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# Effects of the Tumor Promoter TPA on the Induction of DNA Synthesis in Normal and RSV-Transformed Rat Fibroblasts

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Induction of DNA synthesis by the tumor promoter tetradecanoyl phorbol acetate (TPA) was studied in a line of cultured rat fibroblasts (Rat-1) and their Rous sarcoma virus-transformed derivative (Rat-1(RSV)). Following serum deprivation for 54 h to achieve quiescence, semiconservative DNA replication was measured by incubation of cells in BrdUrd and FdUrd after serum stimulation in the presence or absence of TPA. Optimal concentrations of TPA (0.1–0.5 μg/ml) in serum-free medium induced a small increase (10–15%) in the amount of DNA made over a 30-h period in both Rat-1 and Rat-1(RSV) cells. When Rat-1 cells were stimulated by a 4-h serum pulse, 30% of the DNA was replicated by 30 h. If the serum pulse was followed by TPA addition, 70% DNA replication was observed. If the serum pulse was preceded by TPA addition, the onset of DNA synthesis was delayed by several hours, but stimulation of DNA synthesis occurred. In contrast, the Rat-1(RSV) cells did not show an increase in DNA synthesis induced by TPA in similar protocols, but the serum-induced onset of DNA synthesis was delayed by several hours in the presence of TPA. Therefore, TPA acts as a co-inducer of DNA synthesis in the Rat-1 but not in the Rat-1(RSV) cells. The parent alcohol, phorbol, was inactive in Rat-1 cells, but delayed the onset of DNA synthesis in the Rat-1(RSV) cells. We conclude that the co-inducing and delaying activities of TPA on DNA synthesis appear to be distinct and to act at different points in the $G_1$ phase of the cell cycle.

**Key words: tumor promoter, DNA synthesis, transformed cells, serum stimulation**

The concept of tumor promotion was first developed from studies using mouse skin [1–4] and has since been applied to malignant transformation of other tissues [5–7] as well as cells in culture [8–12]. In the classical two-stage system of skin tumor formation,

Abbreviations used: TPA, 12-O-tetradecanoyl-phorbol-13-acetate; BrdUrd, 5′-bromodeoxyuridine; FdUrd, 5′-fluorodeoxyuridine; DMSO, dimethyl sulfoxide; TdR, thymidine; ODC, ornithine decarboxylase.

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a subcarcinogenic dose of a tumor initiator (mutagen) is followed by frequent applications of a tumor promoter to produce both benign and malignant tumors. Tetradecanoyl phorbol acetate (TPA) is the most active tumor-promoting phorbol diester present in croton oil [13], which was used by Mottram [4] to demonstrate the two-stage mechanism of carcinogenesis in mouse skin. Tumor promotion using TPA also has been demonstrated to occur in vitro in 10 $T_{1/2}$ mouse fibroblasts using different initiators [9–11].

In an attempt to elucidate the mechanism of tumor promotion, the effects of TPA on a variety of molecular events in cells in culture have been studied. These include a TPA-induced increase in ODC [14, 15], plasminogen activator synthesis [7], a decrease in epidermal growth factor binding [16, 17], and fibronectin production [18], and the induction of DNA synthesis [11, 14, 19, 20].

Tumor promoters stimulate cell proliferation in epidermal target tissue [21] as well as in primary cultures of mouse epidermal cells [20] and in rodent fibroblasts [11], in which a transient depression in DNA synthesis was also seen. TPA was found to be mitogenic for chick chondroblasts, but not for fibroblasts [22]. TPA has been shown to act as a comitogen with phytohemagglutinin (PHA) in cultures of bovine lymph node lymphocytes [23]. At dose levels of PHA and TPA which were independently inactive as mitogens, when used in combination they were highly effective in promoting lymphocytes to enter into cell replication. It has been suggested that lymphocytes which have been stimulated minimally with PHA depend on TPA to release or activate certain intracellular factors that promote the cell to engage in nuclear and cellular replication processes [22]. In human lymphocytes, however, TPA was found to be mitogenic [23].

Much of the confusion in the literature concerning the mitogenic versus comitogenic capacity of TPA is due, in part, to the addition of TPA to confluent cultures in serum-containing medium. In these instances an apparent mitogenic response might be the result of a TPA-induced sensitization to serum growth factors. In order to begin to investigate these events we studied the effect of TPA on the extent of DNA synthesis in arrested, serum-deprived normal rat fibroblasts (Rat-1 cells), and in cultures which were suboptimally stimulated by serum. DNA synthesis in rat fibroblasts transformed by Rous sarcoma virus (Rat-1(RSV) cells) was also investigated, in order to compare the effect of TPA on cells whose growth control mechanisms are controlled, at least in part, by the *src* gene product.

## MATERIALS AND METHODS

### Chemicals

TPA was purchased from Dr. Peter Borchert, Chemical Carcinogenesis, Eden Prairie, Minnesota, and phorbol from Consolidated Midland Corp., Brewster, New York. [2-$^{14}$C]-Thymidine (43 mCi/mmole) was obtained from Research Products International, Elk Grove, Illinois. Fetal bovine serum was purchased from K. C. Biologicals, Inc., Lenexa, Kansas.

### Cell Culture Techniques

The Rat-1 line used in these experiments refers to the F2408 established line derived from Fischer rat embryo fibroblasts [24]. Untransformed Rat-1 cells and Rat-1 cells transformed by the B-77 strain of Rous sarcoma virus (Rat-1(wt/RSV)) were kindly supplied by J. A. Wyke. The cell lines were propagated in Dulbecco's minimal essential medium containing 10% heat-inactivated (56°C, 30 min) fetal bovine serum at 39°C in a humidified 5% $CO_2$, 95% air atmosphere.

### Measurement of Replicated DNA

This technique has been described in detail [25]. Briefly, the technique requires labeling exponentially growing cells for several generations with [$^{14}$C] thymidine. Upon stimulation from the serum-deficient state, the medium was adjusted to contain 50 $\mu$g/ml 5′-bromodeoxyuridine (BrdUrd) and 0.1 $\mu$g/ml 5′-fluorodeoxyuridine (FdUrd). Newly replicated DNA was then determined after centrifugation of DNA, isolated from these cultures, on neutral CsCl gradients (unsubstituted DNA density 1.70 g/cc, BrdUrd-substituted density 1.75 g/cc). The percentage DNA replicated was then determined by the percentage of DNA which had attained the hybrid density. Under conditions of DNA replication, a complete density shift was observed from 1.70 to 1.75 g/cc, indicating that possible alterations in cell membrane permeability did not cause DNA precursor pool fluctuations which could affect the results reported here.

Each plotted value for percentage DNA replicated, as displayed in the figures, represents the result of a complete CsCl density gradient profile. In several instances three identical culture dishes were analyzed for variations in amount of DNA replicated. Results in our laboratory show the standard error to be less than 1% DNA replicated. It is important to emphasize that neither numbers of cells per plate nor time of isotope incorporation affect the results. All of the values in each figure were determined from cultures that were part of the same experiment.

### Experimental Design

Stock solutions of TPA and phorbol were made up in DMSO and added to cultures to give 0.1% DMSO and final concentrations ranging from 1 ng to 1000 ng/ml of culture medium. Cultures were plated out to give $3 \times 10^5$ cells per 6-cm culture dish (Flow Labs, Rockville, Maryland). [2-$^{14}$C] Thymidine was added at 0.1 $\mu$Ci/ml and 48 h later the medium was exchanged for one lacking serum and isotope. Experiments were begun 54 h later to investigate effects of serum and TPA addition on the induction of DNA synthesis. At this time (t = 0) the cell number reached stable plateau values, and cell viability as determined by colony-forming ability was undiminished. Flow microfluorimetric analysis of both the Rat-1 and Rat-1(RSV) cells demonstrated identical $G_1$-like contents of DNA per cell [26]. At t = 0 BrdUrd and FdUrd were added for 4 h, at which time the medium was replaced with serum-free medium containing TPA or phorbol. In all experiments BrdUrd and FdUrd were present from t = 0 to t = 30.

## RESULTS

### Effect of TPA in Serum-Free Medium

When serum-deprived cultures of Rat-1 and Rat-1(RSV) cells were incubated in serum-free medium containing BrdUrd and FdUrd, 5% and 37% of the DNA, respectively, was replicated over a 30-h period (Fig. 1). Upon addition of various concentrations of TPA in serum-free medium, the total amount of DNA replicated was increased in a dose-dependent manner. The optimal concentration (500 ng/ml) increased the amount of DNA replicated in the Rat-1 cells to a level of 16%. In the Rat-1(RSV) cells, the optimal concentration (100 ng/ml) increased the amount of DNA replicated to 53%. In subsequent experiments the optimal concentrations of TPA derived from Figure 1 were used.

The time course of the onset of DNA synthesis after TPA addition in serum-free medium is shown in Figure 2. In the Rat-1(RSV) cells in serum-free medium alone (with

DMSO), the rate of DNA synthesis is higher (1.6%/h, Fig. 2A) than in the untransformed Rat-1 cells (0.3%/h, Fig. 2B). When TPA is included in the serum-free medium, there is an initial inhibition in the onset of DNA synthesis in the Rat-1(RSV) cells, which is followed by an increased rate beginning at 14 h. The total amount of DNA synthesized at 30 h in the Rat-1(RSV) cells was increased from 40% to 50% by the presence of TPA. When TPA was added to the untransformed Rat-1 cells in serum-free medium, an early increase in DNA synthesis was seen (Fig. 2B), without the initial inhibition which occurred in the Rat-1(RSV) cells.

### Effect of TPA in Medium Containing 20% Serum

Both TPA and fetal calf serum were added simultaneously to quiescent cultures of Rat-1 (Fig. 3A) and Rat-1(RSV) (Fig. 3B) cells. The presence of TPA had no effect in the onset, rate, or extent of DNA synthesis in the Rat-1 cells. However, in the Rat-1(RSV) cells, the presence of TPA delayed the onset of DNA synthesis by about 6 h. The rate of DNA synthesis in the latter case was the same.

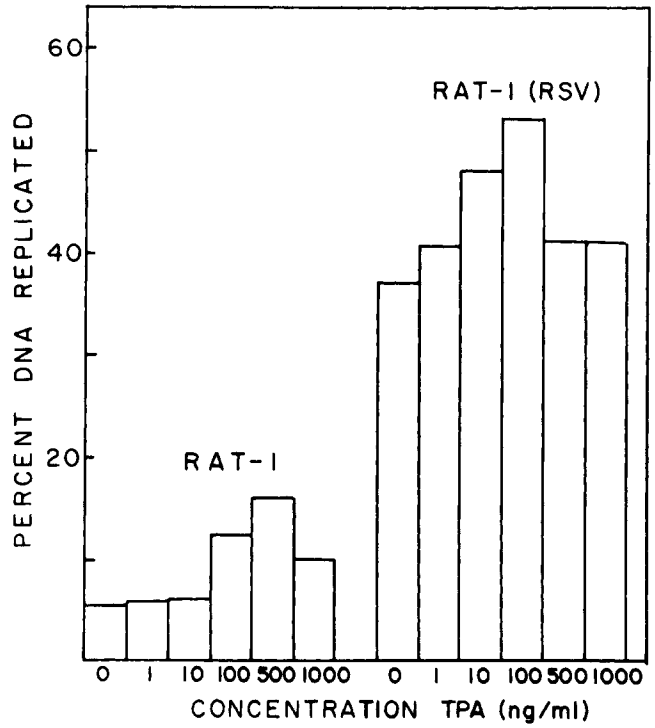


Fig. 1. Stimulation of DNA synthesis in quiescent Rat-1 and Rat-1(RSV) cells by TPA. Cells were seeded at $3 \times 10^5$ per 6-cm dish in medium containing 10% serum and 0.10 $\mu$Ci/ml [$^{14}$C] thymidine. After 48 h the dishes were rinsed briefly with 5 ml serum-free medium, refilled with fresh serum-free medium, and replaced in the incubator for 54 h. At that time fresh serum-free medium containing BrdUrd, FdUrd, 0.1% DMSO, and varying concentrations of TPA was added to each plate. Control cultures lacked TPA but contained DMSO. Thirty hours later cells were harvested from each dish and prepared for CsCl gradient analysis as described in Materials and Methods. Following density gradient centrifugation of DNA isolated from each sample in CsCl, the $^{14}$C activity recovered from the hybrid density peak divided by the $^{14}$C activity recovered from the entire gradient was taken as percentage DNA replicated (ordinate). Examples of DNA distribution in CsCl gradients, for conditions similar to those reported here, have been previously published in detail [25, 27].

### Effect of TPA Under Suboptimal Serum Conditions

Although addition of TPA and serum simultaneously did not enhance the rate or extent of DNA synthesis in Rat-1 cells, the possibility of synergistic effects of TPA by suboptimal conditions of serum stimulation was investigated. Medium containing 20% serum was added to cultures of Rat-1 and Rat-1(RSV) cells for 4 h, at which time the medium was exchanged for one lacking serum but containing TPA or DMSO (for controls). In cultures of Rat-1 cells a 4-h serum pulse followed by serum-free medium and DMSO resulted in 30% DNA replicated at 30 h compared to 70% when TPA was added in serum-free medium at 4 h (Fig. 4A). If phorbol (1 $\mu$g/ml) was added in serum-free medium at 4 h, a delay in the onset of DNA synthesis was seen compared to DMSO controls, although the final amount of DNA replicated at 30 h (30%) was the same as controls. Therefore, in Rat-1 cells TPA but not phorbol acts in a synergistic capacity with serum to induce DNA synthesis.

An identical series of experiments were performed with the Rat-1(RSV) cells. Addition of serum-containing medium for 4 h followed by serum-free medium and DMSO resulted in replication of 60% of the DNA. If TPA was present in the serum-free medium at

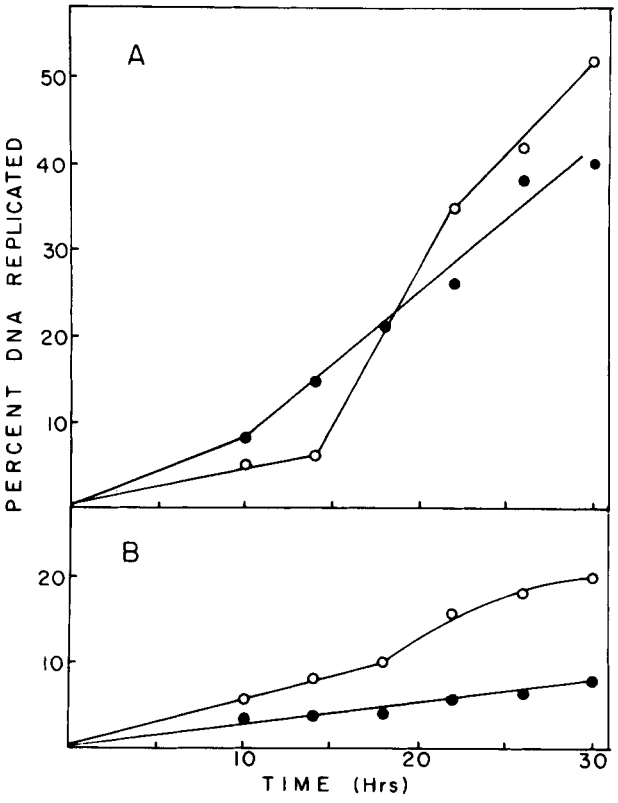


Fig. 2. Time course of DNA synthesis in quiescent Rat-1 and Rat-1(RSV) stimulated by addition of TPA in serum-free medium. Rat-1(RSV) cells (A) and Rat-1 cells (B) were prelabeled in [$^{14}$C] thymidine for 48 h and serum-deprived for 54 h, as described in Figure 1. At that time (t = 0) the medium was replaced by fresh serum-free medium containing 0.1% dimethyl sulfoxide, BrdUrd, FdUrd with TPA (open circles), and 100 ng/ml for Rat-1(RSV) cells (A) or 500 ng/ml for Rat-1 cells (B). Filled circles represent plates treated identically except that TPA was omitted. Percentage DNA replicated was determined by CsCl density gradient analysis of samples at 10, 14, 18, 22, 26, and 30 h later.

4 h there was no change in the rate or extent of DNA synthesis. Identical results were obtained using 500 ng/ml and 1,000 ng/ml TPA (data not shown). However, in the presence of phorbol (1 μg/ml) in the serum-free medium, the onset of DNA synthesis was delayed by 3 h. Therefore, TPA does not appear to stimulate the onset of DNA synthesis in the Rat-1(RSV) cells.

### Effect of Varying Time of TPA Addition

Experiments were performed to determine the time at which TPA was able to exert its comitogenic effect in the Rat-1 cells. When TPA was present in serum-free medium from hour 4 to 5 (Fig. 5A), the amount of DNA replicated was only slightly less (63% versus 70%) than when TPA was present for the entire experiment (Fig. 4A). If the 4-h serum stimulation was preceded by a 1-h treatment with TPA, there was a 4-h delay in the onset of DNA synthesis (Fig. 5A). The amount of DNA synthesized was only slightly less (58% versus 63%). Addition of TPA at 8 h to Rat-1 cells stimulated by serum for 4 h did not alter the rate or extent of subsequent DNA synthesis. From these experiments we conclude that TPA is synergistic with serum when preceded by serum stimulation but is ineffective when added at 8 h. Although TPA is effective as a synergistic inducer of DNA synthesis when addition precedes serum stimulation, a delay in the onset of DNA synthesis occurs.

Experiments similar to the above were conducted using Rat-1(RSV) cells. When the 4-h serum pulse was preceded by a 1-h TPA treatment, the onset of DNA synthesis was de-

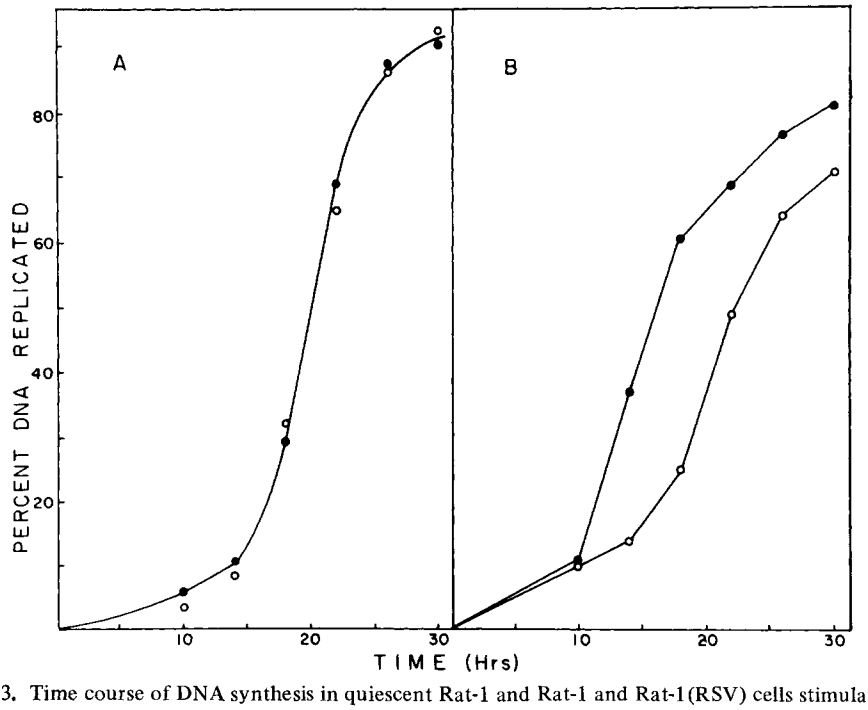


Fig. 3. Time course of DNA synthesis in quiescent Rat-1 and Rat-1 and Rat-1(RSV) cells stimulated by simultaneous addition of TPA and serum. Rat-1 cells (A) and Rat-1(RSV) cells (B) were prelabeled in [$^{14}$C]-thymidine for 48 h and serum-deprived for 54 h, as described in Figure 1. At that time (t = 0) the medium was replaced by fresh medium containing 20% serum, BrdUrd, FdUrd, 0.1% dimethyl sulfoxide with TPA (open circles, 500 ng/ml in panel A or 100 ng/ml in panel B) or without TPA (filled circles). Percentage DNA replicated was determined by CsCl density gradient analysis of samples at 10, 14, 18, 22, 26, and 30 h later.

layed by 3 h. A delay of 6 h was seen if TPA was added at 8 h. From these results we can conclude that addition of TPA retards the onset of DNA synthesis in Rat-1(RSV) cells. The extent of the retardation is increased when TPA is added closer to the point at which DNA synthesis normally commences (8–12 h).

## DISCUSSION

The method which was employed for measuring DNA synthesis using isopycnic density gradient centrifugation of DNA following BrdUrd labeling has been described in several previous publications [25, 27]. The method offers the advantage of measuring the absolute rate of DNA synthesis in cultures over a selected time interval, in contrast to the method of [$^3$H] TdR pulse labeling which provides relative measurements. Such relative measurements do not provide data concerning the amount of DNA replicated. Furthermore, the influence of TPA on the uptake of [$^3$H] TdR into cells must be considered. Comparison of rates of DNA synthesis among different cell lines using [$^3$H] TdR pulse labeling additionally may be complicated by such differential uptake of thymidine.

The same method of analysis of DNA replication employed in the present investigation was used to study the serum requirements for initiation of DNA replication in Rat-1 and Rat-1(RSV) cells [26]. The Rous sarcoma virus transforming function in Rat-1(RSV) cells was able to supplant a serum-dependent process in late $G_1$ which was necessary for the transformed Rat-1 cells to enter S and complete DNA replication. However, serum was necessary initially for both lines of cells to move toward the $G_1$/S boundary when a relative state of quiescence had been induced by serum deprivation.

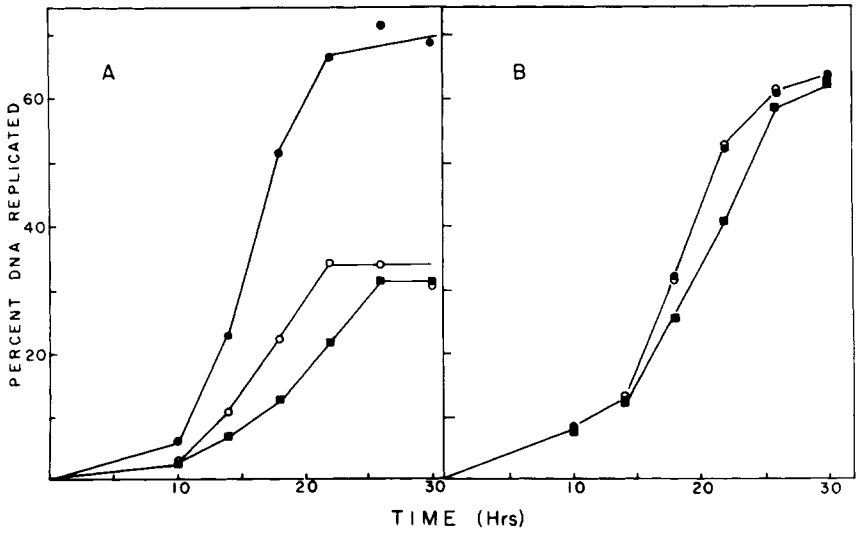


Fig. 4. Time course of DNA synthesis in quiescent Rat-1 and Rat-1(RSV) cells stimulated by a pulse of serum and followed by addition of TPA. Rat-1 cells (A) and Rat-1(RSV) cells (B) were prelabeled in [$^{14}$C] thymidine for 48 h and serum-deprived for 54 h, as described in Figure 1. At that time (t = 0), medium containing 20% serum, BrdUrd, and FdUrd was added to all plates. At t = 4, the medium was removed, and plates were filled with fresh serum-free medium containing BrdUrd, FdUrd, and 0.1% dimethyl sulfoxide (○) or with the latter solution containing 1 μg/ml phorbol (■) or TPA (●). The TPA concentration was 500 ng/ml in dishes containing Rat-1 cells (A) and 100 ng/ml in dishes containing Rat-1(RSV) cells (B). Percentage DNA replicated was determined by CsCl density gradient analysis of samples at t = 10, 14, 18, 22, 26, and 30 h.

In serum-free medium, TPA caused a small dose-dependent increase in the rate of DNA synthesis in serum-deprived Rat-1 and Rat-1(RSV) cells.

In contrast, O'Brien and Diamond [14] reported that TPA, when added to confluent quiescent cultures of normal or transformed hamster embryo cells, neither produced an increase in cell number nor in the percentage of [$^3$H] TdR-labeled nuclei, and did not stimulate the incorporation of [$^3$H] TdR. A delay in the onset of DNA synthesis was observed in TPA treated Rat-1(RSV) cells but not in Rat-1 cells. A similar observation was made by Fusenig and Samsel [28], who found that optimal concentrations of TPA in mouse dermal fibroblasts caused an initial short-term inhibition of DNA synthesis followed by stimulation, with a maximum at 24 h after addition, as measured by [$^3$H] TdR incorporation and labeling indices.

In the untransformed Rat-1 cells, the only conditions which led to significant TPA stimulation were those in which addition of TPA was temporally associated with a brief exposure (1 h) to serum. One explanation of the necessary time coincidence of treatment for a co-inducing effect is that TPA could induce sensitization to serum growth factors which might remain associated with the cell after the shift to serum-free medium. In contrast, TPA was unable to stimulate if its addition was delayed until 4 h after the end of the serum pulse. In the above system, DNA replication normally begins at 14 h after serum

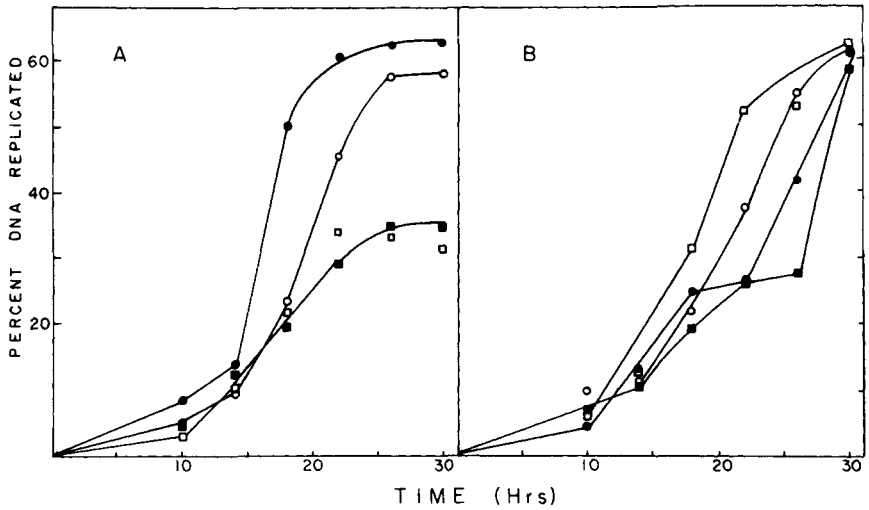


Fig. 5. Time course of DNA synthesis in quiescent Rat-1 and Rat-1(RSV) cells stimulated by a pulse of serum and a 1-h treatment with TPA before or after the serum pulse. Rat-1 cells (A) and Rat-1(RSV) cells (B) were prelabeled in [$^{14}$C] thymidine for 48 h and serum-deprived for 54 h, as described in Figure 1. For one set of cultures, at 53 h of serum deprivation (t = −1) the medium was changed for fresh serum-free medium containing TPA for 1 h (○). At the end of 1 h, the medium was removed and cultures were stimulated with fresh 20% serum-containing medium containing BrdUrd and FdUrd for 4 h (t = 0 to t = 4). At t = 4, the serum-containing medium was removed and exchanged for an identical medium lacking serum. Experimental groups were stimulated by TPA for 1 h at different times. For each panel, one set of cultures was stimulated with TPA for 1 h prior to addition of serum (t = −1 to t = 0, ○). One set of cultures was stimulated with TPA for 1 h immediately after removal of the serum-containing medium (t = 4 to t = 5, ●). One set of cultures was stimulated with TPA for 1 h from t = 8 to t = 9 (■). One set of cultures was not stimulated at any time by TPA (□). For cultures receiving TPA, the medium contained BrdUrd, FdUrd, and either 500 ng/ml (Rat-1 cells, panel A) or 100 ng/ml (Rat-1(RSV) cells, panel B) TPA in 0.1% dimethyl sulfoxide. Percentage DNA replicated was determined by CsCl density gradient analysis of samples at t = 10, 14, 18, 22, 26, and 30 h.

stimulation [26]. Therefore, the stimulatory effects of TPA on cellular events in the Rat-1 cells do not occur within 6 h prior to the onset of DNA synthesis.

The inhibitory effects of TPA are temporally distinct from the stimulatory effects in the untransformed Rat-1 cells. TPA will inhibit only if present prior to, but not after, serum exposure. Furthermore, the inhibitory actions of TPA affect only the time of onset of DNA synthesis but not the extent. When added before a 4-h serum pulse, therefore, TPA both delays the onset and increases the amount of DNA replicated in the cultures. We therefore suggest that TPA acts on two distinct cellular regulatory mechanisms in the $G_1$ phase of the cell cycle of untransformed Rat-1 cells.

The RSV-transformed Rat-1 cells were not responsive to the DNA-stimulating effects of TPA with serum, but in serum-free medium a slight stimulation of DNA synthesis was seen when TPA was added to quiescent cultures. Therefore, it appears that RSV-transformed Rat-1 cells are refractory to the DNA-stimulating effects of TPA but do respond to the retardation of DNA synthesis induced by TPA. The mechanisms of DNA synthesis delay in the Rat-1 and Rat-1(RSV) cells may differ, since TPA is most effective in delaying the onset of DNA synthesis when added before serum stimulation in the Rat-1 cells and just before the onset of DNA synthesis in the Rat-1(RSV) cells.

It was of interest to discover that the parent alcohol phorbol, which is inactive as a tumor promotor and comitogen in other systems [21], caused a delay in the onset of serum-induced DNA synthesis in both untransformed and RSV-transformed Rat-1 cells. The possibility exists that in experiments utilizing TPA, the active inhibitor of DNA synthesis is phorbol, which is either present as a contaminant or is produced through metabolism of TPA. Experiments currently are in progress to test these possibilities.

How TPA and other tumor promotors are able to promote the appearance of neoplasms in initiated cells is unknown. It is significant that the two-stage mechanism of carcinogenesis has been demonstrated in cultured cells as well as in animal models. Using cultured 10 $T_{1/2}$ mouse cells, Peterson et al [11] found that TPA treatment of log-phase cells induced a transient inhibition of [$^3$H]TdR incorporation. Since phorbol did not produce these effects, the authors suggested that the inhibition of DNA synthesis was associated with the process of promotion. Although the results of the present investigation do not address the question of tumor promotion per se, the different TPA-induced responses of normal and RSV-transformed cells may help clarify the role of induced DNA synthesis in the conversion of initiated cells to malignantly transformed cells.

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