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Temperature-Sensitive Simian Virus 40 Mutant Defective in a Late Function

SAUL KIT, S. TOKUNO, K. NAKAJIMA, D. TRKULA, AND D. R. DUBBS

Division of Biochemical Virology, Baylor College of Medicine, Houston, Texas 77025

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A temperature-sensitive simian virus 40 (SV40) mutant, tsTNG-1, has been isolated from nitrosoguanidine-treated and SV40-infected African green monkey kidney (CV-1) cultures. Replication of virus at the nonpermissive temperature (38.7 C) was 3,000-fold less than at the permissive temperature (33.5 C). Plaque formation by SV40tsTNG-1 deoxyribonucleic acid (DNA) on CV-1 monolayers occurred normally at 33.5 C but was grossly inhibited at 38.7 C. The time at which virus replication was blocked at 38.7 C was determined by temperature-shift experiments. In shift-up experiments, cultures infected for various times at 33.5 C were shifted to 38.7 C. In shift-down experiments, cultures infected for various times at 38.7 C were shifted to 33.5 C. All cultures were harvested at 96 hr postinfection (PI). No virus growth occurred when the shift-up occurred before 40 hr PI. Maximum virus yields were obtained at 96 hr PI when the shift-down occurred at 66 hr, but only about 15% of the maximum yield was obtained when the shift-down occurred at 76 hr PI. These results indicate that SV40tsTNG-1 contains a conditional lethal mutation in a late viral gene function. Mutant SV40tsTNG-1 synthesized T antigen, viral capsid antigens, and viral DNA, and induced thymidine kinase activity at either 33.5 or 38.7 C. The properties of the SV40 DNA synthesized in mutant-infected CV-1 cells at 33.5 or 38.7 C were very similar to those of SV40 DNA made in parental virus-infected cells, as determined by nitrocellulose column chromatography, cesium-chloride-ethidium bromide equilibrium centrifugation, and by velocity centrifugation in neutral sucrose gradients. Mutant SV40tsTNG-1 enhanced cellular DNA synthesis in primary cultures of mouse kidney cells at 33.5 and 38.7 C and also transformed mouse kidney cultures at 36.5 C. SV40tsTNG-1 was recovered from clonal lines of transformed cells after fusion with susceptible CV-1 cells and incubation of heterokaryons at 33.5 C, but not at 38.7 C.

A direct way of relating viral gene functions to biochemical changes in infected cells is to study temperature-sensitive mutants of a virus. A temperature-sensitive mutant is one that produces an altered gene product (protein) that is functional at one temperature but not at another. By asking whether a given biochemical change occurs under conditions where the mutated viral gene product is not functional, it can be established whether the viral gene product is required for the biochemical change to occur.

By using temperature-sensitive mutants, three or four polyoma virus genes have so far been identified (2, 5-7, 22). Plaque morphology mutants of simian virus 40 (SV40) have also been described, some of which replicate very poorly at the elevated temperature of 40 to 41 C (14, 18, 19, 22, 23). However, use of the latter SV40 mutants has been somewhat limited because of the deleterious effects of supranormal temperatures upon the host cells.

In this report, we describe the properties of an SV40 mutant, tsTNG-1, which replicates normally at 33.5 C, but not at 38.7 C. The mutant was isolated from SV40-infected African green monkey kidney (CV-1) cultures that had been treated with nitrosoguanidine. The data to be presented demonstrate that viral replication is markedly inhibited at 38.7 C, but that induction of early SV40 functions, replication of SV40 deoxyribonucleic acid (DNA), and formation of viral capsid (V) antigens do take place.

MATERIALS AND METHODS

Cell lines. CV-1, an established line of African green monkey kidney cells, was grown in "R5a" medium containing 10% calf serum and 0.5% lactalbumin hydrolysate (13), or in Eagle's minimal essential

medium (MEM; Auto POW, Flow Laboratories, Inc., Rockville, Md.) supplemented with 10% calf serum. CV-1 cells were subcultured at weekly intervals. Primary mouse kidney cell cultures were prepared from 10- to 14-day-old Swiss mice (Texas Inbred Mouse Co., Houston, Texas). Five-day-old cultures containing 3×10^6 cells per culture were transformed by infecting them with SV40tsTNG-1 at an input multiplicity of 61 plaque-forming units (PFU)/cell (4). Transformed cells were routinely grown in monolayer cultures at 36.5 C and were subcultured twice weekly. The transformed cells were also capable of growth at 38.7 C for three to four generations. The transformed cell line was cloned at passages 6 and 17 in medium containing 1% SV40 antisera. Transformed cell lines contained SV40 T antigen, as determined by immunofluorescence (IF), but did not contain detectable infectious virus. Cell-free extracts were prepared from cultures of transformed cells which were incubated at 36.5 C for 4 days after subculturing and then were incubated at 33.5 C for 6 days. No SV40 was detected after plating the cell extracts on CV-1 monolayers at either 33.5 or 36.5 C.

Virus. SV40 was grown and assayed in monolayer cultures of CV-1 cells (13). Parental SV40 clone 307L, which produces large clear plaques on CV-1 cells, was assayed at 36.5 C or 33.5 C. Mutant SV40tsTNG-1 was assayed at 33.5 C and produces small indistinct plaques at this temperature. Sendai virus was grown in the allantoic cavity of 10- to 11-day-old embryonated eggs (3).

Virus growth curves. Seven-day-old monolayer cultures of CV-1 cells in 2-oz prescription bottles (ca. 60 ml) were infected at a multiplicity of five PFU per cell in a 0.2-ml volume for 2 hr at 36.5 C. The cultures were washed three times with isotonic-glucose-saline solution (GKN). Then 0.5 ml of SV40 antiserum (diluted 1 part to 9 parts of medium) was added, and the cultures were incubated for 30 min at 37 C. (The SV40 antiserum was prepared in horses against SV40 strain Rh 911; Flow Laboratories, Inc., Rockville, Md.) After washing cultures three times with GKN to remove the antiserum, growth medium (5.0 ml) was added and the cultures were incubated at either 33.5 or 38.7 C. At the times indicated in Fig. 1, two cultures were removed from the incubator and frozen. Cells and supernatant fluid were harvested from each culture. The cells were disrupted by sonic treatment, and the virus yields were assayed on CV-1 monolayers. Parental SV40 clone 307L and mutant SV40tsTNG-1 were assayed at 33.5 C.

For the temperature-shift experiments, cultures were treated in a similar manner, except that at the times indicated in Fig. 2 two cultures were shifted from incubators of one temperature to the other. At 96 hr postinfection (PI), all cultures were frozen and thawed once prior to harvesting cells and supernatant fluid.

Complement fixation (CF) test. Preparation of cell extracts and demonstration of SV40 T antigen by the CF test have been described (13, 17).

IF test. The presence of SV40 T antigen in cells transformed by SV40 was demonstrated by indirect IF (12) by using sera from hamsters bearing SV40 transplant (virus-free) tumors and fluorescein-labeled goat antihamster globulin. V antigen was demonstrated by direct IF by using fluorescein-labeled SV-40-hyperimmune monkey sera (20).

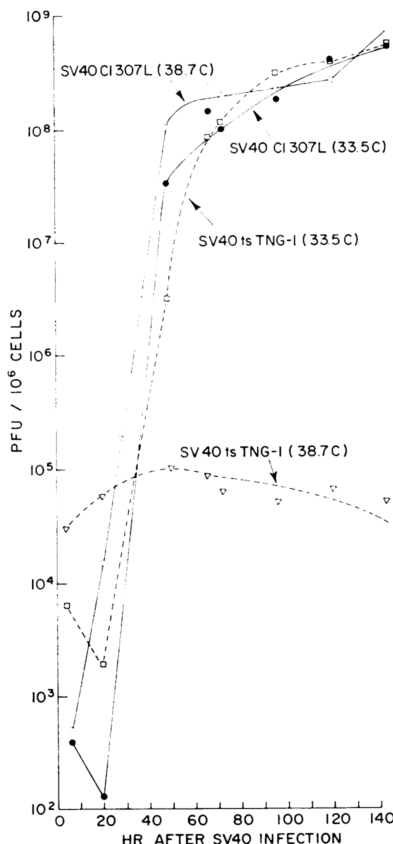

FIG. 1. *Growth of parental SV40 clone 307L and mutant SV40tsTNG-1 in CV-1 cells at 33.5 and 38.7 C. At the times indicated, two infected cultures for each point were frozen. Both viruses were assayed at 33.5 C.*

Preparation and assay of infectious SV40 DNA. SV40 DNA was extracted from infected CV-1 cells and separated from cellular DNA by the method of Hirt (8). Extracts were deproteinized with an equal volume of phenol and then dialyzed against 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0 ($1 \times$ SSC), until the dialysate was free from phenol, as determined by absorption measurements at 260 nm. Unfractionated DNA samples and samples fractionated by equilibrium or velocity centrifugation were assayed for infectivity by the diethylaminoethyl-dextran method on CV-1 monolayers (15).

Fractionation of DNA preparations. Closed-circular double-stranded SV40 ^{3}H-DNA (form I) was separated from nicked-circular (form II) or from linear double-stranded DNA by nitrocellulose chromatography (17), or by equilibrium centrifugation in cesium chloride (CsCl) solutions (density of 1.57 g/cm^3) containing 300 μg of ethidium bromide (EtBr) per ml (9). Samples studied by the dye-buoyant density method were centrifuged at 20 C for 44 hr at 120,000

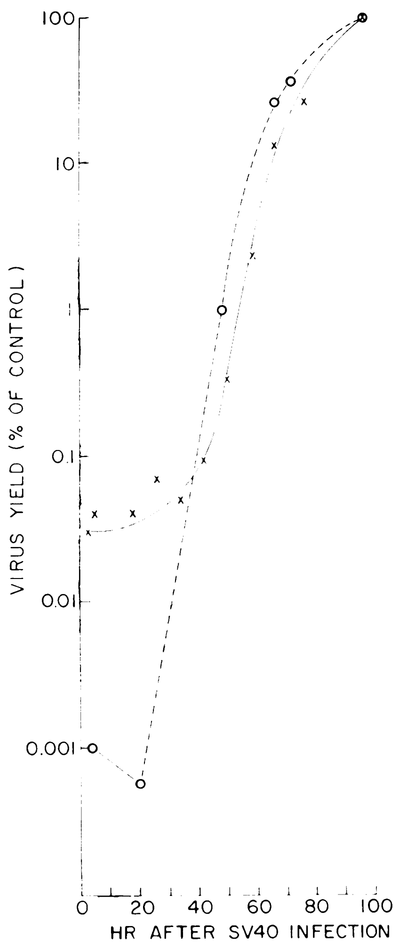

FIG. 2. *Temperature shift-up experiment. CV-1 cultures were infected with SV40tsTNG-1 and incubated at 33.5 C. For curve* X, *two infected cultures were shifted to 38.7 C at the times indicated and incubated until 96 hr PI. For curve* O, *infected cultures were frozen at the times indicated. The virus yields are presented as the per cent yield of control SV40tsTNG-1-infected cultures incubated 96 hr at 33.5 C.*

$\times$ *g* by using rotor 50Ti and a Spinco L2 centrifuge. EtBr was removed from DNA solutions by extracting two times with an equal volume of isoamyl alchohol, followed by ether extraction. The extraction procedure with isoamyl alcohol and ether was repeated, and the DNA solutions were dialyzed against 1 $\times$ SSC containing 0.001 M ethylenediaminetetraacetic acid (EDTA), and then against 1 $\times$ SSC without EDTA. Control experiments demonstrated that the EtBr concentration was reduced below the level detectable by spectrophotometric measurement at 476 or 284 nm and that the infectivity of SV40 DNA after removal of EtBr was the same as that of DNA which had not been exposed to EtBr.

Heavy and light DNA fractions obtained by the dye-buoyant density procedure and freed of EtBr as described above were in some instances further analyzed by centrifugation through 5 to 30% (w/v) sucrose gradients. Sucrose solutions were dissolved in 0.1 M sodium chloride, 0.001 M EDTA, 0.01 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, *p*H 7.2. DNA samples (0.1 ml) were layered on top of the gradient and centrifuged at 5 C for 210 min at 100,000 $\times$ *g* by using an SW39 or an SW50 rotor and a Spinco L2 centrifuge.

Ten-drop fractions were collected from the gradients on Whatman no. 4 filter paper. The papers were washed with cold 5% trichloroacetic acid and cold ethyl alcohol and assayed for radioactivity by liquid scintillation counting (17).

Enzyme assay. Thymidine kinase activity of uninfected and SV40-infected CV-1 cells was assayed with ^{3}H-deoxyuridine as nucleoside substrate (13).

Materials. Optical grade CsCl was obtained from the Harshaw Chemical Co., Cleveland, Ohio, and thymidine-*methyl-^{3}H* (14 to 18 Ci/mM) from New England Nuclear Corp., Boston, Mass. Nitrosoguanidine (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine) was purchased from the Aldrich Chemical Co., Inc., Milwaukee, Wis.

RESULTS

Treatment of SV40-infected cell cultures with nitrosoguanidine. Nitrosoguanidine treatment of SV40-infected CV-1 cells was used to induce SV40 mutants. Selection of this mutagen was based upon the observations of Cerda-Olmedo and Hanawalt (1) that nitrosoguanidine inflicts very high levels of mutagenesis with little lethality and preferentially mutagenizes the region of replication of the bacterial chromosome. Also, Kao and Puck (10, 11) had found that nitrosoguanidine is an efficient mutagen for mammalian cells. Cell cultures infected with parental SV40 clone 307L at an input multiplicity of five PFU/cell were treated at 2 hr after infection with 15 μg of 1-β-D-arabinofuranosylcytosine (ara-C) per ml to synchronize the replication of SV40 DNA. At 26 hr, the medium was removed, the cultures were washed, and fresh medium containing deoxycytidine (45 μg/ml) was added to reverse the ara-C block of DNA replication. Nitrosoguanidine (100 μg/ml) was added to replicate cultures for 30 min at 0, 30, 60, 90, and 120 min, respectively, after the addition of deoxycytidine. After the 30-min nitrosoguanidine pulse, the cultures were again washed, fed with fresh growth medium containing deoxycytidine, and incubated at 37 C until 56 hr PI. The SV40 yields (assayed at 37 C) of nitrosoguanidine-treated cultures were 8 to 16% of the yields obtained from untreated cultures. The SV40 mutant described in this report was derived from cultures pulsed with nitrosoguanidine at 120 to 150 min after the reversal of the ara-C block.

Isolation of virus mutants. To select virus mutants, harvests from cultures mutagenized with nitrosoguanidine were treated by sonic oscillation for 1 min at 10 kc at 4 C and were plated on CV-1 monolayers. They were overlaid with agar-medium and incubated at 33.5 C until small plaques (1 mm in diameter) developed. The periphery of each plaque was marked, and then the plates were shifted to 38.7 C for 4 days. The majority of plaques enlarged at the high temperature. Those plaques which failed to enlarge (12 of 1,137) were picked with a capillary pipette and resuspended in 2 ml of medium. After dispersion by freeze-thaw and sonic oscillation for 1 min, 0.1-ml samples of undiluted fluid were plated on each of four CV-1 monolayers. Two of these were incubated at 33.5 C and two at 38.7 C. Samples which produced confluent destruction at 33.5 C, and less than 100 plaques at 38.7 C, were titrated at the two temperatures. For the initial sample of virus mutant SV40tsTNG-1, the ratio of plaques formed at 33.5 to 38.7 C was 120. The mutant virus was plaque-purified a second time, and virus pools were prepared. After four passages in CV-1 cells, the titer of SV40tsTNG-1 was 1.7×10^8 PFU/ml at 33.5 C, and less than 10^3 PFU/ml at 38.7 C. Parental SV40 clone 307L gives the same titer when assayed at either temperature.

Growth of SV40 clone 307L and SV40tsTNG-1 at two temperatures. Parental SV40 clone 307L grows equally well at 33.5 and 38.7 C, reaching titers greater than 10^8 PFU/10^6 cells (Fig. 1). At 33.5 C, the eclipse is somewhat longer, and maximum yields were obtained about 10 to 20 hr later than at 38.7 C. Growth of mutant SV40tsTNG-1 resembled that of SV40 clone 307L at the low temperature (33.5 C), but very little increase in SV40tsTNG-1 titer was observed at 38.7 C (Fig. 1).

Temperature-shift experiments. To determine the time at which replication of mutant SV40tsTNG-1 was blocked at the high temperature (38.7 C), cultures infected with the mutant virus at a multiplicity of 5 PFU per cell were incubated at 33.5 C. At various times after infection, two cultures were shifted to 38.7 C. All of the cultures were harvested at 96 hr PI. The results of this experiment are shown in Fig. 2, along with the growth curve of SV40tsTNG-1 at 33.5 C. No virus growth occurred when infected cultures were shifted from 33.5 to 38.7 C before 40 hr PI. A comparison of the two curves indicates that when cultures were shifted between 40 and 96 hr, no new virus maturation occurred, for the virus yields of the shifted cultures were almost identical with those of cultures frozen at the indicated times. These results suggest (i) that virus replication is blocked in a late function, and (ii) that the product of the late function does not accumulate, but rather, the product is used as rapidly as it is synthesized.

Shift-down experiments were also performed. Cultures infected with SV40tsTNG-1 were incubated at 38.7 C. At various times after infection, two cultures were shifted to the lower temperature (33.5 C). When cultures were shifted to 33.5 C at any time between 2 and 66 hr PI, maximum virus yields were obtained by 96 hr PI. However, when cultures were shifted from the high to the low temperature at 76 hr PI, about 15% of the maximum virus yield was obtained. These results also indicate that a late function is blocked at the high temperature.

Induction of T antigen and thymidine kinase activity. Two early SV40 functions are induction of T antigen and thymidine kinase activity (4, 13, 17). The capacity of SV40tsTNG-1 to induce these functions was studied by infecting confluent monolayer cultures of CV-1 cells at 33.5 and 38.7 C. Enzyme and T antigen assays were then performed at various times from 16 to 66 hr PI. Both T antigen and thymidine kinase activity were induced by the mutant at either 33.5 or 38.7 C.

Synthesis of SV40 DNA at 33.5 and 38.7 C. To determine the total amount of SV40 DNA made at the high and low temperatures, CV-1 cells were infected with parental SV40 clone 307L or SV40tsTNG-1 at either 33.5 or 38.7 C. At various times after infection, the DNA was labeled with ^{3}H-thymidine for 4 hr, and then the SV40 DNA was separated from the bulk of the cellular DNA by the selective precipitation method of Hirt (8). Supernatant fractions from the Hirt extract were assayed for total infectious DNA at 33.5 C (Fig. 3), and at 38.7 C (Fig. 4). In addition, the samples were studied by nitrocellulose column chromatography (17) to demonstrate that heat-resistant, double-stranded ^{3}H-DNA, similar to form I SV40 DNA, had been synthesized.

The 33.5-C assays (Fig. 3) show that parental SV40 clone 307L and mutant SV40tsTNG-1 DNA species were synthesized somewhat earlier when cells were infected at 38.7 than at 33.5 C. Also, the total infectious DNA reached a maximum by about 50 hr after infection at 38.7 C and then declined somewhat. However, with infections at 33.5 C, the total infectious SV40 DNA continued to increase from 50 to 72 hr after infection.

Figure 4 shows that parental SV40 clone 307L DNA synthesized at either 38.7 or 33.5 C could be assayed at 38.7 C. In contrast, the infectious DNA synthesized by SV40tsTNG-1 at either temperature formed few plaques on CV-1 monolayers when assayed at 38.7 C.

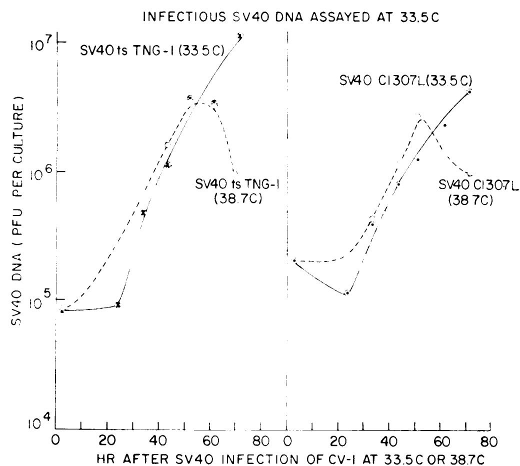

FIG. 3. *Synthesis of infectious DNA in CV-1 cells (8.2 × 10⁶ cells per culture) infected with parental SV40 clone 307L or SV40tsTNG-1 (input multiplicity of 20 PFU/cell) at 33.5 or 38.7 C. DNA extracts were prepared from infected cells at the indicated times and assayed at 33.5 C on CV-1 monolayer cultures.*

Radioactivity measurements provided further evidence that SV40 DNA was synthesized by SV40tsTNG-1 at the nonpermissive temperature. The Hirt supernatant fractions obtained from the cultures that had been labeled with ^{3}H-thymidine for 4 hr prior to harvest were boiled for 10 min, cooled, and applied to nitrocellulose columns (17). The heat-resistant, superhelical SV40 DNA which is double-stranded was not retained by the nitrocellulose. It was shown that during infection at 38.7 C with either parental SV40 or mutant SV40tsTNG-1, the rate of synthesis of heat-resistant DNA increased to a maximum at about 44 to 52 hr after infection and then declined. After infections at 33.5 C, radioactivity in heat-resistant DNA increased from 24 to 52 hr, but the rate of synthesis remained high through 72 hr after infection. Very little radioactivity was found in the Hirt supernatant fractions from uninfected cells.

Properties of the labeled DNA in the Hirt extracts. We wondered whether SV40 DNA abnormal in size or form might be generated during infection of cells by mutant SV40tsTNG-1 under nonpermissive conditions. Since infectious DNA, but not infectious virus, was made, it seemed possible that the processing of the DNA might be abnormal. Therefore, the properties of the labeled DNA in the Hirt extracts were examined further.

Chosen for study was a period in infection when the rate of SV40 DNA synthesis was rapidly increasing and when maturation of infectious particles would normally be occurring. Replicate cultures of CV-1 cells were infected for 26 hr at

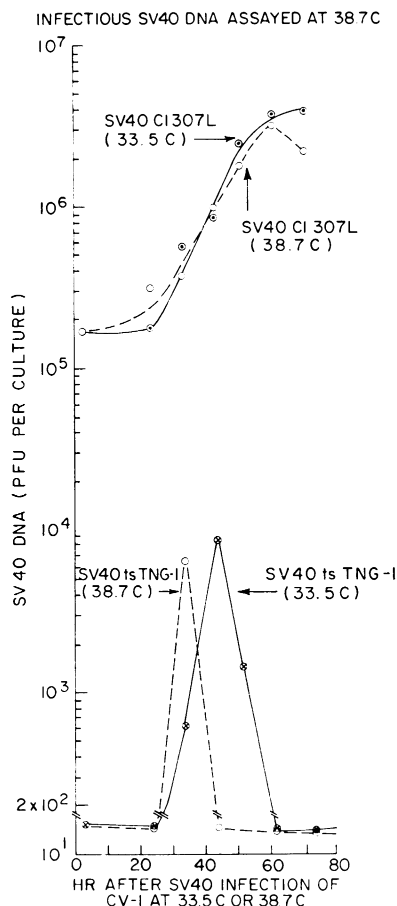

FIG 4. *Synthesis of infectious DNA in CV-1 cells infected with parental SV40 clone 307L or SV40tsTNG-1 at 33.5 or 38.7 C. DNA extracts were prepared from infected cells at the indicated times and assayed at 38.7 C on CV-1 monolayer cultures.*

33.5 or 38.7 C. The cultures were then labeled for 4 hr with ^{3}H-thymidine. The medium was changed and cycloheximide (25 μg/ml) was added to half of the cultures to inhibit protein synthesis. It was anticipated that cycloheximide would stop capsid protein formation in treated cultures, at the same time reducing further SV40 DNA synthesis (16). If abnormal SV40 DNA were synthesized at nonpermissive temperatures in mutant-infected cultures, the altered properties might be accentuated when capsid protein synthesis was shut off. Incubation was continued for a 5-hr chase period in the absence of ^{3}H-thymidine. The low-molecular-weight DNA was then extracted from the cells by the Hirt procedure (8). About 1.3 to 1.8 times as much DNA was labeled by the 26- to 30-hr pulse and 5-hr chase at 38.7 C, as at 33.5 C. The cyclo-

heximide treatment, however, did not significantly change the total amount of radioactivity in the Hirt supernatant fractions.

The radioactive DNA extracts were fractionated by equilibrium centrifugation in cesium chloride density gradients containing ethidium bromide. Figure 5 shows the properties of extracts from cells infected with mutant SV40tsTNG-1. Similar results were obtained with extracts from cells infected with parental SV40 clone 307L. "Heavy" and "light" fractions, respectively, represent superhelical DNA and nicked-circular plus linear DNA. Of the total ^{3}H-DNA, 77 to 89% was found in the heavy density peak (superhelical DNA). This distribution of radioactivity in DNA was found in cultures incubated at 33.5 and 38.7 C, with or without cycloheximide addition during the 5-hr chase period.

The sizes of the DNA species in the heavy and light peaks were next analyzed by velocity sedimentation in neutral 5 to 30% sucrose gradients. This was done to determine whether oligomeric circular forms of SV40 DNA were synthesized by mutant SV40tsTNG-1. Analyses of the heavy fractions (fractions 14 to 19 of Fig. 5) obtained by dye-buoyant density centrifugation showed that this ^{3}H-DNA sedimented with the same velocity as an SV40 form I marker DNA (21*S*). Thus, the form I DNA synthesized at 38.7 or 33.5 C by SV40tsTNG-1 consisted almost entirely of monomeric circles. The same result was obtained with parental SV40 DNA.

When the DNA from the light peaks shown in Fig. 5 was centrifuged in neutral sucrose gradients, two radioactivity peaks were observed (Fig. 6). The slow peak sedimented at about 16*S*. The faster peak was heterogeneous and had an average sedimentation coefficient of about 36*S*. The faster (36*S*) peak, but not the 16*S* peak, was also obtained when DNA from Hirt extracts of uninfected CV-1 cells was centrifuged in sucrose gradients (Fig. 6d). Both peaks were found in DNA samples obtained from cells infected at 38.7 or 33.5 C by either SV40tsTNG-1 or parental SV40 clone 307L. The radioactivity of the faster

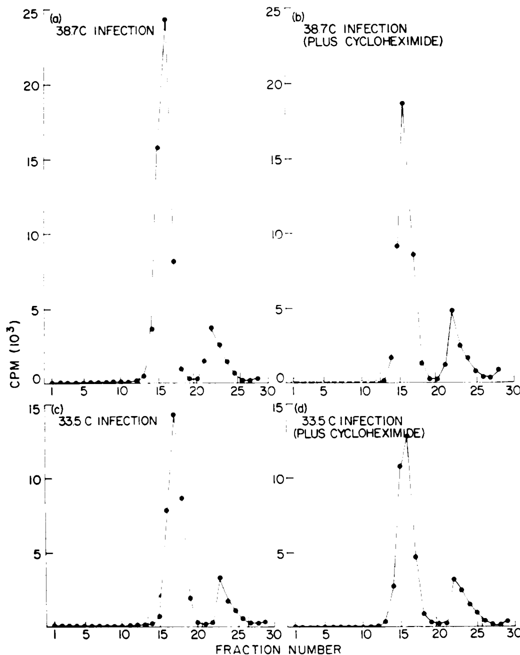

FIG 5. *Radioactivity distribution after dye-buoyant density gradient centrifugation of sodium dodecyl sulfate-extracted DNA. Replicate cultures of CV-1 cells were infected with mutant SV40tsTNG-1 at either 38.7 C (Fig. 5a, 5b) or 33.5 C (Fig. 5c, 5d) for 26 hr, then incubated with ^{3}H-thymidine (5 μCi and 0.5 μg/ml) for 4 hr. The media were removed and fresh media were added. Cycloheximide (25 μg/ml) was added to half of the cultures (Fig. 5b, 5d), and incubation was continued for 5 hr at 38.7 or 33.5 C. Low-molecular-weight DNA was extracted by the method of Hirt (8). Samples were centrifuged at 20 C in CsCl-EtBr. Twenty-drop fractions were collected and 20-μliter portions were assayed for radioactivity.*

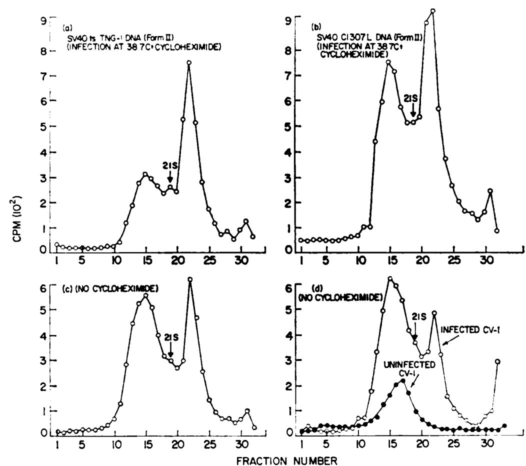

FIG. 6. *Sedimentation velocity analysis in 5 to 30% (w/v) neutral sucrose gradients of nicked circular and linear DNA from uninfected and SV40-infected CV-1 cells. Fractions 20 to 26 of the "light" DNA fraction purified by CsCl-EtBr equilibrium centrifugation (see Fig. 5) were centrifuged. The distributions of radioactivity of fractions from CV-1 cells infected at 38.7 C with mutant SV40tsTNG-1 are shown in Fig. 6a and 6c; radioactivity distribution of cells infected with parental SV40 clone 307L is shown in Fig. 6b and 6d. The radioactivity distribution of low-molecular-weight DNA from uninfected CV-1 cells is also shown in Fig. 6d. The arrow marks the position of marker SV40 form I DNA (21S).*

peak relative to the slower peak was reduced when infected cultures were treated with cycloheximide at 30 to 35 hr after infection. Possibly, the addition of cycloheximide for 5 hr caused some of the 36*S* DNA to be degraded to smaller pieces.

The light DNA fractions (Fig. 5) that had been fractionated by sucrose gradient centrifugation were also assayed on CV-1 monolayers at 33.5 C. The peaks of infectivity, representing 73.2% of the total infectivity, were found in fractions corresponding to the 16*S* peaks (Fig. 6). Another 20.5% of the infectivity was found in the intermediate fractions (about 21*S*) and may have been part of the leading front of the 16*S* peak or replicative intermediates. Few plaques were found in the fractions from the fast peak. These results suggest that the DNA in the fast (36*S*) peak from the light density DNA was mostly cellular and that the DNA from the slow (16*S*) peak contained minimally nicked forms of SV40 DNA.

Formation of V antigen. The formation of V antigen by SV40tsTNG-1-infected cells at the permissive and nonpermissive temperatures was demonstrated by direct immunofluorescence by using SV40-immune green monkey serum conjugated with fluorescein isothiocyanate. Under the conditions used, the antiviral monkey serum failed to stain SV40-transformed mouse cells containing SV40 T antigen. Cover slips of CV-1 cells infected with mutant SV40tsTNG-1 were incubated at either 33.5 or 38.7 C for 2 to 72 hr. At a multiplicity of 50 PFU/cell, essentially all of the cells were positive for both T and V antigen at 72 hr PI at 33.5 C, and at 48 hr PI at 38.7 C. At multiplicities of 5.6, 1.3, and 0.3 PFU/cell, only a fraction of the cells was positive for T antigen at either 33.5 or 38.7 C, but approximately the same proportion of cells at either temperature was positive for V antigen. The staining for V antigen could not be diminished or abolished by pretreating the SV40tsTNG-1-infected cells for 1 hr with unconjugated SV40 hamster tumor serum.

Ara-C has been shown to inhibit the synthesis of SV40 DNA (13) and SV40 coat protein antigen, but not the synthesis of SV40 T antigen (20). To substantiate the fact that SV40 V antigen is synthesized in SV40tsTNG-1-infected cells at the nonpermissive temperature, the effect of ara-C on the induction of SV40 T and V antigen was studied at 33.5 and 38.7 C. CV-1 cells on cover slips were infected with SV40tsTNG-1 at a multiplicity of about one PFU/cell. After 1 hr of adsorption at 36.5 C, medium either with or without 15 μg of ara-C per ml was added. Then the cultures were incubated at either 33.5 or 38.7 C. At the times indicated in Table 1, cells on cover slips were fixed in acetone and used for IF. The results

TABLE 1. *Induction of T and V antigen synthesis in CV-1 cells infected with mutant SV40tsTNG-1 at 33.5 and 38.7 C*[a]

Times post-infection	Incubation temp	Per cent of cells[b] positive for			
		T antigen		V antigen	
		No ara-C	With ara-C	No ara-C	With ara-C
hr	*C*				
2	33.5	0		0	
20		0.4	0.9	0	0
28		4.2	7.0	0	0
48		12.6	16.7	5.6	0.1[c]
72		15.6	11.4	13.3	0.05[c]
2	38.7	0		0	
20		8.5	10.9	1.6	0.08[c]
28		19.9	17.4	3.4	0.05[c]
48		24.7	13.8	15.9	0.1[c]
72		18.7	11.4	24.8	0.09[c]

[a] Petri dishes of CV-1-containing cover slips were infected at a multiplicity of about 1 PFU/cell with SV40tsTNG-1. Virus was allowed to adsorb for 1 hr at 36.5 C. Medium (5 ml) either with or without ara-C (15 μg/ml) was added, and dishes were incubated for indicated times at 33.5 or 38.7 C.

[b] At least 500 cells were scored for each determination.

[c] Very faint staining.

(Table 1) indicate that (i) V antigen is synthesized at either 33.5 or 38.7 C in the absence of ara-C, but is not synthesized at either temperature in the presence of ara-C; (ii) T antigen is synthesized at both temperatures whether ara-C is present or not; (iii) the per cent of T antigen-positive cells increases between 20 and 72 hr PI at 33.5 C and between 20 and 48 hr PI at 38.7 C; (iv) T antigen-positive cells are more prevalent than V antigen-positive cells at 20 to 28 hr PI; and (v) the per cent of V antigen-positive cells at 72 hr PI equals the per cent of T antigen-positive cells at 48 hr PI.

Induction of cellular DNA synthesis. In primary mouse kidney cultures infected with parental SV40, little or no viral DNA replication takes place. Nevertheless, cellular DNA synthesis is enhanced (17). To determine whether mutant SV40tsTNG-1 also induced cellular DNA synthesis, 5-day-old cultures of primary mouse kidney cells (3.3×10^6 cells per culture) were infected at input multiplicities of 150 PFU/cell with parental and mutant SV40 strains and incubated at 33.5 and 38.7 C. The cultures were pulse labeled with ^{3}H-thymidine from 40 to 44 hr and from 64 to 68 hr PI; the total DNA was extracted and hydrolyzed, and the incorporation of ^{3}H-

thymidine into DNA was determined. After infection by either parental or mutant SV40, the formation of ^{3}H-DNA was stimulated 2.5- to 4.3-fold at 33.5 and at 38.7 C. These results indicate that mutant SV40tsTNG-1 can induce cellular DNA synthesis at the nonpermissive and permissive temperatures.

Transformation of mouse kidney cells by SV40tsTNG-1. Parental SV40 clone 307L is capable of transforming mouse kidney cells in culture (4). To demonstrate that mutant SV40-tsTNG-1 also had this ability, primary cultures of mouse kidney cells were infected with virus, incubated at 36.5 C for 18 hr, and subcultured. The cultures were fed twice weekly for 2 to 3 weeks until transformed colonies developed. Thereafter, transformed cells, mKS(tsTNG-1), were subcultured at 36.5 C at 4 to 7 day intervals as described previously (3, 4). Nine clonal lines of mKS(tsTNG-1) cells were isolated. The clonal lines all contained the SV40 T antigen, but infectious virus was not detected in cell-free extracts.

To determine whether SV40 could be rescued from the clonal mKS(tsTNG-1) cells, they were fused with CV-1 cells in the presence of ultraviolet-irradiated Sendai virus. Cell mixtures were then plated with freshly trypsinized CV-1 cells, overlaid with agar, and further incubated at either 33.5 or 38.7 C [frequency of induction test (3)]. When rescue experiments were performed with mKS-U13, a mouse kidney line transformed by SV40 clone 307L, the same number of infectious centers were produced at the two temperatures. However, SV40 infectious centers were not detected when the rescue experiments with mKS-(tsTNG-1) clones were performed at the nonpermissive temperature of 38.7 C. Nevertheless, SV40 was rescued at the permissive temperature (33.5 C) from seven of nine mKS(tsTNG-1) clones. The frequency of induction varied from 2 to 60 infectious centers per 10^{5} transformed cells in the fusion mixture. Infectious centers appearing at 33.5 C in rescue experiments of each of the seven mKS(tsTNG-1) clones were picked and assayed for SV40 on CV-1 monolayers at the two temperatures. The rescued virus formed plaques at 33.5 C but failed to form plaques at 38.7 C. These results demonstrate that the mKS-(tsTNG-1) cells were indeed transformed by the mutant SV40tsTNG-1 and not by a revertant or contaminating wild-type SV40. Moreover, they provide another example that SV40 rescued from a transformed cell resembles the virus used to initiate the transformation.

DISCUSSION

A temperature-sensitive mutant, SV40tsTNG-1, has been isolated after nitrosoguanidine treatment of SV40-infected CV-1 cultures. The mutant replicates in a normal manner at the permissive temperature of 33.5 C but not at the nonpermissive temperature of 38.7 C. For the induction of the mutant, SV40 DNA synthesis was first synchronized by ara-C treatment of infected cultures. Deoxycytidine was then added to reverse the ara-C block and to initiate DNA replication. At various times after SV40 DNA synthesis had started, the infected cultures were treated for 30 min with nitrosoguanidine. This procedure was based upon the finding that nitrosoguanidine preferentially mutagenizes the replication region of DNA (1). The procedure used here may be useful in inducing sequential mutations at various sites on the SV40 DNA.

In the present study, nitrosoguanidine mutagenesis was used to obtain a conditional lethal mutant defective in a late function. The data that have been presented show that the mutant SV40-tsTNG-1 was capable of replicating its DNA at 38.7 C and that the early functions of inducing T antigen, thymidine kinase, and cellular DNA synthesis were expressed. The DNA synthesized at 38.7 C was fully infectious at 33.5 C but did not plaque well at 38.7 C. The viral DNA synthesized in SV40tsTNG-1-infected cells was studied by various biophysical methods. It was similar to DNA from parental SV40 in its resistance to denaturation at 100 C and in dye-buoyant density centrifugation. The possibility was considered that the processing of the SV40tsTNG-1 DNA might be abnormal at the nonpermissive temperature. However, sucrose sedimentation velocity studies demonstrated that practically all of the form I DNA consisted of monomeric circles (21*S*), and that almost all of the infectivity of the nicked forms sedimented at about 16 to 21*S*.

The DNA studies and the temperature-shift experiments suggest that a late function is defective when SV40tsTNG-1 replicates at 38.7 C. It does not appear, however, that formation of capsid proteins is blocked. The IF studies of viral antigens indicate that antigen(s) (coat protein) reacting with viral antisera is synthesized in cells infected at 38.7 C with SV40tsTNG-1. This suggests that this mutant produces noninfectious particles at the nonpermissive temperature, since antisera made against purified virus generally react well only with particles. Consistent with this interpretation, electron microscopy studies of thin sections of SV40tsTNG-1-infected cells have shown that structures which resemble virus particles are made at both 33.5 and 38.7 C (S. Kit, D. Zarling, and D. R. Dubbs, *unpublished data*). Further experiments are needed to determine the amounts and properties of the individual virion-peptide chains synthesized under nonpermissive

conditions in SV40tsTNG-1-infected cells and to determine whether these proteins are assembled properly at 38.7 C.

Mutant SV40tsTNG-1 transforms mouse kidney cultures at 36.5 C. Heterokaryons of mKS-(tsTNG-1) and CV-1 cells incubated at the permissive temperature (33.5 C) yielded infectious virus, but heterokaryons incubated at the nonpermissive temperature (38.7 C) did not. However, merely incubating mKS(tsTNG-1) cells alone at 33.5 C does not lead to virus production. The mouse cells transformed by the temperature-sensitive SV40 mutant have different properties from mouse cells transformed by temperature-sensitive polyoma mutants. Cells transformed by polyoma mutant Ts-a initiate infectious virus formation when shifted from a nonpermissive to a permissive temperature (24), but cells transformed by SV40-tsTNG-1 do not. The difference arises from the fact that mouse cells are permissive for polyoma but not for SV40 replication. Therefore, in addition to the temperature shift-down, the susceptible monkey cell protoplasm must be supplied for SV40 rescue to occur.

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