Molecular Cloning of Gene Sequences Regulated by Tumor Promoters and Mitogens through Protein Kinase C

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cDNA clones representing genes whose expression is modulated by treatment with mitogens and tumor promoters were isolated and characterized. TPA-S1 corresponds to an mRNA species whose abundance was increased markedly within 1 h of exposure to the tumor promoter 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), and TPA-R1 represents an mRNA that was decreased in TPA-treated cells. The induction of TPA-S1 was blocked by actinomycin D but was not affected by cycloheximide, and it was specific for phorbol esters with tumor-promoting activity. The role of protein kinase C in the induction of TPA-S1 is supported by the following lines of evidence. (i) Agents that activated protein kinase C (TPA, platelet-derived growth factor, and diacylglycerol) also increased TPA-S1 mRNA levels. (ii) A potent PKC inhibitor blocked the induction of TPA-S1. (iii) Down-regulation of PKC activity, by treatment of cells with TPA for 24 h, resulted in a loss of responsiveness to TPA-S1 induction by subsequent TPA treatment. DNA sequence analysis of TPA-S1 predicts a cysteine-rich, secreted protein with a molecular weight of 22.6×10^3 that exhibits homology with sequences representing a protein with human erythroid-potentiating activity and protease inhibitory activity.

Studies on the effects of the potent tumor promoter 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) have demonstrated that it induces a highly pleiotropic response, including effects on cell morphology, membrane transport, phospholipid metabolism, protein phosphorylation, growth factor receptor binding, cell proliferation and differentiation, chromosomal stability, induction of expression of viral genes, enhancement of malignant transformation by chemicals, viruses, and radiation, and transient mimicry of the transformed phenotype (reviewed in references 6, 7, 13, 59). TPA also induces several mitogen-responsive genes, including *c-myc* (21), *c-fos* (21), actin (21), ornithine decarboxylase (40), a mitogen-regulated protein (43) that is homologous to a prolactin-related sequence termed proliferin (31), a secreted lysosomal protein (MEP) (19), and T-cell growth factor (10). TPA also induces the expression of metallothionein (1), vimentin (50), plasminogen activator (60), transglutaminase (61), prolactin (35), and specific interferons (2). In addition, TPA and epidermal growth factor (EGF) have been shown to share inducible regulatory sequences for prolactin gene expression (56). The expression of other genes, such as collagen (53) and glycophorin (49), is inhibited by TPA treatment.

Protein kinase C (PKC), a Ca^{2+} -activated and phospholipid-dependent enzyme, has been shown to be the high-affinity receptor for TPA (reviewed in reference 38) and is activated in response to TPA binding (8). The activation of PKC results in the phosphorylation of multiple substrates, including growth factor receptors, cytoskeletal proteins, and a retinoid-binding protein, and also in the autophosphorylation of PKC (reviewed in reference 39). The endogenous activator of PKC is thought to be diacylglycerol, generated by the hydrolysis of phosphatidylinositol 4,5-diphosphate (5). A synthetic diacylglycerol, 1-oleoyl-2-acetyl-glycerol

(OAG), has been shown to activate PKC both in subcellular systems and in intact cells (26).

An important question is whether the activation of PKC mediates the effects of TPA on mitogenesis, differentiation, and gene expression. Activators of PKC, such as OAG, can mimic many of the effects of TPA in vitro (15, 47). Also, inhibitors of PKC block the effects of TPA on the induction of human T-cell lymphotropic virus type I (HTLV-I) antigen expression (37) and block TPA induction of differentiation in HL60 cells (33). Recent studies indicate that additional intermediate factors are involved in signal transduction from the plasma membrane to the nucleus, based on evidence that neither TPA (32) nor PKC (39) is present in appreciable amounts in the nucleus. It should also be stressed that PKC activation may not be sufficient to explain all of the effects of TPA. For example, the compound bryostatin is a potent activator of PKC, yet, in contrast to TPA, it does not induce the differentiation of HL60 cells (29). Also, the induction of multinucleated cell formation in HTLV-1-infected cells by TPA is not affected by PKC inhibitors (37).

The objective of the present studies was to identify DNA sequences whose expression is modulated in cells undergoing a mitogenic response to TPA treatment. Previous investigators have screened cDNA libraries by differential hybridization to identify sequences induced in response to various factors, including serum (30), platelet-derived growth factor (PDGF) (11), and interferon (17), and also to identify sequences distinguishing transformed and normal cells (51). In this paper we describe the isolation and characterization of a cDNA clone corresponding to a gene whose expression is increased in C3H 10T1/2 cells in response to TPA, a cDNA representing a gene whose abundance is decreased by TPA, and evidence for the role of PKC in their regulation.

MATERIALS AND METHODS

Cell culture. C3H 10T1/2 mouse embryo fibroblasts (45) were kindly provided by Catherine Reznikoff (University of

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Wisconsin). Cells were cultured within passage 12 in Dulbecco modified Eagle medium (Gibco Laboratories) with 10% fetal bovine serum (FBS; Flow Laboratories) or platelet-depleted horse serum (Hyclone), penicillin (25 U/ml), and streptomycin (25 µg/ml). Routine cultures were maintained in an incubator at 37°C with 5% CO₂. The large-scale isolation of RNA needed for constructing and screening a cDNA library from these cells was done with tissue culture plastic roller bottles (Corning). Cultures were rotated at 2 rpm on a Bellco roller bottle apparatus at 37°C in 120 ml of medium with 10% FBS and gassed with CO₂. In all of these studies, the cell culture medium was changed every 3 days.

Reagents. The chemicals used in these studies were obtained from the following sources: dimethyl sulfoxide (DMSO), Aldrich Chemicals; TPA, mezerein, and 4- α -phorbol didecanoate (4 α -PDD), LC Services; all *trans*-retinoic acid, Eastman Kodak; EGF, Collaborative Research; H7 and HA1004, Seikagaku America, Inc.; A23187, colchicine, cycloheximide, actinomycin D, OAG, and albumin (fraction V), Sigma Chemical Co. OAG-albumin complex was prepared by mixing 1.1 mg of OAG per ml of 0.5 mM albumin solution to obtain a clear solution. Human PDGF, purified from serum (12), was generously provided by Larry Witte. Restriction enzymes, DNA polymerases, T4 DNA ligase, and DNA-modifying enzymes were purchased from New England Biolabs or Boehringer Mannheim. Reverse transcriptase was purchased from Life Sciences.

RNA isolation. Cells were scraped from culture plates or roller bottles into cold phosphate-buffered saline and pelleted by centrifugation. The cell pellet was dissolved in 4 M guanidinium thiocyanate, and the RNA was isolated by centrifugation through a 5.7 M CsCl gradient (9). The polyadenylated [poly(A)⁺] fraction was isolated following two rounds of selection through an oligo(dT)-cellulose column (3).

cDNA library construction. C3H 10T1/2 cells were grown to confluence, and 48 h after the last medium change the cultures were treated with either 0.1% DMSO or 100 ng of TPA per ml of medium for 4 h. Poly(A)⁺ RNA was isolated as described above and then used to construct a cDNA library, essentially by previously described methods (22, 42). First-strand cDNA was synthesized from 10 µg of poly(A)⁺ RNA with reverse transcriptase, followed by second-strand synthesis with DNA polymerase I. The double-stranded cDNA was blunt-ended with T4 DNA polymerase, the internal *Eco*RI sites were methylated, and then *Eco*RI linkers were ligated and activated. The cDNA fragments were ligated into the *Eco*RI site of the phage cloning vector λ GT10. Recombinant phage were identified and amplified on the *Escherichia coli* *hflA* mutant strain. The resulting cDNA library contained 10⁵ recombinants.

Differential screening of cDNA library. The cDNA library from TPA-treated cells was screened for induced sequences by differential hybridization analysis. Initially 3 $\times$ 10⁴ recombinant phage were screened (3,000 phage per 150-mm plate). A series of four nylon filters (HyBond; Amersham Corp.) were pulled from each plate (4). The samples were hydrolyzed in 0.5 M NaOH–1.5 M NaCl and then neutralized in 0.5 M Tris (pH 7.5)–1.5 M NaCl and cross-linked by UV irradiation. The ³²P-labeled cDNA probes were synthesized by reverse transcription of 1 µg of poly(A)⁺ RNA from control or TPA-treated cultures in 100 mM Tris (pH 8.3)–100 mM KCl–10 mM MgCl₂–100 ng of actinomycin D per µl–10 mM dithiothreitol–2 U of RNasin RNase inhibitor (Promega Biotec) per µl–0.1 µg of oligo(dT) per µl–1 mM each dCTP, dGTP, and TTP–125 µCi of [³²P]dATP (800 Ci/mmol; Amersham)–1 U of reverse transcriptase per µl (20 µl total

reaction volume). The reaction mixtures were incubated at 42°C for 2 h. The cDNA-RNA duplex was treated with 0.25 M NaOH for 1 h at 65°C to hydrolyze the RNA. The sample was then neutralized, and unincorporated nucleotides were separated on a Sephadex G-100 column. Duplicate filters from each plate were hybridized to the control or TPA-treated cDNA probes in 50% formamide–5 $\times$ SSPE–1% sodium dodecyl sulfate–5 $\times$ Denhardt solution–10% dextran sulfate–20 µg of salmon sperm DNA per ml at 42°C for 72 h. The filters were then washed with 0.1 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 68°C and autoradiographed. Phage clones demonstrating differential hybridization between filters from the control and TPA probes were isolated and rescreened with the same probes. The first filter pulled from each plate was hybridized to the control probe to reduce the number of false-positives on the initial screening.

RNA analysis. The RNA samples were electrophoresed through 1% agarose-formaldehyde gels and blotted onto nylon membranes in 10 $\times$ SSC. The blots were hybridized to nick-translated probes (46) in 50% formamide–5 $\times$ SSPE–5 $\times$ Denhardt solution–10% dextran sulfate–20 µg of salmon sperm DNA per ml and then washed with 0.1 $\times$ SSC at 68°C and autoradiographed.

DNA analysis. Chromosomal DNA was isolated from C3H 10T1/2 cells by extraction of the guanidinium thiocyanate supernatant solution from the large-scale RNA preparation. The supernatant was extensively dialyzed against 10 mM Tris–1 mM EDTA (pH 7.9), treated with 100 µg of proteinase K per µl, and then phenol extracted and ethanol precipitated. The high-molecular-weight DNA was digested with a series of restriction enzymes, electrophoresed on a 1% agarose gel, blotted onto a nylon membrane by alkaline transfer in 0.4 M NaOH–0.6 M NaCl, and then neutralized in 0.5 M Tris (pH 7.0)–1.0 M NaCl. DNA blots were hybridized and autoradiographed under the same conditions as described for the RNA analysis.

SP6 transcription. The TPA-S1 insert was cloned into the *Eco*RI site of pGEM-1 plasmid (Promega-Biotec), which contains the SP6 promoter region, to generate the recombinant plasmid designated pTPA-S1. pTPA-S1 was digested with *Pvu*II, which linearizes the plasmid downstream from the polylinker region. The linear band was isolated by gel electrophoresis and purified on an Elutip-D column (Schleicher & Schuell). SP6 RNA transcripts were synthesized in a solution containing 40 mM Tris (pH 7.5), 6 mM MgCl₂, 10 mM dithiothreitol, 2 mM spermidine, 2 U of RNasin (Promega Biotec) per µl, 0.5 mM each ATP, CTP, and UTP, 0.1 to 0.5 mM GTP, 0.5 mM m⁷GpppG (Pharmacia) 0.1 µg of bovine serum albumin per µl, 1.0 µg of pTPA-S1, and 10 U of SP6 RNA polymerase (Boehringer Mannheim) in a 20-µl reaction volume. The reaction mixture was incubated at 40°C for 1 h, and then an additional 10 U of SP6 polymerase and 40 U of RNasin were added and incubated for 1 h. The reaction mixture was phenol extracted and ethanol precipitated three times with 2 M ammonium acetate to remove unincorporated ribonucleotides.

In vitro translation. SP6 transcripts were translated with a reticulocyte lysate (Promega Biotec), 20 µM amino acid mixture (without methionine), 1 µCi of [³⁵S]methionine (1,100 Ci/mmol; New England Nuclear Corp.) per µl, 1 U of RNasin per µl, and 1 to 5 µg of RNA (50 µl total reaction volume). The reaction mixture was incubated at 30°C for 2 h. A control reaction mixture containing no RNA was included. Molecular weight standards were prepared from a series of RNAs from brome mosaic virus. Portions of these samples were analyzed by electrophoresis on a 15% acrylamide gel and autoradiography.

FIG. 1. Effect of TPA on expression of TPA-S1 and TPA-R1 transcripts. Poly(A)⁺ RNA was isolated from postconfluent C3H 10T1/2 cells 4 h after treatment with either 0.1% DMSO (control) or 100 ng of TPA per ml (in 0.1% DMSO). Each lane contains 10 μ g of poly(A)⁺ RNA that was electrophoresed through a 1.0% agarose-formaldehyde gel, blotted onto nylon membrane, hybridized to ³²P-labeled probes of TPA-S1 or TPA-R1, and then autoradiographed. The locations of rRNA bands are indicated.

RESULTS

Construction and differential screening of a cDNA library from TPA-treated cells. A λ GT10 cDNA library was synthesized from poly(A)⁺ RNA obtained from quiescent C3H 10T1/2 cells 4 h after treatment with TPA (100 ng/ml). This library contained 100,000 recombinant clones. To isolate TPA-induced sequences, 30,000 phage were screened by differential hybridization with [³²P]cDNA probes prepared from poly(A)⁺ RNA from control and TPA-treated cells as described in Materials and Methods. Several clones were detected, two of which were isolated and characterized in detail. One of these corresponded to an RNA species that

was induced by TPA treatment and the other to an RNA species inhibited by TPA treatment. Figure 1 shows hybridization of the induced clone (TPA-S1) and the inhibited clone (TPA-R1) to poly(A)⁺ RNA from control cells and from cells treated with TPA for 4 h. TPA-S1 hybridized to a 0.8-kilobase (kb) transcript that was induced about 20-fold in response to TPA treatment (as determined by densitometric scan). TPA-R1 hybridized to a 4-kb transcript whose abundance was markedly decreased in response to TPA.

Kinetics of TPA-S1 induction. Analysis of the time course of induction of TPA-S1 (Fig. 2) showed that an increase in the abundance of the corresponding RNA transcript could be detected by 1 h and that the maximum increase occurred by 9 h after TPA treatment. The amount of TPA-S1 RNA returned to basal levels by 24 h. These experiments were conducted in postconfluent cultures in which the medium was changed 48 h prior to TPA treatment.

To evaluate the specificity of TPA-S1 induction, cells were treated with either DMSO, TPA, mezerein, 4 α -PDD (a nonpromoting phorbol ester), or the calcium ionophore A23187 for 4 h (Fig. 3A). The induction of TPA-S1 was specific for tumor-promoting phorbols, and ionophore-induced Ca²⁺ influx had no effect. We have, however, observed that the levels of mRNA for ornithine decarboxylase were increased by A23187 treatment, as well as by TPA and mezerein (data not presented). Replacing the culture medium with fresh medium containing 10% FBS resulted in an increased level of TPA-S1 mRNA, but to a lesser degree of induction than that obtained with TPA. In a second induction experiment, cells were treated with mitogenic doses of human PDGF, TPA, FBS, or calf serum (CS). To reduce the effect of the PDGF that is present in serum, the cells were maintained in culture medium containing 5% platelet-depleted horse serum for 48 h prior to treatment. PDGF treatment gave the same degree of TPA-S1 induction as TPA (Fig. 3B). The addition of 5% CS also showed a moderate increase. The RNA sample from the FBS group was partially degraded, so that the degree of TPA-S1 induction could not be accurately determined (Fig. 3B). Subsequent experiments demonstrated that 5% CS and 5% FBS induced TPA-S1 to the same extent. Murine EGF also induced an increase in TPA-S1 transcripts (Fig. 3C).

Effects of various inhibitors on the induction of TPA-S1 transcripts. The increase in TPA-S1 RNA caused by TPA was inhibited by actinomycin D treatment but not by cyclo-

FIG. 2. Time course of induction of TPA-S1 RNA. RNA was isolated from postconfluent C3H 10T1/2 cultures at various times after treatment with either 0.1% DMSO or TPA (100 ng/ml). Each lane contains 20 μ g of total cellular RNA that was electrophoresed, blotted, and then hybridized to a ³²P-labeled TPA-S1 probe and autoradiographed.

FIG. 3. Specificity of TPA-S1 induction by mitogens and tumor promoters. Postconfluent C3H 10T1/2 cultures were treated with (A) 0.1% DMSO, TPA (100 ng/ml), mezerein (100 ng/ml), 4 α -PDD (100 ng/ml), or 1.0 μ M A23187 in 0.1% DMSO; (B) untreated control, TPA (100 ng/ml in 0.1% DMSO), human PDGF (30 ng/ml), 5% FBS, or 5% CS in 5% platelet-derived horse serum; or (C) 0.1% DMSO, TPA (100 ng/ml), or EGF (100 ng/ml) in 10% FBS. Total RNA was isolated after a 4-h treatment with the various agents, and then 10 μ g of each sample was electrophoresed, blotted, hybridized to a 32 P-labeled TPA-S1 probe, and autoradiographed.

heximide, nor did cycloheximide alone induce an increase in the amount of this RNA species (Fig. 4). These results suggest that the induction by TPA was not mediated by de novo synthesis of specific proteins. The induction of TPA-S1 by TPA was not affected by treatment of the cells with all-*trans* retinoic acid (Fig. 4), an inhibitor of many of the biochemical effects of TPA and of the promotion of tumors by TPA in the mouse skin model (57). Assays of higher concentrations of retinoic acid were limited by cell toxicity. TPA-S1 levels were also not affected by colchicine, an inhibitor of microtubule assembly and of TPA induction of ornithine decarboxylase activity (41).

Specificity of TPA-R1 decrease for tumor promoters and mitogens. Parallel studies with the TPA-R1 sequence, whose mRNA levels were decreased by TPA treatment, demonstrated some similarities to the induction of TPA-S1 expression in terms of specificity (Fig. 5), since both TPA and mezerein caused a decrease in TPA-R1 RNA levels and 4 α -PDD, cycloheximide, retinoic acid, and colchicine had no

appreciable effect. Treatment with the calcium ionophore A23187, however, resulted in a decrease in TPA-R1 levels which was approximately equal to the reduction caused by TPA. It is of interest that treatment with actinomycin D blocked the ability of TPA to cause a decrease in the amount of TPA-R1 RNA (Fig. 5B). Treatment of cells with PDGF, EGF, CS, or diacylglycerol had no significant effect on TPA-R1 RNA levels (data not shown).

Role of PKC in the induction of TPA-S1. The potential role of PKC in the induction of TPA-S1 by TPA was assessed by treating cells with the synthetic diacylglycerol OAG, an activator of PKC in vivo and in vitro (26). Treatment with OAG (40 μ g/ml) complexed with bovine serum albumin to increase solubility resulted in a significant induction of TPA-S1 (Fig. 6). We also assessed the effects of two protein kinase inhibitors, 1-(5-isoquinolylsulfonyl)-2-methylpiperazine (H7) and *N*-(2-guanidinoethyl)-5-isoquinoline sulfonamide hydrochloride (HA1004). Both compounds demonstrate similar K_i values for the inhibition of protein kinase A, protein kinase G, and myosin light-chain kinase, but H7 is a more potent inhibitor of PKC than is HA1004 (23). When tested at 50 μ M, H7 inhibited the induction of TPA-S1 by TPA, whereas the same concentration of HA1004 showed no effect (Fig. 6). Neither compound alone reduced the basal level of TPA-S1 mRNA.

Chromosomal DNA analysis. Southern blot analysis of C3H 10T1/2 chromosomal DNA demonstrated that TPA-S1 hybridized to single fragments generated by a series of restriction enzymes that did not recognize sites within the TPA-S1 cDNA (Fig. 7). The exception was *Bam*HI, which appeared to recognize a restriction site within the intron regions of the genomic sequence. These results suggest that TPA-S1 is a single-copy gene with a maximum size of 4 kb, based on the size of the *Hind*III fragment.

Nucleotide sequence analysis of TPA-S1. The *Eco*RI-*Pst*I fragments of TPA-S1 were subcloned into M13mp18 and mp19 phage vectors, and both strands were then sequenced by the dideoxy method. Sequencing of both strands revealed two consecutive methionine codons near the 5' end, with an upstream stop codon (Fig. 8). The sequence contained an open reading frame of 615 base pairs (bp), which predicted a peptide of 205 amino acids with a molecular weight of

FIG. 4. Effects of various inhibitors on TPA-S1 RNA levels. Postconfluent C3H 10T1/2 cells were treated with either 0.1% DMSO, TPA (100 ng/ml), cycloheximide (CH) (10 μ g/ml), TPA (100 ng/ml) plus cycloheximide (10 μ g/ml), actinomycin D (200 ng/ml) (Act D), 1 μ M all-*trans* retinoic acid (RA), or 0.1 or 0.01 μ g of colchicine (Colc) per ml. Total RNA was isolated 4 h after treatment, and 10 μ g of each sample was electrophoresed, blotted, hybridized to a 32 P-labeled TPA-S1 probe, and autoradiographed.

FIG. 5. Effects of tumor promoters and various inhibitors on levels of TPA-R1 RNA. Postconfluent C3H 10T1/2 cells were treated with various agents as in Fig. 3A (A) or as in Fig. 4 (B). Total RNA was isolated 4 h later, and 10 μ g of each sample was electrophoresed, blotted, hybridized to a 32 P-labeled TPA-R1 probe, and autoradiographed.

22,600. The amino terminus of this sequence contained a hydrophobic region (Fig. 8, amino acid region -24 to -1), similar to leader sequences found in secreted proteins (58). The sequence contained a cysteine-rich region in the carboxy terminus (Fig. 8, amino acid region 124 to 173) and had 12 cysteine residues overall, suggesting that this peptide has a high degree of secondary structure. Potential glycosylation sites were also identified (25) (Fig. 8, amino acid regions 30 to 32 and 78 to 80). A signal for polyadenylation (AATAAA) was not identified in the cDNA, suggesting that it may have been truncated during the construction of the cDNA library. A comparison of the nucleotide and predicted protein sequence to the National Biomedical Research Foundation (NBRF) data base demonstrated strong homology (98%, DNA) to an incomplete murine cDNA sequence that hybrid selects an RNA species that, when translated *in vitro*, generates a protein with beta interferon activity (52). This partial sequence showed only weak homology to another

FIG. 6. Effect of PKC activators and inhibitors on expression of TPA-S1. Postconfluent C3H 10T1/2 cells were treated with either (A) 0.1% DMSO, TPA (100 ng/ml) (in 0.1% DMSO), or OAG complexed to BSA (40 μ g/ml) or (B) TPA (100 ng/ml), 50 μ M H7, or 50 μ M HA1004 (in 0.1% DMSO), alone or in combination with TPA. The control culture received no treatment. Total RNA was isolated after a 4-h treatment with the various agents, and 10- μ g samples were electrophoresed, blotted, hybridized to a 32 P-labeled TPA-S1 probe, and autoradiographed.

murine beta interferon cDNA clone (24) that has 63% nucleotide and 48% amino acid homology to human beta-1 interferon. It is possible, therefore, that TPA-S1 codes for a novel form of beta interferon. TPA-S1 also exhibited significant homology (76%, DNA and protein) to a human cDNA sequence that corresponds to a protein that has erythroid-potentiating activity (EPA) (18) and also to a cDNA that corresponds to an inhibitor of metalloproteinases (14).

In vitro translation of SP6 transcripts of TPA-S1. The TPA-S1 cDNA insert was subcloned into the plasmid pGEM-1 (pTPA-S1), which contains the promoter region for the procaryotic SP6 polymerase. SP6 transcripts for pTPA-S1 were generated from the linearized plasmid with SP6 polymerase. The protein corresponding to the pTPA-S1 RNA was synthesized in a reticulocyte lysate reaction mixture containing [35 S]methionine. The translation products were compared with molecular weight standards synthesized from brome mosaic virus RNA (Fig. 9). Analysis of the translation products on a 15% acrylamide gel, followed by autoradiography, showed that the SP6 transcripts from TPA-S1 generated a protein of about 22 kilodaltons, which was the size predicted from the nucleotide sequence of

FIG. 7. Analysis of mouse chromosomal DNA for sequences homologous to TPA-S1. High-molecular-weight DNA was isolated from C3H 10T1/2 cells and then digested with various restriction enzymes (Bm, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*I; Bs, *Bst*EI; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; P, *Pvu*II; X, *Xba*I). Samples (10 μ g) were electrophoresed through a 1.0% agarose gel, alkaline-blotted onto a nylon membrane, hybridized to a 32 P-labeled TPA-S1 probe, and autoradiographed. Sizes are indicated (in kilobases).

TPA-S1 (Fig. 8). The efficiency of translation was not significantly affected by the concentration of GTP relative to the m⁷pppG capping structure.

DISCUSSION

The cellular response to mitogens and tumor promoters involves alterations in a number of physiologic processes, which has been termed the mitogenic program (58a). Included in this response are effects on the expression of genes that are, in some cases, cell type specific as well as genes that may play a more general role in the mitogenic process. Previous studies have elucidated several growth factor-responsive genes and demonstrated that the expression of these genes is controlled by various, and in some cases multiple, mechanisms of regulation. These studies have provided evidence that control of the mitogenic program involves *cis*-acting regulatory DNA sequences, *trans*-acting transcriptional control proteins that can either activate or inhibit gene transcription, alterations in ion flux and intracellular calcium mobilization, and the activation of specific protein kinases (see Introduction).

The present paper describes the characterization of two genes, TPA-S1 and TPA-R1, whose mRNA levels are modulated by the tumor promoter TPA. The increase in TPA-S1 mRNA levels induced by TPA was inhibited by actinomycin D, suggesting that this increase requires *de novo* RNA synthesis, but further studies are required to establish the precise mechanism. The results obtained with cycloheximide (Fig. 3B) provide evidence that the TPA-induced increase in TPA-S1 RNA levels does not require protein synthesis and is not stimulated by inhibition of protein synthesis. The latter finding is in contrast to other mitogen-responsive genes, such as *c-fos*, *c-myc*, and actin, in which the corresponding mRNA levels are increased by inhibitors

of protein synthesis and are superinduced by cotreatment with growth factors and protein synthesis inhibitors (16, 20, 27, 34). Furthermore, the induction of *c-myc* by growth factors in PC12 cells requires protein synthesis (20), suggesting that synthesis of a *trans*-acting protein mediates the activation of *c-myc* in this cell type.

PKC has been implicated in a number of physiologic processes, including signal transduction in response to growth factor receptor binding, stimulation of neurotransmitter release, control of normal cell proliferation, and tumor promotion (39). A question addressed in the present study is whether activation of PKC mediates the ability of TPA to alter the expression of TPA-S1 and possibly other genes whose expression is modulated by tumor promoters. We found that TPA-S1 expression was also induced by mezerein, the diacylglycerol OAG, serum, and PDGF (Fig. 3). These results are consistent with a role for PKC in this process, since mezerein and OAG are direct activators of PKC and serum and PDGF can indirectly activate PKC through phospholipase C-stimulated hydrolysis of phosphatidylinositol 4,5-diphosphate and the release of diacylglycerol (5, 48). The hydrolysis of phosphatidylinositol 4,5-diphosphate by phospholipase C also yields the compound inositol 1,4,5-triphosphate, which stimulates the intracellular mobilization of Ca²⁺ from storage sites in the endoplasmic reticulum (55). Since some of the effects of TPA can be mimicked by treatment with the calcium ionophore A23187 (38), an alternative mechanism of signal transduction during mitogenesis is via a transient increase in intracellular Ca²⁺. We found, however, that A23187 had no effect on TPA-S1 mRNA levels, indicating that an increase in cytosolic Ca²⁺ is not sufficient to modulate the expression of this gene. Our studies on the effects of protein kinase inhibitors demonstrated that H7, which is a more potent inhibitor of PKC than HA1004 (23), was more potent than HA1004 as an inhibitor of TPA induction of TPA-S1 transcription (Fig. 6). These results are also consistent with the hypothesis that PKC activation is involved in mediating the effects of TPA on TPA-S1 expression. Also consistent with this mechanism are recent studies indicating that when cells are pretreated for 24 h to induce downregulation of PKC (47), subsequent exposure to TPA fails to induce the expression of TPA-S1 RNA (unpublished data).

We found that the decrease in TPA-R1 RNA in response to TPA was not mimicked by PDGF, EGF, serum, or diacylglycerol and that the ability of TPA to elicit this response was not inhibited by the protein kinase inhibitors H7 and HA1004 (data not shown). It is possible, therefore, that PKC does not play a direct role in mediating this response.

At present it is not clear how the activation of PKC leads to an alteration in the abundance of TPA-S1 RNA. Studies with fluorescence-labeled TPA (32) and with antibodies to PKC (39) have failed to reveal a significant concentration of PKC in the nucleus. One possibility is that the proteolytic fragment of PKC enters the nucleus and interacts directly with chromatin. This mechanism is supported by the recent nucleotide sequence analysis of PKC (44), which reveals a consensus DNA-binding region in the corresponding protein. An alternative mechanism is that activation of PKC leads to the phosphorylation of specific DNA-binding proteins, resulting in the binding of these proteins to the regulatory regions of a specific set of genes and enhancement of transcription. The results of recent studies on the activation of DNA-binding proteins for the immunoglobulin promoter region by mitogens are consistent with this mechanism (54).

FIG. 8. Nucleotide and predicted amino acid sequence of TPA-S1. The *EcoRI-PstI* fragments of TPA-S1 were subcloned into the M13 cloning vectors mp18 and mp19, and both strands of each clone were sequenced by the dideoxy method. The 730-bp cDNA insert contained an open reading frame of 615 bp (205 amino acids). Amino acid region -24 through -1 represents the putative amino-terminal signal sequence of the nascent protein. Cysteine residues are indicated (*). The underlined regions represent potential glycosylation sites.

Comparisons of the nucleotide and predicted amino acid sequences of TPA-S1 to the NBRF data base reveal homologies to the following sequences: (i) human EPA, a protein which stimulates the proliferation of erythroid precursor cells in vitro and is induced by TPA in HL60 cells (18) (76% homology for both DNA and protein); (ii) a tissue inhibitor of metalloproteinases (TIMP [14]), a protein that is induced by

FIG. 9. Analysis of in vitro translation products generated from SP6 transcripts of TPA-S1. SP6 RNA transcripts from pTPA-S1 were synthesized by SP6 polymerase. Samples of the SP6 RNA were incubated in an in vitro translation reaction with rabbit reticulocyte lysate with [³⁵S]methionine. A portion of each reaction mixture was electrophoresed on a 15% acrylamide gel. Lane 1, control sample (no RNA); lanes 2 and 3, separate preparations of SP6 RNA transcripts from pTPA-S1 (0.5 and 0.1 mM GTP, respectively). The gel was then dried and autoradiographed. Molecular weight markers (in thousands) were proteins synthesized from specific brome mosaic virus RNA species (Promega Biotec).

TPA and interleukin 1 in human fibroblasts (36) (76% homology for both DNA and protein); and (iii) a murine cDNA sequence (116-bp coding region) that codes for a protein whose biologic activity resembles that of beta interferon (52) (98% homology at the DNA level), although the predicted sequence of this protein is not homologous to other mouse or human interferon sequences (24). Comparisons of the protein sequence for EPA, TIMP, and TPA-S1, as predicted from their cDNA sequences, indicate that the molecular weights of these proteins would be comparable. These proteins also show exact alignment of the corresponding cysteine residues, suggesting that the secondary structures of these proteins are very similar. In addition, the strong homology between these sequences in the amino-terminal leader sequence suggests that the protein coded for by TPA-S1 is also a secreted protein. It is possible, therefore, that TPA-S1 is the mouse homolog of the human EPA and TIMP sequences. The significance of the homology of TPA-S1 to a murine sequence that apparently codes for a protein with beta interferon activity is unclear. However, the induction of a beta interferon sequence by TPA is consistent with previous evidence that TPA and other mitogens (PDGF, interleukin 1, and tumor necrosis factor) can induce interferons and various lymphokines (2, 28, 62). Studies are now in progress to assess the biologic activities of the TPA-S1 sequence following its expression in appropriate recipient cells.

Although the biologic significance of the increase in TPA-S1 RNA and the reduction of TPA-R1 RNA by TPA remains to be determined, these sequences should serve as useful probes for delineating the specific pathways of signal transduction related to the actions of growth factors and tumor promoters.

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ADDENDUM IN PROOF

Since the submission of this paper, we became aware of two other reports describing cDNA clones with sequences related to TPA-S1. Edwards et al. (Nucleic Acids Res. 14:8863-8878, 1986) described a growth-responsive murine gene (16C8), which is identical to TPA-S1 at the amino acid level. Carmichael et al. (Proc. Natl. Acad. Sci. USA 83:2407-2411, 1986) described cDNA clones encoding a human collagenase inhibitor (HCI) whose amino acid sequence is 75% homologous to that of TPA-S1.

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