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Genetic Analysis of Simian Virus 40

III. Characterization of a Temperature-Sensitive Mutant Blocked at an Early Stage of Productive Infection in Monkey Cells[1]

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A temperature-sensitive mutant of simian virus 40 (SV40), *ts*101*, has been characterized during productive infection in monkey kidney cells. The mutant virion can adsorb to and penetrate the cell normally at the restrictive temperature, but cannot induce the synthesis of cellular deoxyribonucleic acid (DNA) nor initiate the synthesis of SV40-specific tumor, virion, or U antigens or viral DNA. First-cycle infection with purified *ts*101* DNA is normal at the restrictive temperature, but the resulting progeny virions are still temperature-sensitive. The mutant neither complements nor inhibits other temperature-sensitive SV40 mutants or wild-type virions. The affected protein in the *ts*101* mutant may be a regulatory structural protein, possibly a core protein, that is interacting with the viral DNA.

Several functional classes of temperature-sensitive mutants have been described for the papovaviruses, simian virus 40 (SV40) and polyoma virus (6, 7, 9, 22, 30, 38, 39). This paper characterizes a temperature-sensitive SV40 mutant, *ts*101*, which is blocked at an early step during productive infection of monkey cells. [A uniform nomenclature has been proposed for the classification of SV40 mutants (35). The nomenclature used in this paper corresponds to that nomenclature and indicates that the mutant is temperature-sensitive (ts), noncomplementing (*), and has the unique isolation number, 101.] An accompanying paper (34) describes this mutant during abortive infection of Balb/3T3 cells. To the best of our knowledge, no papovavirus mutant of this type has been described previously.

MATERIALS AND METHODS

Cell lines and medium. CV-1 monkey cells (21) were maintained and used for viral assay in microcultures and petri dishes as previously described (33). TC7 cells are a cloned subline derived from CV-1 cells (J. Robb, *manuscript in preparation*). The TC7 cells had no detectable mycoplasma contamination by ^{3}H-uridine incorporation (reference 40; kindly performed by Carl Anderson, Cold Spring Harbor) or by direct culture [kindly performed by Walter James, National Institutes of Health (NIH)].

[1] An abstract of some of this material has been presented previously (J. A. Robb. 1970. J. Cell Biol. 47: part 2, p. 172a).

Virus stocks. The small plaque strain (SV-S) of SV40 (37) was used as wild type (WT). WT, *ts*101*, and the temperature-sensitive mutants *tsA7*, *tsB2*, *tsB8*, and *tsB11* [kindly supplied by P. Tegtmeyer (39) and formerly called NTG-7, NTG-2, NTG-8, and NTG-11, respectively] were grown in TC7 cells at 33 C at a multiplicity of infection (MOI) of 0.005. A summary of their phenotypes is given in Table 1. All virus stocks were free from detectable mycoplasma contamination by direct culture (kindly performed by Walter James, NIH). End-point dilution titration was performed as previously described (33).

Serological reagents. SV40 tumor (T) antigen, virion (V) antigen, and the SV40 U-antigen described by Lewis et al. (25) were assayed by the immunofluorescent technique adapted to microculture as previously described (33). SV40 U antigen-positive, T antigen-negative monkey serum was kindly supplied by A. Lewis (NIH). Fluorescein-conjugated rabbit anti-monkey (rhesus)-globulin globulin was obtained from Sylvana Co., Millburn, N.J. Rabbit anti-SV40 immune serum was obtained from Grand Island Biological Co., Grand Island, N.Y.

Multiplicity-dependent leakage. Microtest plates (Falcon) (containing 60 wells per plate) were inoculated with virus mixed with 10^3 TC7 cells (in 2 μliters of medium with 5% FBS per well) at MOI values of 100, 10, 1.0, 0.1, and 0.01 and were incubated at 40 or 31 C. After 24 hr, 0.01 ml of medium containing 5% FBS and 6% rabbit anti-SV40 serum was placed in each well. The plates were fixed (after 72 hr at 40 C and after 120 hr at 33 C) and stained for T or V antigen.

Preparation of radiolabeled virus. For protein

TABLE 1. *Phenotypes of wild-type and mutant virus at 41 C in TC7 cells*[a]

Virus	Virion infection				DNA infection		
	TFU	Viral DNA[b]	VFU	IU	TFU	VFU	PFU
WT	WT	WT	WT	WT	WT	WT	WT
*ts*101*	1% WT	1% WT[c]	1% WT	0.1% WT	WT	WT	0.1% WT
tsA7	80% WT	<1% WT	<1% WT	0.1% WT	5% WT	<1% WT	ND
tsB2	WT	WT	0	0.1% WT	WT	0	ND
tsB8	WT	WT	WT	0.1% WT	WT	WT	ND
tsB11	WT	WT	0	0.1% WT	WT	0	ND

[a] Abbreviations: TFU, tumor antigen-forming units during first cycle infection; VFU, virion antigen-forming units during first cycle infection; IU, infectious units as derived from end-point dilution titrations; PFU, plaque-forming units as derived from plaquing titrations; WT, wild-type levels; ND, not done.

[b] Ability to synthesize infectious viral deoxyribonucleic acid (DNA) at 41 C after virion infection (assayed by measuring at 33 C the number of TFU and VFU contained in the 41 C Hirt supernatant fluid).

[c] Actual amounts of WT and 101 DNA I were measured (see Fig. 4).

labeling of virus, WT and *101* virions were grown at a MOI of 0.01 for 6 days at 33 C. The infected cells were lysine depleted for 24 hr, and ^{14}C-lysine was added to a final concentration of 1.0 μCi/ml (Amersham/Searle, 350 Ci/μmole). After freezing and sonic treatment (33), Nonidet P-40 was added to a final concentration of 0.1%. The virus was then purified by banding through sucrose on a CsCl cushion as described by Ozer (29) and by rebanding to equilibrium in CsCl. The purified WT and *101* virus had 10^6 counts/min and 10^9 infective units (IU) per ml. For nucleic acid labeling, WT virus was grown with 10 μCi of [*methyl-*^{3}*H*]thymidine per ml (New England Nuclear, 5 mCi/0.0606 mg) and *101* with 2 μCi of [*2-*^{14}*C*]-thymidine per ml (New England Nuclear, 250 μCi/1.12 mg). The virus preparations contained 10^{10} IU of ^{14}C-*101* virus per ml with 13,000 counts per min per ml, and 7×10^8 IU of ^{3}H-WT virus per ml with 250,000 counts per min per ml.

Virus adsorption. (i) Single-cycle T, U, and V antigen synthesis was determined after adsorption at the restrictive temperature and incubation at the permissive temperature. Petri dishes (Falcon; 32-mm) containing confluent TC7 cells (4×10^5 cells per dish) were infected with WT or *101* virions at a MOI of 0.1 in 0.1 ml of medium per dish. The dishes were incubated at 41 or 33 C and agitated every 10 min. After 60 min, all dishes were washed twice with 25 C phosphate-buffered saline (PBS), and 2 ml of medium containing 4% FBS was added. The dishes with virus adsorbed at 41 C were incubated at 41 or 33 C, and those with virus adsorbed at 33 C were also incubated at 41 or 33 C. The cells were fixed and stained for T, U, or V antigen after 48 hr at 41 C and after 96 hr at 33 C.

(ii) The second adsorption assay depended on the measurement of biologically active virus which had become attached to cells during the adsorption period and could be released by freezing and sonic treatment. An 0.10-ml sample of either WT or *101* virions was adsorbed at a MOI of 1 in 32-mm plastic petri dishes containing 6×10^5 confluent TC7 cells. The virions were adsorbed for 45 min at 41 C and for 75 min at 32 C and then rinsed three times with PBS at 27 C. An amount (0.5 ml) of medium with 5% FBS was added to each dish, and all dishes were frozen. After thawing, the cell lysate was scraped, sonically treated, filtered, and titered on TC7 cells at 32 C.

(iii) Adsorption of radiolabeled virions was performed in replicate 32-mm glass petri dishes containing confluent TC7 cells (6×10^5 cells per dish). The dishes were infected with 0.5 ml of lysine-radiolabeled virus per dish (about 55,000 counts per min per dish). The dishes were incubated at 41 C, and the supernatant medium was removed at varying times after virus application. The monolayer was rinsed four times with PBS, these rinses were added to the supernatant medium, and the mixture was treated with 10 ml of cold 10% trichloroacetic acid and filtered through a Whatman GF/C glass-fiber filter (Arthur H. Thomas, Co., Philadelphia, Pa.). The filter was washed with 0.1 N HCl and 95% ethanol and air dried. The cell monolayers were scraped and treated as above. The filters were counted in a Nuclear-Chicago Mark I scintillation system. As controls, petri dishes were rinsed once with medium containing 2% FBS and were carried through the entire procedure.

Penetration of WT and 101 virions at 40 C. Penetration of virions was determined in two ways: (i) by the sensitivity of adsorbed virions to neutralizing antisera, and (ii) by the resistance of adsorbed thymidine-radiolabeled virus to trypsin and deoxyribonuclease treatment. In the first assay, WT or *101* virions were adsorbed at an MOI of 1 in 32-mm plastic petri dishes containing confluent TC7 cells (4×10^5 cells/dish) at 40 C for 45 min with agitation every 10 min. The dishes were rinsed at 40 C with 40 C PBS and received 2 ml (per dish) of 40 C medium containing 5% FBS with or without 6% rabbit anti-SV40 serum. All dishes were incubated for 96 hr at 32 C, and stained for T or V antigen.

In the second penetration assay, thymidine-radiolabeled WT and *101* virus were mixed so that 0.4 ml

contained 2,100 counts/min of ^{3}H-WT and 1,370 counts/min of ^{14}C-*101* virus. Glass petri dishes containing confluent TC7 cells (4×10^6 cells per 32-mm dish) were infected with 0.4-ml fractions of the double-labeled virus preparation. Adsorption was allowed to proceed at 41 or 33 C with samples removed at various times. The plates were rinsed twice with PBS, and the cells were removed with EDTA-trypsin [0.5 g of trypsin/liter, 0.5 mM ethylenediaminetetracetic acid (EDTA)] in Puck's saline A (31). The cell suspension was made with 0.033 M $MgCl_2$, 0.4 mg of soybean trypsin inhibitor per ml (Worthington), and 0.02 mg of deoxyribonuclease I per ml (Worthington), and treated for 20 min at 27 C. Under these conditions, 75% of 1.2 optical density units (at 260 nm) of calf thymus deoxyribonucleic acid (DNA) is rendered perchloric acid-soluble in 2 min. After low-speed centrifugation, the cells were suspended in 0.5 ml of 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride and counted.

Induction of cellular DNA synthesis. Replicate 32-mm plastic petri dishes containing confluent TC7 cells (9×10^5 cells/dish) were prepared. After being rinsed once with PBS, the cultures were infected with 0.1 ml of viral lysate (MOI = 10) or mock TC7 cell lysate. Control dishes were immediately incubated with ^{14}C-thymidine medium at 40 or 33 C (see below). The infected dishes were incubated at 33 C for 90 min with agitation every 10 min. After adsorption, the dishes were rinsed twice with PBS, 2 ml of medium without FBS was added, and incubation was continued for 48 hr at 40 C and for 72 hr at 33 C. Some dishes were fixed and stained for V antigen. All the remaining dishes, after rinsing, were exposed to 1.5 μCi of [^{14}*C-2*]thymidine per ml (43 mCi/mmole; New England Nuclear) in 0.5 ml of medium without serum. The plates were incubated for 90 min at 40 C or for 120 min at 33 C, rinsed twice with PBS, and the DNA was extracted by the method of Hirt (17). The Hirt supernatant fluid was mixed at 4 C with cold 20% trichloroacetic acid to a final concentration of 5%; the precipitates were treated with 4 ml of cold 5% trichloroacetic acid, resuspended by vigorous mixing, and counted.

Viral DNA isolation. Viral DNA was isolated for use in the DNA infection of TC7 cells and for use as an assay for the induction of viral DNA synthesis after virion infection of TC7 cells. Confluent TC7 cells were infected with either WT or *101* virions at an MOI of 10 and incubated at 32 C for 6 days. Viral DNA was fractionated from high-molecular-weight cellular DNA (17). Viral DNA I was purified by CsCl density centrifugation with ethidium bromide by the method of Radloff et al. (32).

Radiolabeled viral DNA was isolated as an assay for the induction of viral DNA synthesis by infecting confluent TC7 cells at 40 C with either WT or *101* virions at an MOI of 15. After 45 min of adsorption at 40 C, the cells were rinsed twice with 40 C PBS, 40 C medium containing 5% FBS was added, and the cells were incubated at 40 or 31 C. Radioactive thymidine (2 μCi of [*2-*^{14}*C*]-thymidine per ml, 59 mCi/mmole, Amersham Searle, Des Plains, Ill.) was added to the 40 C flasks 10 hr after infection and to the 31 C flasks (2 μCi of [*methyl-*^{3}*H*]-thymidine/ml, 5 mCi/mmole, Amersham Searle) 23 hr after infection. The viral DNA was extracted by the method of Hirt (17) 36 hr after infection for the 40 C monolayers and 72 hr for the 31 C monolayers.

Infection of cells with viral DNA. Confluent monolayers of TC7 cells in 32-mm plastic petri dishes were infected with either WT or *101* DNA I by the method of McCutchen and Pagano (28). The DNA dilutions in medium without serum were brought to 400 μg/ml in diethylaminoethyl (DEAE) dextran (2×10^6 molecular weight; Pharmacia), and 0.08-ml samples were used per dish. Dishes were adsorbed for 25 min with 5-min agitations at either 40 or 31 C. After adsorption, the dishes were rinsed twice with 40 C PBS and assayed for single-cycle T and V antigen synthesis, plaque-forming capacity (33), and temperature sensitivity of the progeny virions.

Temperature shift-down experiments. TC7 cells were infected with *101* virions at an MOI of 0.5 using microtest plates and incubated at 40 or 31 C. After 24 hr, 0.01 ml of 40 C medium containing 5% FBS and 6% rabbit anti-SV40 serum was added and incubation resumed. The 31 C plates were fixed at 96 hr. At various times, the 40 C plates were fixed or shifted to 31 C and incubated an additional 96 hr before fixation.

Temperature shift-up experiments. The first experiment concerned the ability of the mutational block in *101* virions to be overcome at 31 C. TC7 cells were mixed with WT or *101* virions at a MOI of 0.2 and plated in Micro Test plates. After 48 hr of incubation at 31 C, 0.01 ml of medium containing 5% FBS with or without 2×10^{-4} M cycloheximide (Sigma) was added, and all plates were shifted to 40 C except for the 31 C controls. At various times, plates were fixed and stained for T, U, and V antigens.

The second experiment was concerned with the role of protein synthesis in overcoming the mutational block at 31 C. The adsorption period in this experiment was only 45 min on confluent monolayers, whereas in the above experiment the virions were mixed in suspension with the cells and left with the cells for the duration of the experiment. Replicate 32-mm petri dishes containing confluent TC7 cells (5×10^5 cells per dish) were infected with 0.10 ml of virus at an MOI of 0.1 for 45 min at 27 C with agitation every 5 min. After adsorption, the dishes were rinsed once with PBS, and medium containing 5% FBS and 6% rabbit anti-SV40 serum with or without 2×10^{-4} M cycloheximide was added to each dish. At various times, the dishes were rinsed twice in PBS and shifted to 40 C or left at 31 C after the addition of medium with 5% FBS and 6% anti-SV40 serum, but without cycloheximide. The total incubation time prior to staining for each dish was equivalent to about 72 hr at 40 C in the absence of cycloheximide.

Complementation assay. Virus stocks were diluted in medium containing 5% FBS with 5×10^5 TC7 cells/ml to twice the desired multiplicity. For single infection, the stocks were mixed 1:1 with medium

containing 5×10^5 TC7 cells/ml. For double infections, two virus stocks were mixed 1:1 with each other. The final virus mixtures were plated in Micro Test plates and incubated for 72 hr at 33 C or for 42 and 58 hr at 41.5 C for T or V antigen staining, respectively. Complementation occurred if the number of antigen-positive nuclei in the double infection was at least threefold greater than the number obtained by adding the values in their single infections.

RESULTS

Temperature dependence and plaque morphology. The ratios between the propagative titers (multiple cycles, IU/ml, and plaque-forming units/ml) of *101* at 31 to 33 C and 40 to 41 C were 10^3 to 10^4 in primary African green monkey kidney (AGMK) cells (kindly performed by K. Takemoto and C. Scher), CV-1, and TC7 cells, whereas the same ratios for WT virions were 1 to 10. A small plaque similar to WT was formed by *101* virions or DNA I on primary AGMK, CV-1, and TC7 cells at 31 C.

Heat stability of 101 virions. Less than 50% inactivation of WT and *101* virions occurred after 2 hr of incubation at 50 C. After 1 hr at 60 C, however, WT and *101* virions had lost 99% of their infectivity. The rates of inactivation were equal. The temperature sensitivity of *101* cannot be explained as resulting from instability of the whole virion at 41 C in contrast to known SV40 capsid mutants (30, 39).

Early location of the 101 block in the replicative cycle. The mutation in *101* produces a block in viral replication at the restrictive temperature at some point prior to the initiation of T antigen synthesis. The data in Table 2 show that first-cycle V and T antigen synthesis were equally inhibited, indicating that the affected function was required for the initiation of both an early function (T antigen) and a late function (V antigen). The data also indicate that *101* exhibits a low multiplicity-dependent leakage. Even with an MOI of 100 (first row, Table 2), only 2 to 3% of the cells infected with *101* virions at the restrictive temperature expressed either viral function.

TABLE 2. *Multiplicity-dependent leakage of 101 virions at 40 and 31 C in TC7 cells*

Multiplicity of infection	Tumor antigen-forming units per well[a]		Virion antigen-forming units per well[a]	
	40 C	31 C	40 C	31 C
100	32[b]	TM[c]	24	TM
10	14	TM	8	TM
1	1.4	112	1.0	100
0.1	0	13	0.2	10
0.01	0	1.1	0	0.9

[a] Cells per well = 10^3.
[b] Standard error of the mean = ±15%.
[c] TM, Too many to count.

Time course of appearance of WT and 101 virion-initiated T and V antigen synthesis. To determine whether any abnormality in the infection at permissive temperature could be detected, the time course of appearance of T and V antigen synthesis at permissive temperature was examined. The results given in Fig. 1 demonstrate that there was no significant difference.

Virion adsorption and initiation of T, U, and V antigen synthesis. A mutant virion unable to adsorb, penetrate, or uncoat normally at the restrictive temperature would manifest the *101* phenotype. Three different techniques were used to evaluate the ability of *101* virions to adsorb at the restrictive temperature. (i) Temperature-shift experiments were carried out assaying the ability of WT and *101* virions to initiate T, U, and V antigen synthesis after adsorption and incubation at permissive and restrictive temperatures. (ii) WT and *101* virions were allowed to adsorb at both temperatures, and the number of biologically active virus adsorbed to the cells was determined. (iii) The rate of radiolabeled virus adsorption was determined for WT and *101* virus at the restrictive temperature. All of these assays indicate that *101* virions adsorb as efficiently as WT virions at the restrictive temperature (Tables 3 and 4; Fig. 2).

Penetration of WT and 101 virions at the restrictive temperature. The penetration of virions into a cell has been defined as the loss of sensitivity of the virions to neutralizing antibody after adsorption of the virions to the cell (4). The data

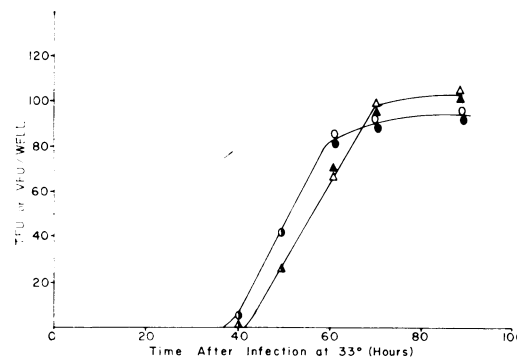

FIG. 1. *Time course of appearance of WT and 101 virion-initiated T and V antigen synthesis at 33 C. TC7 cells were infected in Micro Test plates with WT (●, ▲) or 101 (○, △) virions, incubated at 33 C, and stained for T (●, ○) or V (▲, △) antigen. TFU = T antigen-forming units; VFU = V antigen-forming units.*

TABLE 3. *Virion adsorption and initiation of T, U, and V antigen synthesis after a temperature shift*[a]

Temp virus adsorbed (C)	Temp virus incubated (C)	Antigen-forming units per 32-mm petri dish ($\times 10^{-3}$)								
		Wild type (T^+V^+)			*tsB11* (T^+V^-)			*ts*101* (T^-V^-)		
		T	U	V	T	U	V	T	U	V
41	41	8.6[b]	8.9	9.4	2.6	2.2	0	0.02	0.02	0.03
41	33	13.4	12.8	12.1	3.5	3.1	2.3	12.8	12.1	11.8
33	33	14.1	13.9	13.3	4.6	4.2	3.9	14.5	14.0	13.6
33	41	11.2	11.0	10.8	4.3	4.0	0	0.04	0.03	0.04

[a] T, tumor antigen; V, virion antigen.
[b] Standard error of the mean = ±20%.

TABLE 4. *Adsorption of wild-type and 101 virions onto TC7 cells at 41 and 33 C*

Adsorption temp (C)	Adsorbed virions (IU/ml)[a]			
	Wild type		*101*	
	No cells	Cells	No cells	Cells
33	4.1 $\times 10^{3}$[b]	1.2 $\times 10^{5}$	2.8 $\times 10^{3}$	1.0 $\times 10^{5}$
41	3.7 $\times 10^{3}$	1.7 $\times 10^{5}$	3.2 $\times 10^{3}$	1.3 $\times 10^{5}$

[a] IU, Infectious units.
[b] Standard error of the mean = ±20%.

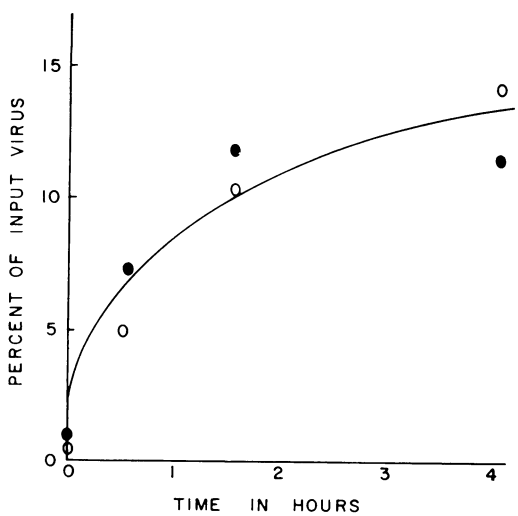

FIG. 2. *Adsorption of WT and 101 virions at 40 C. Radiolabeled WT (●) and 101 (○) virus were adsorbed to TC7 cells at 40 C, and the infected cells were counted for adsorbed virus at the indicated times.*

in Table 5 demonstrate that treatment with SV40 neutralizing serum did lower the number of antigen-positive nuclei, but lowered it equally for both WT and *101* infected cultures. By definition, the *101* virions penetrated the cells normally at the restrictive temperature.

TABLE 5. *Effect of simian virus 40 neutralizing antibody on wild-type and 101 virions after adsorption at 41 C onto TC7 cells*

Antigen	Neutralizing antibody (%)	Antigen-forming units[a]	
		Wild type	*101*
T	0	24,800[b]	23,100
	6	21,300	20,200
V	0	23,300	24,400
	6	20,200	21,700

[a] Per 32-mm petri dish containing 4×10^5 cells.
[b] Standard error of the mean = ±15%.

Penetration can also be assayed by estimating the rate of incorporation of radiolabeled virus into cells. Although a small difference between the rate of penetration of WT and *101* virions is apparent from Fig. 3, we do not believe this difference is significant to understanding the phenotype of *101*. When the radiolabels were reversed, a similar 30% difference in the rate of uptake was noted, but in this case the *101* virions appeared to penetrate more rapidly.

Induction of cellular DNA synthesis. To evaluate the induction of cellular DNA synthesis by *101* virions at the restrictive temperature, confluent monolayers of TC7 cells were mock or viral infected at 40 and 33 C, and the newly synthesized DNA was measured. Table 6 shows that both WT and *101* virions induced comparable levels of both cellular (Hirt precipitate) and viral (Hirt supernatant fraction) DNA synthesis at 33 C. Only WT virions induced cellular and viral DNA synthesis at 40 C. The table also shows that the induction of V antigen synthesis paralleled the induction of cellular and viral DNA synthesis in all cultures. Virion infection with *101* does not induce the synthesis of cellular or viral DNA at the restrictive temperature.

Initiation of viral DNA synthesis. The preceding experiment suggested that very little viral DNA was synthesized at the restrictive temperature after *101* virion infection. The following experiment was performed to confirm these results. TC7 cells were infected with WT or *101* virions and incubated at 40 C with ^{14}C-thymidine or at 31 C with ^{3}H-thymidine. The viral DNA was extracted and analyzed on alkaline sucrose density gradients. Figure 4 shows that WT- and *101*-infected cells synthesized similar amounts of viral DNA I at 31 C, but very little DNA I was synthesized by the *101*-infected cells at 40 C. We conclude that the function inhibited at the restrictive temperature after *101* virion infection must be expressed for viral DNA synthesis to be initiated.

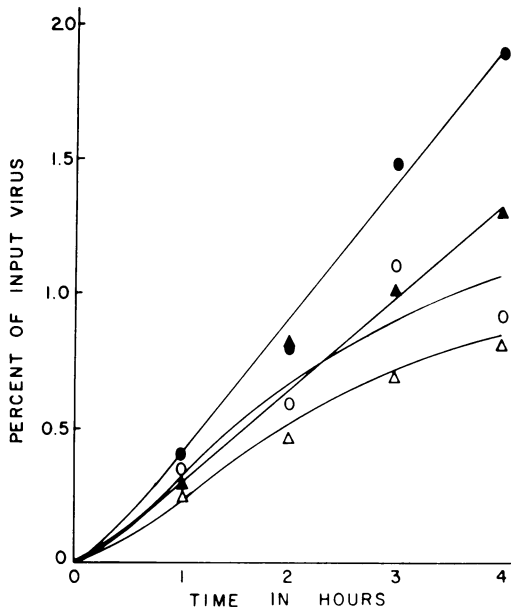

FIG. 3. *Penetration of WT and 101 virions at 40 and 33 C. WT* (○, ●) *and 101* (△, ▲) *radiolabeled virus were adsorbed onto TC7 cells at 40 C* (●, ▲) *or 33 C* (○, △), *and the ability of the virus to penetrate the cell was assayed (see Materials and Methods for details).*

Infection with viral DNA. Surprisingly, first-cycle T and V antigen synthesis, after *101* DNA infection, was normal at the restrictive temperature, although the progeny virus was indistinguishable from *101* virions (i.e., still mutant). Table 7 shows that both the WT and *101* DNA were equally capable of inducing the expression of T and V antigen synthesis at 40 C and that the number of T antigen-positive nuclei was similar to the number of V antigen-positive nuclei for both viruses. This finding is in marked contrast to the inhibition of T and V antigen synthesis at 40 C after infection with *101* virions. Table 7 also shows that the *101* DNA I was defective at 40 C in plaque formation (which requires multiple cycles). Table 8 shows that the progeny virions derived from the cultures infected with *101* DNA I at either 40 or 31 C were as temperature-sensitive as the parental *101* virions. The number of progeny virions produced by the *101* DNA I infection at 40 and 30 C was the same as the number produced by the WT DNA I infections. Thus, after *101* DNA I infection, (i) initial-cycle T and V antigen synthesis was similar to that occurring after WT DNA I infection, although (ii) plaque formation at the restrictive temperature was inhibited, and (iii) the progeny virus formed after DNA infection at either temperature was temperature-sensitive.

The results with purified *101* DNA I were repeated with unfractionated viral DNA (the Hirt supernatant fluid). Other temperature-sensitive mutants known to be blocked after T antigen synthesis, but before or after V antigen synthesis, were used as controls. The first-cycle

TABLE 6. *Induction of cellular and viral deoxyribonucleic acid (DNA) synthesis by wild-type and 101 virions at 40 and 33 C in TC7 cells*

Incubation temp (C)	^{14}C-Thymidine incorporation and virion antigen synthesis										
	Cells at zero time uninfected		Mock infected			Wild-type infected			*101*-Infected		
	Precipitate[a]	Supernatant[a] fraction	Precipitate	Supernatant fraction	VFU[b]	Precipitate	Supernatant fraction	VFU	Precipitate	Supernatant fraction	VFU
33	1,888	72	552	56	0	21,276	12,470	176,000	22,099	9,026	169,000
40	3,213	122	1,490	78	0	26,958	5,500	158,000	2,837	283	4,400

[a] The Hirt precipitate (measured in counts per minute) contains predominantly cellular DNA, whereas the Hirt supernatant fraction (measured as counts per minute) is predominantly viral DNA.

[b] Number of virion antigen-forming units per 32-mm dish.

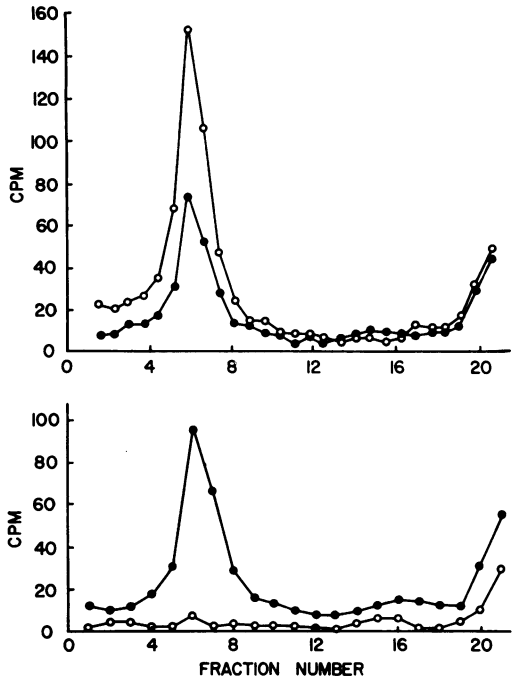

FIG. 4. *WT and 101 viral DNA synthesis at 40 and 31 C after virion infection of TC7 cells. TC7 cells were infected with WT (upper figure) or 101 (lower figure) virions and incubated at 40 or 31 C. The viral DNA was labeled with ^{14}C-thymidine at 40 C (○) and ^{3}H-thymidine at 31 C (●). Hirt supernatant fluids containing viral DNA were prepared and mixed so that the mixture from the WT- and 101-infected cultures contained ^{14}C-thymidine DNA (cells incubated at 40 C) and ^{3}H-thymidine DNA (cells incubated at 31 C). The mixtures were denatured by treatment for 15 min at 25 C with 0.1 N NaOH and 0.05 mM EDTA. Samples of 0.33 ml of the denatured samples were placed on 4.8-ml alkaline sucrose gradients (5–20% sucrose in 0.7 M NaCl, 0.3 N NaOH, 1 mM EDTA, 10 mM tris(hydroxymethyl)aminomethane, pH 12.5) and centrifuged at 40,000 rev/min for 4 hr at 8 C in a SW-50.1 rotor in a Spinco L2-ultracentrifuge. Fractions of approximately 0.2 ml were collected, and samples were counted in Bray's solution in a Nuclear-Chicago Mark I scintillation system. Corrections for spillage and quenching were performed. Centrifugation is from right to left.*

infection at the restrictive temperature with *101* DNA was normal. Infection with *tsB8* and *tsB11* DNA produced first-cycle blocks at the restrictive temperature similar to those produced after virion infection (Table 9). DNA dilutions (crude or purified) of 1:5 and 1:500 were used to insure that at least one dilution would be below the saturating levels for DNA infection. Aaronson and Martin (1) have shown that the number of T antigen-positive nuclei after SV40 DNA infection of human fibroblasts is linearly related to the concentration of DNA and reach a maximum of approximately 1 to 5%. A similar finding has been observed in CV-1 cells (S. Kit, *personal communication*).

Stability of the 101 genome in the host cell at the restrictive temperature. One interpretation of the results presented thus far is that the DNA of *101* virions is so altered that it is immediately degraded at 40 C by the host cell after "uncoating" within the nucleus. Possibly DEAE dextran stabilizes the DNA to such degradation. Figure 5 shows that the biological integrity of the infecting *101* virions, as assayed for their capacity to initiate T and V antigen synthesis at permissive temperature, was progressively lost by preincubation at the restrictive temperature. Nonetheless, as much as 20% of the capacity to initiate V antigen synthesis survived for up to 3 days, making it unlikely that the results of the preceding section are explicable in terms of some artifact of DNA stabilization by DEAE dextran.

Overcoming the temperature-sensitive block by incubation at 31 C prior to temperature shift to 40 C. Figure 6 demonstrates that after a 48-hr incubation period at 31 C, WT virions express nearly 60% of the input tumor antigen-forming units (TFU) within 24 to 26 hr after the shift to 40 C, whereas *101* virions only expressed 15 to 20% of the input TFU. The results obtained for U and V antigen (not shown) were very similar to those for T antigen, except the V antigen curve was displaced to about 10 hr later than the T and U antigen curves. Thus, a stage in the replication of the temperature-sensitive mutant can be reached at the permissive temperature, after which viral production ceases to be temperature sensitive.

In an experiment parallel to that of Fig. 6, cycloheximide was added at the time of the temperature shift, and, as expected, less than 5% of the cells subsequently became T, U, and V antigen-positive. None of these antigens disappeared with further incubation at 40 C. Once synthesized, these antigens therefore do not rapidly decay at the restrictive temperature, in contrast to the V antigen of *tsB8* (Table 10).

Requirement for protein synthesis at 31 C in overcoming the temperature-sensitive block. To test if more than passive uncoating (e.g., proteases already present in the cell) was required to overcome the *101* mutational block at 31 C, the requirement for protein synthesis was investigated. Figure 7 shows that the *101*-infected cultures treated with cycloheximide were incapable of V antigen synthesis after the removal of cycloheximide and shift to 40 C. The control cultures incubated with *101* virions at 33 C, but without cycloheximide, synthesized V antigen

after the shift to 40 C. The fact that 72 hr of preincubation at 33 C was required for all of the input *101* virions to overcome their block is in agreement with the results of the preceding section (Fig. 6). Similar kinetic results have been obtained with *tsB2*, *tsB8*, and *tsB11* (P. Tegtmeyer, *personal communication*). Protein synthesis at the permissive temperature is therefore required to overcome the temperature-sensitive block in *101* virions. Once the affected viral function is activated at the permissive temperature, the remainder of the lytic cycle proceeds in a normal manner at the restrictive temperature.

Complementation studies. Co-infection studies

TABLE 7. *Wild-type and 101 DNA I infection of TC7 cells at 40 and 31 C*[a]

DNA dilution	Wild type						*101*					
	40 C			31 C			40 C			31 C		
	T[b]	V	PFU titer (PFU/ml)	T	V	PFU titer (PFU/ml)	T	V	PFU titer (PFU/ml)	T	V	PFU titer (PFU/ml)
1:5	2,215	2,006	TM	1,821	1,682	TM	1,976	2,094	62	2,206	2,125	TM
1:500	233	210	1.2×10^4	89	79	1.5×10^4	281	254	10	152	168	1.9×10^4

[a] Abbreviations: DNA, deoxyribonucleic acid; T, tumor antigen; V, virion antigen; PFU, plaque-forming units; TM, too many to count.

[b] Standard error of the mean = ±10%. Measured as antigen-forming units/32-mm petri dish.

TABLE 8. *Temperature sensitivity of progeny virions produced at 40 and 31 C after wild-type and 101 DNA I infection of TC7 cells*[a]

Temp lysate tested (C)	Wild type						*101*					
	Parental		Progeny from DNA infection at				Parental		Progeny from DNA infection at			
			40 C		31 C				40 C		31 C	
	TFU/well	VFU/well	TFU/well	VFU/well	TFU/well	VFU/well	TFU/well	VFU/well	TFU/well	VFU/well	TFU/well	VFU/well
31	92[b]	85	88	81	90	86	77	82	86	90	79	85
40	86	79	78	87	85	82	0.8	1.1	1.0	0.9	1.2	1.0

[a] Abbreviations: DNA, deoxyribonucleic acid; TFU, tumor antigen-forming units; VFU, virion antigen-forming units.

[b] All stocks diluted so as to yield 80 to 90 TFU or VFU/well at 31 C. Standard error of the mean = ±10%.

TABLE 9. *First-cycle infection of TC7 cells at 41 and 33 C with crude DNA from wild-type, tsB8, tsB11, and ts*101 virion-infected TC7 cells at 33 C*[a]

Temp of incubation[b] (C)	Antigen-forming units per 32-mm dish of cells infected with							
	WT (T^+V^+)		*8* (T^+V^+)		*11* (T^+V^-)		*101* (T^-V^-)	
	T	V	T	V	T	V	T	V
33	1,312	1,177	1,432	1,367	1,376	1,286	1,782	1,862
41	1,286	1,195	1,306	1,236	1,325	2	1,726	1,694

[a] Abbreviations: DNA, deoxyribonucleic acid; WT, wild type; T, tumor antigen; V, virion antigen.

[b] Incubations were carried out for 96 hr at 33 C or for 42 hr at 41 C. Mutant *tsB8* is V-positive when incubated for 40 to 44 hr at 41 C, but the capsid disintegrates upon further incubation at this temperature (39).

[c] Standard error of the mean = ±15%.

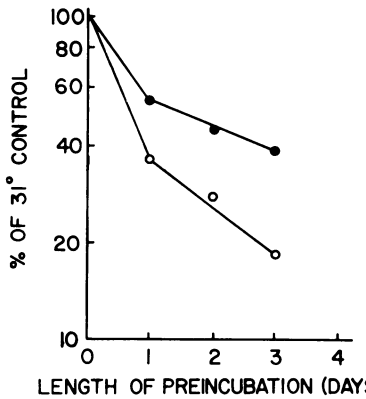

FIG. 5. *Temperature shift-down experiment with 101 virions. TC7 cells were infected with 101 virions and incubated at 40 or 31 C. Anti-SV40 immune serum was added at 24 hr. At the indicated times, the cells incubated at 40 C were either fixed and stained for T or V antigen, or shifted to 31 C and stained for T antigen (●) or V antigen (○) after a further 96-hr incubation period. The 31 C control plates had 75 TFU and 76 VFU per well. The 40 C control plates had 2 TFU or VFU per well.*

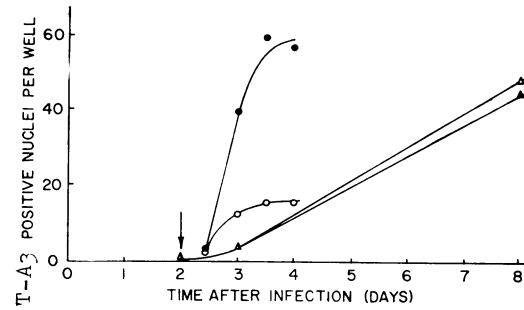

FIG. 6. *Temperature shift-up experiment. TC7 cells were infected with WT (●) or 101 (○) virions and incubated for 48 hr at 33 C. They were then shifted to 40 C and stained for T antigen at the indicated times. Control WT- and 101-infected cultures (▲ and △, respectively) were incubated at 33 C.*

were performed to determine whether other temperature-sensitive viral mutants would complement *101*. Rather than employing the standard complementation assay, which measures single cycle progeny virion yield by plaque assay, a fast, sensitive, and accurate complementation microassay was developed dependent upon the expression of first-cycle T or V antigen synthesis after co-infection at the restrictive temperature.

The advantages, disadvantages, and limitations of the microcomplementation assay are also illustrated by the results presented in Table 10. This experiment has been repeated four times with similar results. The assay of V antigen after co-infection detected complementation among *tsA7* and *tsB2*, *tsB8*, and *tsB11* as expected from the results of Tegtmeyer and Ozer (39) and Tegtmeyer (*personal communication*), who have designated their mutants as belonging to complementation groups *A* or *B* (35). Complementation detected by staining for T antigen could not be observed because all of the *tsB* mutants are blocked after the synthesis of T antigen. Thus, although the microcomplementation assay has the advantage of speed [requiring incubation for only 2 days instead of 2–3 weeks for the plaque assay technique (7, 39)], it has the disadvantage of not detecting complementation by T or V antigen staining if either of the mutants in question is blocked after T or V antigen synthesis, respectively. The assay is also unreliable if high multiplicity dependent leakage is encountered.

The microcomplementation assay also appears to be more sensitive than the standard first-cycle progeny virus yield assay for the detection of negative complementation (inhibition of complementation). Negative complementation occurred (Table 10) between *tsB2* and *tsB11* and their parental WT strain, WT-T. The failure of Tegtmeyer and Ozer (39) to detect negative complementation may reflect the fact that the progeny yield at the restrictive temperature was very low in their single infections. Further decrease in the number of progeny in the double infection was not detectable. The significance of this negative complementation remains obscure, and requires verification by a modified plaque assay.

Neither positive nor negative complementation was observed when *101* virions were co-infected with other temperature-sensitive mutants or WT virions (Table 10). Since *101* failed to complement mutants of two mutually complementing classes (*A* and *B*), *101* must be categorized as noncomplementing, hence the designation *ts*101* (35). One possible reservation is that the WT strain from which *101* was derived and the strain from which Tegtmeyer's mutants (39) were derived were independently isolated.

DISCUSSION

A temperature-sensitive mutant of SV40, *ts*101*, has been isolated and characterized during productive infection in monkey cells. The ratio between its propagative titers at permissive (31–33 C) and restrictive (40–41 C) temperatures is 10^3 to 10^4. Mutant virions adsorb to and penetrate the cell normally at the restrictive temperature. The induction of cellular DNA synthesis and the synthesis of T, U, and V antigens and viral DNA are blocked at the restrictive temperature after *101* virion infection.

TABLE 10. *Complementation microassay at 41 C*[a]

Infecting virus	MOI[b]	Antigen-forming units per well bottom[c]				VFU mixed infection at 41 C/ ΣVFU single infections at 41 C
		TFU		VFU		
		33 C[d]	41 C[e]	33 C[d]	41 C[f]	
WT	3	183[g]	172	146	136	
WT-T[h]	4	218	226	201	191	
7	4	331	132	325	26	
2	20	318	326	310	0	
11	3	175	164	162	0	
8	3	187	176	182	0[i]	
101	20	319	3.8	327	3.4	
*7*x*2*					208	8.0
*7*x*11*					114	4.4
*2*x*11*					0	—[k]
*7*x*101*					27	0.92
*2*x*101*					0.4[j]	0.12[j]
*11*x*101*					0.2[j]	0.06[j]
*8*x*2*					60[k]	—[k]
*8*x*11*					0	—
*8*x*7*					151	5.8
*8*x*101*					0.4	0.12
*8*xWT					139	1.0
*2*xWT					129[j]	0.96[j]
*11*xWT					102[j]	0.75[j]
*7*xWT					172	1.1
*101*xWT					207	1.5
WTxWT-T					327	1.0
*101*xWT-T					185	0.95
*2*xWT-T					68[j]	0.34[j]
*11*xWT-T					87[j]	0.45[j]
*7*xWT-T					173	0.80
*8*xWT-T					188	1.0

[a] Abbreviations: MOI, multiplicity of infection; TFU, tumor antigen-forming units; VFU, virion antigen-forming units; WT, wild type.

[b] Mixed infection MOI was equal to single infection MOI for each virus.

[c] There were 325 to 350 cells per well bottom.

[d] Fixed at 72 hr.

[e] Fixed at 42 hr.

[f] Fixed at 58 hr.

[g] Standard error of the mean = ±10%.

[h] Tegtmeyer's parental WT from which *2*, *7*, *8*, and *11* are derived (38, 39).

[i] At 40 to 48 hr at 41 C, *8* is V antigen-positive, but by 58 hr the V antigen has decayed [capsids have fallen apart (39)] and the infected nuclei are only faintly V antigen-positive.

[j] Although the *2*x*101* and *11*x*101* crosses seem to indicate negative complementation, the number of positive nuclei counted was not sufficient to give statistically significant results. Curiously, *2* and *11* seemed to negatively complement WT-T but not our WT.

[k] These nuclei were only faintly positive and because of the faint background from *8* alone (see footnote *i*) we cannot be sure whether *8* and *2* are in separate complementation groups or not.

When infections are carried out with WT and *101* DNA, however, there is no difference between initial-cycle T and V antigen synthesis for the two viruses at either 40 or 31 C, but plaque formation by the *101* DNA at 40 C is blocked. Progeny virions produced after *101* DNA infection at 40 or 33 C are indistinguishable from parental *101* virions.

Since the mutant is temperature-sensitive, we assume that the affected macromolecule is a protein, although temperature-sensitive transfer ribonucleic acid (RNA) mutants are known (36). We propose that the *101*-affected protein is a virion structural protein, because first-cycle infection with purified *101* DNA (free from virion proteins) is normal at the restrictive temperature. An alternate hypothesis, that *101* encodes a temperature-sensitive protein which is

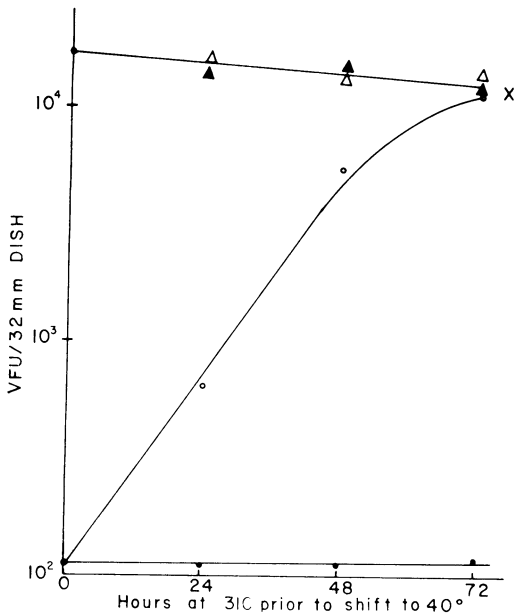

FIG. 7. *Temperature shift-up experiment with cycloheximide. TC7 cells were infected with WT (△, ▲) or 101 (○, ●) virions without cycloheximide (○, △), or with cycloheximide (●, ▲). The infected cells were incubated for the indicated times prior to the removal of the cycloheximide and shift to 40 C. The cells were fixed and stained for V antigen after a total incubation equivalent to 72 hr at 40 C. X = WT- and 101-infected dishes left for 6 days at 31 C without cycloheximide or treated with cycloheximide for 3 days at 31 C with a subsequent 6-day incubation period at 31 C without cycloheximide.*

required to completely free the viral DNA from the core proteins (partial denudation having been accomplished by the host), seems unlikely because *101* is noncomplementing (see below).

Available data indicate that the SV40 virion contains six structural proteins: a major coat protein of about 43,000 daltons, a variable component of about 32,000 daltons, a minor protein that sometimes is isolated bound to the viral DNA (8) of about 23,000 daltons, and three basic histone-like core proteins of 11,000 to 16,000 daltons (8, 11).

Several arguments would suggest that the affected *101* virion protein is not one of the major capsid components, but a core protein. (i) Adsorption and penetration of *101* virions at the restrictive temperature are normal. (ii) *101* Virions are as resistant as WT virions to heat inactivation. (iii) Once V antigen synthesis (capsid synthesis) is initiated at the permissive temperature, it continues normally at the restrictive temperature, and the immunofluorescence does not decrease with time after the shift. (iv) Barbanti-Brodano et al. (2) and Hummeler et al. (18) have suggested that SV40 virions are partially or completely uncoated within the nucleus of permissive monkey cells. Preliminary experiments suggest that WT and *101* virions move into the cell nucleus in similar amounts and at similar rates at 40 C (K. Huebner and J. Robb, *unpublished data*). Both the WT and *101* DNA were found to become deoxyribonuclease-sensitive within the nucleus at 40 C, suggesting that partial nuclear uncoating of *101* virions does occur at restrictive temperature. Furthermore, *101* virions penetrated the Chinese hamster cell nucleus at 40 C as efficiently as WT virions, although the input *101* DNA did not become covalently linked to the cell DNA and T antigen synthesis was markedly inhibited at both permissive and restrictive temperatures (reference 16; K. Hirai, J. Robb, and V. Defendi, *unpublished data*).

These results are consistent with the hypothesis that the major capsid proteins of *101* are not affected and that *101* virions can be normally processed by the host cell at the restrictive temperature to the point of partial denudation of the viral DNA. If *101* has a virion component alteration, that alteration most likely affects one of the core proteins. A role for a core protein in penetration or gene expression has recently been proposed for the coliphage MS2 (23) and R17 (24). Such an hypothesis is also consistent with our finding that *101* is noncomplementing.

Co-infection studies with our microcomplementation assay revealed that *101* virions exhibited neither positive nor negative complementation with other temperature-sensitive mutants or WT virions. These results have been confirmed by the standard single-cycle virus yield complementation assay (reference 39, and P. Tegtmeyer, *personal communication*). The mutational block in the *101* virions, therefore, cannot be overcome at the restrictive temperature by a diffusable viral product derived from a different co-infecting viral genome or from a viral-induced cell product. This result makes it unlikely that the temperature-sensitive *101* protein is necessary for the complete removal of all virion proteins from the viral DNA. Furthermore, the *101* virions do not produce an inhibitory product at the restrictive temperature because they do not inhibit other co-infecting viruses (i.e., the *101* mutation is not *trans*-dominant). The affected *101* protein acted only on the DNA with which it was associated in the virion, hence it is a *cis*-acting protein. *Cis*-acting proteins may be regulatory proteins in bacteriophage (26, 27).

The temperature-shift experiment with cyclo-

heximide (Fig. 7) suggests that protein synthesis is required to overcome the block created by the temperature-sensitive defective *cis*-acting protein in *101* virions. That is, the mutational block is not overcome in the presence of cycloheximide even after 72 hr of incubation at 31 C. Whether the protein synthesis required is viral or cell directed is not known.

At least five models, having precedents in bacterial and bacteriophage systems, could explain our findings with *ts*101*. (i) A replicon attachment model (19) would predict that the affected structural virion protein played a role in the attachment of the viral genome to some host site. Our finding that infection at the restrictive temperature with crude or purified mutant SV40 DNA is not inhibited does not support this model. (ii) The affected structural protein could be required for the efficient initiation of transcription from the viral genome, possibly a sigma factor (41). Because mutant DNA infects the cell normally at the restrictive temperature, the affected virion structural protein may not be essential for first-cycle initiation. (iii) The affected protein could be an endonuclease necessary for the conversion of supercoiled form I viral DNA to nicked, open circular form II DNA before transcription can be initiated (3, 14). There are no data for or against this model. (iv) The affected protein in *101* could be a positive effector regulating the initiation of transcription from the viral DNA. Activation of this effector could require the binding of some small molecule such as cyclic adenosine monophosphate or ppGpp (5). High concentrations of dibutyryl adenosine 3′:5′-cyclic monophosphate with or without theophylline have not been able to overcome the mutational block in *101* virion-infected cells at the restrictive temperature (J. Robb, *unpublished data*). (v) Finally, the affected protein in *101* could normally have some type of repressor function (20). Recent work by Herzberg and Winocour (15) suggests that there is only one monkey RNA polymerase binding site on the supercoiled SV40 DNA I molecule. A *101* virion core protein could conceivably block this RNA polymerase binding site at restrictive temperature and inhibit subsequent transcription after partial uncoating has occurred. Green, Miller, and Hendler (reference 12, and M. Green, *personal communication*) using polyoma virus, and Huang, Estes, Nonoyama, and Pagano (reference 8, and *personal communication*) using SV40 virus have isolated a viral DNA-nucleoprotein complex from infected cells or virions, respectively. They tested the complex for transcription capacity in an in vitro RNA-synthesizing system using *Escherichia coli* or mammalian RNA polymerase. The complex had a much lower capacity for synthesizing RNA than did the purified form I DNA without any attached protein. Experiments are underway to determine if *101*-specific RNA is synthesized at restrictive temperature.

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ADDENDUM IN PROOF

Huang, Estes, Nonoyama, and Pagano have further characterized the SV40 virion structural proteins and have investigated the ability of the core proteins to regulate in vitro transcription (J. Virol. 9:923–929, 930–937).

LITERATURE CITED

1. Aaronson, S. A. 1970. Susceptibility of human cell strains to transformation by simian virus 40 and simian virus 40 deoxyribonucleic acid. J. Virol. 6:470–475.
2. Barbanti-Brodano, G., P. Swetly, and H. Koprowski. 1970. Early events in the infection of permissive cells with simian virus 40: adsorption, penetration, and uncoating. J. Virol. 6:78–86.
3. Blair, D. G., D. B. Clewell, D. J. Sheratt, and D. R. Helinski. 1971. Strand-specific supercoiled DNA-protein relaxation complexes: comparison of the complexes of bacterial plasmids $ColE_1$ and $ColE_2$. Proc. Nat. Acad. Sci. U.S.A. 68:210–214.
4. Darnell, J. E., and T. K. Sawyer. 1960. The basis for variation in susceptibility to poliovirus in HeLa cells. Virology 11: 665–675.
5. de Crombrugghe, B., B. Chen, M. Gottesman, I. Pastan, H. E. Varmus, M. Emmer, and R. L. Perlman. 1971. Regulation of lac mRNA synthesis in a soluble cell-free system. Nature N. Biol. 230:37–40.
6. DiMayorca, G., J. Callender, G. Martin, and R. Giordano. 1969. Temperature-sensitive mutants of polyoma virus. Virology 38:126–133.
7. Eckhart, W. 1969. Complementation and transformation by temperature-sensitive mutants of polyoma virus. Virology 38:120–125.
8. Estes, M. K., E.-S. Huang, and J. S. Pagano. 1971. Structural polypeptides of simian virus 40. J. Virol. 7:635–641.
9. Fried, M. 1970. Characterization or a temperature-sensitive mutant of polyoma virus. Virology 40:605–617.
10. Gershon, D., L. Sachs, and E. Winocour. 1966. The induction of cellular DNA synthesis by simian virus 40 in contact-inhibited and in X-irradiated cells. Proc. Nat. Acad. Sci. U.S.A. 56:918–925.
11. Girard, M., L. Marty, and F. Suarez. 1970. Capsid proteins of simian virus 40. Biochem. Biophys. Res. Commun. 40:97–102.
12. Green, M. H., H. I. Miller, and S. Hendler. 1971. Isolation of a polyoma-nucleoprotein complex from infected mouse-cell cultures. Proc. Nat. Acad. Sci. U.S.A. 68:1032–1036.
13. Hatanaka, M., and R. Dulbecco. 1966. Induction of DNA

synthesis by SV40. Proc. Nat. Acad. Sci. U.S.A. 56:736–740.
14. Helinski, D. R., and D. B. Clewell. 1971. Circular DNA. Annu. Rev. Biochem. 40:899–912.
15. Herzberg, M., and E. Winocour. 1970. Simian virus 40 deoxyribonucleic acid transcription in vitro: binding and transcription patterns with a mammalian ribonucleic acid polymerase. J. Virol. 6:667–676.
16. Hirai, K., J. Lehman, and V. Defendi. 1971. Integration of simian virus 40 deoxyribonucleic acid into the deoxyribonucleic acid of primary infected Chinese hamster cells. J. Virol. 8:708–715.
17. Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. 26:365–369.
18. Hummeler, K., N. Tomassini, and F. Sokol. 1970. Morphological aspects of the uptake of simian virus 40 by permissive cells. J. Virol. 6:87–93.
19. Jacob, F., S. Brenner, and F. Cuzin. 1963. On the regulation of DNA replication in bacteria. Cold Spring Harbor Symp. Quant. Biol. 28:329–348.
20. Jacob, F., and J. Monod. 1961. On the regulation of gene activity. Cold Spring Harbor Symp. Quant. Biol. 26:193–211.
21. Jensen, F. C., A. J. Girardi, R. V. Gilden, and H. Koprowski. 1964. Infection of human and simian tissue cultures with Rous sarcoma virus. Proc. Nat. Acad. Sci. U.S.A. 52:53–59.
22. Kit, S., S. Tokuno, K. Nakajima, D. Trkula, and D. R. Dubbs. 1970. Temperature-sensitive simian virus 40 mutant defective in a late function. J. Virol. 6:286–294.
23. Kozak, M., and D. Nathans. 1971. Fate of maturation protein during infection by coliphage MS2. Nature N. Biol. 234:209–211.
24. Krahn, P. M., R. J. O'Callaghan, and W. Paranchych. 1972. Stages in phage R17 infection. VI. Injection of a protein and RNA into the host cell. Virology 47:628–637.
25. Lewis, A. M., M. J. Levin, W. H. Wiese, C. S. Crumpacker, and P. H. Henry. 1969. A nondefective (competent) adenovirus-SV40 hybrid isolated from the AD. 2-SV40 hybrid population. Proc. Nat. Acad. Sci. U.S.A. 63:1128–1135.
26. Lindahl, G. 1970. Bacteriophage P2: replication of the chromosome requires a protein which acts only on the genome that coded for it. Virology 42:522–533.
27. Lindahl, G., Y. Hirota, and F. Jacob. 1971. On the process of cellular division in *Escherichia coli*: replication of the bacterial chromosome under control of prophage P2. Proc. Nat. Acad. Sci. U.S.A. 68:2407–2411.
28. McCutchen, J. H., and J. S. Pagano. 1968. Enhancement of the infectivity of simian virus 40 deoxyribonucleic acid with diethylaminoethyldextran. J. Nat. Cancer Inst. 41: 351–357.
29. Ozer, H. L. 1972. Synthesis and assembly of simian virus 40. I. Differential synthesis of intact virions and empty shells. J. Virol. 9:41–51.
30. Ozer, H. L., and K. K. Takemoto. 1969. Site of host restriction of simian virus 40 mutants in an established African green monkey kidney cell line. J. Virol. 4:408–415.
31. Puck, T. T., S. J. Cieciura, and A. Robinson. 1958. Genetics of somatic mammalian cells. III. Long-term cultivation of euploid cells from human and animal subjects. J. Exp. Med. 108:945–956.
32. Radloff, R., W. Bauer, and J. Vinograd. 1967. A dye-bouyant-density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells. Proc. Nat. Acad. Sci. U.S.A. 57:1514–1521.
33. Robb, J. A., and R. G. Martin. 1970. Genetic analysis of simian virus 40. I. Description of microtitration and replica-plating techniques for virus. Virology 41:751–760.
34. Robb, J. A., H. S. Smith, C. D. Scher. 1972. Genetic analysis of simian virus 40. IV. Inhibited transformation of Balb/3T3 cells by an early mutant. J. Virol. 9:969–972.
35. Robb, J. A., P. Tegtmeyer, R. G. Martin, and S. Kit. 1972. Proposal for a uniform nomenclature for SV40 mutants. J. Virol. 9:562–563.
36. Smith, J. D., L. Barnett, S. Brenner, and R. L. Russel. 1970. More mutant tyrosine transfer ribonucleic acids. J. Mol. Biol. 54:1–14.
37. Takemoto, K. K., R. L. Kirschstein, and K. Habel. 1966. Mutants of simian virus 40 differing in plaque size, oncogenicity, and heat sensitivity. J. Bacteriol. 92:990–994.
38. Tegtmeyer, P., C. Dohan, and C. Reznikoff. 1970. Inactivating and mutagenic effects of nitrosoguanidine on simian virus 40. Proc. Nat. Acad. Sci. U.S.A. 66:745–752.
39. Tegtmeyer, P., and H. Ozer. 1971. Temperature-sensitive mutants of simian virus 40: infection of permissive cells. J. Virol. 8:516–524.
40. Todaro, G. J., S. A. Aaronson, and E. Rands. 1971. Rapid detection of mycoplasma-infected cell cultures. Exp. Cell Res. 65:256–257.
41. Travers, A. A. 1970. Positive control of transcription by a bacteriophage sigma factor. Nature (London) 225:1009–1012.