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Polyomavirus-Plasmid Recombinants Capable of Replicating Have an Enhanced Transforming Potential

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The frequency of transformation of rodent fibroblasts by polyomavirus is enhanced by a viral gene product, large T-antigen. However, this effect of large T-antigen cannot be demonstrated with pBR322-cloned viral DNA. Recently, it was discovered that pBR322 contains *cis*-acting sequences inhibitory to DNA replication in mammalian cells. Because polyomavirus large T-antigen is required for viral DNA replication, we examined the possibility that our inability to demonstrate a requirement for large T-antigen in transformation with pBR322-cloned viral DNA was due to the failure of the chimeric DNA to replicate in the transfected cells. To this end we constructed polyomavirus recombinant molecules with a plasmid (pML-2) that lacks these "poison" sequences and measured their capacity to transform cells. Here we report that recombinant plasmids capable of replicating in the transfected cells transform these cells at frequencies approximately sixfold greater than their replication-defective counterparts.

Polyomavirus replicates in mouse cells and transforms a wide variety of other rodent cells (16). The transforming potential of the virus resides in its early transcription unit, which encodes three proteins or tumor antigens (T-antigens) (7, 8, 13, 14). Genetic and biochemical analyses of their function in transformation have revealed that large T-antigen enhances the initiation of transformation (2, 3, 5) but is not required to maintain the transformed state (6). The latter function is performed by middle T-antigen (10, 15) and may be aided by small T-antigen or a part of large T-antigen (12). The mechanism by which large T-antigen enhances the frequency of transformation is not clear. Large T-antigen is required during the lytic cycle to initiate each new round of DNA replication (4), and we have proposed that this protein functions during transforming infections to increase the dose of viral DNA per cell (6). This would enhance the probability of integration of viral DNA into cellular DNA, a necessary step to stabilize the transformed state. This proposal was made to explain our inability to show a requirement for large T-antigen after DNA-mediated transformation with polyomavirus-recombinant plasmids. We suspected that the use of relatively large quantities of DNA during transfection overcame the need for large T-antigen. However, the inability of recombinant viral genomes to replicate in mammalian cells regardless of the presence of large T-antigen could also account for our observations. The latter explanation was lent credence by the discovery that pBR322 (the vector for the viral DNA) contains *cis*-acting, "poison" sequences that retard replication of DNA in mammalian cells (9). The isolation of new plasmids derived from pBR322 that lack poison sequences (i.e., pML-2) has allowed us to test this hypothesis.

To assess the relationship between the replicating capacity of a recombinant plasmid and its transforming potential, we constructed a variety of recombinant DNAs and measured their capacity to replicate in mammalian cells and to transform these cells. The compositions of the various recombinant plasmids used for this investigation are described in Table 1. They fall into several classes, including those composed of pBR322 DNA and viral DNA that is either capable of encoding functional large T-antigen (pPB1a and pPtsa at the permissive temperature) or incapable of doing so (pPR1a and pPtsa at the nonpermissive temperature), and those composed of pML-2 DNA and viral DNA that can either encode functional large T-antigen (pdPB1a and pdPtsa at the permissive temperature) or cannot (pdPR1a and pdPtsa at the restrictive temperature). To determine whether these recombinant genomes could replicate in permissive mouse cells or semipermissive Rat-1 cells, we transfected these cells with the plasmid DNAs, extracted low-molecular-weight DNA 72 h later, and assessed the state of methylation of the recombinant DNA as an indirect measure of replication after digestion with *Dpn*I and *Bcl*I, followed by Southern blotting-hybridization (11). Plasmid DNA that is propagated in *Esche-*

TABLE 1. Composition of polyomavirus-plasmid recombinants[a]

Recombinant plasmid	Vector	Composition
pPB1a[b]	pBR322	Entire polyomavirus genome cloned at its *Bam*HI site in the + orientation
pPR1a	pBR322	Entire polyomavirus genome cloned at its *Eco*RI site in the + orientation
pPtsa	pBR322	Entire genome of the *tsA* mutant of polyomavirus cloned at its *Bam*HI site in the − orientation
pdPB1a[b]	pML-2	Entire polyomavirus genome cloned at its *Bam*HI site in the + orientation
pdPR1a	pML-2	Entire polyomavirus genome cloned at its *Eco*RI site in the + orientation
pdPtsa	pML-2	Entire genome of the *tsA* mutant of polyomavirus cloned at its *Bam*HI site in the + orientation

[a] The structure of the recombinant plasmids and the orientation of the viral sequences in comparison with the vector sequences were determined by restriction endonuclease analyses. A + indicates that the early region of polyomavirus is in the same transcriptional orientation as the *bla* gene of the vector. A − denotes the opposite orientation.

[b] This recombinant contains a deletion of about 500 base pairs that straddles the *Hin*dIII site in the late region of polyomavirus.

richia coli is methylated by the cell at adenine residues that lie within the sequence GATC. Such methylated sequences are recognized and cleaved by *Dpn*I. However, DNA that is isolated from bacteria and subsequently replicated in mammalian cells is not methylated and therefore becomes resistant to cleavage by *Dpn*I. Similarly, *Bcl*I recognizes and cleaves DNA within the sequence TGATCA, but in contrast to *Dpn*I, this enzyme cleaves only DNA which is not methylated at the adenine residue shown above. Therefore, by digesting the recombinant DNA that was recovered after transfection of mammalian cells with *Dpn*I and *Bcl*I (the latter cleaved all the recombinant DNAs shown in Table 1 once), it was possible to distinguish the replicated DNA from the input DNA that did not replicate. Replicated DNA appeared as a band that comigrated with linear recombinant DNA. Any unreplicated DNA appeared as lower molecular-weight fragments because each of the recombinant plasmids described previously contained many sites of cleavage for *Dpn*I. The results of this analysis are shown in Fig. 1. Recombinant plasmids capable of encoding functional large T-antigen (pPB1a, pPtsa at 32°C, and pdPB1a and pdPtsa at 32°C) were all capable of replicating in permissive mouse cells. However, among these, those which used pML-2 as a vector replicated significantly more efficiently than those that used pBR322 as a vector (Fig. 1). Note that pPB1a and pdPB1a each contain a deletion of approximately 500 base pairs in the late region of the viral sequences, in comparison with pPR1a and pdPR1a. This deletion did not affect the expression of the early region, nor did it influence the replication of the recombinant plasmids. Not surprisingly, recombinant plasmids incapable of encoding functional large T-antigen (pPR1a, pdPR1a, and pPtsa and pdPtsa at the restriction temperature) did not replicate in the permissive host. Results identical to these were obtained when calcium phosphate was used as the facilitator instead of DEAE-dextran.

To determine whether these observations were also true for semipermissive Rat-1 cells, the host for transformation, we also measured the replicating capacity of pPtsa and pdPtsa DNA at the permissive and nonpermissive temperatures in these cells. Again, we observed that functional large T-antigen is required for replication in these semipermissive cells and that pBR322 sequences significantly inhibit the replication of the recombinant plasmid DNAs (Fig. 2). Note, however, that semipermissive Rat-1 cells did not support the replication of the various recombinant DNAs as efficiently as permissive mouse cells (compare Fig. 1 and 2). We estimate that permissive mouse cells contain from 100 to 200 replicated DNA molecules per cell at 72 h posttransfection, whereas semipermissive Rat-1 cells contain 1% of that amount. These calculations were made by assuming that all the cells take up the transfected DNA and by reference to markers of known quantity analyzed on the same gel.

To determine whether a relationship existed between the capacity of a recombinant plasmid to replicate in Rat-1 cells and its capacity to transform these same cells, we next measured the specific transforming activity of each of the recombinant plasmids described previously. For these measurements we calculated the specific transforming activity of the DNA over a range of DNA concentrations (from 2 to 100 ng of DNA per 3×10^5 cells) in which doubling the amount of DNA transfected resulted in a doubling of the number of foci (10). This activity was determined in triplicate for each of three DNA concentrations, and the experiments were repeated three or four times, depending on the comparisons being made. Because large numbers of plates had to be used to measure accurately the

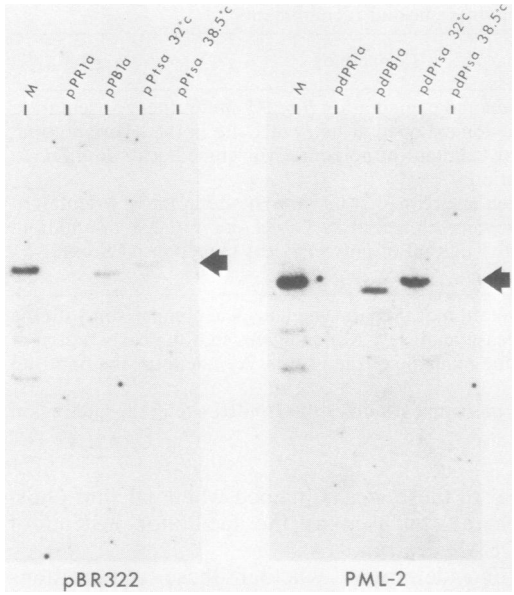

FIG. 1. Replication of various polyomavirus-plasmid recombinant DNAs in mouse 3T3 cells. Polyomavirus recombinant plasmid DNAs were isolated from *E. coli* DH-1 with the Triton-lysis protocol and purified by cesium chloride-ethidium bromide equilibrium centrifugation as previously described (6, 10). Of each supercoiled DNA species, 50 ng was used directly to transfect NIH 3T3 cells with DEAE-dextran as the facilitator. The cells were incubated at 32°C (pPtsa and pdPtsa), 37°C (pPR1a, pPB1a, pdPR1a, and pdPB1a), or 38.5°C (pPtsa and pdPtsa) in a humidified $-CO_2$ incubator for 72 h, and then low-molecular-weight DNA was isolated. The recovered DNA was digested with *Bcl*I and *Dpn*I and electrophoresed through a 1.0% (wt/vol) agarose gel; the DNA fragments were then transferred to a nitrocellulose sheet and hybridized with ^{32}P-labeled pPR1a DNA. After the nitrocellulose sheet was washed, it was autoradiographed for 24 h with intensifying screens. Photographs were subsequently made from a single audioradiogram and divided into the panels shown above for illustrative purposes. The bottoms of the autoradiograms, which show the unreplicated DNA, were cropped from the final photograph. They revealed that half of the total DNA remaining after 72 h had not replicated. The arrows to the right of the panels labeled pBR322 and pML-2 refer to the positions of linear pPtsa and pdPtsa DNA, respectively. The lane labeled M in the pBR322 panel contains, from top to bottom, linear pPtsa DNA (10^{-4} μg), supercoiled pPtsa DNA (10^{-5} μg), and linear polyomavirus DNA (10^{-5} μg) as molecular weight markers. The lane labeled M in the pML-2 panel contains, from top to bottom, linear pdPtsa DNA (5×10^{-4} μg), supercoiled pdPtsa DNA (10^{-5} μg), and linear polyomavirus DNA (10^{-5} μg).

specific transforming activities of the various recombinant plasmids, we performed the experiments in groups of two to four DNAs. The error margin within each experiment was approximately 20%. The results are summarized in Table 2. The specific transforming activities of replication-defective plasmids that are capable of encoding functional large T-antigen were nearly the same as those of plasmids that are incapable of doing so (Table 2, experiments 1 and 2). Note that meaningful comparisons among the transforming activities of various DNAs can only be made within an experiment and not between experiments. By contrast, the specific transforming activities of recombinant

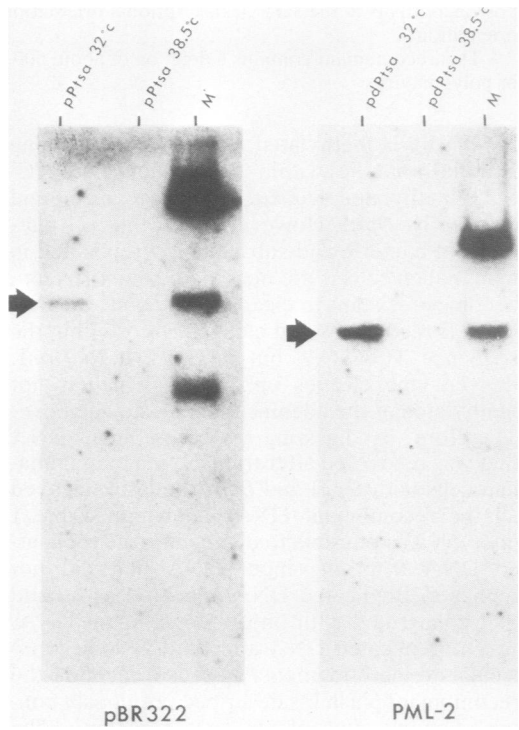

FIG. 2. Replication of pPtsa and pdPtsa DNA in Rat-1 cells at the permissive and nonpermissive temperatures. Rat-1 cells were transfected with 500 ng each of pPtsa and pdPtsa DNA, and the replication of the DNA was assessed as described for Fig. 1. The arrow at the left of the panel labeled pBR322 refers to the position of linear pPtsa DNA, and the arrow to the left of the panel labeled pML-2 refers to the position of linear pdPtsa DNA. The lanes labeled M contain a *Sal*I partial digest of 10^{-5} μg of pPtsa DNA (pBR322) or pdPtsa DNA (pML-2). *Sal*I cleaves both of the aforementioned recombinant plasmids once. The top band in each of the lanes labeled M corresponds to nicked circular DNA, the middle band corresponds to linear DNA, and the fastest-migrating species is supercoiled DNA. Note that the digest in lane M (pML-2) contains very little supercoiled pdPtsa DNA. The nitrocellulose sheet was exposed to film for 6 days with intensifying screens. Photographs were subsequently made from a single audioradiogram and divided into the panels shown above.

TABLE 2. Transforming activity of various polyomavirus-plasmid DNAs[a]

Expt	Plasmid	Temp (°C)	Capacity to replicate in Rat-1 cells	Capacity to produce functional large T-antigen	Specific transforming activity (foci per μg)[b]
1	pPB1a	37	−	+	3,300
	pPR1a	37	−	−	2,900
2	pPtsa	32	−	+	6,100
	pPtsa	38.5	−	−	5,800
3	pdPB1a	37	+	+	16,000
	pdPR1a	37	−	−	2,300
	pdPtsa	32	+	+	15,000
	pdPtsa	38.5	−	−	2,500
4	pdPB1a	37	+	+	13,000
	pPB1a	37	−	+	2,700
	pdPR1a	37	−	−	3,100
	pPR1a	37	−	−	2,800

[a] Rat-1 cells were seeded at a density of 3×10^5 cells per 100-mm-diameter plate and transfected 12 to 16 h later with calcium phosphate as the facilitator.

[b] The specific transforming activity of each DNA was calculated from titration curves obtained by transfection in triplicate with three different amounts of DNA (2 to 100 ng per plate) under conditions where doubling the quantity of DNA transfected resulted in a doubling of the number of foci. For pBR322-based plasmids these amounts of DNA were 10, 50, and 100 ng, whereas for pML-2-based vectors they were 2, 5, and 10 ng of DNA per 3×10^5 cells. Each plate contained an average of between 20 and 300 foci, depending upon the nature and amount of DNA used.

plasmids capable of replicating and, of necessity, encoding functional large T-antigen were sixfold greater than those of their replication-defective counterparts (Table 2, experiment 3). This effect is related to the replicating properties of the recombinant plasmids and not to the sequences of the vector because direct comparison of the specific transforming activities of pPR1a and pdPR1a DNA or of pPB1a and pdPB1a revealed that only DNA capable of replicating (pdPB1a) transformed cells at elevated levels of activity (Table 2, experiment 4). These results establish a correlation between the capacity of polyomavirus-plasmid recombinants to replicate in Rat-1 cells and their capacity to transform these cells, and they suggest to us that the function of large T-antigen in the initiation of transformation is to promote replication of the transforming DNA. Thus, the number of copies of transforming DNA per cell or the structure of the DNA may be altered, and this could increase the probability of integration.

Our results confirm and extend previous observations which showed that mutations that affect the functioning of large T-antigen also affect the initiation of transformation by the virus. Comparisons of the frequency of transformation of hamster cells with the *tsA* mutant of polyomavirus (2, 3, 5) or of Rat-1 cells with the DNA of the *tsA* mutant (1) have shown that from 100 (for the former transformation) to 20 (for the latter transformation) more transformants arise at the permissive as opposed to the nonpermissive temperature. Using molecularly cloned polyomavirus DNA, we found a sixfold effect of large T-antigen on the establishment of transformation. We believe that these quantitative differences are related to the replicating properties of the DNAs and the degree of permissiveness of the host cells.

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LITERATURE CITED

1. **Della Valle, G., R. G. Fenton, and C. Basilico.** 1981. Polyoma large T-antigen regulates the integration of viral DNA sequences in the genome of transformed cells. Cell **23**:347–355.
2. **Di Mayorca, G., J. Callender, J. Marin, and R. Giordano.** 1969. Temperature-sensitive mutants of polyoma virus. Virology **38**:126–133.
3. **Eckhart, W.** 1969. Complementation and transformation by temperature-sensitive mutants of polyoma virus. Virology **38**:120–125.
4. **Francke, B., and W. Eckhart.** 1973. Polyoma gene function required for viral DNA synthesis. Virology **55**:127–135.
5. **Fried, M.** 1965. Isolation of temperature-sensitive mutants of polyoma virus. Virology **25**:669–671.
6. **Hassell, J. A., W. C. Topp, D. B. Rifkin, and P. E. Moreau.** 1980. Transformation of rat embryo fibroblasts by cloned polyoma virus DNA fragments containing only part of the early region. Proc. Natl. Acad. Sci. U.S.A. **11**:3978–3982.
7. **Hutchison, M. A., T. Hunter, and W. Eckhart.** 1976. Characterization of T-antigens in polyoma-infected and transformed cells. Cell **15**:65–77.
8. **Ito, Y., N. Spurr, and R. Dulbecco.** 1977. Characterization of polyoma virus T-antigen. Proc. Natl. Acad. Sci. U.S.A. **74**:1259–1263.
9. **Lusky, M., and M. Botchan.** 1981. Inhibition of SV40 replication in simian cells by specific pBR322 DNA sequences. Nature (London) **293**:79–81.
10. **Mes, A.-M., and J. A. Hassell.** 1982. Polyoma viral middle T-antigen is required for transformation. J. Virol. **42**:621–629.
11. **Peden, K. W. C., J. M. Pipas, S. Pearson-White, and D. Nathans.** 1980. Isolation of mutants of an animal virus in bacteria. Science **209**:1392–1396.
12. **Rassoulzadegan, M., A. Cowie, A. Carr, N. Glaichenhaus, R. Kamen, and F. Cuzin.** 1982. The roles of individual polyoma virus early proteins in oncogenic transformation. Nature (London) **300**:713–718.
13. **Silver, J., B. Schaffhausen, and T. L. Benjamin.** 1978. Tumor antigens induced by nontransforming mutants of polyoma virus. Cell **15**:485–496.

14. **Smart, J. E., and Y. Ito.** 1978. Three species of polyoma virus tumor antigens share common peptides near the amino termini of the proteins. Cell **15**:1427–1437.
15. **Triesman, R., U. Novak, J. Favaloro, and R. Kamen.** 1981. Transformation of rat cells by an altered polyoma virus genome expressing only the middle T-protein. Nature (London) **292**:595–600.
16. **Tooze, J. (ed.).** 1981. DNA tumor virus, part 2, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.