NOTES

Replication of Chinese Hamster Embryo Cells Transformed by Temperature-Sensitive T-Antigen Mutants of Simian Virus 40

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Chinese hamster embryo cells transformed by simian virus 40 temperature-sensitive T-antigen mutants replicated when confluent at 40.5°C, regardless of the selection method, selection temperature, or virus strain used.

Continual expression of T antigen appears to be required for simian virus (SV40)-induced transformation because a variety of cell types transformed at permissive temperatures by SV40 mutants which encode a temperature-sensitive T antigen (*tsA* mutants) return to a more normal phenotype at restrictive temperatures (2, 5, 9, 14). Reduced [³H]thymidine incorporation by some of these "normal" *tsA* mutant-transformed cell lines at the restrictive temperature suggests that the cells have entered a normal resting state in the G₁ (G₀) phase of the cell cycle and has been taken as evidence that T antigen maintains the transformed state by directly initiating cell DNA synthesis (1, 3, 8).

We have recently shown that Chinese hamster (ChH) embryo cells can be transformed by SV40 *tsA* mutants at 37°C and revert to "normal" at 40.5°C (11). However, in contrast to the results obtained with other cell types in other laboratories, normal *tsA* mutant-transformed ChH embryo cells do not G₁ arrest when confluent on plastic at 40.5°C. Replication continues, and the monolayers are maintained at low density by balanced cell replication and cell death. This dissociation between cell replication and transformation questions whether transformation is controlled by T antigen at the level of initiation of DNA synthesis.

In this study, we investigate the following possible explanations for the difference in replication of SV40 *tsA* mutant virus-transformed ChH embryo cells and cells derived in other laboratories. (i) The method used to select the transformed cells could influence their response to the mutation (10, 13). (ii) Transformation at 37°C, instead of a lower temperature, could select cells less responsive to the mutation. (iii) The location of the mutation in the *tsA* strain that we used could produce a change in T antigen not observed in the T antigens of other mutants. We find that none of these possibilities

explains the temperature insensitivity of *tsA* mutant-transformed ChH embryo cell replication.

The influence of selection method was tested by infecting sparse or confluent ChH cells with plaque-purified *tsA58* or wild-type (WT) virus (multiplicity of infection = 10) at 37°C. The cells were maintained in these growth states for 2 weeks after infection and then rapidly passaged until they became 100% T antigen positive and transformed. If the cell growth state during transformation had produced A-type (temperature-independent) cells which replicate when confluent at the restrictive temperature, N-type (temperature-independent) selection might yield cells capable of arresting in G₁. Clonal analysis was not performed, since it had been reported that polyoma *tsA* mutant infection of growing or confluent normal rat cell cultures gives rise to 100% A-type or 100% N-type transformants, respectively (10).

The nuclear immunofluorescence of the *tsA*-transformed ChH embryo cells selected by either method was reduced at high temperature. Cell morphology and cell underlapping returned to more normal patterns at 40.5°C as well. As shown in Table 1, both the A-type and N-type *tsA* mutant virus-transformed cell lines were temperature sensitive for growth in low (1%) serum. These characteristics were temperature independent in WT virus-transformed cells. Despite their temperature-sensitive T antigen, morphology, and growth, the *tsA* mutant-transformed cells incorporated [³H]thymidine and had many S and G₂ + M phase cells when confluent at high temperature. Balanced cell replication and cell death maintained their low-density monolayers at 40.5°C (data not shown). Similar results were obtained when high (10%) serum was used. Thus, the selection method was not a major factor in the cell behavior observed. Others have also reported that selection method

has little effect on the temperature sensitivity of *tsA* mutant-transformed ChH cell lines (6).

The effect of selection temperature was investigated first by evaluating the ability of *tsA58* to initiate transformation at 33 or 37°C. Selection of rare and perhaps less temperature-sensitive transformants at 37°C would be suggested if the transformation frequency were lower at 37 than at 33°C. Overgrowth of a normal cell monolayer by transformed cells in low serum was the transformation assay (5). Figure 1 shows that the frequency of transformation by *tsA58* is approximately equivalent at both 33 and 37°C, whereas 40.5°C is clearly a nonpermissive temperature. Thus, transformation at 37°C is not a rare event.

To determine whether ChH cells selected at 33 or 37°C had different replication patterns at 40.5°C, cell lines were isolated at either temper-

ature. Confluent monolayers were infected (multiplicity of infection = 10) for 1 h at 37°C and then shifted to 33°C or held at 37°C. The cells were first subcultured 18 h after infection, then twice a week until they became 100% T antigen positive and appeared transformed. The resulting uncloned *tsA* mutant-transformed cell lines exhibited temperature-dependent changes in cell morphology and saturation density. Syrian hamster fibroblasts transformed by SV40 *tsA* mutants at 37°C or derived from tumors induced in hamsters (body temperature = 37°C) also have a temperature-sensitive phenotype (2). However, our *tsA* mutant ChH transformed cell lines differ in that they have reduced T-antigen immunofluorescence and fail to enter a resting state at the nonpermissive temperature (Table 2). Similar results were obtained with cells trans-

TABLE 1. Effect of selection method on the temperature sensitivity of SV40-transformed ChH embryo cells

Selection method	Transforming virus	Saturation density (no. of cells/cm ² × 10 ⁻⁴) ^a			Labeled nuclei (%) ^b			Cells in S phase (%) ^c		
		37°C	40.5°C	Ratio 37°C/40.5°C	37°C	40.5°C	Ratio 37°C/40.5°C	37°C	40.5°C	Ratio 37°C/40.5°C
A type	None	6	5	1.2	7	5	1.4	4	3	1.3
	WT	13	11	1.2	36	42	0.9	23	28	0.8
	<i>tsA58</i>	18	4	4.5	45	39	1.2	17	15	1.1
N type	WT	19	21	0.9	31	39	0.8	18	24	0.8
	<i>tsA58</i>	17	6	2.8	29	36	0.9	14	17	0.8

^a Cells maintained at 37°C were seeded at 2×10^4 /cm² into Eagle minimal essential medium containing 10% fetal bovine serum overnight. The cultures were refed with medium containing 1% serum, and half were shifted to 40.5°C. The medium was not changed during the 6-day experiment. Daily cell counts were performed using a Coulter Counter.

^b Autoradiographic determination of [³H]thymidine (1 μ Ci/ml) uptake in 1-h pulse at time of saturation density.

^c Determined by analysis of DNA content, using a Flow microfluorometer as previously described (12).

FIG. 1. Transformation of ChH cell monolayers at various temperatures by SV40 in low serum. Confluent normal cell monolayers were established in minimum essential medium containing 5% fetal bovine serum. They were mock infected or infected with 20 PFU of *tsA58* or WT virus per cell at 37°C for 1 h, shifted to 33, 37 or 40.5°C and fed with minimum essential medium containing 2% fetal bovine serum twice a week. Three weeks later, they were fixed in methanol and stained with Giemsa.

TABLE 2. *Effect of selection temperature and virus strain on the temperature sensitivity of SV40-transformed ChH embryo cells*

Selection temp	Transforming virus	Saturation density (no. cells/cm ² × 10 ⁻⁴) ^a			Labeled nuclei (%) ^b			Cells in S phase (%) ^c		
		PT	RT	Ratio PT/RT	PT	RT	Ratio PT/RT	PT	RT	Ratio PT/RT
37°C	0	8	6	1.3	6	4	1.5	4	5	0.8
	WT	30	26	1.2	27	36	0.8	35	48	0.7
	<i>tsA58</i>	25	6	4.2	23	40	0.6	28	34	0.8
	<i>tsA30</i>	30	9	3.3	33	42	0.8	28	38	0.7
33°C	<i>tsA58</i>	31	5	6.2	30	41	0.7	29	35	0.8

^a The procedure was the same as in Table 1, except that the cultures were maintained and tested in 5% serum. The permissive temperature (PT) was the temperature at which the cells were selected, maintained, and tested. The restrictive temperature (RT) was 40.5°C in all cases.

^{b,c} Same as Table 1.

formed by *tsA30*, which maps in a different *Hind*II + III restriction fragment than *tsA58* (4).

These studies demonstrate that the selection temperature, the selection method after infection, or the virus strain used probably does not account for the observed differences in our results and those of others. Although it is difficult to understand why ChH embryo cells should differ significantly from Syrian hamster cells (3) or ChH lung fibroblasts (2, 8), it is possible that DNA synthesis of certain cell types is more sensitive to any residual T-antigen activity or to mitogenic stimulation of small t antigen (7, 12). Alternatively, T antigen could be important for viability of SV40-transformed cells at high temperature, with cell replication occurring secondary to loss of cells from the monolayer. Others have also noted decreased viability of SV40 *tsA* mutant-transformed rat cells, but not normal or WT virus-transformed rat cells, at high temperature (13). Cocultivation of the *tsA* mutant-transformed ChH embryo cells on normal ChH embryo cell monolayers has been found to prevent both the persistent cell replication and cell death (Robinson and Lehman, manuscript in preparation) which will provide a system in which to investigate the reason for the apparent dissociation between T-antigen control of cell replication and transformation.

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