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Tumor Promoter 12-*O*-Tetradecanoylphorbol 13-Acetate Stimulates Simian Virus 40 Induction by DNA-Damaging Agents and Tumor Initiators

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Simian virus 40 (SV40)-transformed Syrian hamster kidney cells produce infectious SV40 virus particles after treatments which damage DNA, such as UV irradiation or mitomycin C treatment. We have found that the induction of SV40 by DNA-damaging agents is greatly stimulated when a typical tumor promoter, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), is present in the medium. Phorbol, which has a molecular structure similar to TPA but does not have any tumor-promoting activity, showed no such stimulatory effect on SV40 induction. This apparent synergistic effect of DNA-damaging agents and tumor promoter (TPA) was more pronounced when a tumor initiator, benzo [*a*]pyrene or 2-acetamidofluorene, was combined with TPA. The effect of TPA on UV-triggered SV40 induction was greatly influenced by the timing of TPA addition to the culture medium, which was most efficient when addition of TPA was 5 to 20 h before UV irradiation. The effect of TPA, however, was not observed in SV40 rescue from hamster cells by cell fusion with permissive monkey (C7) cells.

It has been known for a long time that bacteriophage is induced from lysogenic bacteria after treatment of the lysogens with DNA-damaging agents such as UV light or mitomycin C. The mechanism of the phage induction process is now quite well understood, especially in bacteriophage λ induction (9). In mammalian cells, similar effects of DNA-damaging agents have been reported in simian virus 40 (SV40) induction from virus-transformed Syrian hamster cell lines. UV light, X-ray, or mitomycin C treatment of the transformed cells induces a low level of infectious SV40 particles (3, 5, 10). There is evidence that under these inducing conditions, SV40 genomes are physically excised from the host chromosomes in which the virus genomes have been integrated (8). More recently, it was reported that various chemical and physical carcinogens (tumor initiators) cause SV40 DNA amplification in Chinese hamster embryo cells transformed by SV40 (6). In this case, the DNA amplification did not result in the rescue of infectious virus particles or the formation of complete viral DNA molecules. On the other hand, Epstein-Barr virus is induced from certain cell lines by tumor promoters such as 12-*O*-tetradecanoylphorbol 13-acetate (TPA) (14). Epstein-Barr virus induction, however, is apparently not affected by DNA-damaging agents, including a variety of tumor initiators (13).

In this paper, we report that SV40 is induced from Syrian hamster kidney cells (E line) in a synergistic manner in the presence of a tumor promoter (TPA) and DNA-damaging agents, including tumor initiators.

MATERIALS AND METHODS

Materials. TPA was purchased from P. Borchert (Eden Prairie, Minn.). Phorbol was a generous gift from M. Terada and T. Sugimura of the National Cancer Center of Japan. Eagle essential medium was obtained from Nissui Seiyaku (Tokyo). Fetal calf serum and calf serum were purchased from Flow Laboratories, Inc., McLean, Va. and Alpha (Granite) Biosupply (Tokyo), respectively. Agar (Noble agar) was obtained from Difco Laboratories, Detroit, Mich.

Cells and cell culture. SV40-transformed Syrian hamster kidney cells (E line, kindly provided by P. H. Black, Institution, City, State) were cultured in plastic petri dishes (60 by 15 mm) in Eagle minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum at 37°C in humidified air with 5% CO_2. C7 (African green monkey kidney) cells were grown in a similar way but with 10% heat-inactivated calf serum in Eagle MEM.

Infectious virus assay. The cells were collected from petri dishes with a rubber policeman and suspended in phosphate-buffered saline (NaCl, 137 mM; KCl, 4.2 mM; Na_2HPO_4, 9.6 mM; KH_2PO_4, 1.1 mM). After freezing and thawing (−80 and 37°C), the samples were sonicated (Kubota, model 200 M; 1.8 A, 5 min) and centrifuged (1,200 × *g*, 5 min), and the supernatant fractions were used for the virus assay. The cell-free extracts (200 μl each), after appropriate dilution, were mixed with C7 cells (4×10^5 to 5×10^5 cells) which had been grown to confluence. After 2 h of virus absorption with occasional gentle rocking, the samples

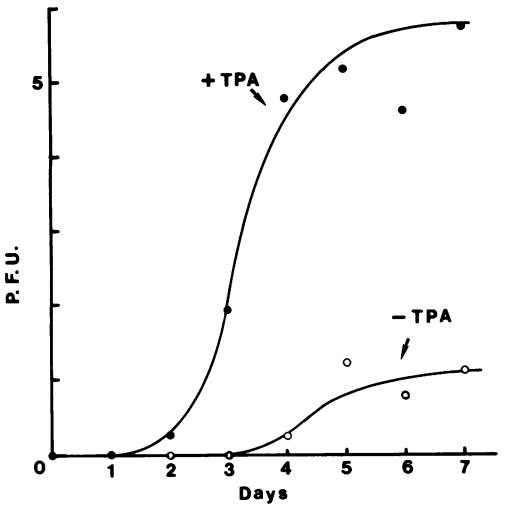

FIG. 1. Effect of a tumor promoter (TPA) on SV40 induction by UV irradiation. SV40-transformed Syrian hamster kidney cells (E line) were inoculated at a density of 3×10^5 cells per ml into plastic petri dishes, each with 5 ml of fresh medium. After 2 days at 37°C, the medium was removed by suction, and the cells were rinsed once with phosphate-buffered saline. The samples were placed under a germicidal lamp (Toshiba-GL15, 15 W) at a distance of 52 cm for 15 s, which gave a UV intensity of 20 J/m^2. After irradiation, fresh medium (5 ml) was added for further incubation at 37°C in the absence or presence of TPA (150 ng/ml). At various intervals, the cells were collected and suspended in 1 ml of phosphate-buffered saline. After freezing, thawing, and sonication, the samples were centrifuged, and the supernatant fractions were assayed for infectious virus. For details, see the text.

were transferred to 5 ml of solid medium (MEM supplemented with 10% heat-inactivated calf serum and 0.9% Noble agar [Difco]). After 5 days at 37°C, 3 ml of the same medium containing 5% calf serum was overlaid on top of the solid medium. The samples were further incubated for 3 days under the same conditions, and 2 ml of MEM (0.9% agar) containing neutral red (0.016%) was added to each dish. The number of plaques was scored next day. Usually, the dilution of the original extracts was made to give between 10 to 100 plaques per dish, and samples with standard infectious SV40 viruses were always included in the assay. The number (PFU) of plaques shown in the figures is expressed as the number per 0.1 mg of protein of the treated cells. On average, 0.1 mg of protein was equivalent to 3×10^5 to 5×10^5 cells, and each dish (5 ml) contained up to 1.5×10^7 cells. The protein concentrations of the fractions were determined by the Lowry procedure with bovine serum albumin as the standard protein.

RESULTS AND DISCUSSION

When SV40-transformed Syrian hamster cells (E line) were irradiated with UV light (20 J/m^2), SV40 infectious particles were produced after 5 days of incubation (Fig. 1). However, when the UV-irradiated cells were incubated in the presence of a tumor promoter (TPA; 150 ng/ml), the induction of SV40 infectious particles was increased more than fivefold. TPA alone did not induce any appreciable number of virus particles. The stimulatory effect of TPA on SV40 induction as a function of TPA concentration is shown in Fig. 2. The stimulation of SV40 induction by TPA increased almost linearly as TPA concentration in the medium increased up to 100 ng/ml and gradually leveled off thereafter (Fig. 2). The number of plaques produced by UV was in the same range or slightly lower (to 20%) than what other workers had obtained (3, 5, 10). Although we often found that the level of SV40 infectious particles induced by UV irradiation alone varied substantially, depending on the cell preparations, TPA always expressed the stimulatory effect shown in Fig. 2. The concentrations of TPA for the appreciable stimulation of SV40 induction were the same as or one order higher than the range of TPA concentrations which most efficiently affect transformation (7) and cellular differentiation in vitro (1, 2, 11, 12). Figure 3 shows the effect of TPA on SV40 induction as a function of UV dose. When 100 ng of TPA per ml was present in the medium, the maximum SV40 induction was observed when 20 J of UV per m^2 was given to SV40-transformed (E line) cells. Figure 3 shows that phor-

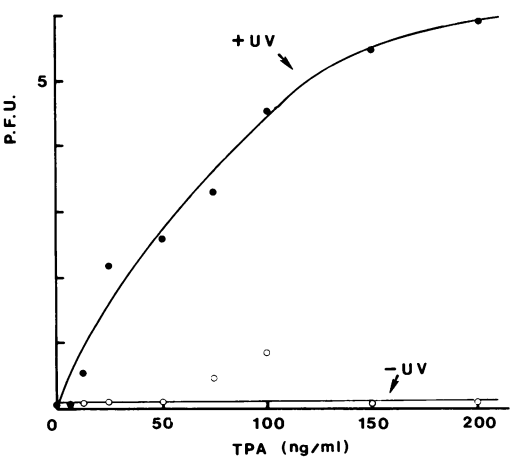

FIG. 2. Effect of TPA concentration on SV40 induction with UV light. SV40-transformed Syrian hamster kidney cells (E line) were grown, and a portion of the sample was irradiated with UV light (5 J/m^2) as described in the legend of Fig. 1. The samples were then incubated for 5 days in the presence of various concentrations of TPA as indicated on the abscissa. On day 5, the cells were disrupted and the number of infectious virus particles was scored as described in the text. The number (PFU) of plaques is expressed as the number per 0.1 mg of protein of the treated cells.

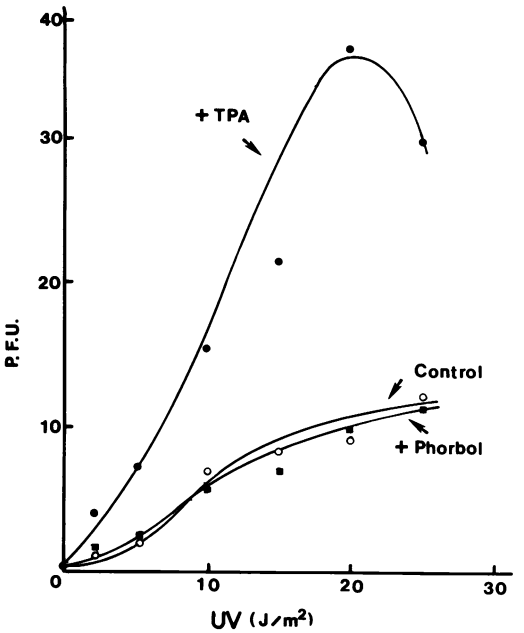

FIG. 3. Effect of TPA and phorbol on SV40 induction as a function of UV dose. SV40-transformed Syrian hamster kidney cells (E line) were grown and irradiated with UV light at various intensities as shown on the abscissa. The cells were then incubated in the presence of TPA (100 ng/ml) or phorbol (100 ng/ml, kindly provided by M. Terada and T. Sugimura, National Cancer Center Institute, Tokyo). On day 5, the cells were disrupted by sonication, and the number of infectious virus particles in the extracts was assayed. The number (PFU) of plaques is expressed as the number per 0.1 mg of protein of the treated cells. For details, see the text.

bol, which has a molecular structure similar to TPA but does not have any tumor-promoting activity, showed no stimulatory effect on the SV40 induction caused by UV irradiation.

The stimulatory effect of TPA on SV40 induction was not limited to the SV40 induction with UV irradiation. SV40 induction in mitomycin C-treated cells was also stimulated by TPA (data not shown). Moreover, tumor initiators which usually have little effect on SV40 induction exhibited an inducing effect on SV40 induction when combined with TPA. As seen in Table 1, benzo [*a*]pyrene or 2-acetamidofluorene demonstrated little SV40-inducing activity by themselves, but substantial SV40 induction was observed when TPA was present in the medium. Again, phorbol did not express any appreciable synergistic activity with tumor initiators in SV40 induction.

The effect of TPA on UV-triggered SV40 induction was greatly affected by the timing of TPA addition to the culture medium. The stimulatory effect of TPA was most pronounced when TPA was added 5 to 20 h before UV irradiation (Fig. 4). Addition of TPA more than 20 h after UV irradiation had little effect on SV40 induction. These kinetic patterns suggest that TPA induces a cellular reaction(s) which potentiates the cells to induce SV40 upon UV irradiation.

It has been known that SV40 in semipermissive and nonpermissive cells is efficiently rescued (induced) when these cells are fused with permissive cell lines such as African green monkey cells (4). However, SV40 rescue in Syrian hamster cells by cell fusion with monkey cells (C7) was not affected over a wide range of TPA concentrations (data not shown). Thus, the effect of TPA on SV40 induction was apparently limited to the virus induction triggered by DNA-damaging agents, including tumor initiators.

The experimental results described above demonstrate that a tumor promoter (TPA) greatly stimulates SV40 induction by UV irradiation or by tumor initiators in Syrian hamster kidney cells. Since TPA alone did not induce SV40, TPA must be acting somehow in a synergistic way with those DNA-damaging agents in the SV40 induction process. The timing of TPA addition in relation to the timing of UV irradia-

TABLE 1. Effect of TPA and phorbol on the induction of SV40 by benzo[*a*]pyrene and 2-acetamidofluorene[a]

Addition (μg/ml)	Concn (μg/ml)	Plaques		
		Control	TPA (100 ng/ml)	Phorbol (100 ng/ml)
None		<1.0	<1.0	<1.0
Benzo[*a*]pyrene	3.75	<1.0	7.6	2.3
	7.50	<1.0	18.9	3.6
	15.00	<1.0	9.9	1.2
2-Acetamidofluorene	0.5	1.1	6.7	3.3
	5.0	1.3	50.5	6.8
	50.0	1.3	15.5	5.8
UV (20 J/m²)		9.2	35.7	9.8

[a] Confluently grown SV40-transformed Syrian hamster kidney cells (E line) were trypsinized and transferred to a cell density of 3×10^5/ml in fresh Eagle MEM supplemented with 10% heat-inactivated fetal calf serum. After incubation for 2 days at 37°C, the medium was removed by suction and the cells were rinsed once with phosphate-buffered saline. Portions of the samples were treated with UV light (20 J/m²) as described in the legend of Fig. 1. Immediately after addition of fresh medium, 2-acetamidofluorene (Sigma Chemical Co., St. Louis, Mo.), benzo[*a*]pyrene (Sigma), TPA (100 ng/ml), or phorbol (100 ng/ml) was added, and the samples were incubated for 5 days. The number (PFU) of plaques is expressed as the number per 0.1 mg of protein of the treated cells. For details, see the text.

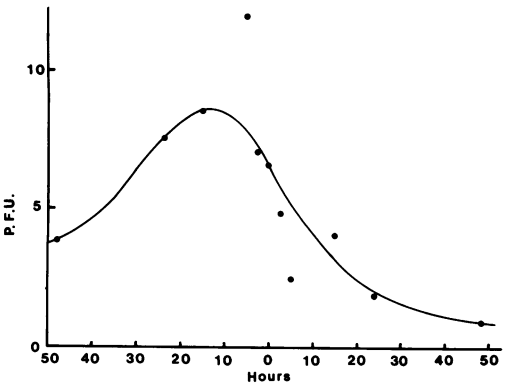

FIG. 4. Effect of timing of TPA addition on the induction of SV40. TPA (100 ng/ml) was added to the Syrian hamster cell culture at various intervals before and after UV irradiation (5 J/m^2). The cells were collected on day 5, and the number of infectious SV40 virus particles was scored. Time 0 indicates the time of UV irradiation. Timing of TPA addition before UV irradiation (to the left of 0) and after UV irradiation (to the right of 0) is indicated as a function of time (hours) on the abscissa. The number (PFU) of plaques is expressed as the number per 0.1 mg of protein of the treated cells. For details, see the text.

tion is critical in expressing its stimulatory effect (Fig. 4). It seems likely that potentiation of the cells by TPA has to be matched with the reaction triggered by DNA-damaging agents to express the maximum SV40 induction. Although the nature of these intracellular reactions induced by TPA- and DNA-damaging agents is not clear, it is interesting to note that Epstein-Barr virus, which is persistently present in the cells in nonintegrated form, is efficiently induced by tumor promoters alone. These results have led us to speculate that although the effect of DNA-damaging agents may be related to the excision of SV40 genomes from host chromosomes, the effect of TPA may be related to the derepression of the SV40 genomes which have been repressed. The synergistic effect observed here for SV40 induction by DNA-damaging agents (including tumor initiators) and TPA may reflect the effect of these agents on the two processes, excision and expression, both required for infectious SV40 particles to be produced.

The synergistic effect of tumor initiators and promoters for SV40 induction presented in this paper is similar, at least ostensibly, to the effect of these agents on tumorigenesis, in which both initiators and promoters are required to complete a tumorigenic process. However, in tumorigenesis, the effect of tumor promoters is seen most prominently when treatment of the cell (or tissues) with promoters (often repeatedly) follows treatment with tumor initiators. As described above, the SV40 induction was stimulated much more efficiently when TPA was added to the medium before treatment of the hamster cells with DNA-damaging agents or tumor initiators. The biological significance of the difference in timing of the treatment with TPA to obtain the most significant effect in tumorigenesis and SV40 induction is not clear at present. In any event, the SV40 induction described here may be used as a simple model system in studying the biological roles played by tumor initiators and promoters in tumorigenesis.

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