

Characterization of Conventional Protein Kinase C (PKC) Isootype Expression during F9 Teratocarcinoma Differentiation

OVEREXPRESSION OF PKC α ALTERS THE EXPRESSION OF SOME DIFFERENTIATION-DEPENDENT GENES*

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F9 teratocarcinoma is a useful model for studying early embryogenesis since these cells can differentiate into primitive or parietal endoderm under the influence of retinoic acid or retinoic acid and cyclic AMP, respectively. We have found that three isoforms of protein kinase C (PKC α , β , and γ) were expressed in undifferentiated stem cells. When the cells were treated with retinoic acid either alone or in the presence of cAMP for 120 h, PKC α mRNA and protein levels increased, whereas those of PKC β and PKC γ became undetectable. These changes began within 24 h of drug treatment and were complete by 48–72 h. In order to determine the functional significance of the induction of PKC α during F9 differentiation, we established two stable transfectants that overexpressed PKC α protein between 4- and 5-fold compared to wild type cells. Characterization of these cell lines revealed an altered pattern of expression of some of the markers of F9 differentiation. The clone that had the highest amount of PKC α protein constitutively expressed mRNA for type IV collagen and c-Jun, which are not normally expressed until 24–48 h of treatment with differentiation agents. In the other overexpressing clone, these markers were induced much faster than in wild type cells. The growth rate of both overexpressing clones was less than wild type cells, while the expression of the PKC β protein in these clones was similar to the levels found in differentiated F9 cells. However, other markers of differentiation, including the cellular morphology and levels of pST6-135 and c-myc RNA, responded to agents identically in both wild type and PKC α -overexpressing clones. Therefore, overexpression of PKC α is not sufficient to induce full differentiation of F9 cells. However, our data suggest that certain pathways that lead to the expression of differentiation-dependent genes are regulated by PKC α protein levels.

F9 teratocarcinoma cells are murine embryonal cells, which, when treated with retinoic acid (RA),¹ can be induced to differentiate into primitive endoderm. Cyclic AMP (cAMP) and RA treatment of F9 stem cells results in further differentiation into parietal endoderm with concomitant loss of malignant potential (1). This differentiation process can be monitored by fol-

lowing the expression of several specific markers. The production of Type IV collagen, laminin, and c-Jun is induced with differentiation (2, 3), while the stage-specific embryonic antigen disappears from the cell surface (4) and the levels of pST6-135 and c-Myc are dramatically decreased (5, 6).

Protein kinase C is an intracellular calcium, phospholipid-dependent enzyme, which is activated by diacylglycerol and is believed to be involved in regulating a wide variety of cellular activities. It is a multigene family consisting of 10 known isoforms (7, 8). Some of these isoforms (ϵ , δ , η , and θ) do not require calcium for activity, while others (λ and ζ) do not require calcium or diacylglycerol for activity. PKC β 1 has been shown to induce cell proliferation when overexpressed in some fibroblast cell lines (9, 10). However overexpression of PKC α in melanoma cells produced a differentiated phenotype (11), while 12-O-tetradecanoylphorbol-13-acetate, which also activates PKC, has been shown to induce differentiation of HL60 cells (12). It is possible that the seemingly opposite effects induced by PKC activation may be due to the differential expression, activation, and translocation of the particular isoforms. It has been shown that PKC α , β , and γ proteins are localized within different regions of embryonic tissue (13). However, the function of these proteins during embryonic development is still unknown. Using F9 teratocarcinoma as a model of early embryonic development, we determined which conventional PKC isotypes (α , β , and γ) were expressed and whether their expression changed as a function of differentiation. After discovering that PKC α was induced during F9 differentiation, we assessed its role in this process by transfecting and overexpressing PKC α in undifferentiated stem cells.

EXPERIMENTAL PROCEDURES

Cell Culture—F9 cells were grown on tissue culture dishes coated with 0.1% gelatin in minimal essential medium (MEM) supplemented with 3000 mg/liter glucose, 50 units/ml penicillin G, 50 μ g/ml streptomycin sulfate, 2 mM L-glutamine, 2 mM sodium pyruvate, and 10% heat-inactivated, iron-supplemented bovine calf serum (Sterile Systems, Logan, UT) in a 7% CO₂, 93% air incubator at 37 °C. RA (Fluka) was dissolved in 100% ethanol at a concentration of 10⁻² M and then diluted to a final concentration of 10⁻⁷ M in MEM. 8-Br-cAMP (Sigma) was prepared at a final concentration of 0.5 mM in high glucose MEM and 3-isobutyl-1-methylxanthine (IBMX) (Sigma) was prepared at a final concentration of 0.2 mM. All manipulations involving RA were performed in subdued light to prevent photo-oxidation.

Induction of Differentiation—F9 cells were treated with RA or RA and cAMP/IBMX for various times to induce differentiation. Cells were treated for 6, 12, 24, 48, 72, or 120 h with RA alone to induce a primitive endoderm-like state or with RA, 8-Br-cAMP, and IBMX to produce parietal endoderm-like cells.

Transfection—F9 cells were transfected with 20 μ g of the YK504 plasmid (14), containing full-length PKC α cDNA driven by an SV40 promoter (15), and 2 μ g of a plasmid encoding the neomycin resistance gene (pSVneo) in 100-mm dishes by the calcium phosphate precipitation/glycerol shock procedure as described previously (16). 2

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¹ The abbreviations used are: RA, retinoic acid; PKC, protein kinase C; MEM, minimal essential medium; IBMX, 3-isobutyl-1-methylxanthine; 8-Br-cAMP, 8-bromo-cyclic AMP; kb, kilobase(s).

days later, cells were replated in high glucose MEM containing 350 µg/ml geneticin sulfate (G418). 12 days later, clones were selected and expanded. Cells transfected with pSVneo alone and selected for G418 resistance served as a negative control in most experiments.

Northern (RNA) Blot Analysis—RNA was isolated by an acid phenol method as described previously (17) or by LiCl precipitation (18). In some cases, poly(A)⁺ RNA was isolated by passing total RNA over an oligo(dT) column as described by Aviv and Leder (19). The RNA was then fractionated by size on 1% agarose gels containing formaldehyde and transferred to Hybond-N nylon membranes. The membranes were baked for 2 h in a vacuum oven at 80 °C and prehybridized for 4–16 h in 50% formamide, 5 × SSC, 5 × Denhardt's solution, 0.1% SDS, and 20 µg/ml single-stranded herring sperm DNA. ³²P-Labeled cDNA probes were then incubated with the membranes in fresh hybridization solution for 48 h at a specific activity of 1×10^6 cpm/ml. Blots were washed three times for 30 min each in 0.6 × SSC and 0.1% SDS, and once in 0.1 × SSC and 0.1% SDS for 15 min at 42 °C. The blots were exposed to Kodak XAR film in a cassette for 2–21 days. The ³²P-labeled cDNA probes were labeled by random priming using Amersham Corp.'s Multiprime kit. The amount of different mRNAs was quantitated by imaging the autoradiogram with a Molecular Dynamics computing densitometer, making sure that the signals were within the linear range of the instrument. The data were expressed as the ratio of the specific mRNA to the mRNA for the internal control, glyceraldehyde-3-phosphate dehydrogenase. Levels of glyceraldehyde-3-phosphate dehydrogenase did not vary as a function of F9 differentiation.

cDNA Probes—The following cDNA clones were used as probes in Northern blot analysis: phPKC- α 7 (1.29-kb insert), phPKC- β 1-15 (1.7-kb insert), and phPKC- γ 6 (1.4-kb insert) from the American Type Tissue Collection (ATCC, Rockville MD) (20); type IV collagen from the authors' laboratory (Dr. R. Niles) and Dr. S. Farmer (Boston University School of Medicine); c-Myc (1.7-kb insert) from Dr. K. Marcu (SUNY, Stonybrook) (21); Jac7 (c-Jun) from Dr. D. Nathans; and pHcGAP (glyceraldehyde-3-phosphate dehydrogenase) from ATCC (22).

Western Blot Analysis—Cells were plated at 3×10^5 cells/dish onto gelatinized 100-mm dishes. After 24 h the medium was removed, and the cells were refed with experimental medium for the indicated times. Cells were washed two times with phosphate-buffered saline solution and then lysed in 0.5 ml of lysis buffer (10 mM Tris, pH 7.5, 1 mM EDTA, pH 8.0, 10% glycerol, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 50 µg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride). Cells were then sonicated three times for 10 s each with a Tekmar sonic disruptor at power setting 60. Protein concentration was determined by use of the Pierce BCA protein assay kit. 100 µg of protein was separated on 7.5% sodium dodecyl sulfate-polyacrylamide gels. The proteins were transferred to a Hybond-C extra nitrocellulose membrane (Amersham) by electroblotting in transfer buffer (39 mM glycine, 40 mM Tris base, 0.0378% SDS, 20% methanol) (16). The membrane was incubated in blocking solution (10% nonfat dry milk in Tris-buffered saline, pH 8.0, containing 0.2% Tween 20) for 1 h and then incubated with 5 µg/ml primary antibody for 2 h, followed by three 10-min washes in blocking solution. The secondary antibody was then added at a 1:3000 dilution and incubated for 1 h. The blots were then washed several times in Tris-buffered saline plus 0.2% Tween 20. The antibody conjugate was reacted, and the signal was detected with Amersham's enhanced chemiluminescence kit. The amount of PKC α or β was determined by imaging the autoradiograms with a Molecular Dynamics densitometer, making sure that the signals were within the linear range of the instrument.

Growth Curves—Cells were plated at 1×10^4 cells/dish onto gelatinized 60-mm dishes. After 24 h, the medium was removed and the cells were refed with differentiation medium. At the indicated time points, the cells were washed twice with phosphate-buffered saline solution, trypsinized, and diluted into MEM. Viable cells were counted using a hemocytometer.

All experiments were repeated at least three times with similar qualitative results.

RESULTS

PKC α , - β , and - γ Isoforms Are Differentially Expressed during F9 Differentiation—Poly(A)⁺ RNA from F9 cells treated with RA, cAMP, or RA plus cAMP for 5 days was hybridized with PKC α , - β , and - γ cDNA probes (Fig. 1A). Stem cells (lane 1) were found to express two messages for PKC α at 10.8 and 3.8 kb, respectively, with the smaller message being more abundant. These cells also expressed a 10.9-kb PKC β mRNA and a

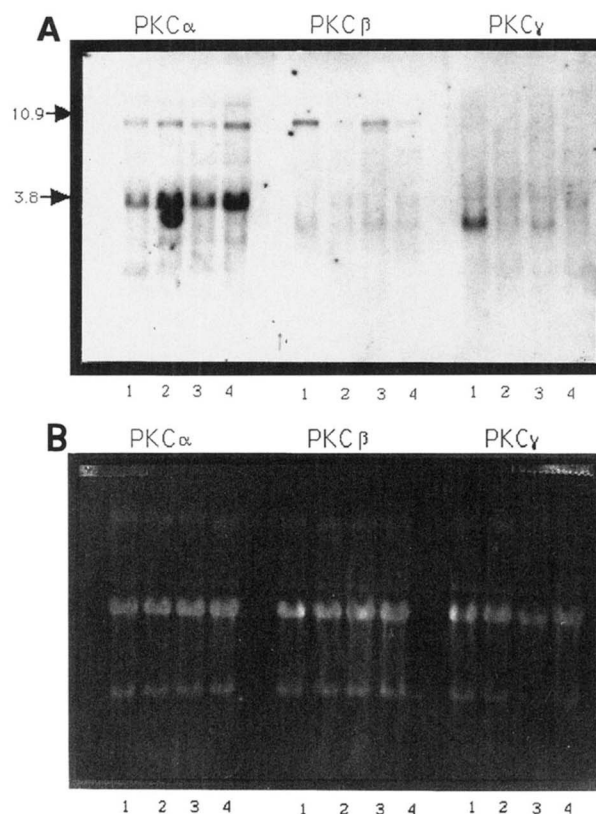


FIG. 1. Expression of PKC α , - β , and - γ mRNA in stem and differentiated F9 cells. Cells were grown in high glucose MEM with 10% bovine calf serum for 5 days without treatment (lane 1), with 0.5 mM 8-Br-cAMP and 0.2 mM IBMX (lane 2), with 10^{-7} M RA (lane 3), or with 10^{-7} M RA plus 8-Br-cAMP/IBMX (lane 4). Cells were washed two times with ice-cold phosphate-buffered saline solution, and RNA was isolated by guanidine monothiocyanate/LiCl precipitation as described under "Experimental Procedures." Poly(A)⁺ RNA was purified over an oligo(dT) column. 7 µg of poly(A)⁺ RNA was separated in 1% agarose gels and transferred to Hybond-N nylon membranes. A, blots hybridized with ³²P-labeled PKC α , - β , or - γ cDNA inserts for 48 h and exposed to film for 21 days. B, photograph of the ethidium bromide-stained gel illustrating the amount of RNA in each lane.

3.5-kb PKC γ mRNA. Upon treatment with cAMP (lane 2) or RA + cAMP (lane 4), both of which induce differentiation into parietal endoderm (23), the amount of PKC α mRNA increased while the amount of PKC β and PKC γ mRNA decreased. When the cells were treated with RA alone, which induces differentiation into primitive endoderm, PKC α mRNA increased and PKC β and γ mRNA decreased (lane 3) but to a lesser extent than in the parietal endoderm-like cells. The gel was stained with ethidium bromide and photographed (Fig. 1B) to show the relative amount of RNA from each sample.

To determine if differentiation-dependent changes in PKC mRNA levels were indicative of changes in protein levels, we performed Western blot analysis of F9 protein extracts using monoclonal, isotype-specific antibodies (Fig. 2). The level of PKC α protein in RA-treated and RA + cAMP-treated cells began to increase by about 24 h and reached a maximum by 72–120 h. There was also a small elevation of PKC α protein in control cells at the later time points (Fig. 2). After 5 days of treatment, PKC α was increased by 14-fold in parietal endoderm-like cells (RA + 8-Br-cAMP/IBMX) and 6-fold in the primitive endoderm-like cells (RA) compared to stem cells. Treatment of F9 cells with RA+cAMP also decreased PKC β protein levels (Fig. 3). At 48 h of incubation, PKC β protein in RA + cAMP-treated cells was only 50% of that found in control cells. The level of PKC γ could not be determined because none of the commercially available PKC γ -specific antibodies had the

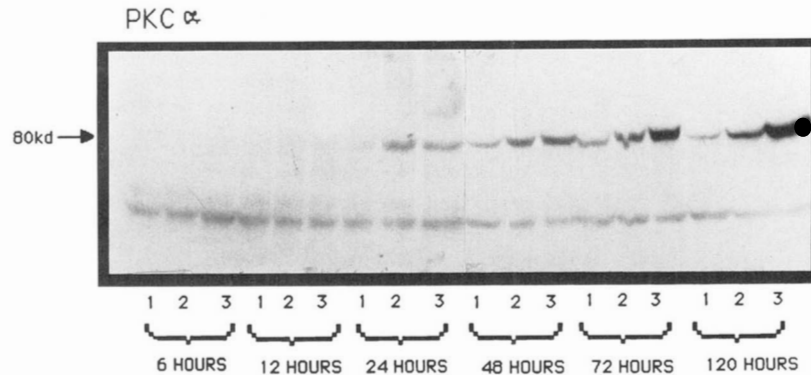


FIG. 2. Time-dependent changes in PKC α protein during F9 differentiation. Proteins were isolated from cells left untreated (1), treated with 10^{-7} M RA (2), or treated with 10^{-7} M RA plus 0.5 mM 8-Br-cAMP and 0.2 mM IBMX (3) at the indicated time points and then separated on 7.5% denaturing SDS-polyacrylamide gels. Equal amounts (100 μ g) of protein from each sample were electrophoretically transferred to a nylon membrane and then incubated with anti-PKC α antibody from Seikagaku. Antigen/antibody complexes were detected with Amersham's enhanced chemiluminescence kit. Quantitation of the blot was achieved through the use of a Molecular Dynamics computerized densitometer.

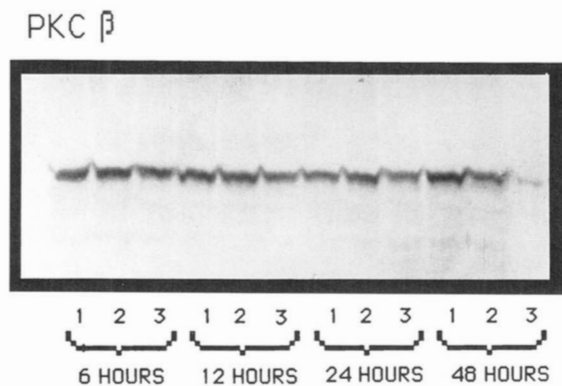


FIG. 3. Time-dependent change in PKC β protein during F9 differentiation. Conditions used for differentiation were as described in Fig. 2. 100 μ g of protein from each sample was analyzed for PKC β protein level by Western blotting using a monoclonal anti-PKC β -specific antibody from Seikagaku, followed by enhanced chemiluminescence. The autoradiogram was imaged with a Molecular Dynamics densitometer and the relative amount of PKC β in each sample determined.

sensitivity or specificity required for detection in F9 cells.

Establishment of Stable Transfectants Overexpressing PKC α —To determine the relationship between the increase in PKC α and the differentiation program, we established stable PKC α F9 transfectants. A plasmid containing the full-length PKC α cDNA driven by an SV40 promoter (YK504) was cotransfected with a plasmid encoding the gene for neomycin resistance (pSVneo) at a ratio of 10:1. 50 G418-resistant colonies were selected and screened by Western blot analysis using a monoclonal antibody to PKC α . Two clones that overexpressed PKC α were identified. Southern blot restriction enzyme analysis confirmed that these clones had integrated the exogenous PKC α gene (data not shown). Fig. 4 is a Western blot of the proteins isolated from the clones YK504-1 and YK504-25 and a clone selected for G418 resistance from F9 cells transfected only with pSVneo. Clone YK504-1 expressed PKC α at a level 5.4-fold higher than the controls and YK504-25 at a level of 4.4-fold higher than that of the neomycin-resistant clone as determined by scanning laser densitometry. RA treatment did not increase PKC α levels in YK504-25 cells but did increase PKC α levels by 2-fold in both YK504-1 and neomycin-resistant (control) cells. When all three clones were treated with RA and cAMP for 120 h, the level of PKC α increased by 2-fold in YK504-25, while there was no further increase in YK504-1 beyond that induced by RA alone. The control clone had almost a 10-fold increase in the level of PKC α .

Characterization of PKC α -overexpressing Cell Lines—Both PKC α clones were morphologically indistinguishable from F9

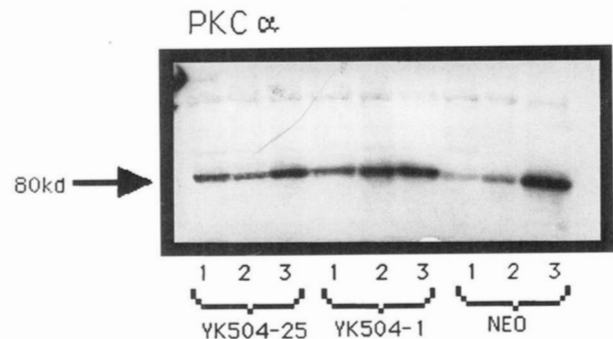


FIG. 4. PKC α protein levels in transfected clones. F9 cells were cotransfected by calcium phosphate precipitation with 2 μ g of SV40Neo DNA and 20 μ g of YK504 DNA. 48 h later, the cells were split into medium containing 350 μ g/ml of gentamycin sulfate (G418), and after 2 weeks 50 clones were selected and screened by Western blot analysis as described in legend to Fig. 2. Two clones were found to stably overexpress PKC α between 4.7-fold (YK504-25) and 5.4-fold (YK504-1) relative to neomycin-transfected control cells. Lane 1 contains proteins from untreated cells. Lanes 2 and 3 contain proteins extracted from cells treated with 10^{-7} M RA (lane 2) or with 10^{-7} M RA plus 0.5 mM 8-Br-cAMP/0.2 mM IBMX (lane 3), respectively, for 120 h. 100 μ g of protein were loaded in each lane.

stem cells (not shown). However, both PKC α clones grew more slowly than the wild type F9 cells or neomycin-resistant clones. The YK504-1 cells had approximately the same growth rate as RA + cAMP-treated wild type F9 cells (Fig. 5).

The expression of several genes serve as markers for the differentiation process in F9 cells. The markers are indicative of different points in the differentiation process; therefore, comparison of the two PKC α cell lines with the wild type and/or neomycin-resistant clones was used to determine the point at which PKC α may be involved in differentiation.

We have shown that PKC β is down-regulated during F9 differentiation (Figs. 1 and 2). We therefore examined the expression of the PKC β protein in wild type, neomycin-resistant, and PKC α -overexpressing clones (Fig. 6). The amount of PKC β in both PKC α -overexpressing clones was decreased by 82–90% compared to either untreated neomycin-resistant or wild type cells (Fig. 6). Treatment of all cells with RA+cAMP for 5 days resulted in an additional decrease of PKC β protein in YK504-25 and YK504-1 cells and a dramatic decrease in both wild type and neomycin-resistant cells.

Changes in several RNA transcripts including increases in type IV collagen (2) occur somewhat later in the differentiation pathway and require new protein synthesis for induction of their mRNAs. Type IV collagen mRNA was induced by about 4-fold at 72 h of treatment with differentiation medium in the

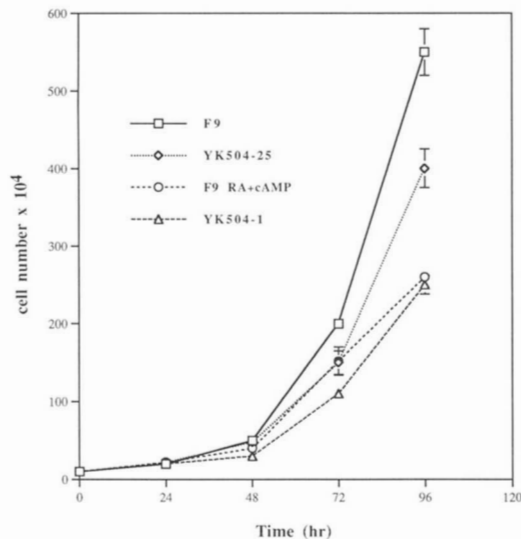


FIG. 5. Growth of wild type and PKC α -overexpressing F9 clones in the absence and presence of differentiation inducers. F9 cells and YK504 clones were seeded at 1×10^4 cells/60-mm tissue culture dish. The cells were grown for 24 h in normal medium. The cells from the zero time point were harvested, and the remaining dishes were refed with either control medium or 10^{-7} M RA plus cAMP (DM). At 24, 48, 72, and 96 h, the cells were counted. These data are presented as the mean of triplicate dishes. *, $p < 0.005$ (means were determined to be significantly different from F9 control cells by ANOVA followed by Newman-Keuls multiple comparisons).

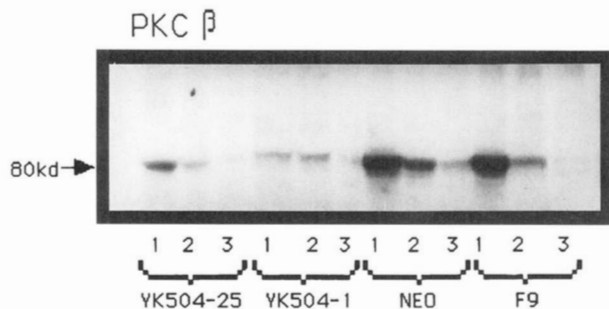


FIG. 6. PKC β protein levels in control and differentiated F9 clones. Proteins were extracted from untreated and differentiated clones and control cells (F9 and neomycin-resistant clones), and Western blot analysis was performed as described for Fig. 2. The cells were differentiated by a 5-day treatment with 10^{-7} M RA (lane 2) or with 10^{-7} M RA plus 0.5 mM 8-Br-cAMP/0.2 mM IBMX (lane 3). Untreated samples are shown in lane 1. The relative amount of PKC β in the various cells treated with or without maturational agents was determined by densitometric analysis of the autoradiogram.

neomycin control cells (Fig. 7). Type IV collagen mRNA was constitutively expressed in clone YK504-1 and was induced only 1.4-fold during 72 h of RA + cAMP treatment. Although type IV collagen mRNA was not constitutively expressed in YK504-25 cells, the message was induced more quickly than in neomycin-resistant cells, with the maximum amount of type IV collagen mRNA achieved within 24 h of treatment.

The expression of *c-jun* and *c-myc* proto-oncogenes have been shown to change with differentiation. *c-myc* mRNA levels have been shown to decrease within 24 h of RA + cAMP treatment in F9 cells (5). *c-myc* mRNA is present in the PKC α -overexpressing clones at a level similar to wild type cells, and its rate of decrease does not appear to be different than in F9 control cells (data not shown). The proto-oncogene *c-jun* is induced in differentiated F9 cells (3, 24). In neomycin-resistant cells, *c-jun* mRNA was barely detectable until 72 h of RA+cAMP treatment. At this time, *c-jun* mRNA was induced by 8–9-fold (Fig. 8). Within 24 h of treatment of clones YK504-1 and YK504-25 with the differentiating agents, *c-jun* mRNA was induced to

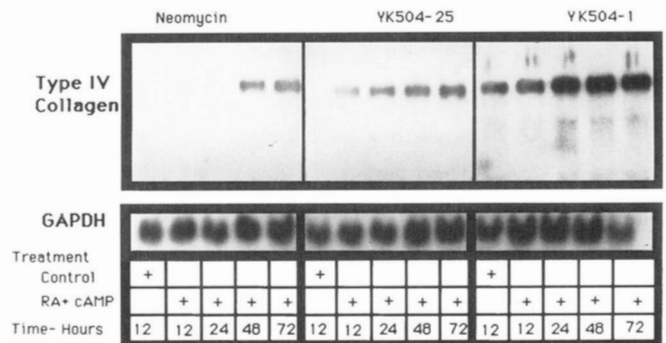


FIG. 7. Temporal expression of type IV collagen RNA during differentiation of F9 clones. PKC α clones YK504-1 and YK504-25 and neomycin-resistant cells were either untreated or treated with differentiation medium containing RA and cAMP/IBMX for 12, 24, 48, or 72 h. RNA was isolated and analyzed by Northern blotting. The RNA was hybridized to a type IV collagen ³²P-labeled cDNA. The blots were exposed to film for 1–7 days. The amount of glyceraldehyde-3-phosphate dehydrogenase RNA serves as a control for equal loading and transfer of RNA.

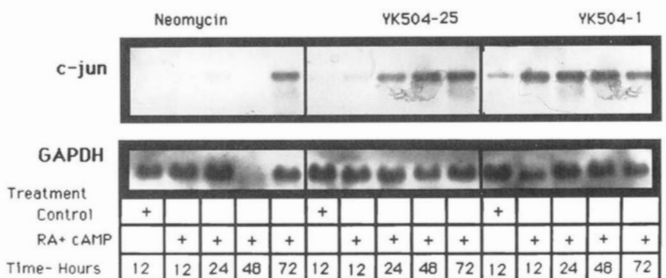


FIG. 8. Induction of *c-jun* mRNA in F9 clones. A neomycin-resistant clone and the two PKC α -overexpressing clones were incubated with or without 0.1 μ M RA + 0.5 mM 8-Br-cAMP/0.2 mM IBMX for the indicated times. RNA from these cells was analyzed for *c-jun* expression by Northern blotting. Each lane contained 20 μ g of RNA. Hybridization with the cDNA for glyceraldehyde-3-phosphate dehydrogenase was used to correct for equal loading and specificity of RNA changes. The relative amount of *c-jun* mRNA was determined by computerized densitometric analysis of the autoradiogram correcting for the amount of glyceraldehyde-3-phosphate dehydrogenase.

near-maximal levels. The *c-jun* message in the PKC α -overexpressing clones remained at this high level for the duration of the treatment period.

DISCUSSION

In this study we have shown that F9 teratocarcinoma stem cells express at least three different isoforms of PKC. As the cells are induced to differentiate, PKC α mRNA and protein levels increase, while PKC β and PKC γ mRNA and PKC β protein levels decrease. There have been several studies relating PKC expression to differentiation of F9 cells. Kraft and Anderson (25) could not find any PKC enzyme activity in F9 stem cells; however, when these cells were treated with 0.1 μ M RA, a time-dependent increase in cytosolic PKC enzyme activity was observed. These data were confirmed by Snoek *et al.* (26), who also demonstrated that RA induced about a 5-fold increase in phorbol ester receptors (suggesting an increase in the amount of PKC). It should be noted that in both of these studies, cAMP was not added to the cells, and therefore the cells differentiated into primitive endoderm but not parietal endoderm. There is a paucity of information regarding changes in PKC isotype expression during embryogenesis. Spasi *et al.* (27) analyzed human brain RNA and found that expression of PKC α and PKC β genes could be detected as early as 6 weeks postconception. However, the PKC γ gene was not expressed during embryonic and early fetal development but was expressed abundantly in neonates. Yoshida *et al.* (28) confirmed the above findings for

rat brain development. They found that PKC γ was not expressed until later in development (3 weeks of gestation).

Many tissues have been found to express novel PKCs, *i.e.* those isoforms that do not require calcium for activity (ϵ , δ , ζ , η , λ , and θ) (29–32). It was not determined in this study whether any novel PKCs are expressed in F9 cells and whether their expression changes as a function of differentiation.

Because the transcript level for all three PKC isoforms is very low in F9 stem cells, we were not able to address the mechanism by which RA and RA + cAMP regulate the change in mRNA expression of these genes. It appears unlikely that induction of PKC α is a direct response to RA, because the change in PKC α protein levels occurs relatively late (24 h). In B16 melanoma cells, which also respond to RA treatment by inducing PKC α , it was shown that the effect of RA required new protein synthesis and was primarily regulated at the post-transcriptional level (33).

In light of the differential regulation of PKC isoform expression during F9 differentiation, we decided to examine the role of PKC α by establishing stable F9 transfectants that overexpress PKC α . Two clones were isolated that express 4.7 and 5.4 times more PKC α than wild type stem cells. Examination of a variety of differentiation markers (morphology, type IV collagen, PKC β , c-Myc, and c-Jun) revealed that some of the markers in the PKC α -overexpressing clones were either constitutively expressed, or the changes in expression were induced more rapidly than in either wild type or neomycin-resistant clones treated with RA + cAMP. However, differentiation-induced changes in other markers including pS16–135 (data not shown), c-Myc, and morphology were not markedly different from wild type cells. We do not believe that the changes described in the PKC α transfectants were due to clonal selection, since both PKC α -overexpressing clones, which were independently isolated, exhibited similar properties; indeed, the degree to which they expressed differentiation markers correlated with the level of PKC α overexpression. In addition, several independently isolated neomycin-resistant clones behave like wild type F9 cells in the expression of their differentiation-dependent markers.

Since PKC α was induced with differentiation and overexpressing clones had slower growth rates than wild type cells, it can be postulated that the PKC α isoform plays an anti-proliferative role in F9 cells. It has been reported in several other cell lines that increases in PKC α are concomitant with growth inhibition and differentiation (33, 34). Overexpression of PKC α in B16 melanoma cells resulted in growth inhibition, induction of a differentiated phenotype, and a decrease in tumorigenicity (11). However, it is unlikely that all the changes in the differentiation markers found in the PKC α -overexpressing cells were due to a slower growth rate, since several studies have shown that inhibition of F9 cell growth is not sufficient to trigger differentiation (35, 36).

Both PKC β and PKC γ gene expression decrease during F9 differentiation. Since we were unable to find a commercially available PKC γ isotype-specific antibody that was sensitive and specific enough for use in the F9 system, we concentrated on PKC β . The 5' regulatory region of the human PKC β gene has been sequenced, and both positive and negative cis acting elements have been identified (37). One of the negative elements (N1) contains several AP1 binding sites with a 1-base mismatch. If mouse PKC β genes contain these same elements, it may be the N1 region that is responsible for the down-regulation of PKC β in F9 cells. PKC is known to stimulate the synthesis of c-Jun (38–40). c-Jun can bind either as a homodimer or heterodimer with c-Fos to AP1 DNA elements (41–47). Although previous reports have not determined which PKC

isoform is responsible for induction of c-Jun, we have shown in this report that overexpression of PKC α is sufficient to rapidly induce the expression of c-jun mRNA in F9 cells, in response to differentiation medium (Fig. 8). Treatment of the cells with differentiation medium may allow for the activation of PKC, which has already accumulated in the overexpressing clones, and this may cause the early induction of c-Jun. This increase in c-Jun may be responsible for binding to the negative AP1-containing element in the PKC β gene, thus causing its down-regulation.

PKC β induction or overexpression is correlated with differentiation and growth inhibition in some cell lines (48) and associated with growth and transformation in others (9). PKC β expression has been shown to be developmentally regulated in rats, with levels increasing after birth (28). In both differentiated wild type F9 cells and in PKC α -overexpressing cells, PKC β expression is down-regulated. Since both of these cell lines have slower growth rates than undifferentiated cells, it is possible that PKC β helps to maintain the stem cell state by increasing the expression of growth-associated genes. This hypothesis could be tested experimentally by establishing F9 clones that overexpress PKC β . These cells should be at least partially resistant to induction of differentiation by RA + cAMP.

We conclude from this study that the induction of PKC α and the decrease in PKC β and PKC γ are important steps in the differentiation of F9 stem cells into parietal endoderm-like cells. The intricate balance between the steady-state levels of PKC α , β , and γ may serve to maintain the stem cell phenotype. As differentiation begins, PKC α is induced, which in turn appears to be a controlling element in the pathway that regulates the expression of certain differentiation-dependent genes. At the same time, PKC α , via induction of c-Jun, may also down-regulate PKC β expression, further distorting the balance between the isotypes required for maintaining stem cell proliferation.

REFERENCES

- Strickland, S. (1981) *Cell* **24**, 277–278
- Wang, S. Y., LaRosa, G. J., and Gudas, L. J. (1985) *Dev. Biol.* **107**, 75–86
- Yang-Yen, H. F., Chiu, R., and Karin, M. (1990) *New Biol.* **2**, 351–361
- Solter, D., and Knowles, B. B. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 5565–5569
- Finklestein, R., and Weinberg, R. A. (1988) *Oncogene Res.* **3**, 287–292
- Levine, R. A., LaRosa, G. J., and Gudas, L. J. (1984) *Mol. Cell. Biol.* **4**, 2142–2150
- Nishizuka, Y. (1992) *Science* **258**, 607–614
- Ohno, S., Kawasaki, H., Imajoh, S., Suzuki, K., Inagaki, M., Yokokura, H., Sakoh, T., and Hikida, H. (1987) *Nature* **325**, 161–166
- Housey, G. M., Johnson, M. D., Hsiao, W. L. W., O'Brian, C. A., Murphey, J. P., Kirchmeier, P., and Weinstein, I. B. (1988) *Cell* **52**, 343–354
- Persons, D. A., Wilkison, W. O., Bell, R. M., and Finn, O. J. (1988) *Cell* **52**, 447–458
- Gruber, J. G., Ohno, S., and Niles, R. (1992) *J. Biol. Chem.* **267**, 13356–13360
- Kuo, J. F., Shoji, M., Kiss, Z., Girard, P. R., Deli, E., Oishi, K., and Vogler, W. R. (1989) *Adv. Exp. Med. Biol.* **255**, 8–28
- Otte, A. P., Kramer, I. M., and Durston, A. J. (1991) *Science* **251**, 570–573
- Ohno, S., Akita, Y., Konno, Y., Imajoh, S., and Suzuki, K. (1988) *Cell* **53**, 731–741
- Takebe, Y., Seiki, M., Fujisawa, J., Hoy, P., Yokota, K., Arai, K., Yoshida, M., and Arai, N. (1988) *Mol. Cell. Biol.* **8**, 446–472
- Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., pp. 1630–1636, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Chomczynski, P., and Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- Cathala, G., Savouret, J. F., Mendez, B., West, B. L., Karin, M., Martial, J. A., and Baxter, J. D. (1983) *DNA (N.Y.)* **2**, 329–335
- Aviv, H., and Leder, P. (1972) *Proc. Natl. Acad. Sci. U. S. A.* **69**, 1408–1412
- Parker, P. J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M. D., and Ullrich, A. (1986) *Science* **233**, 853–859
- Stanton, L. W., Watt, R., and Marcu, K. B. (1983) *Nature* **303**, 401–406
- Tso, J. Y., Sun, X. H., Kao, T., Reece, K. S., and Wu, R. (1985) *Nucleic Acids Res.* **13**, 2485–2502
- Goldstein, B., Rogelj, S., Siegel, S., Farmer, S. R., and Niles, R. M. (1990) *J. Cell. Physiol.* **143**, 205–212
- deGroot, R. P., Schoorlemmer, J., van Genesen, S. T., and Kruijer, W. (1990) *Nucleic Acids Res.* **18**, 3195–3202
- Kraft, A. S., and Anderson, W. B. (1983) *J. Biol. Chem.* **258**, 9178–9183
- Snoek, G. T., Mummery, C. L., van der Brink, C. E., van der Saag, P. T., and deLaat, S. W. (1986) *Dev. Biol.* **115**, 282–292

27. Sposi, N. M., Bottero, L., Cossu, G., Russo, G., Testa, U., and Peschle, C. (1989) *Mol. Cell. Biol.* **9**, 2284–2288
28. Yoshida, Y., Huang, F. L., Nakabayashi, H., and Huang, K. P. (1988) *J. Biol. Chem.* **263**, 9868–9873
29. Bacher, N., Zisman, Y., Berent, E., and Livneh, E. (1991) *Mol. Cell. Biol.* **11**, 126–133
30. Koide, H., Ogita, K., Kikkawa, U., and Nishizuka, Y. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 1149–1153
31. Ogita, K., Miyamoto, S., Yamaguchi, K., Koide, H., Fujisawa N., Kikkawa, U., Sahara, S., Fukami, Y., and Nishizuka, Y. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 1592–1596
32. Powell, C., Leng, L., Dong, L., Kiyokawa, H., Busquets, X., O'Driscoll, K., Marks, P. A., and Rifkind, R. A. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 147–151
33. Rosenbaum, S. E., and Niles, R. (1992) *Arch. Biochem. Biophys.* **294**, 123–129
34. Tanaka, Y., Yoshihara, K., Tsuyuki, M., Itaya-Hironaka, A., Inada, Y., and Kamiya, T. (1992) *J. Biochem. (Tokyo)* **111**, 265–271
35. Campisi, J., Gray, H. E., Pardee, A. B., Dean, M., and Sonenshein, G. E. (1984) *Cell* **36**, 241–247
36. Strickland, S., and Mahdavi, V. (1978) *Cell* **15**, 393–403
37. Niino, Y. S., Ohno, S., and Suzuki, K. (1992) *J. Biol. Chem.* **267**, 6158–6163
38. Hallahan, D. E., Virudachalm, S., Beckett, M., Sherman, M. L., Kufe, D., and Weichselbaum, R. R. (1991) *Int. J. Radiat. Oncol. Biol. Phys.* **21**, 1677–1681
39. Mufson, R. A., Szabo, J., and Eckert, D. (1992) *J. Immunol.* **148**, 1129–1135
40. Rubin, E., Kharbanda, S., Gunji, H., Weichselbaum, R., and Kufe, D. (1992) *Cancer Res.* **52**, 878–882
41. Chiu, R., Boyle, W. J., Meek, J., Smeal, T., Hunter, T., and Karin, M. (1988) *Cell* **54**, 541–552
42. Halazonetis, T. D., Georopoulos, K., Greenberg, M. E., and Leder, P. (1988) *Cell* **55**, 917–924
43. Kouzarides, T., and Ziff, E. (1988) *Nature* **336**, 646–654
44. Nakabeppu, Y., Ryder, K., and Nathans, D. (1988) *Cell* **55**, 907–915
45. Rauscher, F. J., Cohen, D. R., Curran, T., Bos, T. J., Vogt, P. K., Bohmann, D., Tjian, R., and Franza, B. R. J. (1988) *Science* **240**, 1010–1061
46. Sassone-Corsi, P., Sisson, J. C., and Verma, I. M. (1988) *Nature* **334**, 314–319
47. Sassone-Corsi, P., Lamph, W. W., Kamps, M., and Verma, I. M. (1988) *Cell* **54**, 553–560
48. Choi, P. M., Tchou-Wong, K. M., and Weinstein, I. B. (1990) *Mol. Cell. Biol.* **10**, 4650–4657