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Homologous Recombination Between Transfected DNAs

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An extensive analysis of the fate and structure of polyomavirus-plasmid recombinant molecules transfected into Rat-1 cells has revealed that the DNA often becomes integrated within transformed cell DNA in a head-to-tail tandem arrangement. This occurs independently of the replicative capacity of the transforming DNA and is facilitated by the use of large quantities of DNA during transfection. These observations have led us to suggest that head-to-tail tandems are formed by homologous recombination between transfected DNAs either before or after integration within cellular DNA. To test this hypothesis, we have measured the transforming activity of pairs of mutant, nontransforming, recombinant plasmid DNAs that carry different lesions in the transforming gene of polyomavirus. The results show that, although the individual mutant DNAs are incapable of transformation, transfection with pairs of mutant DNAs leads to the formation of transformed cells at high frequency. Moreover, there is a direct relationship between the distance between the lesions in pairs of mutant DNAs and their transforming activity. Finally, analyses of the structures of integrated recombinant plasmid DNAs and the viral proteins within independent transformed cells prove that recombination occurs between the mutant genomes to generate a wild-type transforming gene.

Polyomavirus, one member of the papovaviruses, is a small double-stranded DNA tumor virus that is capable of replicating in mouse cells and transforming other rodent cells in culture. The transforming potential of the virus resides in the early transcription unit which encodes three tumor antigens, large, middle, and small T antigen (28). Large T antigen facilitates the initiation of transformation (8, 9, 12), whereas middle T antigen is required to maintain the transformed phenotype (3, 10, 14, 16, 20, 22, 23, 25, 27, 29). The role of small T antigen in transformation is not known. Stable cellular transformation is achieved by the integration of the viral sequences within cellular DNA. Recombination between the cellular and viral genomes does not appear to involve specific sites, nor are regions of extensive homology required (15). One distinguishing characteristic of polyomavirus-transformed cells, especially transformed rat cells, is the arrangement of the integrated viral sequences within transformed cell DNA in a head-to-tail tandem array (1, 2, 4, 13, 19). This arrangement of integrated viral sequences is not only unique to polyomavirus-transformed cells but also occurs in simian virus 40-transformed cells (5, 18) and in cells transformed with viral recombinant DNA (24). The formation of tandemly arranged, integrated viral DNA may be stimulated by large T antigen (7), perhaps indirectly, but is not absolutely dependent on the activity of this protein (24). We have suggested that tandemly integrated sequences are formed by recombination between transforming DNAs (24), and have now formally tested this possibility.

To determine whether homologous recombination can occur after transfection of mammalian cells, we used pairs of polyomavirus-plasmid recombinant DNAs that bear physically separated mutations within their transforming sequences (20, 29). These recombinant DNAs are incapable of independently transforming cells because their mutations completely inactivate the activity of middle T antigen, the transforming protein (20, 29). We reasoned that if homologous recombination could occur between the mutations borne by different nontransforming mutant DNAs after transfection of cells, then wild-type (wt) transforming genomes would result. The latter, when integrated and expressed, should yield wt middle and small T antigen and result in cellular transformation. Moreover, we expected that the greater the distance between the mutations carried by the mutant pairs, the greater the frequency of occurrence of transformed cells. The structures of the various recombinant plasmid DNAs employed in this study are shown in Fig. 1B and C, together with a physical map of that part of the polyomavirus early transcription unit which they all contain (Fig. 1A). Two different vectors were used to

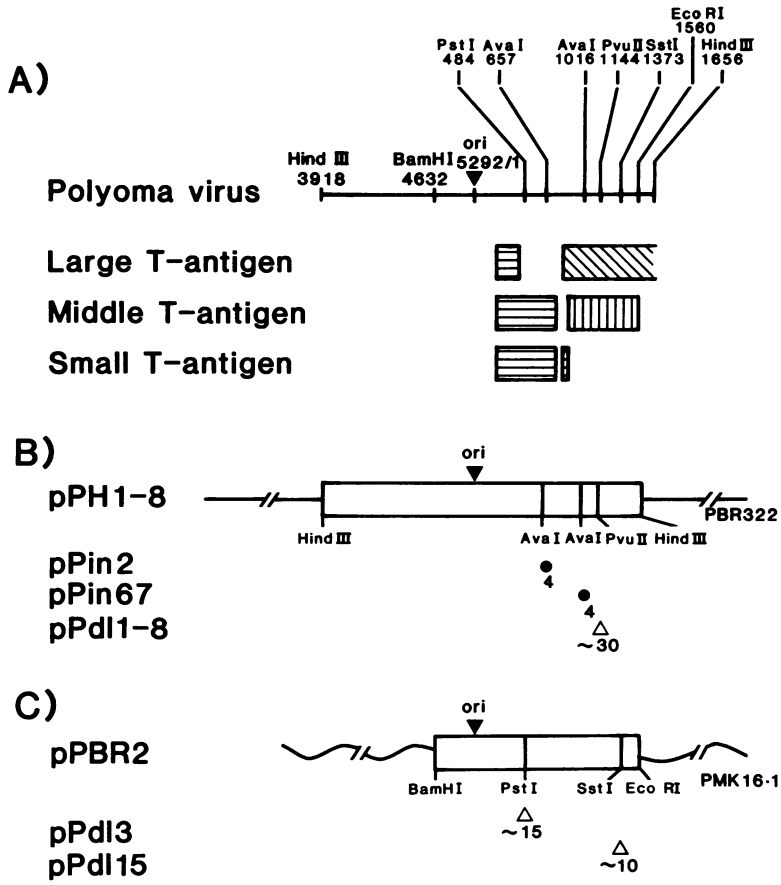

FIG. 1. Physical map of the polyomavirus early region important for transformation and the location of mutations that render the DNA nontransforming. A linear map of the polyomavirus genome between the two *Hind*III sites at nucleotide positions 3918 and 1656 is shown (A) together with the location of relevant restriction endonuclease cleavage sites. The solid triangle marks the junction of nucleotide numbers 5292 and 1, which is thought to be near the site from which bidirectional DNA replication initiates (ori). Below the linear map are displayed the coding sequences for the polyomavirus tumor antigens (T antigens). Note that all three T antigens share sequences at their amino termini (the blocked areas with the horizontal lines), but as a result of RNA splicing, the reading frame is altered for large and middle T antigens. Therefore, all three T antigens differ in amino acid sequence following the splice junction in their respective mRNAs. The amino acid blocks are not joined in the illustration, the better to display their origin from the DNA sequences shown above them. Note that the coding sequences for large T antigen continue beyond nucleotide position 1656. The structure of the parental plasmids, pPH1-8 and pPBR2, and the mutant genomes derived from them are shown in B and C. pPH1-8 is composed of polyomavirus sequences between the *Hind*III sites (illustrated as a blocked area in B), which were cloned within the *Hind*III site of pBR322 DNA (B, thin line). The mutant genomes shown below the structure of pPH1-8 (pPin2, pPin67, and pPdl1-8 in B) were derived from pPH1-8 by altering the *Ava*I or *Pvu*II cleavage sites within the viral sequences (20). pPin2 contains a 4-bp insertion (●) that destroys the *Ava*I site at nucleotide position 657. pPin67 contains a 4-bp insertion that destroys the *Ava*I site at nucleotide position 1016. pPdl1-8 contains a deletion (△) of about 30 bp that removes the *Pvu*II site at nucleotide position 1144. pPBR2 (C) is composed of the *Bam*HI-to-*Eco*RI fragment of polyomavirus DNA (blocked area) and the *Bam*HI-to*Eco*RI fragment of pMK16.1 (wavy lines). The two mutants derived from pPBR2, pPdl3, and pPdl15, carry deletions of about 15 and 10 bp that remove the *Pst*I and *Sst*I sites, respectively.

construct the recombinant genomes, pMK16.1 and pBR322. These vectors are homologous to each other over only a part of their DNAs (they share sequences that include part of the tetracycline resistance marker). The mutant genomes were constructed as described previously (20). Shown in Table 1 is the outcome of an experiment in which Rat-1 cells were transfected with the mutant recombinant DNAs, either alone or in pairs, and the number of transformed cells

TABLE 1. Transformation of Rat-1 cells with mutant pairs of nontransforming polyoma virus recombinant DNAs[a]

Recombinant DNA	Approx distance between mutations (bp)	Foci per dish	
		Separate dishes	Avg
pPin2		0,0,0,0	0
pPin67		0,0,0,0	0
pPdl3		0,0,0,0	0
pPdl1-8		0,0,0,0	0
pPdl15		0,0,0,0	0
pPin2 and pPin67	360	19,19,21,23	21
pPdl3 and pPdl1-8	660	19,25,34,35	28
pPdl15 and pPdl3	870	74,88,104,125	98
pPH1-8		460,492,521	491
pPBR2		445,480,493	473

[a] Rat-1 cells(3×10^5 to 5×10^5) per 10-cm-diameter plastic dish) were transfected with 1 μg of each mutant DNA alone or in pairs, with calcium phosphate used to facilitate DNA uptake as described (14, 20). The cultures were stained with Giemsa, and foci were counted after 10 to 14 days.

that resulted scored as foci. Transfection of Rat-1 cells with each mutant DNA alone (1 μg of mutant DNA per 5×10^5 cells) yielded no foci. By contrast, transfection with mixtures of two mutant DNAs (1 μg of each species per 5×10^5 cells) yielded a large number of foci, and the greater the distance between the mutations borne by the mutant DNA pairs, the higher the frequency of occurrence of foci (Table 1). As a control, two wt recombinant plasmid DNAs, the parents of the mutant DNAs, were also tested for transforming potential. One μg of each of these DNAs yielded approximately 500 foci. By comparison, from 20 to 100 foci resulted from transfection with pairs of mutant DNAs, depending upon the distance between the mutations carried by each DNA (Table 1).

The transformed cells that arose after transfection with pairs of nontransforming recombinant DNAs could have resulted from complementation or recombination between the mutant DNAs. To determine which of these explanations was correct, we isolated six independent transformed cell lines (three lines from the cross pPdl 3 × pPdl 1-8 and three lines from the cross pPin2 × pPin67) and examined the structure of the integrated viral sequences for recombinant restriction enzyme fragments. In addition, we isolated the viral proteins from these cell lines and measured their molecular weights by polyacrylamide gel electrophoresis. Each of the mutant recombinant DNAs that we employed in transformation assays bears a lesion that destroys a restriction endonuclease cleavage site. For example, pPdl1-8 carries a deletion of about 30 base pairs (bp) that removes a *Pvu*II cleavage site, whereas pPdl3 has a deletion of about 15 bp that includes a *Pst*I cleavage site (Fig. 1B and C; Fig. 2, panel 1A and B). If recombination should occur between these markers before or after integration to generate a wt genome, then this could be recognized by cleavage of the transformed cell DNA with *Pvu*II and *Pst*I. A fragment of 660 bp would result comigrating with a fragment obtained by cleaving one of the parental, wt, DNAs (i.e., pPBR2 or pPH1-8) with these same enzymes (Fig. 2, panel 1C). Such recombinant fragments would be diagnostic of a recombination event. Application of this method to analyze the integrated viral DNA in three cell lines isolated after transfection with pPdl1-8 and pPdl3 revealed the presence of recombinant DNA fragments in each of these cell lines (Fig. 2, panel 1D). Similarly, when this method was used to analyze the integrated viral DNA present in three transformed cell lines that resulted from the cross pPin2 × pPin67 (Fig. 2, panel 2A through C), we again observed the presence of recombinant DNA fragments that could only have been generated by recombination between the lesion in each mutant DNA to yield a wt transforming gene (Fig. 2, panel 2D).

Finally, we analyzed the viral proteins in each of the six cell lines described above. This was accomplished by incubating the cells with [^{35}S]methionine, isolating the total complement of cellular proteins and then immunoprecipitating the viral proteins by reaction with antiserum obtained from rats bearing tumors induced by polyomavirus-transformed cells. The spectrum of labeled proteins that are immunoprecipitated with the antiserum from tumor-bearing rats by comparison with those that react with antiserum from normal rats are shown in Fig. 3. Each of the six transformed cell lines synthesized full-length, middle, and small T antigens. The synthesis of complete middle T antigen by each of these cell lines could only occur by expression of a wt coding region that resulted from homologous recombination between the transfected mutant DNAs.

The analysis of integrated viral sequences and viral proteins in cells transformed with pairs of mutant nontransforming DNAs leads us to conclude that homologous recombination can occur between transfected DNAs. The frequency of homologous recombination is difficult to measure accurately, but our results suggest that it is relatively high. For example, as many as 100 foci resulted from transfection with pairs of mutant DNAs whose lesions are separated by about 900 bp. By comparison, approximately 500 foci resulted after transfection with a nearly equivalent amount of wt DNA. The mechanism by which

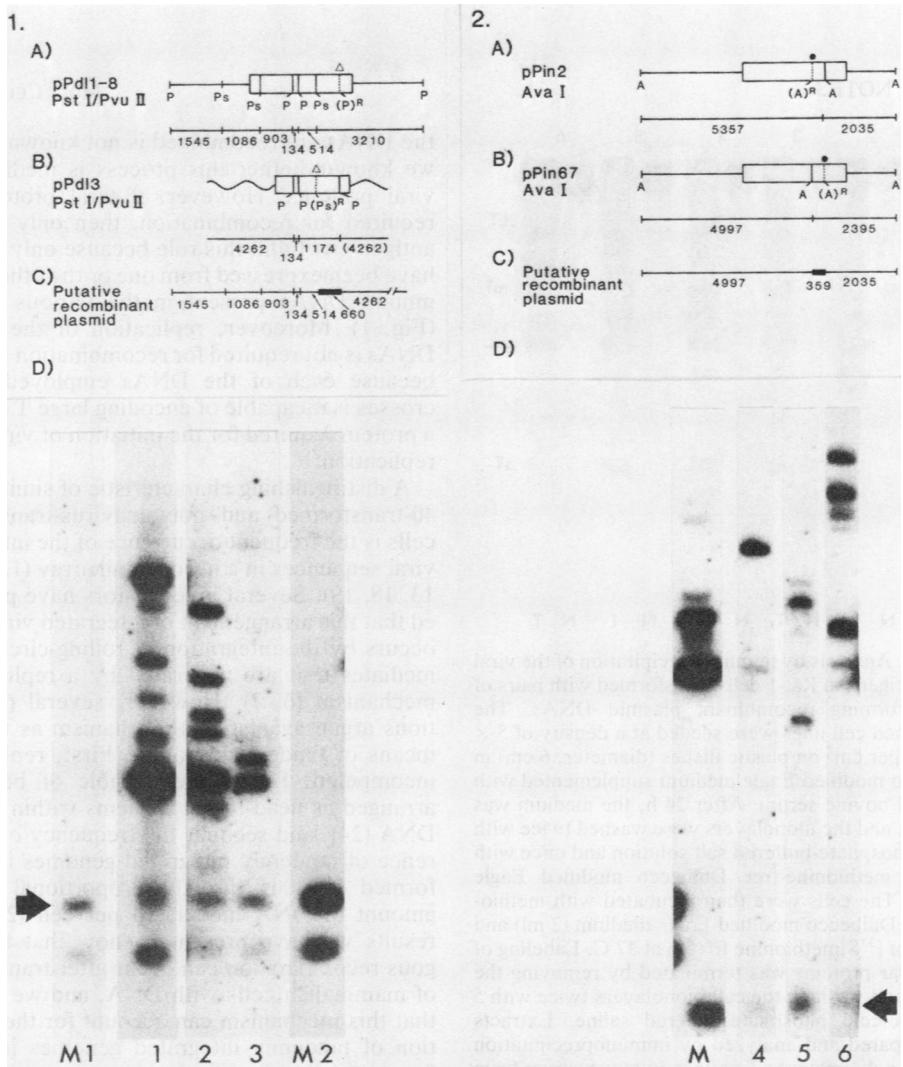

FIG. 2. Analysis by Southern blotting and hybridization of the cellular DNA of Rat-1 cells transformed with pairs of nontransforming mutant recombinant plasmids. Panel 1 shows the structures of pPdl1-8 (A) and pPdl3 (B) DNA, the sites of cleavage by *Pst*I (Ps) and *Pvu*II (P), and the lengths of the resulting fragments in bp. The superscript R denotes that this site is resistant to cleavage by the restriction endonuclease. If a recombination event should occur between the deletions borne by the mutant DNAs to generate a wt transforming gene, then its structure would resemble that shown in C. Note that the *Pst*I-*Pvu*II restriction endonuclease cleavage map of this putative recombinant plasmid (panel 1C) is different from that of its parents (shown in A and B). In particular, a new viral fragment of 660 bp (the darkened area) would occur after cleavage of this putative recombinant DNA with *Pst*I and *Pvu*II that would comigrate with a similar fragment obtained by cleaving the wt polyomavirus sequences (i.e., pPBR2 or pPH1-8) with these same enzymes. In panel D are shown the results of cleaving with *Pst*I and *Pvu*II of 10 μg of cellular DNA from three independent transformed cell lines (numbered 1, 2, and 3) that resulted from transfection with a mixture of pPdl3 and pPdl1-8 DNAs followed by agarose gel electrophoresis, blotting, and hydrization to a ^{32}P-labeled probe that contained only viral sequences between nucleotide positions 5262 and 1144. The lanes marked M1 and M2 contained 10^{-6} and 10^{-5} μg, respectively, of pPBR2 DNA that had been cleaved with *Pst*I and *Pvu*II and analyzed on the same gel. The two fragments that hybridized to the probe in the marker lanes are 660 and 514 bp long. The arrow indicates the position of the 660-bp fragment that is diagnostic of a recombination event which occurred between the deletions borne by pPdl1-8 and pPdl3. In panel 3, the *Ava*I (marked by A) restriction endonuclease cleavage maps of the mutant nontransforming DNAs of pPin2 (A), pPin67 (B), and a putative recombinant transforming DNA (C) are shown. The darkened area in C represents the 359-bp fragment that would be diagnostic of a recombination event between the lesions borne by the two mutant DNAs. The results of cleaving the cellular DNAs from three independent transformed cell lines (numbered 4, 5, and 6) with *Ava*I followed by agarose gel electrophoresis, blotting, and hybridization to a ^{32}P-labeled viral probe (viral sequences between nucleotide positions 5262 and 1144) are shown in panel 2D. Lane M contained 10^{-5} μg of pPBR2 DNA cleaved with *Ava*I. The arrow denotes the position of the 359-bp fragment that is diagnostic of a recombination event between the mutations borne by pPin2 and pPin67. The symbols used to denote the nature of the mutations are described in the legend to Fig. 1. The autoradiograms shown in panels 1 and 2 were originally part of a single autoradiogram which was subsequently cut for illustrative purposes and because the various lanes were exposed for different periods. Panel 1D, lanes 1 and M2, were exposed for 2 days; lanes M1, 2, and 3 were exposed for 18 days. The nitrocellulose sheet was exposed for 18 days to obtain the autoradiogram shown in panel 2D.

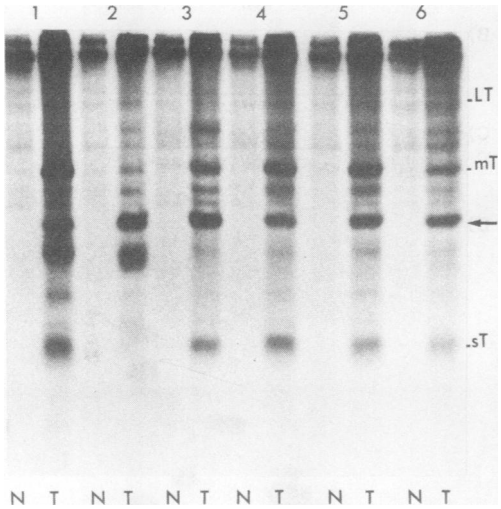

FIG. 3. Analysis by immunoprecipitation of the viral tumor antigens in Rat-1 cells transformed with pairs of nontransforming recombinant plasmid DNAs. The transformed cell lines were seeded at a density of 5×10^4 cells per cm^2 on plastic dishes (diameter, 6 cm) in Dubbecco modified Eagle medium supplemented with 10% fetal bovine serum. After 24 h, the medium was removed, and the monolayers were washed twice with 5 ml of phosphate-buffered salt solution and once with 5 ml of methionine-free Dulbecco modified Eagle medium. The cells were then incubated with methionine-free Dulbecco modified Eagle medium (2 ml) and 400 μCi of [^{35}S]methionine for 5 h at 37°C. Labeling of the cellular proteins was terminated by removing the medium and washing the cell monolayers twice with 5 ml of ice-cold phosphate-buffered saline. Extracts were prepared and analyzed by immunoprecipitation with anti-polyomavirus T antigen immune serum from rats bearing tumors induced by polyomavirus-transformed rat cells as described (19). The immunocomplexes formed with normal serum (N) and antitumor serum (T) were electrophoresed through 10% polyacrylamide-sodium dodecyl sulfate gels, and the [^{35}S]methionine-labeled proteins were visualized by autoradiography. The [^{35}S]methionine-labeled tumor antigens from mouse cells lytically infected with polyomavirus were processed as described above and were run on the same gel as markers. Their positions are indicated by the lettering on the right of the autoradiogram: LT, large T antigen; mT, middle T antigen; sT, small T antigen. The arrow marks a cellular protein immunoprecipitated by this particular preparation of antiserum. This cellular protein is not induced by viral transformation because it is equally abundant in untransformed Rat-1 cells. Lanes 1 through 3 contain the proteins that are immunoprecipitated by normal (N) and anti-T antigen (T) antiserum from three independent transformed cell lines transfected with a mixture of pPdl3 and pPdl1-8 DNA. Similarly, lanes 4 through 6 contain the immunoprecipitated proteins from three independent transformed cell lines that resulted form transfection with a mixture of pPin2 and pPin67 DNA. The cell lines labeled 1 through 6 here are the same as those designated 1 through 6 in Fig. 2.

the DNAs are recombined is not known, nor do we know whether this process is mediated by viral proteins. However, if viral proteins are required for recombination, then only small T antigen can fulfill this role because only it could have been expressed from one or the other of the mutant DNAs present in the various crosses (Fig. 1). Moreover, replication of the mutant DNAs is not required for recombination to occur because each of the DNAs employed in the crosses is incapable of encoding large T antigen, a protein required for the initiation of viral DNA replication.

A distinguishing characteristic of simian virus 40-transformed and polyomavirus-transformed cells is the frequent occurrence of the integrated viral sequences in a head-to-tail array (1, 2, 4, 5, 13, 18, 19). Several investigators have postulated that this arrangement of integrated viral DNA occurs by the integration of rolling-circle intermediates that are generated by a replicational mechanism (6, 7). However, several observations argue against this mechanism as the sole means of tandem formation. First, replication-incompetent DNAs are capable of becoming arranged as head-to-tail tandems within cellular DNA (24), and second, the frequency of occurrence of tandemly integrated genomes in transformed cells is directly proportional to the amount of DNA inoculated per cell (24). The results we have presented show that homologous recombination can occur after transfection of mammalian cells with DNA, and we suggest that this mechanism can account for the formation of tandemly integrated genomes in transformed cells. It is not clear to us whether homologous recombination between the transfected species occurs before or after their integration within cellular DNA. To resolve this question, we are currently attempting to isolate recombinational intermediates. Finally, the observations we have made are not unique to polyomavirus recombinant DNAs and Rat-1 cells but appear to be generally true of other animal cells transfected with a variety of DNA molecules (11, 17, 21, 26, 30, 31).

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LITERATURE CITED

1. **Basilico, C., S. Gattoni, D. Zouzias, and G. Della Valle.** 1979. Loss of integrated viral DNA sequences in polyoma transformed cells is associated with an active viral A function. Cell 17:645–659.
2. **Basilico, C., D. Zouzias, G. Della Valle, S. Gattoni, V. Colantuoni, R. Fenton, and L. Dailey.** 1979. Integration

and excision of polyoma virus genomes. Cold Spring Harbor Symp. Quant. Biol. **44**:611–619.
3. **Benjamin, T.** 1970. Host-range mutants of polyoma virus. Proc. Natl. Acad. Sci. U.S.A. **67**:394–399.
4. **Birg, F., R. Dulbecco, M. Fried, and R. Kamen.** 1979. State and organization of polyoma virus DNA sequences in transformed rat cell lines. J. Virol. **29**:633–648.
5. **Botchan, M., W. Topp, and J. Sambrook.** 1976. The arrangment of simian virus 40 sequences in the DNA of transformed cells. Cell **9**:269–287.
6. **Chia, W., and P. Rigby.** 1981. Fate of viral DNA in nonpermissive cells infected with simian virus 40. Proc. Natl. Acad. Sci. U.S.A. **78**:6638–6642.
7. **Della Valle, G., R. Fenton, and C. Basilico.** 1981. Polyoma large T-antigen regulates the integration of viral DNA sequences into the genome of transformed cells. Cell **23**:347–355.
8. **DiMayorca, G., J. Callender, G. Maria, and R. Giordano.** 1969. Temperature-sensitive mutants of polyoma virus. Virology **38**:126–133.
9. **Eckhart, W.** 1969. Complementation and transformation by temperature-sensitive mutants of polyoma virus. Virology **38**:120–125.
10. **Feunten, J., L. Sompayrac, M. Fluck, and T. Benjamin.** 1976. Localization of gene functions in polyoma virus DNA. Proc. Natl. Acad. Sci. U.S.A. **73**:4169–4173.
11. **Folger, K. R., E. A. Wong, G. Wahle, and M. R. Capecchi.** 1982. Patterns of integration of DNA microinjected into cultured mammalian cells: evidence for homologous recomination between injected plasmid DNA molecules. Mol. Cell. Biol. **2**:1372–1387.
12. **Fried, M.** 1965. Isolation of temperature-sensitive mutants of polyoma virus. Virology **25**:669–671.
13. **Gattoni, S., V. Colantuoni, and C. Basilico.** 1980. Relationship between integrated and nonintegrated viral DNA in rat cells transformed by polyoma virus. J. Virol. **34**:615–626.
14. **Hassell, J., W. Topp, D. Rifkin, and P. 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. **77**:3978–3982.
15. **Hayday, A., H. E. Ruley, and M. Fried.** 1982. Structural and biological analysis of integrated polyoma virus DNA and its adjacent host sequences cloned from transformed rat cells. J. Virol. **44**:67–77.
16. **Israel, M., D. Simmons, S. Mourihan, W. Rowe, and M. Martin.** 1979. Interrupting the early region of polyoma virus DNA enhances tumorigenicity. Proc. Natl. Acad. Sci. U.S.A. **76**:3713–3716.
17. **Jackson, D. A.** 1980. Assays for genetic recombination in mammalian cells, p. 65–78. *In* R. Baserga, C. Croce, and G. Rovera (ed.), Introduction of macromolecules in viable mammalian cells. Alan R. Liss, Inc., New York.
18. **Ketner, G. and T. Kelly.** 1976. Integrated SV40 sequences in transformed cell DNA. Analysis using restriction endonucleases. Proc. Natl. Acad. Sci. U.S.A. **73**:1102–1107.
19. **Lania, L., M. Griffiths, B. Cooke, Y. Ito, and M. Fried.** 1979. Untransformed rat cells containing free and integrated DNA of a polyoma nontransforming (hr-t) mutant. Cell **18**:793–802.
20. **Mes, A.-M., and J. A. Hassell.** 1982. Polyoma viral middle T-antigen is required for transformation. J. Virol. **42**:621–629.
21. **Miller, C. K., and H. M. Temin.** 1983. High-efficiency ligation and recombination of DNA fragments by vertebrate cells. Science **220**:606–609.
22. **Moore, J., K. Chowdhury, M. Martin, and M. Israel.** 1980. Polyoma large tumor antigen is not required for tumorigenesis mediated by viral DNA. Proc. Natl. Acad. Sci. U.S.A. **77**:1136–1140.
23. **Novak, U., S. Dilworth, and B. Griffin.** 1980. Coding capacity of a 35% fragment of the polyoma virus genome is sufficient to initiate and maintain cellular transformation. Proc. Natl. Acad. Sci. U.S.A. **77**:3278–3282.
24. **Pomerantz, B., M. Naujokas, W. Muller, A.-M. Mes, M. Featherstone, P. Moreau, and J. Hassell.** 1983. DNA-mediated transfer of genes to mammalian cells: evidence for homologous recombination between transfected DNAs, p. 256–261. *In* M. Pearson and N. Sternberg (ed.), Gene transfer and cancer. Raven Press, New York.
25. **Silver, J., B. Schaffhauser, and T. Benjamin.** 1978. Tumor antigens induced by nontransforming mutants of polyoma virus. Cell **15**:485–496.
26. **Small, J., and G. Scangos.** 1983. Recombination during gene transfer into mouse cells can restore the function of deleted genes. Science **219**:174–176.
27. **Staneloni, R., M. Fluck, and T. Benjamin.** 1977. Host range selection of transformation-defective hr-t mutants of polyoma virus. Virology **77**:598–609.
28. **Tooze, J.** 1981. DNA tumor viruses, part 2, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. **Treisman, 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.
30. **Upcroft, P., B. Carter, and C. Kidson.** 1980. Mammalian cell functions mediating recombination of genetic elements. Nucleic Acids Res. **8**:5835–5844.
31. **Wake, C. T., and J. H. Wilson.** 1979. Simian virus 40 recombinants are produced at high frequency during infection with genetically mixed oligomeric DNA. Proc. Natl. Acad. Sci. U.S.A. **76**:2876–2880.