QUANTITATIVE CORRELATION BETWEEN SIMIAN VIRUS 40
T-ANTIGEN SYNTHESIS AND LATE VIRAL GENE EXPRESSION IN
PERMISSIVE AND NONPERMISSIVE CELLS

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ABSTRACT

The time-course of intranuclear Simian virus 40 (SV40) tumor (T) antigen synthesis and accumulation in permissive CV1 monkey cells and nonpermissive 3T3 mouse cells has been studied by immunofluorescence and cytofluorometry. CV1 cells accumulate T antigen continuously over a period of 48 h after infection, whereas in 3T3 cells the T-antigen content remains about constant and at a comparatively low level. Only those CV1 cells which have attained a threshold concentration of intranuclear T antigen synthesize viral capsid proteins (V antigen). In nonpermissive 3T3 cells, the T-antigen threshold value for the onset of V-antigen synthesis is higher than in CV1 cells and is never reached by infected cells. However, 3T3 cells microinjected with sufficient amounts of SV40 DNA easily surpass this value and behave permissively.

KEY WORDS

SV40 antigen cytofluorometry
SV40 T-antigen quantitation
SV40 gene expression microinjection

MATERIALS AND METHODS

Cells

CV1 cells, an African green monkey kidney cell line (13), Balb/c 3T3 cells, and SV80 cells, a human transformed cell line (20, kindly provided by M. Osborn), were grown in Dulbecco's modified Eagle medium (Grand Island Biological Co., Grand Island, N. Y.) containing 10% newborn calf serum (Gibco Bio-Cult, Glasgow, Scotland).

Virus and Viral DNA

Wild type (wt) SV40 strain 777 and the mutant strain tsA7 virus were plaque-purified three times before use. SV40 empty capsids were prepared from virus stocks by CsCl equilibrium density gradient centrifugation (3). The empty particle band (buoyant density 1.29-1.30) was recycled through CsCl equilibrium density gradients twice. SV40 DNA was isolated from the virus by sodium...
Dodecyl sulfate-phenol extraction (15) and further purified by CsCl-ethidium bromide equilibrium density gradient centrifugation (10), yielding the supercoiled circular DNA form I and the nicked circular DNA form II. For microinjection, SV40 DNA I was dissolved at a concentration of 1 mg/ml in 0.01 M Tris-HCl, pH 7.4.

Microinjection Procedure

Cells grown on glass slides were microinjected with 10 to 2 x 10^11 ml of injection solution per cell with our glass capillary microinjection technique (7, 9).

Detection of Viral Antigens

SV40 T and V antigens were determined by the direct immunofluorescence technique with fluoresceine-conjugated hamster anti-T IgG (kindly provided by G. Fey) and rhodamine-B conjugated guinea pig anti-V IgG (Berlin), respectively. Cells on glass slides were fixed in acetone-methanol (2:1) at -20°C for 10 min, air-dried, covered with the appropriate antibody or a double concentrated mixture of both antibodies (total volume 10 μl), incubated in a humid chamber at 37°C for 45 min, and extensively washed with phosphate buffer. Fluorescence was observed with a Zeiss universal fluorescence microscope equipped with a mercury HBO 100W/2 lamp, a fluorescence condensor III RS and Zeiss filter sets code nos. 48 77 10 and 48 77 15 for fluoresceine and rhodamine, respectively.

Cytofluorometry

For cytofluorometric quantitation of intranuclear T antigen, confluent cultures of CV1 or 3T3 cells, grown on glass slides, were infected. After an adsorption time of 2 h, cells were washed and further incubated in serum-free medium until fixation and staining.

The T-antigen concentration was measured in arbitrary units (AU) with a Zeiss fluorescence photometer equipped with an operation and control unit, an electrically operated shutter, a light modulator, and a digital printer XP2. Each single cell nucleus was first adjusted by phase contrast and transmitted light, then measured with 7 ms excitation time and reflected light, the surroundings being masked by insertion of a diaphragm. Fig. 1 demonstrates that the intranuclear T-antigen fluorescence of a single SV80 cell remains nearly constant when measured frequently with short excitation times, but falls off when continuously excited. In all quantitation experiments, identically stained semiconfluent SV80 cells served as a biological standard whose intranuclear T-antigen fluorescence was 13-27 AU (mean value 22 AU) under the conditions used, irrespective of whether cells were stained for T and V antigen simultaneously or for T antigen alone. With the direct immunofluorescence technique, the measured AU correlates linearly with the amount of the antigen (17).

RESULTS

Time-Course of Intranuclear T- and V-Antigen Accumulation in CV1 Cells

In CV1 cells, infected with 100 plaque-forming units (PFU) of SV40 777 per cell, intranuclear T antigen is first demonstrable 12 h post infection. At this early time, 4-5% of the infected cells exhibit intranuclear T-antigen fluorescence as tested by the direct immunofluorescence technique. 24-h after infection, 99% of the cells are T-antigen-positive. At lower multiplicities of infection (MOI) (MOI:10 or 1 PFU/cell), the onset of T-antigen accumulation is delayed and the number of T-antigen-positive cells is lower (Fig. 2).

However, independent of the MOI, both the number of T-antigen-positive cells and the intensity of the intranuclear T-antigen-specific fluorescence increase as the incubation time is prolonged. To quantitate this increase, we measured the intranuclear T-antigen fluorescence in SV40-infected CV1 cells in AU, using a microscope fluorescence photometer. Fig. 3 illustrates the changes during the first 48 h in the intranuclear T-antigen concentration in CV1 cells infected with 100 PFU per cell. 12 h after infection, when 4-5% of the cells are T-antigen-positive (Fig. 2), 70% of these cells already have antigen concentrations up to 20 AU (indicated by the bars in Fig. 4).

No significant differences were noted in T-antigen fluorescence as a function of the multiplicity of infection (MOI:10 or 1 PFU/cell, respectively). However, a considerable number of T-antigen-positive cells already have antigen concentrations up to 20 AU (indicated by the bars in Fig. 4).

**Figure 1** Decrease of SV80 intranuclear T-antigen fluorescence with prolonged excitation time. Single SV80 cells were excited over a 5-min period either every 10 s for 7 ms (●) or continuously (○).
FIGURE 2 Time-course of SV40 T-antigen synthesis in CV1 cells infected with 100 (○), 10 (▲), or 1 (■) PFU per cell.

FIGURE 3 Time-course of SV40 T-antigen concentration in CV1 cells infected with 100 PFU per cell. For each time point, >100 randomly chosen cells were measured. Columns represent the T-antigen fluorescence of 70% of the cells; minimal and maximal values are given by the bars. The T-antigen content of SV80 reference cells is inserted. The dotted line represents the threshold value of T antigen below which V antigen is not detectable. Values given are reproducible within ±10%.

Fig. 3). 24 h post infection, when ~99% of the cells are T-antigen-positive, 70% have a fluorescence intensity of 26–46 AU. After this time, the number of T-antigen-positive cells does not change, but T-antigen concentration per cell increases continuously for 48 h after infection. Observation was stopped at this point, since onset of cell lysis alters the initial measuring conditions. With lower input multiplicities (10, 1.0, 0.1 PFU/cell), intranuclear T-antigen accumulation proceeds more slowly, as shown in Fig. 4 for a MOI of 1 PFU per cell.

SV40 capsid protein (V antigen) synthesis is only demonstrable after the onset of viral DNA replication (2, 21). To measure the number of capsid molecules necessary to obtain a detectable intranuclear V-antigen antibody reaction, empty SV40 capsids were suspended in injection buffer at different concentrations and microinjected into the nuclei of CV1 cells. Immediately after injection, cells were fixed and stained with rhodamine-B-conjugated anti-V serum. The minimal concentration of capsid protein required for a detectable immunofluorescence reaction is 4 mg/ml. Under these conditions, ~500–1,000 empty SV40 particles are microinjected into a recipient cell. In SV40-infected CV1 cells, this intranuclear V-antigen concentration is obtained ~6 h after T-antigen appearance (Figs. 2 and 5).

Quantitative Correlation between Intranuclear T-Antigen Accumulation and V-Antigen Synthesis

When SV40-infected cells were stained with a mixture of fluoresceine-conjugated anti-T serum...
and rhodamine-conjugated anti-V serum, a quantitative correlation between intranuclear T-antigen concentration and V-antigen synthesis was implied. Independent of the MOI, V-antigen-positive cells always showed a strong intranuclear T-antigen-specific fluorescence (Fig. 6). To test whether a measurable correlation exists between the relative amount of intranuclear T-antigen and V-antigen synthesis, the T-antigen concentration of V-antigen-positive CV1 cells was measured in AU at different times after infection. Independent of the MOI (100, 10, 1, 0.1 PFU/cell), the minimal concentration of T antigen in V-antigen-positive cells is 28 AU (dotted line in Figs. 3 and 4), but the higher the MOI, the sooner this value is attained. However, not all CV1 cells having this amount of T antigen are necessarily V-antigen-positive. The distribution of intranuclear T-antigen concentration and its correlation with V-antigen synthesis is illustrated in Fig. 7 for a population of 300 CV1 cells 32 h after infection with 10 PFU per cell.

To test whether the continuous intranuclear T-antigen accumulation is due to the increase of viral DNA template molecules, we measured the T-antigen content of infected CV1 cells blocked for DNA synthesis by 5-fluorodeoxyuridine (FUDr; 15 μg/ml medium; 0–48 h post infection). Control experiments with autoradiography revealed no incorporation of [3H]thymidine at these conditions (data not shown). As expected (14), V antigen is not detectable at any time, but T-antigen accumulation is unaffected by the presence of the drug (data not shown). CV1 cells infected with the early temperature-sensitive mutant virus tsA7 and continuously held at 41.5°C also surpass the T-antigen threshold concentration of 28 AU without synthesizing V antigen in detectable amounts.

**Time-Course of T- and V-Antigen Synthesis in 3T3 Cells**

Mouse cells (3T3) infected with SV40 virus by the conventional virus adsorption method are nonpermissive for SV40 (21). Fig. 8 shows the time-course of T-antigen formation in 3T3 cells infected with 500 and 50 PFU/cell. With these MOI's, T-antigen formation is first demonstrable 17 and 24 h, respectively, after infection. Only with the high MOI and an incubation time of at least 65 h do nearly 100% of the infected cells become T-antigen-positive, but V antigen was not demonstrable at any time after infection. Furthermore, the mean value of the intranuclear T-antigen concentration does not exceed that of SV80 cells (Fig. 9). As reported recently, 3T3 cells support early and late SV40 gene expression either after microinjection of at least 200–400 SV40 DNA I molecules or after microinjection of early virus-specific RNA together with 20–40 DNA I molecules per cell (6). Fig. 10 shows the time-course of T- and V-antigen formation after microinjection of 2,000–4,000 SV40 DNA I molecules into the nuclei of 3T3 cells. 70% of the injected cells had a T-antigen concentration between 40 and 59 AU 48 h after microinjection (Fig. 9). The fluorescence intensity of 3T3 cells microinjected with a known number of purified T-antigen molecules is comparable to the fluorescence intensity of microinjected CV1 cells (A. Graessmann et al., manuscript in preparation). In this nonpermissive cell type, the minimal concentration of T antigen in V-antigen-positive cells turns out to be 52 AU (dotted line in Fig. 9). With 100–200 SV40 DNA molecules transferred per recipient cell, the intranuclear T-antigen concentration is always lower than 40 AU. No V-antigen synthesis can be detected in these cells at any time tested.

**DISCUSSION**

The subdivision of the SV40 infection cycle into an early and a late phase is not merely a phenomenological description, since late SV40 gene expression depends on sufficient synthesis of the early virus-coded protein (T antigen, A protein)

**Figure 5** Time-course of SV40 V-antigen synthesis in CV1 cells infected with 100 (•), 10 (▲), or 1 (●) PFU per cell.
in terms of both quantity and quality. This investigation has yielded strong evidence that late viral gene expression (V-antigen synthesis) depends upon a threshold concentration of intranuclear T antigen. The double-staining experiments show that a T-antigen content equivalent to 28 AU is the minimal concentration attained before the onset of V-antigen synthesis in monkey kidney (CV1) cells. Preliminary results obtained by microinjection of the purified T antigen indicate that...
cellular and viral DNA synthesis. However, induction of cellular DNA synthesis can be separated from viral DNA replication experimentally, e.g.: (a) at the nonpermissive temperature, early ts mutants stimulate cellular but not viral DNA synthesis (1) although these cells surpass the 28 AU T antigen concentration; (b) stimulation of cellular DNA synthesis requires a significantly lower T-antigen concentration than viral DNA synthesis as shown by microinjection of purified T antigen (A. Graessmann et al., manuscript in preparation); (c) induction of cellular but not viral DNA synthesis can be obtained by microinjection of an SV40 DNA fragment lacking 20% of the

FIGURE 7 Distribution of intranuclear SV40 T-antigen concentration and correlation of T-antigen content to V-antigen appearance in CV1 cells 32 h after infection with 10 PFU per cell. 300 double-stained cells were measured for T-antigen fluorescence and checked for V antigen simultaneously. Hatched areas represent percent of total cells which are T- and V-antigen-positive.

FIGURE 8 Time-course of SV40 T-antigen synthesis in 3T3 cells infected with 500 (c) or 50 (h) PFU per cell. This value corresponds to about $10^6$ T-antigen molecules (A. Graessmann et al., manuscript in preparation).

Since intranuclear T-antigen formation is not affected in cells blocked for DNA synthesis, the accumulation of these high amounts of T antigen is not a consequence of an increase in the number of viral template molecules, but is rather needed for the onset of viral DNA replication as a prerequisite for late viral gene expression (2, 7).

It is still unknown how T antigen stimulates cellular and viral DNA synthesis. However, induction of cellular DNA synthesis can be separated from viral DNA replication experimentally, e.g.: (a) at the nonpermissive temperature, early ts mutants stimulate cellular but not viral DNA synthesis (1) although these cells surpass the 28 AU T antigen concentration; (b) stimulation of cellular DNA synthesis requires a significantly lower T-antigen concentration than viral DNA synthesis as shown by microinjection of purified T antigen (A. Graessmann et al., manuscript in preparation); (c) induction of cellular but not viral DNA synthesis can be obtained by microinjection of an SV40 DNA fragment lacking 20% of the

FIGURE 9 Time-course of SV40 T-antigen concentration in 3T3 cells infected with 500 PFU per cell. Intranuclear T-antigen fluorescence of 3T3 cells microinjected with 2,000-4,000 SV40 DNA I molecules is additionally given (— ). Details as described in the legend to Fig. 3.

FIGURE 10 Time-course of T- and V-antigen synthesis in 3T3 cells microinjected with SV40 DNA I. DNA concentration was 1 mg/ml, corresponding to 2,000-4,000 DNA I molecules transferred per recipient cell. Each time point is based on a count of 200 cells. T antigen (△), V antigen (△).
early region (11; C. Mueller et al., manuscript in preparation). From these observations, at least two alternatives can be deduced for the possible mode of T-antigen action: (a) cellular and viral DNA synthesis are stimulated in distinct ways, maybe even by different sites; and (b) a common function induces cellular and viral DNA synthesis, although with different specificities.

Late SV40 gene expression in the "nonpermissive" mouse cell needs a T-antigen threshold concentration of \( \sim 52\text{AU} \). This amount is easily obtained by microinjection of large numbers of SV40 DNA molecules, but not by conventional virus infection (Fig. 9). In contrast to permissive cells (Fig. 2), 3T3 cells do not accumulate T antigen continuously, even when infected with 500 PFU per cell. These cells may synthesize T antigen less efficiently and/or degrade it at a higher rate than monkey cells. Since the intranuclear presence of at least 250-500 viral particles or viral DNA molecules overcomes nonpermissiveness (6, 8), a very early event in the infectious cycle (adsorption, penetration, intracellular release) must be additionally involved in the nonpermissive type of response in infected mouse cells.

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