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Transformation of BALB/c-3T3 Cells by *tsA* Mutants of Simian Virus 40: Effect of Transformation Technique on the Transformed Phenotype

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Simian virus 40 *tsA*-transformed BALB/c-3T3 cells isolated as foci of overgrowth in liquid medium were compared with those isolated as colonies in soft agar. Efficiencies of transformation were equivalent in the two procedures. Cells isolated as foci were able to grow in agar and vice versa. No difference in temperature sensitivity of the transformed phenotype was detected when *tsA* transformants selected in agar were compared with those selected as foci. The use of the two different transformation procedures, then, did not form the basis for generation of different transformed phenotypes, and transformants generated in both ways were dependent upon expression of the *A* gene for maintenance of the transformed state.

Recent studies (1-3, 5, 6, 10) indicate that the protein encoded by the Simian virus 40 (SV40) *A* gene (T antigen) is required for maintenance of several parameters of transformation in certain types of SV40-transformed cells. In these studies, cells transformed by SV40 *tsA* mutants at the permissive temperature were assayed for various manifestations of the transformed phenotype at the permissive and nonpermissive temperatures. In our analysis (1) of SV40-transformed BALB/c-3T3 mouse cells, 15 of 16 *tsA* transformants were temperature sensitive for ability to overgrow a monolayer of normal cells, whereas 3 of 3 *wt* transformants were not. Cuzin and colleagues (7, 9), however, have reported that rat fibroblasts transformed by *tsA* mutants (or *tsa* mutants of polyoma virus) may exhibit either a temperature-sensitive or a temperature-resistant phenotype, depending upon the procedure used for transformation. Procedures, such as cloning in agar, which arrest the growth of normal cells appear to favor isolation of temperature-resistant transformants (A type), whereas procedures, such as isolation of foci in liquid, which permit division of cells after infection appear to favor the isolation of temperature-sensitive transformants (N type) (8). Since the BALB/c-3T3 transformants we analyzed were isolated as foci of dense overgrowth in liquid, a procedure which did not arrest growth of normal cells, the present study was initiated to examine the effect of transformation procedure on the phenotype of *tsA*-transformed mouse cells.

BALB/c-3T3 cells were infected with either *wt* or *tsA* virus. As we had previously demonstrated that the position of the mutation site within various *tsA* mutants did not influence the transformed phenotype (1), only one *tsA* mutant, *tsA*58 (obtained from Peter Tegtmeyer), was used. Infection and subsequent manipulations were performed in a manner analogous to that employed by Seif and Cuzin (9) and Rassoulzadegan et al. (7) in the production of A- and N-type transformants of Fr-3T3 rat cells by polyoma or SV40 infection. Briefly, cells growing at 33°C were infected at multiplicities of 0.08 to 50 PFU per cell. Twenty-four hours later the cells were trypsinized and plated at a density of 2×10^3 cells per cm^2 in tissue culture dishes containing liquid medium (1) or at the same density in soft agar (4). After 4 to 6 weeks at 33°C, dense foci overgrowing the monolayers in liquid medium and macroscopically visible colonies growing in agar were scored as transformants. The number of transformants detected by each assay procedure is shown in Table 1. Transformation occurred with approximately the same frequency (about 10^5 PFU per transforming unit) in both procedures.

Several isolated colonies were picked under both assay conditions, cloned twice at 33°C in liquid and in soft agar, respectively, and shown by indirect immunofluorescence assay to contain SV40 T antigen. A total of 15 transformants isolated as foci in liquid (11 transformed by *tsA*58 and 4 transformed by *wt* SV40) and 14 selected in agar (8 transformed by *tsA*58 and 6 transformed by *wt*) were studied in more detail. These transformants are enumerated in Table 2 together with the virus-to-cell ratio at which the original infections were performed.

To assess the role of the SV40 *A* gene product in the maintenance of the transformed state, the various transformed lines were tested for tem-

TABLE 1. *Transformation as assessed by formation of foci of overgrowth on plastic and colonies in soft agar*

Virus	Multiplicity of infection	Transformed foci for colonies/10^5 cells[a]	
		Foci of overgrowth	Colonies in agar
wt	50	17	18
	10	3	3.5
	2	1.5	1.5
	0.4	0.5	0.5
tsA58	10	8	14.5
	2	2.5	9.5
	0.4	2	1.5
	0.08	0	1

[a] Average of duplicate infections.

perature sensitivity of the transformed phenotype. This was done by plating the transformed cells on confluent monolayers of normal cells and comparing their abilities to form colonies at the permissive and nonpermissive temperatures (1). Untransformed BALB/c-3T3 cells plated on plastic form colonies a single cell thick; when seeded onto a confluent monolayer they are unable to form colonies. Transformed cells, on the other hand, have the ability to overgrow one another and can form dense colonies when seeded onto a normal monolayer. The results of a series of monolayer overgrowth experiments are shown in Table 2. In these experiments 33°C was taken as the permissive temperature, and 39.3 to 39.5°C was taken as the restrictive temperature. Comparison of the efficiency of plating, or colony-forming ability, on monolayers at the permissive temperature with that at the nonpermissive temperature revealed that the permissive-nonpermissive ratios were 5 or less for the majority of *wt* transformants and 60 or greater for the majority of *tsA* transformants. The method of transformation appeared to have little effect on the transformed phenotype; most cells transformed by *tsA* mutants were temperature sensitive, whereas those transformed by *wt* were relatively temperature resistant, regardless of the transformation procedure.

As an additional test of the temperature sensitivity of the transformed phenotype, we tested these cell lines for anchorage dependence of multiplication at the permissive and restrictive temperatures. For this purpose cells were suspended as previously described (1) in either agar or purified agarose. All transformed lines, whether originally selected as foci in liquid or as colonies in agar, grew in agar (efficiency of plating ranging from 5 to 50% versus 0.001% for normal 3T3) at 33°C. Conditions satisfactory for testing growth at the restrictive temperature,

TABLE 2. *Effect of temperature on the abilities of focus-selected and agar-selected transformed cells to grow on normal monolayers*

Virus	Selection	Multiplicity of infection	Line	Efficiency of plating[a]
tsA58	Foci of overgrowth	10	A58F10a	≥125, ≥1,000
			A58F10c	≥1,000, ≥250, ≥1,000
			A58F10d	325
			A58F10e	≥1,000
			A58F10f	225
			A58F10g	≥1,000
		2	A58F2a	400
			A58F2b	≥1,000
			A58F2c	450
			A58F2d	430
		0.4	A58F.4a	625
	Colonies in agar	10	A58A10a	≥1,000, ≥1,000
			A58A10b	≥1,000, ≥270
			A58A10c	≥1,000, ≥1,000
		2	A58A2a	≥625
			A58A2b	≥60, ≥40
			A58A2c	≥300, ≥1,000
		0.4	A58A.4b	≥1,000, ≥1,000
		0.08	A58A.08a	≥1,000, ≥1,000
wt	Foci of overgrowth	50	WtF50a	1.2, 1.7
		10	WtF10a	1.2, 1.8
			WtF10b	1.8, 1.1
			WtF10c	1.4
	Colonies in agar	50	WtA50a	3.7, 5.0
			WtA50b	2.3
			WtA50c	3.0, 10.0
		10	WtA10a	5.0, 5.9
			WtA10b	3.0, 3.0
			WtA10c	20.0, 5.0

[a] Ratio of the efficiency of plating on a normal monolayer obtained at the permissive temperature to that obtained at the nonpermissive temperature. For those lines which were assayed more than once, the result of each experiment is shown.

however, could not be achieved. Agar at 39.5°C proved too stringent (many *wt* transformants did not grow), whereas assays in agarose gave variable results (some *wt* transformants grew poorly when plated at 39.5°C, whereas some plated with high efficiency at 39.5°C on some occasions but poorly on others). In this regard it may be noted that BALB/c-3T3 cells grow poorly in liquid medium at temperatures above 40°C; somewhat lower temperatures may be harmful to BALB/c-3T3 derivatives plated in semisolid medium.

We have used, then, two methods for the isolation of SV40-transformed BALB/c-3T3 cells. In one, infected cells are plated in liquid medium under conditions which permit normal cells to undergo multiple rounds of division. In

the other, cells are plated in soft agar, a circumstance which arrests the growth of normal cells. Several observations indicate that the transformed phenotype is not differentially affected by these two procedures. First, the frequency of transformation did not differ appreciably in the two methods. Second, all cell lines isolated as foci were able to grow well in agar at the permissive temperature, and all cell lines selected in agar were capable of efficiently forming colonies of overgrowth on normal cells at this temperature. Finally, no difference in temperature sensitivity of the transformed phenotype (assessed by the ability to overgrow a normal monolayer) could be detected when *tsA* transformants isolated in agar were compared with those isolated as foci. The latter finding is in contrast to that of Rassoulzadegan et al. (7), who found that two of three SV40 *tsA*-transformed rat lines selected as foci were temperature sensitive, whereas four of four *tsA* transformants selected in agar were temperature resistant. As the infection and subsequent manipulations were performed similarly in the two studies, the cause of this discrepancy is not obvious, but it may be related to differences in virus-cell ratios at the time of infection or to the nature of the host cells used. In regard to the former, it may be noted that the *tsA* transformants analyzed here were infected at multiplicities of 0.08 to 10 PFU per cell, whereas Rassoulzadegan et al. (7) transformed at multiplicities of infection of 30 to 100. It is conceivable that transformation at the higher multiplicities of infection results in a greater frequency of multiple integrations of SV40 DNA molecules in nongrowing cells than in cells which can divide before integration. This in turn might lead to increased levels of T-antigen synthesis in cells in which growth is arrested soon after infection. We have previously noted (1) that the temperature-resistant phenotype in one *tsA* transformant was correlated with an increased intracellular level of T antigen. In regard to differences in the host cell, it is of interest that R. G. Martin, V. P. Setlow, and C. A. Edwards (J. Virol., in press) have observed that the majority of *tsA*-transformed Chinese hamster lung cells display a temperature-sensitive phenotype irrespective of the state of growth of cells after infection.

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