

## Accelerated Onset of Viral Transcription in Adenovirus-Infected HeLa Cells Treated with the Tumor Promoter 12-*O*-Tetradecanoyl-Phorbol-13-Acetate

TIMOTHY H. CARTER,<sup>1\*</sup> ZAHRA ZAKERI MILOVANOVIC,<sup>1</sup> LEE E. BABISS,<sup>2</sup> AND PAUL B. FISHER<sup>2</sup>

*Department of Biological Sciences, St. John's University, Jamaica, New York 11439<sup>1</sup>; and Department of Microbiology, Cancer Center, College of Physicians and Surgeons, Columbia University, New York, New York 10032<sup>2</sup>*

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When adenovirus type 5-infected HeLa cells were exposed to the tumor promoter 12-*O*-tetradecanoyl-phorbol-13-acetate, short pulse-labeling with [<sup>3</sup>H]uridine *in vivo* and [<sup>3</sup>H]UTP incorporation by isolated nuclei *in vitro* were both consistent with a decreased latent period before initiation by RNA polymerase at early viral promoters. Acceleration was not dependent upon concurrent protein synthesis and could not be attributed to rapid entry of virus into the cell nucleus. 12-*O*-tetradecanoyl-phorbol-13-acetate suppressed the transcription-delay phenotype of the E1a mutant, hr1, without restoring its ability to replicate.

Diterpene phorbol ester tumor promoters, such as 12-*O*-tetradecanoyl-phorbol-13-acetate (TPA), increase the transformation frequency of cells infected with type 5 adenovirus (Ad5) (9, 10), simian virus 40 (25), polyoma (29), Epstein-Barr virus (36), and bovine papilloma virus (1). In a number of virus-transformed cells, exposure to TPA has been shown to increase the amount of viral RNA (1, 2, 17, 18, 31, 32, 35), and in uninfected cells, TPA rapidly induces the synthesis of specific RNAs (13, 15, 34) and polypeptides (7, 21, 22). An understanding of the mechanism by which TPA induces gene expression may therefore be crucial to understanding its role as a tumor promoter (14).

We have shown that TPA accelerates Ad5 replication in HeLa cells and that early viral mRNA appears in cytoplasm ahead of schedule when TPA is added at the time of infection (11). To test the hypothesis that accelerated production of Ad5 mRNA resulted from early onset of transcription, we measured incorporation of [<sup>3</sup>H]uridine into viral RNA during a 5-min pulse at various times after infection at 33°C (Fig. 1A). Early viral RNA was detected in TPA-treated cells 2 to 3 h before it was detected in samples from control cultures. Specific radioactivities of RNA from treated and control cells were similar at each time point, suggesting that increased labeling of viral RNA did not result from changes in transport of [<sup>3</sup>H]uridine or from alterations in the UTP pool size or availability that favored mRNA synthesis nonspecifically. In seven independent experiments, including the ones shown in Fig. 1, viral RNA could be detected an average of 2 h earlier in TPA-treated cells than in controls. This represents a significant acceleration, since the normal latent period is only 4 to 5 h at 33°C. In this respect, TPA action resembles that of the Ad5 E1a regulatory product (5, 26), the absence of which (in E1a mutants such as hr1) considerably delays early gene expression (12, 19, 20, 26).

When the experiment shown in Fig. 1A was repeated in the presence of 25 µg of cycloheximide per ml, TPA still accelerated the onset of RNA synthesis from all early regions (Fig. 1B). In this case, however, the maximal rates of synthesis for all early regions in TPA-treated cells were also 2 to 3 times greater than in the controls. The magnitude of this difference was variable in different experiments and was dependent upon the time after infection at which RNA was

analyzed. In two additional experiments similar to the one shown in Fig. 1B, the rate of total adenovirus RNA synthesis in TPA-treated cells was two- and sixfold greater than in controls at 7.5 h postinfection (h.p.i.). At 6 h.p.i., the differences were 3.4- and 17.7-fold, respectively. Comparison of panels A and B of Fig. 1 shows that cycloheximide alone did not increase the rate of early RNA synthesis compared with untreated controls.

A possible explanation for the early start of viral transcription in TPA-treated cells is that viral genomes enter the nuclei of infected cells more rapidly. However, when HeLa monolayers were infected with purified virus grown in the presence of [<sup>3</sup>H]thymidine, association of label with the nuclear fraction was linear throughout the first 6 h, and the kinetics of uptake were not altered by TPA treatment (Fig. 2).

Although TPA primarily changes the timing of early Ad5 gene expression, rather than its maximal rate, the effect is seen as an increased rate of viral RNA labeling during the interval from 6 to 7 h.p.i. (Fig. 1). We therefore used differences in the rate of viral RNA synthesis at 7 h.p.i. to measure the degree of acceleration of early transcription by TPA and other phorbol esters. The strong tumor promoters TPA and phorbol didecanoate both enhanced RNA labeling at 7 h.p.i., whereas phorbol and 4- $\alpha$ -phorbol-didecanoate, both inactive as tumor promoters on mouse skin (33), caused no enhancement (data not shown).

The effect of TPA on viral transcription was confirmed in nuclei isolated from infected cells at 7 h.p.i. and incubated *in vitro* under conditions that measure activity of RNA polymerase capable of initiating *de novo* on endogenous viral DNA (Table 1). During a 1-h incubation, nuclei from TPA-treated cells synthesized from 4 to 7 times the amount of viral RNA synthesized by control nuclei. The effect of TPA was specific for virus transcription, because the specific radioactivity of total labeled RNA was not increased by TPA. TPA also did not alter the rate or amount of viral transcription when added directly to isolated nuclei from either TPA-treated or control cultures, suggesting that the intact cell is required for the TPA effect on viral transcription (unpublished data).

Two viral genes control the timing and amount of Ad5 early transcription. Mutants in E1a (e.g., hr1) cause delayed onset of transcription (5, 12, 18), whereas E2a mutants (e.g.,

\* Corresponding author.

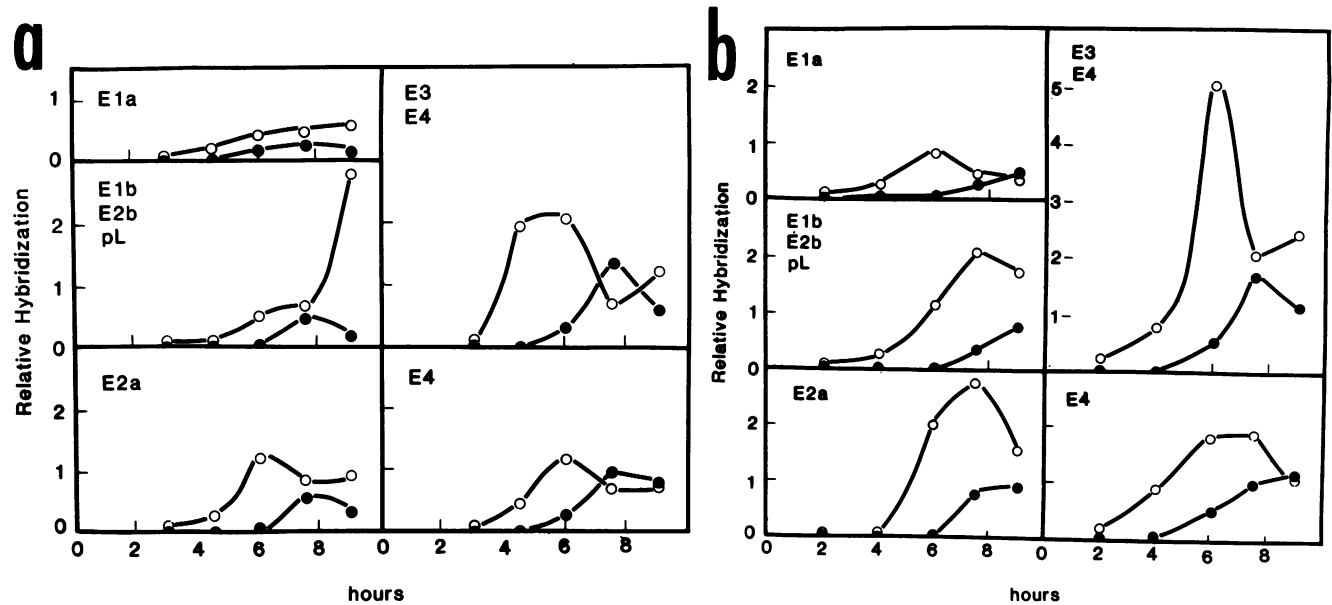


FIG. 1. Effect of TPA on rate of early transcription in HeLa cells infected by wild-type Ad5. HeLa monolayers were infected with 100 to 200 PFU per cell and pulse-labeled with 500  $\mu\text{Ci}$  of [ $^3\text{H}$ ]uridine per ml for 5 min at the indicated times. Whole-cell RNA was purified by hot phenol extraction (30) and hybridized to *KpnI* restriction endonuclease fragments of Ad5 DNA bound to nitrocellulose filters (11). The standard deviation of duplicate hybridization values was  $\pm 9.2\%$ . TPA (100 ng/ml) was added after a 1-h virus adsorption (open circles); 0.01% dimethyl sulfoxide was added to a second set of cultures at 1 h.p.i. (closed circles). Legends in the upper left hand corner of each panel indicate the transcription unit or units encoded by each *KpnI* fragment: E1a = fragment H, 0 to 6 map units (m.u.); E1b, E2b, pL (late promoter) = fragment A, 6 to 23.5 m.u.; E2a = fragment E, 62 to 72.5 m.u.; E3, E4 = fragment D, 81 to 93.5 m.u.; E4 = fragment G, 93.5 to 100 m.u. Transcription is expressed as "relative hybridization," which is calculated by dividing the percentage of input counts per minute hybridized by the relative length of the transcription unit or units encoded by each fragment. (a) No cycloheximide added. (b) Cycloheximide (25  $\mu\text{g}/\text{ml}$ ) added to both TPA- and dimethyl sulfoxide-treated cells at 1 h.p.i.

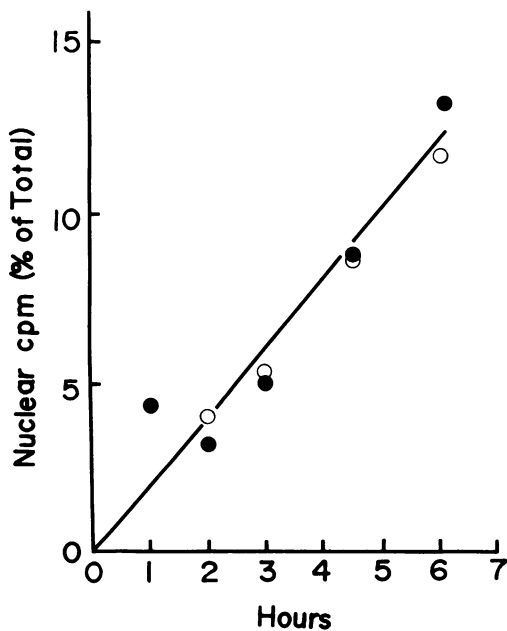


FIG. 2. Rate of association of labeled viral DNA with the nuclear fraction of HeLa cells. Ad5 was labeled in vivo by exposure to 50  $\mu\text{Ci}$  of [ $^3\text{H}$ ]thymidine per ml (40 to 60 Ci/mmol; Amersham Corp.) from 10 to 40 h.p.i., purified (8), and dialyzed against phosphate-buffered saline in 50% glycerol at 4°C overnight. The viral DNA had a specific radioactivity of  $6.15 \times 10^4$  cpm/ $\mu\text{g}$ . Labeled virions were diluted in Dulbecco modified Eagle medium containing 2% fetal

TABLE 1. Effect of TPA exposure in vivo on prolonged viral transcription in "initiating" nuclei in vitro<sup>a</sup>

Expt	Labeling time (min)	% Viral RNA in cells treated with:		TPA/DMSO ratio
		DMSO	TPA	
1	10	$1.6 \times 10^{-2}$	$7.3 \times 10^{-2}$	4.6
	60	$1.2 \times 10^{-2}$	$7.3 \times 10^{-2}$	6.1
2	10	$4.7 \times 10^{-2}$	$3.1 \times 10^{-1}$	7.2
	60	$7.0 \times 10^{-2}$	$3.3 \times 10^{-1}$	4.7

<sup>a</sup> HeLa monolayers were infected and treated with TPA or DMSO as described in the legend to Fig. 1. Nuclei were isolated at 7 h.p.i., and in vitro transcription reactions were carried out according to Manley et al. (23, 24) with minor modifications. In each experiment, half of the reaction was stopped at 10 min and the remainder was stopped after 60 min. RNA was extracted with hot phenol and hybridized to denatured whole Ad5 DNA on nitrocellulose filters (6). Percent viral RNA was calculated as [(counts per minute hybridized - counts per minute bound to blank filter)/total cpm]  $\times$  100. When nuclei were incubated for 60 min, the total amount of viral RNA synthesized increased threefold over the amount at 10 min (data not shown). DMSO, Dimethyl sulfoxide.

bovine serum and adsorbed to HeLa monolayers for 1 h, after which the cells were treated with either 100 ng of TPA per ml (open circles) or 0.01% dimethyl sulfoxide (closed circles). At the indicated times, cells were fractionated by Nonidet P-40 treatment and centrifugation (11), and the radioactivity associated with the nuclear fraction was determined after TCA precipitation.

TABLE 2. Effect of TPA on early Ad5 transcription in HeLa cells infected by hr1 at 33°C<sup>a</sup>

Early region	% Input cpm ( $\times 10^3$ ) in HeLa cells infected with:			
	Wild type		hr1	
	-TPA	+TPA	-TPA	+TPA
1a	0.24	0.34	0.45	0.83
1b	0.49	0.47	0.24	0.75
2a	0.16	1.85	0.12	2.65
3	0.40	1.47	0.21	1.92
4	0.23	1.31	0.01	0.18

<sup>a</sup> HeLa monolayers were infected with 10 PFU of wild type or hr1 per cell, treated (+) or not treated (-) with TPA or dimethyl sulfoxide at 1 h.p.i., and labeled for 5 min at 7 h.p.i. with [<sup>3</sup>H]uridine. Whole-cell RNA was extracted with hot phenol and hybridized to denatured, cloned restriction endonuclease fragments of Ad5 DNA on filters (E1a, 0 to 4.5 m.u.; E1b, 4.5 to 8.0 m.u.; E2a, 59.5 to 78.5 m.u.; E3, 75.9 to 84.0 m.u.; E4, 98.3 to 100 m.u.). The results from two separate experiments, each hybridized in duplicate, were averaged. Specific activity of the RNA ranged from  $1.9 \times 10^4$  to  $3.4 \times 10^4$  cpm/ $\mu$ g, and input counts per minute ranged from  $3.1 \times 10^6$  to  $12.7 \times 10^6$  per hybridization. Background was 20 to 30 cpm.

H5ts125) allow prolonged transcription (6, 8, 28) and increased stability (3, 27) of mRNA. As expected from the cycloheximide results, H5ts125 did not prevent the acceleration of early transcription onset at 39.5°C (data not shown). In hr1-infected HeLa cells, TPA allowed expression of most early regions by 7 h.p.i. at 33°C, at rates equal to or exceeding those in wild-type cells treated with TPA, whereas hr1-infected cells without TPA synthesized little or no early RNA (Table 2). Northern blots of cytoplasmic polyadenylated RNA from region E3 were consistent with these results (data not shown). Although TPA was able to correct the transcription-delay phenotype in hr1-infected HeLa cells, it did not suppress the replication defect of hr1. The titers of lysates from TPA-treated HeLa cells, determined on the permissive 293 cell line, were less than twice that of control lysates without TPA. Comparison of lysate titers on 293 and HeLa cells showed that most, if not all, of the difference in virus yield between TPA-treated and control cells could be explained by revertants in the hr1 stock (unpublished data).

Like TPA, cycloheximide can also overcome the delay in Ad5 early gene expression in hr1-infected HeLa cells (26). The increased maximal rate of transcription in TPA-treated cells blocked with cycloheximide (Fig. 1B) suggests that the effect of TPA on early viral transcription may be analogous to that of the E1a gene product, and the ability of TPA to overcome the early inhibition of viral transcription in hr1-infected cells is consistent with this hypothesis. Results to be published elsewhere show that TPA can also suppress the cold-sensitive defect of hr1 in cell transformation (4, 16).

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