that RA directly induces mRNA for the β form of the retinoic acid receptor (RARβ), which is undetectable in untreated B16 mouse melanoma cells. Therefore, one possibility is that the induction of PKCα is mediated through the RARβ receptor, which must first be autoinduced by RA. The induction of PKCα mRNA appears to be primarily post-transcriptional, since RA treatment did not alter the stability of the PKCα mRNA. Moreover, RA increases the rate of transcription only 2- to 3-fold, which is not enough to account for the 10- to 12-fold increase in steady state mRNA levels. The primary method of PKCα regulation must be post-transcriptional, either enhanced RNA processing or increased transport out of the nucleus.

## Role of PKCα in the Differentiation Program

B16 cells transfected with and overexpressing PKCα exhibit phenotypes very similar to those of wild type cells treated with RA. PKCα overexpressing clones showed slower monolayer growth rates, diminished capabilities to form viable colonies in soft agarose, and increased melanin contents as well as secretion. When injected into syngeneic mice, PKCα overexpressing clones produced smaller tumors and had longer latency times than control cells (73).

RA has been repeatedly shown to induce differentiation in several different types of cancer cells (114, 120, 121, 122, 124, 213). The mechanism by which RA induces differentiation is still largely unknown. Prolonged phorbol ester treatment of B16 cells depletes the cells of PKCα and counteracts the effects of RA treatment. In the presence of PDB, RA still stimulated PKC expression relative to cells treated with PDB alone; however, the absolute amount was considerably less than in control cells or cells treated with RA alone (156). These findings provide strong evidence that PKCα plays a key role in RA induced B16 cell differentiation.

### Enrichment of PKCα in Nucleus

Gruber et al. (72) have shown that RA increases PKCα protein levels in the cytosol, membranes, and nuclei. RA increased the amount of nuclear PKCα approximately 15-fold. RA increased PKCα levels in the membranes and cytosol approxomately 5-fold over control cells. Thus, RA induced a selective enrichment in nuclear PKCα. Translocation of PKC to the nucleus in response to phorbol esters and/or other mitogens seems to vary greatly depending on the PKC isoform and the host cell.

## EXPERIMENTAL OBJECTIVE

The major part of this dissertation has focused on the effect of RA on AP-1 expression and transcriptional activity in B16 mouse melanoma cells. An initial study in the laboratory showed that RA inhibits cell growth and induces differentiation in B16 melanoma cells. RA-induced differentiation is preceded by 8 fold induction of PKCα protein level, the only conventional isoform expressed in these cells. They also express mRNA for PKCε, δ, and ζ. None of these PKC mRNA species were induced during RA treatment of cells (Huang and Niles, unpublished data). Gruber

et al. have shown that RA induces an enrichment of PKCα in the nucleus. The role of PKCα in the nucleus is not known. The hypothesis is that PKC regulates gene expression by modulating the activity of transcription factors. In light of the RA-induced PKC enrichment in the nucleus, we studied the effect of RA on the AP-1 transcription factor.

The second part of this dissertation has focuses on the effect of receptor-specific retinoid analogs on PKCα expression and AP-1 transcriptional activity. The goal of this study was to determine which receptors are involved in RA-induced PKCα expression and AP-1 transcriptional activity. Retinoid analogs specific for RARα (AM580) (40), RARγ (SR11254) (40), RXR (SR11246) (39), and RXR/RARβ (SR11346) (unpublished) were used in these experiments.

# METHODS AND MATERIALS

## Cells and culture condition

B16-F1 mouse melanoma cells were grown in a humidified atmosphere of 7% $CO_2$/93% air at 37°C in Dulbecco's modified Eagle's medium (DMEM) containing 1 g/liter of glucose and supplemented with 10% heat-inactivated bovine calf serum (HyClone Lab. Inc., Logan, UT.), 50 Units of penicillin-G, and 50 μg streptomycin sulfate/ml.

## Treatment of Cells with RA, PDB, bisindolylmaleimid and retinoid analogs

All-trans-RA was obtained from Fluka (New York). All the experiments involving the use of RA were conducted in subdued light to prevent photo-oxidation. A concentrated stock solution of RA (10 mM) was prepared in ethanol and stored at 4 °C. New RA stock was prepared every 10 days. The stock solution was directly added to cell culture medium to yield the desired final concentration. Cells were seeded at the densities indicated and 24 h later were refed with growth medium containing ethanol (control) or various concentrations of RA. At 24 and 48 h time points, cells were harvested and assayed as described below.

Phorbol dibutyrate (PDB) was obtained from Sigma Chemical Co., St. Louis, MO and dissolved in DMSO. PDB stock solution (10 mM) was prepared and stored at -20 °C. Cells were treated either chronically or acutely with 1 μM PDB and assayed as described below.

Bisindolylmaleimid (Boehringer Mannheim, Indianapolis, IN) is a specific and potent PKC inhibitor. Stock solution (5 mM) was prepared in DMSO and stored at -20 °C, protected from light. For *in vitro* PKC enzyme activity assay, 0.1, 0.5 and 1 μM concentrations were used while 2.5 μM concentration was used for the selective analysis of PKC-mediated processes in the cellular system.

Retinoid analogs (AM580, SR11254, SR11246, and SR11346) and 9-cis RA were provided by Marcia Dawson, SRI International, Menlo Park, CA. The stock solutions ($10^{-2}$ M) were prepared in DMSO and stored at -20 °C. Cells were treated with different concentrations of these retinoids ($10^{-6}$, $5X10^{-7}$, $10^{-7}$, $10^{-8}$, $10^{-9}$ M) and subjected to either western blot analysis for PKCα expression or luciferase assay for the induction of AP-1 activity. All the experiments involving the use of these retinoids were conducted in subdued light. When the combination of any two of these analogs were used in the experiment, $0.5X10^{-7}$ M concentration of each analog was added to achieve $10^{-7}$ M final concentration.

## RNA Isolation and Northern Blot Analysis

RNA was isolated by a single step method as described previously (34). Cells were lysed in a denaturing solution containing 4 M guanidinium thiocyanate. The lysate was mixed sequentially with 2 M sodium acetate, pH 4, phenol, and chloroform/isoamyl alcohol. The resulting mix was centrifuged, yielding an upper aqueous phase containing total RNA. In this single-step extraction, the total RNA was separated from proteins and DNA that remain in the interphase and in the organic phase. Following isopropanol precipitation, the RNA pellet was redissolved in denaturing solution (containing 4M guanidium thiocyanate), reprecipitated with isopropanol, and washed with 75% ethanol. The RNA was quantitated spectrophotometrically at $A_{260}$ and stored at -70 °C.

The RNA was fractionated by size on 1% agarose gels containing formaldehyde and transferred to Hybond-N nylon membranes by downward alkaline blotting (33). The transferred RNA was crosslinked to the membrane by UV light. The membrane was prehybridized for 1 h in 6X SSC and 2% SDS. $^{32}$P-labeled cDNA probes were then incubated with the membranes in fresh hybridization solution for 20 h at specific activity of 1 X $10^6$ cpm/ml. Blots were washed three times for 15 min each in 1 X SSC - 0.1% SDS, 0.5 X SSC - 0.1% SDS, and 0.2 X SSC - 0.1% SDS, respectively. The blots were exposed to Kodak XAR film with an enhancing screen in a cassette for 2-5 days at -70 °C. All the cDNA probes were

labeled by using the prime-a-gene labeling system (Promega). The amounts of different mRNAs were quantitated by imaging the autoradiograms with a Molecular Dynamics computing densitometer, making sure that the signals were within the linear range of the instrument and normalizing to glyceraldehyde 3-phosphate dehydrogenase as an internal standard.

**cDNA Probes**

The following cDNA clones were used as probes in Northern blot analysis: Clones pGEM-4 c-jun (rat) contains complete 1.8 kb c-jun (rat) cDNA inserted within Eco RI restriction sites (191,192). Clone pSP65 c-fos (rat) contains full length 2.2 kb c-fos (rat) cDNA inserted within Eco RI restriction sites (38). Clone pHcGAP (glyceraldehyde-3-phosphate dehydrogenase) was purchased from ATCC (219).

**Western Blot Analysis For c-Jun, c-Fos, and PKC$\alpha$**

2.5 X $10^5$ cells were plated onto 100-mm dishes. The following day, cells were refed with DMEM with or without RA or PDB, depending on the experiment.

For Jun and Fos protein analysis, cells were washed two times with PBS, harvested at different time points and nuclear extracts prepared by a modification of the method of Schreiber et al. (169). A cell pellet containing $10^7$ cells was

resuspended in 400 μl of buffer containing 10mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1% (vol/vol) aprotinin by pipetting repeatedly using a Pipetman (Rainin, Woburn, MA, USA) fitted with a cut-off tip. Cells were chilled on ice for 15 min and then gently lysed by the addition of 0.6% (vol/vol) Nonidet P-40, followed by mixing by inversion several times. Nuclei were pelleted at 200 X g for 5 min in a microcentrifuge. The nuclei were lysed by shaking for 30 min in 100 ml of ice-cold 20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1% (vol/vol) aprotinin and 10% (vol/vol) glycerol at 4 °C. The lysate was then microcentrifuged at 12000 X g for 10 min and the supernatant aliquoted into a new microcentrifuge tube, supplemented with 0.025 mg/ml leupeptin and stored at -80 °C as a nuclear extract.

For PKCα western blot analysis, cells were washed with cold PBS and harvested in 250 μl of lysis buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 1% glycerol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 50 μg/ml aprotinin, 0.5 mM PMSF). Cells were lysed on ice by three consecutive 10-sec sonications with a Tekmar® sonic disruptor at power setting 60. The total cell lysate was stored at -80 °C.

Protein concentration was determined by using the Pierce BCA protein assay kit. Proteins (50 μg) were separated by 10% SDS-PAGE. The proteins were transferred to a Hybond-C extra nitrocellulose membrane (Amersham) by using a semi-dry transfer cell (Bio-Rad). The membrane was blocked overnight with 5%

nonfat dry milk in Tris-HCl-buffered saline containing 0.2% Tween 20 (TBST). The membrane was incubated with 1 μg/ml of polyclonal anti-c-jun antibody (Ab-2, Oncogene Science) or with 1 μg/ml of polyclonal anti-c-fos antibody (Ab-2, Oncogene Science) for one hour. Then membrane was washed three times in TBST and incubated with 1:3000 dilution of secondary antibody (Horseradish peroxidase [HRP]-linked anti-rabbit IgG from Amersham, USA) for one hour. The membrane was washed 3-5 times in TBST and visualized by using the ECL kit from Amersham (Chicago, IL).

For PKCα western blot analysis, 1 μg/ml of monoclonal anti PKCα III antibody (UBI) was used as primary antibody and HRP-linked anti-mouse IgG (Amersham, USA) was used as secondary antibody.

**RT-PCR**

The RNA PCR core kit provided by Perkin-Elmer (Norwalk, CT) was used for these assays. 1 μg of RNA was converted to cDNA by reverse transcription (RT) with Moloney murine leukemia virus reverse transcriptase using oligo dT (1.5 mM) as the primer. The 30-μl reaction contained 1X PCR buffer II, 3 mM $MgCl_2$, 0.5 mM each dATP, dTTP, dCTP, dGTP, and 30 Units RNasin. RNA was denatured at 70 °C for 3 min and cooled on ice before adding all the reagents. The samples were incubated first for 45 min at 42 °C, and then for 10 min at 80 °C.

For PCR amplification of specific cDNAs, 50 μl reactions were prepared on ice and contained 1X PCR buffer II, 1.5 mM $MgCl_2$, 0.2 mM each dATP, dCTP, dTTP, and dGTP, 2.0 units of Taq Polymerase, 1.0 mM each primer and 10% of the cDNA synthesized in the RT reaction. Primer sequences for c-jun (5' ACC CAG TTC TTG TGC CCC AA 3'), Jun B (5' AAA CCC ACC TTG GCG CTC AA 3') and Jun D (5' CCG GAT CTT GGG CTG CTC AA 3') were provided by Dr. Steven Estus, Univ. of Kentucky (50). All the reactions were covered with a drop of mineral oil, and subjected to 17-25 PCR cycles. The typical reaction conditions were 1 min at 95 °C, 1 min at 55 °C, and 2 min at 72 °C. The amplified cDNA products were separated by electrophoresis on 7.5% polyacrylamide gel, stained with ethidium bromide and visualized by UV light. c-Jun, Jun B, and Jun D cDNAs (from ATCC) were used as positive controls. Primers specific for β-actin were used to normalize the RNA in each RT reaction. Reactions without reverse transcriptase and without cDNA were used as negative controls.

## Gel Mobility Shift Assay

An oligonucleotide containing one AP-1 site was synthesized by the Marshall University Molecular Biology core facility. This oligonucleotide was labeled using a $^{32}$P dCTP (Amersham Chicago, IL) by a Klenow fill-in reaction. Nuclear extracts (10 μg) were incubated with incubation buffer (2 mM Tris-Cl (pH 7.5), 0.2 mM EDTA, 8 mM NaCl, and 12% (vol/vol) glycerol), 0.5 μg poly (dI-dC), and $^{32}$P-labeled oligonucleotide (30,000 cpm). The total volume of each reaction mixture was brought to 25 μl by adding distilled water. For the competition assay, 10-, 20- and 50- fold excess of unlabeled oligonucleotide was added just before adding the labeled oligo. The reaction mixture was incubated at room temperature for 20 min. In the supershift experiment, 1-2 μg of anti c-jun or anti c-fos antibody (Santa Cruz) was added to the reaction mixture and incubated at room temperature for an additional 45 min. All the samples were loaded on a 5% non-denaturing polyacrylamide gel. The gel was electrophoresed at 150 V using 0.25X TBE until the dye front was 3 cm from the bottom. The gel was dried and exposed to Kodak XAR film.

**Transient Transfection**

Four tandem repeats of the consensus AP-1 site, 5'-TGAGTCA-3', between *Mlu* I and *Bgl* II restriction sites was synthesized by the Marshall University Molecular Biology core facility. This was inserted in the appropriate restriction sites of the pGL2 vector upstream of an SV40 promoter followed by the gene encoding luciferase. Plasmid with or without the AP-1 elements was transfected into B16 cells via the calcium phosphate DNA precipitation method (200) together with plasmid containing SV-40-β-gal (to correct for transfection efficiency). B16 cells were seeded ($3 X10^5$ cells/dish) into 60-mm tissue culture plates 1 day prior to transfection. On the day of transfection, cells were refed with complete growth medium 4 h prior to the procedure. Transfections included 4 μg of the plasmid vector pGL2 with or without insert and 1 μg of pSV-β-galactosidase (Promega, Madison, WI). Cells were incubated with the plasmid DNAs for 16 h. Cells were then washed twice with PBS and treated with different concentrations of RA for 24 h, with 1 μM PDB for the last 3 h before harvest, or for 24 h depending on the experiment. Cells were washed twice with PBS, harvested, and assayed for luciferase and for β-galactosidase using the Promega kits. Luciferase assays were evaluated in the linear range and values were normalized to β-galactosidase activity. All transfections were performed in triplicate and all the experiments were repeated three to five times.

## Stable Transfection

B16 cells (in 100 mm dishes) were transfected with 10 μg of pCMV-67 plasmid, containing a dominant negative c-jun cDNA driven by a CMV promoter (kindly provided by Dr. Birrer, NCI) (190) and 1 μg of a plasmid encoding the neomycin resistance gene (pSVneo). The DNA was precipitated with calcium phosphate and the cells were incubated with the precipitates for 20 h. Cells were then refed with fresh medium for 2 more days and replated in normal growth medium containing 500 μg/ml geneticin sulfate (G418) (GIBCO BRL, Grand Island, N. Y.). Ten to twelve days later 40 clones were selected and expanded. Cells transfected with SVneo alone and selected for G418 resistance served as a negative control.

## Enzyme Activity of Partially Purified PKC

Culture conditions were the same as described for western blot analysis. Cells were lysed on ice with 20 mM Tris, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol, 5% Triton X-100, 0.5 mM PMSF, and 10 μg/ml aprotinin. Complete cell disruption was further ensured by three consecutive 10-s sonications with a Tekmar sonic disruptor at power setting 60. The total cell lysate was centrifuged at 12,000 X g for 15 min. The supernatant was loaded onto a DEAE-cellulose anion

exchange column (Cellex-D, Bio-Red) previously equilibrated with column buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 1 mM DTT). The column was washed with 5 volumes of column buffer. The PKC fraction was eluted with 2.5 column volumes of column buffer containing 100 mM NaCl and concentrated with a Centricon-10 microconcentrator (Amicon, Bedford, MA). Protein concentrations of samples were determined by the Pierce BCA assay. Samples were diluted to equal protein concentrations and assayed with a commercially available PKC assay system (Amersham, Chicago, IL) + 0.2 μCi of γ-[$^{32}$ P] ATP in the presence and absence of 12-O-tetradecanoylphorbol-13-acetate (TPA) and phosphatidylserine. The system utilizes synthetic, PKC-specific, substrate peptides which become phosphorylated with the radiolabeled phosphate group from (γ-$^{32}$P)ATP. At the end of the reaction, the radiolabeled peptide was separated from unincorporated $^{32}$P by the use of peptide affinity paper. The degree of phosphorylation was determined by liquid scintillation counting. Enzyme activity was calculated from counts/min taking into account the specific activity of the radioisotope and reaction time. Specific enzyme activity was obtained by substracting the CPM obtained in the absence of lipid mixture from that obtained in the presence of the lipid mixture.

# RESULTS

## AP-1 transcriptional activity

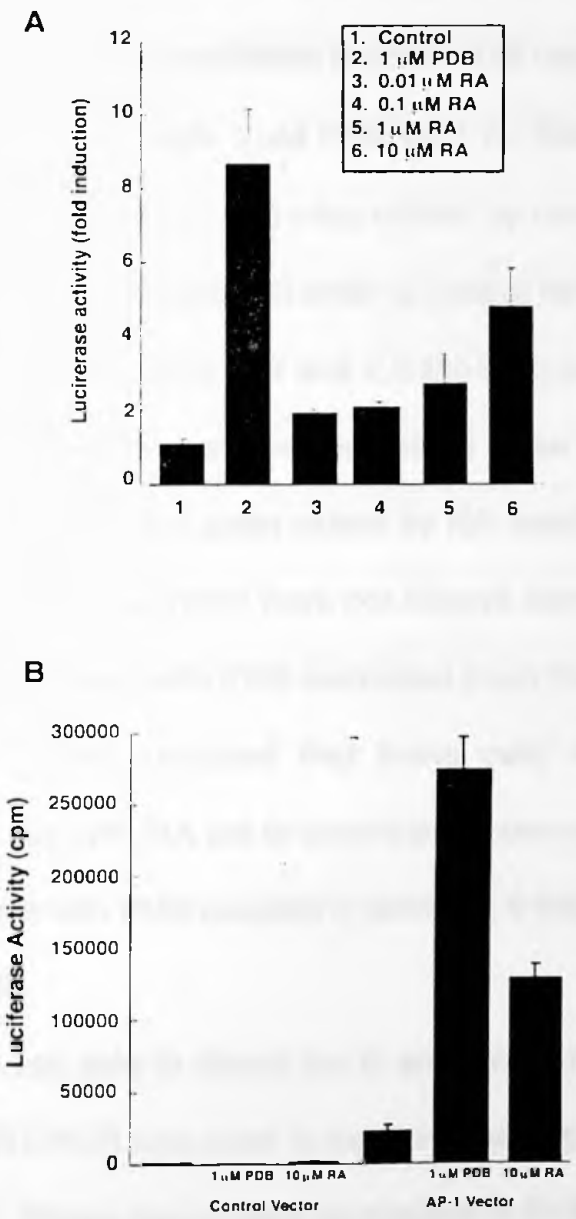
Phorbol ester-activated PKC stimulates AP-1 acitvity (20). In light of the large increase in PKCα protein in RA-treated B16 melanoma cells (156), and a selective enrichment of nuclear-associated PKCα (72), I investigated whether RA might also increase AP-1 transcriptional activity. B16 cells were transfected with either a PGL2 plasmid containing 4 tandem AP-1 consensus sequences inserted 5' to an SV40 promoter driving the expression of luciferase, or with a PGL2 plasmid lacking the AP-1 sequences. Cells were also co-transfected with a plasmid encoding β-galactosidase to correct for transfection efficiency. Transfected cells were treated for 24 h with or without the various concentrations of all-trans RA shown in Fig. 5A. As a positive control, one group of transfected cells was treated with 1 μM PDB for the last 2 h of incubation. At the end of the incubation (a total of 48 h from the time of transfection), all cells were harvested and assayed for luciferase and β-galactosidase activity. A 2 h treatment with PDB stimulated luciferase activity and thus AP-1 activity by 10 fold. RA increased luciferase activity by two-fold at the lowest concentration (10 nM) and the fold stimulation increased to four-fold at the highest concentration (10 μM). Cells transfected with the PGL2 plasmid lacking the AP-1 sites showed no change in luciferase activity with either 24 h RA or 2 h PDB treatment (Fig. 5B).

Figure 5. **A. AP-1 activity in control, PDB- and RA-treated B16 cells**

B16-F1 cells were transfected via the calcium phosphate method with the AP-1-SV40-leuciferase vector or PGL-2 without insert and plasmid encoding β-gal as described under materials and methods. Cells were treated with either vehicle (0.1% DMSO), different concentrations of RA (0.01, 0.1, 1, 10 μM ) for 24 h, or 1 μM PDB for 2 h just prior to harvesting the cells. 48 h after transfection all groups were harvested and assayed for luciferase and for β-galactosidase. Luciferase assays were evaluated in the linear range and values were normalized to β-galactosidase activity. All transfections were performed in triplicate and the entire experiment was repeated four times. The data presented is the mean ± SEM (error bars) of triplicate dishes for each treatment group from a representative experiment.

**B.** Vector without AP-1 binding sites (first three bars) was transfected and treated with either PDB or RA as described above.

## Figure 5


A
Lucirerase activity (fold induction)
12
10
8
6
4
2
0
1
2
3
4
5
6
1. Control
2. 1 μM PDB
3. 0.01 μM RA
4. 0.1 μM RA
5. 1 μM RA
6. 10 μM RA
B
Luciferase Activity (cpm)
300000
250000
200000
150000
100000
50000
0
1 μM PDB
10 μM RA
1 μM PDB
10 μM RA
Control Vector
AP-1 Vector

**Effect of RA and PDB on mRNA expression of jun and fos family members**

Since the AP-1 transcription complex is usually composed of c-jun homodimers or c-jun/c-fos heterodimers, I examined the possibility that the RA and/or PDB-induced increase in AP-1 activity could be due to an increase in the expression of one of these transcription factors. B16 cells were treated with 10 μM RA for either 24 or 48 h or with 1 μM PDB for 1 h. RNA was then extracted and analyzed for the amount of c-jun and c-fos mRNA by northern blot. The blots were also probed for GAPDH expression in order to control for the equality and specificity of any changes. Two messages (3.2 and 2.6 kb) for c-jun were found in B16 cells. The 2.6 kb message was the predominant form in these cells. The amount of c-jun mRNA was not altered to any great extent by RA treatment (Fig. 6A). The small increases seen in this experiment were not always seen in replicate experiments. In contrast, a 1 h treatment with PDB increased c-jun mRNA by 1.8 fold. Northern analysis of c-fos mRNA indicated that these cells express a 2.1 kb mRNA. Treatment of the cells with RA led to a consistent decrease in c-fos mRNA levels, while a 1 h treatment with PDB resulted in almost a 5-fold increase the amount of c-fos mRNA (Fig. 6B).

Since I was not able to detect jun B and jun D mRNA by northern blotting, semi-quantitative RT-PCR was used to examine the expression of these jun family members (Fig. 7). These genes were expressed in B16 cells, but after ensuring a linear signal (by varying the number of PCR cycles) and correcting for β-actin levels, I found that 24 or 48 h of RA treatment did not change junB or jun D mRNA levels.

Figure 6. **The effect of RA and PDB on c-jun and c-fos mRNA level in B16 cells.**

B16 cells were cultured and treated with 10 μM of RA for 24 and 48 h or 1 μM PDB for 1 h. Cells were washed twice with ice-cold phosphate-buffered saline solution and RNA was isolated by a single step method. RNA (25 μg) from each treatment group was analyzed for c-jun (Fig. 6A) and c-fos (Fig. 6B) expression by northern blotting. Blots were stripped and reprobed with GAPDH as an internal control. Time of the blot being exposed to X-ray film: A, 1 day; B, 3 days. The relative amount of c-jun and c-fos mRNA was determined by computerized densitometric analysis of the autoradiogram correcting for the amount of GAPDH.

## Figure 6A

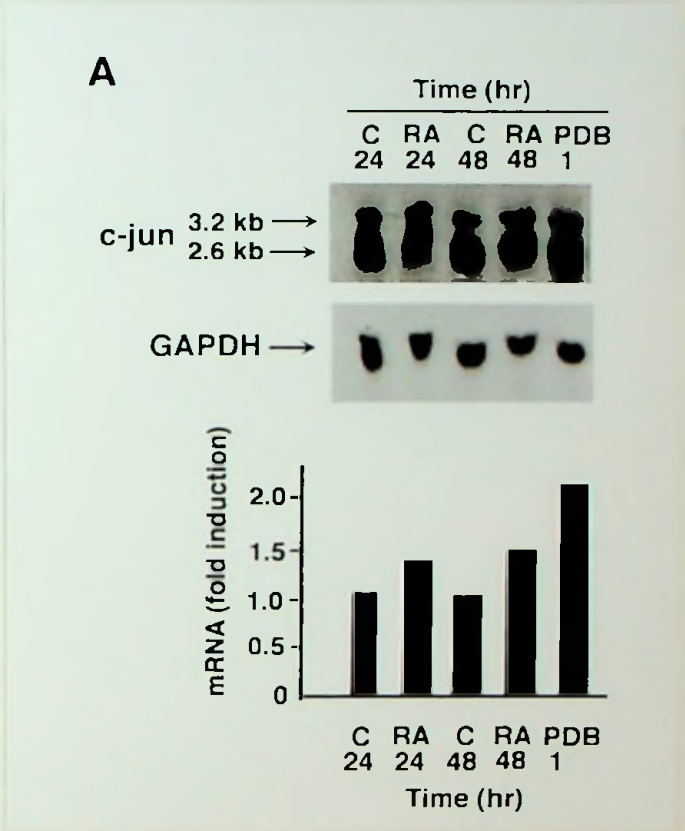

A
Time (hr)
C 24
RA 24
C 48
RA 48
PDB 1
c-jun
3.2 kb
2.6 kb
GAPDH
mRNA (fold induction)
2.0
1.5
1.0
0.5
0
C 24
RA 24
C 48
RA 48
PDB 1
Time (hr)

**Figure 6B**

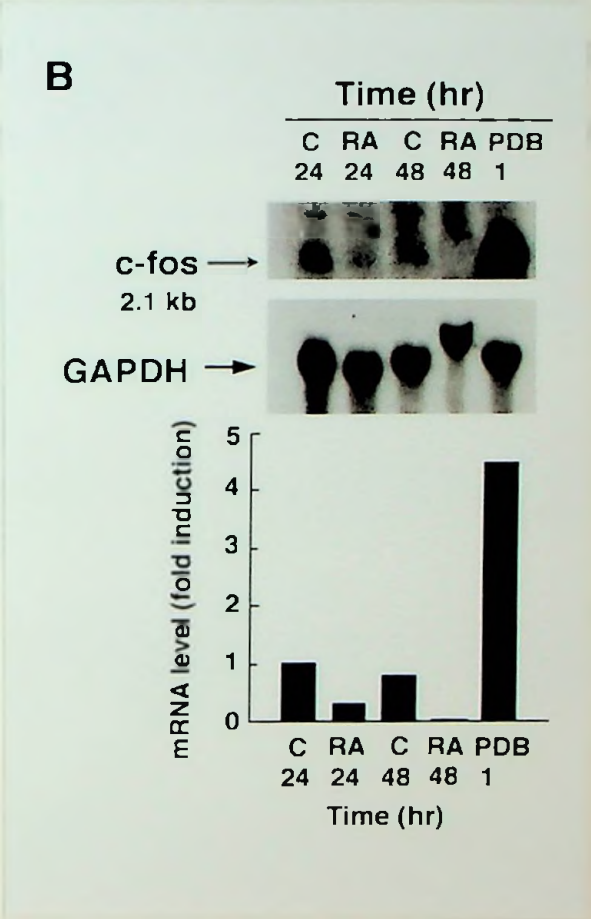
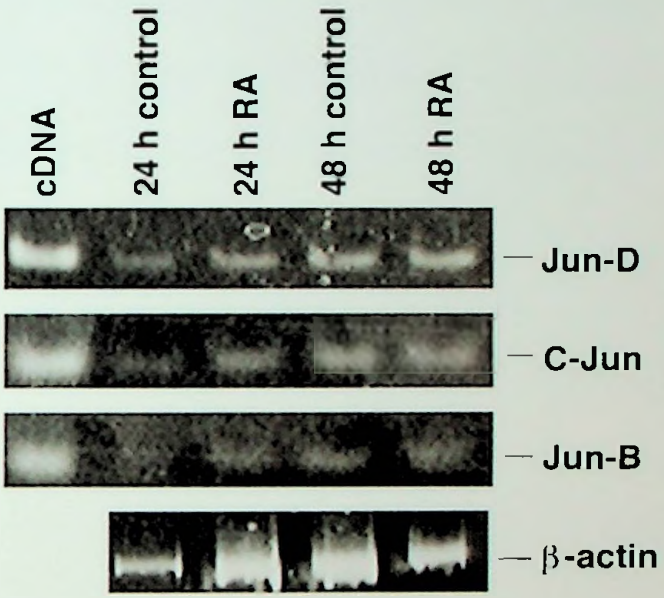

B
Time (hr)
C 24
RA 24
C 48
RA 48
PDB 1
c-fos
2.1 kb
GAPDH
mRNA level (fold induction)
0
1
2
3
4
5
C 24
RA 24
C 48
RA 48
PDB 1
Time (hr)

**Figure 7.** **Detection of jun B and jun D by RT-PCR.**

Cells were treated with 10 μM RA for 24 and 48 h and RNA was isolated from each sample as described in methods and materials. RT-PCR was performed as described in methods and materials. cDNAs for c-jun, jun B and jun D were used as positive controls for PCR. β-actin was used as internal control. Samples were visualized on 5% polyacrylamide gel stained with ethidium bromide.

**Figure 7**


cDNA
24 h control
24 h RA
48 h control
48 h RA
Jun-D
C-Jun
Jun-B
β-actin

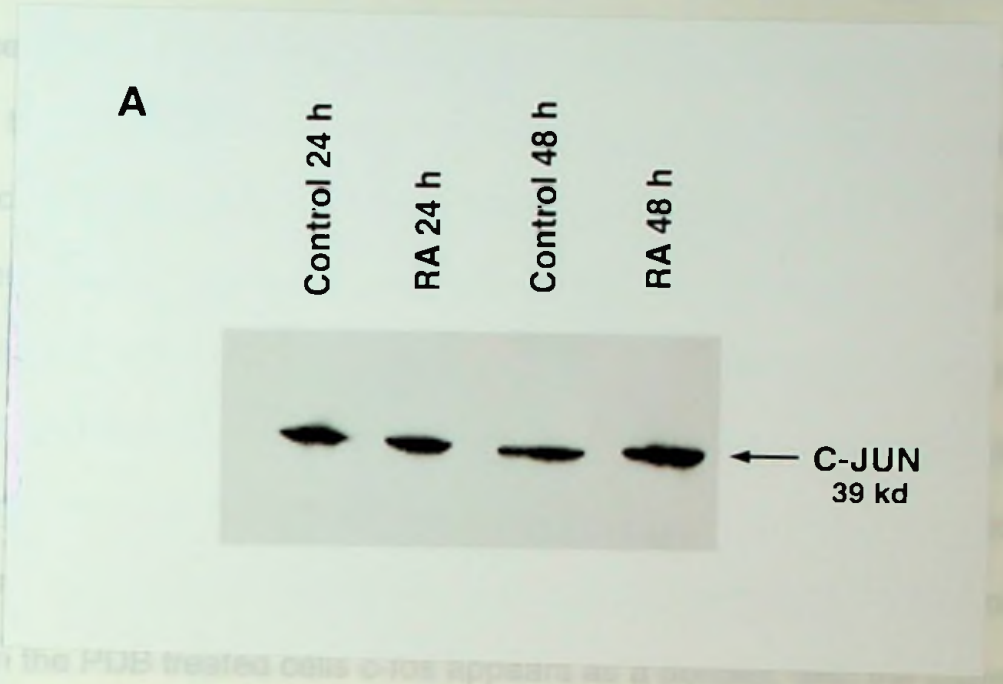
**Effect of RA on c-jun and c-fos protein level**

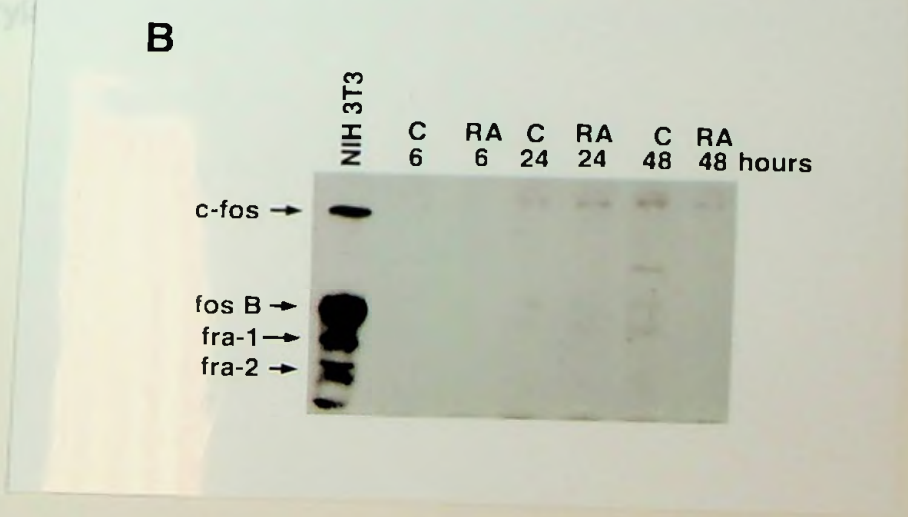
To determine if the changes in RNA levels found above were reflected in changes in the amount of c-jun or c-fos protein, B16 cells were treated for different times with RA and the amounts of immunoreactive proteins were examined by western blotting. B16 cells were found to contain a 39 kD c-jun immunoreactive protein. The amount of this protein was quite similar in both control and RA-treated cells at 24 and 48 h time points (Fig. 8A). The antibody used to detect c-fos also recognizes fos B as well as fra-1 and fra-2 (Fig. 8B - NIH 3T3 cell extract used as a control). In control cells no signal was detected at the 6 h time point, but these cells had increasing amounts of c-fos protein at 24 and 48 h of incubation. B16 cells treated for 48 h with RA had a markedly decreased amount of c-fos protein compared to the control cells at the same time point (Fig. 8B). The expression of other fos family members was barely detectable in nuclear extracts from B16 cells.

**Figure 8.** **Effect of RA on c-jun and c-fos protein level.**

B16 cells were treated with 10 μM RA for different time points (6, 24, 48 h) and nuclear extracts were prepared. 50 μg of nuclear extract was used to separate nuclear proteins on 10% SDS-PAGE. Western blot analysis for c-jun (Fig. 8A) and c-fos (Fig. 8B) was done by using anti-jun (Ab-2, Oncogene Science) and anti-fos antibody (Ab-2, Oncogene Science), respectively. Proteins were visualized by enhanced chemiluminescence.

Figure 8


A
Control 24 h
RA 24 h
Control 48 h
RA 48 h
C-JUN
39 kd


B
NIH 3T3
C 6
RA 6
C 24
RA 24
C 48
RA 48
hours
c-fos
fos B
fra-1
fra-2

**Time course study of c-fos induction by PDB**

Since PDB induced a large increase in c-fos mRNA, I examined the time course for this induction at both the RNA and protein level in more detail. PDB increased c-fos mRNA by 4-fold at one hour of treatment (Fig. 9A). This increase was transient and returned to the unstimulated level by 12 h despite the continued presence of PDB. Similar to the induction of RNA, PDB also increased the level of c-fos protein within 2 h of treatment (Fig. 9B). The increase in protein was also transient and returned to unstimulated level by 12 h. Fos B and fra-1 proteins were also induced by PDB, but the rate of increase was slower than that for c-fos (Fig. 9B). In the PDB treated cells c-fos appears as a doublet, with the lower band decaying at a faster rate than the upper band. It is possible that the upper band is phosphorylated c-fos whereas the lower band is unphosphorylated c-fos protein.

Figure 9. **Time course study of c-fos induction by PDB in B16 cells**

**A.** Control cells (0 h) or cells treated with 1 μM PDB for different periods of time (0.5, 1, 1.5, 3, 12 h) were harvested, RNA was isolated, and 20 μg/lane of total RNA was subjected to northern blot analysis to determine the amount of c-fos mRNA at each time point. The blot was stripped and reprobed with GAPDH as an internal control. The autoradiogram in the linear range was scanned using a Molecular Dynamics densitometer. C-fos signals were normalized to the GAPDH signals.

**B.** Cells were treated with PDB for different periods of time points (0, 2, 3, 4, 6, 8,12, and 24 h), nuclear protein was extracted, and 50 μg/ lane of nuclear protein was subjected to western blot analysis. The autoradiograms which were in the linear range were scanned using a Molecular Dynamics densitometer. The experiment was repeated several times with similar results.

Figure 9A

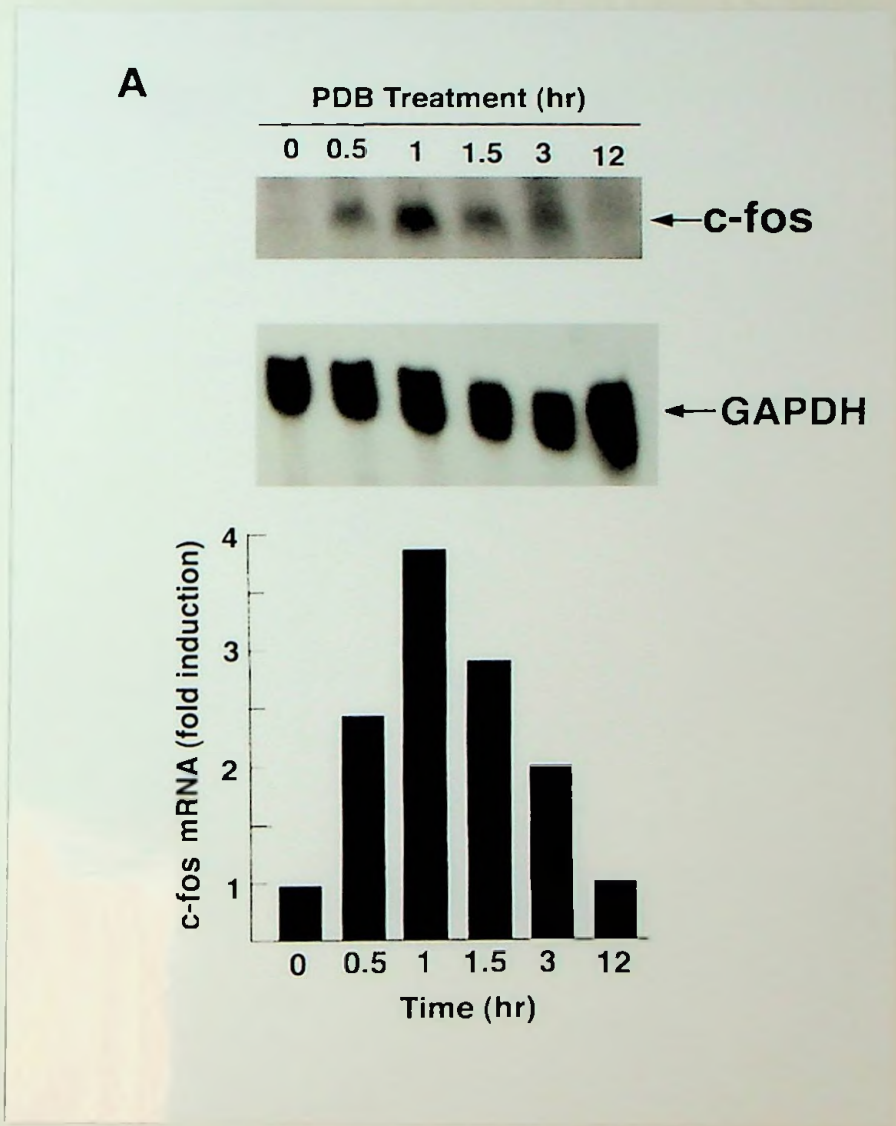
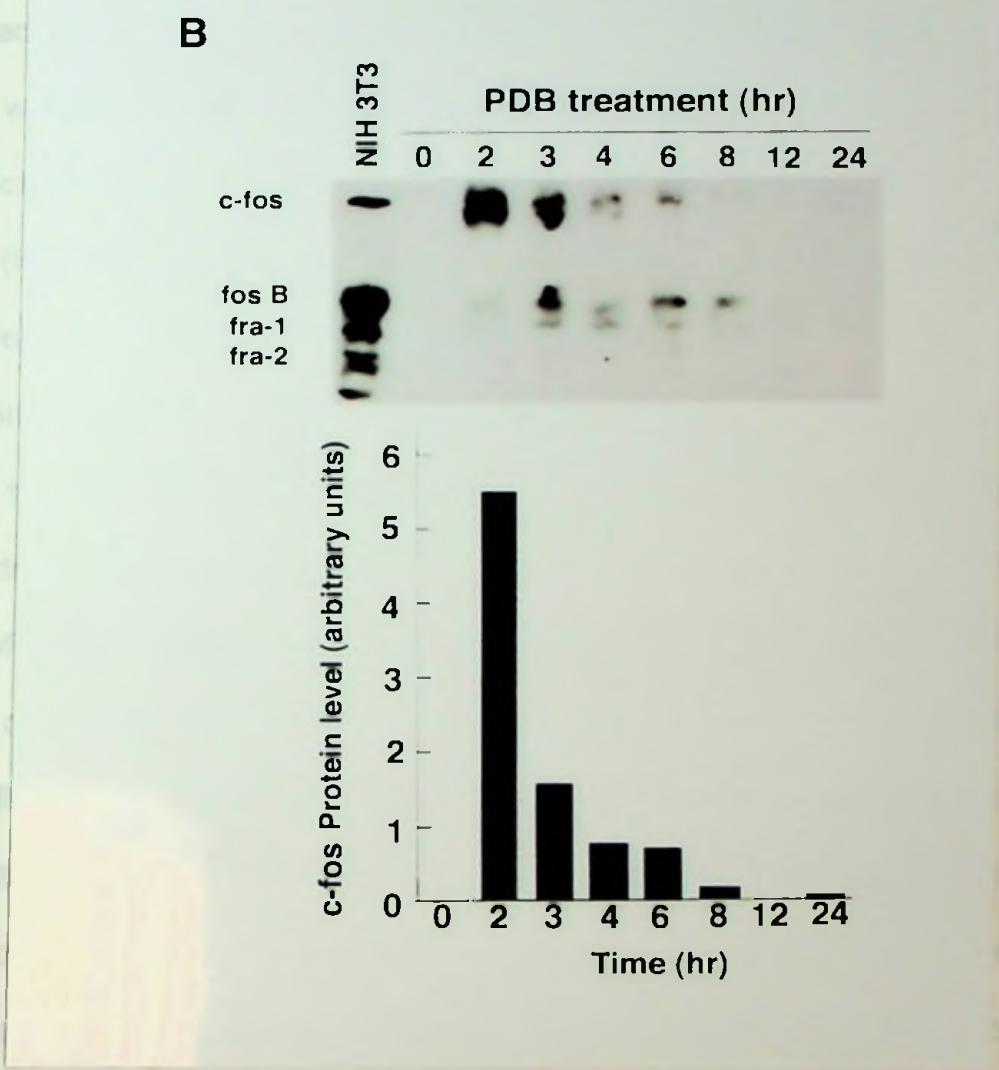

A
PDB Treatment (hr)
0 0.5 1 1.5 3 12
c-fos
GAPDH
c-fos mRNA (fold induction)
4
3
2
1
0 0.5 1 1.5 3 12
Time (hr)

Figure 9B


B
NIH 3T3
PDB treatment (hr)
0 2 3 4 6 8 12 24
c-fos
fos B
fra-1
fra-2
c-fos Protein level (arbitrary units)
0 1 2 3 4 5 6
0 2 3 4 6 8 12 24
Time (hr)

**AP- 1 binding to a DNA consensus site**

Since RA did not increase the amount of any of the AP-1 family members, I explored the possibility that an increase in binding activity to a consensus AP-1 site might account for the RA-induced increase in AP-1 transcriptional activity. A radiolabeled 15 bp oligonucleotide containing one AP-1 consensus site (TRE) was incubated with nuclear extracts from control, RA-treated or PDB-treated B16 cells. Using electrophoretic mobility shift assays, five protein-DNA complexes were observed (Fig. 10A). Complex 5 was absent when nuclear extracts from 24 or 48 h RA-treated cells were analyzed. Nuclear extracts from 48 h RA-treated cells were also missing complex 1, while the binding of complexes 2 and 4 was reduced compared to control cells (Fig. 10A). PDB treatment of B16 cells for 2 h resulted in nuclear extracts that gave a large increase in the intensity of complexes 1 and 2. As the time of PDB treatment was increased, nuclear extracts yielded a less intense signal for complex 1 and 2, but the intensity of complex 4 and 5 increased rather dramatically. Incubation of nuclear extracts with antibodies to c-jun did not change the intensity of any bands, nor were any bands supershifted. In contrast, incubation of the nuclear extracts with antibodies to c-fos decreased the binding activity of complexes 1 and 2 and also induced a formation of a supershifted complex. Competition experiments with non-radioactive wild type and mutant AP-1 oligonucleotides indicated the following sensitivity : complex 1 > complex 4 > complex 2 = complex 3 > complex 5. All 5 complexes were still present at 50-fold excess of the mutant oligonucleotide (Fig. 10B).

**Figure 10. Gel mobility shift analysis of AP-1 binding to the TRE from the collagenase gene**

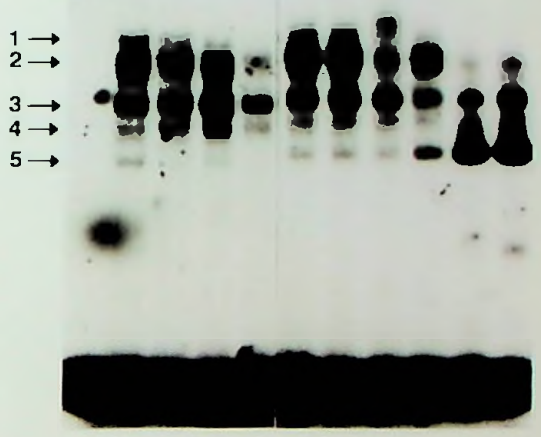
**A.** Ten microgram of nuclear protein from B16 cells treated with 10 μM RA (24 and 48 h), 1 μM PDB (2, 4, 6, and 24 h), or control cells was incubated with [$^{32}$P] TRE (3 x $10^4$ CPM) for 20 min at room temperature, resolved in a 5% non-denaturing polyacrylamide gel in 0.5X TBE, and examined by autoradiography. Some reaction mixtures were incubated for an additional 45 min with 2 μg of anti-c-jun ( lane 7) or anti-c-fos (lane 8) antibody (Santa Cruz).

**B.** 10, 20, or 50 fold excess of non radioactive wild type or mutant TRE oligonucleotide was added immediately after the incubation of radiolabelled oligonucleotide with nuclear extract.

## Figure 10A

A

| AP-1 (oligo) | + | + | + | + | + | + | + | + | + | + | + |
|---|---|---|---|---|---|---|---|---|---|---|---|
| B16 Ctrl 24 h | - | + | - | - | - | - | - | - | - | - | - |
| B16 RA 24 h | - | - | + | - | - | - | - | - | - | - | - |
| B16 Ctrl 48 h | - | - | - | + | - | - | - | - | - | - | - |
| B16 RA 48 h | - | - | - | - | + | - | - | - | - | - | - |
| B16 PDB 2 h | - | - | - | - | - | + | + | + | - | - | - |
| C-jun Ab | - | - | - | - | - | - | + | - | - | - | - |
| C-fos Ab | - | - | - | - | - | - | - | + | - | - | - |
| B16 PDB 4 h | - | - | - | - | - | - | - | - | + | - | - |
| B16 PDB 6 h | - | - | - | - | - | - | - | - | - | + | - |
| B16 PDB 24 h | - | - | - | - | - | - | - | - | - | - | + |

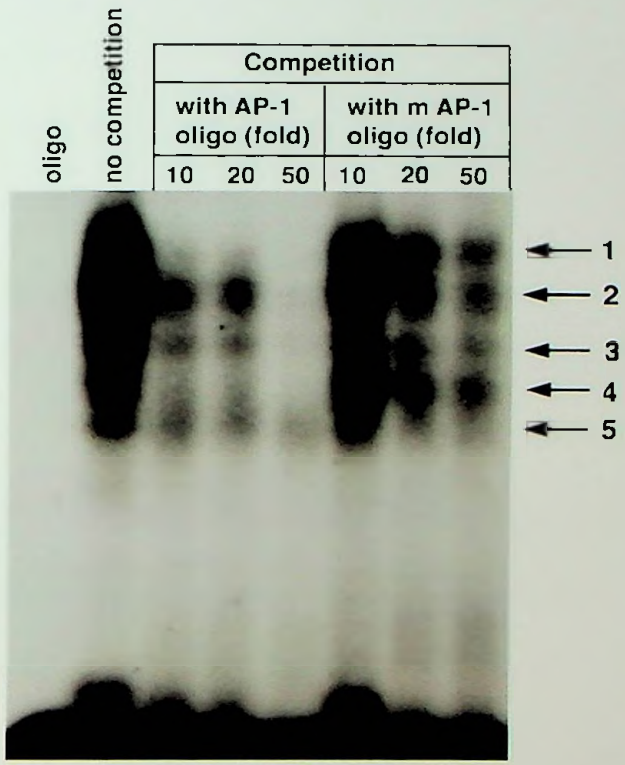


## Figure 10B

**B**

AP-1 oligo - 5' CGCATGAGTCAGACA 3'

mAP-1 oligo - 5' CGCAgGAGTCAGACA 3'



**Role of PKC in RA-induced AP-1 activity**

RA-induced differentiation of B16 cells is accompanied by a large increase in PKCα mRNA and protein. PDB, via its activation of PKC is known to increase AP-1 activity. Therefore I investigated whether PKC was required for the RA-induced increase in AP-1 activity in B16 mouse melanoma cells. B16 cells were transfected with the AP-1-PGL2 reporter plasmid described previously. Following transfection, cells were treated with 10 μM RA, 1 μM PDB (to down regulate PKC) or RA + PDB for 24 h. As a positive control, I also treated transfected cells with PDB for 24 h and during the last 2 h of incubation challenged them with fresh PDB. Fig. 11A shows that down regulation of PKC inhibited both the acute PDB-induced (lane 2 vs 5) and the RA-induced (lane 3 vs 6) increase in AP-1 activity. Western blot analysis (Fig. 11B) shows that RA induced PKCα, long-term PDB treatment depleted the cells of PKCα and RA + PDB-treated cells had PKCα levels slightly lower than control cells but higher than cells treated with PDB for 24 h.

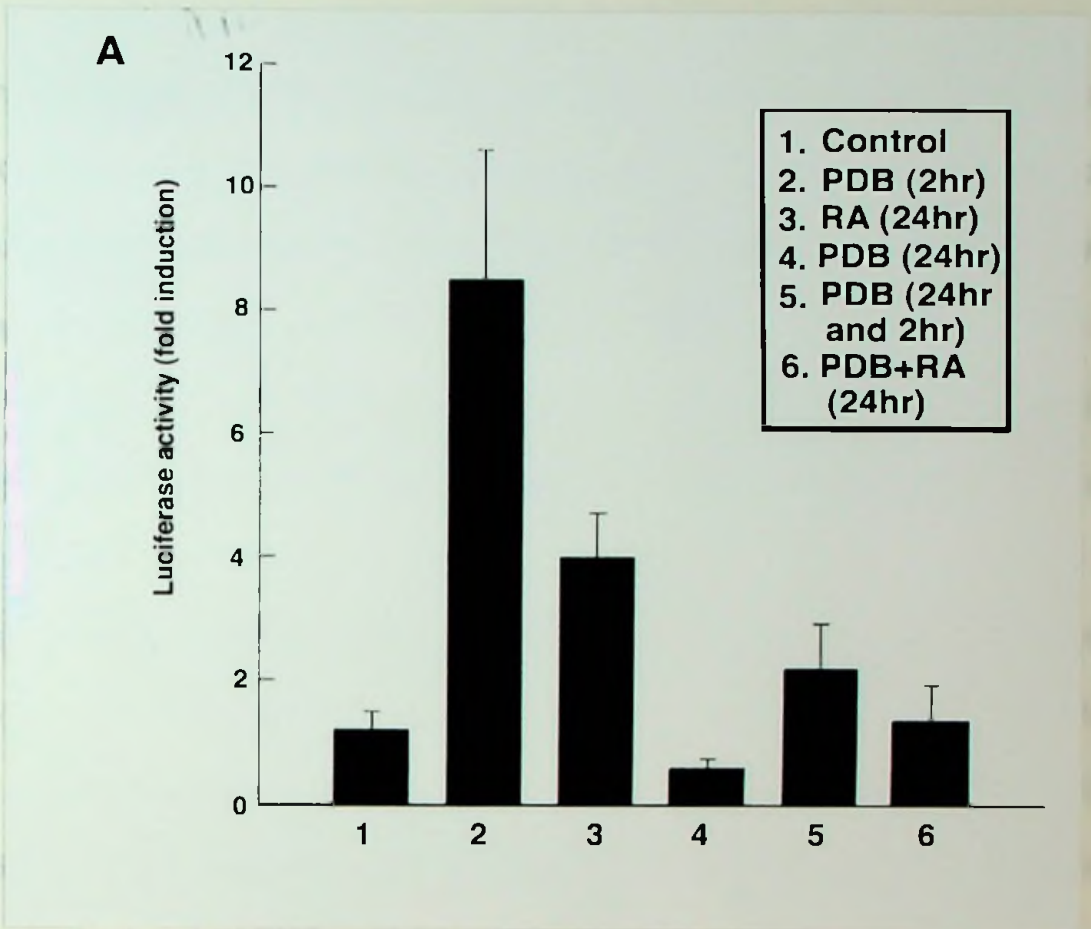
**Figure 11.** **A. Role of PKCα in RA-induced AP-1 activity**

B16-F1 cells were transfected with 4X AP-1-SV40-luc reporter gene and β-gal plasmid as described in methods and materials. After the transfection cells were treated with 1 μM PDB (2 or 24 h), 10 μM RA (24 h) and RA+PDB (24 h). Bar 5 represents the group in which cells were treated with PDB for 24 h and then refed with normal growth medium for 24 h. For the last two hours, cells were again treated with PDB. Cells were harvested and luciferase assay was performed as described in the methods and values were normalized to β-galactosidase activity. All transfections were performed in triplicate and the entire experiment was repeated four times. The data presented is the mean ± SEM (error bars) of triplicate dishes for each treatment group from a representative experiment.

**B. Regulation of PKC expression by RA and PDB**

Cells were treated with 10 μM RA, 1 μM PDB or RA+PDB for 24 h and total cell extracts were prepared. 75 μg of protein/lane was used for western blot analysis. Anti-PKCα monoclonal antibody (UBI) was used to detect the protein.

**Figure 11**


A
Luciferase activity (fold induction)
12
10
8
6
4
2
0
1
2
3
4
5
6
1. Control
2. PDB (2hr)
3. RA (24hr)
4. PDB (24hr)
5. PDB (24hr and 2hr)
6. PDB+RA (24hr)
B
control
RA
PDB
PDB + RA
PKC α

**Effect of bisindolylmaleimid on RA-induced AP-1 activity**

In a separate experiment we tested the effect of inhibition of PKC enzyme activity on the PDB and RA induction of AP-1 transcriptional activity. We found that PKC enzyme activity from B16 cells was reduced 90% by 0.1 μM bisindolylmaleimid and was not detectable at 1.0 μM concentration of this inhibitor (Fig. 12). B16 cells were transfected with the AP-1 reporter plasmid and then treated with RA, PDB, bisindolylmaleimid, or a combination of these compounds. Fig. 13A shows that while bisindolylmaleimid reduced the PDB-induced AP-1 activity from 11-fold down to 4-fold, the RA-induced AP-1 activity was further increased by the enzyme inhibitor (from 5-fold to 8-fold). It has recently been reported that once PKC is activated, it is more susceptible to protease degradation (177). If the enzyme activity is inhibited, then this protein should be less susceptible to degradation. To determine if changes in the level of PKCα might explain the RA + bisindolylmaleimide results, western blot analysis was performed on extracts from cells treated/untreated with 10 μM RA (24 h), 2.5 μM bisindolylmaleimide (24 h) or a combination of these two compounds. There was a small increase in PKCα protein level in inhibitor-treated cells compared to control. PKCα protein level was also somewhat increased in RA + inhibitor -treated cells compared to RA treated cells (Fig. 13B).

**Figure 12. Inhibition of PKC enzyme activity by bisindolylmaleimid**

Cells were harvested and lysed. PKC-enriched fractions were obtained by ion exchange chromatography. These fractions were further concentrated by microfiltration. Equal amounts of protein were assayed using a commercially available PKC assay system (Amersham) in the presence of cofactors phosphatidyl-serine and phorbol ester or in combination with different concentrations (0.1, 0.5 and 1 μM) of bisindolylmaleimid.

**Figure 12**

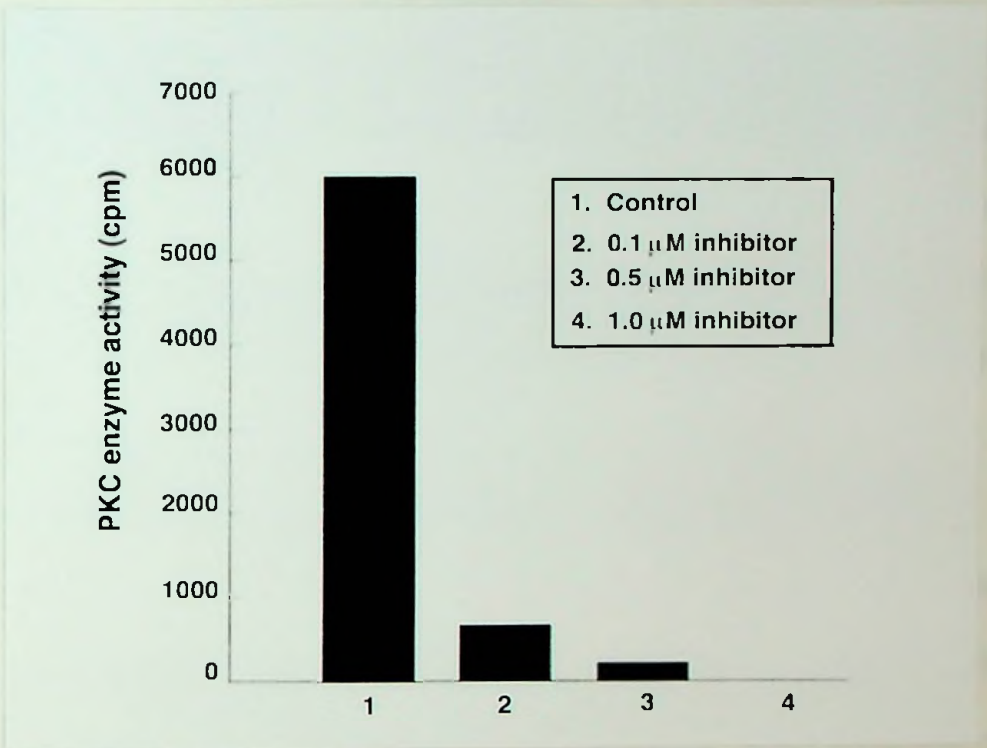

PKC enzyme activity (cpm)
7000
6000
5000
4000
3000
2000
1000
0
1
2
3
4
1. Control
2. 0.1 μM inhibitor
3. 0.5 μM inhibitor
4. 1.0 μM inhibitor

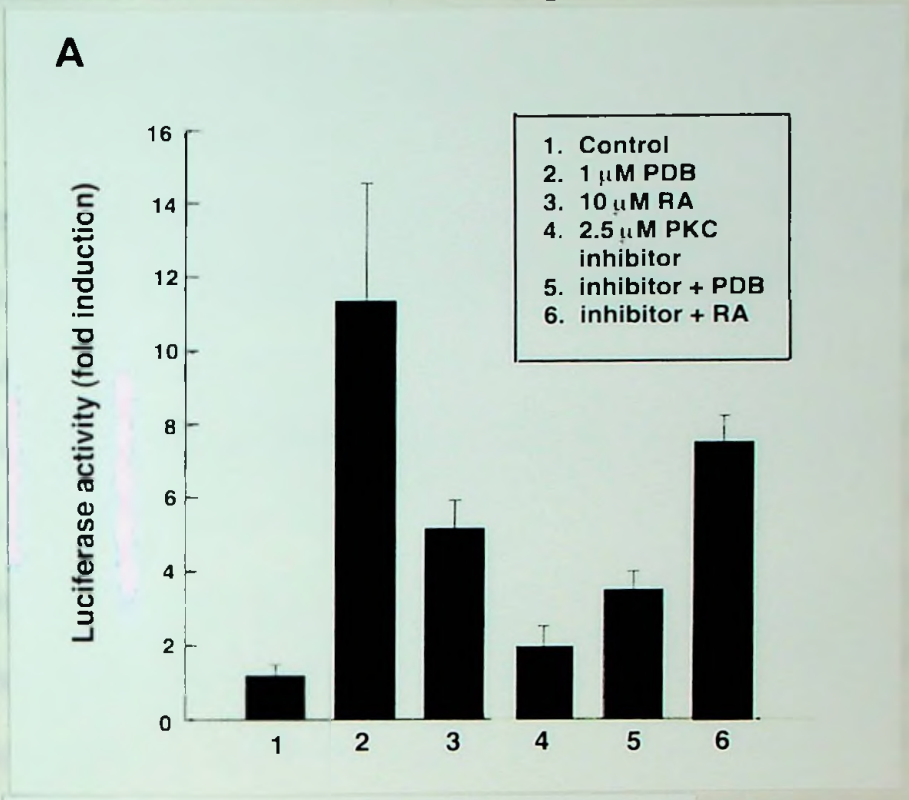
**Figure 13.** **A. Effect of bisindolylmaleimid on RA-induced AP-1 activity**

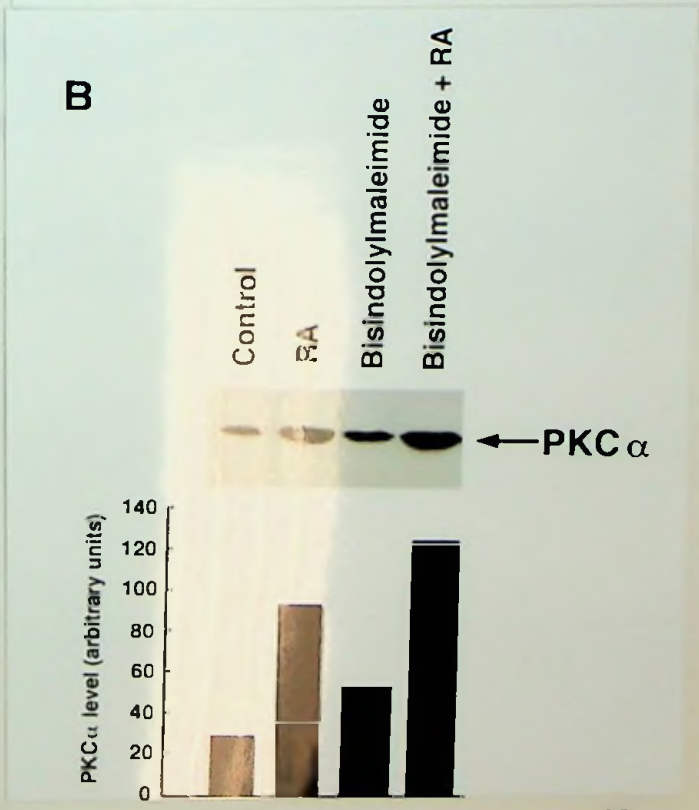
B16-F1 cells were transfected with 4X AP-1-SV40-luc reporter gene and β-galactosidase plasmid as described in methods and materials. After the transfection cells were treated with 1 μM PDB (2 h), 10 μM RA (24 h), 2.5 μM bisindolylmaleimid (24 h), + RA or bisindolylmaleimid + PDB. Cells were harvested and the luciferase assay was done as described previously and values were normalized to β-galactosidase activity. This experiment was performed in triplicate and the entire experiment was repeated four times. The data presented is the mean ± SEM (error bars).

**B. Effect of bisindolylmaleimide on PKCα protein level**

Cells were treated with 10 μM RA, 2.5 μM bisindolylmaleimide or in combination for 24 h and total cell extracts were prepared. 75 μg of protein/lane was used for western blot analysis. Anti-PKCα monoclonal antibody (UBI) was used to detect the protein.

Figure 13


A
Luciferase activity (fold induction)
16
14
12
10
8
6
4
2
0
1
2
3
4
5
6
1. Control
2. 1 μM PDB
3. 10 μM RA
4. 2.5 μM PKC inhibitor
5. inhibitor + PDB
6. inhibitor + RA


B
Control
RA
Bisindolylmaleimide
Bisindolylmaleimide + RA
PKCα
PKCα level (arbitrary units)
140
120
100
80
60
40
20
0

**Isolation of B16 clones expressing dominant negative c-jun**

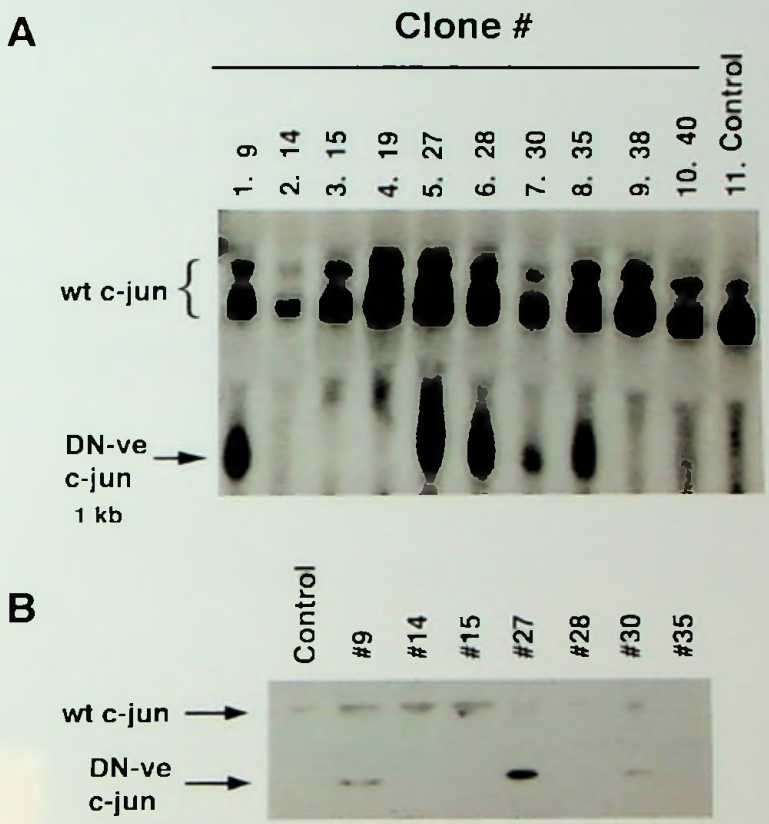
Since RA increases AP-1 transcriptional activity, it is important to study the role of c-jun in RA-induced B16 mouse melanoma differentiation. One approach would be to stably transfect a plasmid containing a dominant negative c-jun into B16 melanoma cells and determine the role of c-jun (by blocking its normal function) in B16 differentiation. I have used pCMV-67 plasmid (kindly provided by Dr. Birrer, NIH) that has amino acids 3-73 deleted in the c-jun cDNA. After transfection and selection in medium containing G418, about 30 clones were analyzed by northern blot analysis (Fig. 14A), and five positive clones were found. The dominant negative c-jun mRNA is about 1 kb in size. Using these five clones along with neo transfected clones and wild type cells, I found three clones that express dominant negative c-jun protein (~ 30 kd), whereas wild type c-jun is expressed in all the clones (Fig. 14B).

**Figure 14. Isolation of B16 clones expressing dominant negative c-jun**

**A.** B16 cells were stably cotransfected with pSVneo and pCMV-67 containing a dominant negative c-jun cDNA (first 3 to 73 amino acids deleted) driven by a CMV promoter. After treating these cells with G418 for 10 days, about 30 clones were selected. These clones were analyzed for c-jun mRNA by northern blot analysis.

**B.** B16 clones found positive by northern blot analysis along with several neo transfected clones were used for western blot analysis to detect the the expression of dominant negative c-jun protein. Cells were harvested, nuclear extracts were prepared and 50 µg /lane was used for western blot analysis using c-jun antiserum (Oncogene Science)

**Figure 14**


A
Clone #
1. 9
2. 14
3. 15
4. 19
5. 27
6. 28
7. 30
8. 35
9. 38
10. 40
11. Control
wt c-jun
DN-ve
c-jun
1 kb
B
Control
#9
#14
#15
#27
#28
#30
#35
wt c-jun
DN-ve
c-jun

**Effect of Retinoid Analogs on the Expression of PKCα Protein Level**

The purpose of this study was to determine the involvement of retinoic acid receptor subtypes (RARα, γ and RXRs) in RA-induced biochemical changes such as induction of PKCα (156) and increase in AP-1 transcriptional activity (72). In this study, six different retinoids were used: All-trans RA, 9-cis RA, AM580 (RARα specific) (40), SR11254 (RARγ specific) (40), SR11246 (RXR specific) (39), and SR11346 (RARβ/RXR specific) (unpublished).

B16 cells were treated with different concentrations ($10^{-9}$, $10^{-8}$, $10^{-7}$, $5x10^{-7}$, and $10^{-6}$M) of all-trans RA, 9-cis RA or all four of the above mentioned analogs for 48 h. At the end of the incubation, cells were harvested, total protein was extracted and 75 μg of total protein was subjected to electrophoresis. Proteins were transferred to nitrocellulose membranes and western blot analysis was carried out using PKCα antibody (UBI-monoclonal PKCα III).

All of the retinoids increased PKCα protein level in a dose-dependent manner but the potency of each compound and the level of induction was different (Fig. 15A and B). Out of all six compounds, 9-cis RA showed the highest fold induction (9 fold at $10^{-6}$ M) in the amount of PKCα protein whereas all-trans RA was the most potent retinoid (5 fold at $10^{-9}$ M). While comparing receptor-selective analogs, AM580 was the most potent analog since the lowest concentration ($10^{-9}$ M) increased the PKCα

protein level by 4 fold. 9-cis RA, SR11254, and SR11346 were effective only at higher concentrations ($10^{-6}$, $5x10^{-7}$, and $10^{-7}$M). At the lowest concentration ($10^{-9}$M), SR11246 gave approximately 3 fold induction. While comparing all the retinoids, the following potency was observed: All-trans RA (5-fold) > AM580 (4-fold) > SR11246 (3-fold) > SR11346 (1.5) ≥ 9-cis RA (1.5) > SR11254. Comparision of these retinoids for their maximum fold induction of PKCα, the following order was observed: 9-cis RA (9-fold) > all-trans RA (6.5-fold) > AM580 (6-fold) > SR11346 (5-fold) > SR11246 (4-fold) ≥ SR11254 (4-fold). Thus, AM580 is the most potent analog and RXR-specific analogs give moderate PKCα induction suggesting the involvement of RARα and RXR in RA-induced PKCα expression.

Combinations of these analogs were tested to detect any synergistic effect (Fig. 15 C). All-trans RA + SR11246 was the most effective combination suggesting that RXR when liganded and dimerized with RARs gives the highest PKCα induction, while RXR homodimer is ineffective (SR11246 + SR11346). The combinations of AM580 + SR11246, AM580 + SR11346 and SR11254 + SR11246 gave higher PKCα induction than any of these analogs alone while the combinations of AM580 + SR1124, SR11254 + SR11346 and SR11246 + SR11346 did not give significantly higher PKCα induction than each analog alone. These results suggest that RARα:RXR heterodimer is dominant over any other combinations used to induce PKCα expression. It also suggests that RARγ can also induce PKCα expression when dimerized with RXR. Moreover, this data clearly indicate that both the receptors in a dimer require ligand for the maximum induction of PKCα.

**Figure 15.** **A. Effect of receptor specific retinoid analogs on PKCα expression**

B16-F1 cells were cultured and treated with different concentrations ($10^{-9}$, $10^{-8}$, $10^{-7}$, 5 X $10^{-7}$, $10^{-6}$ M) of at-RA, 9-cis RA, AM 580 (RARα-specific), SR11254 (RARγ-specific), SR11246 (RXR-specific), or SR11346 (RARβ/RXR-specific) for 48 h. Cells were harvested and total cell extracts were prepared. 75 µg of total protein/lane was used for western blot analysis. Anti-PKCα monoclonal antibody (UBI) was used to detect the protein. The enhanced chemiluminescent method was used to visualized the bands. The experiment was repeated three times.

**B.** Autoradiogram developed in Fig. 15 A was quantitated with densitometry, using a Molecular Dynamics (Sunnyvale, CA) computerized densitometer and represented as a line graph for each compound used in the experiment.

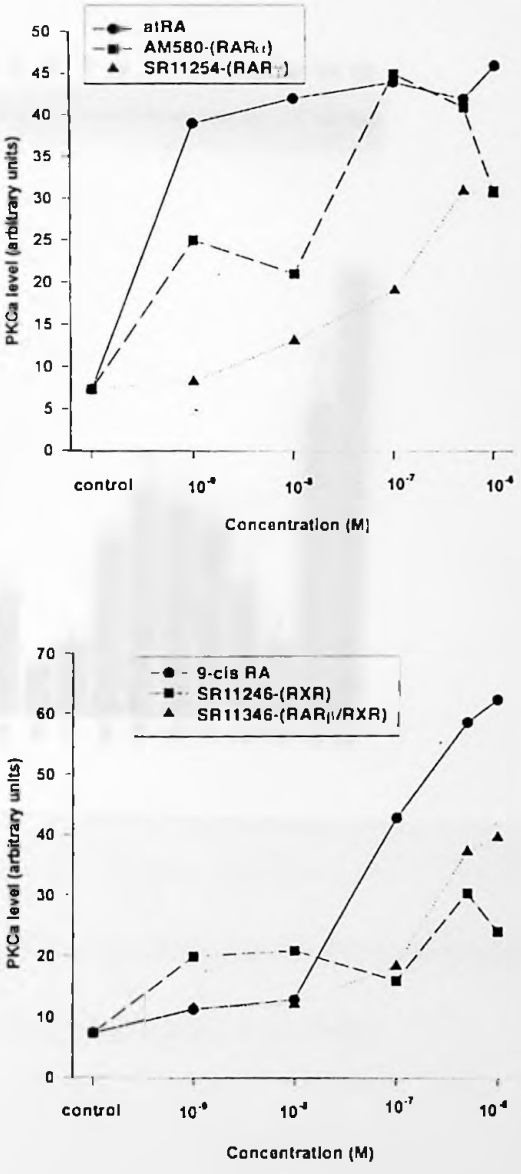
**C.** B16 cells were treated with either different analogs ($10^{-7}$ M concentration) alone or in combinations (final concentration $10^{-7}$ M) for 48 h. Cells were harvested and western blot analysis was done as described in legend 15 A. The experiment was repeated three times and autoradiograms were quantitated with densitometry. The bar graph with standard errors represents the average fold induction from 3 different experiments.

Figure 15A

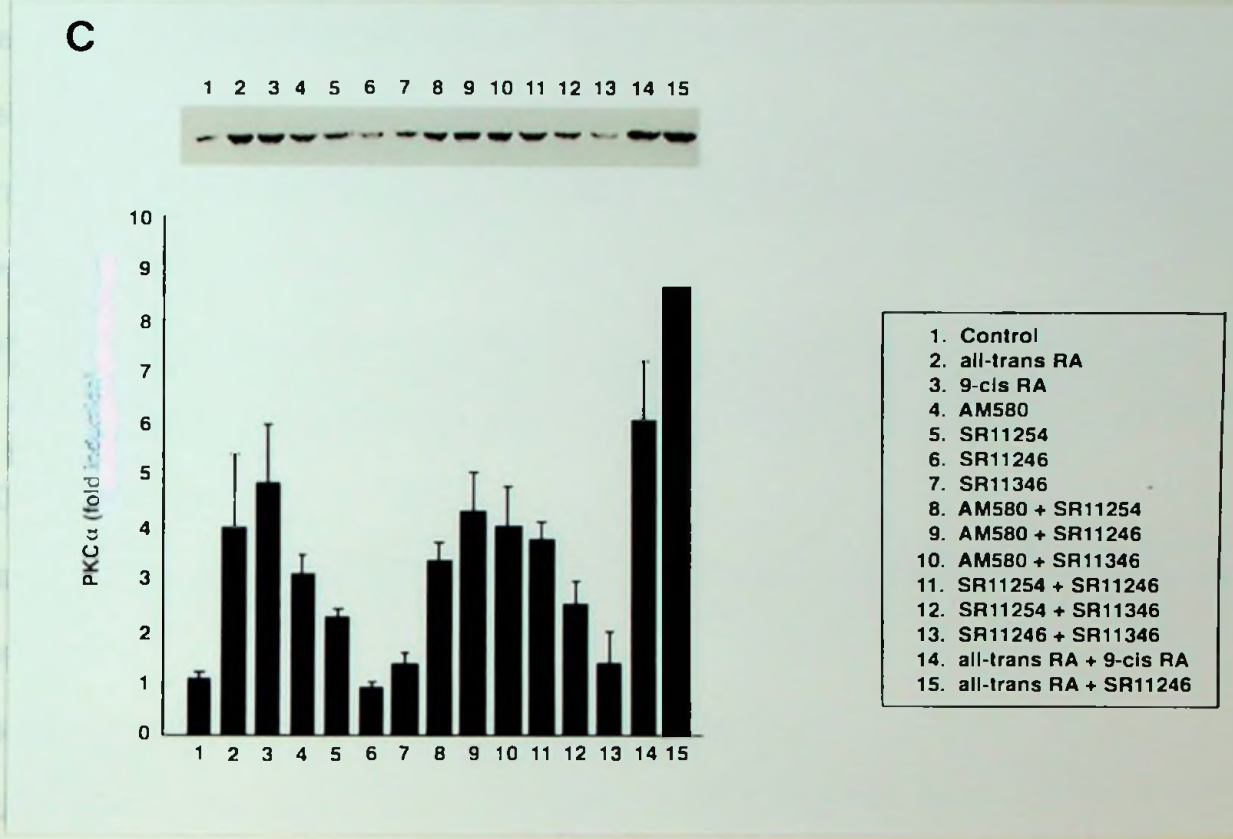

A
A-431 Human carcinoma
Control
10⁻⁹ M
10⁻⁸ M
10⁻⁷ M
5x10⁻⁷M
10⁻⁶ M
all-trans RA
AM580
SR11254
Control
10⁻⁹ M
10⁻⁸ M
10⁻⁷ M
5x10⁻⁷M
10⁻⁶ M
9-cis RA
SR11246
SR11346

## Figure 15B

B


atRA
AM580-(RARα)
SR11254-(RARγ)
PKCa level (arbitrary units)
Concentration (M)
control
9-cis RA
SR11246-(RXR)
SR11346-(RARβ/RXR)
PKCa level (arbitrary units)
Concentration (M)
control

## Figure 15C


C
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
PKC α (fold induction)
0 1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
1. Control
2. all-trans RA
3. 9-cis RA
4. AM580
5. SR11254
6. SR11246
7. SR11346
8. AM580 + SR11254
9. AM580 + SR11246
10. AM580 + SR11346
11. SR11254 + SR11246
12. SR11254 + SR11346
13. SR11246 + SR11346
14. all-trans RA + 9-cis RA
15. all-trans RA + SR11246

**Effect of retinoid analogs on AP-1 Transcriptional Activity**

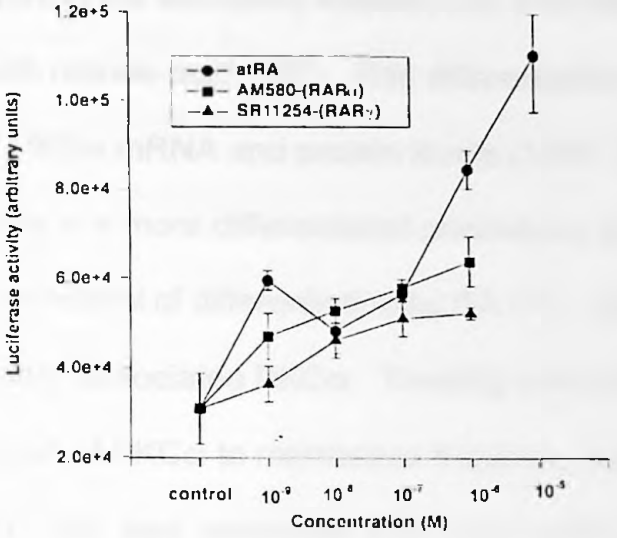
Since all-trans RA increased AP-1 transcriptional activity, retinoid analogs were also tested for their effect on AP-1 activity. Cells were transfected as described before and treated with different concentrations of all six retinoids for 24 h. At the end of the time point, cells were harvested and luciferase activity measured. The experiment was set up in triplicate and repeated three times. All of the analogs induced a dose dependent increase in AP-1 transcriptional activity (Fig.16).

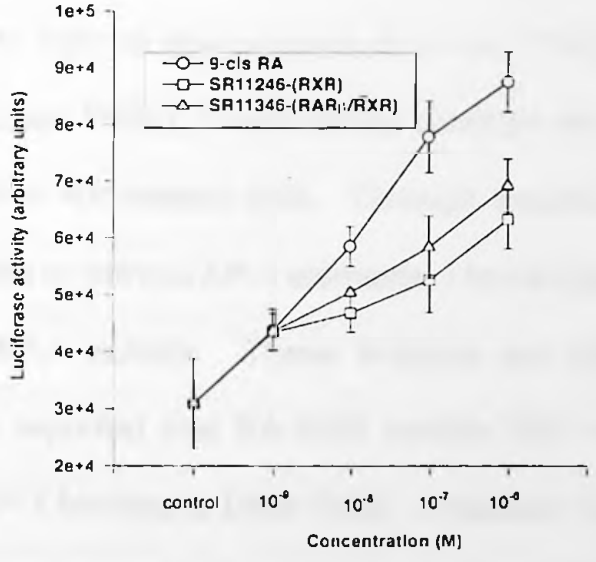
All-trans RA and 9-cis RA increased AP-1 transcriptional activity by 3 fold at $10^{-6}$ M concentration. Among the receptor-selective analogs, AM580 and SR11346 gave the maximum induction (2.2-fold at $10^{-6}$ M). SR11246 gave about 2-fold induction while SR11254 was the least effective (1.7-fold at $10^{-6}$ M). The potency of these retinoids at $10^{-9}$ M concentration was as follows: All-trans RA > AM580 > 9-cis RA $\geq$ SR11246 $\geq$ SR11346 >SR11254. The maximum induction of AP-1 activity by these compounds was as follows: All-trans RA $\geq$ 9-cis RA > SR11346 $\geq$ AM580 > SR11246 > SR11254. The results suggest that retinoid-mediated increase in AP-1 transcriptional activity involved RAR$\alpha$ and RXR.

**Figure 16. Effect of retinoid analogs on AP-1 transcriptional activity**

B16-F1 cells were transfected with a 4X AP-1-SV40-luc reporter gene and β-galactosidase plasmid. Cells were treated with different concentrations of retinoids for 24 h. Cells were harvested and luciferase activity measured as described previously. Values were normalized to β-galactosidase activity. Each treatment was given in triplicate and the entire experiment was repeated three times. The data presented is the mean ± SEM (error bars) of the triplicate dishes for each treatment group from a representative experiment.

## Figure 16


1.2e+5
1.0e+5
8.0e+4
6.0e+4
4.0e+4
2.0e+4
Luciferase activity (arbitrary units)
atRA
AM580-(RARα)
SR11254-(RARγ)
control
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
Concentration (M)


1e+5
9e+4
8e+4
7e+4
6e+4
5e+4
4e+4
3e+4
2e+4
Luciferase activity (arbitrary units)
9-cis RA
SR11246-(RXR)
SR11346-(RARβ/RXR)
control
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
Concentration (M)

# Discussion

Previous work in the laboratory showed that B16 melanoma cells differentiate upon treatment with retinoic acid (157). This differentiation process is accompanied by an increase of PKCα mRNA and protein levels (156). Overexpression of PKCα in these cells results in a more differentiated phenotype, suggesting the importance of this protein in the control of differentiation by RA (73). RA also induces a selective enrichment in nuclear-associated PKCα. Treating cells with an active phorbol ester induced translocation of PKCα to membrane fractions, but had no effect on nuclear PKCα levels (72). RA also increases PKC enzymatic activity in intact cells as determined by phosphorylation of the the PKC-specific endogenous substrate MARCKS (72). In light of the increase in *in situ* PKC enzyme activity and the enrichment of nuclear PKCα, I determined whether AP-1 activity and expression might be changed in RA-treated cells. Through transient transfection of reporter gene constructs with or without AP-1 elements, I found that RA induced a four to five fold increase in AP-1 activity. These findings are controversial since various laboratories have reported that RA-RAR inhibits AP-1-dependent reporter gene expression and AP-1 binding to DNA (205). However, it has recently been shown that RAR transactivation and AP-1 transrepression can be dissociated by certain retinoid analogs (54, 147). Treatment of F9 teratocarcinoma cells with retinoids

which had predominantly AP-1 transrepression activity did not induce differentiation, although there was some growth inhibition (54). Also the RA induction of F9 teratocarcinoma cell differentiation is accompanied by a large increase in AP-1 transcriptional activity (230). It is possible that, in B16 cells with their milieu of nuclear proteins/ transcription factors, the transactivation function of RA-RAR is dominant over the AP-1 transrepression function. Although it is possible that RA increases AP-1 activity independently of its induction of PKCα, the working hypothesis is that the increase in PKCα (especially nuclear-associated) subsequent to RA treatment of B16 melanoma cells leads to the increase in AP-1 activity. In F9 teratocarcinoma cells, overexpression of PKCα was sufficient to rapidly induce the expression of c-jun mRNA in response to differentiation medium (96). The expression of fos and jun was induced during differentiation of M1 leukemic myeloblasts (119). In embryonal carcinoma (EC) cells c-jun expression and TRE binding activity were strongly enhanced by RA (42). Moreover, ectopic expression of c-jun leads to differentiation of P19 EC cells in the absence of RA (46), suggesting the role of c-jun in RA-induced EC cell differentiation. It has been shown that the sequences located between -329 and -293 of the c-jun promoter are responsible for the observed RA effect (45). These protein binding sites do not resemble RA-responsive elements (RARE's). Moreover, there is an evidence that RA induced expression of c-jun is probably mediated by an indirect effect of the RARβ (45).

Since RA increased AP-1 transcriptional activity, I studied the mechanism by which RA altered this activity. Northern blot, western blot, and RT-PCR analysis were performed to study the expression of jun and fos family members in control and RA-treated cells. B16-F1 mouse melanoma cells expressed c-jun, jun B, jun D, and c-fos. Fos B and Fra-1 are barely detected by western blot analysis. More sensitive methods such as RT-PCR may be necessary to determine if these cells express fra-2. The amount of c-jun present in B16 mouse melanoma cells is very high compared to c-fos. The short half life of c-fos compared to c-jun may account for this difference. RA did not increase the expression of fos or jun family members, but instead decreased the expression of c-fos mRNA and c-fos, fos B, and fra-1 proteins. Busam et al. (27) previously reported that RA decreased mitogen induction of c-fos mRNA. Induction of c-jun mRNA was also suppressed, but required higher concentrations of RA and a longer period of incubation. Englaro et al. (49) has shown that the MAP kinase pathway and AP-1 are activated during cAMP-induced melanogenesis in B16 cells but the role of p44MAPK and that of AP-1 in the regulation of melanogenesis remains to be proven. It is also known that RA blocks c-fos induction by inhibiting transcription. This inhibition of transcription occurs through the serum response element (SRE), since the SRE alone was sufficient to confer down-regulation by RA to a minimal c-fos promoter construct (28). Thus, the SRE plays a critical role in the suppression of c-fos transcription by RA. Perez et al.

(183) showed that the repression of c-fos gene expression by triiodothyronine (T3) and RA appears to be exerted through transcriptional interference with the SRE and the AP-1 binding site of the promoter. Since mRNA for jun B and jun D were not detected by northern blot analysis, RT-PCR was performed. The amounts of these messages were not altered by RA treatment of B16 cells (Fig. 7).

Acute PDB treatment consistently induced c-jun mRNA level by 1.8 to 2 fold (Fig.6A) whereas c-fos expression was induced by about 5-fold (Fig. 6B). PDB-induced c-fos expression was much higher than c-jun expression so I performed a time course study on c-fos mRNA and protein expression subsequent to PDB treatment. The maximum c-fos mRNA induction was observed at 1 h and the message was back to control levels by 12 h (Fig. 9A). Western blot anlaysis for c-fos protein induction by PDB correlates very well with northern blot analysis. There was an induction of c-fos protein at 2 h which returned to control levels by 12 h. Moreover, two bands were observed for c-fos. The top band could be the phosphorylated c-fos whereas the bottom band could be the unphosphorylated c-fos protein. Interestingly, PDB also increased the expression of fos B and fra-1 (Fig. 9B). This induction occured at a later time point than c-fos. The maximum induction was observed at 3 h which decreased to control level by 24 h. These data suggest a difference in regulation of c-fos, fos B and fra-1 by PDB. The expression of fra-2 in B16 cells and its induction by PDB need to be confirmed by more sensitive methods such as RT-PCR. Delayed expression of fra-1 and fra-2 with serum

stimulation has previously been reported. A possible explanation may lie in the observation that c-fos can transactivate the fra-1 and fra-2 genes (231). These data suggest that the RA-induced increase in AP-1 transcriptional activity may not involve a c-jun/c-fos form of the AP-1 transcriptional complex. In contrast, PDB may increase AP-1 activity by increasing the expression of c-fos thus producing more stable c-jun:c-fos heterodimers.

Since an increase in members of the AP-1 transcription complex could not explain the RA-induced increase in AP-1 transcriptional activity, I examined the possibility that RA altered AP-1 DNA binding activity. Using a consensus oligonucleotide from the AP-1 site in the collagenase gene and nuclear proteins from untreated B16 cells, five specific protein-DNA complexes were observed (Fig. 10A). Instead of enhancing binding, RA inhibited the appearance of complex number five and with longer times of incubation (48 h) also inhibited the appearance of complex number two. Also the intensity of complexes three and four was reduced by a 48 h treatment of the B16 cells with RA. Short-term (2 h) treatment of cells with PDB increased the intensity of complexes one and two, but with longer times of treatment this change was reversed and the intensity of complexes four and five dramatically increased. Since a c-fos antiserum diminished the intensity of complex two and caused a "supershift" of complex one, I conclude that complexes one and two contain fos family members. A variety of c-jun antisera failed to diminish the appearance of any complexes or to supershift any complex. Some of these same

antisera successfully recognized c-jun on western blots. Since EMSA is conducted under non-denaturing conditions, one explanation of these results is that the epitope recognized by the antibodies is unavailable under the EMSA conditions. Alternatively, c-jun may not be involved in AP-1 complexes using our assay conditions (B16 melanoma nuclear extracts, specific AP-1 oligonucleotide). The acute PDB-induced increase in complex two and its decrease in RA-treated cells probably reflect a change in the expression of AP-1-associated transcription factors regulated through the PKC pathway since long term PDB treatment down regulates PKC. Overall, these results suggest that the RA-induced increase in AP-1 transcriptional activity cannot be explained by an increase in binding activity.

Competition analysis (Fig. 10B) using wild type or mutant non-radioactive AP-1 oligonucleotides suggest that all the complexes are specific for the AP-1 binding site. Wild type AP-1 oligonucleotide (50-fold excess) completely inhibited the binding of the first four complexes whereas the binding of the 5th complex was inhibited in a less effective manner. Mutant oligonucleotide did not compete for binding at 10-fold excess. At 20- and 50-fold excess the binding was competed but not to the same extent as the wild type oligonucleotide competitor.

To study the role of PKC in RA induced AP-1 transcriptional activity, two different strategies were used : 1) Down regulation of PKCα protein level by chronic PDB treatment and 2) Inhibition of PKC enzyme activity by bisindolylmaleimid treatment. Chronic PDB (24 h treatment) inhibited RA-induced expression of PKCα

protein level (Fig. 11B). Thus, PDB antagonizes the action of RA. The current hypothesis for the down regulation of PKCα by chronic PDB is that activation is followed by rapid degradation. When cells are treated with PDB, there is a transient increase in PKC enzyme activity. The major receptor for PDB is PKC. It binds to the C1 region of the PKC molecule and changes the conformation which allows PKC to be activated. However, the V3 region of PKC (which is very susceptible to protease action) is now exposed and is more susceptible to protease degradation. Thus, transient activation leads to the degradation of PKC.

When the cells are transfected with 4X AP-1-luc reporter construct and treated with PDB (24 h), we found that down-regulation of PKC through chronic PDB treatment inhibited both the acute PDB and RA-induced increase in AP-1 transcriptional activity (Fig. 11A). This suggests that PKCα protein plays a role in RA-induced AP-1 transcriptional acitvity.

Bisindolylmaleimid is a specific and potent PKC inhibitor. *In vitro*, it inhibits PKC enzyme activity at as low as 0.1 μM concentration (Fig. 12). When the cells were treated with inhibitor, a higher concentration (2.5 μM) was used since the penetration ability and metabolism into an inactive form inside the cell may limit the actual concentration that reacts with PKC. To determine whether PKC enzyme activity is required for RA-induced AP-1 transcriptional activity, transfected cells were treated with bisindolylmaleimid. Surprisingly, there was 60% increase in basal AP-1 activity (Fig.13A). Moreover, bisindolylmaleimid treatment had an additive effect on

the RA-induced AP-1 activity while inhibiting acute PDB stimulation of AP-1 transcriptional activity by 60%. These data suggest that PKC protein, but not the PKC enzyme activity is required for the RA-induction of AP-1 activity. As expected, inhibitor suppressed PDB-induced AP-1 activity again suggesting that PKC enzyme activity is required for PDB-induced AP-1 activity. Bisindolylmaleimid binds to the C3 region (ATP binding site) of PKC and prevents autophosphorylation of the molecule. It is also suggested that autophosphorylation is required for maturation and activation of PKC (181). Once the PKC is activated, it would be rapidly degraded. When the cells are treated with inhibitor, PKC becomes inactive and is less sensitive to protease degradation.

Cells treated with inhibitor showed about 60% increase in PKCα protein level (Fig. 13B). Inhibitor treatment had an additive effect on RA-induced PKCα expression. These data suggests that the mechanisms by which RA and inhibitor induce PKCα are different. It is known that RA induces PKCα expression at transcriptional and posttranscriptional levels (probably mRNA processing or transport of mRNA from nucleus), whereas inhibitor may induce PKCα levels just by preventing protein degradation. The increased level of PKCα protein by RA and/or inhibitor may be responsible for increased AP-1 transcriptional activity. These data provide evidence for a structural (non-enzymatic) role of PKCα. It has been reported that PKC stimulates phospholipase D activity through a non-enzymatic mechanism (208). Also PKC binds to proteins other than substrates (48). Borchardt et al.

(unpublished data) have suggested that regions within the regulatory domain of PKC can regulate gene expression independent of kinase activity. These data suggest that activation of PKC enzyme activity (via PDB) increases AP-1 activity by a different pathway than that induced by RA and that the two pathways are antagonistic to each other. This might provide a molecular explanation for the antagonist action of phorbol esters and RA on B16 melanoma growth and differentiation (127, 156). Moreover, PDB-induced AP-1 activity is transient, while the RA-induced activity is prolonged. This could allow different sets of genes to be activated resulting in different biological phenotypes induced by these different agents in B16 mouse melanoma cells.

**Study of receptor specific retinoid analogs**

The last part of the dissertation includes the effect of retinoid analogs on PKCα expression and AP-1 activity. PKCα expression was induced by all the analogs used in this study but the potency of each compound was different (Fig. 15A and B). All-trans RA and 9-cis RA act as pan-agonists meaning that they activate both RARs (α,β, and γ) and RXRs (α,β, and γ) (all-trans RA can isomerize to 9-cis RA). All-trans RA was the most potent whereas 9-cis RA gave the maximum induction (9-fold induction at $10^{-6}$ M concentration). In comparing the receptor selective analogs, AM580 was the most potent and also gave the maximum

induction. These data suggest that RARα plays a major role in RA-induced PKCα expression. Moreover, 9-cis RA which activates both RARs and RXRs gave the maximum induction of PKCα suggesting that PKCα induction requires ligand bound to both RAR and RXR. A retinoid specific for RXR receptors (SR11246) also moderately induces PKCα expression. The combination study shows that all-trans RA + SR11246 is the most effective combination (Fig. 15C). Induction of PKCα by AM580 + SR11246 and AM580 + SR11346 suggests that the RARα:RXR dimer plays a very important role in RA-induced PKCα expression. Since, SR11254 + SR11246 also induces PKCα expression, it is suggested that there might be some redundancy between the RARα and RARγ. Both of these receptors are constitutively expressed in B16 cells. It is possible that these receptors increase transcription of RARβ (through βRARE) which then directly increases PKCα expression. These data also indicate that both the partners (RAR:RXR) require ligand for maximum activity. This is in contrast to the hypothesis that only one partner requires ligand for activation and the other remains silent. The role of RARβ in RA-induced PKCα expression is still not clear. The analysis of the promoter region of PKCα gene will help clarify the role of these receptors at the transcriptional level. These receptors might also be involved in the regulation of other proteins that play an important role in the stability and processing of PKCα mRNA.

The effect of these analogs on AP-1 transcriptional activity was also determined (Fig. 16). All the compounds except RARγ gave about 2-fold induction

at the highest concentration ($10^{-6}$ M). All-trans RA and 9-cis RA gave the maximum fold induction. AM580 (RARα-specific) and SR11246 (RXR-specific) also induced AP-1 transcriptional activity in a concentration dependent manner suggesting that RARα and RXR play important roles in RA-induced AP-1 activity.

# FUTURE STUDIES

This dissertation has addressed and answered several questions. However as scientific research generally does, it has raised new issues which still remain to be explored and solved. One aspect of the work presented here focuses on the expression and activity of the AP-1 transcription factor. It is known that PKCα plays a role in RA-induced B16 differentiation. The results presented here suggest that PKC does play a role in RA-induced AP-1 transcriptional activity. Therefore the role of AP-1 transcription factor in the B16 differentiation process should be determined.

The role of c-jun in RA-induced B16 differentiation can be determined by characterizing the clones expressing dominant negative c-jun. These clones can be treated with all-trans RA and checked for AP-1 transcriptional activity. RA treatment of these clones should not induce AP-1 transcriptional activity. RA-treated clones can also be analyzed for the inhibition of cell growth. If c-jun has a role in RA-induced growth inhibition, these clones should have a higher growth rate than wild type cells (or neo transfected cells) treated with RA. Melanin production is another marker of RA-induced cell differentiation. The role of c-jun in RA-induced melanin production can also be determined in these clones.

Although RA-induced AP-1 trancriptional activity correlates well with PKCα expression, I cannot rule out the possibility of the involvement of other PKCs. B16

cells also express novel PKCs (δ, ϵ, ζ). I did not determine if they also play a role in RA-induced AP-1 transcriptional activity. Although less is known about the novel isoforms they no doubt play an important role in cellular processes. Therefore the role of the novel isoforms in the B16 differentiation process and AP-1 transcriptional activity should be determined.

The study of the retinoid analogs showed some interesting preliminary results. It will be very important to expand this study. The effects of receptor-specific retinoid analogs on the expression of RARβ would also provide further insight into the mechanism by which RA induces PKCα expression.

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