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Temperature-Sensitive Growth Regulation in One Type of Transformed Rat Cells Induced by the *tsa* Mutant of Polyoma Virus

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A fibroblast line of the 3T3 type with a low saturation density was established from Fisher rat embryo cells. After infection with either wild-type or *tsa* mutant polyoma virus, transformants were isolated and cloned at 33°C on the basis of their ability either to grow as dense foci on plastic in liquid medium (type N) or to form colonies in soft agar (type A). Polyoma T antigen was detected in all of the transformed lines. The following growth characteristics were studied for both types at 33 and 41°C: saturation density, growth in soft agar and at a low serum concentration, colony-forming ability, and generation time. *tsa*-N transformants behaved at 33°C similarly to transformed cells, but reverted at 41°C to the nontransformed phenotype for all of these characters. *tsa*-A transformants and all of the wild-type transformants exhibited the transformed phenotype at both low and high temperatures. These results led us to distinguish at least two types of virus-induced transformants. In one of them, the activity of the protein affected by the *tsa* mutation appears to be necessary for the expression of several of the characters defining the transformed state.

Converging evidence from a number of laboratories has suggested that the product of simian virus 40 virus gene *A* may play a role in the maintenance of the transformed phenotype (3, 20, 23, 25, 32). Some of the characters that define the transformed state are not expressed at the restrictive temperature in cells transformed by temperature-sensitive (*ts*) mutants of this gene. The principle of this approach is straightforward enough. Nevertheless, answers are still ambiguous, as recently discussed by Dulbecco (5). The most striking discrepancy is found between results obtained for the closely related polyoma virus and simian virus 40.

The early region of both viruses is transcribed in stable transformants from integrated viral genomes (16, 17, 30) and codes in both cases for the respective viral T antigens (1, 26). Studies on the replication of viral DNA in permissive cells infected with *ts* mutants led to the conclusion that at least one of the functions of the early gene product is at the level of the initiation of replication (10, 31). After infection of nonpermissive cells with *ts* mutants at high temperature, the frequency of transformed colonies was in both cases found to be drastically reduced, as compared with wild-type (WT) infection (4, 7, 11, 19, 20, 23).

In spite of all of these similarities, opposite results were obtained when the growth characteristics of cells transformed with *ts* mutants were studied. As indicated above, simian virus 40 *tsA* transformants were found to recover a partially normal phenotype at high temperature. The same experiment with the corresponding *tsa* mutants of polyoma virus had previously led to the opposite conclusion: the transformed phenotype was maintained at high temperature, and the function of the polyoma *a* gene product was therefore considered as necessary for the establishment, but not the maintenance, of the transformed phenotype (4, 7, 11). Kimura then observed that several polyoma *tsa*-transformed rat cell lines were temperature dependent for growth at a low serum concentration (18).

Since polyoma virus and simian virus 40 are very similar viruses, it is important to establish whether they differ with respect to the function(s) involved in cell transformation. Differences in methodologies might alternatively account for some of the apparent discrepancies between results obtained with the two viruses.

It is, for instance, known (13) that the *tsa* mutants of polyoma virus are leaky at 38.5°C, the "restrictive" temperature used in these studies. The level of residual activity of the mutant protein in the transformants cannot be determined and might be sufficient for maintaining the transformed phenotype.

Alternatively, several types of transformation

events, or steps in the transformation process, could occur, due either to a viral function only or to the addition of cellular variations. The transformed phenotype could therefore result from various combinations of virus- and cell-mediated steps, not all of them being under the direct control of a viral gene product. The use of different types of "normal" cells (primary cultures, established lines) or of different ways of selecting transformants (growth in soft agar, as foci in liquid medium, at low serum concentration) may lead to different combinations of such events.

We planned a reinvestigation of the properties of WT and *tsa* polyoma virus transformants. Permanent cell lines were first freshly isolated from inbred rat embryos by the procedure of Todaro and Green (33). These cells, which grow well at 41°C, were cloned and kept at a minimal number of passages in culture. They were infected in parallel with WT and *tsa* polyoma virus. Transformants were isolated at 33°C by using two independent selection procedures: growth in soft agar (22) and ability to form a focus on plastic under liquid medium. Clonal isolates were in each case studied for their growth characteristics at 33 and 41°C.

MATERIALS AND METHODS

Virus. WT polyoma virus (strain A2) and the *tsa* mutant (12) were obtained from M. Fried. After plaque purification, they were grown in secondary mouse embryo cell cultures infected at a low multiplicity (less than 0.1 PFU/cell).

Cells. Primary rat embryo cultures were prepared by mincing 15-day-old embryos of Fisher rats, treating them with trypsin, and plating in Dulbecco-modified Eagle medium (GIBCO H21) supplemented with 10% fetal calf serum. From the primary cultures, permanent lines were established by subculturing them in medium containing 10% calf serum, as described by Todaro and Green (33) for mouse cells. The 3T3 type of schedule (33) was used (transfer every 3rd day at a density of 3×10^5 cells per 6-cm petri plate). The growth rate declined markedly during the first generations and began to rise later in a way quite similar to that reported for mouse cells. A doubling time equivalent to that of the primary culture was reached after 18 to 20 generations. At this time the cells could be cloned. After three successive isolations starting from morphologically flat colonies, an established line designated as Fisher rat 3T3 (FR 3T3) was obtained. These cells were serially transfered at 33°C in medium containing 10% calf serum.

Transformation. Actively growing FR 3T3 cells were infected with a multiplicity of 500 PFU of either WT or *tsa* polyoma virus per cell. Mock-infected controls were treated in the same way, except for the addition of virus. After 1 h at 33°C, fresh medium was added and the plates were further incubated at 33°C. After 24 h, cells were trypsinized, counted, and replated at a density of 5×10^4 cells per 6-cm-diameter petri plate (Nunclon) in liquid medium and at the same density in soft agar (22). No cytopathic effect could be detected in infected cultures at any time after infection. After 3 to 4 weeks at 33°C, the plates of the first group were scored for the presence of dense foci of cells overgrowing the monolayer, and the agar plates were scored for macroscopically visible colonies. Several isolated colonies were picked from different plates in both cases and cloned twice in succession, on plastic and in soft agar, respectively. Cells were thereafter grown at 33°C for a limited number of generations, and stocks were frozen in liquid nitrogen.

Autoradiographic determination of DNA-synthesizing cells. DNA-synthesizing cells were labeled by the addition to the growth medium of [^{3}H]thymidine (C.E.A., France; 10 to 20 Ci/mmol; final concentration, 20 μCi/ml). Cells were washed 60 min later, fixed with ice-cold 5% trichloroacetic acid, and rinsed with absolute ethanol. Ilford K5 emulsion, diluted 1:1 with distilled water, was poured on the plate, and the excess emulsion was carefully drained. After a 48-h exposure, plates were developed, fixed, and observed with a phase-contrast microscope.

Assay for polyoma T antigen. Polyoma T antigen was assayed by immunofluorescence as previously described (26).

RESULTS

Virus-transformed cells have been previously obtained either from primary cultures or from established lines. Both procedures have their disadvantages: since primary cultures are heterogeneous populations of various cell types, it is not clear to what extent transformants isolated under different conditions can be compared. On the other hand, most cell lines have been propagated in a number of laboratories for many generations. They are usually heteroploid and tend to segregate spontaneous transformants at high frequency.

We made the choice of preparing for these studies new fibroblast lines from inbred Fisher rat embryos by the procedure of Todaro and Green (33) (see above).

FR 3T3 cells grow in 10% calf serum only to a low saturation density as do the analogous mouse cell lines (33). Although their growth rate is maximal between 37 and 39°C, they still grow efficiently at 41°C (Fig. 1). They are morphologically homogeneous and show a diploid male rat karyotype ($2n = 42$ chromosomes [14]), with a very small proportion of tetraploid metaphasis. They are strictly anchorage dependent for growth: less than 1 cell in 1×10^6 is able to produce a colony in soft agar. They are not tumorigenic: no tumor could be detected 8 weeks after injecting up to 4×10^6 cells in 2-month-old Fisher rats.

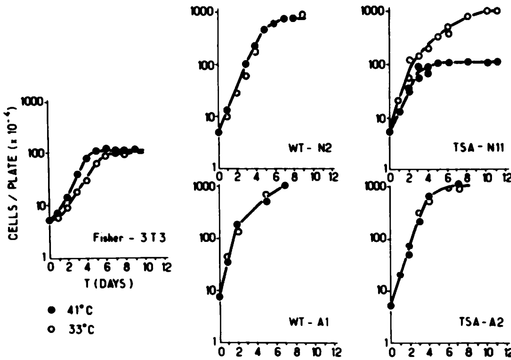

FIG. 1. *Growth at 33 and 41°C of FR 3T3 cells and of representatives of the various classes of polyoma virus transformants. Cells were grown as indicated in footnote a to Table 1. Zero time is the time of the shift to 41°C. At the indicated times, cells were trypsinized and counted.*

Transformation by WT and *tsa* polyoma virus. As described above, transformants of four types were isolated at 33°C from FR 3T3 cells. Clones referred to as WT-A and *tsa*-A were selected and cloned on the basis of their ability to produce colonies in soft agar, after infection with wild type and *tsa* polyoma virus, respectively. WT-N and *tsa*-N similarly refer to the transformants isolated from foci and cloned as dense colonies grown in liquid medium.

Transformation frequencies were ranging from 0.1 to 0.3%, the usual values for polyoma virus transformation of rat cells at this multiplicity (21, 27). Within the broad range of variation inherent to this type of measurement, values were not found to differ in a significant way from one type of transformant to another.

In no case have mock-infected control cells been observed to give rise to a single focus or to a colony in soft agar under the conditions of the experiment.

Saturation density of growth of WT and *tsa* transformants. The saturation density of growth in medium supplemented with 10% calf serum was first measured at 33 and 41°C for three independently isolated transformants of each of the four types, WT-N, WT-A, *tsa*-N, and *tsa*-A.

Plates were seeded at a density of 5×10^4 cells per 6-cm-diameter petri plate and incubated at 33°C for 24 h. Part of them were then shifted to 41°C.

All transformants grew at 33°C up to a cell density at least 10-fold higher than that of FR 3T3 cells (Table 1). An accurate determination of a plateau value was in most cases difficult to obtain: in spite of very frequent medium changes, overcrowding of cells at the maximal densities led to a very rapid exhaustion of the medium and sometimes to the detachment of the cell layer.

The same saturation densities were reached at 33 and 41°C in all cases except the *tsa*-N lines. Three out of three *tsa*-N clones tested were temperature sensitive for their saturation density. The number of cells per plate reached at 41°C the same saturation value as did the nontransformed FR 3T3 cells.

On the basis of these results, one cell line of each class was then studied in more detail, namely, the WT-N2, WT-A1, *tsa*-N11, and *tsa*-A2 lines. The growth rate in 2 and 10% calf serum and the colony-forming ability on plastic surfaces and in soft agar were measured. As shown below, all of these characters were strongly temperature dependent in *tsa*-N11. Whereas the other lines behaved as full transformants at 41 as well as at 33°C, *tsa*-N11 cells behaved at 33°C like the other transformants and at 41°C like the original FR 3T3 cells.

This phenotypic reversion to the "normal" state was subsequently found, with only minor variations, in the two other *tsa*-N lines (*tsa*-N1 and -N3) and in none of the other six lines of the WT-N, WT-A, and *tsa*-A classes (data not shown). Unless otherwise indicated, the results given below must therefore be taken as representative of all of the lines of a given category.

Growth in liquid medium supplemented with 10% calf serum. Growth curves obtained under the conditions described for Table 1 are shown in Fig. 1, and the doubling times during the exponential phase of each curve are indi-

TABLE 1. *Saturation densities at 33 and 41°C of FR 3T3 cells and their transformed derivatives*[a]

Cell line	Saturation density[b] at:	
	33°C	41°C
FR 3T3	5.3	4.8
tsa-N[c]	≥45	4.4–6.8
tsa-A[c]	≥45	≥45
WT-N[c]	≥45	≥45
WT-A[c]	≥45	≥45

[a] Cells were grown at the indicated temperature in medium supplemented with 10% calf serum in 6-cm petri plates (Nunclon). The medium was changed every other day at cell densities below 5×10^4 cells per cm², every day between 5×10^4 and 2×10^5, and twice a day above 2×10^5. Values indicated are those of the growth plateau (see Fig 1). In the case of the phenotypically transformed cells, only minimal values could be measured as explained in the text. For all of the transformed lines, independent measurements were made on three independently isolated clones of each type.

[b] Cells $\times 10^{-4}$ per cm².

[c] Range of measurements on three independent isolates.

cated in Table 2. All of the transformed lines grow at 33°C at a faster rate than do the nontransformed FR 3T3 cells. Generation times are in all cases 12 h, with the only exception being WT-N2 (other N transformants have generation times in the 10- to 12-h range). At 41°C, generation times are reduced by 10 to 20% in all cases, except for *tsa*-N11, whose generation time is increased and reaches the same value as that of the nontransformed line.

The arrest of *tsa*-N11 growth at a low density at 41°C corresponds to a cessation of DNA synthesis. Autoradiographic measurements indicated that less than 1% of the cells in the arrested monolayer incorporated [^{3}H]thymidine during a 1-h pulse at 41°C, whereas 40 to 60% of the nuclei were found labeled in exponential cultures at either 33 or 41°C.

This inhibition of growth may be taken as an indication that the normal control is operating in *tsa*-N11 at 41°C, as it is in FR 3T3. It is of interest to determine whether the transformed type of growth control can be reinstated by shifting the cultures down to 33°C.

Cells of the *tsa*-N11 line were therefore grown at 41°C until they reached their maximal density and then shifted back to 33°C. Although these shifts did not alter the growth of a WT-N line taken as a control, Fig. 2 clearly shows that the inhibition of the *tsa*-N cells was reversible. After a 4-day lag period, these cultures resumed their growth, eventually reaching the same high saturation density as the WT transformant.

Colony-forming ability on plastic surfaces. Table 2 shows that the ability to produce colonies on plastic surfaces at a low cell input was found to be higher for transformants of all types at 33°C than for the FR 3T3 cells at the same passage number. This property is temperature dependent only in the case of the *tsa*-N lines.

Colony-forming ability in soft agar. Table 2 shows that A transformants were found to produce colonies in soft agar with a relatively high efficiency. This result was expected in view of their selection procedure. Less expected was the fact that all of the N transformants could also produce colonies in agar at 33°C, although with a consistently lower efficiency. This plating efficiency was maintained at 41°C for the WT-N cells, but was reduced to less than 1 viable colony per 500 cells for *tsa*-N11. In a separate experiment, no visible colony was produced after seeding 10^5 *tsa*-N11 cells in agar at 41°C, whereas colonies corresponding to 15 to 30% of the cell input were produced on identical plates

TABLE 2. *Generation time and colony-forming ability on plastic and in soft agar of FR 3T3 cells and of transformants of the different classes.*

Cell line	Temp (°C)	Generation time (10% calf serum)[a]		Colony-forming ability on plastic[b]		Colony-forming ability in soft agar[c]	
		h	Ratio of 41/33°C	%	Ratio of 41/33°C	%	Ratio of 41/33°C
FR 3T3	33	25	0.8	4.6	1.0	<0.0001	
FR 3T3	41	19		4.4		<0.0001	
WT-A1	33	12	0.8	19.4	1.1	7.4	1.0
WT-A1	41	10		21.7		7.7	
WT-N2	33	20	0.9	15.2	1.1	4.3	1.0
WT-N2	41	18		16.5		4.5	
tsa-A2	33	12	1.0	19.0	1.0	15.8	1.0
tsa-A2	41	12		18.0		16.0	
tsa-N11	33	12	1.5	14.0	0.2	4.1	≪0.05[d]
tsa-N11	41	18		2.2		<0.2	

[a] For the generation time, the values shown are the minimal doubling times measured during exponential growth under the conditions described in footnote *a* to Table 1.

[b] For the determination of colony-forming ability on plastic, 6-cm petri plates (Nunclon) were each seeded with 1,000 cells in liquid medium containing 10% calf serum. After 24 h at 33°C, part of the plates were shifted to 41°C. The medium was changed twice a week, and the plates were stained with Giemsa after 2 weeks. Values shown are the relative number of colonies as a percentage of the cell inoculum.

[c] For the determination of colony-forming ability in soft agar, 10^6 cells of the nontransformed FR 3T3 line or 500 cells of each of the transformed lines were seeded in soft agar by the method of MacPherson and Montagnier (22). Plates were incubated at 33 and 41°C, and the number of visible colonies was measured after 15 days. Values are expressed as the percentage of cell input.

[d] See text.

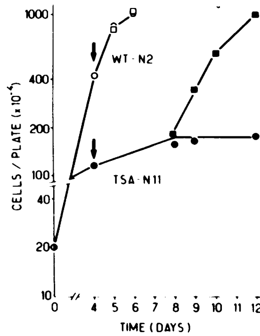

FIG. 2. *Reversibility of the growth arrest at 41°C of tsa-N11 transformants. WT-N2 and tsa-N11 cells were plated at 33°C and shifted up to 41°C at zero time as indicated in footnote a to Table 1. After 4 days (arrows), some of the plates were shifted back to 33°C. Symbols: ●, tsa-N11, 41°C; ○, WT-N2, 41°C; ■, tsa-N11, 41 and then 33°C; □, WT-N2, 41 and then 33°C.*

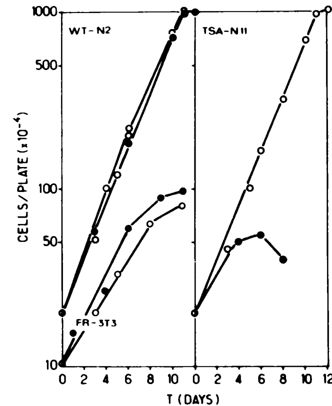

FIG. 3. *Growth of FR 3T3, WT, and tsa-N transformants at 33 and 41°C in medium containing 2% calf serum. Same experiment as those shown in Fig. 1, except that the serum concentration was reduced to 2%. Symbols: ○, 33°C; ●, 41°C.*

incubated at 33°C and on plates seeded with the same number of *tsa*-A2 cells and incubated at either temperature.

Growth at a low serum concentration. Figure 3 shows that FR 3T3 cells grew at a slower rate in medium supplemented with 2%, instead of 10% calf serum. A twofold increase in generation time was observed, in a way quite similar to that previously described for the mouse 3T3 lines (29). The saturation density, however, remained unchanged.

Under these conditions, WT-N2 cells and the A-type transformants (data not shown) were found to grow faster than the normal cells and to reach a higher density. The behavior of the *tsa*-N11 line was again dependent on the temperature: whereas its growth at 33°C was similar to that of the other transformants, cultures shifted to 41°C did not even reach the saturation density observed under these conditions for the nontransformed cells.

The serum requirement, unlike the other growth characteristics studied, therefore appears to be different in *tsa*-N11 at 41°C and in the FR 3T3 line. Whether this applies to all of the *tsa*-N transformants and whether this situation can be found for other transformation criteria are presently under study. Temperature sensitivity for growth in 2% serum has been reported previously for some *tsa* polyoma virus transformants (18).

T antigen. Polyoma T antigen was detected by immunofluorescence in all of the transformed lines at 33°C. The intensity of staining was systematically found to be lower in the A than in the N transformants. After a shift up to 41°C, both WT- and *tsa*-transformed cells remained positive for T antigen, even after 4 to 20 days of continuous growth at this temperature. This observation excludes the possibility of a loss of the viral genome in the *tsa*-A and *tsa*-N transformants during growth at high temperature. We have observed previously that *tsa*-transformed mouse cells produce an antigen that appears to be more labile in extracts than that of WT transformants, when assayed by complement fixation (26), although the cells remain positive at 39°C by immunofluorescence (our unpublished data). Whether the same applies to the *tsa*-A and *tsa*-N transformants is currently under study (P. Gaudray, P. Clertant, R. Seif, and F. Cuzin, manuscript in preparation).

Growth characteristics of *tsa*-N11 at 38.5°C. Since 38.5°C was the restrictive temperature used in previous work on polyoma virus *ts* mutants, we also analyzed the growth properties of *tsa*-N11 at this temperature. The cells were found to grow at this temperature to the same high density as they did at 33°C. Their colony-forming ability was also found to be identical to that observed at lower temperatures.

DISCUSSION

Our results indicate that infection of rat fibroblasts with polyoma virus may give rise to at least two types of transformed lines: the N type, when isolation is made from foci growing at high densities, and the A type, obtained after selection in soft agar. In both, the growth control is permanently modified: the saturation density is higher than in normal cells, the generation time is shorter, the colony-forming ability is increased, and the cells have lost their anchorage dependency and their requirement for high serum concentrations for growth in culture. In spite of this similarity of phenotypes, the mech-

anisms involved seem to be different in the two cell types. In only one of them do all of these transformation characters appear to be dependent on the activity of the protein coded by the virus *a* gene (T antigen), as judged from their temperature-dependent expression in *tsa*-transformed cells.

These results are not, in fact, contradictory to previous data on polyoma virus *tsa* mutants. The *tsa*-transformed lines studied by Fried (11), Di Mayorca et al. (4), and Eckhart (7) have been obtained by selection in soft agar and grown at 38.5°C, both conditions being sufficient for the expression of the transformed phenotype.

In cells isolated as foci after virus infection (type N), the transformed phenotype can be considered as primarily, if not only, under the control of the product of the virus gene *a*. One might object that a temperature-dependent transformed phenotype may also result from *ts* mutations in cellular genes (2, 28). It would be quite unexpected, however, to have such mutational cellular events occurring in all of the cells transformed by *tsa* polyoma virus and only in these cells.

The situation is more difficult to analyze in type A transformants isolated in soft agar. This selection procedure is a complex one, involving both anchorage-independent ability to divide and resistance to agar polyanions (24). Although not apparently dependent on the activity of the *a* gene protein for the expression of the transformed phenotype, these transformants clearly were produced as a consequence of the viral infection, since mock-infected cells never produced a colony in soft agar.

The frequencies of appearance of clones of types A and N were of the same order. This observation argues against the possibility of the *tsa*-A lines being transformed by revertant viral genomes. Morever, like other polyoma virus transformants (9, 21), both the A- and N-transformed cells yield virus upon fusion with permissive mouse cells, and both the *tsa*-A and *tsa*-N transformants produce virus temperature sensitive for growth in mouse cells (R. Seif, M. Berebbi, and F. Cuzin, manuscript in preparation).

The next possibility would be that in the A transformants, the *a* gene product is produced in greater amounts. Maintenance of the transformed phenotype at high temperatures in the case of *tsa* could then be attributed to the known leakiness of the mutation (13). This hypothesis does not seem to be compatible with T-antigen determinations in these clones: in all of the type A transformants so far tested, the amount of T antigen, estimated by complement fixation, appears to be lower than in N transformants (Gaudray et al., manuscript in preparation).

If we assume on this basis that these transformants are not under control of gene *a*, the possibility remains of a control by another viral gene. From what is presently known of polyoma mutants, two other, and certainly different, viral functions may have to be considered: the "hrt function," corresponding to the block occurring in the nontransforming host range mutants mapped in the early region (8, 15), and the late gene product identified by the *ts*3 mutation, whose activity was described as necessary for maintaining some of the transformation characters (6).

It may also be assumed that no viral function is required for maintaining the transformed state in type A cells. Maintenance might result from a permanent modification, initially induced by the virus, of a cellular mechanism. The presence and expression of viral DNA could even become irrelevant for the maintenance of transformation. This hypothesis could account for the occurrence of defective mutations in the integrated viral genomes of polyoma virus-transformed semipermissive hamster cells, which keep nevertheless their transformed characteristics (9). It would be in agreement with various observations that indicate that cellular genetic events can induce, suppress, or modify the expression of transformation (2, 28). Some of the characters of a transformed cell can, in fact, be selected simply by repeated passages in culture. For instance, the colony-forming ability of the freshly established lines is low, and it is increased by transformation (our data and reference 20). It is under the control of a viral gene product in N transformants. On the other hand, cell lines with high colony-forming abilities can be readily selected during culture. Viral transformation at this stage does not further affect this character (29).

The transformed phenotype seems, therefore, to result from a complex interplay between three types of controls: (i) by a virus-coded protein, (ii) by cellular changes specifically induced by the virus, and (iii) by cellular changes selected by growth in culture.

One could, for instance, assume that polyoma virus only transforms the rat cell up to a given transformation step, as previously defined by Vogt and Dulbecco (34), corresponding to the N transformants. Once this phenotype is expressed and only in this case, transformation to the A type could occur, due to an additional and irreversible cellular variation. This model predicts that clones selected in soft agar from a *tsa*-N line should have lost their temperature sensitivity.

An alternative model would consider that for a limited period of time early in the establishment of transformation, some culture conditions, such as the presence of agar polyanions, the absence of growth substratum, or some other aspect of the soft agar environment, could irreversibly change the growth control mechanism. It would make it independent or at least less dependent on the viral *a* function. This hypothesis is formally similar to that recently proposed by Dulbecco (5) as "a positive feedback stabilizing the transformed state."

These alternative models are currently being tested experimentally.

It may also be pointed out that the decrease in generation time that we observed upon transformation of FR 3T3 (see above) has not been observed in previous studies with other established lines (see, for instance, reference 29). The fact that this property is temperature dependent for the *tsa*-N lines strongly suggests that it may be under the control of the viral genome and therefore be considered as a "transformation character." This may be an accidental peculiarity of this system. Alternatively, it could be related to the fact that the FR 3T3 line was very recently established at the time of the virus infection. "Older" established lines, such as hamster BHK and mouse 3T3 cells, have been propagated in culture for many more generations before being transformed, under conditions that clearly must select for fast-growing variants. On such spontaneous partial transformants, no further effect could then be detected after virus infection.

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