Biochemical and Biophysical Research Communications **263,** 28–34 (1999)
Article ID bbrc.1999.1307, available online at http://www.idealibrary.com on IDEAL®

Cloning and Characterization of the Murine PKC α Promoter: Identification of a Retinoic Acid Response Element

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Received July 6, 1999

Protein kinase C (PKC) is a family which consists of multiple isoforms whose distinct physiological roles within the cell are unknown. We have previously demonstrated that levels of PKC α mRNA, protein, and enzyme activity in B16 melanoma cells can be modulated by retinoic acid. We investigated this regulation by cloning and characterizing the promoter region of the murine PKC α gene. A 13 kb mouse genomic fragment containing the 5′ flanking region, first exon, and first intron was isolated and sequenced. Two transcription initiation sites were identified at 919 and 925 bp upstream from the translation start site. The promoter region contained a TATA-like box at −93 bp upstream of the transcription start site, but no CAAT box. Promoter activity differed between cell lines and correlated with the levels of PKC α expressed in these cell lines. Reporter gene assays showed that the region between −179 and −452 bp likely contains a silencer element(s). The promoter activity of a −179 bp fragment in B16 cells was stimulated twofold by retinoic acid. Within this region (−93 to −65 bp) there is a retinoic acid response element. An oligonucleotide spanning this region specifically bound exogenous RAR-RXR heterodimers and endogenous RAR from B16 nuclear extracts. These results suggest that retinoic acid increases PKC α gene expression in B16 cells, at least in part, through direct transcriptional stimulation of its promoter.

Protein kinase C (PKC) is a family of closely related serine/threonine protein kinases. These enzymes play an important role in regulating cell proliferation, tumor promotion and differentiation (1, 2). At least 11 different isozymes exist which are divided into three groups. The conventional PKC isozymes (PKC α, β1, β2, γ) require calcium, diacylglycerol and phospholipid for full enzymatic activity. The novel isozymes (PKC ϵ, δ, μ and theta) are calcium-independent, while the atypical isozyme (PKC-zeta, lambda) do not require either calcium or diacylglycerol (2).

In several different tumor cell lines, induction of differentiation by agents such as retinoids results in a large increase in the levels of PKC (3–7). Retinoic acid-induced differentiation of B16 mouse melanoma cells and F9 teratocarcinoma cells results in a large increase in PKC α mRNA and protein (6, 8, 9). Over-expression of PKC α in both of these cell lines resulted in acquisition of some of the phenotypic changes induced by retinoic acid (6, 10). These results suggest that the increase in PKC α may be an important part of the signal transduction pathway(s) leading to differentiation rather than an end-product marker of the differentiated state of the cell.

The mechanism by which PKC expression is increased in these different cell types is unknown. Retinoic acid increased the transcription rate of the PKC α gene by 2–3 fold in B16 mouse melanoma (8). Based on these findings, we decided to isolate and characterize the 5′ regulatory region of the mouse PKC α gene. We report here the sequence and promoter activity of a 2 kb genomic fragment of this gene, identify a retinoic acid response element that specifically binds RAR-RXR heterodimers, and show that retinoic acid increases the activity of a promoter fragment containing this element.

MATERIAL AND METHODS

Screening of a mouse genomic library. A 129SVJ-mouse library prepared from liver genomic DNA (Stratagene, CA) was screened by

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0006-291X/99 $30.00

standard method (11). Initial screening was performed using a NcoI and PstI fragment of mouse PKC α cDNA as a probe (Fig. 1). Secondary screening was performed using 5′ untranslated region of the PKC α mRNA, i.e. NcoI and EcoRI fragment of mouse PKC α cDNA. Positive clones were isolated and digested with NcoI or XbaI and subcloned into pG13-luciferase reporter vector (Promega, WI) at a NcoI site or into pBluescript vector (Stratagene, CA) at a XbaI site.

DNA sequencing. DNA sequencing was performed by the dideoxy method using a fluorescent labeled primer sequencing kit (Epicenter, USA) and an automated sequencing system (LiCoR, USA). DNA sequence analysis was performed using GeneWorks software (Intelligentics, USA). The putative promoter region was analyzed for transcription factor binding sites by consensus site analysis available through the database of the GCG Wisconsin package.

Primer extension analysis. Primer extension was done using synthesis oligonucleotides. The oligomer sequence as 5′ TGC CTG TGG GTC CAG GTA GCT AGA CAA GGA G 3′ corresponding to positions −800 bp from the translation start site of the mouse PKC α gene. The oligonucleotide was end labeled with γ-[^{32}P] ATP using T_4 polynucleotide kinase. The labeled probe was annealed in 20 μl of hybridization buffer (400 mM NaCl; 40 mM PIPES, pH 6.6; and 1 mM EDTA) to 30 μg of total RNA from NIH-3T3 cells overnight at 55°C after denaturing the mixture for 5 min at 85°C. Following overnight precipitation, the pellet was resuspended in 50 μl of reverse transcription buffer containing 1 mM $MgCl_2$ and 0.5 mM deoxynucleotide 5′-triphosphates and 12.5 U of AMV reverse transcriptase (Takara, Japan). The mixture was incubated at 42°C for 60 min. The extended products were analyzed by electrophoresis on 6% polyacrylamide urea sequencing gels with a sequencing ladder established by use of the same primers and the dideoxy chain termination method. Sau3A digested pBluescript DNA labeled at the 5′ end with polynucleotide kinase was used as size makers. Gels were fixed in 10% acetic acid and 5% methanol and exposed to Kodak XAR5 film or subjected two phosphoimage analysis.

Plasmid construction. A NcoI fragment (2 kb) of the mouse PKC α 5′ regulatory region (Fig. 1) was subcloned into NcoI site upstream of the luciferase reporter gene in the pGL3 vector (Promega, WI). Nested deletions of the 3 kb fragment were made using ExoII-mung bean nuclease (Takara, Japan).

Cell culture. Mouse NIH-3T3, B16-F1 mouse melanoma, COS-7 and 3Y1 rat fibroblast cells were grown in Dulbecco's modified medium (GIBCO/BRL, MD), supplemented with 10% FBS (Sigma, MO), L-glutamine and antibiotics (Penicillin (100 U/ml); streptomycin sulfate (100 μg/ml).

DNA transfection and luciferase assay. Plasmid DNA was purified through use of a Quiagen maxiprep kit (Quiagen, CA). Transfections were performed as described by Chen and Okayama (12) using either calcium phosphate or electroporation. Cells were transfected with 20 μg of the appropriate plasmid DNA. The Sea Pansy luciferase reporter plasmid (pRL-CMV, Promega, WI) was co-transfected (10 ng) to server as an internal control for efficiency of the transfection. After 16 h, the cells were washed with phosphate buffered saline and subjected to various treatments for 24 h. At the end of these treatments, the cells were harvested and processed for dual luciferase assay as per the manufacturer's instructions. At least three independent transfections were performed and the results expressed as the mean +/− SD.

Isolation of nuclei and preparation of nuclear extracts. B16 cells were treated with RA (1 μM) for 24 h. Cells were washed with cold PBS and then scraped with a rubber policeman into cold PBS. The cell suspension was transferred to a centrifuge tube and the cells pelleted by centrifugation at 1000 rpm for 5 min. The cell pellet was resuspended in 1 ml of cold PBS and transferred to a 1.5 ml microfuge tube. Cells were pelleted by a 15 sec centrifugation at 13,000 rpm in the microfuge. The cell pellet was resuspended in 300 μl of solution A (10 mM HEPES, pH 7.9; 10 mM KCl; 0.1 mM EDTA; 0.1 mM EGTA; 1 mM DTT and 5 mM PMSF) and incubation ice for 15 min. At the end of this incubation, 20 μl of 10% NP-40 was added and the microfuge tubes vortexed for 10 sec. This mixture was centrifuged at 13,000 rpm in a microfuge for 1 min. The nuclear pellet was resuspended in 50 μl of ice-cold solution C (20 mM HEPES, pH 7.9; 25% glycerol; 0.4 M KCl; 1 mM EDTA, 0.1 mM EGTA; 1 mM DTT and 5 mM PMSF) and incubated at 4°C with intermittent vortexing for 20 min. The mixture was centrifuged at 13,000 rpm in a microfuge for 5 min. The supernatant was aliquoted and stored at −70°C until use.

Electrophoretic mobility shift assay (EMSA). The double stranded oligonucleotide (5′ AAG CAG GTG AAC TGC AGG TCA AAG 3′) was end labeled with γ-[32P] dCTP (3000 Ci/mmol) by klenow. The labeled oligonucleotide was purified using Sephadex G50 column and resuspended in TE buffer (10 mM Tris, pH 7.5 and 1 mM EDTA) at a concentration of 20,000 cpm/μl.

EMSA reaction contained B16 nuclear extract or sf9 cell extracts from recombinant hRAR α, β and γ or RXR β baculovirus-infected cells, and 0.5× Dignam buffer D (1× Dignam buffer: 20 mM HEPES, pH 7.9; 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF and 0.5 mM DTT) plus 100 ng poly dI:dC and 20,000 cpm of the labeled oligonucleotide in a 25 μl reaction volume. The reactions were incubated for 20 min on ice and immediately resolved at 100 V at room temperature on 6% non-denaturing polyacrylamide gels in 1× TBE buffer. Gels were dried under vacuum at 80°C for 2 h and exposed to Kodak XAR-5 autoradiography film at −80°C.

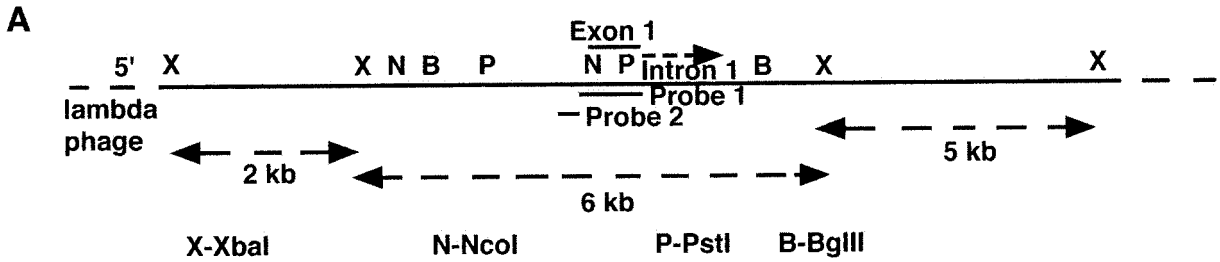
RESULTS

Isolation of a 5′ Promoter/Regulatory Region of the Mouse PKC α Gene

To isolate the promoter region the mouse PKC α gene, we screened mouse liver genomic library constructed in the lambda FixII vector with a fragment from mouse PKC α cDNA (NcoI and PstI, probe 1) (Fig. 1A). Three positive clones were isolated and analyzed further by restriction mapping and Southern blotting using 5′ untranslated region of mouse PKC α (NcoI and EcoRI fragment, probe 2) (Fig. 1A). One 13 kb clone contained the promoter region, one exon coding for the 5′ untranslated region (Exon I) and for the first 51 amino acids of the N-terminal region of PKC α plus part of the first intron. This genomic fragment was subcloned into pGL3-luciferase reporter vector or pBluescript plasmid and detailed restriction maps were constructed. The 5′ terminal region was identified by Southern analysis (data not shown) and sequences data (Fig. 1B). This region contained one exon and one intron. The next coding exon was separated by at least 5 kb of intronic sequence.

Determination of Transcription Start Site

The transcriptional start site was determined by S1 nuclease mapping and primer extension analysis. Multiple bands were protected from S1 nuclease digestion (Fig. 2A). These results were confirmed by primer extension analysis (Fig. 2B). These sites were located 919 and 925 bp respectively upstream of the translational start site. We assigned the first transcription start site as +1. A sequence that resembles a TATA box



B

-1379 GAGCTCTAAT ACGACTCACT ATAGGGCGTC GACTCGATCC CATTTTCCTA GTGTGCTTTG TTCTTCATTT TTCTCCTGCC TCTTCCCTGC TCGCCTCCTT

-1279 GGCCTGGAAG TGCTTAAATT CACTTTCCAC AGAATATCAC CAAGCACCAG CAGTACTGGC TGCGCATGTG TAGCCCTATC AACTTGACAC AAGCTGACAC

-1179 CAGTTGAGAA AATACCTCCA TAAGATTGGA CTGTAGGCAA GCTTGTAGTG CATTTTCCTA GTGACTGATG GAGGAGGGCC CAGTCCATTG TGAGTGGTGT

-1079 TATTCCTGGG CCCATGATCC TGGCTGAGTA AACCAGTAAG TAGCAATCTT CCATGGCCTC TGCAATCAGC TCCTGCCCTC AGGTTCTCTC CTGGCTTGGC
NcoI

-979 TTCTCTCAGT GGGCTGTGAT TCAGGATATG TAAGCCAAAT AAACCCTTTC CTGTTCAAGT TGCCATTGGT CATGGTGTTT CATCACAGCA GTAGTAACCC
AP-1

-879 TAAGACACTT GCTCACCAGA TTGTAAATGG GTCAGTGAAG AGCCTGGTCT ACTGATGGCT ATTCAGTTGC CTGCACTTGT TTAAATGTAA AACCTATCTT

-779 TTCGTCAAGA GTCACAATTA GGCAATCTTG GAAAAGGATA GTCCTTCGTG GCTTATGGGA TATGCTGTGA GCTAGTATTT CAGGCGATTT CCCTCTTTTA
C/EBP

-679 AAGTCTGCAC TTGAGATCTT ACATTCCTGA TCCTCTTGGG ATCACTGGCT GGTGTTACCA TGTCCAAGTT CAAGTATGCA CATTGTCTTA ATGGATTTGT
BglII

-579 ATTCTACAAT AAACGAAGCT GATGCGATGG CTCATCTCTG TAATTCCAGC ACTCAGGAGC TGTGACAGGA GTATTACCTC GAGTTTGGGA GCACCCTAGG
XhoI

-479 CCGATTGGGA CAATGAACTT CTTGTTGGTA GAGGGTTGCT CTGTCCCAGG TTTAGAATCC TCCAGGCAGA CCTAATAAAT ATTCAGCAGG CGCTCGGACA

-379 GCCCAGCCCC AAGTGCGCCC TTTGCATACA TATCCATTCC AGCTACCCCT TCACAAGCAG TTTTACTTGG TAATTTACAA ATGTTCTGGC TGAACTCTAC

-279 ACCTGCCAGT ATTTCAGCAA TTAGCTTAGG ATGCAAAATG ATTTCTAAGT TAAGGTTCTG GACATGTAAA ATCAAGAGAG CCCGATCATC TTCGGGCACC

-179 ACAGCTCTGA TAGCCAACGA ATAACATCAC AAACCAGGGC AAGCCCTTTG GCATCTTACC GGGTTATTAC GACTGTCATA CAAATCTATA TAATAAGCAG
CRE -93 -81

-79 GTGAACTGCA GGTCAAAGAT CGAAGGGATG TAAGAGAGCC GGCAATCAGT TATTCAAAAC CGCTCCCTTT TACCGACTCG GAACTGAGCG AGGGTTAGGG
PstI -65 +1

+22 TTTGGGCATT TTAGTGGCAT AAACGACTAG AATGACATTT TGGTCAGGTG AAAGGTTAAA CGGGAAAGAG GCTGATAAAA AGCCAGCAAA TCCCCCACCC

+122 CCAAATGCCT GTGGGTCCAG GTAGCTAGAC AAGGAGCTAC CTCTGTACCT GCTAAGTGGC ATGGGGGTGG GGGTGGGGAG CGCTCCGTGC CGGGTTCCTT

+222 CGGGGCACAA CTTCCATCCC AGAAAGAGGA AAGCATGAGC AGAGAAGCGT GGTGG AGGAAGGAGTGGTGG GGAGAGGAGG AGGCCCGGCA CCACGAGCGG

+322 AAGGAGAGGA GGAGAAAAAA GAGGGAGGAG GAAGCAGAGG AAAAGGGAGC AAAAAGAAGC GGGCGGCGGA GGAGAAGGAC CCGCCCCTTC CGGCTTCTTC

+422 CTGGGCTGGG CATCTTCAGG GCCTCTCCCC GACCGCCGCC CTCGGCAGTT GGCGGGGCTC ACAGGGGCCC CCCAGTCTGC GTGTCCAGCT CTGTGCAGCG

+522 GAGCGCAGAG GCCCAAGGCT CGCCCGGCGC GCGTCCCCGT CCCGCGCGCT CCTACTCCAG CCCGCGCGCG CGCGTGCACA CATACACACG CTAGCTCTCC
NheI

+622 CGGCATGCGC AGTGGGCGGC GCCGCTCCGG GCCGCCTCTT GCCGCCGCGA GTAGGAAGCG CGAGCGCCGG GCGCCGGGCT GTCAGTGAGC GTGGGGCCAG

+722 CCAGCGAGCC AGAGAGCCGG AGAGAGCCAG AGACAGCGGC TCGGCTCCCA GCTCCGAGTA GCGCAGCGCC CGCCCGGCTC TCCCGGGCCA CCGCCGCCAC

+822 CACCGCACCT CATCAGCACC GCCACCTCGG CCGCCGCCCC CGCCCACCCC GGCCCTCCCC GGCTGCTGCT CCCCGGCGGA GGCAAGAGGT GGTTGGGGGG

+922 AACCATGGCT GACGTTTACC CGGCCAACGA CTCCACGGCG TCTCAGGACA TGGCCAACCG CTTCGCCCGC AAAGGGGCGC TGAGGCAGAA GAACGTGCAT

+1022 GAGGTGAAAG ACCACAAATT CATCGCCCGC TTCTTCAAGC AACCCACCTT CTGCAGCCAC TGCACCGACT TCATCTGGta ggtgcgcgcg gcaagccctg
PstI Exon/Intron

+1122 gggacccaag aaccaccttt gccccggggc tcaggtctgc ttgtccccgg gacttaagct cggggacgtc tctgagcccc ggcgcgtcct tactctcagg

+1222 gccaggactc ctccgagaac ttacagtgcc tggccggagt ccgaactctc gccaggagtg acaggcgcgc acccacgcgg ggacactccc tggaaagtgg

+1322 gcccgcgctg ccacgcatgc cctggtgccc ttgaagggac gcttggctct gcacgggggtg actcggatta aggggaaagt ccctccgccc ctcctatggg

+1422 gcaaaagtct tc +1433

FIG. 1. Restriction map and sequence of a mouse genomic clone containing a portion of the PKC α promoter region. (A) Schematic restriction map of the subcloned genomic PKC α gene showing the position of exon I, intron I, and the 5′ untranslated region. Probe 1 was used for primary screening of the mouse liver genomic library in the lambda FixII vector. Probe 2 was used for secondary screening of the

(TATATAATAA) was located −83 bp upstream of the first transcriptional start site. Inclusion of this sequence in the reporter gene constructs (described below) yielded higher activity than when this sequence was excluded. The 3′ end of exon I was identified at 154 bp downstream of the translational start site. Thus exon I contains 974 bp of non-coding sequence in addition to the 154 bp encoding the first 51 amino acid residues. This 5′ untranslated region is also GC rich.

The 5′ promoter/regulatory region contains consensus-binding sequences for several transcription factors (Fig. 1B). Two E-box sequences were present at −860 and −1213. A consensus retinoic acid response element was found between −65 and −81. This region consists of a direct repeat separated by 5 bp (DR-5). Other potential binding sites include: AP-1, C/EBP, GATA-1, GATA-2, ROR and CRE.

Analysis of Promoter Activity

Various 5′ segments of the protein kinase C α DNA flanking the transcriptional start site were subcloned into a luciferase reporter vector (Fig. 3A). These constructs were transiently transfected into NIH-3T3, B16-F1, COS-7 and 3Y1 cells. The smallest fragment tested, −70 bp, produced promoter activity in all the transfected cells lines (Fig. 3B and C). The −179 bp construct gave the highest promoter activity in all cells. Transfection of the construct that included 452 bp gave decreased promoter activity in all cell lines, compared to the −179 bp fragment, indicating a repressor or silencer region between −179 and −452 bp. Different cell lines gave varying promoter activities with the same constructs. NIH-3T3 and 3Y1 cells gave the greatest activity; COS-7 cells were intermediate, while the lowest activity was observed in the B16-F1 mouse melanoma cells. These promoter activities roughly correlate with the amount of PKC α protein expressed in these cell lines (data not shown).

Retinoic Acid Regulation of PKC α Promoter Activity

We have shown that RA treatment of B16-F1 mouse melanoma cells increases PKC α mRNA and protein levels. Part of this regulation is at the transcriptional level (8). These observations plus the identification of a retinoic acid response element (RARE) in the PKC α promoter region led us to examine the possible regulation of PKC α promoter activity by all trans-retinoic acid (RA). In contrast, the −179 bp fragment which contains the complete DR-5 (RARE) gave a consistent two-fold increase in promoter activity when B16-F1 mouse melanoma cells were transfected with this fragment were treated with RA (Fig. 3D).

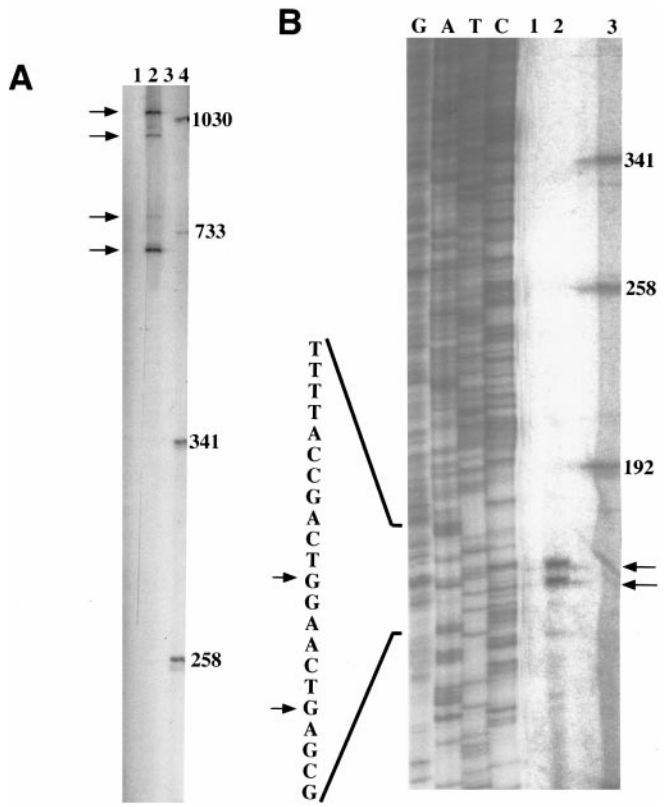


FIG. 2. Identification of transcription initiation sites of the mouse PKC α gene. (A) S1 nuclease mapping analysis: Twenty micrograms of total RNA from NIH-3T3 cells was hybridized with a 5′ end labeled NcoI fragment and digested with S1 nuclease as described in Methods. The reaction products were separated on a 6% polyacrylamide gel. Lane 1, S1 nuclease product in the presence of tRNA; lane 2, S1 nuclease product in the presence of NIH-3T3 mRNA; lane 3, S1 nuclease product in the absence of RNA; lane 4, pBluescript plasmid DNA digested with HaeII and 5′ end labeled to serve as size markers. S1 nuclease protected fragments are indicated by arrow heads on the left of the figure. Size markers are given on the right of the figure. (B) Primer extension analysis: 5′ end labeled complementary synthesis oligonucleotide corresponding to position +224 bp (as indicated in Methods) was annealed to 30 μg of total RNA from NIH-3T3 cells and extended with reverse transcriptase. Extended products were separated by electrophoresis on a 6% polyacrylamide gel. Lane 1 is the primer extension product and lane 2 is pBluescript plasmid DNA digested with HaeII and 5′ end labeled for size markers. Arrowheads on the right of the figure indicate primer extension products and on the left side of the figure the corresponding sequence is given and transcription start sites are shown. Numbers on the right indicate the size markers.

selected clones and for Southern hybridization (data not shown). (B) Nucleotide sequence of the cloned and sequenced PKC α gene containing the part of first intron, first exon, and 5′ untranslated region. Transcription initiation sites as determined by both S1 nuclease mapping and primer extension as indicated as +1. Part of 5′ untranslated region and first exon are identical to the published sequence (13) of mouse PKC α cDNA. Consensus transcription factor binding sites are indicated by underline. The retinoic acid response element is indicated in bold.

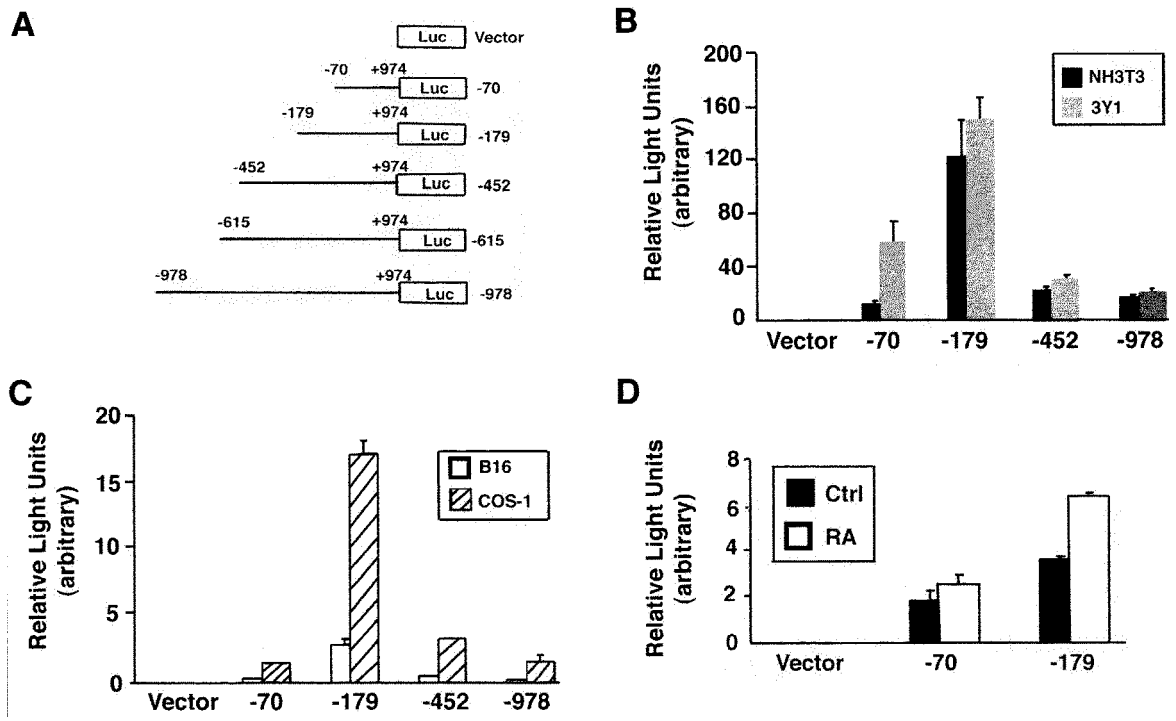


FIG. 3. Mouse PKC α promoter activity and effect of retinoic acid. Mouse PKC α promoter activity was assayed by transcient transfection of the reporter constructs shown in A. Transfection was accomplished by the calcium phosphate method with 20 μg of the various constructs show in A plus 10 ng of Sea Pansy luciferase to serve as a control for transfection efficiency. At the end of the incubation of transfected cells, luciferase activity was determined by use of the dual luciferase assay kit. All data points are from triplicate dishes of cell transfections and experiments were repeated at least twice. (A) Schematic diagram of PKC α promoter fragments used in these experiments. (B) Promoter activity (luciferase) of PKC α promoter fragments in NIH-3T3 cells and 3Y1-rat fibroblast cells. (C) Promoter activity (luciferase) of PKC α promoter fragment in COS-7 and B16-F1 mouse melanoma cells. Note different y-axis scales between B and C. (D) Effect of retinoic acid on promoter activity of the -70 and -179 bp PKC α promoter constructs. Transfected B16 mouse melanoma cells were treated with 1 μM all-trans retinoic acid for 24 h prior to assay for luciferase activity. Note that the -70 bp fragment contains a half-site of the RARE, while the -170 bp fragment contains the entire RARE.

Binding of Retinoic Receptors to a DR-5 Site in PKC α Promoter

In order to determine whether retinoid receptors were capable of specifically binding to the RARE identified in the PKC α promoter; we synthesized a wild type and mutant 24 bp deoxynucleotide spanning this site. Double stranded end labeled wild type oligonucleotides were used in electrophoretic mobility shift assays with either recombinant baculovirus expressed RAR/RXR, or nuclear extracts from B16-F1 cells. Although a protein-DNA complex was formed with RAR α, β or g added alone to the oligonucleotide (Fig. 4), the concentration of sf9 insect cell extract used contains a sufficient amount of an RXR homolog to allow dimerization necessary for DNA binding (14). The greatest amount of complex was formation is seen when RXR β was combined with RAR (Fig. 4). This binding is specific as seen b efficient competition by unlabeled wild type, but not by unlabeled mutant oligonucleotide (Fig. 4). Endogenous RAR/RXR from B16-F1 cells also formed a complex with the wild type oligonucleotide (Fig. 4). Identity of sf9-baculovirus produced RAR and endogenous RAR was confirmed by a "super-shift" of the complex when incubated with RAR antibody. Our laboratory has previously shown that this antibody reacts with all three of the RAR isoforms (14).

DISCUSSION

PKC α expression has been demonstrated to vary as a function of normal *in vivo* differentiation of keratinocytes (7) and colonic epithelial cells (15). Retinoic acid increased the transcription rate of the PKC α gene in B16-F1 mouse melanoma cells, human leukemia HL-60 cells, and human pancreatic carcinoma cells (8, 15, and 16). These reports suggest that the PKC α gene be regulated by a variety of signal transduction pathways. Therefore we decided to isolate and characterize the PKC α gene promoter in order to provide insights into how this gene is regulated.

In this study, we isolated part of the mouse genomic PKC α gene and identified the first exon, boundary between first exon and intron and part of the promoter region. A TATA-like box was found between -87 and

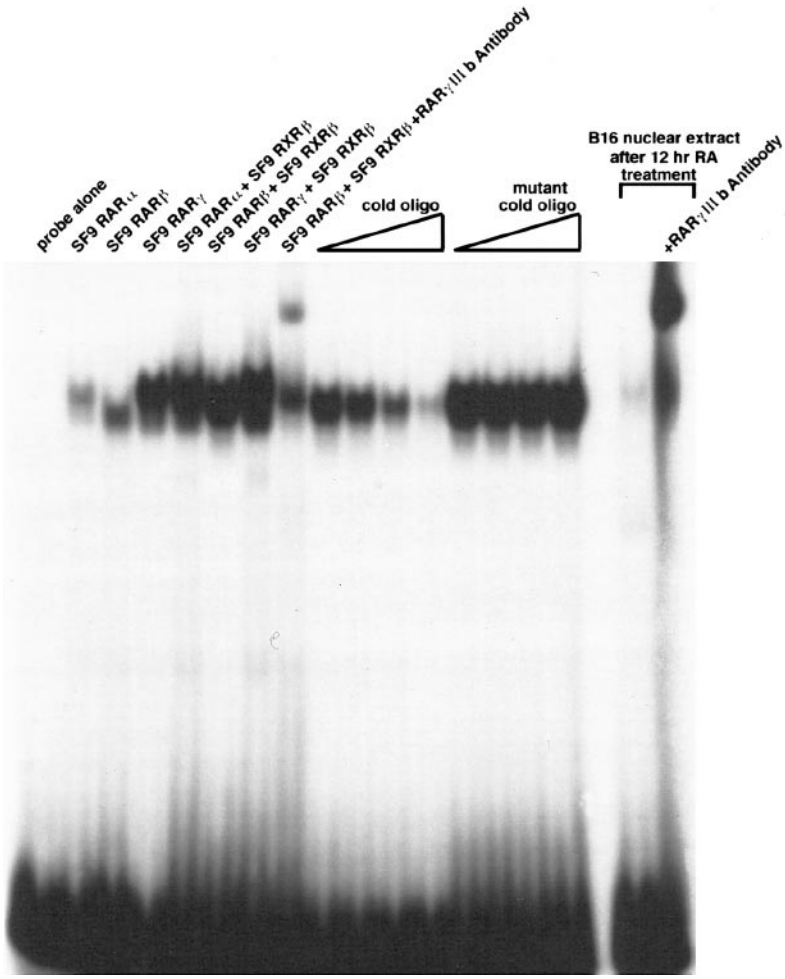


FIG. 4. RAR binding to RARE from the mouse PKC α promoter. An oligonucleotide overlapping the RARE in the mouse PKC α promoter (-65 to -81 bp) was synthesized (5′ AAG CAG GTG AAC TGC AGG TCA AG 3′) along with a mutated version of this oligonucleotide (5′ AAG CTC TCG TAC TGC TCT CGT AAG 3′). The wild type oligonucleotide was end labeled and allowed to react with either 25 μg of nuclear extract from B6-F1 cells or 1 μg of extract from sf9 insect cells expressing the indicated receptor in the absence of presence of increasing amounts of the unlabeled wild type or mutant oligonucleotides. DNA-protein complexes were resolved on nondenaturing polyacrylamide gels and identified by autoradiography. Identity of the protein complex was ascertained by reacting with the RAR Antibody that caused a "supershift" of the complex.

-93 bp from the transcriptional start site, but no CAAT-like box was observed. Several putative motifs for transcription factors were identified. The functionality of these sites awaits further investigation, however it is interesting that the PKC α promoter activity varied significantly among the different cell types used in this study, possibly be due to differences in transcription factor production by the different cell lines.

The only PKC promoters characterized to date have been from the human PKC β and γ genes (17–20). In contrast to the PKC α promoter, the human PKC β gene has a CCAT box but no TATA box (18). The 5′ flanking sequence of the mouse PKC α gene reported here contains an inhibitor element between -179 and -452 bp. Negative elements have also reported in the human PKC β promoter between -411 and 674 bp (18) and between -690 and -3000 bp (19). The mechanism by which these elements exhibit their inhibition of transcription is not known.

Similar to the PKC α promoter, several putative transcription factor consensus sequences were found in the PKC β promoter, such as Sp-1, E boxes, AP-1 and AP-2 sites (17). In addition to these sites, we identified a putative RARE with 5 bp spacing between the consensus repeats. Inclusion of a promoter fragment containing this element (-179 bp) in reporter gene assays resulted in a two-fold stimulation of promoter activity in B16-F1 cells treated with retinoic acid. Interestingly, this corresponds to the RA-induced increase in transcription of the PKC α gene in B16-F1 cells (8). We demonstrated that this element could specifically bind RAR/RXR heterodimers supplied exogenously as well as binding endogenous RAR found in B16-F1 cell nuclear extracts. Despite specific binding to this RARE,

the transcriptional activity of the liganded nuclear retinoid receptors was rather modest. This may reflect the location of the RARE within the promoter (3′ of the TATA box), the amount of RARs expressed in these tumor cells, or the low abundance of coactivators necessary for full transcriptional activity. Lastly, these findings reinforce our previous conclusion (8) that neither transcriptional regulation nor mRNA stability can account for the full induction of PKC α mRNA and protein by retinoic acid in B16-F1 cells. It is likely that retinoids can also influence post-transcriptional activities such as splicing (21–23) and/or nuclear transport.

ACKNOWLEDGMENT

This work was funded in part by a Japan Society for the Promotion of Science in Japan (JSPS) fellowship awarded to Dr. Dinakar Desai.

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