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Simian Virus 40 t Antigen Affects the Sensitivity of Cellular DNA Synthesis to Theophylline

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Cellular DNA synthesis induced by simian virus 40 mutants that lack t antigen is sensitive to concentrations of theophylline that do not affect wild-type-induced synthesis.

Several viable mutants of simian virus 40 (SV40) that contain deletions in the 0.54 to 0.59 region of the genome have been isolated (8), and it has been shown that these mutants have a reduced capacity to transform cells to anchorage-independent growth (1, 9). The mutant viruses code for the synthesis of an intact T antigen (2), but are unable to synthesize a second early gene product, t antigen. The viability of these mutants suggests that the t antigen is not required for productive infection, but that the protein is involved in alterations of the cell that lead to anchorage-independent growth.

The viable deletion mutants, dl-885, dl-888, and dl-890, kindly provided by Thomas Shenk, were compared to the wild-type (WT) virus for their ability to induce host cell DNA synthesis and the enzyme, thymidine kinase, in permissive CV-1 cells. CV-1 cells were grown to confluence in basal minimal medium (BME) containing 5% fetal bovine serum. At confluence, the cells were infected with virus lysates (5 to 10 PFU/cell) or with lysates of uninfected cells. Following a 2-h adsorption period, BME lacking serum was added to the infected cells, and the cells were incubated at 39°C.

To determine thymidine kinase activity, infected cells were extracted at 60 h postinfection by Dounce homogenization, and the 40,000 × *g* supernatant fraction was assayed by standard assay procedures (6). The WT virus and the two deletion mutants, dl-885 and dl-888, induce the appearance of thymidine kinase after infection (Table 1). Although not shown here, a third deletion mutant, dl-890, also induces the enzyme.

Similarly, the viable deletion mutants are able to induce cellular DNA synthesis in CV-1 cells (Fig. 1A). Cells were infected with mock lysate, WT SV40 or dl-888, then placed at 39°C in serum-free medium. The T antigen mutant, *ts*A58 (10), was also included in this experiment, and cells infected with *ts*A58 were incubated at 41°C. Infected cells were pulsed with [^{3}H]thymidine (20 Ci/mmol) for 18-h intervals through the first 72 h of infection, and then cellular and viral DNA were analyzed as described in the figure legend. In this experiment, the deletion mutant and the WT virus induced cellular DNA synthesis to approximately the same extent. In each case, the incorporation of [^{3}H]thymidine was 2.5 to 3 times that of mock lysate-infected cells.

The level of cellular DNA synthesis induced by SV40 and the deletion mutants was examined in cells placed in the presence and in the absence of theophylline. Theophylline is an inhibitor of cyclic AMP phosphodiesterase and has the effect of elevating intracellular cyclic AMP. In many cell lines, increased intracellular cyclic AMP prevents the onset of DNA synthesis induced by serum addition (3, 5) and apparently prohibits the cells from entering the cell cycle from the resting stage, G_0. Similarly, theophylline completely blocks the stimulation of CV-1 cells by serum (unpublished data).

The cellular DNA synthesis induced by SV40 is only slightly affected by concentrations of theophylline that completely inhibit serum stimulation. Infected cells were incubated at 39°C in serum-free medium containing 0, 1.0, or 1.5 mM theophylline. At 36 h postinfection, [^{3}H]thymidine was added to each plate for 18 h, and then the viral and cellular DNA were separated for analysis. The DNA synthesis induced in mock-infected cultures was drastically decreased by 1.0 mM and 1.5 mM theophylline (Table 2). This decrease is equivalent to decreases observed in serum-stimulated CV-1 cells. Cellular DNA synthesis induced by SV40 was only slightly affected by these concentrations of theophylline.

Similar experiments have been performed to monitor DNA synthesis by autoradiography. These experiments have shown that the actual number of CV-1 cells synthesizing DNA decreases in the presence of theophylline (data not

TABLE 1. *Thymidine kinase activity in infected cells*[a]

Virus	Thymidine kinase (pmol of TMP/min per μg of protein)
Mock	15.4
WT SV40	268.0
dl-885	238.0
dl-888	243.0

[a] CV-1 cells were infected and then incubated at 39°C for 60 h. Cells were scraped from 35-mm dishes and extracted in 0.5 ml of Tris-buffered saline by Dounce homogenization (12 strokes). Extracts were centrifuged at 40,000 × *g* for 20 min, and the supernatant fraction was assayed for thymidine kinase. Reaction mixtures contained 6 mM ATP, 3.5 mM $MgCl_2$, 2.5 μCi of [^{3}H]thymidine (20 Ci/mmol), 2 mM dithiothreitol, and 80 mM Tris-hydrochloride (pH 7.8). Incubation was for 20 min at 35°C. After incubation, the mixtures were boiled and then applied to DEAE-cellulose (2.0-cm columns). Unreacted thymidine was washed from the resin with 3.0 ml of water and then the product (TMP) was recovered, using 2.0 ml of 1 M NaCl. Results are expressed as specific activity (pmol/min per μg of protein).

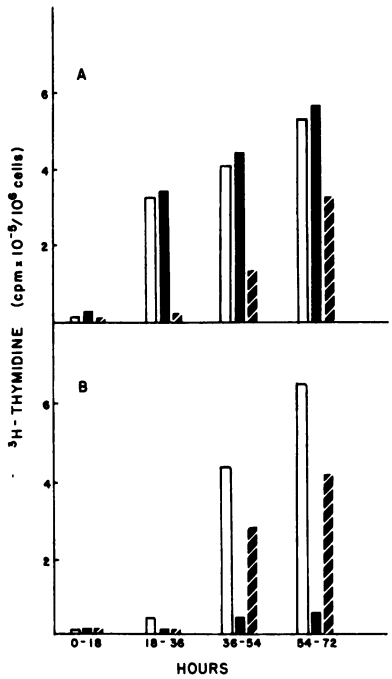

FIG. 1. *Induction of cellular DNA synthesis by WT, tsA58, and dl-888. CV-1 cells were infected with mock lysate, WT SV40, or dl-888, placed in serum-free medium (A) or serum-free medium containing 1.5 mM theophylline (B), and incubated at 39°C. Cells infected with tsA58 were incubated at 41°C. At the times indicated, 4.0 μCi of [^{3}H]thymidine was added to each plate for 18 h. Viral and cellular DNAs were separated by the procedure of Hirt (4), and the DNA fractions were processed as described in Table 2. Values obtained with mock lysate-infected cells were subtracted from each of the experimental values shown in the figure. Open bars, WT virus; closed bars, dl-888; hatched bars, tsA58.*

shown). In contrast, the number of cells induced to synthesize DNA after infection is not affected by the presence of theophylline, and the relative intensity of the labeled nuclei remains approximately the same. The *ts*A mutant, *ts*A58, was used in these experiments at nonpermissive temperature to eliminate viral DNA synthesis.

To compare cellular DNA synthesis induced by the WT virus, *ts*A58, and the deletion mutants, infected cells were incubated in the presence of 1.5 mM theophylline (Fig. 1B), then pulsed for 18-h intervals throughout infection. For the first 36 h, cellular DNA synthesis is minimal in all cultures. After this time, the WT and the *ts*A mutant appear to overcome the inhibition by theophylline, and DNA synthesis equals or even exceeds that observed in the absence of the drug. It is interesting that although the *ts*A mutant induces DNA synthesis more poorly than the WT virus, the induction of cellular DNA synthesis does occur in the presence of theophylline. In contrast, the deletion mutant cannot overcome the theophylline block and DNA synthesis remains at a low level throughout the infection. Similar results have been obtained with all three deletion mutants. The synthesis of viral DNA parallels the synthesis of cellular DNA in these experiments. For example, during the 54- to 72-h pulse, the synthesis of WT viral DNA in the presence of theophylline equalled the synthesis in untreated cultures, but synthesis of dl-888 DNA was inhibited 86% by theophylline.

TABLE 2. *Effect of theophylline on SV40-induced cellular DNA synthesis*[a]

Theophylline concn (mM)	[^{3}H]thymidine incorporation (cpm × 10^{-5}/10^6 cells)		
	Mock lysate	WT SV40	SV40/mock
0	3.14	7.19	2.3
1.0	0.14	6.06	43.3
1.5	0.09	4.44	49.3

[a] CV-1 cells were infected with SV40 or mock lysates, and then incubated in serum-free medium containing 0, 1.0, or 1.5 mM theophylline for 36 h at 39°C; 4.0-μCi of [^{3}H]thymidine was added to each plate for 18 h, at which time the cells were extracted by the procedure of Hirt (4). Cellular DNA was collected by centrifugation, dissolved in 1.0 ml of water, sonically treated to reduce viscosity, and then spotted on glass-fiber filter disks. The filters were washed in three changes of 5% trichloroacetic acid and two changes of 95% ethanol before counting.

It seems unlikely that the results obtained in these experiments are misleading because of the use of [^{3}H]thymidine as a label. Intracellular pools of [^{3}H]thymidine have been measured in infected cells both in the presence and absence of theophylline, and only minor differences are observed (less than twofold). In addition, similar experiments have been performed using [^{32}P]-phosphate to label cellular DNA, and (Table 3) the incorporation of label into cellular DNA follows the same pattern observed with [^{3}H]thymidine incorporation.

The 0.54 to 0.59 deletion mutants are similar, in some respects, to the hr-t class of polyoma mutant. It has been reported (7) that the polyoma hr-t mutants also induce cellular DNA synthesis, but that the induction is transient in that cellular DNA synthesis decreases after the first 24-h period. This situation differs from our observations with the SV40 deletion mutants, because cellular DNA synthesis induced by the mutants parallels that induced by the WT virus for at least 72 h and, in many experiments, the induction by the deletion mutants equals the WT-induced cellular DNA synthesis. However, we do not feel that we are able, at this time, to completely rule out the possibility that t antigen provides a mitogenic effect in addition to the effect of the T antigen during SV40 infection. It seems likely, however, that t antigen is involved primarily in additional cellular alterations that result in the theophylline resistance demonstrated in our experiments.

It is not yet clear whether the differences with respect to response to theophylline are unique to the CV-1 cell line, which is particularly sensitive to theophylline inhibition of serum stimulation. This phenomenon, however, does provide a useful system both for the screening of additional mutants and for probing possible functions of t antigen. Experiments designed to determine the mechanism that leads to theophylline insensitivity of virus-induced cellular DNA synthesis will explore phosphodiesterase levels and the levels of cyclic nucleotides present in infected cells.

TABLE 3. *Incorporation of [^{32}P]phosphate into cellular DNA in the presence and absence of theophylline*[a]

Virus	[^{32}P]phosphate incorporation (cpm × 10^{-5}/10^6 cells)		Ratio of untreated to treated
	Untreated	Treated (1.5 mM theophylline)	
WT SV40	1.23	0.64	1.9
dl-888	0.48	0.02	24.0

[a] CV-1 cells were infected with WT SV40, dl-888, or mock lysates, then placed in serum-free, phosphate-free medium at 39°C in the presence or absence of 1.5 mM theophylline. From 40 to 48 h postinfection, [^{32}P]phosphate (0.2 mCi) was added to each dish, and then the cellular and viral DNA fractions were separated by Hirt extraction (4). Cellular DNA fractions were dispersed by sonic oscillation in 0.01 M Tris-hydrochloride–0.01 M EDTA (pH 7.2). Viral and cellular DNAs were digested with RNAse (pretreated at 100°C to inactivate DNAse), and then extracted with phenol. Portions of the aqueous phases of the phenol extraction were spotted on filters and processed as described in Table 2. Radioactivity in mock-infected samples has been subtracted from the values shown.

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