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Growth Control in Simian Virus 40-Transformed Rat Cells: Temperature-Independent Expression of the Transformed Phenotype in *tsA* Transformants Derived by Agar Selection

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Fisher rat fibroblasts (FR 3T3), transformed with the *tsA30* mutant of simian virus 40 and selected by colony formation in soft agar, maintained the transformed phenotype at high temperature, whereas most transformants isolated from foci were found to undergo a phenotypic reversion toward the normal state in their saturation density, ability to grow in soft agar, and rate of 2-deoxyglucose transport. The temperature-independent phenotype observed in agar-selected transformants was not due to a reversion of the viral mutation. These results, similar to those previously obtained with polyoma virus *tsa* mutants, further suggest that two distinct mechanisms may operate in both cases for maintaining the transformed phenotype. Immunofluorescence studies suggested a different regulation of T antigen synthesis in these two classes of transformants.

Cells transformed by the early *tsA* mutants of simian virus 40 (SV40) have been shown in several instances to express the transformed phenotype at low, but not at high, temperature (1, 2, 7, 8, 12, 16). The conclusion from these studies was that expression of at least part of the transformed phenotype is under control of a virus-coded gene product. Opposite conclusions, however, had been reached for the corresponding early *tsa* mutants of polyoma virus (3-5). In addition, both temperature-dependent and -independent transformants were in fact obtained using a given SV40 *tsA* mutant (1, 16).

We have shown recently (15) that cell lines exhibiting either a temperature-dependent or a temperature-independent phenotype could be derived from the same batches of *tsa* polyoma-infected rat fibroblasts, and that the selection procedure used for isolating the transformants was critical: isolation from colonies grown in soft agar led to temperature-independent transformants (type A), whereas most cell lines derived from foci were strongly temperature dependent (type N). We were therefore led to the conclusion that the transformed phenotype can be maintained by two distinct molecular mechanisms, only one of them being dependent on the viral *a* gene product. This hypothesis is consistent with all previous reports (1-5, 7, 10, 12, 16), since all SV40 *tsA* transformants studied so far have been isolated from foci, whereas polyoma *tsa* transformants have always been derived from agar colonies. It is further confirmed by the present study of wild-type and *tsA30* SV40 transformants derived either from agar colonies or from foci.

(This work constitutes part of the thesis submitted by M.R. for the Doctorat at the University of Nice.)

MATERIALS AND METHODS

Cell lines and viruses. The establishment, the main characteristics, and the growth conditions of the Fisher rat fibroblast (FR 3T3) cell line were previously described (15). Wild-type SV40 and the *tsA30* mutant (17) were grown in CV1 monkey cells at a low input multiplicity (less than 0.01 PFU/cell).

Transformation procedure. Actively growing FR 3T3 cells were infected at 33°C at multiplicities ranging from 30 to 100 PFU/cell. As in the case of polyoma virus infection (15), transformed lines were isolated either from foci (type N) or from colonies grown in soft agar (9) (type A). Four classes of transformants were thus obtained after at least two successive clonal isolations and designated as WT-A, WT-N, *tsA30*-A, and *tsA30*-N, respectively. Transformation frequencies were in the same range (0.4 to 0.8% of the cells infected at a multiplicity of 100 PFU/cell) in all cases.

Polyethylene glycol-mediated cell fusion. Subconfluent cultures were prepared by seeding 5×10^5 cells of each parent in 60-mm plates (Nunclon) at 33°C. After 24 h, the cell layer was washed once with Tris-buffered saline, and a 50% (vol/vol) solution of polyethylene glycol 1000 (Merck) in Dulbecco-modified Eagle medium was added. After 1 min of exposure to polyethylene glycol, the cultures were washed six times with Tris-buffered saline and further incubated in regular culture medium. For plaque assays of infectious centers, the cells were overlaid with agar medium 24 h after polyethylene glycol treatment as described by Mertz and Berg (11).

Transport of 2-deoxyglucose. Cells were seeded on plastic cover slips (Thermanox, Lux Corp.) at a density of 5×10^4 cells per cover slip (9 by 35 mm). They were grown for 70 h at either 33 or 40.5°C, washed with prewarmed phosphate-buffered saline, and incubated in phosphate-buffered saline in the presence of 2-[^{3}H]deoxyglucose (Amersham Radiochemical Centre; 15 to 25 Ci/mmol, diluted in cold deoxyglucose for final concentrations of 0.1 nM and 0.5 μCi/ml) at the same temperature as for cell growth. At the indicated times, cover slips were quickly washed with ice-cold phosphate-buffered saline, dried, and counted in toluene-2,5-diphenyloxazole (PPO)-1,4-bis-[2-(phenyl)-oxazolyl]benzene (POPOP) scintillation fluid.

Assay for SV40 T antigen. SV40 T antigen was detected by immunofluorescence as previously described for polyoma T antigen (13), using an SV40 anti-T serum kindly provided by M. Kress (Institut de Recherches Sur le Cancer Villejuif).

RESULTS

Expression of the transformed phenotype at 40.5°C in N and A transformants. The following characteristics were studied at 33 and 40.5 to 41°C for one out of three or four independent clonal isolates of each type: saturation density of growth in 10% calf serum; ability to grow in soft agar (9); and rate of 2-deoxyglucose uptake (18). Results are presented in Fig. 1 and Table 1 and Fig. 2, respectively. None of these characters appears to be temperature sensitive in the *tsA30*-A1 line. In contrast, *tsA30*-N1 cells, like other *tsA* transformants previously isolated from foci (1, 2, 7, 10, 12, 16), are clearly reverting toward the normal phenotype at 40.5°C.

These studies were subsequently extended to the other two to three lines of each type (data not shown). Four out of four *tsA*-A transformants were found to express the transformed phenotype at 40.5°C. Among three *tsA*-N lines isolated from foci, two were temperature dependent for all the transformation characters studied, whereas the third one behaved like the clones derived by agar selection. The pattern of temperature-dependent versus -independent maintenance in these various transformants thus appears quite similar to that found for transformed lines isolated after *tsa* polyoma virus infection (15). Whether the same phenomenon extends to early mutants other than SV40 *tsA30* and polyoma *tsa* is presently being tested.

SV40 T antigen. SV40 T antigen could be detected by immunofluorescence in all of the transformants at 33°C, as well as by immuno-

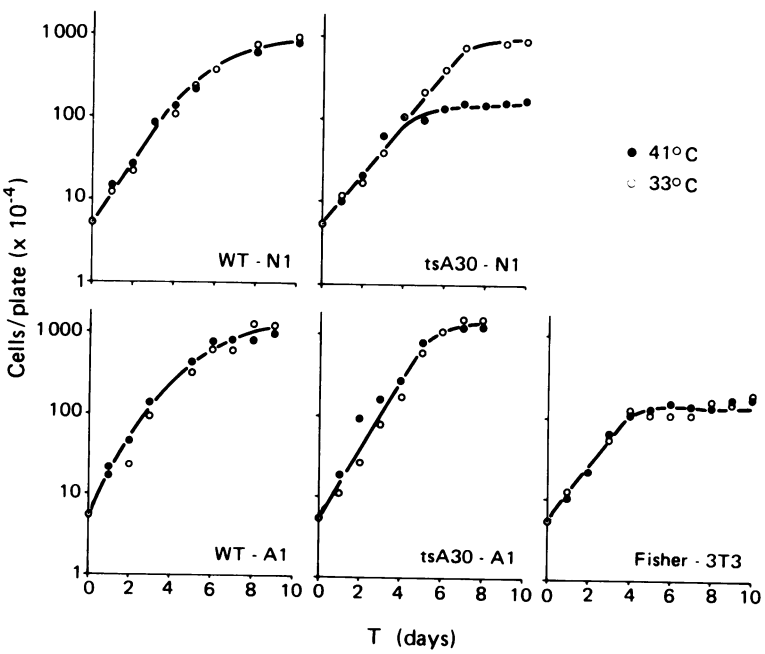

FIG. 1. *Growth at 33 and 41°C of FR 3T3 cells and of representatives of the various classes of SV40 transformants. Cells were seeded at 33°C at a density of 5×10^4 cells per 60-mm petri plate (Nunclon) in Dulbecco-modified Eagle medium (GIBCO) supplemented with 10% calf serum. At 24 h after seeding (time 0), some of the plates were transferred to 41°C. The medium was changed every other day at cell densities below 2×10^6 cells per plate, every day between 2×10^6 and 8×10^6 cells per plate, and twice a day above that density. At the indicated times, cells were trypsinized and counted.*

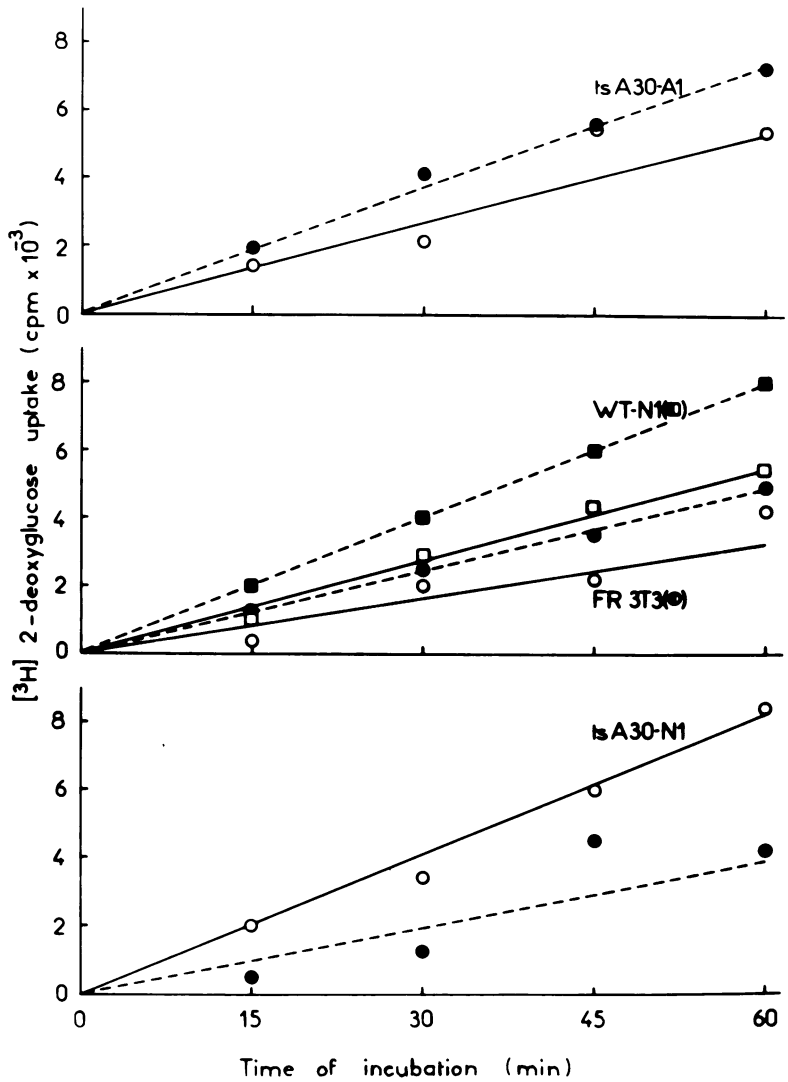

FIG. 2. *Kinetics of 2-deoxyglucose uptake in normal and SV40-transformed FR 3T3 cells. Cells were seeded on cover slips, grown, and assayed for 2-deoxyglucose uptake as indicated in the text. Lines represent the average of several independent measurements. Rates of uptake were not significantly different in WT-A and WT-N transformants. Symbols:* (○——○ *or* □——□) *33°C;* (●- - -● *or* ■- - -■) *40.5°C.*

precipitation of radioactively labeled proteins (6).

Two distinct patterns of immunofluorescent staining were observed. In one set of cell lines, the distribution of the characteristic nuclear staining was homogeneous, all cells being equally labeled. In a second group, a clearly heterogeneous pattern was observed; about half of the cells were stained, and the others were weak to negative. This heterogeneous distribution was not due to a clonal heterogeneity of the population; when several subclones were isolated, all of them still exhibited the same pattern of staining. Experiments are in progress to test the hypothesis that this distribution might correspond to a periodic expression of the antigen in the cell cycle.

All transformants of type A tested were of the homogeneous type, whereas all the *tsA*-N lines, and all but one of the WT-N lines, were heterogeneous.

Presence of an endogenous *tsA* viral genome in the temperature-independent *tsA* transformants. To exclude the hypothesis that the temperature-independent A transformants obtained by agar selection after *tsA* virus infec-

TABLE 1. *Saturation density and ability to grow in soft agar of FR 3T3 cells and transformed derivatives of the various classes*

Cell line	Temp (°C)	Saturation density[a]	Growth ability in agar[b]
FR 3T3	33	5.0	<0.002
FR 3T3	40.5	5.1	<0.002
WT-A1	33	≥45	72
WT-A1	40.5	≥45	72
tsA30-A1	33	≥45	72
tsA30-A1	40.5	≥45	94
WT-N1	33	≥45	24
WT-N1	40.5	≥45	34
tsA30-N1	33	≥45	24
tsA30-N1	40.5	5.5	<0.002

[a] Cells were grown as described in the legend to Fig. 1. Cell densities at the growth plateau are expressed as cells × 10^4 per square centimeter.

[b] Growth ability in agar was measured by seeding 5 × 10^4 cells in soft agar (8) and counting the number of microcolonies per microscope field 8 days later. Values are expressed as the percentage of the cell input.

tion were transformed by spontaneous revertants, the endogenous viral genomes were rescued from these cells by fusion with permissive monkey cells.

Plates were seeded with a 1:1 mixture of CV1 monkey cells and tranformants of the different types, then incubated at 33°C. After 24 h, they were treated with polyethylene glycol (see above), and half of the cultures were shifted to 39.5°C. All plates were overlaid 24 h later with agar medium (11), and plaques were counted after 3 to 4 weeks.

As shown in Table 2, both wild-type and *tsA* transformants yielded infectious virus when kept at 33°C after fusion. Wild-type transformants produced approximately equal numbers of infectious centers at 33 and 39.5°C, whereas none of the *tsA*-A transformants produced any plaque at 39.5°C. Virus produced under these conditions from *tsA*-A as well as from *tsA*-N transformants at 33°C was temperature sensitive for growth when subsequently tested on CV1 cells at 33 and 39.5°C (data not shown). All the *tsA*-A transformants isolated appear therefore to carry mutant endogenous viral genomes.

TABLE 2. *Production of infectious centers upon fusion of agar-selected SV40 transformants with permissive monkey cells*

Cell population[a]	No. of plaques[b] at:	
	33°C	39.5°C
CV1 alone	0	0
WT-A1 + CV1	44	52
tsA30-A1 + CV1	26	0
tsA30-A2 + CV1	134	0
tsA30-A3 + CV1	34	0
tsA30-A4 + CV1	57	0

[a] Cells were seeded and treated with polyethylene glycol as described in the text.

[b] Plaques per petri plate (5 × 10^5 cells of each parent line).

DISCUSSION

Results presented in this report further confirm that the published observations on the maintenance of transformation in polyoma *tsa* and SV40 *tsA* transformants (1–5, 7, 10, 12, 16) are not in fact contradictory. In both cases, two types of transformed cells (A and N) can be induced after virus infection. Type A transformants are exclusively selected by cloning in agar medium. Only in N cells is the activity of the viral gene product affected by the *ts* mutation continuously required for the expression of the transformed phenotype.

A similar situation is thus found in a strictly nonpermissive system (SV40 and rat fibroblast [8, 14]) and in the semipermissive polyoma-rat cell interaction. Previous results on polyoma transformation (15) cannot therefore be explained on the basis of the semipermissivity of the rat cells and of the presence in transformants of autonomous copies of the viral DNA.

In both cases, a critical choice appears to be made after infection, leading to the establishment of either the A or the N state. Results to be reported elsewhere (M. Rassoulzadegan, R. Seif, and F. Cuzin, manuscript submitted for publication) strongly suggest that, after polyoma infection, this choice depends on the growth of the cells at early times after infection. Cells arrested in G0, in the absence of anchorage, as well as in a confluent monolayer on a solid substratum, are converted into A transformants, and actively growing cells are converted into N derivatives.

It was recently suggested by Brockman (1) that the occurrence of a temperature-independent transformed phenotype observed in one *tsA30*-transformed mouse cell line was correlated with an overproduction of T antigen. Such an overproduction was not found in several independent *tsA30*-A FR 3T3 transformants (6). Our results still suggest, however, that distinct modes of regulation of T antigen synthesis might be associated with the A and N mechanisms of maintenance. First, the heterogeneous immunofluorescence staining, which seems to be characteristic of N transformants (see Results), may indicate a cell cycle dependence, which remains to be confirmed. On the other hand, immunoprecipitation studies on radioactively labeled T antigen in A and N transformed cells (6) led us to the conclusion that, at high temperature,

tsA30-A transformants keep synthesizing the various immunoprecipitable T antigenic polypeptides, whereas their synthesis is turned off in *tsA30*-N cells. A complex picture of the regulation of early viral protein synthesis, correlated with the expression of the transformed phenotype, would thus be outlined. This regulation could be dependent in N transformants both on the activity of an early viral gene product and, possibly, on a given phase of the cell cycle.

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