Distinct Functions of Protein Kinase Ca and Protein Kinase Cβ during Retinoic Acid-induced Differentiation of F9 Cells

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Abstract
As F9 embryonal carcinoma cells differentiate into parietal endoderm-like cells, expression of conventional protein kinase C (PKC) changes. Undifferentiated stem cells express PKCβ but not PKCα, whereas differentiated parietal endoderm cells express PKCα but not PKCβ. To determine whether changes in PKCα and/or PKCβ expression control retinoic acid (RA)- and dibutyryl cyclic AMP-induced F9 cell differentiation, we established cell lines stably expressing PKCα, PKCβ, antisense PKCα, or antisense PKCβ RNAs. Constitutive expression of PKCα or inhibition of PKCβ expression in F9 stem cells enhanced RA-induced differentiation, both by increasing total expression and accelerating RA-induced expression of laminin A, B1, B2, and type IV collagen. In addition, expressing PKCβ in a parietal endoderm cell line caused these cells to retrodifferentiate into stem cells. Based on these results, we conclude that PKCβ and PKCα are key targets for RA-regulated gene expression, that PKCα plays an important role in inducing and maintaining the parietal endoderm phenotype, and that PKCβ activity is incompatible with maintaining the differentiated state of these cells.

Introduction
F9 mouse embryonal carcinoma cells have limited developmental potential and exhibit little spontaneous differentiation. When treated with RA, F9 cells differentiate into primitive endoderm-like cells (1, 2), which have the ability to respond to secondary differentiation factors that determine the ultimate differentiated phenotype. Exposure of these primitive endoderm-like cells to agents that elevate intracellular cAMP induces formation of parietal endoderm (1, 2). In the absence of cAMP, aggregates of RA-treated F9 cells differentiate into a cell type resembling the visceral endoderm of the mouse embryo (3, 4).

During F9 differentiation, RA profoundly alters the expression of intracellular signaling pathways. Notable among the reported changes is increases in the AP-1 transcription factor (5–9), increased total PKC activity (10–12), and increased levels of phorbol ester receptors (13). RA also increases the amount and alters the subcellular distribution of protein kinase A, changes that are believed to contribute to the ability of cAMP to induce parietal endoderm differentiation (14).

PKC is a calcium-, diacylglycerol-, and phospholipid-dependent enzyme involved in regulating differentiation and proliferation (15, 16). There are at least 11 distinct genes constituting the PKC family that can be grouped into three subfamilies based on sequence homology and cofactor requirement: the conventional PKCs (α, β1, β2, and γ) are calcium, diacylglycerol, and phospholipid dependent; the novel PKCs (δ, ε, θ, and μ) are diacylglycerol and phospholipid dependent but calcium independent, whereas the atypical PKCs (λ and υ) are phospholipid dependent but diacylglycerol and calcium independent (16). Tumor-promoting phorbol esters are potent diacylglycerol agonists that bind to and activate both conventional PKCs and novel PKCs but not atypical PKCs (17, 18). In addition to activating these enzymes, chronic treatment of cells with phorbol esters down-regulates conventional PKCs and novel PKCs (16).

The increased level of calcium-dependent PKC activity reported during RA- and cAMP-induced differentiation of F9 cells into parietal endoderm results from increased expression of PKCα (11, 12); concurrently, PKCβ expression declines. F9 stem cells and visceral endoderm cells express PKCβ but not PKCα, whereas F9 cells differentiating into parietal endoderm express PKCα but not PKCβ. The transition from PKCβ to PKCα is accompanied by changes in phorbol ester-induced gene expression. Specifically, phorbol ester induces c-fos expression in PKCβ-expressing F9 stem cells but not in PKCα-expressing parietal endoderm cells. In contrast, phorbol ester induces type IV collagen, a parietal endoderm marker gene, in PKCα-expressing parietal endoderm cells.

To establish a functional role for PKCα and PKCβ in mediating parietal endoderm differentiation, we established F9-derived cell lines constitutively expressing PKCα, PKCβ, antisense PKCα, or antisense PKCβ RNAs. Constitutive expression of PKCα in stem cells was sufficient to allow phorbol ester induction of type IV collagen even in the absence of...
RA and cAMP. Furthermore, PKCα expression both accelerated and increased the total expression of laminin A/B1/B2 and type IV collagen in response to RA and cAMP. Antisense PKCα or PKCβ expression decreased RA and cAMP induction of these parietal endoderm markers and alternatively enhanced the expression of visceral endoderm marker genes. In addition, expressing PKCβ in a parietal endoderm cell line gradually caused these cells to retrodifferentiate into stem cells. These results indicate that PKCα is critical in establishing and maintaining the parietal endoderm phenotype, and furthermore, that PKCβ is incompatible with maintaining the differentiated state of these cells. PKCβ expression is associated with maintaining stem cell proliferation and inducing visceral endoderm formation.

Results

Constitutive Expression of PKCβ and PKCα after Stable Transfection of F9 Cells. F9 cells were cotransfected with a plasmid encoding hygromycin resistance and expression plasmids encoding either PKCβ, antisense PKCβ, PKCα, or antisense PKCα cDNA. Expression in each case was controlled by the β-actin promoter, which is expressed in both differentiated and undifferentiated F9 cells (11). Pools of hygromycin B-resistant colonies were isolated. As shown in Fig. 1 and Table 1, when compared with F9 stem cells expressing only the hph gene (H; conferring hygromycin B resistance), expression of PKCβ from the β-actin promoter elevated total PKCβ levels in stem cells by 2.2-fold and allowed continued PKCβ expression following RA + cAMP treatment. Expression of antisense PKCα RNA decreased steady-state PKCβ levels in F9 stem cells by 50% (Fig. 1, Lane 3). Transfection with the PKCα-expressing plasmid resulted in the constitutive expression of PKCα in stem cells. Induction of the endogenous PKCα gene by RA + cAMP was unaffected in cells transfected with PKCβ, antisense PKCβ, or PKCα. Constitutive expression of antisense PKCα RNA partially prevented RA + cAMP-induced PKCα expression (by 70%). Endogenous expression of other PKC isoforms such as PKCγ, δ, ε, and ζ was not altered in any of these cells (data not shown). As we reported previously (11), differentiation-induced changes in PKCβ and PKCα reflect altered mRNA levels (data not shown). Experimental manipulation of PKCβ (either up or down) had little effect on PKCα expression (mRNA or protein). In contrast, expression of PKCα in F9 stem cells decreased PKCβ expression. F9 stem cells transfected with the PKCα plasmid had no detectable PKCβ (Fig. 1, Lane 4). The negative effect of PKCα on PKCβ expression was confirmed in cells expressing antisense PKCα RNA. When these cells were induced to differentiate with RA + cAMP, PKCα expression remained low, and the loss of PKCβ expression was incomplete (Fig. 1, Lane 10). In contrast, constitutive expression of PKCβ did not prevent PKCα expression in differentiating F9 cells (Fig. 1, Lane 7).

PKCβ Enhanced c-fos Expression and PKCα Allowed Phorbol Ester-activated Expression of Collagen IV in F9 Stem Cells. The transition from PKCβ to PKCα in differentiating F9 cells is accompanied by changes in phorbol ester stimulation of target gene expression (11). Specifically, phorbol ester induced c-fos expression in PKCβ-expressing stem cells but not in PKCα-expressing parietal endoderm cells. In contrast, phorbol ester induced type IV collagen, a parietal endoderm marker gene, in F9 stem cells transfected with and expressing PKCα. The causal relationship between PKCβ activity and c-fos expression and between PKCα activity and collagen IV expression was supported by altering expression of PKCβ or PKCα in F9 cells independently of RA + cAMP treatment (Fig. 2 and Table 2). PKCβ expression increased basal expression of both c-fos and c-jun to levels equal to (c-fos) or greater than (c-jun) seen following phorbol ester treatment of F9 stem cells (Table 2; hybridization intensities in each sample were normalized to the L-30 ribosomal protein mRNA control). Decreasing PKCβ by expressing antisense PKCβ RNA had the opposite effect; decreasing both basal and phorbol ester induced expression of c-fos and c-jun. Changes in c-fos/c-jun expression resulted specifically from changes in PKCβ and did not simply reflect increased total PKC activity (11). Expression of comparable levels of PKCα caused little or no alteration of basal c-fos and c-jun expression in F9 stem cells (Fig. 2, Lane 4). Although these results support a link between PKCβ activity and c-fos expression in stem cells, constitutive expression of

Table 1. Relative levels of PKCα and PKCβ expression in transfected F9 cells

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*ND, not detected.
PKCα in RA + cAMP-treated F9 cells did not restore c-fos expression in response to phorbol ester (Fig. 2, Lane 7).

Steady-state c-jun mRNA levels were ~5-fold higher in RA + cAMP-treated, hygromycin-resistant cells than in stem cells (Fig. 2, Lane 1 versus Lane 6; Table 2). Although phorbol ester treatment had only a modest effect on c-jun expression, increasing PKCβ expression resulted in elevated c-jun mRNA, both in stem cells and in differentiated cells (Fig. 2, Lanes 2 and 7; Table 2, 8.2- and 17-fold). PKCα expression in stem cells had no effect on c-jun expression but was associated with substantially increased c-jun expression in RA + cAMP-treated cells (Table 2, 20.7-fold), an effect reversed by antisense PKCα RNA (Table 2, 1.9-fold). Paradoxically, whereas antisense PKCβ RNA expression lowered c-jun levels in stem cells, it actually increased expression in differentiating cells (Table 2, 25.8-fold; an additional 5-fold over the effect of RA + cAMP).

The most plausible explanation for this result is that decreasing PKCβ (and increasing PKCα) enhances the differentiation of F9 cells in response to RA + cAMP. This conclusion was supported by measuring type IV collagen mRNA as a marker of parietal endoderm differentiation. Previously, we demonstrated that expressing PKCα in F9 stem cells resulted in phorbol ester-induced collagen IV expression in the absence of RA-induced differentiation (11). A similar effect of PKCα expression was seen in these experiments (Fig. 2, Lane 4; Table 2, 1.1-fold over basal expression of RA + cAMP-treated F9 cells), and antisense PKCα expression prevented RA + cAMP induction of collagen IV expression (Fig. 2, Lane 10). Further treatment of phorbol ester in differentiated cells with RA + cAMP down-regulated collagen expression in general. This experiment also established a striking antagonistic effect of PKCβ on collagen IV expression. Collagen IV mRNA was induced only slightly by RA + cAMP in cells constitutively expressing PKCβ (Table 2, 0.3-fold), whereas antisense PKCβ enhanced (Table 2, 2.4-fold) the response to RA + cAMP. In this respect, decreasing PKCβ levels (Table 2, 2.4-fold) or increasing PKCα (Table 2, 3.4-fold) had the similar effects on collagen expression.

PKCα Enhanced RA and cAMP-induced Differentiation of F9 Cells into Parietal Endoderm. To determine whether the antagonistic interaction between PKCβ and PKCα was limited to the collagen IV promoter or was an important component of parietal endoderm differentiation, we measured the expression of other differentiation markers in these cell lines. We used metabolic labeling and SDS-PAGE to examine the expression of basement membrane proteins, laminin A, B1/B2, and type IV collagen (Fig. 3) in the cell lines expressing various levels of PKCα and PKCβ. In addition, we measured the induction of the visceral endoderm marker, TTR (20), by RNase protection (Fig. 4).
**A. 5 day treatment**

![Diagram A](image)

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**B. 3 day treatment**

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**Fig. 3.** PKCo or antisense PKCβ expression enhances extracellular basement membrane protein synthesis in differentiated F9 cells. Basement membrane synthesis in hygromycin-resistant control (h), PKCβ (β), antisense PKCbeta (Aβ), PKCo (α), and antisense PKCo (Aα) transfected F9 cells was analyzed by resolving conditioned media (2.2 x 10^6 trichloroacetic acid insoluble cpm/lane) from radiolabeled cells on SDS-PAGE (5%). F9 cells were treated with either ethanol solvent (-RA -cAMP), 5 x 10^-4 m dbcAMP (+cAMP), 2 x 10^-4 m all-trans RA (+RA), or both (+RA +cAMP) for 5 days (A) or 3 days (B). Cells were labeled with ^35S)methionine (50 μCi/ml) for the final 16-20 h. Right, relative migration of molecular weight standards (in thousands). Parietal endoderm differentiation is characterized by the expression of laminin A (M, ~400,000), laminins B1 and B2, and type IV collagen (which all run at about M, 200,000).

**Fig. 4.** PKCo and antisense PKCβ expression inhibit expression of TTR, a visceral endoderm marker. F9 cells were grown as aggregates in suspension for 8 days. Total RNA was isolated, and the expression of the visceral endoderm markers TTR, retinol binding protein (data not shown; results same as TTR), and L-30 (as internal control) was measured in RNAase protection assays. Lane 1, parental F9 control cells (C); Lane 2, hygromycin-resistant control cells (h); Lanes 3-8, parental F9 control (C), hygromycin-resistant control (H), PKCβ (β), antisense PKCβ (Aβ), PKCo (α), and antisense PKCo (Aα) transfected cells treated with 2 x 10^-8 m all-trans RA (+RA). Right, relative migration of molecular weight standards (nucleotides).

The overall pattern of basement membrane protein synthesis (Fig. 3) paralleled that reported above for the type IV collagen mRNA (Fig. 2). After 5 days of treatment with either RA or RA and dbcAMP, in cells constitutively expressing PKCo, laminin and collagen expression were elevated relative to control cells (Fig. 3A, Lanes 3 and 4 compared with Lanes 15 and 16). This was more apparent at 3 days (Fig. 3B, compare Lanes 2 and 6). Similar results were seen in cells in which PKCβ expression was reduced by antisense RNA expression (Fig. 3A, Lanes 11 and 12; Fig. 3B, Lanes 3 and 4) but not as great as increasing PKCo expression. Although overexpression of PKCβ or reduced PKCo did not prevent basement membrane protein synthesis, in both cell lines the response to RA and cAMP was reduced relative to the control cells. Therefore, PKCo expression is an integral component for parietal endoderm differentiation in general and not only for the expression of type IV collagen.

**F9 cells are bipotential.** When aggregated and grown in the presence of RA, F9 cells acquire a visceral endoderm phenotype. The change in PKC expression seen during parietal endoderm formation does not occur during visceral endoderm formation (3, 11). By measuring TTR mRNA levels, we determined the ability of RA-treated aggregates of F9 cells transfected with PKCβ, antisense PKCβ, PKCo, or antisense PKCo to acquire a visceral endoderm phenotype (20). Increasing PKCo or decreasing PKCβ levels inhibited the RA-induced synthesis of TTR (Fig. 4, Lanes 6 and 7). In contrast, increased expression of PKCβ enhanced the expression of TTR in RA-treated F9 aggregates (Fig. 4, Lane 5).

**PKCβ Induced the Gradual Retrodifferentiation of the Parietal Endoderm Cell Line F9RA5.** The above results strongly support a role of PKCo signaling in F9 parietal endoderm differentiation that is distinct from, and most likely antagonistic to, the role of PKCβ signaling. To provide more direct evidence for a negative role of PKCβ in parietal
Endoderm differentiation, we transfected the parietal endoderm cell line, F9RA5, with the PKCβ expression plasmid. With the exception of their ability to proliferate indefinitely, the F9RA5 cells retain all morphological and biochemical characteristics of RA + cAMP-treated F9 cells, including the appropriate PKC expression pattern (Ref. 11; morphological similarity in Fig. 5, A and F). F9RA5 cells were co-transfected with plasmids encoding hygromycin (hph) resistance and either expression plasmids encoding PKCβ and PKCα. Pools of hygromycin-resistant colonies were expanded and frozen. After thawing, transfected F9RA5 cells were grown in media containing 50 μg/ml hygromycin. F9RA5 cells expressing only the hph gene or hph gene + PKCα together retained the morphology of parental F9RA5 cells (data not shown) at all passages. However, F9RA5 cells expressing PKCβ underwent a morphological transition. These cells maintained their parietal endoderm phenotype for passages 1–4 (Fig. 5B). Between passages 5 and 10, these cells underwent a morphological transition, such that around the 11th passage, the entire culture had reacquired a stem cell phenotype (Fig. 5, C and D; morphological similarity in Fig. 5, D and E). By all measurable criteria, the transition was complete. After the 11th passage, PKCβ-expressing F9RA5 cells no longer expressed PKCα (Fig. 6A, Lane 3), type IV collagen (Fig. 6B, Lane 3), or laminins A/B1/B2 (Fig. 6C, Lane 3). In contrast, PKCβ and c-fos were highly expressed. When the phenotypically reverted cells (PKCβ ex-

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**Fig. 5.** PKCβ induces parietal endoderm cells to retrodifferentiate. Exponentially growing F9RA5 control (A), PKCβ-transfected F9RA5 in 3rd passage (B), 7th passage (C), and 11th passage (D), F9 control (E), and F9 cells treated with RA + cAMP for 5 days (F) were photographed using an inverted microscope (×40).

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**Fig. 6.** Parietal endoderm marker gene expression is lost in PKCβ-expressing F9RA5 cells. A, PKCα and PKCβ expression in PKCβ-transfected F9RA5 cells. Whole-cell protein extract (30 μg/lane) from control (C), hygromycin-resistant control (H), and PKCβ-transfected F9RA5 cells (β; in 11th passage) was analyzed for PKCα and PKCβ expression using immunoblotting as described in "Materials and Methods." In Lanes 4–6, cells were treated with 2 × 10⁻⁷ M all-trans RA and 5 × 10⁻⁴ M dbcAMP (+RA/cAMP) for 7 days. B, total RNA (20 μg/ lane) from control (C), hygromycin-resistant control (H), and PKCβ-transfected F9RA5 cells (β; in 11th passage) was analyzed for L-30; collagen IV, c-fos, and c-jun expression using Northern blotting. In Lanes 4–6, cells were treated with 2 × 10⁻⁷ M all-trans RA and 5 × 10⁻⁴ M dbcAMP (+RA/cAMP) for 7 days. L-30 is mRNA expression control. C, basement membrane synthesis in control (C), hygromycin-resistant control (H), and PKCβ-transfected F9RA5 cells (β; in 11th passage) was analyzed for protein expression of laminin A, B1, B2, and type IV collagen as described in "Materials and Methods." In Lanes 4–6, cells were treated with 2 × 10⁻⁷ M all-trans RA and 5 × 10⁻⁴ M dbcAMP (+RA/cAMP) for 7 days.
pressing F9RA5 after 11th passage) were treated with RA and cAMP. PKCβ expression was suppressed, and the expression of PKCα, type IV collagen, and the parietal endoderm phenotype was regained (Fig. 6, Lanes 6). Although PKCα expression was lost in PKCβ-expressing F9RA5 cells, PKCα expression was promptly induced by RA and cAMP treatment (Fig. 6A, Lane 6). c-jun was constantly expressed in F9RA5 cells and was slightly elevated after RA + cAMP treatment, suggesting that PKCβ expression in F9RA5 cells does not affect c-jun expression (Fig. 6B).

Discussion
In this study, we demonstrated that PKCα expression enhanced RA- and cAMP-induced differentiation of F9 mouse embryonal carcinoma cells into parietal endoderm. F9 cells that constitutively express PKCα showed an accelerated response to RA and cAMP in terms of synthesis and secretion of the basement membrane components, laminin A, B1/B2, and type IV collagen. These cells also failed to express TTR after aggregation in the presence of RA. Blocking the RA-induced expression of PKCα with antisense PKCα RNA substantially reduced induction of the parietal endoderm but not the visceral endoderm phenotype. Therefore, PKCα is critical for F9 cell differentiation and for determining which developmental pathway is used by these cells.

PKC represents a family of 11 distinct genes that show tissue and developmental stage-specific expression (16). The complexity of the PKC gene family and the specific expression patterns of members of this family led to the prediction that different PKC isoforms serve distinct, nonredundant functions. In general, PKC regulates growth and differentiation of a diverse group of cell types. In a number of these cases, induction of differentiation correlates with the differential activation of a specific PKC isozyme. Induction of PKCα expression by RA is not unique to F9 cells but also has been reported in vascular smooth muscle cells (21), B16 melanoma cells (22), NT-2 human teratocarcinoma cells (23), and human breast cancer cells (24). PKCα induction by RA (11, 21–24) appears to be a central component of differentiation in multiple cell types.

The mechanism(s) by which PKCα participates in F9 cell differentiation remains to be determined. The increase in steady-state PKCα should increase the basal level of kinase activity leading to increased phosphorylation of cellular substrates that induce the expression of parietal endoderm differentiation markers. Maciaszek et al. (25) and Tahayato et al. (26) demonstrated that phorbol esters and retinoids synergistically activate transcription of multiple promoters, possibly as a result of RA receptor phosphorylation by PKC. Phosphorylation of RA receptors increases their DNA binding activity and transcriptional activation (27, 28). The alteration of the equilibrium between phosphorylation and dephosphorylation of steroid/thyroid hormone receptor family members is proposed to be a major regulator of the balance between cell growth and differentiation (27, 29).

An alternative mechanism for the PKCα induction of F9 cell differentiation involves transcriptional regulation of parietal endoderm marker genes through phorbol ester response elements. Phorbol ester, by activating either conventional or novel PKCs, increases the expression of genes containing TPA-responsive DNA elements in their promoter region (30). TPA-responsive DNA elements are bound by AP-1 transcription factors consisting of either homodimers of Jun family members or heterodimers of Jun and Fos family members (30). The RA- and cAMP-induced differentiation of F9 cells is accompanied by a large increase in AP-1 activity (9) and an accumulation of c-jun mRNA (Fig. 2; Ref. 11). Phorbol esters and AP-1 activity are implicated in the activation of c-jun and laminin B2 gene transcription (9, 31). The transcriptional activation of parietal endoderm marker genes such as the laminins and type IV collagen by RA is indirect, requiring prior new protein synthesis (1, 2, 4, 32). The identity of the new RA-induced gene products that subsequently mediate parietal endoderm gene expression remains unclear. Based on the data presented here, PKCα is a strong candidate for one of these RA target genes.

The second major observation in this study is that PKCβ expression is incompatible with maintenance of the parietal endoderm state. This is supported by three results: (a) PKCβ expression is specifically repressed during parietal endoderm but not during visceral endoderm differentiation (11); (b) reduction of PKCβ with antisense RNA accelerates the response of F9 cells to RA and cAMP (Figs. 2 and 3); and (c) reexpression of PKCβ in the parietal endoderm cell line F9RA5 results in these cells reacquiring a stem cell phenotype (Figs. 5 and 6).

How PKCβ accomplishes this is not clear. Several studies have linked PKCβ positively with cell proliferation (19, 33). PKCβ is expressed in both F9 stem cells and visceral endoderm cells that continue to proliferate but not in parietal endoderm cells that exit the cell cycle. F9RA5 cells, although they morphologically and biochemically resemble parietal endoderm cells, have retained the ability to proliferate. F9RA5 cells do not show the same degree of uncontrolled proliferation as the F9 stem cell, and appear to have distinctly different regulation of cell cycle progression (including altered cyclin D expression and Rb phosphorylation). Overexpression of PKCβ in rat fibroblasts decreases their sensitivity to growth-inhibitory signals and increases their sensitivity to oncogenes (19). We have shown that PKCβ is required for phorbol ester induction of c-fos in F9 cells, and that increasing PKCβ levels increases c-fos expression, even in the absence of phorbol ester induction. This was especially apparent in the retrodifferentiated F9RA5 cells expressing PKCβ. These data support the hypothesis that PKCβ, possibly by deregulating c-fos expression, is critical in maintaining the stem cell phenotype including the characteristic uncontrolled proliferation.

Materials and Methods
Plasmid. The antisense PKCβ plasmid (pβ-actin SP72-As-PKCβ) was constructed by ligating the 2.3-kb EcoRI fragment of PKCβ cDNA from pJ6-PKCβ (19) into the unique EcoRI sites of the pβ-actin SP72 vector. The antisense orientation was confirmed by SmaI restriction digestion. The

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sense and antisense PKCa plasmids (pβ-actin SP72-PKCa and pβ-actin SP72-As-PKCa) were constructed by ligating the 2.4-kb EcoRI fragment of PKCa cDNA from pcMV7-PKCa into the unique EcoRI site of the pβ-actin SP72 vector. The orientation was confirmed by BglII restriction digestion.

**Cell Culture.** The F9 embryonal carcinoma cells (ATCC CRL 1720) were grown on gelatin-coated tissue culture dishes in DMEM supplemented with 10% fetal bovine serum (Upstate Biotechnology, Inc., Saranac Lake, NY). Differentiation was induced by treating monolayers with $2 \times 10^{-7} \text{M RA}$ and $5 \times 10^{-6}$ M dbcAMP (to induce parietal endoderm) or treating aggregates plated on bacteriological Petri dishes with $2 \times 10^{-6} \text{M RA}$ (to induce visceral endoderm formation). The cell line displaying stable parietal endoderm morphology and biochemical properties, F9RA5, was isolated as described previously (11).

**Stable Transfection.** F9 embryonal carcinoma cells were grown in DMEM containing 10% fetal bovine serum, and subconfluent cultures were cotransfected with a hygromycin resistance plasmid (p thygro having hph cDNA) and expression plasmids encoding either PKCB (19), antisense PKCB (As PKCB), PKCa, or antisense PKCa (As PKCa) by DNA-calcium phosphate coprecipitation (34). Following transfection, cells were subjected to hygromycin B selection (150 μg/ml) over 4 weeks. Resistant cells were maintained in DMEM, 10% fetal bovine serum, and 50 μg/ml hygromycin B.

**Western Blot Analysis for PKC Isozyme Expression.** Whole-cell protein extracts were prepared with lysis buffer [20 mM Tris (pH 8), 150 mM NaCl, 10 mM sodium phosphate, 100 mM sodium vanadate, 100 mM ammonium molybdate, 10% glycerol, 1% NP40, and 0.1% SDS] as described previously (24). The protein extracts (30 μg/lane) were separated by SDS-PAGE (10%). Following electrophoretic transfer to Hybond-C extra nitrocellulose, membranes were blocked with 5% nonfat dry milk in PBS. PKC was detected by incubating the membrane with anti-PKC isozyme antibodies (0.5 μg/ml; affinity-purified polyclonal PKCa and PKCB antibodies; Life Technologies, Inc.), followed by extensive washing with PBST (0.05% Tween 20 in PBS) and subsequent incubation with peroxidase-conjugated secondary antibody (1:7500 dilution). PKC bands were visualized by enhanced chemiluminescence (Amer sham Corp.).

**RNA Isolation and Northern Blotting.** The levels of specific RNAs were determined using Northern blots of total RNA isolated as described previously (24). In every case, filters were hybridized to 32P-labeled RNA probes. Probes for L-30, c-fos, c-jun, collagen, and TTR have been described (11, 35). PKCa and PKCB RNAs were detected with riboprobes generated from human cDNA cloned into pSP72 vector. After washing in $2 \times $ SSC/1% SDS and $0.2 \times $ SSC/1% SDS, the Northern blots were exposed to X-ray film at $-80^\circ \text{C}$ with intensifying screens, and the band intensity was quantified by densitometry.

**Analysis of Basement Membrane Protein Synthesis.** Cells were labeled overnight with 35S-methionine (50 μCi/ml; DuPont NEN). Synthesis of type IV collagen and laminin A, B1, and B2 were determined by separating 35S-labeled extracellular proteins on 5% acrylamide gels (SDS-PAGE). Gels were fixed, dried, and exposed to X-ray film at room temperature for 1 day.

**RNase Protection Assay.** TTR RNA levels were determined using an RNase protection assay (36). A 32P-labeled TTR RNA probe was synthesized from TTR cDNA clone. Accurately measured amounts (10 μg/lane) of total RNA were hybridized to the cRNA probe for 16 h at 45°C. Nonhybridized RNA and probe were removed by digestion with RNases A and T1. The hybridized RNA was subjected to separation on a denaturing 10% acrylamide gel. After fixing and drying, the gel was exposed to X-ray film with intensifying screen at $-80^\circ \text{C}$.

**References**