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Conditions Leading to the Establishment of the N (*a* Gene Dependent) and A (*a* Gene Independent) Transformed States After Polyoma Virus Infection of Rat Fibroblasts

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Infection of normal rat fibroblasts (FR 3T3) with the early *tsa* mutant of polyoma virus may lead to either the A or the N phenotype. *tsa*-A transformants, originally derived by agar selection, are not temperature dependent for maintenance of the transformed phenotype, whereas *tsa*-N transformants revert at high temperature to normal growth control. A transformants did not result from an independent cellular mutation selected in agar medium, but rather from a transformation process distinct from that leading to the N state. It occurred in both liquid and agar media when the infected cells were maintained under growth-restricting conditions, such as absence of anchorage and contact inhibition at confluency. N transformation occurred in cells maintained in active growth after virus infection (sparse cultures on a solid substratum). Physiological conditions during a critical period after virus infection thus appear to be a crucial parameter of the transformation process.

Two classes of virus-induced transformants, N and A, have been distinguished in the progeny of rat fibroblasts infected by the *tsa* mutant of polyoma virus (10). N lines were originally derived from foci overgrowing monolayers of normal cells, and A lines were originally derived from colonies grown in soft agar. The expression of various characters defining the transformed state was temperature sensitive in *tsa*-N cells, which thus reverted at 41°C to the phenotype of a normal cell. *tsa*-A transformants, in agreement with previous reports (2-4), exhibited the transformed phenotype at both low and high temperatures. Using the same conditions, temperature-sensitive and temperature-insensitive transformants were obtained after infection of rat cells with the *tsA30* mutant of simian virus 40(8a).

These results could be most simply explained by the hypothesis that growth in agar medium selected for a cellular variation (mutation), which leads to a virus-idependent expression of the transformed phenotype. This hypothesis predicts that subclones from *tsa*-N cells isolated in agar medium should be converted into the A type. We show in the present report that such clones keep, in fact, a *ts* phenotype. A choice between the A and N states therefore appears to be made soon after infection. We show that it occurs, in fact, during the first 5 days at 33°C and primarily depends on the growth potential of the infected cells during that time period.

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MATERIALS AND METHODS

Cells and viruses. The establishment of the FR 3T3 line from Fisher rat embryo cultures, the characteristics of the cell line, and culture conditions have been described previously (10). Wild-type (WT) polyoma virus (A2 strain) and the *tsa* mutant (5) were plaque purified and grown in secondary mouse embryo cells infected at a multiplicity of 0.01 to 0.05 PFU/cell.

Transformation. Viral infection and selection of transformants were performed as described previously (10), except that purified agarose (Agarose A 37 Indubiose, Industrie Biologique Française) was used instead of bacteriological agar for growth of transformants (8).

RESULTS

Characterization of subclones isolated in agar medium from a *tsa*-N transformed line. The *tsa*-N11 transformed cell line, derived from a focus after *tsa* polyoma infection of FR 3T3 cells, has been shown previously to be temperature sensitive for the expression of various characters defining the transformed phenotypes, such as saturation density, generation time, clon-

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ing ability on plastic in 10% calf serum, and growth in 2% serum and in soft agar (10).

To test the hypothesis that growth in agar might select for a cellular variation superimposed to the viral transformation event, and leading to the temperature-independent type A maintenance of the transformed state, *tsa*-N11 cells were seeded at 33°C in soft agar. Three clones derived from individual colonies were designated *tsa*-N11-A1, -A2, and -A3. Their saturation densities and plating efficiencies were measured at both 33 and 41°C. All three clones exhibited the same temperature dependence for these characters as the original *tsa*-N11 line (Table 1).

Growth in soft agar by itself does not appear, therefore, to select for the A phenotype. This conclusion leads to the alternative hypothesis that a choice between the A and N states might be possible only during a limited period of time after virus infection.

Absence of anchorage, which is not a sufficient condition for the A phenotype to occur, does not appear to be necessary either: it will be shown below that A transformants can be obtained by focus selection on a solid substratum, and, conversely, N lines can be obtained by agar selection under the proper conditions.

TABLE 1. *Saturation density and plating efficiency of tsa-N11 cells and their agar-selected clonal derivatives*

Cells[a]	Temp (°C)	Saturation density[b] (cells/6-cm plate)	Plating efficiency[c] (%)
tsa-N11	33	1.1×10^7	17.0
	41	1.5×10^6	3.2
ts-N11-A1	33	1×10^7	12.6
	41	1.1×10^6	4.6
tsa-N11-A2	33	1.5×10^7	14.0
	41	1.2×10^6	2.8
tsa-N11-A3	33	1×10^7	18.8
	41	2×10^6	3.6

[a] *tsa*-N11-A1, -A2, and -A3 subclones were derived from *tsa*-N11 colonies grown in soft agar as described in the text.

[b] Cells were grown at the indicated temperatures in medium supplemented with 10% calf serum in 6-cm petri plates (Nunclon) with regular medium changes. Values indicated are those of the growth plateau.

[c] For the determination of plating efficiency 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.

Occurrence of A transformants in a focus assay. Three independent transformants previously isolated from foci after *tsa* polyoma infection turned out to be temperature-sensitive (N) transformants (10). This result does not obviously imply that only N transformants are selected by this procedure. To obtain statistically significant data, a large number of lines would have to be derived from independent foci. Since this would constitute an expensive and time-consuming experiment, we looked for a quick assay for the ratio of A versus N transformants.

It was previously concluded from temperature shift experiments (4) that establishment of the stably transformed state requires less than 5 days at 33°C. We also know that the established *tsa*-N transformants do not grow at 41°C above the density of a contact-inhibited monolayer and, therefore, are not likely to grow as foci at this temperature. This was confirmed by the results of a reconstruction experiment, in which 100 cells of the established *tsa*-N11 line (10) were seeded at 33°C together with an excess of normal FR 3T3 cells, and part of the plates were shifted at 41°C 5 days later: no foci could be detected at 41°C after 2 weeks, whereas 20 to 30 foci were produced at 33°C.

The number of foci produced by *tsa*-infected cells was then determined either at 33°C or after transferring the cultures to 41°C at least 5 days after infection. Ratios between the frequencies of foci appearing at 41 and 33°C must reflect the ratios of A to total transformants in the progeny of the infected cells.

As shown in Table 2, a reproducible ratio of 0.5 to 0.6 was observed between the number of foci measured at 41°C after shift-up and the number of foci on plates maintained in parallel at 33°C (see also Table 3 and Fig. 1). Similar results were previously mentioned in a report by Kimura and Itagaki (6) on transformation by WT simian virus 40 and the early *ts640* mutant. This observation indicates that about half of the transformants were temperature-independent A lines and therefore suggests that the remainder of the stably transformed population was of the temperature-dependent N type. The validity of this conclusion was confirmed in an experiment described below: foci could be observed on plates which were first shifted to 41°C 9 days after infection, and then back to 33°C 3 days later, under conditions where no foci were produced on the plates left at 41°C.

A ratio of 1 was observed when cells were seeded in agar medium and part of the plates were shifted to 41°C either 5 or 9 days later (Table 2). Only A transformants were thus pro-

TABLE 2. *Transformation of FR 3T3 by WT and tsa polyoma at 33 and 41°C*

Growth conditions after virus infection[a]		Infecting virus				
		WT polyoma		*tsa* polyoma		Mock infection (transformants)
Medium	Temp (°C)	Transformants[b]	Ratio 41/33°C[c]	Transformants	Ratio 41/33°C	
Liquid	33 (20d)	0.40		0.30		<0.005
	41 (20d)	0.35	0.9	0.01	0.03	<0.005
	33 (5d)–41 (15d)	0.36	0.9	0.17	0.57	<0.005
	33 (9d)–41 (11d)	0.40	1.0	0.18	0.60	<0.005
Agar	33 (20d)	0.27		0.40		<0.005
	41 (20d)	0.25	0.9	0.04	0.1	<0.005
	33 (5d)–41 (15d)	0.36	1.3	0.36	0.9	<0.005
	33 (9d)–41 (11d)	0.28	1.0	0.44	1.1	<0.005

[a] Actively growing FR 3T3 cells were infected at 33°C at a multiplicity of 500 PFU of either WT or *tsa* polyoma per cell or were mock infected. Cells were trypsinized 24 h later and seeded at a density of 5×10^4 cells per 6-cm petri plate, in either liquid medium or soft agar. Part of the plates were incubated at 41°C and the remainder, at 33°C. Plates were transferred from 33 to 41°C either 5 or 9 days after infection. Twenty days (d) after infection, cultures in liquid medium were scored for densely grown foci after Giemsa staining, and agar cultures were scored for macroscopically visible colonies.

[b] Relative number of either foci or agar colonies, expressed as percentage of the cell inoculum.

[c] Frequencies reported to the value observed after 20 days at 33°C.

duced under these conditions as previously found by Fried (4), Eckhart (3), DiMayorca and his associates (2), and ourselves (10): a total of seven independent transformed cell lines which were established in our laboratory from colonies grown in agar medium (seeding at day 0 to 1 after infection) were all subsequently identified as A transformants.

A low 41/33°C ratio was observed (Table 2) when *tsa*-infected cells were shifted to high temperature at day 1 after infection. Using agar selection, such a result was obtained originally by Fried (4) and indicates that the viral *a* function is required for the establishment of the stably transformed A state. This requirement is also apparent (Table 2, liquid medium) for the establishment of the A state in a focus assay. It will be shown below that in both cases it is limited to the first 5 days after virus infection.

Establishment of A transformation requires polyoma *a* gene product activity. Shift-up experiments similar to those just described were performed by transferring WT and *tsa*-infected cells from 33 to 39.5°C less than 5 days after infection. A temperature of 39.5°C was chosen in this case as the restrictive temperature for allowing a direct comparison with earlier experiments on *tsa* transformation (2–4), but the same results were obtained upon temperature shifts from 33 to 41°C (data not shown).

The efficiencies of both focus and agar colony formation at high temperature were reduced by about 10-fold when the *tsa*-infected cells were not kept for at least 5 days at the permissive temperature after infection (Fig. 1). Neither transformed foci nor agar colonies were observed on plates which were shifted up at day 1 after infection and back to 33°C 1 to 4 days later. The low 39.5/33°C ratio observed in this case therefore indicates that no stable transformant could be established at high temperature and not, as shown above upon shift-up at later times, that stable, but temperature-dependent transformants were produced. The activity of the early viral protein(s) identified by the *tsa* mutation appears therefore to be required for the establishment of the A phenotype in both the agar and the focus assay. Whether this function of the *a* gene is distinct from the maintenance function previously demonstrated for N transformants (10) remains to be established.

Cells arrested in stage G0 give rise to A transformants, and actively growing cells give rise to N transformants. The sequence of events leading to the A phenotype requires the activity of the viral *a* gene product for initiation of transformation but not for its subsequent maintenance. It consistently occurs in cells which are kept in the absence of anchorage during the first days after infection and in 50 to 60% of the cells undergoing transformation while the cultures are reaching confluency. One might therefore assume that A transformation is characteristic of G0-arrested cells, whereas the N state would be established in cells engaged in their reproductive cycle.

To test this hypothesis, actively growing FR 3T3 cells (2×10^4 cells per 6-cm petri plate) were infected with either WT or *tsa* polyoma at 33°C and thereafter submitted to four different

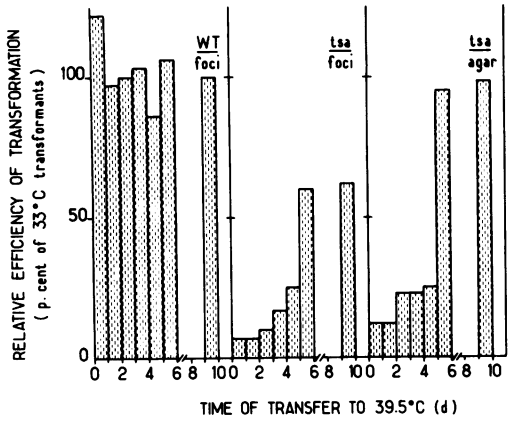

FIG. 1. *Focus formation and colony formation in agar medium at high temperature after tsa polyoma infection. Actively growing FR 3T3 cells were infected at 33°C with either WT or tsa polyoma (500 PFU/cell), or mock-infected (zero time), and seeded at a density of 5 × 10⁴ cells per 6-cm petri plate, either on plastic or in agar medium. Plates were kept at 33°C, and part of them were transferred at 39.5°C at the indicated times. Twenty days (d) after infection, the agar cultures were scored for microscopically visible colonies, and the attached cultures were scored for dense foci after Giemsa staining. In both assays, WT transformation frequencies, relative to the number of infected cells, were 4(±1) × 10⁻³, and frequencies of spontaneous transformation (mock-infected cells) were less than 2 × 10⁻⁵. Results obtained after WT infection and agar selection did not differ significantly from the histogram presented for WT infection and focus selection.*

growth conditions. Part of the cultures were incubated at 33°C for 9 days with regular medium changes as in the previous experiments (Table 3, a). In a second set of cultures (Table 3, b), the cells were trypsinized every third day and replated at a density of 2 × 10⁴ cells per 6-cm plate. In this way, they were never allowed to grow past a density of 10⁵ cells per plate, above which confluent patches of cells are detected. In a third group of cultures (Table 3, c), the infected cells were mixed with an excess of noninfected FR 3T3 cells and replated at a density of 10⁶ cells per plate (confluent monolayer). In Table 3, d), cells were trypsinized 24 h after infection and seeded in agar medium (2 × 10⁴ cells per 6-cm plate). On day 9 after infection, cultures of sets a to d were shifted to 41°C. Twenty days later, the plastic-attached cultures were stained with Giemsa and scored for densely grown foci, whereas macroscopically visible colonies were counted on agar plates.

Results reported in Table 3 indicate that only temperature-independent (A) transformants were produced under conditions of contact inhibition of growth (set c) and of absence of anchorage (set d). The usual 1:2 ratio of N to A clones was found in cells progressively reaching confluency after virus infection (set a), whereas cells maintained at their maximal growth rate (set b) gave rise exclusively to temperature-dependent (N) foci. Dense foci could, however, be observed in plates of set b, which were shifted to 41°C on day 9 and back to 33°C on day 12 after

TABLE 3. *Ratio of temperature-stable (A) to total tsa transformants among foci produced under different growth conditions*

Set[a]	Growth conditions after virus infection[a]	Virus[b]	Temp[c] (°C)	No. of foci (a to c) or colonies (d)[d]	Ratio 41/33°C	Types of transformants[a]
a	Growth on plastic up to confluency	WT	33	75	1.0	A + N
			33, then 41	75		
		tsa	33	64	0.6	
			33, then 41	40		
b	Continuous growth at low cell density	WT	33	48	0.9	N only
			33, then 41	45		
		tsa	33	25	<0.04	
			33, then 41	0		
c	Growth arrested at confluency	WT	33	47	1.1	A only
			33, then 41	54		
		tsa	33	61	1.0	
			33, then 41	63		
d	Growth arrested by seeding in agar medium	WT	33	69	1.0	A only
			33, then 41	71		
		tsa	33	66	1.1	
			33, then 41	73		

[a] See text.
[b] Actively growing cells (2 × 10⁴ cells per 6-cm petri plate) were infected at 33°C at a multiplicity of 500 PFU/cell.
[c] Continuous exposure to 33°C, or transfer to 41°C 9 days after infection.
[d] Average number per 6-cm petri plate.

infection. In all cases, transformation frequencies after WT virus infection were identical at low temperature and after shift-up.

Selection of N transformants in agar medium. Results presented so far strongly suggest that whether or not the cells are under conditions leading to active growth during the first days after infection is the crucial parameter in the A versus N choice, rather than the selection procedure thereafter applied for isolating the transformants. It should then be possible to select N derivatives in agar medium, provided that infected cells have been maintained in active growth prior to their transfer in agar. In the experiments reported in Table 4, cells were infected at 33°C with WT and *tsa* polyoma and subsequently maintained in an active growth state by using the set b schedule of transfers described above. A control set of infected cultures was allowed to reach confluency during the first days after infection (set a). On day 9 after infection, cells were detached with trypsin and seeded in agar medium at a density of 5×10^4 cells per plate, at either 33 or 41°C. The ratios of colonies produced at 33 and 41°C show that 90% of the agar-growing clones correspond under condition b to temperature-dependent *tsa*-N transformants.

DISCUSSION

Transformants of types A and N were originally defined according to the method of isolation, agar selection and focus derivation, respectively (10). Under the conditions used in these experiments, polyoma *tsa*-transformed A clones were subsequently found to be temperature independent for the expression of the transformed phenotype, whereas N transformants were strongly temperature dependent. It now seems most likely that the crucial parameter in the N versus A choice is the physiological state of the cells during the early part of the transformation process, and that no causal relationship exists between the method of selection and the subsequent mode of maintenance. We therefore have to redefine the N and A states, as meaning polyoma *a* gene (or simian virus 40 *A* gene) dependent versus independent for maintenance of the transformed state.

Our present results lead to a more precise definition of the relationship between these states. They first exclude that the A derivatives may result from an additional and independent cellular mutation selected by growth in agar medium. They further suggest that during a critical period after virus infection, alternative sequences of events may lead to either one or the other type of transformant. Type A requires the activity of a *tsa*/mutated gene product for its establishment. This requirement is limited to the period extending from day 1 to 4 after infection at 33°C. It also requires that the cell undergoing transformation is kept during that critical period under conditions where the growth of normal cells is arrested, such as absence of anchorage or contact inhibition in a confluent monolayer. On the other hand, type N transformation occurs in cultures of cells actively dividing after infection. It leads to a state in which a product of the *a* gene (as defined by the *tsa* mutation) must be continuously active for the transformed state to be maintained. Whether this gene product is independently necessary for an initiation step in N transformation cannot be ascertained in view of the observed effect on maintenance.

These results also point to the requirement for precisely defined conditions for viral transformation. In most previous studies, viral transformation was studied after infection of subconfluent cultures, i.e., of mixed populations of growing cells and cells arrested at confluency. Heterogeneity is even more pronounced in the case of primary cultures, where both dividing

TABLE 4. *Ratio of temperature-stable (A) to total tsa transformants among agar colonies produced under different growth conditions*[a]

Set	Growth conditions after virus infection[a]	Virus	Temp (°C)	No. of agar colonies[b]	Ratio 41/33°C	Types of transformants
a	Growth on plastic up to confluency (9 days), then in agar medium	WT	33	109	0.9	A + N
			41	90		
		tsa	33	160	0.8	
			41	130		
b	Continuous growth at low cell density (9 days), then in agar medium	WT	33	58	0.9	Mostly N
			41	51		
		tsa	33	60	0.1	
			41	7		

[a] Same experiment as in Table 3 (a and b), except that cells were transferred in soft agar at either 33 or 41°C 9 days after infection as described in the text.

[b] Average number per 6-cm petri plate (input, 5×10^4 cells).

and nondividing cells are always present. Our results explain, for instance, the variety of transformed phenotypes observed among focus-selected transformants, as in the experiments designed to check the temperature sensitivity of simian virus 40 *tsA* transformants where both temperature-dependent and temperature-independent lines were first obtained (1, 11).

Although the selection procedure per se is not crucial in the N versus A determination, it definitely plays a major role in the choice of the particular set of transformation characters which will be ultimately expressed in a given line (9). The A and N states, as defined by dependence on one particular virus-coded protein for maintenance, would thus have to be considered as independent of the commonly admitted stepwise progression (12) of the transformed phenotype towards the ultimate tumoral stage. Studies in progress in our laboratory (B. Perbal, M. Rassoulzadegan, and F. Cuzin, manuscript in preparation) indicate that several levels of transformation may be observed among transformed rat lines of either the A or the N type.

Why different pathways of transformation are observed in G0-arrested and in actively growing cells is still a matter of speculation. Since in the former case, transformation first requires the G0 block to be suppressed and the cell to be pushed back into the G1 phase, a particular mode of expression might be required for this step, which, once perpetuated in stable transformants, could correspond to the A phenotype. Alternatively, different cellular regulations might act on the expression of the viral genome and result in different patterns of expression and/or integration under different environmental conditions.

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