JOURNAL OF VIROLOGY, Feb. 1980, p. 909-911
0022-538X/80/02-0909/03$02.00/0

Vol. 33, No. 2

Transformation of Rat Fibroblast Cells with Early Mutants of Polyoma (*tsa*) and Simian Virus 40 (*tsA30*): Occurrence of Either A or N Transformants Depends on the Multiplicity of Infection

MINOO RASSOULZADEGAN AND FRANÇOIS CUZIN*

Centre de Biochimie du Centre National de la Recherche Scientifique, Université de Nice, 06034 Nice, France

Infection of rat fibroblasts with early mutants of polyoma virus (*tsa*) or simian virus 40 (*tsA30*) leads to the establishment of either temperature-independent A transformants or N transformants temperature-sensitive for the expression of the transformed phenotype. The choice between the A- and N-transformed states is not only dependent, as we reported previously (Rassoulzadegan et al., J. Virol., **28**:421-426, 1978), on the growth conditions after infection, but is also a function of the multiplicity of infection (MOI); high MOI led to the predominant occurrence of A derivatives, and lower MOI led to that of N transformants.

The involvement of an early viral gene product in the maintenance of the transformed phenotype in cells transformed with polyoma virus and simian virus 40 (SV40) was established from the observation that cell lines transformed with temperature-sensitive (*ts*) mutants of the early complementation groups *a* (polyoma [4-6]) and *A* (SV40 [3, 14]) may be temperature dependent for the expression of various transformation-characteristic properties (1, 2, 8-10, 12, 13, 15). For both viruses, however, two types of transformants were shown to occur after infection with these mutants. Only transformants designated as N type revert at high temperature to the normal phenotype. Transformants of type A, although they carry integrated mutant viral genomes, exhibit a fully transformed phenotype at both low and high temperatures (1, 8, 10, 12, 13).

We reported previously (11) that A and N transformants may be derived from the same batches of infected rat fibroblasts (FR 3T3 [12]) with approximately equal frequencies, and that growth conditions during the first days after infection with polyoma *tsa* were critical for the A versus N determination. Maintaining the cells under growth-inhibiting conditions, such as absence of anchorage or confluency on a solid substrate, led to the predominant occurrence (>90%) of temperature-independent A clones. In contrast, N transformants were almost exclusively produced in the progeny of cells which had been maintained as actively growing sparse cultures on plastic substrate.

These results were reproducibly obtained in all instances where FR 3T3 cells were infected at multiplicities of infection (MOI) between 100 and 500 PFU/cell. However, an effect of the multiplicity became evident when similar experiments were performed on cells infected with polyoma *tsa* at MOI either in the range of 1 to 10 PFU/cell or higher than 500.

In the experiment reported in Table 1, ratios of A to total (A + N) transformants were determined as previously described (11) by comparing the number of transformants (foci or colonies in agarose medium) on plates maintained at 33°C to that measured on parallel cultures shifted from 33 to 40°C 5 to 9 days after infection. Only A transformants register at 40°C, whereas both A and N derivatives produce foci or colonies without anchorage at 33°C. Results were similar to those previously reported in the case of cells infected at an MOI of 100 PFU/cell. Only very few temperature-independent A transformants were obtained when the cells were maintained in active growth during the first 9 days after infection, whereas cells seeded in agarose medium gave rise to a majority of such lines. However, when the MOI was increased up to 1,000 to 2,000 PFU/cell, the proportion of A transformants observed after an initial period of active growth increased, reaching 50% and more. The opposite situation was observed when infection was performed at MOI lower than 10 PFU/cell: a significant proportion of temperature-dependent N transformants were obtained from cells seeded in agarose medium immediately after infection.

These conclusions were independently confirmed by establishing transformed lines at 33°C and determining the growth characteristic of

clonal populations at 33 and 40 to 41°C. The proportion of established transformants exhibiting temperature-dependent growth ability in agarose medium (Table 2) was in good agreement with values reported in Table 1.

Table 2 also shows the result of a similar analysis performed with FR 3T3 cells infected with SV40 *tsA30* at various MOI. As with polyoma *tsa*, N transformants were predominantly obtained by using two specific sets of conditions: (i) infection at 100 to 500 PFU/cell and active growth after infection and (ii) infection at low multiplicities, independent of the growth conditions. Although selection in agarose medium after infection at low MOI was not included in the present work, we know from the results of others (1, 7a) that it leads to the predominant occurrence of N transformants. This is in good agreement with our own previous results which indicate that agarose selection results in the same distribution of A and N phenotypes as focus selection among cells which were seeded at confluency (11).

TABLE 1. *Ratio of temperature-stable* A *to total tsa polyoma transformants among colonies obtained in agarose medium from FR 3T3 cells infected at various multiplicities*[a]

Growth conditions after infection	MOI (PFU/cell)				
	1	10	100	1,000	2,000
Cells maintained under active growth conditions for 9 days at 33°C, then seeded in agarose medium	NT	NT	0.04	0.2	0.5
Cells seeded in agarose medium 2 h after infection	0.3	0.55	0.7	0.8	1.0

[a] Virus infection and culture conditions after infection and measurement of the ratio of A to total transformants were as previously described (11). NT, Not tested.

The present data are consistent with all reports on the occurrence of temperature-dependent and -independent transformants after infection with polyoma *a* and SV40 *A ts* mutants in various cell types. For instance, MOI up to 1,000 PFU/cell were used in the original work of Fried (7) and, together with selection in agar medium, led to the exclusive appearance of A transformants. Previously studied SV40 *tsA*-N lines were derived from cells infected at MOI in the range of 0.1 to 10 PFU/cell and selected as foci (1, 2, 8, 9, 15) as well as agar-grown colonies (see above).

These results should help to define the proper conditions for establishment of transformed cell lines of a given type. They are, on the other hand, still difficult to explain in molecular terms. One possible and trivial hypothesis would be that the *ts* virus stocks contain a small, but sufficient proportion of wild-type revertants, such that transformants generated at high multiplicity might contain both mutated and revertant genomes. To test this hypothesis, we measured the apparent leakiness of polyoma *tsa*

TABLE 2. *Ratio of temperature-independent* A *to total number of transformants established after infection of FR 3T3 cells at various multiplicities with polyoma tsa and SV40 tsA30*[a, b]

Virus	No. of cell lines tested	Growth conditions (first 5 days after infection)	MOI (PFU/cell)		
			1 to 10	100 to 500	1,000 and above
Polyoma *tsa*	27	Agarose medium	4/9	11/12	—[c]
		Active growth	—	0/6	—
Polyoma WT	14	Agarose medium	5/5	6/6	—
		Active growth	—	3/3	—
SV40 *tsA30*	29	Agarose medium	—	4/4	—
		Confluent layer	0/4	7/9	1/1
		Active growth	0/3	0/6	2/2
SV40 WT[d]	8	Agarose medium	—	1/1	—
		Confluent layer	3/3	—	—
		Active growth	3/3	1/1	—

[a] Ratios indicated are the number of transformants of a given class exhibiting temperature-independent agar growth ability to the total number of cell lines established.

[b] Procedures of establishment and cloning of transformants were previously described (10, 12). WT, Wild type.

[c] —, Not tested.

[d] Strain VA 45-54 from which the *tsA30* mutant was originally derived (14).

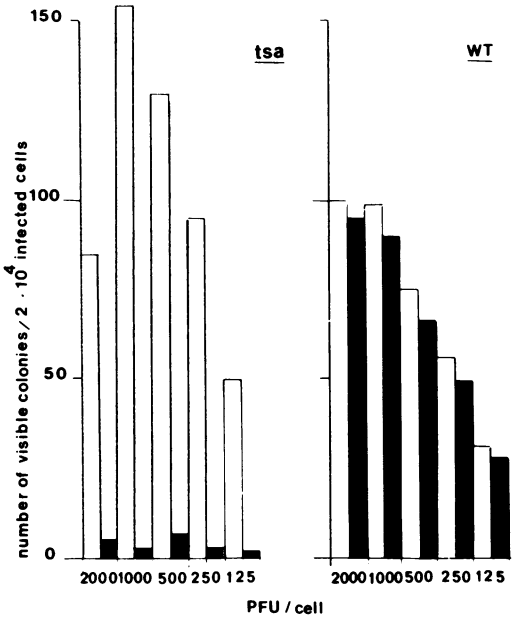

FIG. 1. *Colony formation in agar medium at high and low temperature after tsa and wild-type (WT) polyoma virus infection. Actively growing FR 3T3 cells were infected at 33°C with either wild-type or tsa polyoma virus (125, 250, 500, 1,000, and 2,000 PFU/cell) and seeded at a density of* 2×10^4 *cells per 6-cm petri plate. Half of the plates were kept at 33°C (□), and the remainder were transferred to 41°C (■). Twenty days after infection, the agar cultures were scored for microscopically visible colonies.*

stocks used for transformation as a function of MOI by infecting FR 3T3 cells with serial two-fold dilutions and immediately shifting the cultures to 41°C. Under these conditions, only a limited transforming efficiency was observed at low and intermediate MOI values, corresponding to the known effect of the mutation on the establishment of the transformed state (4, 5, 7, 11). After infection at high MOI values, dominant revertants should be revealed by a significant increase in the number of transformants obtained at high temperature; Fig. 1 shows that this is not the case. Alternatively, the A versus N choice might depend on the rate of accumulation of one or several of the early gene products during the first days after infection. This rate could be affected by both MOI and the physiological state of the cells. Experiments are in progress to test this hypothesis.

The expert technical assistance of C. Bonifacino, L. Carbone, and F. Tillier is gratefully acknowledged.

This work was made possible by grants from the Institut National de la Santé et de la Recherche Médicale (AT 79-114 and CRL 78-1-083) and from the Fondation pour la Recherche Médicale.

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

LITERATURE CITED

1. **Brockman, W. W.** 1978. Transformation of BALB/c-3T3 cells by *tsA* mutants of simian virus 40: temperature sensitivity of the transformed phenotype and retransformation by wild-type virus. J. Virol. **25**:860–870.
2. **Brugge, J. S., and J. S. Butel.** 1975. Role of simian virus 40 gene *A* function in maintenance of transformation. J. Virol. **15**:619–635.
3. **Chou, J. Y., and R. G. Martin.** 1974. Complementation analysis of simian virus 40 mutants. J. Virol. **13**:1101–1109.
4. **DiMayorca, G., J. Callender, G. Marin, and R. Giordano.** 1969. Temperature-sensitive mutants of polyoma virus. Virology **38**:126–133.
5. **Eckhart, W.** 1969. Complementation and transformation by temperature-sensitive mutants of polyoma virus. Virology **38**:120–125.
6. **Fried, M.** 1965. Isolation of temperature-sensitive mutants of polyoma virus. Virology **25**:669–671.
7. **Fried, M.** 1965. Cell transforming ability of a temperature-sensitive mutant of polyoma virus. Proc. Natl. Acad. Sci. U.S.A. **53**:486–491.

7a. **Kelley, S., M. A. R. Bender, and W. W. Brockman.** 1980. Transformation of BALB/c-3T3 cells by *tsA* mutants of simian virus 40: effect of transformation technique on the transformed phenotype. J. Virol. **33**:550–552.

8. **Martin, R. G., and J. Y. Chou.** 1975. Simian virus 40 functions required for the establishment and maintenance of malignant transformation. J. Virol. **15**:599–612.
9. **Osborn, M., and K. Weber.** 1975. Simian virus 40 gene *A* function and maintenance of transformation. J. Virol. **15**:636–644.
10. **Rassoulzadegan, M., B. Perbal, and F. Cuzin.** 1978. Growth control in simian virus 40-transformed rat cells: temperature-independent expression of the transformed phenotype in *tsA* transformants derived by agar selection. J. Virol. **28**:1–5.
11. **Rassoulzadegan, M., R. Seif, and F. Cuzin.** 1978. Conditions leading to the establishment of the N (*a* gene dependent) and A (*a* gene independent) transformed states after polyoma infection of rat fibroblasts. J. Virol. **28**:421–426.
12. **Seif, R., and F. Cuzin.** 1977. Temperature-sensitive growth regulation in one type of transformed rat cell induced by the *tsa* mutant of polyoma virus. J. Virol. **24**:721–728.
13. **Tegtmeyer, P.** 1975. Function of simian virus 40 gene *A* in transforming infection. J. Virol. **15**:613–618.
14. **Tegtmeyer, P., and H. L. Ozer.** 1971. Temperature-sensitive mutants of simian virus 40: infection of permissive cells. J. Virol. **8**:516–524.
15. **Tenen, D. G., R. G. Martin, J. Anderson, and D. M. Livingston.** 1977. Biological and biochemical studies of cells transformed by simian virus 40 temperature-sensitive gene *A* mutants and *A* mutant revertants. J. Virol. **22**:210–218.