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Phenotypic Effects of Overexpression of $PKC_{\beta 1}$ in Rat Liver Epithelial Cells

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We have used a previously described retroviral expression vector pMV7-$PKC_{\beta 1}$ to develop derivatives of two rat liver epithelial cell lines, K16 and K22, that stably express about tenfold-higher PKC activity than control cells. Despite these high levels of PKC, these cells did not exhibit gross morphologic changes, anchorage-independent growth, or tumorigenicity. K16PKC-4 and K22PKC-2, two lines with the highest PKC enzyme activity, were studied further in terms of several responses to the phorbol ester tumor promoter TPA. When treated with 100 ng/ml of TPA, the control K16MV7 and K22MV7 cells displayed a slight change in morphology, whereas the K16PKC-4 and K22PKC-2 cells displayed a marked change in morphology. Northern blot analyses demonstrated that TPA induced increased levels of *fos, myc,* phorbin, and ODC RNAs in control K16MV7 and K22MV7 cells, with maximum induction occurring at about 0.5, 1, 8, and 8 h, respectively. In K16PKC-4 and K22PKC-2 cells, TPA induction of phorbin and ODC RNAs was markedly enhanced, but this was not the case for *myc* and *fos* RNAs. In addition, the levels of *myc* RNA were constitutively higher in both K16PKC-4 and K22PKC-2 cells than in the control cells. Taken together, these results provide direct evidence that PKC plays a critical role in modulating the expression of *myc,* phorbin, and ODC RNAs. On the other hand, overexpression of $PKC_{\beta 1}$ is not itself sufficient to cause cell transformation.

Key words: protein kinase C, TPA, cell transformation, gene expression

Protein kinase C (PKC) is a Ca^{2+}- and phospholipid-dependent serine/threonine protein kinase involved in mediating a wide variety of cellular responses to growth factors, hormones, neurotransmitters, and other modulators of cellular growth control [1–4]. PKC is also a high-affinity receptor for the phorbol ester tumor promoters as well as other agents possessing tumor-promoting activity [5]. The binding of the potent tumor promoter 12-O-tetradecanoyl phorbol-13-acetate (TPA) to PKC activates its kinase activity both in vivo and in vitro [6–8]. The activated enzyme can phosphorylate a number of diverse proteins, including the receptors for epidermal growth factor, insulin, interleukin-2, and transferrin; the Na^+, K^+-ATPase; the glucose transporter; the oncogene protein $pp60^{src}$; and many proteins that appear to play a role in cellular programs of gene expression that control cell proliferation and differentiation [2,3].

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To elucidate further the role of specific forms of PKC in modulating the expression of genes-related to cellular growth control, it would be desirable to generate cell lines that stably produce elevated levels of a specific isoform of this enzyme. Our laboratory has recently cloned a full-length cDNA encoding the β1 subspecies of PKC and inserted it into a retroviral expression vector to yield a construct designated pMV7-PKC$_{\beta 1}$ [9]. In the present study we have utilized this vector system to introduce by transduction this coding sequence into the rat liver epithelial cell lines K16 and K22. These cell lines provide a model system for studying cellular events occurring subsequent to activation of PKC$_{\beta 1}$ by tumor promoters and other agents in an epithelial cell system. We have examined the role of PKC in modulating growth control and the expression of *fos, myc,* phorbin, and ODC genes in these epithelial cells that stably overproduce high levels of PKC$_{\beta 1}$.

MATERIALS AND METHODS

Cells and Culture Conditions

K16 and K22 cells were originally established from the livers of Sprague-Dawley rats, and their properties have been previously described [10]. These cells were routinely cultured in a 1:1 mixture of Basal Minimum Eagle (BME) with Hanks' salts and F-12 Nutrient mixture (HAM) (GIBCO Laboratories, Grand Island, NY) supplemented with 5% fetal calf serum.

Isolation of Cell Lines Stably Overexpressing PKC$_{\beta 1}$

A retrovirus-derived cDNA expression vector designated pMV7 and a construct designated pMV7-PKC$_{\beta 1}$ containing a full-length cDNA encoding the β1 subspecies of PKC were transfected onto subconfluent ψ-2 cells by the calcium phosphate coprecipitation method [11,12]. After 48 h, the medium was harvested, filtered, and used to infect recipient subconfluent K16 and K22 cells with 2 μg/ml polybrene for 48 h. The cells were then trypsinized and replated in culture medium that contained 200 μg/ml of the neomycin derivative G418 (Geneticin). Resistant colonies were cloned and maintained in culture medium containing 50 μg/ml G418.

Purification and Assay of PKC Activity From Tissue Culture Cells

The total PKC activity (membrane-associated and cytosolic) present in cultured cells was determined after partial purification of cellular extracts, as described previously [9]. Briefly, the cell lysate was centrifuged, loaded onto a DEAE Sephacel column previously equilibrated with homogenization buffer, and eluted with 3 ml of homogenization buffer containing 0.5M NaCl. Total protein concentrations were determined by the method of Bradford [13].

The PKC activity present in the above-described partially purified cell extracts was assayed immediately after isolation. Total PKC activity was assayed by using as the phosphoacceptor substrate a synthetic peptide, R-K-R-T-L-R-R-L, corresponding to a region of the EGF receptor, at a final concentration of 100 μM [14]. The general method of assay has been published in detail [15].

RNA Isolation and Northern Blot Analysis

Total RNAs were isolated by the method of Chirgwin et al. [16]. The poly A$^+$ RNA fraction was then isolated by passage of this RNA through oligodeoxythymidylate

cellulose columns (Collaborative Research, Waltham, MA) [17]; 5-μg samples of poly A^+ or 20 μg of total RNA was analyzed by electrophoresis on 1% agarose gels containing 6% formaldehyde and transferred to Hybond-N hybridization transfer membranes (Amersham Corporation, Arlington Heights, IL). The membranes were then irradiated with UV light for 2–5 min. Hybridization to appropriate ^{32}P-labeled probes (see below) and autoradiography were performed according to Wahl et al. [18]. A poly A^- RNA sample was included in each gel to provide molecular size marker (5.0 and 2.0 kilobases). In order to visualize the markers and the amount of RNA present in each lane, the gels were stained with ethidium bromide. The ethidium bromide staining indicated that all lanes contained approximately equivalent amounts of RNA. The relative abundance of specific transcripts in the different lanes was determined by densitometric analysis of the autoradiographs.

Hybridization Probes

The following fragments were used: $PKC_{\beta 1}$, a 720-base Pst I fragment excised from a plasmid designated pS2-RP58 [19]; phorbin, a 730-base EcoR I fragment excised from pTPA-S1 plasmid [20]; c-*myc*, a 1.5-kilobase Pst I fragment excised from a pBR322 clone [21]; ODC, a 2.4-kilobase EcoR I–BamH I fragment excised from pmODC-1 plasmid [22]; v-*fos*, a 1.3-kilobase Bgl II–Pvu II fragment excised from pFBJ-2 plasmid [23]. The purified fragments were ^{32}P-labeled by nick translation [24].

Assays of Growth in Soft Agar

To assess growth in soft agar (anchorage independence), 5×10^4 cells were suspended in 2 ml of 0.3% Bacto-agar (Difco Laboratories, Detroit, MI) in BME + F12 (1:1) medium containing 5% fetal calf serum and overlaid above a layer of 5 ml of 0.5% agar in the same medium, on 60-mm petri dishes. The cells were then overlaid with 2 ml of 0.3% agar once a week. At the end of 30 days, colonies were stained with the vital stain 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolinium chloride hydrate (Sigma Chemical Co., St. Louis, MO) for 48 h at 37°C in an incubator with 5% CO_2 [25].

Nude Mouse Tumorigenicity Assay

Actively growing cells were trypsinized, washed twice with PBS, and resuspended at a concentration of 5×10^7/ml. Approximately 1×10^7 cells were injected subcutaneously into 4–8-week-old Balb/c nu/nu athymic nude mice (Harlan Sprague Dawley). Mice were examined once a week for tumor formation.

RESULTS

Cell Lines That Stably Overproduce PKC

The retrovirus-derived cDNA expression vector that was used for the present studies has been described previously [9]. The vector consists of a modified herpes simplex virus thymidine kinase (tk) promoter and the selectable marker gene *neo*. This structure maintains the functional integrity of the selectable marker without interfering with the expression of an inserted 5′ cDNA sequence. This expression vector has been demonstrated to efficiently transfer various cDNAs into recipient cells by virus-mediated passage, with subsequent high-level expression of the encoded protein [9,26,27].

The pMV7-PKC$_{\beta 1}$ containing the control PKC$_{\beta 1}$ cDNA sequence was transfected onto ψ-2 cells [12] by the calcium phosphate coprecipitation method. After 48 h, the culture medium was collected, filtered, and used to infect recipient subconfluent K16 and K22 rat liver epithelial cells. The cells were then trypsinized and replated in BME + F12 (1:1) medium containing 200 μg/ml G418 for selection. Individual G418-resistant clones were isolated and maintained independently in G418-containing medium. These lines were designated K16PKC-1 through K16PKC-4 and K22PKC-1 through K22PKC-6. In parallel, a set of control K16 and K22 lines was generated by transfection of the plasmid pMV7 (that lacks the PKC$_{\beta 1}$ insert) onto ψ-2 cells, infection of recipient K16 and K22 cells, and selection for G418 resistance as described above for plasmid pMV7-PKC$_{\beta 1}$. These control lines were designated K16MV7 and K22MV7.

To determine the level of PKC activity, each of the cell lines was assayed for Ca^{2+}- and phosphatidylserine-dependent protein kinase activity by using partially purified cell extracts of total (membrane and cytosolic) PKC activity. Cell extracts were prepared 48 h after the cells had reached 75–80% confluence. As shown in Table I, the various derivatives displayed a one- to tenfold increase in total PKC activity. The cell lines K16PKC-4 and K22PKC-2 contained marked increases (about tenfold) in PKC activity when compared to the control lines. These two cell lines displayed essentially the same levels of PKC activity even after continuous growth and serial passage.

To confirm the expression of PKC$_{\beta 1}$ gene in K16PKC-4 and K22PKC-2 cells, the poly A^+ RNA was separated on 1% agarose, 6% formaldehyde gels, blotted onto Hybond-N membrane, and hybridized to a ^{32}P-labeled full-length PKC$_{\beta 1}$ cDNA probe. As shown in Figure 1, K16PKC-4 and K22PKC-2, which contained elevated levels of PKC activity, contained a prominent 6.6-kilobase species, which corresponds to the predicted size for an mRNA transcript that initiates in the 5′ LTR and terminates in the 3′ LTR of the pMV7-PKC$_{\beta 1}$ construct. Neither in K16MV7 nor K22MV7 were we able to detect evidence of an endogenous transcript homologous to the PKC$_{\beta 1}$ probe. Thus, in these cells there is negligible expression of the endogenous gene encoding PKC$_{\beta 1}$.

TABLE I. PKC Activity in Neo$^+$ Clones of K16 and K22 Cells Derived After Transduction With the pMV7 or pMV7-PKC$_{\beta 1}$ Constructs

Cell lines	PKC activity (pmol/min/mg protein)	Fold increase[a]
K16MV7	311	1
K16PKC-1	579	2
K16PKC-2	1,172	4
K16PKC-3	1,761	6
K16PKC-4	2,981	10
K22MV7	234	1
K22PKC-1	720	3
K22PKC-2	2,367	10
K22PKC-3	196	<1
K22PKC-4	309	1
K22PKC-5	316	1
K22PKC-6	77	<1

[a]Compared to corresponding control, either K16MV7 or K22MV7. For additional details see Materials and Methods and Results.

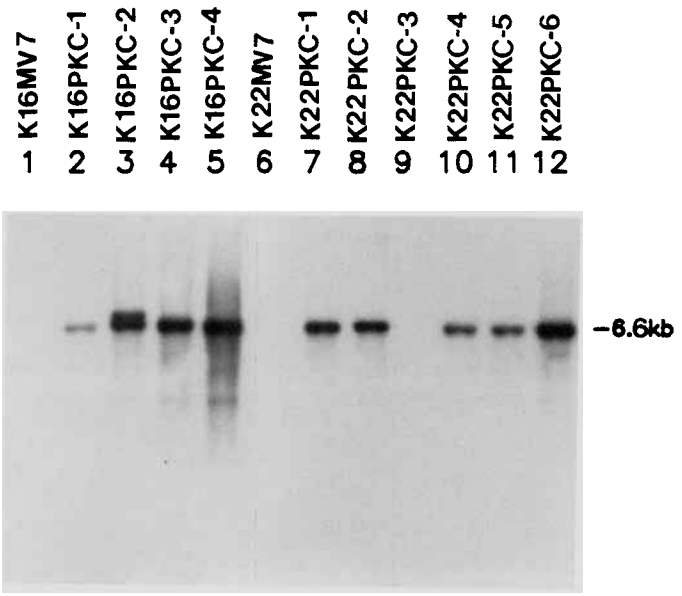

Fig. 1. Northern blot analysis of the expression of the PKC$_{\beta 1}$ gene, using a ^{32}P-labeled full-length RP58 cDNA probe and poly A$^+$ RNA samples from K16MV7, K16PKC clones, K22MV7, and K22PKC clones. For specific details see Materials and Methods.

Phenotypic and Growth Characteristics of PKC-Overproducing Cells

To characterize further the phenotypic changes that occurred in the cell lines that overproduce PKC$_{\beta 1}$, lines K16PKC-4 and K22PKC-2 were first examined in detail with respect to their morphology, as compared to that of the control cell lines K16MV7 and K22MV7. As shown in Figure 2, in the absence of TPA treatment all four cell lines showed the characteristic morphology of monolayer cultures of the normal parental K16 and K22 liver epithelial cell lines. At 4 h after treatment with 100 ng/ml TPA, the control cells displayed slightly elongated and dendritic-like changes. These changes were, however, much more dramatic when the K16PKC-4 and K22PKC-2 cells were treated with TPA. By 24 h following exposure to TPA, the morphology of the control and PKC-overproducing cells had returned to the normal appearance (data not shown). When non-confluent cultures were treated with TPA the changes in morphology were similar to those shown in Figure 2.

It was also of interest to determine the growth rates of these cells in monolayer culture. In the absence of TPA, the control (K16MV7 and K22MV7) and PKC-overproducing (K16PKC-4 and K22PKC-2) cells did not show significant differences in growth rates. The K16PKC-4 and K22PKC-2 cells had a slightly higher saturation density (about 10–15%) than the control cells.

We also assayed these cell lines for their ability to form colonies in soft agar, since with rodent cells the acquisition of anchorage-independent growth often correlates with tumorigenicity [10,28]. When 5×10^4 cells were plated in 0.3% soft agar, neither the control nor the PKC-overproducing cells could grow, with or without the presence of 100 ng/ml TPA in the soft agar medium. The effects of constitutive PKC$_{\beta 1}$ expression on tumorigenicity were also examined by injecting 1×10^7 cells per site subcutaneously into

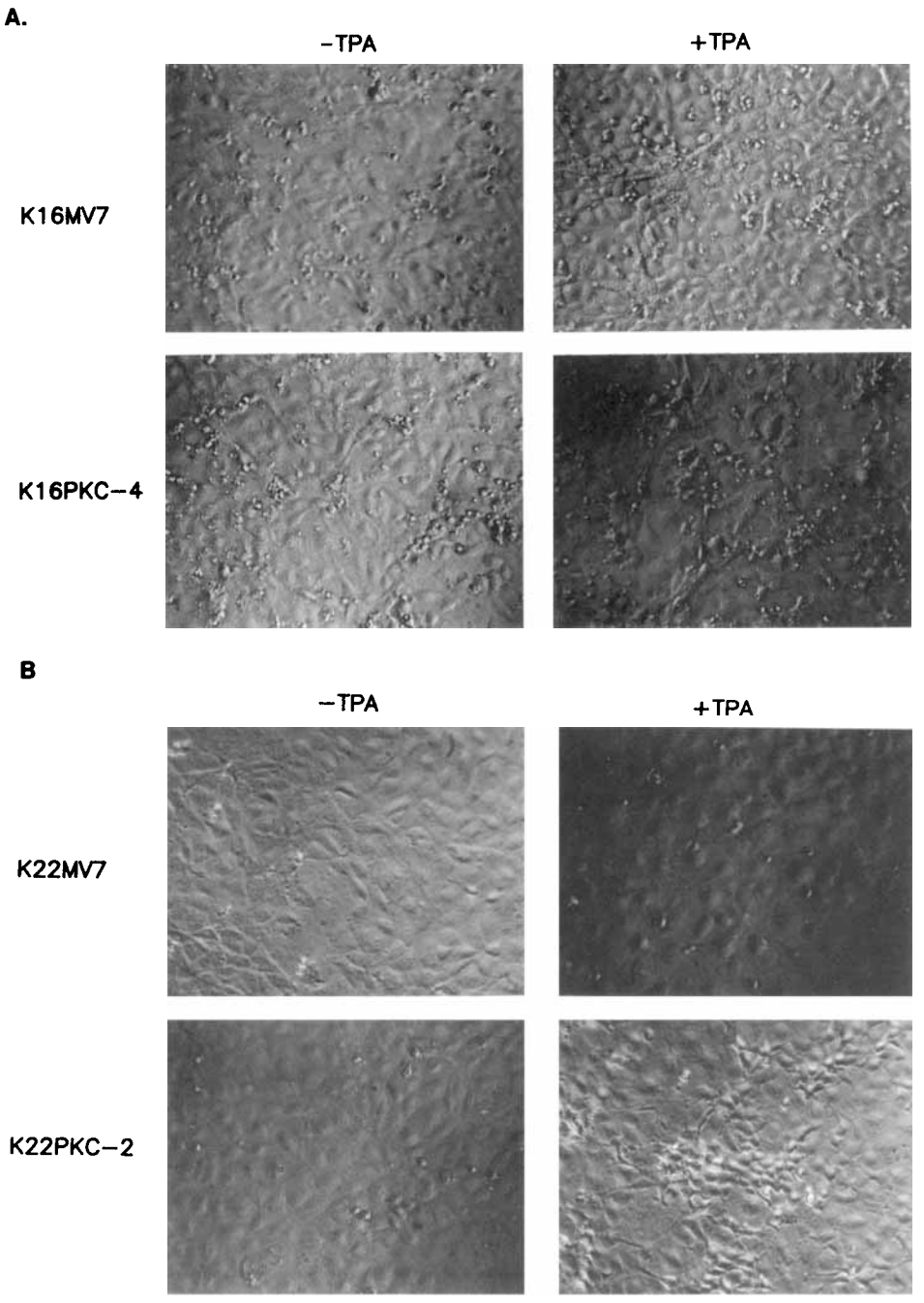

Fig. 2. Morphologic responses of the cell lines to phorbol ester treatment. Semiconfluent cultures of the four indicated cell lines were exposed to 100 ng/ml TPA in 0.01% DMSO solvent (+TPA) or to 0.01% DMSO alone (−TPA), in culture medium containing 5% fetal calf serum. Photographs were taken at 4 h after TPA treatment. **A:** K16MV7 and K16PKC-4. **B:** K22MV7 and K22PKC-2. ×80.

nude mice and monitoring the mice for tumor growth. Both the control and the PKC-overproducing cells failed to show tumor growth in nude mice, up to 8 weeks after injection.

Effects on Gene Expression in Constitutive $PKC_{\beta 1}$-Overproducing Cells

c-*myc*. Northern blot analysis indicated that RNA transcripts of about 2.5 kilobases in length, homologous to c-*myc*, were present in all of the control and $PKC_{\beta 1}$-overproducing cells (Fig. 3). K16PKC-4 and K22PKC-2 constitutively expressed higher levels (about twofold) of this transcript than the corresponding control K16MV7 and K22MV7 cells. Treatment with 100 ng/ml TPA led to a moderate increase in the level of *myc* RNAs in both control and $PKC_{\beta 1}$-overproducing cells, which was maximum (about two to threefold) at 1 h. Constitutive overexpression of $PKC_{\beta 1}$ gene did not, however, enhance this phenomenon.

c-*fos*. Low levels of 2.2-kilobase RNAs homologous to c-*fos* were seen in all of the control and $PKC_{\beta 1}$-overproducing cells (Fig. 3). Treatment with 100 ng/ml TPA led to a dramatic increase in the level of *fos* RNAs in the control K16MV7 and K22MV7 cells, which was maximum (about 16–18-fold) at 0.5 h. In the presence of 100 ng/ml TPA, K16PKC-4 and K22PKC-2 cells also displayed an increase (about 12-fold) in the level of c-*fos* RNAs. This induction was actually somewhat lower than in the control cells.

ODC. ODC-related transcripts that were 2.6 kilobases and 2.4 kilobases in size were seen in all of the control and $PKC_{\beta 1}$-overproducing cells (Fig. 3). Treatment with 100 ng/ml TPA led to a slight increase in the level of ODC RNAs in the control K16MV7 and K22MV7 cells, which was maximum (about 1–1.5-fold) at 8 h. TPA induction of ODC RNAs was enhanced (about 2.5–3-fold) in K16PKC-4 and K22PKC-2 cells.

Phorbin. Phorbin-related transcripts [3,20], which were about 0.8 kilobases in size, were observed in all of the control and $PKC_{\beta 1}$-overproducing cells (Fig. 3). Treatment with 100 ng/ml TPA led to a moderate increase in the level of phorbin RNAs in the control K16MV7 and K22MV7 cells, which was maximum (about 1.5–2.4-fold) at 8 h. This induction was enhanced (about 3.3–5.7-fold) in K16PKC-4 and K22PKC-2 cells.

DISCUSSION

We have used a previously described [9] retroviral expression vector pMV7-$PKC_{\beta 1}$ to develop derivatives of two rat liver epithelial cell lines, K16 and K22, that stably express about tenfold-higher PKC activity than control cells. These PKC-overproducing cells were designated K16PKC-4 and K22PKC-2. Our studies with these cell lines indicate that despite the overproduction of PKC, they did not show gross phenotypic changes or alterations in growth properties in the absence of TPA. These findings in an epithelial cell system differ from those obtained in previous studies with fibroblast cell lines [9,29]. Overproduction of $PKC_{\beta 1}$ in Rat 6 embryo fibroblast cells (R6-PKC3) caused an increased growth rate and higher saturation density, the formation of dense foci in postconfluent cultures, and anchorage-independent growth [9]. Overproduction of PKC_{γ} in NIH3T3 cells also produced changes in cellular morphology and other

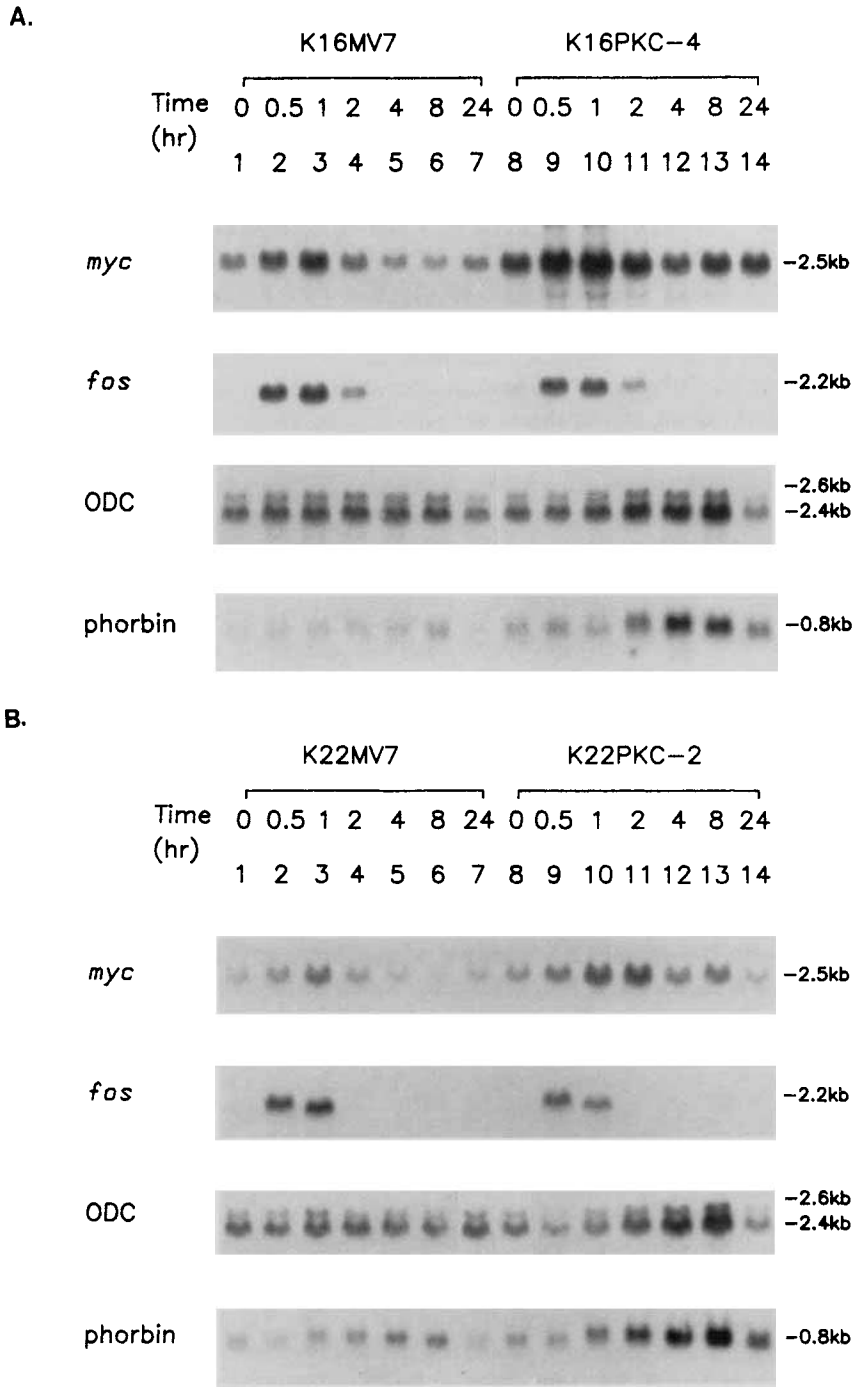

Fig. 3. Northern blot analysis of the expression of c-*myc*, c-*fos*, ODC, and phorbin genes using the corresponding ^{32}P-labeled probes and total RNA samples isolated from confluent (**A**) K16MV7 and K16PKC-4 or (**B**) K22MV7 and K22PKC-2 cells, following treatment with 100 ng/ml TPA for 0, 0.5, 1, 2, 4, 8, and 24 h. For additional details see Materials and Methods.

growth properties and enhanced tumorigenicity in nude mice [29]. In general, it is much more difficult to transform rodent epithelial cell cultures with carcinogens, or transfected oncogenes, than is the case with rodent fibroblast cell lines. Thus, it is not surprising that overproduction of $PKC_{\beta 1}$ in rat liver epithelial cells might cause more subtle cellular changes than those seen in fibroblast cell lines.

Previous studies suggest that PKC is involved in modulating the expression of several protooncogenes and growth-related genes [20,30–33]. The present studies provide further evidence for this role of PKC. Thus, we found that both the K16PKC-4 and K22PKC-2 cells expressed constitutively higher levels of c-*myc* RNAs than the control cells. Previous findings demonstrated that various agonists that activate PKC lead to increased cellular levels of c-*myc* and c-*fos* mRNA [30,31,33]. We also found that TPA induced increased levels of c-*myc* and c-*fos* RNAs in our cell system, but curiously, this induction was not greater in the K16PKC-4 and K22PKC-2 cells than in the control K16MV7 and K22MV7 cells (Fig. 3). These findings suggest that factors in addition to the level of PKC enzyme may influence the expression of these two genes.

Ornithine decarboxylase (ODC) is the first and rate-limiting enzyme in the biosynthesis of polyamines in mammalian cells, and increases in ODC enzyme activity are frequently associated with cell proliferation [34]. Previous studies indicated that TPA induced an increase in steady-state levels of ODC mRNA in primary epidermal cell cultures and T24 cells [32,35]. The downregulation of PKC in T24 cells blocked the ability of TPA to induce ODC mRNA and the induction of ODC enzyme activity [32]. Our studies with K16PKC-4 and K22PKC-2 indicate that the cells that overproduce $PKC_{\beta 1}$ displayed an enhanced induction by TPA of ODC RNA, when compared to the control cells (Fig. 3). These results, taken together, suggest that PKC activation may be an initial event in ODC gene transcription by TPA. Previous studies from our laboratory have demonstrated that increases in expression of the phorbin (TPA-S1) gene are mediated through the PKC pathway [20]. The present studies indicate that the K16PKC-4 and K22PKC-2 cells also display an enhanced induction by TPA of phorbin RNA (Fig. 3). These results, taken together, provide further evidence that PKC activation plays a critical role in regulating the expression of both ODC and phorbin RNAs.

The present studies are the first to examine the effects of overexpression of a specific isoform of PKC in an epithelial cell system. Our findings provide direct evidence that PKC plays a critical role in modulating the expression of c-*myc*, phorbin, and ODC RNAs in these cells. On the other hand, overexpression of $PKC_{\beta 1}$ is not itself sufficient to cause cell transformation. Further studies are in progress to determine whether the overexpression of PKC in these cells sensitizes them to transformation by other agents.

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