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Tumor Promoters Alter the Temporal Program of Adenovirus Replication in Human Cells

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In this study we evaluated the effect of phorbol ester tumor promoters on the kinetics of adenovirus type 5 (Ad5) replication in human cells. When added at the time of infection, 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) accelerated the appearance of an early virus antigen (72,000-molecular-weight [72K] deoxyribonucleic acid-binding protein), the onset of viral deoxyribonucleic acid synthesis, and the production of infectious virus. The appearance of an Ad5-specific cytopathic effect (CPE) was also accelerated in infected cultures exposed to TPA, whereas phorbol, 4α-phorbol-12,13-didecanoate and 4-OmeTPA, which are inactive as tumor promoters, were ineffective in inducing this morphological change. The acceleration of the CPE seen in TPA-treated Ad5-infected cells was not caused by TPA induction of the protease plasminogen activator, since the protease inhibitors leupeptin and antipain do not inhibit the earlier onset of this CPE and, in contrast, epidermal growth factor, which induces plasminogen activator in HeLa cells, does not induce an earlier CPE. Evidence for a direct effect of TPA on viral gene expression was obtained by analyzing viral messenger ribonucleic acid (mRNA) synthesis. TPA accelerated the appearance of mRNA from all major early regions of Ad5, transiently stimulated the accumulation of region III mRNA, and accelerated the appearance of late Ad5 mRNA. Thus, TPA altered the temporal program of Ad5 mRNA production and accelerated the appearance of at least some Ad5-specific polypeptides during lytic infection of human cells. These effects presumably explain the earlier onset of the Ad5-specific CPE in TPA-treated cells and may have relevance to the effects of TPA on viral gene expression in nonpermissive cells carrying integrated viral deoxyribonucleic acid sequences.

The carcinogenic process often results from interaction among multiple and diverse factors and often proceeds through several stages in its development (21). The most extensively studied multistage animal system is the "two-stage" mouse skin carcinogenesis assay, in which two discrete stages, one termed "initiation" and the other "promotion," have been described. In general, initiating chemical carcinogens yield electrophiles which bind covalently to deoxyribonucleic acid (DNA) and other macromolecules and are, therefore, mutagenic. Tumor-promoting agents are not mutagenic, and covalent binding to DNA or other macromolecules does not appear to be a prerequisite for biological activity (for a review, see reference 49). The most potent class of tumor-promoting agents on mouse skin are the phorbol esters, such as 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), and related macrocyclic plant diterpenes (for a review, see reference 45). A significant advance in analyzing the mechanism of action of tumor promoters has been the development of cell culture systems which are responsive to these agents (for reviews, see references 47 and 48). TPA has been found to induce a wide array of effects on cells in culture, some of which mimic the phenotype of cells transformed by chemical carcinogens or viruses. Alterations induced by phorbol ester tumor promoters in cell culture systems include changes in cellular morphology and growth (3, 11, 12, 16, 37, 40, 41, 48); induction and enhancement of anchorage-independent growth in chemically transformed epidermal cells and adenovirus type 5 (Ad5)-transformed rat embryo cells (8, 9, 13, 14, 16); changes in cell surface properties (3, 7, 15, 48); enhanced nutrient transport (12, 31); enhanced growth of normal cells in medium containing low extracellular Ca^{2+} (18); induction of various enzymes, such as plasminogen activator and ornithine decarboxylase (48-51, 54); induction of arachidonic acid release and pros-

taglandin synthesis (35, 39); and a decrease in epidermal growth factor (EGF)-receptor binding (4, 17, 30, 44). TPA and related compounds have also been found to be potent regulators of terminal differentiation in a wide spectrum of cell culture systems (for a review, see references 35 and 47). The cell culture effects of phorbol esters usually require only nanogram quantities, and a good but not absolute correlation exists between biological activity on mouse skin and potencies in cell culture.

We have previously demonstrated that TPA enhances viral transformation of rat embryo cells when the cells are grown in the continuous presence of the tumor promoter after infection with a temperature-sensitive mutant of Ad5 (H5*ts*125) (20). More recent studies have indicated that TPA can also enhance Epstein-Barr virus transformation of human lymphocytes (53), simian virus 40 transformation of Chinese hamster lung cells (34), and polyoma virus transformation of rat cells (43). In addition, phorbol esters have been found to induce the replication of Epstein-Barr virus and other oncogenic herpesviruses in virus-producing cell lines (55, 56), enhance Epstein-Barr virus antigen and DNA synthesis in virus-producer and nonproducer cell lines (27, 33, 55), and stimulate the synthesis of mouse mammary tumor virus in a mouse mammary tumor virus-producing C3H mouse mammary tumor cell line (1). In the adenovirus-rat embryo system, TPA enhances expression of the transformed phenotype in cells containing integrated Ad5 DNA (13, 14, 16, 20). It is not known, however, whether TPA enhancement of viral transformation results from a primary effect of TPA on the expression of viral genes or host genes involved in maintenance of the transformed state. To gain further insight into the effects of TPA on viral and host gene expression, we have investigated the effects of TPA on the replicative cycle of Ad5 during permissive infection of HeLa cells.

MATERIALS AND METHODS

Cells and viruses. HeLa cells, obtained from M. S. Horowitz, were the plating line described by Williams (52). They were serially passaged in Dulbecco-modified Eagle minimal essential medium supplemented with 10% calf serum (Flow Laboratories). Nunc 35-mm plates were seeded with $\sim 1.5 \times 10^6$ cells and used on the next day, when they were subconfluent.

The wild-type virus was a plaque-purified isolate obtained from H. S. Ginsberg and was used at an early passage.

Infection protocol. Subconfluent HeLa cells in 35-mm dishes were washed once with Hanks balanced salt solution, inoculated with 0.2 ml of the appropriate viral dilution or mock-infected, and after 2 h of adsorption overlaid with either infecting fluid (28), which contains 7.5% chicken serum (GIBCO Laboratories, Grand Island, N.Y.), or Dulbecco-modified Eagle minimum essential medium supplemented with 2% fetal bovine serum (Flow). Various compounds were added either at the time of overlay or at subsequent intervals, but in all cases whenever a medium change occurred it was performed on both treated and untreated cultures.

Chemicals and isotopes. For information on the sources and preparation of the tumor promoters and their analogs and inhibitory compounds see reference 13. [^{3}H]uridine was obtained from New England Nuclear Corp. (80 Ci/mmol). [^{3}H]thymidine was obtained from New England Nuclear Corp. (40 to 60 Ci/mmol).

Cell fractionation. The method for cell fractionation was modified from that described earlier (5). After isotopic labeling, the cell monolayer was rinsed twice with cold TBS [10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.4–0.14 M NaCl–1.5 mM $MgCl_2$] and treated with 5 ml of 0.1% Nonidet P-40 in TBS on ice for 2 to 5 min, until free-floating nuclei could be produced by a sharp blow to the side of the flask. The lysates were then decanted and centrifuged at $250 \times g$ for 5 min at 0°C to remove the nuclei. The supernatant solution containing cytoplasm was further centrifuged at $18,000 \times g$ for 20 min at 0°C to remove residual nuclei, debris, and mitochondria; made 0.6% sodium dodecyl sulfate; and frozen at −20°C.

RNA purification. Ribonucleic acid (RNA) was first purified by extraction with phenol-chloroform-isoamyl alcohol and recovered by ethanol precipitation (10). Polyadenylic acid [poly(A)]-containing RNA was then isolated by the method of Gielen et al. (23), except that all steps were carried out at 4°C. Briefly, ethanol-precipitated RNA was dissolved in 1 ml of 10 mM Tris-hydrochloride (pH 7.4)–0.5 M NaCl and passed through an oligodeoxythymidylic acid-cellulose column (Collaborative Research, Inc., Waltham, Mass.) equilibrated at 4°C with the same buffer. After washing with 3 column volumes of starting buffer, poly(A)-containing RNA was eluted with four successive 1.0-ml washes with 10 mM Tris-hydrochloride, pH 7.4. Eluted RNA was used for hybridization after partial alkaline hydrolysis. Of the [^{3}H]uridine incorporated into cytoplasmic RNA in a 2-h period, 20 to 30% was recovered in the low-salt wash.

RNA hybridization to restriction endonuclease fragments of Ad5 DNA. *Hin*dIII restriction endonuclease fragments of Ad5 DNA were isolated by agarose gel electrophoresis and batch-eluted from hydroxylapatite. Poly(A)-containing cytoplasmic RNA was hybridized to an excess of DNA bound to nitrocellulose filters in Denhardt buffer containing 2× SSC (SSC = 0.15 M NaCl–0.015 M sodium citrate) (2). Each filter contained 2.5-μg equivalents of a *Hin*dIII-cleaved DNA fragment. Hybridization reactions were incubated at 67°C for 48 h. The filters were washed, treated with ribonuclease, dried, and counted for radioactivity as described previously (5).

RESULTS

Effects of tumor-promoting agents on HeLa cell morphology and the CPE induced

by adenovirus infection. TPA induces a distinctive morphological change in uninfected HeLa cells and markedly accelerates the appearance of an adenovirus-induced cytopathic effect (CPE) (Fig. 1). The latter effect is illustrated graphically in Fig. 2 for a set of plates incubated at 39.5°C where the TPA-treated cells demonstrated a CPE at 21 h postinfection (p.i.), whereas the untreated controls did not develop CPE until 30 h p.i. At 32°C the treated and untreated cells developed a CPE at 27 h and 45 h p.i., respectively. Good correlation was observed between the promoting activity on mouse skin of a series of diterpene esters and induction of both the morphological change in uninfected HeLa cells and the accelerated CPE in cells

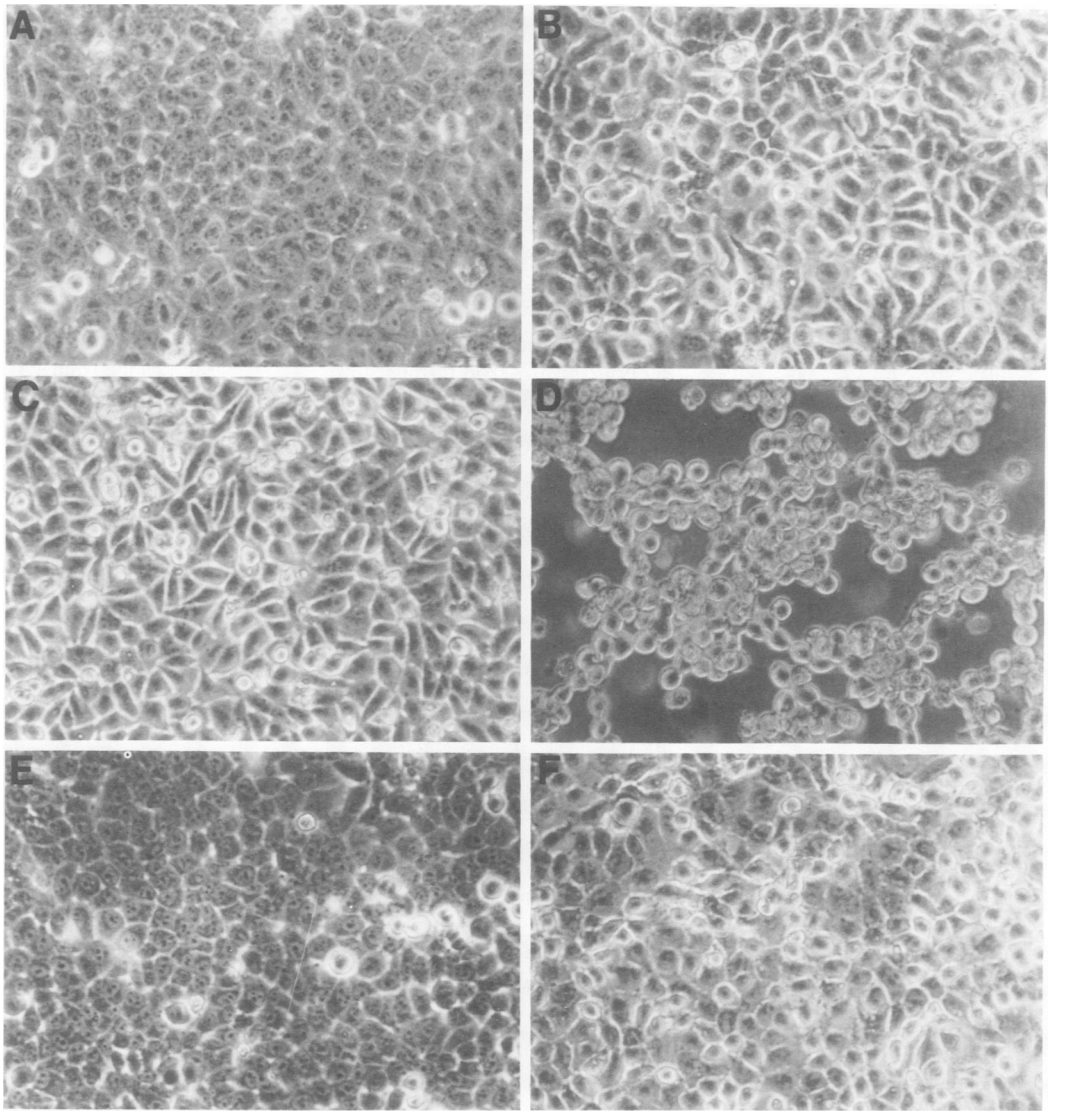

FIG. 1. *Effect of TPA and phorbol on the morphology of uninfected and Ad5-infected HeLa cells. Cells were seeded in 35-mm tissue culture dishes at 32°C and mock-infected or infected with 10 plaque-forming units of Ad5 per cell. At 16 h p.i., the various compounds were added and the plates were grown at 39°C for 24 h and photographed with phase-contrast optics (150× magnification). (A) Mock-infected; (B) Ad5-infected; (C) mock-infected cells grown in 100 ng of TPA per ml for 24 h; (D) Ad5-infected cells grown in the presence of 100 ng of TPA per ml for 24 h; (E) mock-infected cells grown in the presence of 200 ng of phorbol per ml for 24 h; (F) Ad5-infected cells grown in 200 ng of phorbol per ml for 24 h.*

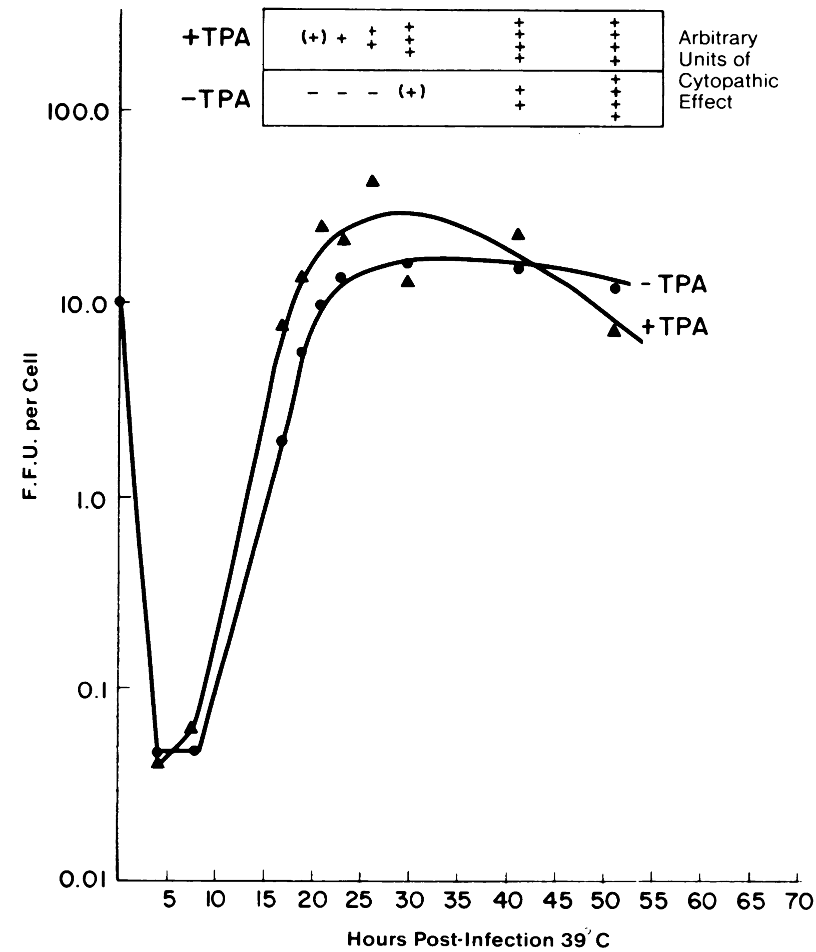

FIG. 2. *Growth curve of Ad5 in HeLa cell monolayers and the change in gross morphology in the presence and absence of TPA. Replicate dishes of HeLa cells were infected with Ad5 at a multiplicity of infection of 10 focus-forming units per cell. After 2 h of adsorption, they were overlaid with growth medium with or without 100 ng of TPA per ml and incubated at 39.5°C. At intervals, the dishes were examined microscopically to determine the development of CPE (expressed in arbitrary units at the top of the figure), and individual dishes were frozen and thawed to release intracellular virus. The titer of virus was determined by an indirect immunofluorescent focus assay.*

infected with Ad5 (Table 1). In the case of cultures infected with virus and incubated at 39.5°C, the accelerated CPE was observed in cells treated with as little as 3 ng of TPA per ml. Infection of nonpermissive murine cell lines (either B16 melanoma or C3H 10T1/2 fibroblasts), adenovirus-transformed rat embryo cells, or the chemically transformed W8 rat liver cells with Ad5 in the presence of TPA did not result in a CPE (unpublished data).

Previous studies have indicated that the polypeptide hormone EGF and the bee venom polypeptide melittin share a number of biological properties with the phorbol esters (13, 29, 36). EGF induced a morphological change in uninfected HeLa cells but only slightly accelerated the CPE induced by Ad5 infection, even when tested at a high concentration (100 ng/ml) (Table 1). In contrast, melittin (2 μg/ml) did not induce a morphological change in uninfected HeLa cells, but it accelerated the appearance of the Ad5-induced CPE. The latter effect was similar to that obtained with 100 ng of the weak tumor promoter phorbol-12,13-dibenzoate per ml (Table 1). These results suggest that the morphological change induced by TPA in uninfected HeLa cells and the acceleration of the Ad5 CPE occur through different mechanisms.

TABLE 1. *Effects of tumor promoters, inhibitors of tumor promotion, and various hormones on the morphology of uninfected HeLa cells and the development of a CPE in Ad5-infected HeLa cells*

Addition to culture[a]	Morphological changes[b]	
	Uninfected	Infected
None	−	−
TPA (10 ng/ml)	+	++++
PDD (10 ng/ml)	+	++++
PDB (100 ng/ml)	+	++
PDB (10 ng/ml)	−	−
Phorbol (200 ng/ml)	−	−
4-*O*-meTPA (100 ng/ml)	−	−
4α PDD (100 ng/ml)	−	−
EGF (100 ng/ml)	+	±
Estradiol (2 μg/ml)	−	+
Testosterone (2 μg/ml)	−	+
Melittin (2 μg/ml)	−	++
Leupeptin (50 μg/ml)	−	−
TPA (100 ng/ml) + leupeptin (50 μg.ml)	+	++++
Antipain (50 μg/ml)	−	−
TPA (100 ng/ml + antipain (50 μg/ml)	+	++++
trans-RA (5 μg/ml)	−	−
TPA (100 ng/ml) + *trans*-RA (5 μg/ml)	+	+++±

[a] PDD, Phorbol-13,13-didecanoate; PDB, phorbol-12,13-dibenzoate; 4αPDD, 4α-phorbol-12,13-didecanoate; EGF, epidermal growth factor; *trans*-RA, *trans*-retinoic acid.

[b] HeLa cells growing in 35-mm plates (~1.2 × 10^6 cells) were either mock infected or infected with 10 plaque-forming units of Ad5 per cell and incubated for 16 h at 32°C. Then the various compounds were added to the infecting fluid and the cells were either left at 32°C or shifted to 39.5°C. The morphological change in uninfected cells refers to a change from an epithelioid to a more fibroblastic morphology (see Fig. 1). The morphological change in Ad5-infected cells is designated ++++ (maximum CPE) to − (no CPE).

Vitamin A (*trans*-retinoic acid) and various protease inhibitors inhibit certain in vitro and in vivo effects of tumor promoters (for a review, see reference 45). Quigley (40) has found that phorbol ester-induced morphological changes in Rous sarcoma virus-transformed chicken embryo fibroblasts are inhibited by certain protease inhibitors, including leupeptin, and has suggested that these morphological changes may be caused by a direct action of plasminogen activator. For these reasons we tested the ability of *trans*-retinoic acid (1 to 10 μg/ml) and the protease inhibitors antipain and leupeptin (50 μg/ml) to inhibit the TPA-induced morphological changes in both uninfected and Ad5-infected HeLa cells. We found that these compounds had little or no influence on either of these TPA effects (Table 1). Thus, although TPA induces plasminogen activator in HeLa cells (50), it appears that plasminogen activator does not mediate the morphological changes observed in HeLa cells. The fact that EGF induces plasminogen activator in HeLa cells (29) but does not accelerate the onset of Ad5-induced CPE in the same cells also provides evidence that plasminogen activator does not play a role in this phenomenon.

Effects of TPA on the adenovirus replication cycle. The TPA-induced acceleration of the onset of CPE in Ad5-infected HeLa cells led us to investigate the possible effects of TPA on viral replication. When tested over a range of 1 to 1,000 ng/ml TPA failed to enhance the total yield of infectious virus from HeLa cells in monolayer culture (Table 2). In a series of seven additional experiments, TPA gave either no or at most a two- to threefold increase in final yield of infectious virus (see, for example, Fig. 2 and 3). This is in contrast to the effects of TPA on Epstein-Barr virus production (55, 56). We next examined the time course of virus replication. In the first experiment, illustrated in Fig. 2, HeLa cells in monolayer culture were inoculated with virus at a multiplicity of infection of approximately 10 plaque-forming units per cell, and after 2 h of adsorption, the plates were overlaid with infecting medium with or without TPA (100 ng/ml). The plates were incubated at 39.5°C, individual dishes were removed at various time intervals, and frozen at −20°C, and the viral yields were subsequently titrated by fluorescent focus assay. The progression of CPE was noted also. It is apparent that TPA caused an acceleration of the development of infectious virus by some 2 h at mid-experimental times (Fig. 2). In a similar experiment conducted at

TABLE 2. *Response of final viral yield to increasing doses of TPA*[a]

TPA dose (ng/ml)	Final viral yield[b]
0	10
1	12
3	12
10	8
30	11
100	10
300	10
1,000	12

[a] HeLa cells in 35-mm plastic dishes were infected with Ad5 and treated with various doses of TPA as described in footnote *b* of Table 1. Cells were harvested after 72 h of incubation at 32°C. Virus was released from the cells by six cycles of freezing and thawing and titrated on KB cells by fluorescent focus assay. Each point is the average of two plates.

[b] Focus-forming units per milliliter (×10^7).

32°C, the acceleration of virus development was even more pronounced (4 to 5 h) than at 39.5°C (Fig. 3). This effect is reproducible at both temperatures. The onset of CPE in Ad5-infected, TPA-treated cells was a relatively late effect, since it was first observable at a time when infectious virus was being produced (Fig. 2). This raised the possibility that the TPA effect on CPE was mediated by a direct interaction of the compound with late viral antigens in the infected cells. To test this possibility, cultures were infected with virus as in the 32°C experiment described above, but the addition of TPA was delayed until 4, 6, 8 and 23 h p.i. We found that the onset of the CPE was progressively delayed the later the TPA was added and, in the case of the 23-h-p.i. addition, occurred at the same time as in the non-TPA-treated controls. We also found that the direct addition of diethylaminoethyl column-purified preparations of Ad5 hexon, penton, and fiber polypeptides, at concentrations ranging from 0.04 to 27 μg/ml, either alone or in combination, did not induce a CPE in TPA-treated uninfected cells or accelerate the TPA-induced early onset of CPE in virus-infected, TPA-treated cells. These two sets of results provide indirect evidence that TPA must be added to HeLa cultures at early times after Ad5 infection to cause an acceleration of the onset of CPE and that this effect is not

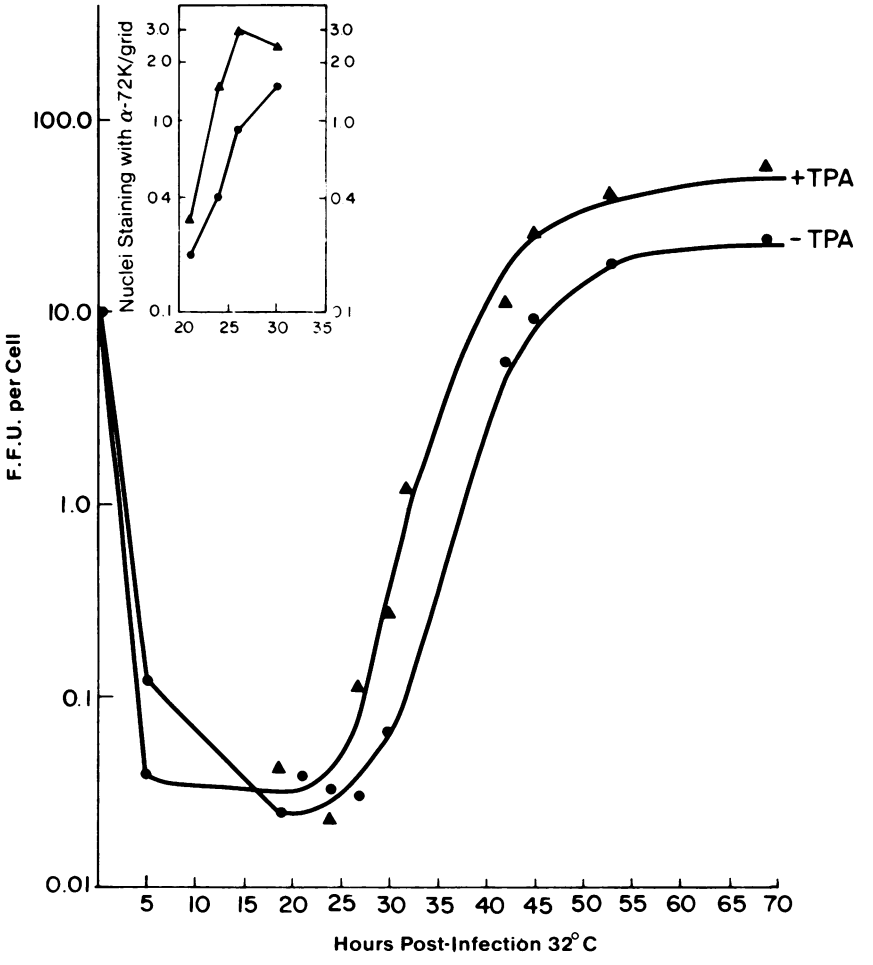

FIG. 3. *Growth curve of Ad5 in HeLa cell monolayers and the presence of 72K DBP in the presence or absence of TPA. For the growth curves, dishes were treated as described in the legend to Fig. 2, except that the temperature of incubation was 32°C. For measurement of adenovirus DBP, HeLa cells on glass cover slips were infected with virus at a multiplicity of infection of 0.2 focus-forming units per cell and overlaid as above. At intervals, cover slips were removed, the cells were fixed by air drying and acetone treatment, and the 72K DBP was visualized by using an indirect fluorescence technique with rabbit anti-DBP (a gift of H.S. Ginsberg).*

mediated by a direct interaction between TPA and the viral antigens that accumulate at late times during viral synthesis.

To examine possible effects of TPA on early events in the replicative cycle of Ad5, we studied the synthesis of the 72,000-molecular-weight (72K) DNA-binding protein (DBP), an early Ad5 gene product whose role in DNA synthesis and transformation is well documented. Two sets of HeLa cell cultures were prepared, one containing glass cover slips and the other without. At confluence, the dishes containing cover slips were infected with Ad5 at a multiplicity of infection of approximately 0.2 plaque-forming units per cell, whereas the parallel set without cover slips were infected at 10 plaque-forming units per cell. The latter were harvested and titrated as described above to follow the 32°C time course of viral replication in cultures treated with TPA and in controls. As mentioned earlier, the viral replication cycle was accelerated some 5 h at mid-exponential times by TPA treatment (Fig. 3). At intervals, the parallel cultures containing cover slips were processed by fixing the cover slips in acetone and staining with a rabbit antiserum directed against DBP. Indirect immunofluorescence, with a fluoresceinated goat anti-rabbit antiserum, was observed with an ultraviolet light microscope. TPA-treated cultures displayed a DBP appearance 3-h earlier than the control cultures, and the number of cells displaying DBP fluorescence reached a maximum at 27 to 30 h p.i. in TPA-treated cultures, whereas in the controls the maximum occurred after 30 h (Fig. 3, insert). In addition, the appearance of the "mature" form of antigen-antibody complexes, i.e., dense masses of brightly staining material (24), occurred in some cells at 24 h in TPA-treated and at 27 h in untreated cells. In other experiments employing hybridization of newly synthesized pulse-labeled viral DNA to adenovirus DNA immobilized on filters (6), we have demonstrated that the onset of viral DNA synthesis is also accelerated by TPA treatment (data not shown). Host cell DNA synthesis in uninfected cells is not affected by TPA-treatment (data not shown). These results provide further evidence that the TPA-mediated enhancement of Ad5 replication occurs during the early phase of viral replication, probably before the onset of viral DNA synthesis.

Effects of TPA on adenovirus messenger RNA (mRNA) production. Our findings that TPA caused an acceleration of the appearance of Ad5 DBP, the onset of viral DNA synthesis, and the appearance of infectious virus in HeLa cells infected with Ad5 suggested that TPA might enhance the transcription of early genes of Ad5 and thus accelerate the entire cycle of viral replication. To examine this possibility, Ad5-infected cell monolayers were incubated at 32°C and labeled with [^{3}H]uridine for successive 2-h periods during the first 10 h p.i. Polyadenylated [poly(A)$^+$] RNA was isolated from the cytoplasm and hybridized to an excess of filter-bound *Hin*dIII fragments of Ad5 DNA (Fig. 4). RNA hybridizing to the early regions Ia, II, III, and IV (fragments $G_{0.0\text{-}8.0}$, $A_{50.1\text{-}73.6}$, $B_{73.6\text{-}89.1}$, and $F_{89.1\text{-}97.1}$, respectively) was detected 2 h earlier in TPA-treated cells than in untreated cells. Except for their earlier appearance in TPA-treated cells, the production of virus RNA from regions I, II, and IV followed similar overall kinetics in treated and untreated cells. However, in addition to appearing earlier in TPA-treated cells, the amount of RNA from region III was transiently increased more than threefold by TPA. In both TPA-treated and control cells, RNA from the early regions Ia and III was detected 2 h earlier than RNA from regions II and IV. Little or no hybridization to late regions of Ad5 DNA was detected, but a small amount of hybridization to the fragment containing early regions Ib and IIb ($E_{8.0\text{-}17.0}$ and $C_{17.0\text{-}31.5}$) was consistently seen during the first 10 h of infection in TPA-treated cultures.

In additional studies the 2-h labeling periods were spaced so as to encompass the first 20 h p.i. and thus include the onset of late virus gene expression (Fig. 5). Again, the production of early Ad5 mRNA's was accelerated in TPA-treated cells during the first 10 h postinfection. Between 10 and 15 h p.i., hybridization of mRNA from TPA-treated cells to Ad5 DNA fragments A, B, and E increased sharply; this was followed at later times by an increase in hybridization to fragment D, which contains late viral DNA sequences, and to fragment C, which contains the late promoter in addition to sequences expressed in low abundance at early times (32). A similar pattern of hybridization was observed with mRNA obtained from control cells, but these mRNA's appeared approximately 4 h later than in the TPA-treated cells. Hybridization to the early regions Ia and IV and to the late region $H_{31.5\text{-}37.3}$ did not increase sharply between the 10- and 22-h interval with mRNA obtained from either the TPA-treated or control cells.

DISCUSSION

Although tumor-promoting agents have been shown to exert a spectrum of effects on cells in culture, the critical biochemical alterations relevant to their biological actions are not known (for reviews, see references 29, 47, and 48). Recent studies have indicated that TPA and related macrocyclic plant diterpenes can enhance

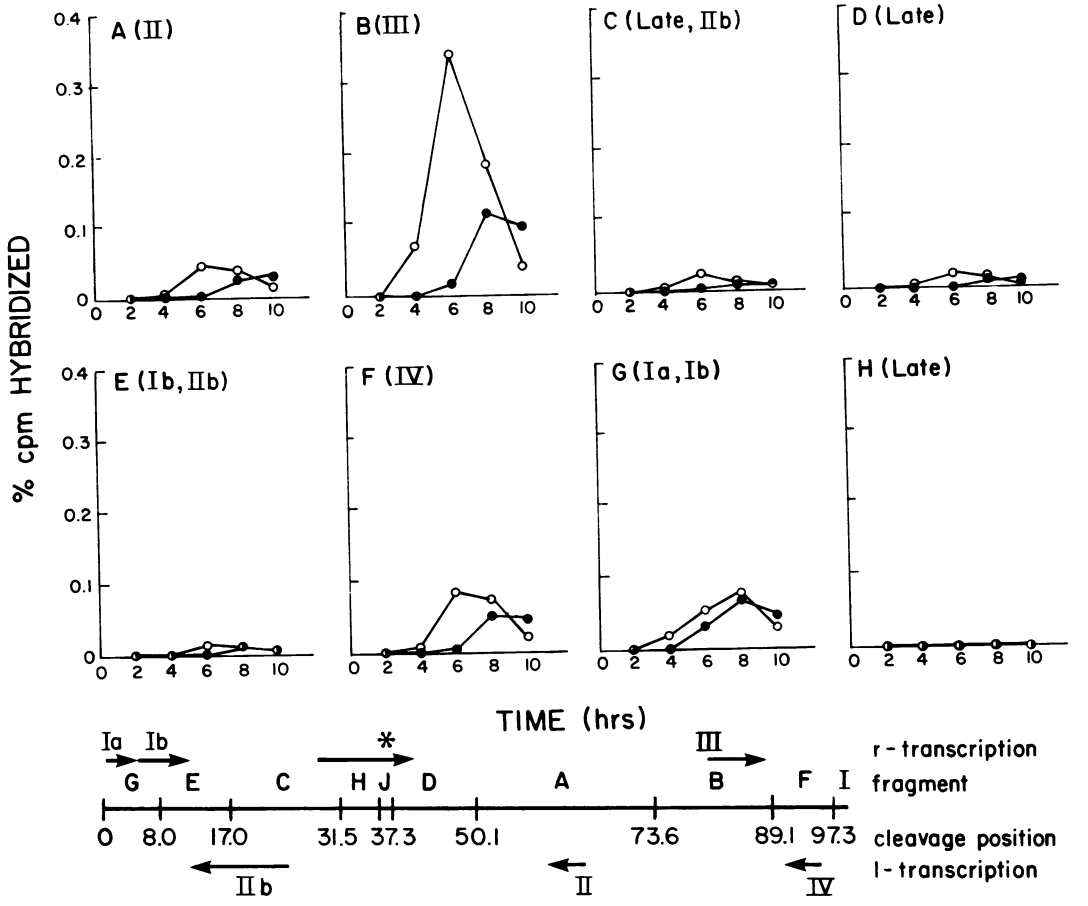

FIG. 4. *Effect of TPA on Ad5 poly(A)$^+$ RNA (2 to 10 h p.i.). Confluent HeLa monolayers were infected with 100 plaque-forming units of Ad5 per cell at 32°C. At intervals 50 μCi of [^{3}H]uridine per ml (80 Ci/mmol, New England Nuclear) was added and incubation continued for 2 h. Cytoplasmic poly(A)$^+$ RNA was isolated by phenol extraction and oligodeoxythymidylic acid-cellulose chromatography as described in the text. Thirty percent of the cytoplasmic RNA labeled from 5 to 7 h p.i. was retained on the column; the percentage of poly(A)$^+$ RNA at each time did not differ significantly in the TPA-treated and control cultures. Specific activity of the poly(A)$^+$ RNA was 3 × 10^4 to 4 × 10^4 cpm/μg. Poly(A)$^+$ RNA was hybridized to separated HindIII restriction endonuclease fragments of Ad5 DNA bound to nitrocellulose filters (2.5-μg equivalent per filter). Hybridization is expressed as percentage of total counts per minute in the hybridization reaction that bound to the filter. Each point on the graph represents the midpoint of a 2-h labeling period. The lower portion of the figure shows the location of early mRNA on the Ad5 HindIII restriction endonuclease cleavage map. Fragments are designated by letter in order of decreasing size. Map positions are shown as the percentage of genome length; the positions of major early mRNA groups are indicated by arrows identified by roman numerals. Asterisk refers to an immediate early transcript.*

viral transformation (20, 34, 43, 46, 53), alter viral gene expression in cells containing integrated viral genomes (1, 13, 14, 16, 26, 27, 33, 55, 56), and enhance viral DNA replication in infected cells (27, 33). The ability of TPA to alter phenotypic properties in normal chicken embryo fibroblasts and Rous sarcoma virus-transformed chicken embryo fibroblasts does not appear to involve an effect of this agent on the level of protein kinases encoded by the viral *src* or endogenous *sarc* genes (25).

In the present study we have demonstrated that TPA accelerates the appearance of a CPE in Ad5-infected HeLa cells (Fig. 1). This effect is not blocked by leupeptin, antipain, or *trans*-retinoic acid (Table 1) and does not appear to be a consequence of TPA induction of plasminogen activator. It may involve an alteration in the cytoskeleton, since TPA has been shown to induce cytoskeletal changes in chicken embryo fibroblast cells, and this effect also is not blocked by protease inhibitors (41). In the case of Ad5-

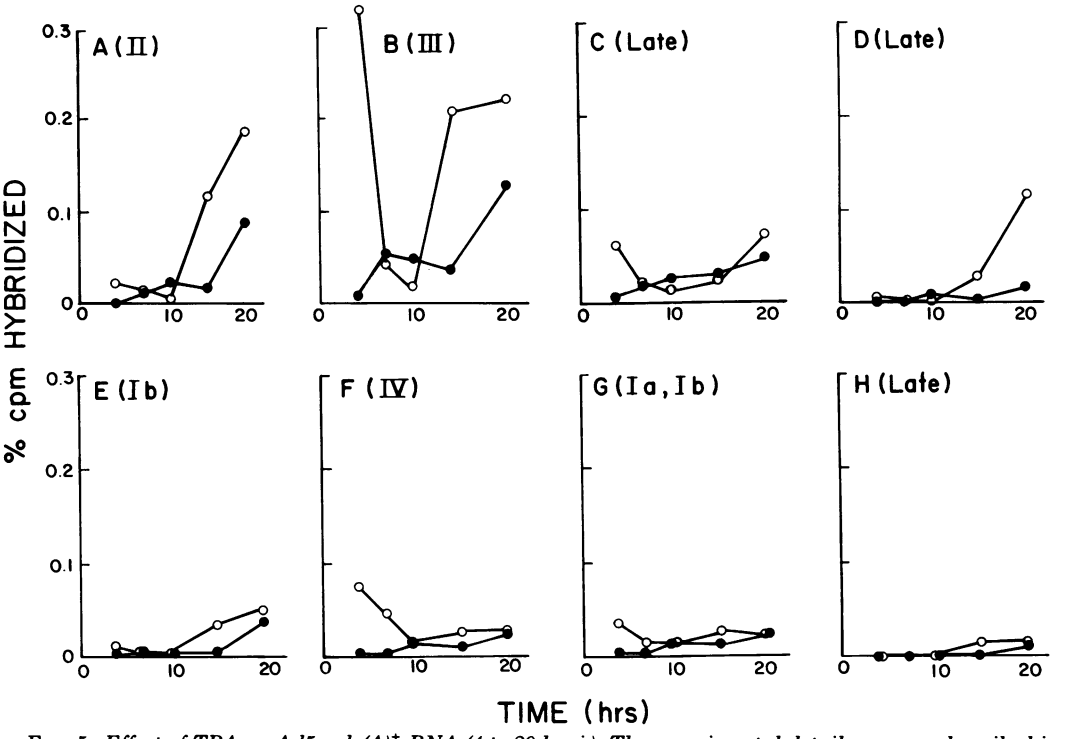

FIG. 5. *Effect of TPA on Ad5 poly(A)$^+$ RNA (4 to 20 h p.i.). The experimental details were as described in the legend to Fig. 4.*

infected cells, the TPA-induced acceleration of CPE appears to be related to an acceleration of the viral replicative cycle (Fig. 2 and 3). We have found that TPA treatment of Ad5-infected cells accelerates the following events: the appearance of the early viral antigen, designated 72K DBP; the onset of viral DNA replication; the production of infectious viral progeny; and the appearance of specific viral mRNA's (Fig. 4 and 5).

Our nucleic acid hybridization results demonstrate that production of specific viral mRNA's is dramatically affected at early times after exposure of cells to TPA (Fig. 4 and 5). TPA accelerated the appearance of cytoplasmic poly(A)$^+$ virus mRNA's from all but one of the major early regions, i.e., region Ib. Labeling of poly(A)$^+$ RNA from early region III was also transiently stimulated more than threefold by TPA. This region encodes a small glycoprotein (42). We are currently examining the possibility that TPA enhancement of the synthesis of this glycoprotein plays a role in the accelerated CPE seen in adenovirus-infected cells exposed to TPA.

Our data suggest that although TPA accelerates the appearance of viral mRNA's, it does not induce late viral DNA sequences to be abnormally expressed at early times in the replicative cycle, although this requires further study. We did detect hybridization of poly(A)$^+$ cytoplasmic RNA to *Hin*dIII fragments C and D at early times. This region has been found recently to contain early genes in addition to VA RNA [which is not poly(A)$^+$] and the major late promoter. The two early regions, IIb (22) and an "immediate early" region (32, 43a, 46a) which maps between coordinates 29 and 41, are not normally expressed in an abundance expected to be detectable in our experiments. Thus, TPA may stimulate expression of these regions also.

We have observed a reproducible decrease in virus mRNA labeling in TPA-treated cultures after the initial stimulation (Fig. 4). This may reflect either regulatory events that normally occur early in lytic infection (2, 5, 38) or a transient effect of TPA itself, perhaps similar to that observed with TPA induction of plasminogen activator or ornithine decarboxylase (for a review, see reference 48).

Virus DNA replication was detected at 8 h p.i. in TPA-treated cells, about 2 h earlier than in untreated cells (data not shown). Because the increase in viral RNA production between 10 and 20 h p.i. (Fig. 5) included sequences present

in high abundance primarily in late viral RNA (those hybridizing to fragment D), this secondary increase is most likely to be the result of viral DNA replication and the consequent transcription of late viral genes.

The ability of TPA to alter cellular levels of discrete classes of mRNA has recently been reported in other systems (1, 26). TPA treatment of normal NIH/3T3 cells for 4 to 6 h increases the levels of mRNA for a 35,000-dalton extracellular glycoprotein (the major excreted glycoprotein) which is synthesized in large amounts by Kirsten sarcoma virus-transformed cells (26). Enhanced production of mouse mammary tumor virus RNA occurs in a TPA-treated, mouse mammary tumor virus-producing C3H mouse mammary tumor cell line (1). In this system, dexamethasone was also capable of enhancing viral mRNA production and the combination of dexamethasone and TPA further increased mouse mammary tumor virus RNA production.

In previous studies we have demonstrated that certain clones of rat embryo cells transformed by a temperature-sensitive mutant of Ad5 (H5*ts*125) exhibit progressive alterations in their phenotype with continued subculture or exposure to TPA (13, 14, 16, 19). In a series of H5*ts*125-derived subclones, we demonstrated that progression did not involve an alteration in the pattern of viral DNA integration in the host cell genome, when using the restriction enzymes *Eco*RI and *Xba*I (14).

The studies mentioned above and those reported in this manuscript suggest that TPA-enhanced progression of the H5*ts*125-transformed clones might involve an alteration in the expression of integrated H5*ts*125 sequences, at the level of either transcription or RNA processing or both. Studies on the effects of TPA on viral gene expression in transformed cells containing integrated viral DNA should therefore be of interest and may shed light on mechanisms of chemical-viral synergy in the process of cell transformation.

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