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"Early" simian-virus-40-specific RNA contains information for tumor antigen formation and chromatin replication

(microinjection of simian virus 40 cRNA/T-antigen/DNA synthesis)

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ABSTRACT Simian virus 40 (SV40) induces tumor (T)-antigen formation, chromatin replication, and mitosis in primary mouse kidney cells arrested in G_0 phase of the mitotic cycle. The temporal and quantitative relation between these early virus-specific reactions led to the hypothesis that the early SV40 mRNA contains information necessary for T-antigen formation and induction of cellular DNA synthesis. To get direct experimental evidence for this hypothesis, the early strand of SV40 DNA was transcribed *in vitro* by *Escherichia coli* DNA-dependent RNA polymerase and the SV40-specific cRNA was transferred by microinjection into epitheloid cells of confluent primary mouse kidney cultures. T-antigen formation and stimulation of DNA synthesis were investigated in the recipient cells. The experimental results obtained agree with the hypothesis that T-antigen is a virus-coded protein and that the early virus-specific mRNA contains information necessary for stimulation of cellular DNA replication in the arrested cells.

The infectious agent of simian virus 40 (SV40) is the virus DNA. The manner in which the virus genes are expressed is highly cell dependent. Cells of the native host (monkey cells) support early and late viral gene expression at a high efficiency (productive infection) while mouse kidney cells are nonpermissive for SV40 (abortive infection) (1, 2). However, the following sequence of events can be observed in both productive and abortive infected cells: (*a*) synthesis of early virus-specific mRNA, (*b*) formation of tumor (T)-antigen, (*c*) increase of cellular RNA synthesis, (*d*) induction of chromatin replication and mitosis (3).

The temporal and quantitative relation between the synthesis of the early virus-specific RNA and the subsequent sequence of events led to the hypothesis that the early virus-specific RNA may contain information necessary for T-antigen formation and chromatin replication (3, 4).

In the present study, we have tested this hypothesis by a more direct experimental approach. For this purpose, SV40-specific RNA, complementary to the early viral DNA strand, was synthesized *in vitro* by *Escherichia coli* DNA-dependent RNA polymerase (5). Following thorough removal of the SV40 DNA by exhaustive DNase treatment and equilibrium density centrifugation in Cs_2SO_4 (6) the cRNA preparation was injected into epitheloid cells of confluent primary mouse tissue culture by our microinjection technique (7, 8) and cRNA-induced T-antigen formation and DNA synthesis were investigated.

Abbreviations: SV40, simian virus 40; T-antigen, tumor-antigen; PFU, plaque-forming units; DNase, deoxyribonuclease; RNase, ribonuclease; DNA I, the supercoiled, covalently closed circular, double-stranded form of DNA; SSC, standard saline–citrate solution (0.15 M sodium chloride–0.015 M sodium citrate, pH 7); 4 × SSC means that the concentration of the solution used is four times that of the standard saline–citrate solution.

MATERIALS AND METHODS

Preparation of Supercoiled SV40 DNA. Cultures of confluent TC7 cells, a subline of CV1 (African green monkey kidney cells), grown in 100 mm plastic petri dishes, were infected with SV40 (777) at a multiplicity of 0.1 plaque-forming units (PFU) per cell. After an adsorption period of 2 hr, cells were washed and covered with serum-free Eagle's medium. DNA was extracted from these cells by the selective extraction method of Hirt (9) 48 hr after infection. The extract was phenol treated (80% phenol in 1 M Tris·HCl, pH 8.0) and nucleic acids present were precipitated with ethanol at −20°. The precipitate was collected by centrifugation, washed with ethanol, dried, dissolved in 0.1 × SSC (1 × SSC: 0.15 M NaCl, 0.015 M Na-citrate), treated with RNase (Worthington, 50 μg/ml, 30 min at 37°), phenol treated, and reprecipitated with ethanol. For separating the contaminant cellular DNA the precipitate then was layered onto a neutral sucrose gradient (5–20% weight/volume, 1 M NaCl, 0.001 M EDTA, 0.001 M Tris·HCl, pH 7.4) (10). Fractions corresponding to viral DNA were pooled and further purified by CsCl–ethidium bromide equilibrium centrifugation (initial density = 1.56 g/ml, 100 μg of ethidium bromide per ml in 0.02 M Tris·HCl, pH 8.0) as described (10, 11).

Transcription of Wild-Type SV40 DNA I. The sigma-factor-containing DNA-dependent RNA polymerase was used for SV40 DNA transcription (Miles Laboratories, *E. coli* K-12 RNA polymerase, grade II). The transcription reaction mixture (100 μl) contained 10 μg of SV40 DNA I (the supercoiled covalently closed circular form), 10 μg of RNA polymerase, 0.15 M KCl, 0.04 M Tris·HCl pH 8.0, 5% glycerol, 0.01 M $MgCl_2$, 1.5 mM 2-mercaptoethanol, 0.4 mM each of ATP, GTP, CTP, UTP, and 0.01 mM each of [8-^{3}H]GTP, [5-^{3}H]CTP, [5-^{3}H]UTP (Amersham Buchler, 15 Ci/mmol). The nucleoside triphosphates were added after preincubation of SV40 DNA and RNA polymerase at room temperature for 10 min. After 60 min of incubation at 37°, 10 μg of electrophoretically purified DNase (Worthington) was added to the reaction mixture and further incubated for 30 min at 37°. Then 0.1 × SSC and 5% sodium dodecyl sulfate were added to a final concentration of 0.01 × SSC and 1% sodium dodecyl sulfate. The assay was extracted two times with 1 volume of phenol–hydroxyquinoline (80% phenol in H_2O vol/vol and 1% hydroxyquinoline weight/volume) (12) and precipitated from the aqueous phase at −20° with 2 volumes of ethanol (95%) and 1/10 volumes of 1 M NaCl. To further reduce traces of any contaminating SV40 DNA, the cRNA preparation was subsequently subjected to Cs_2SO_4 equilibrium density gradient centrifugation (see Fig. 1) (6, 13). The average yield of cRNA was 80–100 μg with a specific activity of 1.5 to 2 × 10^5 cpm/μg of cRNA. Self-an-

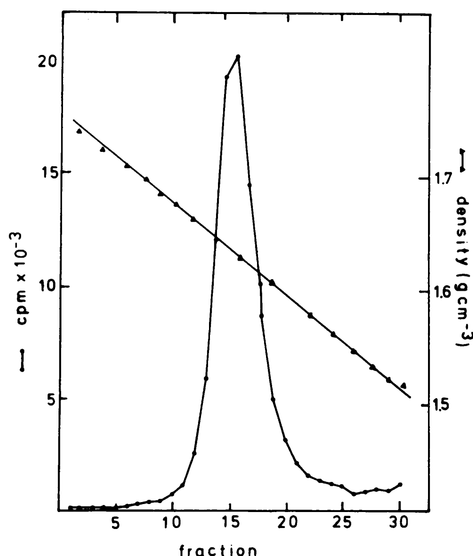

FIG. 1. Cs_2SO_4 equilibrium density gradient centrifugation of SV40 cRNA. The initial density in a solution of EDTA-Tris (10^{-3} M EDTA, 10^{-2} M Tris, pH 7.2) was 1.62 g/cm^3. The gradient was centrifuged in a Spinco SW 65 rotor at 30,000 rpm for 72 hr at 5°. The gradient was fractionated from the bottom of the tube and fractions 12-19 were collected. Cs_2SO_4 was removed by passing the pooled fractions through a Sephadex G-25 column and by exhaustive dialysis against diluted injection buffer.

nealing experiments were done in 4 × SSC at 72° for 20 hr as described elsewhere (5). Total cellular RNA and polyadenylylated RNA were isolated from TC7 cells (14, 15).

Microinjection of Nucleic Acids. Primary mouse kidney cells, prepared from 10-day-old mice (NMRI) (16), were grown in 100-mm plastic petri dishes on small glass slides (10 × 50 mm) subdivided in squares of 1 mm^2. The transfer of nucleic acids into the cells was performed under a microscope by help of glass microcapillaries (7, 8). The capillaries, having a diameter of about 0.5 μm at the tip, were placed in a micromanipulator (Leitz). The micromanipulator is equipped with a suction and pressure source. Unless indicated otherwise, nucleic acids were dissolved in injection buffer (0.048 M K_2HPO_4, 0.014 M $NaH_2PO_4 \cdot 2H_2O$, 0.0045 M KH_2PO_4, pH 7.2) at a concentration of 1 mg/ml. Fig. 2 illustrates four steps of the microinjection procedure.

Preparation of Micro Glass Capillaries. The capillaries were prepared from glass tubes (SGC, Scientific Inc., 1.2 mm, 1.5 mm). The required shape and diameter of the capillary tip were obtained by melting the crude capillary at a capillary puller (made in our laboratory). Before use, the tips of the capillaries were treated with hydrofluoric acid (50% in H_2O vol/vol, 2 sec) and washed two times with distilled water, ethanol, tetrahydrofuran, and dimethyldichlorsilane (0.5% in tetrahydrofuran vol/vol), again washed with tetrahydrofuran, and ethanol, and then sterilized for 4 hr at 130° (17).

RESULTS

Induction of SV40 T-antigen formation in primary mouse kidney cells by microinjection of SV40-specific cRNA

The early strand of SV40 DNA I was transcribed *in vitro* by *E. coli* DNA-dependent RNA polymerase (5) and the tem-

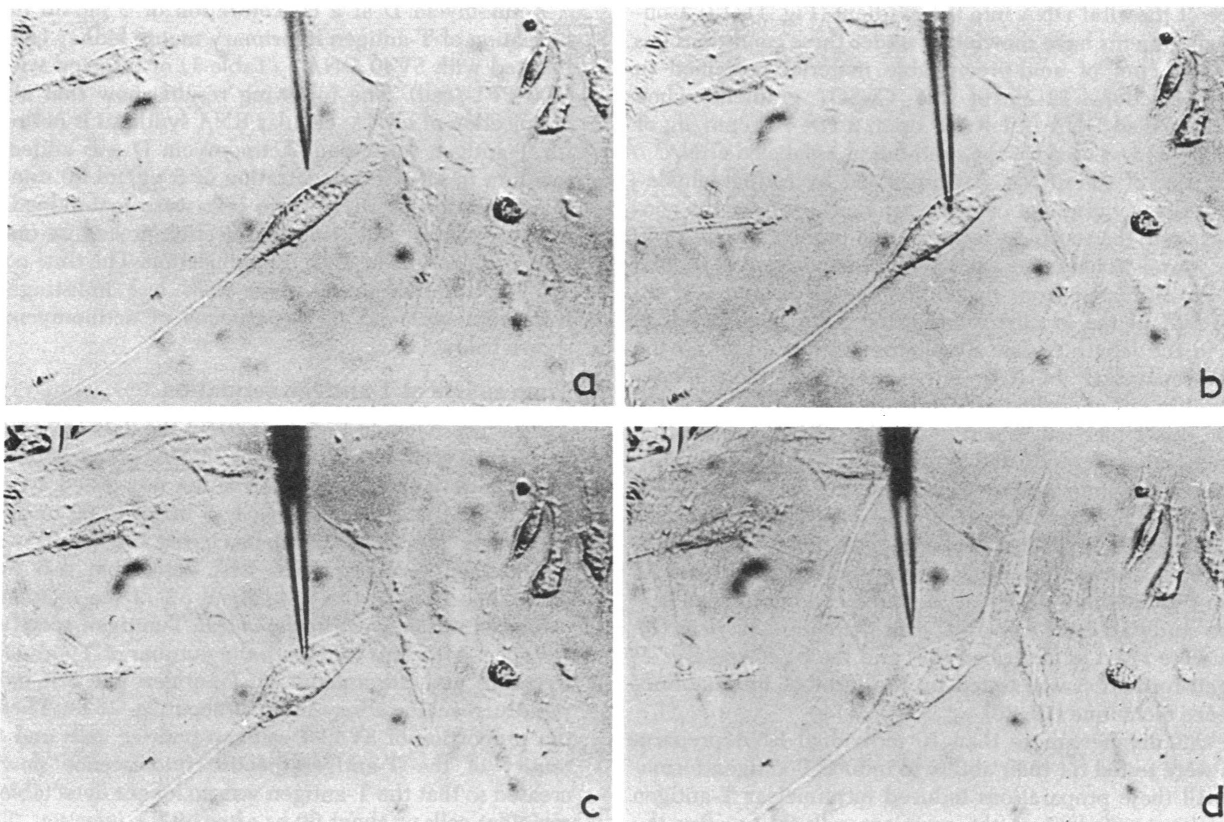

FIG. 2. Microinjection technique: (a) the capillary near the recipient cell; (b) the tip of the capillary inside of the cell; (c) the cell immediately after injection; (d) recipient cell after injection, with the capillary outside of the cell.

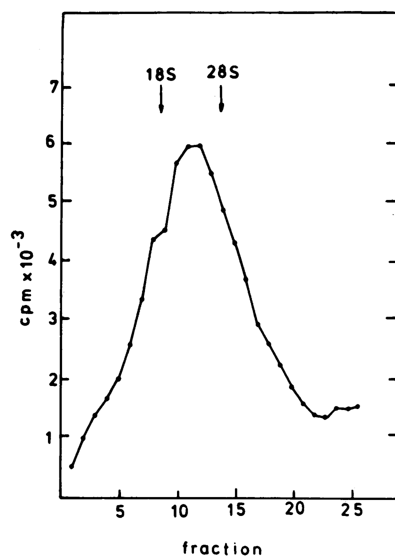

FIG. 3. Velocity sedimentation of SV40 cRNA in a sucrose density gradient (7–27% sucrose weight/volume in high-salt formaldehyde solution: 1.1 M HCHO, 90 mM Na_2HPO_4, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.7). The gradient was centrifuged in a Spinco SW 65 rotor at 49,000 rpm for 3 hr at 20°. Before centrifugation, SV40 cRNA was heated for 5 min at 80° in low-salt formaldehyde solution (1.1 M HCHO, 4.5 mM $Na_2H_2PO_4$, 0.5 mM NaH_2PO_4, 1 mM EDTA, pH 7.7).

plate was thereafter thoroughly removed by exhaustive DNase treatment as described in *Materials and Methods*. In order to further remove any contaminating traces of SV40 DNA, the cRNA preparation was centrifugated to equilibrium in Cs_2SO_4 at a density high enough to exclude the entrance of the viral DNA into the gradient (Fig. 1) (13). Control experiments have shown that under these conditions less than 100 cpm of acid-precipitable material remained in pooled fractions 12–19 of the Cs_2SO_4 gradient when [^{3}H]dT-labeled DNA (2.1 × 10^5 cpm; 2.1 × 10^4 cpm/μg of SV40 DNA) was used for the synthesis of unlabeled cRNA.

The size of the cRNA was estimated by formaldehyde–sucrose density gradient centrifugation (18). Under the conditions used, the complete transcription product of one SV40 DNA strand would have a sedimentation coefficient of 26 S (18). As estimated from the sedimentation profile (Fig. 3) about 50% of the *in vitro* synthesized RNA corresponds at least to the length of one SV40 strand. Depending on the RNA preparation, 14–18% of the cRNA remained RNase-resistant in self-annealing experiments.

For microinjection experiments the cRNA preparation was not further fractionated. Only epitheloid cells from confluent primary mouse kidney cultures were used as recipient cells. The volume transferred into the cytoplasm of the cells was about $1 - 2 \times 10^{-8}$ μl per cell.

After microinjection, cells were further incubated in serum-free medium at 37° at the same conditions used earlier to study abortive infection with the complete virus (3). Cells were fixed as described (19) and the SV40-specific T-antigen formation was tested by the indirect immunofluorescence technique (19, 20).

In this manner, more than 10 individual RNA preparations were tested for their ability to induce T-antigen formation. All these preparations induced intranuclear T-antigen formation in 60–80% of the recipient cells 24 hr after the microinjection. V-antigen (capsid protein) synthesis was demonstrable neither in primary mouse kidney cells nor in TC7 cells, 24, 48, and 72 hr after cRNA microinjection. If polyoma virus DNA was used as template, polyoma-virus-specific T-antigen, but not SV40-specific T-antigen, was synthesized (21). As indicated in Table 1, the biological activity of the cRNA was destroyed by RNase treatment. Cycloheximide (20 μg/ml) also inhibited T-antigen formation in cRNA-injected cells. Upon microinjection, neither total RNA nor polyadenylylated RNA isolated from TC7 cells induced T-antigen formation.

Actinomycin D at a concentration of 5 μg/ml inhibited formation of T-antigen in primary mouse kidney cells either injected with SV40 DNA I (Table 1) or infected with SV40 (200 PFU/cell). The following results show that after microinjection of cRNA, cellular RNA synthesis is not required for T-antigen formation. Actinomycin D was added to the medium to a final concentration of 5 μg/ml 30 min before microinjection of cRNA. The cells were maintained in this medium during and after the injection procedure until fixation 5, 10, and 15 hr after microinjection. The time course of T-antigen formation in these cells was indistinguishable from that observed in the absence of actinomycin D, as shown below.

Table 1. SV40 T-antigen formation in primary mouse kidney cells

Injection of	% Cells with T-antigen formation*
SV40 cRNA (0.5 mg/ml)	41
SV40 cRNA (0.5 mg/ml) in actinomycin-D-treated cells	41
SV40 cRNA (0.5 mg/ml) in cycloheximide-treated cells	0
SV40 DNA I (1 mg/ml) in actinomycin-D-treated cells	0
SV40 DNA I (0.1 mg/ml)† + TC7 cell RNA (1 mg/ml) in actinomycin-D-treated cells	0
SV40 cRNA, RNase treated (8)	0
TC7 cell RNA	0

* Each measurement is based on a count of 300 injected cells. Cells were fixed and stained 15 hr after injection.
† Without actinomycin D this DNA concentration induces T-antigen synthesis in 100% of the injected cells.

Time course of T-antigen formation

The results shown in Fig. 4 represent the data from three experiments with three different cRNA preparations. After cRNA injection, T-antigen formation was tested every hour during the first 5 hr, then every 5 hr up to 60 hr and then once every day. The experiments lasted 160 hr.

T-antigen formation was first detectable 4–5 hr after cRNA injection. At this time about 5% of the recipient cells exhibited rather weak intranuclear T-antigen-specific fluorescence. After this time both the number of T-antigen-positive cells and the amount of T-antigen per cell increased rapidly, reaching a maximum at about 24–26 hr. Thereafter, the proportion of SV40 T-antigen-positive cells and the intensity of the T-antigen-specific fluorescence slowly decreased so that the T-antigen was no longer detectable in the recipient cells at about 60 hr after cRNA injection. The cell density remained indistinguishable in injected and in the uninjected regions throughout the incubation period.

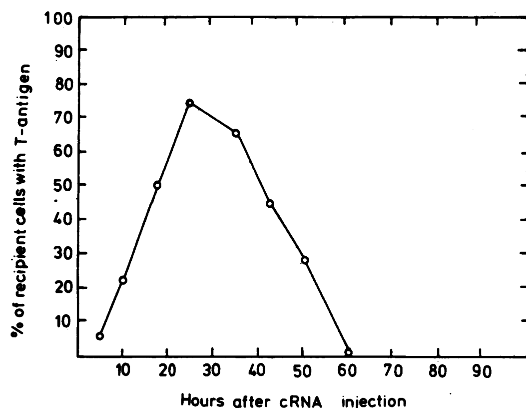

FIG. 4. Time course of T-antigen formation. Each time point is based on a count of 600 injected cells.

[^{3}H]Thymidine uptake induced by SV40 cRNA

Since SV40 induces chromatin replication in primary mouse kidney cells (3), we investigated whether the early SV40-specific RNA is involved in the process of induction of DNA synthesis. In order to answer this question, we injected SV40 cRNA synthesized *in vitro* into epitheloid cells of confluent primary mouse kidney cultures. At different times after injection (see Table 2) cells were pulse labeled with [^{3}H]dThd (0.1 μCi/ml) for 5 hr periods and for cumulative labeling from 0 to 25 hr after injection.

To correlate DNA synthesis with formation of T-antigen, fixed cells were stained with fluorescein-conjugated antibody against T-antigen and then processed for autoradiography. Three independent control experiments were performed and these involved injection of (*a*) injection buffer alone, (*b*) total cellular RNA from TC7 cells (1 mg/ml), and (*c*) polyadenylylated cytoplasmic RNA from TC7 cells (1 mg/ml).

The data summarized in Table 2 show that only SV40 cRNA induced DNA synthesis. Cumulative labeling experiments have shown that all cells induced for T-antigen synthesis were additionally induced for DNA synthesis at the first 25 hr following cRNA injection.

Pulse-labeling experiments with [^{3}H]dThd have shown that the first significant increase in the proportion of DNA-synthesizing cells was observed about 10 hr after cRNA injection. The maximum proportion of DNA-synthesizing cells was reached between 15 and 20 hr. During this time interval 85% of the T-antigen-positive cells were also stimulated for DNA synthesis while only 3.2% of the cells in control experiments incorporated [^{3}H]dThd.

Table 2. Percentage of injected cells stimulated for DNA synthesis

[^{3}H]dThd labeling (hr after injection)	Cells injected with	
	SV40 cRNA*	Control substances†
5–10	6	4
10–15	57	5
15–20	83	3
20–25	55	3
0–25	100	9

* Each point is based on a count of 600 injected cells. The values represent T-antigen-positive cells stimulated for DNA synthesis (the total number of T-antigen-positive cells at each time point was counted as 100%).

† Each point is based on a count of about 1000 injected cells. Control experiments involved injection of (*a*) injection buffer, (*b*) total cellular RNA from TC7 cells, (*c*) polyadenylylated cytoplasmatic RNA from TC7 cells. The number of cells stimulated for DNA synthesis was similar in all control experiments.

DISCUSSION

At the experimental conditions used, the *E. coli* DNA-dependent RNA polymerase transcribes preferentially the early (minus) strand of the SV40 DNA I (5). Following transcription, the DNA template was removed by exhaustive pancreatic DNase treatment and Cs_2SO_4 equilibrium density gradient centrifugation. The biological activity of the cRNA was tested by microinjection into cells of confluent primary mouse kidney cultures and was found to induce T-antigen formation. The ability to induce T-antigen formation was destroyed by a prior treatment of the cRNA with RNase.

The time course study indicated that T-antigen formation appears first at 4–5 hr after cRNA injection. Thereafter, the proportion of T-antigen-containing cells increased rapidly, reaching a maximum of 60–80% at 24–26 hr, followed by a decrease in the proportion of T-antigen-positive cells so that about 60 hr after cRNA injection none remained. The disappearance of T-antigen-positive cells was not due to a loss of the recipient cells since the cell density remained unchanged in the injected and uninjected regions of the slides throughout the incubation time.

In cells treated with actinomycin D to block cellular RNA synthesis, the time course of the appearance of T-antigen remained unchanged. In contrast actinomycin D prevented T-antigen formation in cells either injected with SV40 DNA I or infected with SV40, presumably by an inhibition of "early" SV40 mRNA synthesis. In contrast, the inhibition of protein synthesis by cycloheximide added to the cells after cRNA injection prevented T-antigen formation in these cells.

From these data we conclude that SV40 T-antigen requires cRNA translation but not a *de novo* RNA synthesis in the injected cells. Our results show that all cells synthesizing T-antigen within the first 25 hr after cRNA injection are also stimulated for DNA synthesis. The time course of T-antigen formation and that of stimulation of cellular DNA synthesis show striking similarity to that observed in cells infected with infectious virus (3).

These results support the hypothesis that SV40-specific T-antigen is a virus-coded protein and that the early virus-specific mRNA contains information necessary for stimulation of cellular chromatin replication.

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