Proc. Natl. Acad. Sci. USA
Vol. 80, pp. 4354–4358, July 1983
Cell Biology

Expression of the large T protein of polyoma virus promotes the establishment in culture of "normal" rodent fibroblast cell lines

(oncogenic transformation/genetics/immortalization)

MINOO RASSOULZADEGAN*, ZOREH NAGHASHFAR*, ALISON COWIE†‡, ANTONY CARR†, MILAINE GRISONI*, ROBERT KAMEN†‡, AND FRANÇOIS CUZIN*

*Centre de Biochimie du Centre National de la Recherche Scientifique, Université de Nice, 06034 Nice, France; and †Transcription Laboratory, Imperial Cancer Research Fund Laboratories, London WC2A 3PX, England

Communicated by Renato Dulbecco, April 4, 1983

ABSTRACT **Transfer into mouse and rat embryo fibroblasts in primary culture of cloned polyoma virus genes encoding only the large T protein led to the establishment of flat colonies in sparse subcultures at a frequency equal to that of transformation by wild-type virus. Cell lines could be derived from such colonies and maintained in culture for large numbers of generations without entering crisis. They exhibited a normal phenotype, by the criteria of growth on plastic to a low saturation density and of anchorage dependency. However, they required a lower serum concentration for growth than spontaneously established 3T3 cells. Similar results were obtained after transfer of recombinant DNA molecules encoding only the amino-terminal 40% of the large T protein, suggesting that this "immortalization" function corresponds to the activity of an amino-terminal domain of the protein. Immunoprecipitation analysis of T antigens in cell lines established after transfer of the full-size and of the truncated large T genes demonstrated the expression of the full-size large T protein and of a M_r 40,000 antigen expressed from the amino-terminal part of the gene, respectively. After transfer of a "large T only" plasmid that carries a *tsa* mutation, cell lines were established at 33°C with the same efficiency as with the wild-type large T gene, but their growth was arrested after a shift to 40°C, with a progressive loss in cell viability. This result indicates a continuous requirement for a large T function in the maintenance of "immortality."**

Three distinct proteins—large T, middle T, and small T—are encoded by alternate translational reading frames in the early region of the genome of polyoma virus. The genetic information is resolved by different splicing events, which produce three distinct mRNA species from a common precursor transcript (see ref. 1 for review and Fig. 1). Recent studies using recombinant polyoma virus genomes that each encode only one of these proteins led to the identification of independent functions for two proteins, large T and middle T, in the acquisition of the transformed phenotype (2, 3). In established rat fibroblast lines (F2408 and FR3T3), expression of the middle T protein conferred on cells the lack of topoinhibition and of anchorage dependency characteristic of tumor cells, but in a manner dependent upon the concentration of serum factors. This dependence could be complemented by transfer of a viral gene encoding either the full-size large T protein or only its NH_2-terminal 40%. On the other hand, a decreased dependence on serum factors was separately conferred on FR3T3 cells by transfer either of the full-size large T gene or only of its amino-terminal region. As expected from previous studies on the polyoma *hrt* mutants (4–6), expression of the large T protein alone was not sufficient to induce either focus formation or colony formation in agarose medium.

Different results were obtained when rat embryo fibroblasts in primary cultures were used instead of cells of a line previously selected for an unlimited growth potential in culture (7). Transfer into these primary cells of the "middle T only" plasmid did not produce stable transformants, even in the presence of high serum concentrations (3). This result is consistent with the independent observation that unlike wild-type polyoma DNA (8), DNA encoding only the middle T protein did not induce tumors upon injection into animals (9).

Because transformed cells derived from embryo fibroblasts after infection with the wild-type virus have acquired the ability of indefinite growth in culture (10), these results may suggest that "immortality" is not conferred on cells by expression of middle T only but requires either the large T or perhaps the small T protein. We asked whether the recombinant DNAs separately encoding these two proteins could promote the establishment of permanent lines. A convenient assay was provided by the observation that rodent embryo fibroblasts are unable to grow in culture at low cell densities (7), whereas established cell lines are able to form clonal colonies when seeded as sparse subcultures.

MATERIALS AND METHODS

Cell Cultures. Rat and mouse embryo fibroblast primary cultures (11) were prepared in Dulbecco's modified Eagle's medium (DME medium) supplemented with 10% fetal calf serum (GIBCO) from 15-day embryos of Fischer rats and from 12-day embryos of Swiss (OF1) mice. Subconfluent cultures were trypsinized and aliquots of 10^8 cells were frozen in liquid nitrogen. Aliquots were thawed and cells from one tube were seeded in four 10-cm Petri plates in DME medium containing 10% newborn calf serum (GIBCO). Three days later, the cells were transferred at a density of 5×10^5 cells per 60-mm plate or 2×10^5 cells per 35-mm plate and were used the following day for either DNA transfection or protoplast fusion. Cell lines established from these cultures and lines FR3T3 (12) and 3T6 (7) were propagated in DME medium supplemented with 10% newborn calf serum.

Gene Transfer Procedures. Both the calcium phosphate coprecipitation technique (13) and fusion with bacterial protoplasts (14) were used for gene transfers.

Recombinant Polyoma Virus Genomes. Recombinant polyoma DNA genomes listed in Table 1 have been described (2,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: DME medium, Dulbecco's modified Eagle's medium; SV40, simian virus 40.

‡ Present address: Genetics Inst., 225 Longwood Ave., Boston, MA 02115.

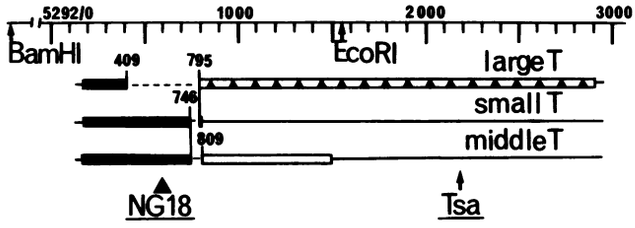

FIG. 1. The early region of polyoma virus. Linearized map of the early region DNA, marked with conventional nucleotide numbers (17) and the cutting sites for endonucleases *Bam*HI and *Eco*RI. Coding regions for the three early proteins are boxed, nontranslated regions are represented by continuous lines, and introns are represented by dashed lines.

3, 15, 16), except for plasmid pLTtsa, which was constructed by exchanging the *Bam*HI–*Eco*RI regions (Fig. 1) of plasmids pPyLT1 and pPYtsa [genomic DNA of the polyoma *tsa* mutant (18) inserted at the *Bam*HI site of pBR322].

Immunoprecipitation Analysis of Viral Proteins (T Antigens). Immunoprecipitation analysis of the viral proteins was performed as described (3).

RESULTS

Colony Formation by Rat Embryo Cells After Transfer of Polyoma Virus Genes Encoding Only the Large T Protein. Bacterial plasmids carrying polyoma virus sequences that encode either large T, middle T, or small T (Table 1) were transferred into rat embryo fibroblasts by the protoplast fusion technique (14) and the cells were seeded 24 hr later at a density of 10^3–10^4 cells per 10-cm Petri dish. One month later, the plates were examined after Giemsa staining. As shown in Fig. 2 and Table 2, only very limited growth was observed when the cells had received either no DNA (not shown) or pBR322 DNA, the apparent efficiency of colony formation being equal or less than 10^{-4}. As indicated below, these "colonies" are not likely to correspond to truly established clones. In parallel cultures derived from cells that had received DNA of the large T only pPyLT1 plasmid, significant numbers of colonies of actively growing cells were observed. The same result was observed after transfer of plasmid pNG1, which also encodes the large T protein only (15). The plating efficiency was $0.5–1.5 \times 10^{-3}$, comparable to the transformation efficiency of wild-type polyoma DNA in the same cell system ($1–1.5 \times 10^{-3}$). Unlike the dense colonies produced by transfer of wild-type polyoma DNA (plasmid pPY1), these colonies displayed a flat morphology. Transfer of plasmids encoding either middle T only (pPyMT1) or small T only (pPyST1) had no effect above the background frequency observed with pBR322 DNA.

Table 1. Coding capability of the recombinant plasmids

Plasmid	Polyoma virus genotype*	Coding capability (early genes)	Ref.
pPY1	Wild type	Large T, middle T, small T	14
pPyLT1	Deletion nucleotides 410–794	Large T	15
pNG1	NG18 *hrt* mutant	Large T	14
pMC1	*Bam*HI–*Eco*RI region of NG18 *hrt* DNA	NH_2-terminal 40% of large T	14
pLT214	*Bam*HI–*Eco*RI region of pPyLT1 DNA	NH_2-terminal 40% of large T	3
pLTtsa	pPyLT1 deletion of *tsa* mutant	Thermolabile large T	This work
pPyMT1	Deletion nucleotides 747–808	Middle T	2
pPyST1	Deletion nucleotides 747–794	Small T	3

*See Fig. 1; nucleotide numbers are according to ref. 17.

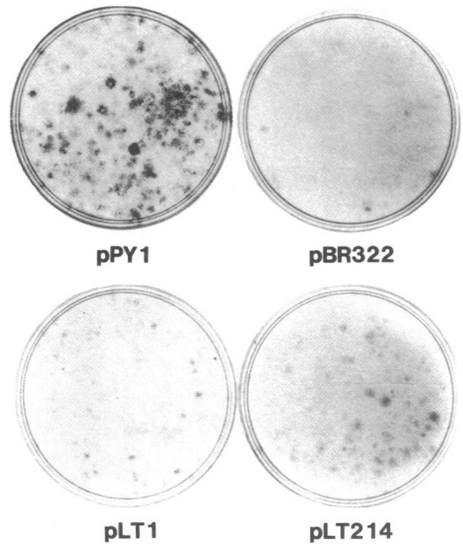

FIG. 2. Colony formation by rat embryo fibroblasts after transfer of wild-type and "large T only" polyoma plasmids. Fibroblasts from rat embryo primary cultures were seeded at a density of 10^4 cells per plate after transfer by protoplast fusion of the indicated plasmids (see Table 1). Giemsa staining after 5 wk at 37°C.

Colony Formation by Rat Embryo Cells After Transfer of a Recombinant Polyoma Virus Genome Encoding Only the Amino-Terminal Part of Large T. In the same series of experiments, colony formation was monitored after transfer into primary rat embryo cells of plasmid pLT214. This plasmid (Table 1) includes the viral sequences from the *Bam*HI to the *Eco*RI sites of plasmid pPyLT1, covering the promoter region and the 5′ 40% of the early coding sequences (Fig. 1). As in plasmid pPyLT1, the only available protein reading frame is that of the large T protein. After transfer into FR3T3 rat cells of this plasmid [or of the equivalent plasmid pMC1 (see Table 1)], a unique T-antigen species was observed (refs. 3 and 15; see Fig. 3, lane 6). It is phosphorylated *in vivo* as the full-size large T protein and has an apparent molecular weight (M_r 40,000) close to that predicted from the nucleotide sequence of the large T-coding regions between the amino-terminal ATG and the *Eco*RI site (M_r 36,700). pLT214 was as efficient as pPyLT1 in inducing colony formation by primary rat cells (Table 2) and the same result was observed for pMC1 (data not shown). Therefore, the func-

Table 2. Plating efficiency of rat embryo fibroblasts after transfer of viral genes encoding one of the early proteins

Plasmid*	Plating efficiency, colonies per 10^4 cells†	Morphology of colonies
pBR322	0–1	—
pPY1	12–15	Dense
pPYLT1	6–8	Flat
pPyMT1	0–1	—
pPyST1	0–0	—
pLT214	5–12	Flat
pLTtsa	12–15‡	Flat

*See Table 1.
†Extreme values observed in three independent experiments (two plates per plasmid in each experiment).
‡Selection at 33°C (see text).

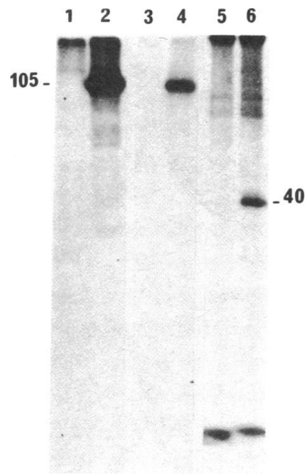

FIG. 3. Detection of polyoma large T antigen by immunoprecipitation in representative cell lines established after transfer of viral early genes. Cells were grown in DME medium supplemented with 10% newborn calf serum. They were labeled with [^{32}P]orthophosphate, and extracts were prepared and analyzed by immunoprecipitation with anti-polyoma T antigen serum as described (3); the indicated electrophoretic mobilities corresponding to apparent M_rs of 105,000 and 40,000 (shown as $M_r \times 10^{-3}$) were deduced from the position of protein markers on the same gels (not shown). See Table 3 for derivation of cell lines. Lane 1, PY-RAT-1, control serum; lane 2, PY-RAT-1, immune serum; lane 3, LT-RAT-2, control serum; lane 4, LT-RAT-2, immune serum; lane 5, 214-RAT-1, control serum; and lane 6, 214-RAT-1, immune serum.

tion of large T involved in induction of colony formation by primary cells is likely to be mediated by an amino-terminal domain of the protein. The same genetic localization was observed for the large T function necessary for the expression of a fully transformed serum-independent phenotype in lines derived from FR3T3 cells (3, 15).

Establishment of Phenotypically Normal Rat Fibroblast Lines. After protoplast-mediated transfer of large T-coding sequences into rat embryo cells (Table 2), cells were picked from several colonies and first seeded in a limited area of a Petri plate, by using a 4-mm-diameter stainless steel cylinder. Extensive growth of the population was then observed and serial transfers could be started. Representative lines (Table 3) have been maintained for >120 cell generations in culture (40 weekly transfers) without any apparent decrease in cell viability or in growth rate, which could indicate the occurrence of a crisis period. As expected (7), untreated rat embryo cultures could not be maintained for more than four to six transfers before a complete arrest of cell division. Similarly, no further growth was observed when cells were taken from the areas of cell growth observed after seeding at low density either untreated cells or cells that had received pBR322 DNA (Table 2). It is likely that these background colonies do not correspond to the development of established clones from isolated cells but rather to restricted growth in areas where the local density happened to be sufficient for the division of embryonic cells.

Table 3. Growth properties of wild-type transformants and LT-RAT cell lines

Cell line*	Saturation density†	Growth ability in agarose medium, %‡
FR3T3	1.5×10^6	<0.002
PY-T21	$>1.5 \times 10^7$	100
PY-RAT-1	$>1.5 \times 10^7$	100
LT-RAT-1	2×10^6	<0.002
LT-RAT-2	8×10^5	<0.002
NG-RAT-1	2×10^6	<0.002
214-RAT-1	1×10^6	<0.002
LTa-RAT-1	9×10^5	<0.002
LTa-RAT-2	5×10^5	<0.002
LTa-RAT-3	3×10^5	<0.002

* Line PY-T21 was derived from FR3T3 cells after transfer of wild-type polyoma genes (3); PY-RAT-1, LT-RAT-1 and -2, NG-RAT-1, 214-RAT-1, and LTa-RAT-1, -2, and -3 were derived from embryo fibroblasts after transfer of plasmids pPY1, pPyLT1, pNG1, pLT214, and pLTtsa, respectively (see Tables 1 and 2).

† Number of cells per 6-cm Petri plate 15 days after seeding at a density of 2×10^4 cells per plate; growth temperature was 37°C in all cases, except for the LTa-RAT lines (33°C).

‡ Cells (5×10^4) were seeded in agarose medium and microcolonies were counted per microscope field after 8 days at 33°C; values are expressed as % of the cell input.

Seven rat lines (Table 3) and three mouse lines (see below) independently established by this procedure were studied. Growth properties in medium containing 10% newborn calf serum were in all cases similar to those of established normal lines, such as FR3T3: in attached cultures, cell division was arrested at a low saturation density corresponding to a complete monolayer and cells seeded in suspension in agarose medium did not divide. However, unlike FR3T3 cells, these cells could grow in medium supplemented with only 0.5% calf serum (Fig. 4), a property that was previously found associated with the expression of the large T protein in FR3T3 cells (3). Although the growth rates were slower in low serum medium, the cultures reached the same saturation density as in 10% serum. Immunoprecipitation experiments performed on representative cell lines (Fig. 3) indicated the presence of the expected T antigen species—namely, the M_r 105,000 full-size large T or the M_r 40,000 truncated protein, depending on the plasmid used for establishment (see above). This result suggests that the continuous expression either of the full-size or of a truncated form of the large T protein may be related with the property of these cells of permanent growth in culture. This point was unambiguously demonstrated by the experiments using a mutated genome encoding a thermolabile large T protein.

Temperature-Dependent Growth Properties of Rat Cell Lines Derived After Transfer into Embryo Fibroblasts of a Large T Gene Carrying the *tsa* Mutation. To determine whether the presence of the large T protein is continuously required for growth in culture of LT-RAT cells, a recombinant DNA was constructed that carries the *tsa* mutation (18) and thus encodes an altered form of large T unstable at high temperature (refs. 19 and 20; see ref. 1 for review). Plasmid pLTtsa includes the polyoma sequences between the *Bam*HI and the *Eco*RI sites of plasmid pPyLT1, which cover the promoter region and the

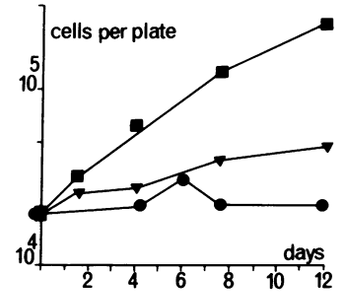

FIG. 4. Growth of LT-RAT cells in DME medium supplemented with 0.5% calf serum. Rat embryo fibroblasts from secondary cultures (▼) and cells of lines FR3T3 (●) and LT-RAT-2 (■) (see Table 3) were seeded at a density of 2×10^4 cells per 60-mm Petri plate in DME medium supplemented with 0.5% newborn calf serum. At the indicated times, cells were trypsinized and counted.

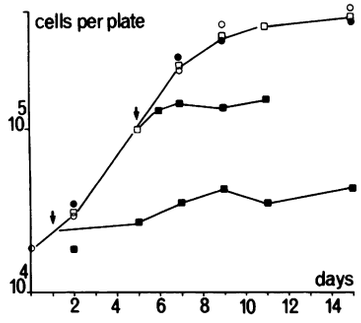

FIG. 5. Temperature-dependent growth of LTa-RAT cells. Growth of LTa-RAT-1 cells at 33°C (□) and after shifts to 40°C (■) either at day 1 or at day 5 (arrows) (see legend of Fig. 3). Cells of line LT-RAT-1 were used as control (○, 33°C; ●, 40°C).

proximal 1,305 nucleotides of the early coding region, linked to the *Eco*RI to *Bam*HI fragment from the *tsa* mutant, where the mutation has been mapped (21) (see Fig. 1). The presence of the *tsa* mutation in the recombinant DNA was ascertained by immunofluorescence measurements of T antigen synthesis after transfer into permissive 3T6 mouse cells at either 33 or 39°C (data not shown).

After transfer into primary rat embryo fibroblasts, selection by colony formation at 33°C produced the same proportion of established clones as with pPyLT1 DNA (Table 2). It was not possible to check for a differential effect of the temperature on the initial plating efficiency because this efficiency was decreased at 39°C, even for pPyLT1 DNA. Three independent cell lines (LTa-RAT-1, -2, and -3) were established and further propagated at 33°C from colonies induced by pLTtsa DNA. These cells grew at 33°C with the same normal phenotype as the lines established after transfer of pPyLT1 plasmid (Table 3). Cultures were then shifted to 40°C, a temperature at which rat fibroblast cells, although growing at a slightly slower rate than at 37°C, can be maintained for large numbers of generations without any apparent deleterious effect (see Fig. 5, LT-RAT-1 cells). Growth of the three LTa lines was arrested at 40°C after, at most, one residual doubling, as shown in Fig. 5 for LTa-RAT-1 cells. The cells became larger and remained attached to the plastic substratum. The viability of the cells at

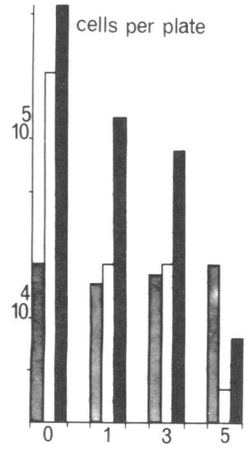

FIG. 6. Resumption of growth of LTa-RAT cells at low temperature after various times at 40°C. LTa-RAT-1 cells were seeded at 33°C and shifted to 40°C after 1 day, as in the experiment shown in Fig. 5. Before the shift (0) and after either 1, 3, or 5 days at 40°C, cultures were trypsinized and the cells were counted (hatched bars) and seeded in two plates, which were further incubated at 33°C. The number of cells per plate was determined 7 days (open bars) and 12 days (closed bars) later.

Table 4. Transformation of large T-established rat embryo fibroblasts by the middle T only gene

Cell culture*	Number of transformants† pPY1	pPyMT1
Rat embryo fibroblasts	15	<0.5
FR3T3	32	25
LT-RAT-1	15	17
LT-RAT-2	NT	10

NT, not tested.
*See Table 3.
†Number of foci per 10^5 cells after transfer by protoplast fusion of the indicated plasmid DNA.

40°C progressively decreased, as shown by growth measurements after replating at 33°C (Fig. 6). By contrast, the viability of control LT-RAT-1 cells measured in an identical manner was unaffected by incubation at 40°C. An activity of the full-size large T protein in these lines thus appears to be continuously required for their growth in culture. The fact that the *tsa* mutation maps within the COOH-terminal half of large T (21) is not contradictory with the attribution of the immortalization function to an NH_2-terminal domain of the protein, because the whole protein is unstable and quickly turning over at high temperature (19, 20).

Polyoma DNA Encoding Only the Middle T Protein Can Transform Embryonic Cells After Prior Establishment by the Large T-Coding Sequences. Our initial assumption was that the failure of plasmid pPyMT1, encoding only middle T, to transform primary embryo fibroblasts was the lack of an immortalization function. This now appears to be a large T function. One may then expect the permanent cell lines established by transfer of the large T-coding sequences to be susceptible to transformation by plasmid pPyMT1. This prediction was confirmed by the experiment summarized in Table 4: the efficiency of focus formation after transfer of plasmid pPyMT1 into LT-RAT cells was in the same range as that observed for FR3T3 cells. Transformed lines were derived from such foci. These cells, like those previously established from FR3T3 cells by either simultaneous or successive transfer of the middle T and the large T genes (3), exhibited the same high saturation density and high efficiency of cloning in agarose medium as wild-type polyoma transformants, both in high and low serum medium.

This result would suggest that simultaneous transfer of

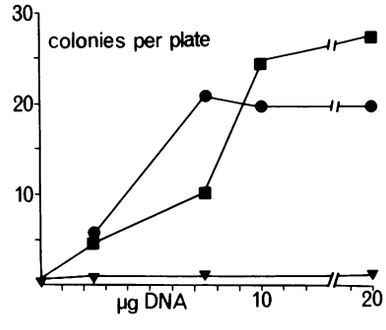

FIG. 7. Colony formation by isolated mouse embryo fibroblasts after transfection with polyoma genes encoding either a complete or a truncated large T protein. Mouse embryo fibroblast cells (10^6) were seeded per 60-mm Petri plate and transfected the next day by the calcium phosphate coprecipitation procedure with the indicated amounts of either calf thymus (▼), pNG1 (■), or pMC1 (●) DNA (see Table 1). Twenty-four hours later, the cells were trypsinized and replated at a density of 10^4 cells per plate. Colonies were counted on Giemsa-stained plates after 4 wk.

pPyMT1 and pPyLT1 into primary embryo cells would be sufficient to induce transformation. However, in repeated attempts we were unable to confirm this expectation. We shall report elsewhere results of complementation studies indicating that all three early proteins, including small T, must be simultaneously expressed to efficiently establish the transformed state under these conditions. Further investigation of alternative growth conditions for the establishment of transformation is necessary to determine if the small T requirement can be circumvented, as it is the case for transformation by simian virus 40 (SV40) (22).

Establishment of Mouse Fibroblast Lines from Primary Embryo Cell Cultures. Colony formation at low cell density was monitored after transfer into mouse fibroblasts in primary culture of plasmids carrying the various polyoma early genes. As shown in Fig. 7, results were essentially the same as with the rat fibroblasts: both the DNAs encoding the full-size large T protein (pNG1) and those encoding only its amino-terminal 40% (pMC1) induced colony formation within the same range of frequencies as in the rat embryo cell cultures. Three independent cell lines were independently established. None of them could grow past confluency on plastic plates or in suspension in agarose medium, and all three expressed the expected T antigen species.

DISCUSSION

Expression of a modified polyoma genome encoding only the middle T protein leads to transformation of established rat cells but not of primary rat embryo fibroblasts (3). This result suggested to us that expression of the middle T protein alone might not confer on primary cells an unlimited growth potential in culture (immortality), as observed after transformation with the wild-type virus (10). This hypothesis was confirmed by using colony formation in sparse subcultures as an assay for immortalization. It was further possible to derive established cell lines from the colonies grown after transfer of reconstructed viral genomes encoding either the full-size large T protein or only its amino-terminal region. The low saturation density and inability to grow in suspension of these lines made them comparable to the spontaneously established normal lines of the 3T3 type (7). We conclude from these experiments that the large T protein is both necessary and sufficient for the immortalization of rodent fibroblasts. Moreover, cell lines established by a modified viral genome uniquely encoding a thermolabile large T protein were unable to grow and became progressively nonviable at the restrictive temperature. This strongly suggests that expression of the viral function is continuously required for such lines to grow in culture.

These results confirm previous evidence that at least two of the viral early gene products, the nuclear large T protein and the membrane-associated middle T protein, act in the control of cellular growth. We have previously shown that in one class of polyoma-transformed FR3T3 cells, the large T protein is necessary for the complete expression of the transformed phenotype (12, 15). More recently, we have shown that the same protein confers on established FR3T3 cells the decreased dependence on serum factors characteristic of polyoma transformants (3). In fact, a relative independence on serum factors is also observed in cell lines derived from embryonic cells after transfer of large T-coding sequences. All of these large T-dependent alterations of growth control appear to require only the presence of the amino-terminal region of the protein.

While this work was in progress, it was shown by Petit *et al.* (23) that the *tsA* mutants of SV40 virus confer immortality in culture on rodent embryo fibroblasts in a temperature-dependent manner, and furthermore, it was independently demonstrated by Clayton *et al.* (24) and by Colby and Shenk (25) that expression of an amino-terminal domain of the SV40 large T antigen is sufficient to promote establishment of fibroblast cell lines in culture. These results confirm, and extend to a virus closely related to polyoma, our conclusion that the large T protein is composed of functionally distinct domains and that the amino-terminal part mediates important interactions with the cell genome. However, a remarkable difference between the two viral systems is that cell lines established after transfer of either a complete or a truncated SV40 large T gene exhibited various levels of transformation, whereas their polyoma counterparts were fully normal, except for their lower requirement in serum factors. This is consistent with the fact that, unlike SV40-transforming functions that are mostly exerted by the large T protein (22), polyoma-transforming functions are distributed between the products of two distinct genes (large T and middle T), which could be physically separated by the construction of the intronless genomes.

1. Ito, Y. (1980) in *Viral Oncology*, ed. Klein, G. (Raven, New York), pp. 447–473.
2. Treisman, R., Novak, U., Favaloro, J. & Kamen, R. (1981) *Nature (London)* **292**, 595–600.
3. Rassoulzadegan, M., Cowie, A., Carr, A., Glaichenhaus, N., Kamen, R. & Cuzin, F. (1982) *Nature (London)* **300**, 713–718.
4. Benjamin, T. L. (1970) *Proc. Natl. Acad. Sci. USA* **67**, 394–399.
5. Fluck, M. M. & Benjamin, T. L. (1979) *Virology* **96**, 205–228.
6. Lania, L., Griffiths, M., Cooke, B., Ito, Y. & Fried, M. (1979) *Cell* **18**, 793–802.
7. Todaro, G. J. & Green, H. (1963) *J. Cell Biol.* **17**, 299–313.
8. Israel, M. A., Simmons, D. T., Hourihan, S. L., Rowe, W. P. & Martin, M. A. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 3713–3716.
9. Asselin, C., Gélinas, M. & Bastin, M. (1983) *Mol. Cell. Biol.*, in press.
10. Vogt, M. & Dulbecco, R. (1963) *Proc. Natl. Acad. Sci. USA* **49**, 171–179.
11. Risser, R. & Pollack, R. (1979) *Virology* **92**, 82–90.
12. Seif, R. & Cuzin, F. (1977) *J. Virol.* **24**, 721–728.
13. Graham, F. L. & Van der Eb, A. J. (1973) *Virology* **52**, 456–467.
14. Rassoulzadegan, M., Binétruy, B. & Cuzin, F. (1982) *Nature (London)* **295**, 257–259.
15. Rassoulzadegan, M., Gaudray, P., Canning, M., Trejo-Avila, L. & Cuzin, F. (1981) *Virology* **114**, 489–500.
16. Tyndall, C., La Mantia, G., Thacker, C. M., Favaloro, J. & Kamen, R. (1981) *Nucleic Acids Res.* **9**, 6231–6250.
17. Soeda, E., Arrand, J. R., Smolar, N., Walsh, J. E. & Griffin, B. E. (1979) *Nature (London)* **283**, 445–453.
18. Fried, M. (1965) *Virology* **25**, 669–671.
19. Ito, Y., Spurr, N. & Dulbecco, R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1259–1263.
20. Silver, J., Schaffhausen, B. & Benjamin, T. L. (1978) *Cell* **15**, 485–496.
21. Thomas, T., Vollmer, P. & Folk, W. R. (1981) *J. Virol.* **37**, 1094–1098.
22. Martin, R. G., Petit Setlow, V., Edwards, C. A. F. & Vembu, D. (1979) *Cell* **17**, 635–643.
23. Petit, C. A., Gardes, M. & Feunteun, J. (1983) *Virology*, in press.
24. Clayton, C. E., Murphy, D., Lovett, M. & Rigby, P. W. J. (1982) *Nature (London)* **299**, 59–61.
25. Colby, W. W. & Shenk, T. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 5189–5193.