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Genetic Analysis of Simian Virus 40

IV. Inhibited Transformation of Balb/3T3 Cells by a Temperature-Sensitive Early Mutant

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The temperature-sensitive early mutant, *ts*101*, was characterized during productive infection in monkey cells, and the results are presented in an accompanying paper. This paper demonstrates that although *101* mutant virions adsorb normally to confluent Balb/3T3 mouse cells at both permissive (33 C) and restrictive (38.5 C) temperatures, T antigen synthesis and transformation, abortive and stable, are inhibited at both temperatures (host-range inhibition). T antigen synthesis is temperature sensitive, whereas abortive and stable transformation are not. Clones of *101*-transformed Balb/3T3 cells were isolated, and virus was rescued from all clones at both permissive and restrictive temperatures. The rescued virus was as temperature sensitive as the original transforming *101* virions.

A temperature-sensitive, heat-stable, small-plaque, early mutant of simian virus 40 (SV40) (*ts*101*) was characterized during productive infection in monkey cells, and the results are in the accompanying paper (7). The initiation of cell deoxyribonucleic acid (DNA) synthesis and the synthesis of SV40 T, U, and V antigens and viral DNA were blocked at restrictive temperature after virion infection, even though adsorption and cell penetration were normal. First-cycle T and V antigen synthesis in the monkey cells after infection with purified *101* DNA I at restrictive temperature was normal. The progeny virions produced by the DNA infections at permissive and restrictive temperatures could not be distinguished from *ts*101*. This paper examines the ability of *101* virions to initiate T antigen synthesis and to transform, both abortively (8) and stably (12), nonproductive mouse cells.

MATERIALS AND METHODS

Culture techniques. Balb/3T3 clone A31 cells (1) were maintained in Dulbecco-modified Eagle medium and supplemented with 10% calf serum (Colorado Serum Co.) and antibiotics. TC7 cells (7) are a cloned subline of CV-1 (4) monkey cells. These cells were cultured and used for viral assay as described in the accompanying paper (7). Mycoplasma contamination was not detected in either cell line by the ^{3}H-uridine incorporation assay (11). The small-plaque strain (SV-S) of SV40 (9) is the wild-type strain (WT), and the *ts*101* (*101*) mutant is described in the accompanying paper (7). Virus stocks were grown at 33 C in TC7 cells (7), and no mycoplasma contamination was detected by direct culture (kindly performed by Walter James, National Institutes of Health).

Assay of SV40 tumor and virion antigens. SV40 tumor (T) antigen and virion (V) antigen were assayed by the immunofluorescent technique adapted to microculture as previously described (6). The hamster anti-SV40 T antigen serum was adsorbed on confluent Balb/3T3 cells for 30 min at 37 C prior to use.

Adsorption techniques. Virion adsorption using low multiplicity at 33 and 38 C was performed by the procedure used for productive infection (7). Adsorption using high multiplicity at 33 C was performed by infecting confluent Balb/3T3 cells at a multiplicity of infection (MOI) of 30 for 75 min with agitation every 10 min. The cells had been confluent for 3 days prior to infection (5×10^{5} cells per 32-mm petri dish). These conditions were identical to those used in the T antigen and transformation experiments below. After adsorption, the monolayers were rinsed two times with 27 C serum-free medium (SFM) and then treated in either of two ways. (i) A 1.0-ml amount of SMF was added, and the cells were scraped and frozen at −20 C. (ii) The cells were removed with trypsin (10), centrifuged, suspended in 1.0 ml of SFM, and frozen at −20 C. All cell lysates were end-point dilution titered on TC7 cells at 33 C. As controls,

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32-mm petri dishes were rinsed once with medium containing 10% calf serum and were carried through the entire procedure.

T antigen and transformation experiments. The ability of WT and *101* virions to initiate T antigen synthesis was determined after virion infection of confluent monolayer cultures of Balb/3T3 cells at a MOI of 30 at 33 and 38 C. After adsorption at 33 C, the monolayers were either directly incubated at 33 and 38 C, or the cells were removed with trypsin (10). The trypsin-treated cells were replated undiluted, or diluted 1:2 and 1:5 and incubated at 33 and 38 C. The percentage of T antigen-positive cells was determined at a magnification of 625× with a 40× water-immersion objective directly in the 32-mm petri dishes. The ability of WT and *101* virions to abortively (8) or stably (10) transform confluent Balb/3T3 cells was assayed at 38 and 33 C with a MOI of 30.

Virus rescue. The release of infectious virus from transformed Balb/3T3 cells after fusion to permissive TC7 cells with the aid of ultraviolet (UV)-inactivated Sendai virus was performed in the following manner (3, 5, 13). Transformed cells were mixed with TC7 cells in equal numbers (5×10^6 cells each), and 6,000 hemagglutinin units of UV-inactivated Sendai virus was added to 1 ml of cell mixture. The cell-virus mixture was incubated at 0 C for 10 min and then at 37 C for 30 min with frequent shaking. Equal volumes of the cell-virus mixture (1×10^6 cells) were placed in T-30 flasks (Falcon) with 5 ml of medium containing 5% fetal bovine serum (FBS) and incubated at 33, 38 or 40 C.

RESULTS

The data in Tables 1 and 2 demonstrate that there was no difference between WT and *101* virion adsorption at low (1) or high (30) MOI at 33 or 38 C. Postadsorption treatment of the cells with trypsin reduced the number of adsorbed virions similarly for WT and *101*. We conclude that *101* virion adsorption is normal at 33 and 38 C. As shown in Table 3, the ability of *101* virions to initiate T antigen synthesis was inhibited 8 to 20 times at 33 C and 400 to 500 times at 38 C. Removal of the cells with trypsin, with or without subsequent dilution, made no difference in the degree of this inhibition. The 1:2 and 1:5 dilutions would more nearly correspond to the state of the *101*-infected cells in the abortive and stable transformation assays. The initiation of T antigen synthesis after *101* virion infection in the abortive cycle is not only temperature sensitive, but is host-range inhibited in comparison to the lytic cycle (i.e., at 33 C there is no difference between WT and *101* virion infection in TC7 monkey cells; reference 7).

The data in Table 4 demonstrate that both abortive and stable transformation of confluent Balb/3T3 cells by *101* virions were inhibited at both 33 and 38 C (host-range inhibition), but were not temperature sensitive. The differences between the 33 and 38 C values for either WT or *101* are not significant. Two clones of *101*-transformed cells from a 33 C stable transformation

TABLE 1. *Adsorption of wild-type (WT) and 101 virions at high multiplicity onto confluent Balb/3T3 cells at 33 C*

Cells	Trypsin treatment[b]	No. of virions adsorbed per 32-mm dish (IU/ml $\times 10^{-4}$)[a]	
		WT	*101*
Control	0	0.18[c]	0.21
Control	+	0.20	0.24
Balb/3T3	0	5.0	17
Balb/3T3	+	1.2	1.4

[a] Multiplicity of infection = 30; IU = infective unit.

[b] The petri dishes (± cells) were either scraped with a rubber policeman or treated with trypsin prior to virus titration.

[c] Standard error of the mean = ±25%.

TABLE 2. *Adsorption of wild-type (WT) and 101 virions at low multiplicity onto confluent Balb/3T3 cells at 40 and 33 C*

Cells	No. of virions adsorbed at the given temperature per 32-mm dish (IU/ml $\times 10^{-4}$)[a]			
	40 C		33 C	
	WT	*101*	WT	*101*
Control	0.9	1.0	0.8	1.2
Balb/3T3	8.8	9.4	15	13

[a] Infectious units (IU) (4.0×10^5) applied per dish (multiplicity of infection = 1); standard error of the mean = ±25%.

TABLE 3. *Wild-type (WT) and 101 virion initiation of T antigen synthesis in confluent Balb/3T3 cells*

Virus	% T-positive nuclei							
	NT[a]		T-ND[b]		T-1:2[b]		T-1:5[b]	
	33 C	38 C	33 C	38 C	33 C	38 C	33 C	38 C
WT	44[c]	41	47	37	33	25	28	30
101	3.2	0.10	2.4	0.08	4.1	0.06	2.8	0.07

[a] NT = Cells not removed with trypsin after adsorption.

[b] T-ND, T-1:2, and T-1:5 = cells removed with trypsin and replated undiluted, or diluted 1:2 or 1:5.

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

assay were isolated and recloned. Upon Sendai virus-mediated fusion with productive TC7 cells, both clones released virus at 33, 38, and 40 C. All the rescued viruses were similar to the original transforming *101* mutant, as shown in Table 5. Neither of the clones released virus spontaneously or synthesized V antigen at any of the three temperatures. Preliminary data indicate that the appearance of T antigen in these two clones is markedly inhibited at 38 and 40 C, but reappears when the cells are shifted to 33 C (J. Robb, *unpublished data*). Wild-type transformed cells do not display this temperature-dependent modulation of T antigen synthesis.

DISCUSSION

Although *ts*101* virions adsorb normally to abortive Balb/3T3 cells, they are inhibited in the initiation of T antigen synthesis and in their ability to abortively and stably transform the cells at both 33 and 38 C. This inhibition is host-range in comparison to the lytic infection in monkey cells where no inhibition occurs at the permissive temperature (7). A working hypothesis to explain this host-range inhibition is that a mouse function interacts with the *101* mutant virion protein in a less efficient manner than with the comparable WT virion protein. The possible mechanisms of this interaction are discussed in the accompanying paper (7).

In addition, the initiation of T antigen synthesis is not only host-range, but also temperature sensitive. When the data in Tables 3 and 4 are compared, the same percentage of T-positive WT or *101*-infected cells (about 10–20%) were stably transformed at 33 and 38 C. The host-range inhibition produced a decrease in the absolute number of *101*-transformed clones. Abortive transformation at 33 and 38 C displayed similar relative differences between WT and *101*. Therefore, once the host-range inhibition is overcome, the ability of the virion to transform the cell is similar for WT and *101*.

The ability of the *101* virion to abortively and stably transform is similar at 33 and 38 C, in contrast to the marked inhibition of T antigen synthesis at 38C. A similar observation has been made with the temperature-sensitive polyoma mutant, TS-a, for the induction of cellular DNA synthesis (reference 2 and W. Eckhart, *personal communication*). These findings suggest that the viral function responsible for the initiation of T antigen synthesis, or the T antigen itself, may not be required for abortive and stable transformation. An alternative is that only an undetectable amount of the T antigen initiation function, or T antigen itself, is sufficient for transformation to occur. First cycle and transforming infections with WT and *101* DNA are being examined.

TABLE 4. *Abortive and stable transformation of confluent Balb/3T3 cells with wild-type (WT) and 101 virions at 38 and 33 C*

Virus	Abortive transformation (no. of divisions per viable cell)		Stable transformation (frequency of transformation)	
	38 C	33 C	38 C (%)	33 C (%)
Mock	0.7[a]	0.3	0	0
WT	15	35	5.1	3.5
101	5.2	2.3	0.6	0.2

[a] Standard error of the mean = ±25%.

TABLE 5. *Temperature sensitivity of virus rescued from 101-transformed Balb/3T3 clones*

Virus	Temp of rescue (C)	No. of VFU/well[a]	
		40 C	33 C
WT[b]		38[c]	41
101		0.1	44
Rescued from clone 1	33	0.1	44
	38	0.3	39
	40	0.2	47
Rescued from clone 2	33	0.3	43
	38	0.4	47
	40	0.2	38

[a] V antigen-forming unit (VFU) assayed on TC7 cells.

[b] Wild type.

[c] All viral stocks were diluted so as to produce about 40 VFU per well at 33 C.

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