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Temperature-Sensitive Mutants of Simian Virus 40: Infection of Permissive Cells

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Ten temperature-sensitive mutants of simian virus 40 have been isolated and characterized in permissive cells. The mutants could be divided into three functional groups and two complementation groups. Seven mutants produced T antigen, infectious viral deoxyribonucleic acid (DNA), and structural viral antigen but predominantly the empty shell type of viral particles. Two mutants produced T antigen and infectious viral DNA, but, although viral structural protein(s) could be detected immunologically, no V antigen or viral particles were found. These two functional groups of mutants did not complement each other. A single mutant was defective in the synthesis of viral DNA, viral structural antigens, and viral particles. T antigen could be detected in infected cells by fluorescent antibody but was reduced by complement fixation assay. This mutant stimulated cell DNA synthesis at the restrictive temperature and complemented the other two functional groups of mutants.

Simian virus 40 (SV40) may cause productive infection of permissive African green monkey kidney (AGMK) cells (36) or transforming infection of restrictive mouse cells (1, 2, 39). Cells from cultures of human or rabbit origin regularly undergo either productive or transforming infection (2, 19, 33-35). Early in productive infection, new antigens (T antigen, U antigen) of unknown function appear in the nuclei of infected cells (11, 20, 26, 27, 30). Soon thereafter, the synthesis of cellular and viral deoxyribonucleic acid (DNA) is stimulated (10, 13, 17, 28, 40). Only after viral DNA synthesis has begun can viral structural protein (V antigen) and particles be detected in infected cells which subsequently degenerate (11, 22, 27). In transforming infection, the production of early viral-induced antigens (3, 20, 26, 30, 31) is followed by stimulation of cellular DNA synthesis, but the synthesis of viral DNA is absent or very limited (10, 14, 17). V antigen and particles usually cannot be detected in the transformed cells (3, 11, 27, 30), some of which attain the characteristics of tumor cells. Evidence suggests that the DNA of the virus is integrated into the DNA of the cell and is responsible for the continued production of early virus-induced antigens in the transformed cells (9, 23, 32). One or more of the viral functions required for replication of the virus in permissive cells may be responsible for initiating and perhaps maintaining the transformation of restrictive cells.

The study of conditional lethal mutants of SV40 should better define the role of each essential viral gene in both productive and transforming infection. This communication describes some of the functional characteristics of 10 temperature-sensitive (*ts*) mutants of SV40 in permissive cells and the results of mixed infection experiments designed to measure complementation between these mutants.

MATERIALS AND METHODS

Cell culture. AGMK cell lines AH (12) or Vero (5), cultivated in Eagle's basal medium (BME) with 2 to 10% fetal calf serum, were used for all experiments. Similar results were obtained in both cell lines.

Virus. The isolation of parental, wild-type clone SV40-W, the method of intracellular mutagenesis by nitrosoguanidine (NTG), and the isolation of *ts* mutants of SV40 have been previously described (38). The isolation of multiple replicas of the same mutant after intracellular mutagenesis was avoided by the isolation of single mutants from any one mutagenized culture. NTG-1 to -12 are the first 12 consecutive mutants obtained by these procedures. Stocks of virus were prepared by inoculating cell cultures with virus aspirated from well-isolated viral plaques. The cultures were incubated in BME with 2% fetal calf serum at 32 C for 2 to 3 weeks, frozen and thawed twice, clarified by centrifugation at 1,200 × *g*, and stored at −60 C.

Infection of cells. Monolayer cultures were inoculated with virus at input multiplicities specified in each experiment. After adsorption for 2 hr at 39 or 41 C, the cells were washed three times with Hanks salt solution to remove unadsorbed virus, and BME with 10% fetal calf serum was added to the cultures. Unless otherwise noted, the cultures were harvested by freezing and thawing after 3 days of incubation at 39 or 41 C (restrictive temperatures) and after 5 to 6 days of incubation at 32 C (permissive temperature).

Temperature control. In all experiments, infected cultures were incubated in water-jacketed CO_2 incubators (National Appliance Co., Portland, Ore.). Strict temperature control at 41 to 41.3 C was necessary. At lower temperatures, leakiness of some of the mutants increased markedly. At higher temperatures, cell monolayers could not be maintained, and the yield of wild-type virus was reduced. It should also be noted that different continuous lines of AGMK cells vary in their heat stability. Monolayers of AH cells, in BME with 10% fetal calf serum, remain intact with little observable change after 3 days of incubation at 41 C, whereas BSC-1 (16) monolayers undergo significant degenerative changes after 1 day at the same temperature.

Assay of infectious virus. Virions were plaque-assayed as previously described (38). Viral DNA was extracted from infected cells by the method of Hirt (15) and assayed for the presence of infectious DNA by the method of McCutchen and Pagano (21).

Preparation of marker SV40 DNA. Marker SV40 DNA was obtained from purified virions. Stocks of virus were grown in the presence of 60 μCi of carrier-free ^{32}P-phosphoric acid per ml. The virus was concentrated in polyethylene glycol and isolated by repeated isopycnic centrifugation in CsCl by the method of Friedmann and Haas (8). DNA was extracted from the virions by phenol saturated with 1 M NaCl, 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.4), and 0.01 M ethylenediaminetetraacetate (EDTA). After two extractions, the aqueous phase was dialyzed overnight at 4 C in 0.15 M NaCl, 0.01 M sodium phosphate (PBS), *p*H 7.4) to remove the residual phenol.

Synthesis of viral and cellular DNA. Monolayer tube cultures were infected with virus stocks diluted 10^{-1} in BME with 2% fetal calf serum, which had been depleted of growth stimulatory factors by preincubation on AH cells for 5 days. At appropriate times, infected cells were exposed to medium containing 5 to 20 μCi of ^{3}H-thymidine per ml of depleted medium prior to extraction of DNA by the method of Hirt (15). To measure viral DNA synthesis, samples (0.1 ml) of supernatant fluid were layered onto 3 ml of neutral CsCl (ρ = 1.50 to 1.52 g/cm^3 in 0.01 M Tris, *p*H 7.4, 0.001 M EDTA) and covered with 1 ml of mineral oil. The samples were centrifuged at 25 C for 4 hr at 35,000 rev per min in a Spinco model SW39L rotor. Fractions (0.1 ml) were collected from the bottom of the polyallomer centrifuge tube, precipitated with 5% trichloroacetic acid in the presence of 20 μg of carrier yeast ribonucleic acid (RNA) per ml, collected on nitrocellulose filters, and assayed for radioactivity in Triton-toluene (25) by liquid scintillation counting. Samples of cellular DNA were prepared from the pellet fraction of the method of Hirt. The pellets were resuspended in PBS and twice extracted with phenol. The aqueous layer of the extract was then dialyzed in PBS. A portion of the pellet DNA was treated with 0.3 M KOH overnight at 37 C, trichloroacetic acid-precipitated, and assayed for incorporation of ^{3}H-thymidine to determine the total quantity of pellet DNA. A second portion of pellet samples was analyzed by neutral CsCl velocity sedimentation as described above to determine what proportion of the total pellet DNA consisted of viral DNA. As previously indicated by Hirt (15), as much as 30% of the viral DNA was occasionally found in the pellet.

FA tests. For immunofluorescence (FA) tests, the presence of SV40 T antigen in infected cells was demonstrated by the indirect technique by using sera from hamsters bearing virus-free transplanted tumors and goat fluorescein-conjugated anti-hamster globulin by the method of Pope and Rowe (26).

CF test. Complement fixation (CF) analysis of sonically treated infected cell material was performed as previously described for T antigen (24). Viral antigen (V antigen) was assayed with antiserum against purified viral particles, and capsid antigens were quantitated with antiserum against sodium dodecyl sulfate- (SDS) or alkaline- (*p*H 10.5) disrupted viral particles. Details of preparation of antisera and their specificities will be presented elsewhere (Ozer and Tegtmeyer, *manuscript in preparation*).

Synthesis of viral particles. The production of viral particles was determined by pulsing infected cells with 10 μCi of ^{3}H-lysine per ml for 2 to 16 hr at 2 days postinfection at 39 or 41 C and 4 days postinfection at 32 C. Viral particles were isolated from the 10,000 $\times$ *g* supernatant fluid of sonically treated cells in a CsCl cushion (ρ = 1.32 g/cm^3) as described by Ozer (*manuscript in preparation*).

The fractions corresponding to intact virions and empty shells were collected separately, dialyzed against 0.01 M sodium phosphate (*p*H 7.2), and analyzed for V antigen by CF and for radiolabeled capsid protein by electrophoresis on SDS acrylamide gels (Ozer, *manuscript in preparation*). Radioactivity was determined by liquid scintillation spectrometry in Triton-toluene.

Complementation. Complementation at 41 C was measured by inoculating mixedly infected tube cultures with 1 plaque-forming unit (PFU) of each of two mutants to be tested per cell. Two singly infected control cultures received the same multiplicity of each mutant. After adsorption, washing, and incubation at 41 C for 3 days, the samples were freeze-thawed and titered at 32 and 39 C. Complementation indexes were measured as the ratio $[(X + Y)_{32\ C} - (X + Y)_{39\ C}]/[(X)_{32\ C} + (Y)_{32\ C}]$ where X and Y are yields of two mutant strains grown at 41 C and assayed at the temperatures indicated in the subscripts.

RESULTS

Plaquing characteristics of parental wild-type and mutant virus. The efficiency of plaque forma-

tion by parental SV40-W in AH cells was similar after 20 days at 32 C and 10 days at 39 C. All of the *ts* mutants exhibited a greater efficiency of plaquing at 32 than at 39 C by a factor of 10^4 or more (Table 1). After prolonged incubation at 39 C, six mutants produced a reduced number of small, poorly defined plaques. The remaining six mutants completely lost the ability to form plaques even when shifted down to 32 C after incubation at 39 C for 8 days, indicating that the virus-cell complexes became irreversibly nonfunctional at the restrictive temperature.

Growth characteristics. Table 2 lists the yields of virus after a single cycle of growth at the permissive and restrictive temperatures after infection at input multiplicities of 10 to 50 PFU/cell. The yields are described as the products of single-growth cycles on the basis of the high input multiplicity of infection, since most of the cells have been shown to be infected after the initial exposure to virus. (FA assay of the number of cells producing T antigen at 24 hr after exposure at 41 C and 48 hr after exposure at 32 C under the same experimental conditions has demonstrated that more than 95% of the cells were actually infected at an input multiplicity of 10 PFU/cell.) Infection of tube cultures by parental SV40-W and mutant viruses produced similar yields of total infectious virions after 6 days at 32 C. In infections with wild-type virus, the yields were approximately equal at 32, 39, and 41 C. Two of the mutants, NTG-5 and 10, produced almost normal yields of virus at 39 and 41 C indicating a high degree of leakiness or temperature sensitivity only in plaque-producing capacity. For this reason, these two mutants were not further characterized. The other mutants produced yields which were 5- to 10,000-fold lower at 39 than at 32 C. The degree of leakiness at 39 C correlated well with the presence or absence of delayed plaque production at 39 C (Tables 1 and 2). At 41 C, the yields of all mutants except NTG-5 and -10 were more than 1,000-fold lower than yields at 32 C. All of the mutants caused extensive cytopathic effect at 41 C at high input multiplicities of infection.

Replication of infectious DNA. The yields of infectious viral DNA extracted from cells after a single-growth cycle at 32, 39, and 41 C are also listed in Table 2. Parental wild-type virus and all mutants except NTG-7 replicated approxi-

TABLE 1. *Plaque characteristics of wild-type and mutant simian virus 40 (SV40) stocks propagated at 32 C*

Virus	Efficiency of plating[a] 39 C/32 C	Time of plaque appearance in days	
		32 C	39 C
SV40-W	1.2	20 (I)[b]	10 (I)
NTG-1[c]	$<10^{-5}$	20 (I)	No plaques[d]
NTG-2	$<10^{-4}$	20 (I)	17 (M)
NTG-3	$<10^{-4}$	20 (IF)	24 (MF)
NTG-4	$<10^{-4}$	20 (I)	No plaques
NTG-5	$<10^{-4}$	20 (I)	17 (MF)
NTG-6	$<10^{-4}$	20 (LF)	24 (MF)
NTG-7	$<10^{-4}$	20 (I)	17 (MF)
NTG-8	$<10^{-5}$	20 (I)	No plaques
NTG-9	$<10^{-5}$	20 (I)	No plaques
NTG-10	$<10^{-4}$	20 (I)	24 (MF)
NTG-11	$<10^{-5}$	20 (I)	No plaques
NTG-12	$<10^{-5}$	20 (I)	No plaques

[a] Number of plaques in AH cell monolayers after 10 days at 39 C per number of plaques after 20 days at 32 C.

[b] Plaque morphology. M, minute; I, intermediate size; L, large; F, fuzzy.

[c] Nitrosoguanidine.

[d] No plaques after 24 days.

TABLE 2. *Yield of virions and of infectious DNA from one cycle of growth at permissive and restrictive temperatures after infection of AH cells by virions at input multiplicities of 10 to 50 plaque-forming units (PFU) per cell*

Virus[a]	MOI[b]	Yield $\log_{10}$ PFU/ml					
		Virion[c]			DNA[d]		
		32 C	39 C	41 C	32 C	39 C	41 C
SV40-W	50	8.2	8.4	8.0	5.5	5.3	5.3
NTG-1	50	8.0	4.6	ND[e]	5.3	5.7	ND
NTG-2	50	8.4	7.3	4.6	5.6	5.3	5.0
NTG-3	10	7.1	6.1	3.6	5.2	5.5	5.3
NTG-4	50	8.3	4.8	ND	5.4	5.5	5.3
NTG-5	10	7.8	7.4	6.9	5.0	4.9	4.2
NTG-6	10	8.0	6.3	4.6	5.0	5.4	4.9
NTG-7	10	7.8	7.1	4.4	5.6	4.8	2.1
NTG-8	10	7.5	4.0	ND	5.0	5.1	5.0
NTG-9	10	7.6	ND	4.6	5.3	ND	5.2
NTG-10	10	7.0	ND	7.2	5.3	ND	4.8
NTG-11	10	7.3	3.9	3.4	5.3	5.3	5.2
NTG-12	10	7.3	ND	4.3	5.6	ND	5.7

[a] SV40, simian virus 40; NTG, nitrosoguanidine.

[b] Multiplicity of infection.

[c] Infected cultures were harvested after 6 days of incubation at 32 C and after 3 days of incubation at 39 and 41 C and assayed for total virus production.

[d] Infected cultures were harvested after 4 days of incubation at 32 C and after 2 days of incubation at 39 and 41 C and assayed for the production of intracellular infectious DNA.

[e] Not done.

mately the same number of infectious DNA molecules at each temperature. Production of infectious DNA by NTG-7 was more than 1,000-fold lower at 41 than at 32 C.

Production of virus-sized DNA and induction of cellular DNA synthesis. Since NTG-7 replicated very little infectious viral DNA, experiments were carried out to determine whether this mutant induced the synthesis of noninfectious, virus-sized DNA. Infected cells were labeled with ^{3}H-thymidine at various times after infection. Supernatant fluid fractions from Hirt extracts of the cells infected by NTG-7 at 41 C were analyzed by velocity sedimentation in neutral CsCl gradients. No incorporation of isotope into viral DNA could be detected, either after labeling with 20 μCi of ^{3}H-thymidine per ml during a period corresponding to rapid wild-type DNA synthesis (Fig. 1) or after labeling with 5 μCi of ^{3}H-thymidine per ml for successive 24-hr periods during the 4 days after infection (Fig. 2). In parallel experiments at 32 C, NTG-7 produced approximately the same quantity of viral DNA as wild-type virus.

The Hirt pellet fractions from the same experiments were processed as described above, and cellular DNA synthesis was determined by measuring the total ^{3}H-thymidine incorporation into trichloroacetic acid-precipitable material with appropriate correction for the presence of virus-sized DNA. NTG-7 induced cellular DNA synthesis at 41 C at approximately the same rate as wild-type virus (Fig. 2).

Synthesis of T antigen. For FA assay, AH cells

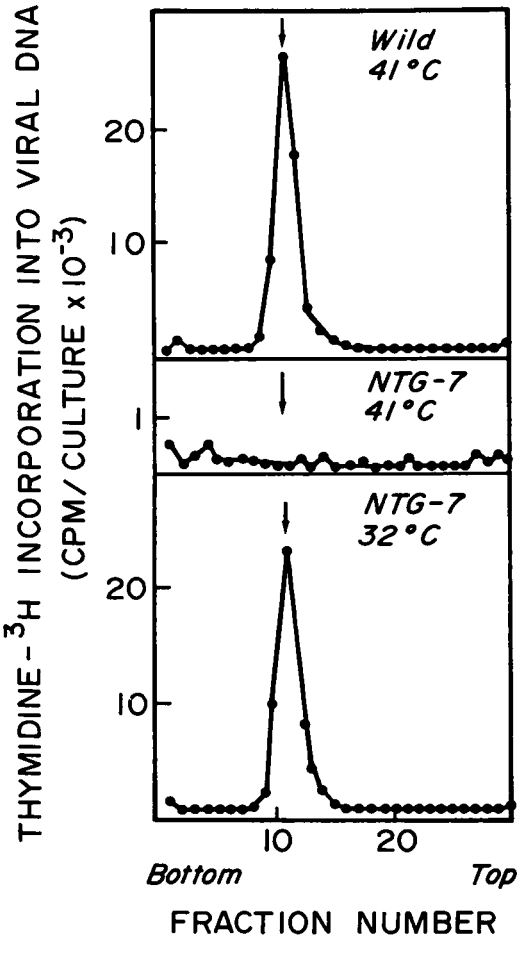

FIG. 1. *Velocity sedimentation analysis of the synthesis of virus-sized DNA in cells infected by wild-type virus and NTG-7 at an input multiplicity of 10 plaque-forming units/cell. Infected tube cultures were labeled with 20 μCi of ^{3}H-thymidine per ml from 45 to 48 hr postinfection at 41 C and from 66 to 72 hr postinfection at 32 C. At the end of the labeling period, viral DNA was selectively extracted by the method of Hirt, mixed with ^{32}P-marker DNA, and centrifuged through a neutral CsCl gradient as described in Materials and Methods. Arrow indicates the position of ^{32}P-marker DNA obtained from purified viral particles.*

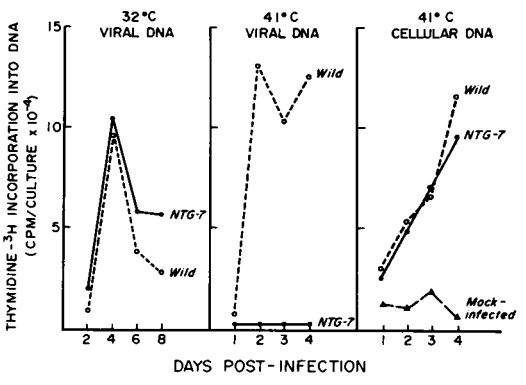

FIG. 2. *Synthesis of viral and cellular DNA in cells infected by wild-type virus and NTG-7 at an input multiplicity of 10 plaque-forming units/cell. Monolayer tube cultures were infected with one of each of the virus stocks diluted 10^{-1} in depleted medium or were inoculated with depleted medium alone (mock infected). The cultures were labeled with 5 μCi of ^{3}H-thymidine per ml during four, successive, 48-hr periods at 32 C and during four successive 24-hr periods at 41 C at the indicated times. At the end of the labeling periods, viral and cellular DNA were extracted by the method of Hirt. Supernatant fluid fractions were sedimented through neutral CsCl gradients. The total quantity of ^{3}H-thymidine label present in DNA cosedimenting with ^{32}P-marker DNA was plotted above as viral DNA. The pellet fractions were processed as described in Materials and Methods. The total ^{3}H-thymidine label in the pellet fraction with appropriate correction for the presence of virus-sized DNA in each sample is plotted above as cellular DNA.*

growing on cover slips were inoculated at a multiplicity of 0.5 PFU/cell. Assay of the number of cells producing T antigen was performed at 24 hr after infection at 39 or 41 C and 48 hr after infection at 32 C. Wild-type virus and all of the mutants induced the production of T antigen in approximately the same number of cells at both restrictive and permissive temperatures. No attempt was made to quantitate the intensity of fluorescent staining. For CF assay, monolayers of Vero cells growing in 32-oz prescription bottles were inoculated at a multiplicity of 1 to 10 PFU/cell. After 3 days of incubation at 39 or 41 C and 5 days of incubation at 32 C, the infected cells were scraped off the glass, pelleted at 1,200 × *g*, resuspended in 1 ml of PBS, and after sonic treatment assayed for T antigen by CF (Table 3). All of the mutants produced antigen at the restrictive temperature, although cells infected by NTG-7 contained proportionally less at 41 C in a number of experiments.

Synthesis of V antigen and viral particles. Samples prepared for CF analysis of T antigen were also assayed for V antigen with antiserum prepared against purified viral particles. This antiserum has been shown to react with both viral particles and isolated capsomeres (Ozer and Tegtmeyer, *manuscript in preparation*). Three groups of mutants were found. The first group (NTG-1, -3, -4, -6, -8, -9, -12) produced a high titer of V antigen in cell lysates at 41 C. In all cases, 85 to 95% of the V antigen sedimented after centrifugation at 100,000 × *g* for 1 hr, as observed for V antigen in cells infected by wild-type virus. When sonically treated extracts of cells infected by wild-type virus were analyzed in CsCl cushions, visible bands corresponding to both intact virions and empty shells were present; however, only a band corresponding to empty particles was observed in cushions of cells infected by the first group of mutants. Lysates of cells infected by wild-type virus and by NTG-1 and NTG-8 were further analyzed after collection of the regions of the cushions containing particles (Table 4). In cells infected by wild-type virus and labeled with ^{3}H-lysine, high titers of V antigen were observed in the regions of both types of viral particles. Analysis of these fractions by SDS polyacrylamide gel electrophoresis confirmed that radioactive lysine had been incorporated into the major capsid protein (molecular weight 45,000). On the other hand, at the restrictive temperature, both NTG-1 and NTG-8 produced appreciably less V antigen at the position of intact virions than at the position of empty shells. This result was also confirmed by experiments in which the amount of ^{3}H-lysine-labeled major capsid protein in the same gradient fractions was determined.

Cells infected by the second group of mutants (NTG-2, -11) at 41 C contained very little or no detectable V antigen when tested with antisera prepared against purified virus; however, these

TABLE 3. *Complement fixation analysis of antigens produced in Vero cells infected by wild-type and mutant virus at an input multiplicity of 1 to 10 plaque-forming units (PFU) per cell*[a]

Expt	Virus[b]	Temp (C)	Yield $\log_{10}$ PFU/ml	CFU titer[c]		
				T	V[d]	Capsid[e]
I	SV40-W	32	7.5	64	32	
		39	7.6	64	64	
	NTG-1	32	7.7	64	128	
		39	4.2	128	128	
II	SV40-W	32	6.2	NT[f]	64	
		41	6.3	32	64	
	NTG-2	32	6.5	>16	128	
		41	2.9	>16	2	16
	NTG-3	32	5.5	NT	128	
		41	2.0	32	32	
	NTG-4	32	6.5	NT	128	
		41	3.0	32	32	
	NTG-6	32	5.5	NT	128	
		41	2.3	64	64	
	NTG-8	32	6.3	NT	64	
		41	2.9	64	32	
III	SV40-W	32	6.6	NT	64	
		41	6.7	16	64	
	NTG-7	32	7.0	NT	64	
		41	3.5	4	<2	<2
	NTG-9	32	6.6	128	32	
		41	4.3	32	32	
	NTG-11	32	6.3	128	64	
		41	2.5	32	<2	16
	NTG-12	32	6.3	128	64	
		41	3.3	32	32	

[a] Infected cultures were harvested after 5 days of incubation at 32 C and after 3 days of incubation at 39 and 41 C.

[b] SV40, simian virus 40; NTG, nitrosoguanidine.

[c] T, V, and capsid antigens were assayed by complement fixation test. The titers are expressed as the reciprocal of the highest dilution giving 3+ fixation.

[d] Antiserum prepared against untreated, purified virions reacts with viral particles and capsomeres.

[e] Antiserum prepared against purified virions treated with sodium dodecyl sulfate or alkaline conditions reacts with nontreated particles as well as the products of treated particles. It does not react with capsomeres.

[f] Not tested.

TABLE 4. *Particle formation in Vero cells infected with wild-type and mutant virus at an input multiplicity of 1 to 10 plaque-forming units (PFU) per cell at 41 C*

Virus[a]	Intact virions		Empty shells	
	CFU[b]	Counts[c]	CFU	Counts
Expt I[d]				
SV40-W	256	10,000	256	4,500
NTG-1	32	850	256	1,550
NTG-2	2	65	2	230
NTG-7	<2	100	<2	370
Uninfected	<2	10	<2	100
Expt II[e]				
SV40-W	256	28,000	256	21,000
NTG-7	<2	220	<2	1,100
NTG-8	16	10,500	128	75,000
Uninfected	<2	100	<2	1,500

[a] SV40, simian virus 40; NTG, nitrosoguanidine.
[b] Complement-fixing units. V-antigen titer per 25 lambda fraction.
[c] Counts per minute in the viral particle fractions of CsCl cushions migrating as the major capsid protein on sodium dodecyl sulfate-acrylamide analysis (normalized per milligram of total cell protein analyzed).
[d] Cells were pulsed with ^{3}H-lysine from 48 to 50 hr postinfection, followed by addition of a 1,000-fold excess of unlabeled lysine for 16 hr.
[e] Cells were labeled with ^{3}H-lysine from 48 to 64 hr postinfection.

infected cells contained capsid antigens when tested with antisera prepared against purified virions treated with SDS or alkali (Table 3). Most of this antigen was not sedimentable at 100,000 × *g*. Studies with ^{3}H-lysine-labeled, NTG-2-infected cells showed correspondingly negligible radioactive capsid protein in the CsCl cushion (Table 4).

The third group of mutants was represented by NTG-7. Although reduced quantities of T antigen were produced in infected cells at 41 C, no V antigen or capsid antigens were detectable by CF assay. In addition, negligible radioactive capsid protein counts were found in CsCl cushions prepared with lysates of ^{3}H-lysine-labeled infected cells.

Heat inactivation. Virions of wild-type and mutant virus produced at 32 C were tested for thermal stability at 41 C to determine the effect of heat lability on the results of experiments carried out at the restrictive temperature. Virus stocks in BME with 10% fetal calf serum were incubated at 41 C for 3 days and titered. Wild-type and mutant virus stocks were not significantly inactivated under these conditions except for NTG-8 which decreased almost 100-fold in titer, indicating that heat lability of virion particles was not the cause of the *ts* character.

Temperature-shift experiments. The time of expression of the *ts* defect was investigated by incubating cells infected by a representative of each mutant group at the permissive temperature for varying periods of time and subsequently shifting the infected cells to the restrictive temperature for completion of the growth cycle (Fig. 3).

The critical period of the growth cycle for each mutant at 32 C was assumed to begin when virus production was no longer completely inhibited by a shift to the restrictive temperature and to end when further incubation at 32 C no longer significantly increased the yield of virus after a subsequent shift-up. The critical period began between 24 and 36 hr in cells infected by NTG-7 (group III) and between 36 and 48 hr in cells infected by NTG-4 (group I) and NTG-11 (group II) and ended between 96 and 120 hr for each of the three representative mutants. At 32 C without shift-up, single-cycle growth curves of the mutants and wild-type virus were similar.

Complementation studies. The yield from mixed

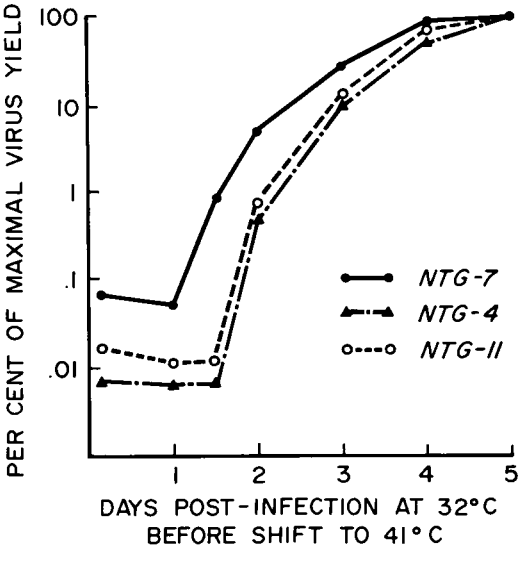

FIG. 3. *Virus production by wild-type and mutant virus at the restrictive temperature after an initial incubation at the permissive temperature. A series of monolayer cultures infected at an input multiplicity of 10 plaque-forming units/cell were incubated at 32 C. At the indicated intervals, the cultures were shifted to 41 C. At 5 days after inoculation, all monolayers were frozen and thawed and titered for virus yield by plaque assay.*

infection by two mutants was compared to the sum of the yields of infection by each mutant alone at 41 C at input multiplicities of 1 PFU/cell. FA assay of the number of cells producing T antigen at 24 hr after exposure to 1 PFU/cell under the same experimental conditions indicated that approximately 25% of the cells were actually infected with one or more viruses. Assuming no barrier to double infection, 6% of the total cells would be expected to be mixedly infected in the complementation tests. All yields produced at 41 C were titered at 32 C. In addition, the mixed infection yields were assayed at 39 C to correct for increases in yield secondary to reversion or recombination, which were not found to be significant at 41 C. NTG-7 complemented all of the other mutants by a factor of 100- to 620-fold. The yields of mixed infection varied from 2 to 10% of the yield of wild-type infection at 41 C. None of the other groups of mutants complemented each other, and none of the NTG mutants complemented a *ts* mutant (*hts-1*) isolated by Robb and Martin (29). No significant inhibition of the production of wild-type virus was observed in any of the mixed infections at input multiplicities of 50 PFU/cell. Representative results are presented in Table 5.

Infectivity of mutant DNA. NTG-7 and NTG-11 both were found to induce production of T antigen at 41 C by FA assay, suggesting that the infecting virions were transported into cells and at least partially uncoated under restrictive conditions. However, since both mutants produced no detectable V antigen or viral particles, it seemed possible that the genome of the infecting virion was not completely expressed because the virion DNA was not completely uncoated at 41 C. AH cells were therefore infected with purified viral DNA, and the yield from a single-growth cycle was assayed to determine whether incomplete uncoating of the virions was responsible for the defective function at 41 C. Infection with viral DNA of both mutants was found to be blocked at the restrictive temperature as with intact virions (Table 6), indicating that the *ts* step remained defective subsequent to complete uncoating of the viral genome.

TABLE 5. *Complementation by mutants at the restrictive temperature after infection of AH cells at an input multiplicity of 1 plaque-forming unit (PFU) per cell*[a]

Virus[b]	41 C yield $\log_{10}$ PFU/ml		Complementation index[c]
	Assayed at 32 C	Assayed at 39 C	
Expt I			
NTG-7	3.0		
SV40-W	7.0		
NTG-8	3.2		
NTG-11	2.8		
NTG-7 × SV40-W	7.0	7.2	<1
NTG-7 × NTG-8	5.5	<4.0	120
NTG-7 × NTG-11	6.0	<4.0	620
Expt II			
NTG-11	2.3		
SV40-W	6.1		
NTG-1	2.0		
NTG-8	2.2		
NTG-11 × SV40-W	6.0	6.2	<1
NTG-11 × NTG-8	2.7	<1.0	1.2
NTG-11 × NTG-1	2.0	<1.0	<1

[a] Infected cultures were harvested after 3 days of incubation at 41 C and were plaque-assayed at 32 and 39 C.

[b] NTG, nitrosoguanidine; SV40, simian virus 40.

[c] As described in Materials and Methods.

TABLE 6. *Yield of virions from one cycle of growth at permissive and restrictive temperatures after infection of AH cells by extracted viral DNA*[a] *at an input multiplicity of 0.1 to 0.5 (PFU) plaque-forming unit per cell*[b]

Viral DNA[c]	Yield $\log_{10}$ PFU/ml	
	32 C	41 C
SV40-W with DEAE-D	6.5	6.4
Without DEAE-D	<2.0	
NTG-7 with DEAE-D	6.5	3.2
Without DEAE-D	<2.0	
NTG-11 with DEAE-D	5.8	2.1
Without DEAE-D	<2.0	

[a] Viral DNA was extracted from purified virions as described in Materials and Methods [preparation of marker simian virus 40 (SV40) DNA].

[b] Infected cultures were harvested at 5 days after inoculation at 32 C and 3 days after inoculation at 41 C.

[c] Abbreviations: NTG, nitrosoguanidine; with DEAE-D, viral DNA diluted in phosphate-buffered saline (PBS) containing 1 mg of diethylaminoethyl dextran DEAE (D) per ml (molecular weight 2×10^6); without DEAE-D, viral DNA diluted in PBS without DEAE-D.

DISCUSSION

The studies reported here indicate that the genetic analysis of SV40 functions is feasible by using conditional lethal mutants. Twelve *ts* mutants of SV40 have been isolated; 10 of these were found to be suitable for further characterization. The mutants can be divided into three groups on the basis of their functional defects (Table 7) and into two groups by complementation tests.

Group I mutants produce T antigen, infectious viral DNA, V antigen, and empty shells, but reduced infectious virions and isolatable intact virions. Temperature-shift experiments suggest that a late function in viral synthesis is affected. The heat lability of one member of this group indicates that a structural protein is altered. These findings are consistent with other studies suggesting that structural viral protein is produced late in infection after the onset of viral DNA synthesis (11, 27). This group of mutants is functionally very similar to previously described *ts* mutants of SV40 (18, 24, 37).

In infection by the second group of mutants, T antigen and infectious viral DNA are produced, but no V antigen and no physical particles can be detected. Antisera produced against purified virions which had been treated with SDS or alkaline *p*H do react to a limited extent with lysates of cells infected by these mutants, suggesting that unassembled and possibly altered capsid proteins are produced in infected cells. Temperature-shift experiments indicate that a late function is defective.

No complementation between functional groups I and II has been detected, although unambiguous complementation was observed between representatives of both groups with functional group III under the same experimental conditions. Other conditions for complementation tests, including increased multiplicity of infection, must be employed to rigorously rule out complementation between groups I and II and between members of each group. If the defects observed in these two mutant groups are secondary to alterations in the same structural protein, markedly different physiological manifestations have been observed. Members of group I have been shown to synthesize empty shells, whereas mutants of group II are unable to produce temperature-stable capsomeres at the restrictive temperature (Ozer and Tegtmeyer, *manuscript in preparation*). Although mutants of groups I and II produce infectious DNA at 41 C, the synthesis of abnormal forms of DNA and the altered processing of viral DNA have not yet been excluded.

Group III, represented by NTG-7, is defective in the synthesis of viral DNA at the restrictive temperature, providing direct evidence that a viral function is required for the replication of SV40 DNA. A similar functional defect can be demonstrated when extracted mutant DNA is used to infect cells, indicating that the defect is still present after the virus has been completely uncoated. The finding that T antigen is present by FA assay but reduced in CF assay requires further clarification. Additional studies of defective viral DNA replication at the restrictive temperature may provide evidence indicating which specific function is affected. It is significant that NTG-7 stimulates cell DNA synthesis to the same extent that wild-type virus does. On the basis of present data, NTG-7 is similar in its *ts* function to the TS-a (6, 7) and HA-25 (4) mutants of polyoma virus. Studies on transforming infection by NTG-7 are in progress.

TABLE 7. *Functional defects of simian virus 40 temperature-sensitive (ts) mutants at 41 C*[a]

Virus group[b]	T antigen	Viral DNA	Capsid antigen[c]	V antigen[d]	Viral particles	Time of *ts*-defect[e]
I. NTG-1, -3, -4, -6, -8, -9, -12	+	+	ND	+	Empty shells	Late
II. NTG-2, -11	+	+	+	−	−	Late
III. NTG-7	±	−	−	−	−	Early

[a] +, Normal; −, defective.
[b] NTG, nitrosoguanidine.
[c] Capsid antigen, antigen reacting with antiserum prepared against purified virions treated with sodium dodecyl sulfate or alkali.
[d] V antigen, antigen reacting with antiserum prepared against untreated purified virions.
[e] Late, greater than 36 hr; early, less than 36 hr as determined by temperature-shift experiments.

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