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Transformation stimulates glucose transporter gene expression in the absence of protein kinase C

(oncogenes/*fps* gene/*ras* gene)

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ABSTRACT **The rat brain glucose transporter (GT) gene is rapidly activated coincident with the initiation of growth in response to oncogenic transformation or the addition of growth factors to quiescent fibroblasts. The latter response has been shown to be mediated by protein kinase C-dependent and -independent pathways. We studied the role of protein kinase C in the transformation-induced activation of the GT gene. Transformation of fibroblasts by either the v-*fps* or the Ki-*ras* oncogene rapidly increased the levels of GT mRNA. Either viral oncogene remained capable of stimulating the GT gene after depletion of cellular protein kinase C by prolonged pretreatment of fibroblasts with phorbol 12-myristate 13-acetate. These data indicate that protein kinase C is not required for the rapid activation of gene transcription by oncogenic transformation.**

Protein kinase C (PKC), a serine/threonine-specific kinase and the receptor for tumor-promoting phorbol esters (1, 2), is a key enzyme in the control of cellular proliferation and differentiation (3, 4). Substantial evidence has accumulated leading to the suggestion that activation of PKC in the absence of exogenous phorbol esters is causally related to oncogenic transformation (5–8). Transformation by *ras* or tyrosine kinase oncogenes is associated with an elevation in diacylglycerol, the endogenous activator of PKC (6, 7); overexpression of PKC results in disordered growth and increased tumorigenicity (9, 10); depletion of cellular PKC markedly reduces the mitogenic activity of Ha-*ras*-encoded protein in 3T3 fibroblasts (11); and the transcriptional activation of several transformation-responsive genes is mediated by the same cis-acting DNA regulatory sequences that confer sensitivity to phorbol esters (12, 13). In spite of these data, a major unresolved question is whether PKC is necessary for the rapid, direct activation of gene expression by viral oncogenes or is involved in more "distal" aspects of transformation.

The rat brain glucose transporter (GT) gene encodes an integral membrane glycoprotein responsible for the facilitated transfer of hexoses across the plasma membrane (14–16). It has long been recognized that virally transformed cells possess elevated rates of hexose uptake (17, 18). Stimulation of quiescent fibroblasts with serum, purified growth factors, or phorbol 12-myristate 13-acetate (PMA) or transformation by a temperature-sensitive (ts) variant of Fujinami sarcoma virus (FSV) increases GT gene transcription within 30 min (19–21). Previously, we demonstrated that there exist PKC-dependent and -independent pathways for the activation of GT gene expression (21). Now we have studied involvement of the PKC pathway in transformation-induced GT gene expression by utilizing rat fibroblasts transformed by ts oncogenic retroviruses.

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MATERIALS AND METHODS

TS225 cells (22), which express a ts FSV, or rat 3Y1 fibroblasts, the nontransformed parent line from which TS225 cells were derived, were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (GIBCO) at 39°C. Transformation was induced by transfer of the cells to 32°C with or without a change of medium, as indicated. Rat fibroblasts (ts6-315 cells) transformed by a ts mutant of Kirsten murine sarcoma virus (23) were grown to confluence in DMEM with 10% calf serum at 40.5°C and shifted to 32°C to induce transformation. For PKC-depletion experiments, cells were incubated for 24 hr in the presence of 4 μM PMA in DMEM containing 0.1% fraction V bovine serum albumin (BSA) and 10 mM Hepes (pH 7.4).

Total RNA was isolated by guanidinium isothiocyanate/cesium chloride centrifugation (24) and assayed for GT mRNA by formaldehyde/agarose gel electrophoresis and Northern blot analysis (25). Alternatively, cytoplasmic RNA was isolated by cell lysis in nonionic detergent and submitted to slot blot analysis (21). In either case, the blots were probed with the 2.6-kilobase, near full-length rat brain GT cDNA at high stringency as described (16, 21). Slot blots were quantified with a Hoefer GS-300 scanning densitometer.

For determination of levels of PKC, the enzyme was partially purified as described (26). In brief, TS225 cells were scraped into 1 mM EGTA/25 mM Tris·HCl, pH 7.6, containing leupeptin at 50 μg/ml and were disrupted by 50 strokes of a Dounce homogenizer. The extract was centrifuged at 150,000 × *g* for 30 min to yield a supernatant that constituted the cytoplasmic fraction. The pellet was resuspended in the above buffer plus 1% (vol/vol) Triton X-100 by Dounce homogenization, incubated on ice for 1 hr, and cleared by centrifugation as above. The detergent-extracted supernatant was considered the membrane fraction. PKC was purified by DEAE-cellulose chromatography, and the enzyme, eluted with 100 mM NaCl (26), was assayed using lysine-rich histones as a substrate (27). For Western immunoblot, 20 μg of partially purified PKC was submitted to SDS/polyacrylamide electrophoresis and transfer to nitrocellulose, which was probed with antibody raised against a consensus peptide present in the enzyme (28).

RESULTS AND DISCUSSION

TS225 cells were grown at a nonpermissive temperature (39°C) prior to transfer to a permissive one (32°C). Total RNA was isolated and assayed for GT mRNA by blot hybridization analysis (Fig. 1*A*). By 24 hr after temperature-shift, GT mRNA levels were 10- to 20-fold higher than in cells maintained at 39°C, an increase comparable to that in cells (FSV9)

Abbreviations: BSA, bovine serum albumin; FSV, Fujinami sarcoma virus; GT, glucose transporter; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; ts, temperature-sensitive.

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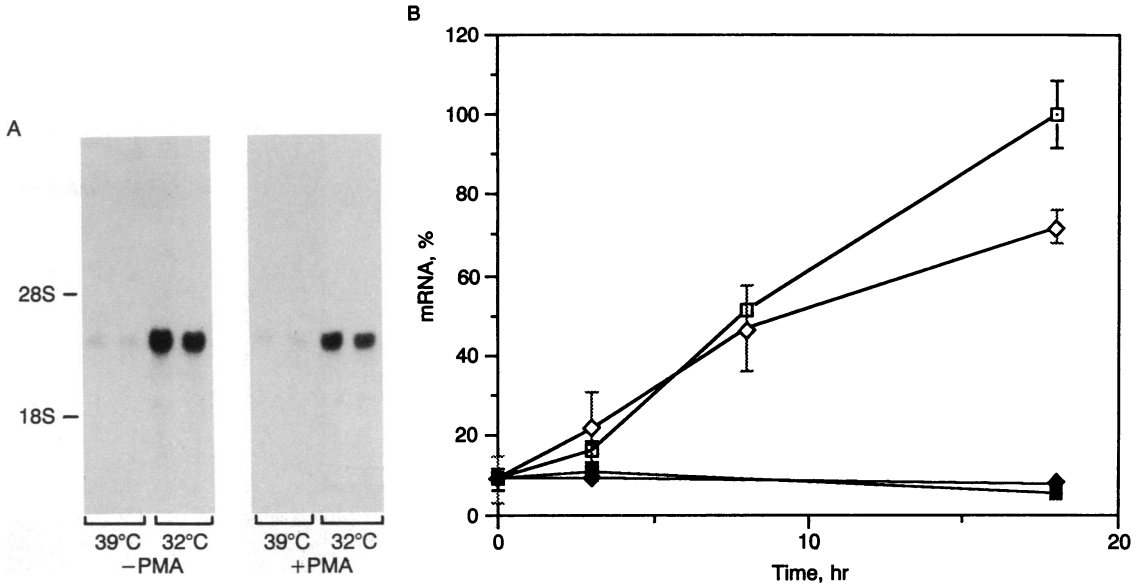

FIG. 1. Induction of GT mRNA in TS225 cells by transformation. (*A*) TS225 cells (4×10^5 cells per 90-mm culture dish), grown to confluence for 8 days at 39°C, were incubated in the presence or absence of 4 μM PMA for 24 hr. The cultures were either maintained at the same temperature or shifted to 32°C for another 18 hr without medium change. Total RNA (12 μg per lane) was analyzed by Northern blot. The positions of 28S and 18S ribosomal RNA are indicated. (*B*) TS225 cells (5×10^4 cells per well in 6-well plates), grown to confluence for 7 days at 39°C, were preincubated in the presence (■, ⊡) or absence (◆, ◇) of 4 μM PMA for 24 hr. The culture medium was replaced with fresh DMEM containing 0.1% BSA and 10 mM Hepes (pH 7.4), and the cultures were either maintained at the same temperature (■, ◆) or grown at 32°C (⊡, ◇). At the times indicated, cytoplasmic RNA was isolated for slot blot analysis. The GT mRNA levels were quantified by scanning densitometry of slot blots. Values were normalized to that of the cells incubated at 32°C for 18 hr without PMA pretreatment and represent the means ± SD (n = 4).

transformed by a wild-type FSV (19). Next, TS225 cells were grown to confluence at 39°C and incubated for another 24 hr in the presence of 4 μM PMA. This procedure depleted cellular PKC to levels representing <3% of the enzyme activity in untreated cells: PKC activity in PMA-nontreated and -treated TS225 cells was 147.4 units per 10^8 cells (140.4 units in the cytosol fraction and 7.0 units in the membrane fraction) and 4.9 units per 10^8 cells (2.6 units in the cytosol fraction and 2.3 units in the membrane fraction), respectively. In addition, after induction of transformation by incubation of the cells for 5 hr at 32°C, PKC activity remained low in down-regulated cells: 179.8 units per 10^8 cells (177.8 units in the cytosol fraction and 2.0 units in the membrane fraction) and 10.3 units per 10^8 cells (18.0 units in the cytosol fraction and 2.3 units in the membrane fraction) in cells untreated and previously exposed to PMA, respectively. The values after down-regulation probably represent the limits of detection for this assay (27). The pattern of distribution of PKC in TS225 cells and the response to chronic PMA exposure are similar to that described in other cell lines (26). Furthermore, determination of immunoreactive PKC protein by Western blot using an antibody directed against a PKC consensus peptide (28) indicated a complete loss of the relevant 78-kDa protein species, which remained undetectable even after shift of the cells to 32°C (data not shown). Thus, the protocol for PKC down-regulation appeared to induce a complete or near-complete loss of the enzyme.

Depletion of PKC did not, however, prevent the increase in GT mRNA when cells were subsequently grown at 32°C for 24 hr (Fig. 1*A*). Cytoplasmic RNA was isolated by cell lysis in nonionic detergent at the times indicated and submitted to slot blot analysis (21). Pretreatment with PMA was without effect on both the basal level of the mRNA at 39°C and the time course of the change in GT mRNA levels (Fig. 1*B*). With or without PMA pretreatment, an increase in GT mRNA was noted as early as 3 hr after transfer to the lower temperature. PKC-depleted cells displayed an unimpaired GT mRNA response to transformation when PMA was present during the incubation at the permissive temperature (Fig. 1*A*) as well as when the cells were changed to phorbol ester-free medium concurrently with transfer to 32°C (Fig. 1*B*).

Further evidence was sought that long-term treatment with PMA did, in fact, eliminate the regulation of GT mRNA mediated by PKC-dependent pathways. Rat 3Y1 fibroblasts, the nontransformed parent line from which TS225 cells were derived, were grown to confluence at 39°C and then shifted to 32°C simultaneously with addition of 200 nM PMA or 15% calf serum. Consistent with our previous work using other nontransformed fibroblast lines (21), PMA or calf serum induced rapid but transient expression of the GT gene, achieving maximal levels 3–4 hr after stimulation (Fig. 2*A*). The change in incubation temperature did not significantly affect the response. Pretreatment of the cells with PMA for 24 hr completely blocked the induction of GT mRNA in response to a subsequent challenge with 200 nM PMA (Fig. 2*B*). This provides additional evidence that the prolonged exposure of the cells to PMA did eliminate the PMA-dependent pathway. An analogous experiment was performed using TS225 cells maintained at a nonpermissive temperature. PMA pretreatment entirely eliminated the PMA-stimulated expression of the gene (Fig. 2*C*). Thus, in the nontransformed 3Y1 cells at 32°C as well as the TS225 cells grown at the nonpermissive temperature, PMA increased GT gene expression and down-regulation of PKC completely obliterated the response in both cell lines.

To provide further evidence that TS225 cells grown at 32°C also have a PKC-dependent mechanism for the activation of the GT gene and, more importantly, to demonstrate that this pathway is completely inhibited by prolonged prior incubation with PMA, we took advantage of the observation that the increase in GT mRNA in response to PMA or serum occurs more rapidly than that after stimulation by transformation (compare Figs. 1*B* and 2*A*). This is probably due to the delay required for the synthesis of an active *fps* gene product in the induced ts cells. TS225 cells were grown at 39°C and then transferred to 32°C in the presence or absence of 200 nM

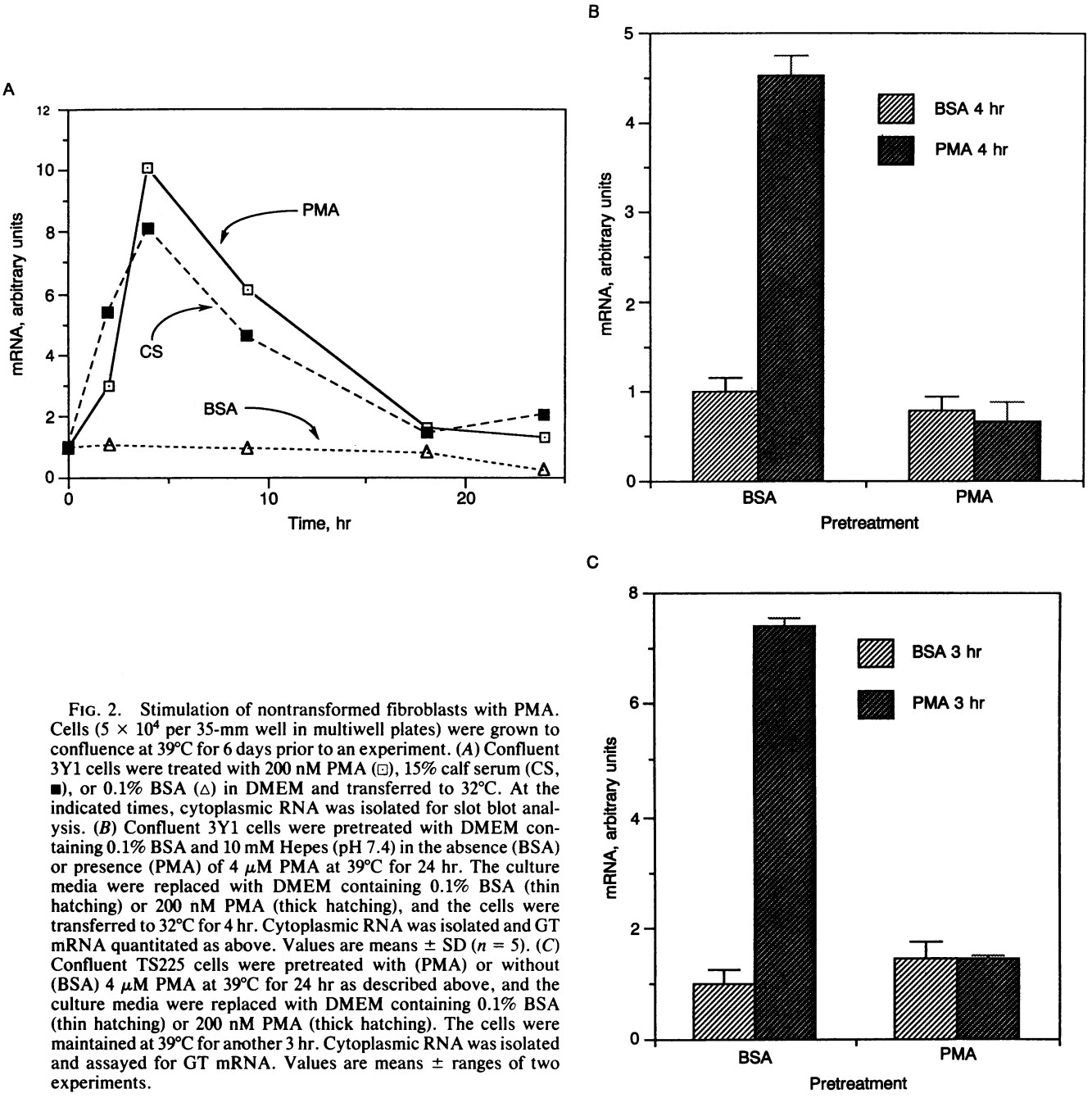

FIG. 2. Stimulation of nontransformed fibroblasts with PMA. Cells (5×10^4 per 35-mm well in multiwell plates) were grown to confluence at 39°C for 6 days prior to an experiment. (*A*) Confluent 3Y1 cells were treated with 200 nM PMA (⊡), 15% calf serum (CS, ■), or 0.1% BSA (△) in DMEM and transferred to 32°C. At the indicated times, cytoplasmic RNA was isolated for slot blot analysis. (*B*) Confluent 3Y1 cells were pretreated with DMEM containing 0.1% BSA and 10 mM Hepes (pH 7.4) in the absence (BSA) or presence (PMA) of 4 μM PMA at 39°C for 24 hr. The culture media were replaced with DMEM containing 0.1% BSA (thin hatching) or 200 nM PMA (thick hatching), and the cells were transferred to 32°C for 4 hr. Cytoplasmic RNA was isolated and GT mRNA quantitated as above. Values are means ± SD ($n = 5$). (*C*) Confluent TS225 cells were pretreated with (PMA) or without (BSA) 4 μM PMA at 39°C for 24 hr as described above, and the culture media were replaced with DMEM containing 0.1% BSA (thin hatching) or 200 nM PMA (thick hatching). The cells were maintained at 39°C for another 3 hr. Cytoplasmic RNA was isolated and assayed for GT mRNA. Values are means ± ranges of two experiments.

PMA. The active phorbol ester significantly accelerated the activation of the GT gene, producing a >2.5-fold increase in GT mRNA at 3 hr compared to cells temperature-shifted in the absence of PMA; there was no difference at 18 hr (Fig. 3*A*). This time course of induction was compared to that of cells similarly treated at 32°C but which had been depleted of PKC by pretreatment with PMA. The early induction of GT mRNA was completely abolished by down-regulation of PKC, whereas the more delayed increase was observed irrespective of whether PMA was present during preincubation at 39°C (Fig. 3*B*).

These results indicate that (*i*) TS225 cells possess a PMA-sensitive, PKC-dependent pathway for increasing GT mRNA, (*ii*) that PMA down-regulation obliterates PKC activity and this pathway, and (*iii*) that under these conditions, the active v-*fps* oncogene is still capable of stimulating the GT gene.

Since the evidence for involvement of PKC in the transformation of cells by v-*ras* is stronger than that for protein-tyrosine kinase oncogenes, we asked whether depletion of cellular PKC interfered with *ras*-induced transcriptional activation. Rat fibroblasts (ts6-315 cells) transformed by a ts mutant of Kirsten murine sarcoma virus (23) were grown to confluence at a nonpermissive temperature (40.5°C) and shifted to 32°C for 24 hr. GT mRNA levels were 2- to 3-fold higher than in cells that had been kept at 40.5°C (Fig. 4). The lesser degree of GT gene activation in ts6-315 cells compared to TS225 cells is probably related to "leakiness" of the ts Ki-*ras*, since, even at the nonpermissive temperature, the cells expressing *ras* displayed a moderately abnormal morphology. Confluent ts6-315 cells grown at 40.5°C were pretreated with 4 μM PMA for 24 hr prior to shifting to a temperature of 32°C. Though PMA alone increased the level of GT mRNA somewhat, transformation still elevated the transcript concentration 2- to 3-fold (Fig. 4).

The findings presented here clearly demonstrate that oncogene products belonging to two different classes, protein-tyrosine kinases and guanine nucleotide-binding proteins, are

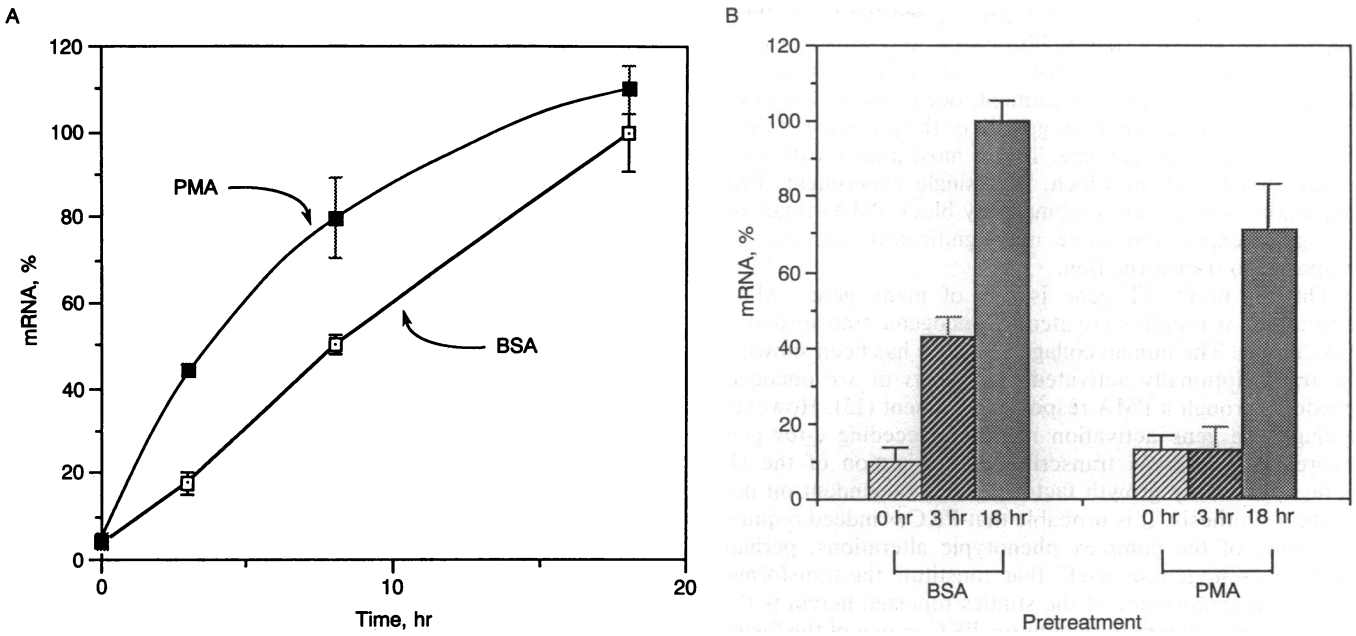

FIG. 3. Effect of PMA on the GT mRNA accumulation induced by transformation in TS225 cells. Confluent cultures of TS225 cells were prepared in 6-well plates at 39°C as described in Fig. 1. (*A*) Cultures were preincubated in DMEM containing 0.1% BSA and 10 mM Hepes (pH 7.4) at 39°C for 24 hr. Medium was replaced with fresh medium containing (■) or not containing (□) 200 nM PMA and the cultures were transferred to 32°C. At the times indicated, cytoplasmic RNA was isolated and submitted to slot blot analysis. GT mRNA was quantitated as described in Fig. 1 and expressed relative to the value 18 hr after transformation without PMA. Values are the means ± ranges of two experiments. (*B*) Cultures were preincubated in DMEM containing 0.1% BSA and 10 mM Hepes (pH 7.4) in the presence or absence of 4 μM PMA at 39°C for 24 hr. The media of all cultures were replaced with DMEM containing 200 nM PMA, 0.1% BSA, and 10 mM Hepes (pH 7.4), and the cultures were transferred to 32°C. At the indicated times, cytoplasmic RNA was isolated and analyzed for GT mRNA. Values are means ± SD (n = 4).

capable of stimulating GT gene expression through signaling mechanisms not dependent on PKC. In these studies, we have gone to great lengths to demonstrate that prolonged pretreatment with PMA does indeed obliterate the PKC-dependent pathways. First, we detected no significant PKC in the depleted cells by enzyme assay or immunoblot. Previous work indicated that one predominant isoform of PKC is expressed in both normal and transformed fibroblasts (29). Further, the ability of PMA to activate GT gene expression is completely abolished by the down-regulation scheme used in these studies (Figs. 2 and 3), indicating that any remaining PKC would have to be insensitive to the phorbol ester or

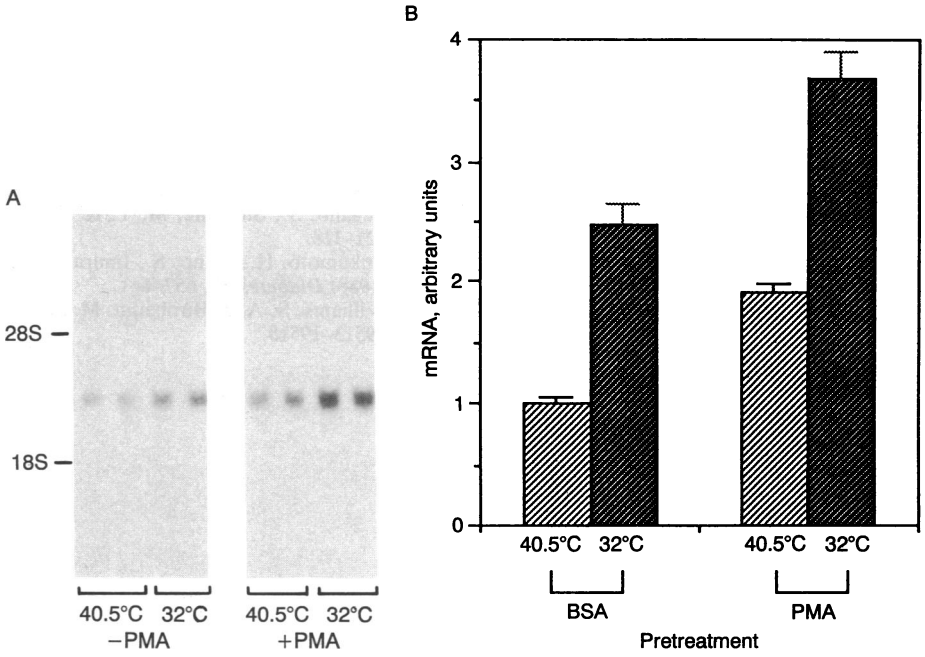

FIG. 4. Induction of GT mRNA by the *ras* gene product. ts6-315 cells (4×10^5 per 90-mm culture dish) were grown to confluence (6 days) at 40.5°C. Cultures were incubated in the presence or absence of 4 μM PMA for 24 hr. The cultures were either maintained at 40.5°C or shifted to 32°C and were incubated for another 24 hr. (*A*) RNA (10 μg) was subjected to Northern blot analysis as described in Fig. 1. (*B*) RNA (4 μg) was analyzed by the slot blot method for quantitation of GT mRNA as described in Fig. 1. Values for means ± range of two experiments.

incapable of activating the GT gene. Importantly, we have shown that the capacity of fibroblasts to respond to PMA with an increase in GT mRNA, as well as the obliteration of this pathway by PMA pretreatment, occurs in cells independently of the temperature of growth or the presence of the ts FSV in the cellular genome. This is most dramatically illustrated in Fig. 3*B*, in which, in a single experiment, PKC depletion can be seen to completely block PMA-stimulated GT gene expression while not significantly affecting the response to transformation.

The rat brain GT gene is one of many genes whose expression is rapidly activated by oncogenic transformation (13, 30–33). The human collagenase gene has been shown to be transcriptionally activated by the *ras* or *src* oncogene product through a PMA-responsive element (13). However, collagenase gene activation requires preceding c-*fos* gene expression, whereas transcriptional activation of the GT gene, at least by growth factors, is not dependent on new protein synthesis. It is probable that PKC is indeed required for some of the complex phenotypic alterations, perhaps including mitogenesis itself, that constitute the transformed state. The importance of the studies reported herein is that they delineate the requirement for PKC in one of the fastest and apparently most direct nuclear effects of transformation known. GT gene activation is virtually coincident with development of v-*fps* protein-tyrosine kinase activity and, by analogy with growth factor-stimulated gene transcription, probably does not require intermediary new protein synthesis (19, 21). Thus, the present studies demonstrate that at least one of the immediate nuclear effects of activated oncogenes does not require PKC. In this context, it will be interesting to characterize the promoter of the GT gene (34, 35) and to map the important cis-acting DNA regulatory elements that confer inducibility by transformation.

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