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Stimulation of RNA synthesis in isolated nuclei by partially purified preparations of simian virus 40 T-antigen

(gene regulation/transformation/gene transcription/*A* gene)

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ABSTRACT **T-Antigen was partially purified from nuclei of cells transformed by simian virus 40 (SV40). When nuclei isolated from either rat liver or quiescent hamster cells were preincubated with T-antigen preparations, there was a marked stimulation of RNA synthesis in an *in vitro* assay, up to 150% above control levels. The stimulation of RNA synthesis was inhibited by hamster antiserum against T-antigen but not by normal hamster serum. When the T-antigen preparations were fractionated on glycerol gradients, the fractions containing complement-fixing activity with antiserum to T-antigen also had the highest stimulatory activity on nuclear RNA synthesis. T-Antigen was also partially purified from nuclei of cells transformed by a temperature-sensitive *A* mutant of SV40. When preincubated up to 2 hr at 50°, the T-antigen preparation from these temperature-sensitive *A* mutants was rapidly inactivated, in terms of both complement-fixing activity and ability to stimulate RNA synthesis in isolated rat liver nuclei. Under the same conditions of preincubation, T-antigen preparations from cells transformed by wild-type SV40 maintained their complement-fixing activity and ability to stimulate RNA synthesis. These results suggest that the biological action of T-antigen may be exerted at the level of transcription.**

There is considerable evidence that the *A* gene product of the simian virus 40 (SV40) genome is required for (*i*) the initiation of SV40 DNA replication (1), (*ii*) the transcription of late SV40 genes (2), (*iii*) the establishment and maintenance of transformation (3–5), and (*iv*) the stimulation of cellular DNA synthesis that occurs after SV40 infection (ref. 6; see also review by Levine, ref. 7). There is also substantial evidence that the main, if not the only, product of the *A* gene is an 80,000–100,000 molecular weight protein, identifiable with the T-antigen (8–11). Recently Graessmann and Graessmann (12) synthesized *in vitro* RNA from the early strand of SV40 DNA, which is believed to code for the *A* gene product (see review by Acheson, ref. 13). This *in vitro* synthesized RNA (cRNA) was microinjected into primary mouse kidney cells and shown to cause the appearance of T-antigen and the stimulation of cellular DNA synthesis in host cells. Graessmann and Graessmann (12) concluded that their results supported the hypothesis that SV40-specific T-antigen provides the necessary information for the stimulation of cellular DNA synthesis.

It has been known for several years that stimulation of DNA synthesis in mammalian cells is preceded by a variety of sequential metabolic steps (see review by Baserga, ref. 14), among which an increase in RNA synthesis is one of the earliest and most striking events (see *Discussion*). Because, in SV40-infected cells, the appearance of T-antigen precedes by some hours the stimulation of host DNA synthesis (15–17), it is possible that T-antigen may act on the early events of the pre-replicative phase, i.e., RNA synthesis, rather than directly on DNA replication. The present communication reports our studies on the effect of partially purified T-antigen preparations on *in vitro* RNA synthesis in isolated nuclei.

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METHODS AND MATERIALS

Cells. SV80 cells were obtained through the courtesy of D. M. Livingston (Sidney Farber Cancer Center, Boston, MA) and were grown in Dulbecco's medium, as described by Jessel *et al.* (18). Temperature-sensitive (ts) AF8 cells, a mutant of baby hamster kidney (BHK) cells, originally obtained from Claudio Basilico (New York University), were grown as previously described (19). F5-1 cells were obtained through the courtesy of Barbara Knowles (Wistar Institute, Philadelphia, PA). Chinese hamster lung (CHL) cells (T-antigen-negative), CHL wt 15 cells (transformed by wild-type SV40) and CHL-A239L1 (transformed by a ts *A* mutant of SV40) were kindly given by R. H. Martin (National Institutes of Health).

Isolation of Nuclei. Nuclei from either rat liver or quiescent AF8 cells were prepared by the method of Marzluff *et al.* (20), with slight modifications. Cells were Dounce homogenized in breaking buffer [10 mM Tris·HCl, pH 8.0/2 mM $Mg(OAc)_2$/3 mM $CaCl_2$/0.5 mM dithiothreitol]. The broken cells were centrifuged for 5 min at 1500 rpm in an International PR-6 centrifuge. The nuclei were suspended in suspension buffer (10 mM Tris·HCl, pH 8.0/150 mM NaCl/2 mM $MgCl_2$) by vortexing. Equal volumes of 1% (vol/vol) Triton X-100 in suspension buffer and 1.5 M sucrose in suspension buffer were added, followed by vigorous vortexing for 1 min. The nuclei were then sedimented by centrifugation for 5 min at 1500 rpm in an International PR-6 centrifuge. Following the centrifugation the nuclei were resuspended in TGMED [50 mM Tris·HCl, pH 8.0/20% (vol/vol) glycerol/5 mM $Mg(OAc)_2$/0.1 mM EDTA/1.5 mM dithiothreitol].

Assay for Nuclear RNA Synthesis. The method of Marzluff *et al.* (20) was also followed for the determination of RNA synthesis *in vitro*. The assay (dependence on the four ribonucleoside triphosphates, inhibition by actinomycin D, etc.) has already been discussed by Marzluff *et al.* (20), and in papers from our laboratory (19, 21). The final volume of the assay was 125 μl and each assay contained 5 μg of DNA as nuclei. All assays were performed at 37° and terminated after 15-min incubation. The control values were always three to four times the background cpm.

Preparation of T Protein. The method was essentially the one described by Jessel *et al.* (18), except that the agarose column step was omitted. Nuclei were isolated from SV80 cells (or other cells listed above) by homogenization of washed cells in three volumes of buffer A (see *Buffers* below) with eight strokes of a Dounce homogenizer at 4°. Nuclei were washed with

Abbreviations: SV40, simian virus 40; CHL, Chinese hamster lung; ts, temperature-sensitive; wt, wild-type; TGMED, Tris/glycerol/magnesium/EDTA/dithiothreitol.

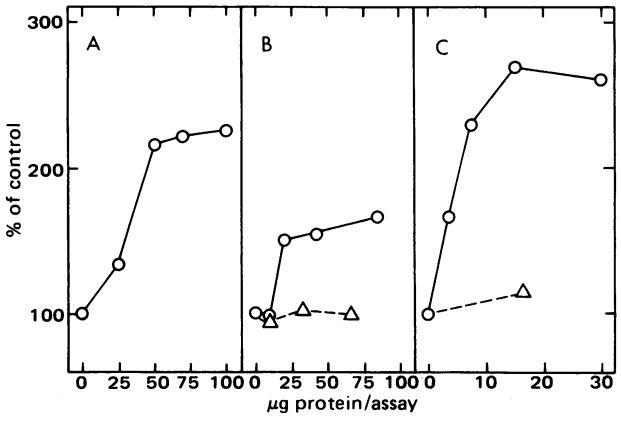

FIG. 1. Effect of preincubation with T-antigen preparations on RNA synthesis of isolated nuclei. On the abscissa is the amount of protein added; the ordinate is [^{3}H]UTP incorporated into RNA per μg of DNA, expressed in percent of control (no T preparation added) values. (*A*) AF8 nuclei and extract from F5-1 cells—controls 180 cpm/μg of DNA; (*B*) AF8 nuclei and (O—O) extract from SV80 cells or (Δ---Δ) extract from CHL cells (mock T)—controls 215 cpm/μg of DNA; (*C*) rat liver nuclei and (O—O) extract from SV80 cells or (Δ---Δ) mock T—controls 458 cpm/μg of DNA. Preincubation was 30 min, assay was stopped at 15 min. Background was subtracted from control values.

buffer A and suspended in buffer B, followed by sonication in a Branson Sonifier model W 185 at 70 W for 60 sec at 4°. The nuclear lysate was centrifuged for 30 min at 40,000 rpm at 4° in a Beckman 50 Ti rotor. The supernatant was brought to 60% saturation with ammonium sulfate at 4°. The precipitate was pelleted by centrifugation for 20 min at 30,000 rpm at 4° in a 50 Ti rotor, dissolved in buffer C, and dialyzed for 3 hr against two changes of 100 volumes of buffer C. After the removal of insoluble material by centrifugation, clear supernatant was applied to a column of DEAE-cellulose (DE-52, Whatman, Piscataway, NJ), equilibrated in buffer C. The column was then washed with two column volumes of buffer C and eluted with four column volumes of a linear gradient between 0 and 0.4 M NaCl dissolved in buffer C. A single peak of T-antigen activity was collected and precipitated by the addition of solid ammonium sulfate. The pellet was collected by centrifugation, dissolved in TGMED, dialyzed for 3 hr against two changes of 500 volumes of TGMED, and frozen in liquid nitrogen until used. These preparations of T-antigen did not contain detectable amounts of DNA. Some preparations of T-antigen contained considerable amounts of RNA polymerase activity [as assayed with poly(dAT)] and weak DNase activity (conversion of circular SV40 DNA to linear form). These activities, however, were completely destroyed by heating at 50° for 30 min, while stimulatory activity on RNA synthesis in isolated nuclei was maintained without loss of activity (not shown).

Complement Fixation. The complement fixation test for T-antigen was carried out by the microtiter technique of Sever (22).

Antisera. Anti-T sera were obtained from hamsters carrying tumors induced by the inoculation of F5-1 cells. All sera were heated at 56° for 30 min and were centrifuged for 2 hr at 45,000 rpm at 4° in a 50 Ti rotor prior to use, to inactivate complement and anticomplementary factors.

Glycerol Gradient Centrifugation. Linear glycerol gradients (10–30% glycerol in TGMED) were overlaid with 0.25 ml of T preparation, and centrifuged at 49,000 rpm at 4° for 3.5 hr in a Beckman SW 50.1 rotor. After centrifugation, fractions were collected from the bottom and each fraction was assayed for T-antigen in the complement fixation test, for template activity, and for absorbance.

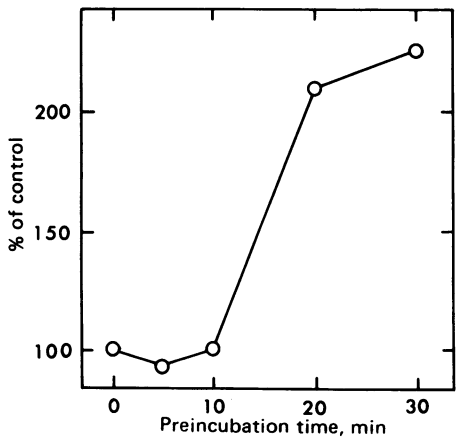

FIG. 2. Effect of time of preincubation with T-antigen on RNA synthesis of nuclei isolated from quiescent AF8 cells. Time of preincubation is on the abscissa, RNA synthesis is on the ordinate (incubation time 15 min). T-Antigen preparation (7.5 μg per assay) was from SV80 cells; control value was 170 cpm/μg of DNA.

Buffers. A: 0.01 M Tris·HCl (pH 7.0), 0.01 M NaCl, 0.0015 M $MgCl_2$, 0.001 M dithiothreitol, and phenylmethylsulfonyl fluoride at 0.3 mg/ml. B: 0.02 M Tris·HCl (pH 8.0), 0.06 M NaCl, 0.001 M dithiothreitol, 10% glycerol, 0.01% Triton X-100, and phenylmethylsulfonyl fluoride at 0.3 mg/ml. C: 0.05 M Tris·HCl (pH 8.0), 0.001 M dithiothreitol, and 10% glycerol.

RESULTS

Effect of T-Antigen Preparations on Nuclear RNA Synthesis. Fig. 1 shows the effect of preincubation with T-antigen preparations on RNA synthesis of nuclei isolated from either quiescent AF8 cells (Fig. 1 *A* and *B*), or rat liver (Fig. 1*C*). The T-antigen preparation partially purified from either F5-1 cells (Fig. 1*A*) or SV80 cells (Fig. 1 *B* and *C*) stimulated RNA synthesis in isolated nuclei. Similarly treated preparations obtained from T-antigen-negative growing CHL cells (mock T) or AF8 cells (not shown) did not stimulate RNA synthesis in isolated nuclei. However, T-antigen preparations from CHL wt 15 cells (SV40-transformed CHL cells) and from CHL A239L1 cells (ts *A* SV40-transformed CHL cells) did stimulate RNA synthesis in isolated nuclei (not shown). Under the incubation conditions used in these experiments, the increase caused by T-antigen preparations in RNA synthesis was 100% α-amanitin resistant, which persisted even in the presence of RNase inhibitors and was completely inhibited by actinomycin D, 10 μg/ml (not shown). The product of the assay was totally digestible by RNase. In agreement with the α-amanitin resistance, it can be shown that partially purified preparations of T-antigen stimulate RNA synthesis in isolated nucleoli. However, the data on nucleoli, together with the detailed characterization of the product, will form the subject of another communication. The assay was not affected by the presence of an inhibitor of proteolysis (phenylmethylsulfonyl fluoride). The experiments in Fig. 1 were repeated several times. The term "RNA synthesis" is used here for its convenient brevity, in agreement with other investigators (20, 23, 24), but other authors have used the term "endogenous RNA polymerase activity" (25–27) for the same assay.

To obtain a good stimulation of RNA synthesis, nuclei have to be preincubated with T-antigen preparations for at least 20 min (Fig. 2). The preincubation was carried out in the assay buffer but without the ribonucleoside triphosphates so that no RNA synthesis was taking place during the preincubation. The experiments without preincubation or with 30 min preincubation have been repeated several times. In all subsequent ex-

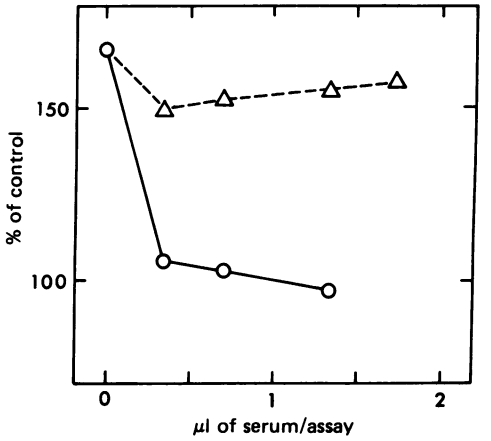

FIG. 3. Effect of anti-T sera on stimulation of RNA synthesis in isolated rat liver nuclei. RNA synthesis assay was carried out as described in Figs. 1 and 2, except that the T-antigen preparation (7.5 μg per assay) from SV80 cells was previously treated with either anti-T serum (O—O) or normal hamster serum (Δ---Δ). Control value (no T) was 880 cpm/μg of DNA; stimulated control (T added but no sera) was 1464 cpm/μg of DNA.

periments, unless otherwise specified, preincubation was 30 min and incubation time was 15 min.

Effect of Anti-T Serum on Stimulation of Nuclear RNA Synthesis. T-Antigen preparations were reacted with hamster anti-T serum overnight at 4° and centrifuged, and the supernatant was used for RNA synthesis assay. The supernatant was found to be inactive in stimulating RNA synthesis (Fig. 3). When normal hamster serum was added to T-antigen preparations and the mixture was centrifuged, the supernatant was still active in stimulating nuclear RNA synthesis (Fig. 3). According to Carroll and Smith (11), the same cellular proteins are nonspecifically precipitated by normal hamster serum and anti-T serum, but only T-antigen is specifically precipitated by anti-T serum. Table 1 shows the effect of different anti-T sera and normal hamster sera on the stimulation of *in vitro* RNA synthesis by T-antigen preparations. Despite some variability, it is obvious that anti-T sera do inhibit the stimulation of RNA

Table 1. Effect of various hamster sera on the stimulation of RNA synthesis by T-antigen preparations

T-preparation, μg protein/assay	Serum		[³H]UTP incorporation, % of control
	Sample	μl/assay	
0	—	0	100
10	—	0	160
10	NHS 1	1.4	170
10	NHS 1	0.7	179
10	NHS 2	1.4	149
10	NHS 2	0.7	147
10	ATS 1	1.4	97
10	ATS 1	0.7	102
10	ATS 3	1.4	112
10	ATS 3	0.7	124
10	ATS 4	1.4	91
10	ATS 4	0.7	88

T-preparation = T-antigen preparation from SV80 cells; NHS, sera from normal hamsters; ATS, hamster anti-T sera. Control value was 130 cpm/μg of DNA after subtraction of a background of 15 cpm. Background was also subtracted from all other values. All values were the average of four determinations.

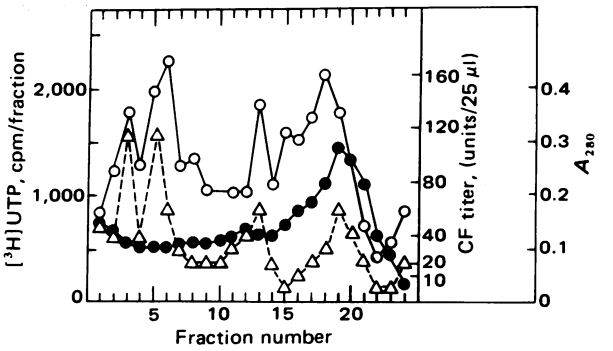

FIG. 4. Glycerol gradient of T-antigen preparations from SV80 cells. Abscissa, fraction number. Ordinates: ●—●, A_{280}; Δ---Δ, complement-fixing (CF) activity determined as described in *Methods and Materials;* O—O, incorporation of [³H]UTP into RNA in nuclear assays (with rat liver nuclei) as described in Figs. 1 and 2.

synthesis by T-antigen preparations, while normal hamster sera have no effect.

Fractionation of T-Antigen on Glycerol Gradients. Fig. 4 shows a 10–30% glycerol gradient of partially purified preparations of T-antigen. T-Antigen (as determined by complement fixation) sediments in three different peaks, plus a fourth peak that sediments with the bulk of the other proteins. Carroll *et al.* (28) also showed four peaks of T-antigen by sucrose gradient centrifugations. The various fractions of the gradients were tested for their ability to stimulate nuclear RNA synthesis. Fig. 4 shows that the RNA-stimulating activity corresponded with the peaks of complement-fixing activity.

Stimulation of RNA Synthesis with ts *A* Mutants. T-Antigen was partially purified from CHL A239L1 cells, a cell line developed by Martin and collaborators (5) by transformation of CHL cells with a ts *A* mutant of SV40. T-Antigen in these cells is temperature sensitive (5). When preincubated at 50° in TGMED, T-antigen from CHL A239L1 cells was rapidly inactivated both in its complement-fixing activity and in its ability to stimulate RNA synthesis in isolated nuclei (Fig. 5). Under the same conditions of preincubation T-antigen preparations from CHL wt 15 cells (transformed by wild-type SV40) maintained their ability to stimulate nuclear RNA synthesis and to fix complement (Fig. 5). Notice also that preincubation at 25° does not affect either the wild type or the ts *A* mutant.

DISCUSSION

When resting cells are stimulated to proliferate, one of the earliest responses is an increase in RNA synthesis, which occurs several hours before the onset of DNA synthesis. This increase in RNA synthesis has been reported in every population of resting cells stimulated to grow (see review by Baserga, ref. 14). Suffice it to say here that it has been detected in whole cells (29), in isolated nuclei (21), in nuclear monolayers (30), and in isolated chromatin (31). According to some authors, the synthesis of both α-amanitin-resistant and α-amanitin-sensitive RNA species is increased (27, 32, 34), but it is generally believed that most of this increase can be attributed to ribosomal RNA synthesis (30–36). The present results show that RNA synthesis in nuclei isolated from resting cells can be increased by preincubation with a partially purified preparation of SV40 T-antigen. The increase is 100% α-amanitin-resistant, although in control nuclei the percentage of α-amanitin-resistant RNA synthesis is 40% (not shown). These experiments are in agreement with the findings of Weil *et al.* (37) and May *et al.* (38) that quiescent cells infected with SV40 (or polyoma virus) have an increased synthesis and an increased amount of cellular RNA.

Because the T-antigen preparation was not pure, one cannot

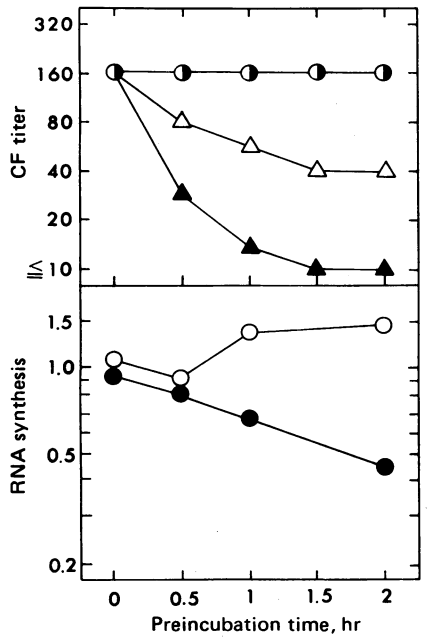

FIG. 5. Effect of preincubation at 50° on complement-fixing activity and RNA-stimulating activity of T-antigen. Preparations from a ts *A* SV40-transformed cell line. (*Upper*) Complement-fixing (CF) activity of T-antigen preparations. ◑—◑, T-Antigen preincubated at 25°, either wild type or ts *A*; △—△, wild type at 50°; ▲—▲, ts *A* at 50°. (*Lower*) Nuclear RNA synthesis expressed as ratio of ts *A*/wild-type T-antigen. ○—○, Preincubation at 25°; ●—●, preincubation at 50°. Abscissa for both *upper* and *lower* is time of preincubation at either 25° or 50°.

say at this point that T-antigen stimulates RNA synthesis. However, our results have the same validity as other data on biological functions of T-antigen (as for instance, its ability to bind to DNA) that have also been carried out with partially purified preparations (18, 28, 39).

The major objection to an effect of T-antigen on RNA synthesis is that a number of nuclear proteins have been reported to stimulate nuclear or chromatin template activity (34, 40–42), and that these proteins may be responsible for the increased RNA synthesis in nuclei preincubated with the T-antigen preparation. Leaving aside trivial explanations (such as DNA or RNA polymerase activity), the following findings lend support to the role of T-antigen in the stimulation of nuclear RNA synthesis: (*i*) T-antigen preparations from three different cell lines are active, while similar nuclear preparations from T-antigen-negative cells are inactive; (*ii*) antisera to T-antigen (but not normal hamster serum) inhibit the stimulatory effect of T-antigen preparations; (*iii*) on glycerol gradients, stimulatory activity and T-antigen (as determined by complement fixation) cosediment in four different peaks; (*iv*) stimulation of nuclear RNA synthesis is temperature sensitive when T-antigen preparations are obtained from cells transformed with a ts A mutant of SV40; and (*v*) a partially purified preparation of T-antigen, kindly given to us by R. H. Martin (National Institutes of Health), gave results similar to our preparation, although the methods of purification were different (not shown). Despite these encouraging findings, final confirmation that T-antigen stimulates cellular RNA synthesis must await its satisfactory purification.

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