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Simian Virus 40-Host Cell Interactions.

I. Temperature-Sensitive Regulation of SV40 T Antigen in 3T3 Mouse Cells Transformed by the *ts*101* Temperature-Sensitive Early Mutant of SV40

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BALB/3T3 and Swiss/3T3 mouse cells transformed at permissive temperature (33 C) by the early temperature-sensitive mutant of simian virus 40 (SV40), *ts*101*, exhibited a temperature-dependent modulation of SV40 tumor (T) antigen as assayed by immunofluorescence. The percentage of T antigen-positive nuclei in *ts*101* transformed cells was reduced at restrictive temperature (39 C) when compared to 33 C and to wild-type SV40 transformed cells at either 33 C or 39 C. The percentage of T antigen-positive nuclei in *ts*101* transformed cells returned to the 33 C control level when the cells were shifted from 39 to 33 C. The *ts*101* transformed cells could be superinfected with wild-type, but not *ts*101*, virions at 39 C as assayed by an increase in T antigen-positive nuclei.

The regulatory interactions between simian virus 40 (SV40)-directed and cell-directed functions in transforming infections and transformed cells are poorly understood (1, 2, 4). Temperature-sensitive virus and cell mutants are facilitating the understanding of the viral and cellular regulatory mechanisms that are operative in papova virus transformed cells (3, 5, 7, 9, 10). The temperature-sensitive ts3 mutant of polyoma produces at permissive temperature transformed BHK hamster cells that possess a partially temperature-dependent transformed phenotype (3, 5), demonstrating the requirement for a viral gene in maintaining some manifestations of the transformed phenotype. At restrictive temperature the ts3 transformed cells exhibit a density dependent inhibition of cellular DNA synthesis and nontransformed Concanavalin A agglutination capacity. Polyoma-specific tumor (T) antigen has not been assayed in the ts3 transformed cells. Wild-type SV40 transformed 3T3 cells possessing a cellular temperature-sensitive mutation and some temperature-dependent transformed characteristics have been isolated (7, 9, 10). At least one cellular gene is, therefore, also required for maintaining some manifestations of the transformed phenotype. Although cellular DNA synthesis, saturation density, serum growth factor requirements, and Concanavalin A binding capacity are temperature-sensitive in these cells, SV40 T antigen is not.

The *ts*101* early mutant of SV40 is affected in a virion protein that must be removed or activated, or both, before abortive or stable transformation can occur (13, 14). I now demonstrate that this virion protein plays a regulatory role in transformed cells as manifested by the temperature-dependent appearance of SV40 T antigen in *ts*101* transformed BALB/3T3 and Swiss/3T3 cells.

Plastic 32-mm petri dishes (Falcon) were used for all experiments. Cell lines were passaged in 250-ml plastic flasks (Falcon) at 37 C using 0.05% trypsin (Worthington) 0.02% ethyleneglycol-bis-(beta-amino-ethyl ether)*N*,*N'*-tetraacetic acid (EGTA) for cell removal. Medium was changed twice weekly. All experiments were performed in WEDCO (Silver Spring, Md.) humidified air incubators, using powdered Dulbecco-modified Eagle medium (Flow Laboratories, Rockville, Md.), supplemented with 1.5 g of D-glucose per liter, 300 mg of arginine-hydrochloride per liter, 1.5 g of glutamine per liter, 20 mg of histidine-hydrochloride per liter, 25 μg of chlortetracycline per ml (Aureomycin HCl, Lederle Labs, Pearl River, N. Y.), and 9% calf serum (Colorado Serum Co., Denver, Colo.), and buffered at pH 7.4 with 60 m*M* *N*-Tris glycine (Tricine, Sigma Chemical Co., St. Louis, Mo.) and 0.5 g of $NaHCO_3$ per liter. All four wild-type transformed clones (11-A-8, SEA-47, and BTW-1) were independently derived from SV-S (15) wild-

type SV40 infected BALB/3T3, clone A31 cells and were kindly provided by Helene Smith. The *ts*101* transformed clones were independently derived from either BALB/3T3, clone A31 cells (BTM-101-1, BTM-101-2, BALB transformed mutant-101) or Swiss/3T3 cells (STM-101-13, Swiss transformed mutant) and have been described previously (14). Wild-type and mutant viruses were rescued from each of the respective wild-type and mutant transformed clones (14, and Helene Smith, personal communication).

The *ts*101* temperature-sensitive mutant was derived from the wild-type SV-S (15) virus and was shown to be inhibited at restrictive temperature after cytoplasmic penetration and prior to T-antigen synthesis (13, 14). Wild-type and *ts*101* virus was grown at 33 C in monkey TC7 cells (J. A. Robb and K. Huebner, in press) from an MOI of 0.005 and was endpoint dilution titered as previously described (12). Cells were fixed and stained directly in the plastic petri dishes for SV40 T antigen by the immunofluorescent technique as previously described (11, 12), using hamster anti-SV40 T-antigen ascitic fluid (Flow Laboratories) and rabbit anti-hamster-globulin globulin (Sylvana, Co., Millburn, N. J.). Both antibody preparations were fluorescein conjugated. The percentage of T antigen-positive nuclei per culture dish was determined from 2,000 cells for each experimental point by using a ×40 water immersion objective.

Virion infection was accomplished by adsorption for 2 h at 25 C in 0.10 ml of serum-free medium (SFM) per 32-mm petri dish with 15-min agitations. Cells were infected with DNA in 32-mm petri dishes by the method of McCutchen and Pagano (6) using CsCl-ethidium bromide density centrifugation purified wild-type and *ts*101* DNA I (8, 13) and a final concentration of 400 μg of DEAE-dextran per ml (2×10^6 daltons; Pharmacia). The DNA I concentration was measured by optical density at 260 nm, and 0.2 μg of DNA I in 0.10 ml of SFM per petri dish was adsorbed for 20 min at 25 C with 2-min agitations. After adsorption, all cultures were rinsed once with SFM, growth medium was added, and the cultures were incubated at 39 C. After 72 h the cells were fixed and stained for T antigen.

Temperature-shift experiments were performed as follows. Growing and confluent replicate cultures of the various transformed clones were incubated in 32-mm petri dishes at 33 and 39 C. After 72 h some cultures were stained for T antigen, while the remaining cultures were shifted from 33 to 39 C or from 39 to 33 C. No significant differences were observed between growing and confluent cultures. The shifted cultures were stained for T antigen 72 h after the shift in order to allow T antigen to maximally appear in the down-shift or to disappear in the up-shift. The data in Table 1 indicate that three of the wild-type transformed lines had little change in the percentage of T-positive nuclei, regardless of the temperature. One wild-type clone (BTW-1) had some reduction of T-positive nuclei at 39 C. All three *ts*101* transformed clones had a significant reduction in T-positive nuclei at 39 C even after the shift from 33 C. The residual T-positive nuclei in the *ts*101* transformed cells were not reduced in intensity suggesting an all or none regulation. The total number of cells per culture was similar for all seven lines at the beginning of the experiments and at the respective times of fixation. Reductions to 2 to 5% T antigen-positive nuclei were obtained after 4 days of incubation at 39 C in the *ts*101* transformed clones, but the prolonged incubation in the Tricine medium at 39 C was equally toxic to both wild-type and *ts*101*-transformed cells, causing a decrease in the number of viable cells as assayed by trypan blue exclusion and cloning efficiency. An 80% decrease in T antigen per milligram of protein was observed by complement fixation in the BTM-101-1 line at 39 C, as compared to 33 C (kindly performed by Harvey Ozer). The wild-type transformed line, BTW-1, had no reduction in T antigen-specific activity although some decrease was observed in T antigen-specific fluorescence at 39 C. The STM-101-13 and BTM-101-2 lines were not tested.

The appearance of T antigen at 39 C in the *ts*101*-transformed cells was inhibited after 72 h of incubation. An inhibitor of T antigen synthesis could explain the reduction in T-positive nuclei. Superinfection experiments were

TABLE 1. *Temperature-sensitive regulation of SV40 T antigen in ts*101-transformed 3T3 cells*

Trans. clones	T antigen-positive nuclei (%)[a]			
	33 C	39 C	39 to 33 C	33 to 39 C
11-A-8(WT)	98[b]	93	97	94
SEA-45(WT)	97	95	96	92
SEA-47(WT)	93	89	96	91
BTW-1(WT)	94	58	96	71
BTM-101-2	84	28	95	32
BTM-101-1	95	13	86	14
STM-101-13	89	14	72	11

[a] Percentage of T antigen-positive nuclei determined after 72 h at 33 and 39 C and again at 72 h after the temperature shifts.

[b] Values are the average of duplicate cultures counting 2,000 cells per culture. SEM, ±15%.

performed as follows. Replicate 32-mm petri dishes were inoculated with the various clones and allowed to incubate 18 h at 37 C prior to infection with wild-type and *ts*101* virions and DNA I. Growing and confluent cultures produced similar results. The data in Table 2 indicate that the *ts*101* transformed clones were superinfected by wild-type virions, but not by *ts*101* virions as previously observed in acutely infected BALB/3T3 cells (14). A higher percentage of T-positive nuclei were present in the wild-type virion infected *ts*101*-transformed cells than was expected from the wild-type infected nontransformed BALB/3T3 cells (Table 2; wild-type infected BALB/3T3 was 26%, BTM-101-1 wild-type minus BTM-101-1 mock = 51%, STM-101-13 wild-type minus STM-101-13 mock = 60%) suggesting an increased efficiency of wild-type virion infection in the *ts*101*-transformed cells. The origin of the T antigen in the wild-type virion infected cells could not be determined, because the T antigen regulated by the *ts*101* genome could not be differentiated from the T antigen produced by the superinfecting wild-type genome.

The *ts*101* DNA I infection of productive monkey TC7 cells was similar to the wild-type DNA I infection at 41 C, although the *ts*101* virion infection was inhibited 1,000-fold (13). The DNA I infection experiments were undertaken to investigate whether *ts*101* DNA I infection was host-range or temperature sensitive, or both, in BALB/3T3 cells as was *ts*101*-virion infection (14). The results given in Table 2 show that there were no differences between wild-type and *ts*101* DNA I infections at 39 C demonstrating that the *ts*101* mutant protein acts as a virion protein in both abortive and productive infections. The percentage of T-positive cells can only be increased to about 1% in BALB/3T3 cells regardless of the amount of DNA I used for infection. Because of this low efficiency of DNA I infection, no detectable effects were observed in the DNA I infected transformed cells due to the large number of pre-existing T-positive nuclei.

The *ts*101* mutation affects an early stage of the SV40 infectious cycle in productive monkey cells (13) and abortive mouse cells (14). At restrictive temperature in monkey cells, *ts*101* virions are similar to wild-type virions in adsorption and cytoplasmic penetration (13), in nuclear penetration (K. Hirai, J. Robb, and V. Defendi, manuscript submitted for publication), and in susceptibility to deoxyribonuclease digestion within the nucleus. However, the integration of virion DNA (K. Hirai, J. Robb, and V. Defendi, manuscript submitted for publication), the synthesis of SV40 messenger RNA (J. Robb and R. Lopez, manuscript in preparation), T antigen, viral DNA synthesis, and virion (V) antigen (13) are inhibited. When monkey cells are infected at restrictive temperature with *ts*101* DNA I purified free of virion proteins, the first cycle infection is normal, but the progeny viruses are still mutant (13). Although the initiation of T-antigen synthesis in BALB/3T3 cells acutely infected with *ts*101* virions is both host range inhibited (T-positive wild-type nuclei at 33C/*ts*101* nuclei at 33 C = 10 to 20) and temperature-sensitive (T-positive *ts*101* nuclei at 33C/*ts*101* nuclei at 39 C = 50, wild-type 33 C/wild-type 39 C = 1), abortive, and stable transformation are only host-range inhibited (14). Both types of transformation frequency are decreased 10-fold compared to wild-type virion infection at 33 and 39 C. My working hypothesis is that a virion protein is the affected protein in the *ts*101* mutant and this protein must be removed or activated, or both, before SV40 gene activity can be initiated (13). The identity of the mutant virion protein is presently not known.

The data presented in this investigation indicate that the appearance of T antigen in *ts*101* virion transformed BALB/3T3 and Swiss/3T3 cells is temperature sensitive, suggesting that the regulation of T antigen in the transformed

TABLE 2. *Superinfection of normal and transformed mouse 3T3 cells at 39 C with wild-type and ts*101 virions and DNA I*

Cells	T antigen-positive nuclei at 72 h after infection (%)				
	Mock infection	Virion infection[a]		DNA I infection[a]	
		Wild type	*ts*101*	Wild type	*ts*101*
BALB/3T3	0	26[b]	0.02[c]	0.11[c]	0.13[c]
SEA-45 (WT)	91	94	92	90	92
BTM-101-1	21	72	20	22	18
STM-101-13	19	79	17	19	21

[a] Virion MOI = 100; 0.2 μg of DNA I was adsorbed per culture.
[b] Values are the average of duplicate cultures counting 2,000 cells per culture.
[c] These values were determined by counting 20,000 cells per culture. SEM, ±15%.

cells is mediated by the *ts*101* protein, a virion protein. The T antigen produced after *ts*101* infection of productive monkey TC7 and abortive mouse BALB/3T3 cells is probably not itself temperature sensitive for the following reason. When wild-type and *ts*101* virion or DNA infected TC7 or BALB/3T3 cells are shifted from permissive to restrictive temperature after the initiation of T-antigen synthesis (12 to 48 h), the T antigen in both *ts*101* and wild-type infected cells continues to accumulate equally for at least 48 h as assayed by immunofluorescence.

The most likely explanation for the disappearance of T antigen at 39 C in the *ts*101* transformed cells is the inhibition of T-antigen synthesis, although an increased rate of degradation cannot be ruled out by these data. The fact that the expression of T antigen is somewhat variable at 39 C in one *ts*101* transformed clone (BTM-101-2) and one wild-type transformed clone (BTW-1) suggests that a cellular function(s) may be involved in the regulation of T-antigen synthesis and that this function(s) may be variable between transformed cell lines. Another possibility to be considered is the cells transformed by *ts*101* virions at 33 C may be a special population as compared to wild-type transformed cells because of the host-range inhibition of stable transformation by *ts*101* virions at 33 C (14). If this possibility is true, the cellular component in the regulation of T antigen may qualitatively or quantitatively, or both, differ from that in wild-type transformed cells (i.e., the observed differences between *ts*101* and wild-type transformed cells could reflect an altered cellular functon that originally permitted stable transformation by *ts*101* virions).

The *ts*101* protein is probably a *cis*-acting protein, acting upon the virion DNA with which it is associated, because *ts*101* neither complements nor can be complemented by other temperature-sensitive SV40 mutants in mixed virion infections at restrictive temperature (13, 16). In support of this viewpoint is the finding that T antigen is synthesized at restrictive temperature in the *ts*101* transformed cells when they are superinfected by wild-type virions. The regulatory effect of the *ts*101* protein on T antigen synthesis may be that of an inhibitor acting on the resident genome rather than that of a diffusible inhibitor, although these data do not rule out a diffusible cellular inhibitor that acts only on the endogenous or integrated *ts*101* genome.

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