Polyoma Viral Middle T-Antigen Is Required for Transformation

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To determine whether small or middle T-antigen (or both) of polyoma virus is required for transformation, we constructed mutants of recombinant plasmids which bear the viral oncogene and measured the capacity of these mutants to transform rat cells in culture. Insertion and deletion mutations in sequences encoding small and middle T-antigens (79.7, 81.3, and 82.9 map units) rendered the DNA incapable of causing transformation by the focus assay. Similar mutations in sequences that encoded middle but not small T-antigen (89.7, 92.1, and 96.5 map units) generally abolished the transforming activity of the DNA. However, two mutants (pPdl1-4 and pPdl2-7) that carried deletions at 92.1 map units retained the capacity to transform cells; pPdl1-4 did so at frequencies equal to those of the parental plasmid, whereas pPdl2-7 transformed at 10% the frequency of its antecedent. From these studies we conclude that small T-antigen alone is insufficient to cause transformation and that middle T-antigen is required for transformation, either in combination with small T-antigen or by itself.

Polyoma virus is capable of causing tumors in rodents and in transforming cells in culture. The oncogenic capacity of the virus resides in its DNA, and the maintenance of cellular transformation is mediated by proteins encoded therein. Genetic and biochemical studies have revealed that the viral oncogene is localized within the early region. This area of the viral genome encodes three known proteins: large (100 kilodaltons), middle (55 kilodaltons), and small (22 kilodaltons) T-antigens (reviewed in reference 21). These tumor antigens are translated from three related but differentially spliced mRNAs that are derived from the same transcription unit (24). The coding sequences for small and middle T-antigens are located exclusively in the proximal part of the early region, 74 to 99 map units (m.u.), whereas those encoding large T-antigen are situated in the proximal and distal (99 to 26 m.u.) parts of the early region. Genetic analyses have defined two functions within the early region required for transformation and tumorigenesis. Mutants within these two functions complement each other for transformation, and their mutations physically map in different areas of the early region. The host-range, transformation-defective mutant class (hr-t) completely fails to transform cells in vitro or induce tumors in rodents (4, 40). Their alterations map in the proximal portion of the early region and affect the primary structure of small and middle T-antigens but not large T-antigen (10, 28, 34, 38). ts-a mutants define the other transforming function encoded in the early region. ts-a mutants transform cells at a lower frequency at the nonpermissive than at the permissive temperature (8, 9, 12). Their mutations map within sequences known to encode only large T-antigen (28), and cells infected with ts-a mutants synthesize an altered T-antigen (22, 30, 32). Generally, cells transformed by ts-a mutants at the permissive temperature retain the transformed phenotype when transferred to the nonpermissive temperature (8, 9, 11, 12, 36). The ts-a function is therefore not required to maintain the transformed state but facilitates its establishment. Della Valle et al. (7) have suggested that large T-antigen promotes transformation by enhancing integration of viral DNA.

That large T-antigen plays no role in maintaining the transformed phenotype was further suggested by other observations. First, several investigators failed to find mRNA sequences complementary to the distal portion of the polyoma viral early region in some transformed cell lines (2, 14, 25). Moreover, large T-antigen could not be detected in a number of cell lines transformed by the virus (19). Israel et al. (20) then demonstrated that the tumorigenicity of polyoma viral DNA was enhanced by cleavage of the molecule in the distal portion of the early region. Direct evidence that large T-antigen is not required for the initiation or maintenance of transformation came from the observation that subgenomic fragments of viral DNA comprising sequences from only the proximal portion of the early region are sufficient for transformation (17, 29). The smallest continuous tract of polyoma

viral DNA capable of causing transformation contains all of the sequences required for the transcription and translation of small and middle T-antigens, but lacks sequences that encode the carboxy-terminal portion of large T-antigen. These observations have served to focus attention on the role of small and middle T-antigens in cellular transformation. To determine whether small or middle T-antigen (or both) is required for transformation, we altered the coding sequences for these proteins by in vitro mutagenesis of recombinant plasmids bearing the transforming gene. Mutants of the recombinant plasmids were cloned in *Escherichia coli* and tested for their capacity to transform Rat-1 cells. Our results demonstrate that small T-antigen alone is incapable of establishing the transformed state, and that middle T-antigen is essential for this process.

MATERIALS AND METHODS

Cell culture and transformation assay. Rat-1 cells were grown on plastic petri dishes, using Dulbecco's modification of Eagle medium supplemented with 10% (vol/vol) fetal bovine serum and antibiotics. Cells were maintained at 37°C in a humidified CO_2 atmosphere. Transfection of Rat-1 cells with plasmid DNA, purified from cesium chloride gradients, was performed by using a modification of the calcium phosphate technique (42). The cells were then incubated for 10 to 21 days, and transformants were scored as dense foci.

Molecular cloning of viral DNA. All research involving cells, viruses, and plasmids was performed in accordance with the Medical Research Council of Canada Guidelines for the Handling of Recombinant DNA Molecules and Animal Viruses and Cells. The plasmids used were pPH1-8 (17) and pPBR2. pPBR2 is composed of the *Bam*HI/*Eco*RI (58.5 to 100/0 m.u.) fragment of polyoma viral DNA inserted in the large *Bam*HI/*Eco*RI digest product of the plasmid pMK16.1 (13, 23).

Mutagenesis, transformation, and plasmid isolation. The mutagenesis protocol used was similar to the method developed to construct simian virus 40 mutants (37). Full-length linear molecules were obtained by cleaving plasmid DNA with multicut restriction endonucleases in the presence of ethidium bromide (31). These permuted linears were then eluted from agarose gels by electrophoresis (43). The free ends of the molecule were modified by treatment with S1 nuclease (Boerhinger), Bal 31 nuclease (Bethesda Research Laboratories), or reverse transcriptase (a gift from P. Beard) under conditions described elsewhere (1, 15, 33). Plasmids were recircularized, using T4 DNA ligase (Bethesda Research Laboratories). Transformation of calcium chloride-treated, competent *E. coli* X1776 with plasmid DNA was performed as described (41). After transfection, plasmid DNA was obtained from drug-resistant colonies by a modification of a previously described technique (6). Cloned, mutated DNAs were identified and characterized by restriction endonuclease digestion. All restriction endonucleases except *Pvu*II were purchased from Bethesda Research Laboratories. *Pvu*II was obtained from New England Biolabs. Restriction endonuclease digestions were performed according to the manufacturer's specifications.

RESULTS

In vitro mutagenesis and isolation of mutants. Two parental plasmids carrying fragments of polyoma viral DNA were used as substrates for in vitro mutagenesis. One of these plasmids, pPH1-8 (Fig. 1a), contains the *Hin*dIII-l fragment of polyoma viral DNA (45 to 1.4 m.u.) inserted at the *Hin*dIII site of the plasmid pBR322. The second recombinant plasmid, pPBR2 (Fig. 1b), contains the *Bam*HI/*Eco*RI fragment of polyoma viral DNA (58.5 to 100/0 m.u.) within the large *Bam*HI/*Eco*RI double-digest product of the plasmid pMK16.1 (13, 23). Both of these recombinant plasmids contain the sequences which code for the polyoma viral small and middle T-antigens, and both have been shown by the focus assay to efficiently cause transformation of Rat-1 cells in culture at the same frequency.

To determine whether small or middle T-antigen is required for transformation, the coding sequences of the T-antigens were mutated. Because the coding sequences of the proximal portion of small and middle T-antigens overlap, only two different classes of mutants could be obtained: those having altered coding sequences for both small and middle T-antigens, and those carrying lesions in coding sequences of middle T-antigen alone. To create deletions or insertions within sequences known to encode polyoma viral small or middle T-antigen, one or the other of the parental plasmid DNAs described above was digested singly with one of several multicut restriction endonucleases under conditions designed to favor linearization of the plasmid DNA. The ends of the linear DNA were then modified enzymatically, and the resulting mutated DNAs were cyclized with T4 ligase. Individual mutated plasmid DNAs were then cloned by transformation of *E. coli* and characterized by restriction endonuclease digestion. In this way, mutations were introduced within coding sequences at 79.7 (*Pst*I), 81.3 (*Sst*I), 82.9 (*Ava*I), 89.7 (*Ava*I), 92.1 (*Pvu*II), and 96.5 (*Sst*I) m.u.

PvuII. The restriction endonuclease *Pvu*II cleaved pPH1-8 DNA at four sites (Fig. 1a); one site was located within pBR322 sequences, two sites were located within noncoding sequences in polyoma viral DNA (67.4 and 70.0 m.u.), and one site (92.4 m.u.) was located within sequences which encoded middle but not small T-antigen. Deletions of various sizes were created about the site at 92.1 m.u. by digesting populations of *Pvu*II-cleaved, permuted linear molecules of pPH1-8 DNA with the Bal 31 nuclease. Individ-
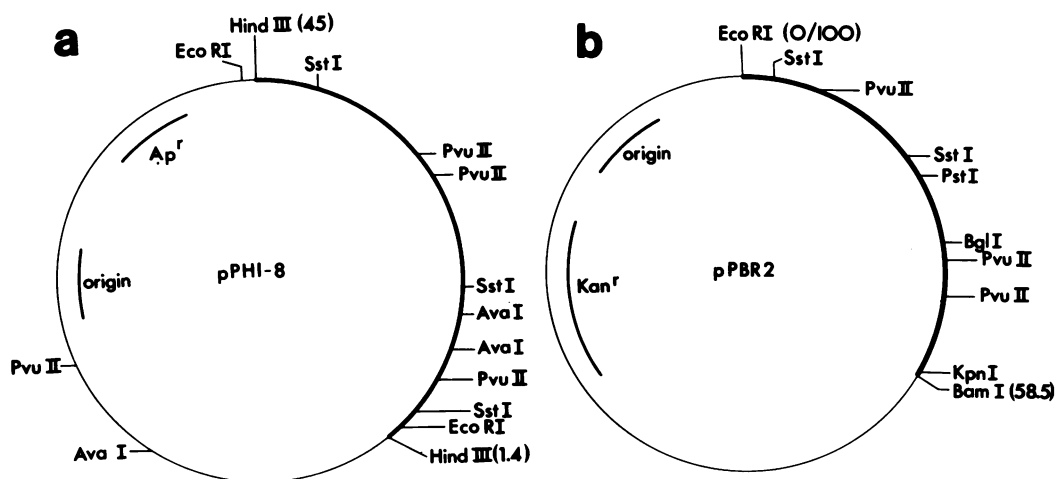
FIG. 1. Maps of restriction endonuclease cleavage sites in pPH1-8 and pPBR2 DNA. (a) pPH1-8 contains the HindIII-1 fragment of polyoma viral DNA (thick line) inserted in the plasmid pBR322. Mutations were generated at the SstI, PvuII, and AvaI sites. (b) pPBR2 is composed of the BamHI/EcoRI (58.5 to 100/0 m.u.) fragment of polyoma viral DNA (thick line) inserted in the BamHI/EcoRI digestion product of the plasmid pMK16.1. Mutations were generated at the SstI and PstI sites.

ual mutated DNAs were obtained as described previously and characterized by digestion with PvuII to determine the site of the deletion (Fig. 2a) and with SstI to estimate the size of the deletion (Fig. 3a). Nine mutants were characterized. All contained deletions (of approximately 30 to 400 base pairs [bp]) at 92.1 m.u. (Fig. 3a).

AvaI. pPH1-8 DNA contained three AvaI sites (Fig. 1a), one in pBR322 sequences and two in polyoma viral sequences. The sites in polyoma viral DNA occurred at 82.9 m.u., which fell in the coding sequences of both small and middle T-antigens, and at 89.7 m.u., in the coding sequences for middle T-antigen. Insertions of 4 bp were created at these sites by back-filling the 5' projections left by AvaI hydrolysis with reverse transcriptase. Two mutants of the recombinant plasmid pPH1-8 were characterized. One of these, pPin 67, contained a mutation at 82.7 m.u., whereas the other, pPin2, was mutated at 89.7 m.u. (Fig. 2b). We infer that both of these mutants carried insertions at these sites because digestion of their DNA with several different restriction endonucleases yielded fragments which comigrated with those of the parental plasmid from which they were derived (i.e., SstI; Fig. 3b) and because the mutagenesis protocol used would strongly favor the isolation of insertion mutations. However, until these mutant DNAs are sequenced, we cannot rule out the possibility that they carry small deletions at the AvaI sites.

SstI. The recombinant plasmid pPBR2 contained two sites of cleavage for SstI (Fig. 1b). One of these sites was at 81.3 m.u., within

coding sequences for small and middle T-antigens; the other mapped within coding sequences for the distal portion of middle T-antigen at 96.5 m.u. Small deletions were created at these locations by digesting permuted linear pPBR2 DNA molecules generated by SstI with the S1 nuclease. SstI left 4-bp, 3' projections which served as a substrate for S1. A number of mutants were isolated and digested with SstI (Fig. 2c; pPdl1, pPdl9, and pPdl15). All of these mutants were resistant to SstI cleavage at one site or the other and were cleaved to yield linear molecules of about the same size as pPBR2 DNA. By doubly digesting the mutant DNAs with SstI and KpnI (which cleaved polyoma viral DNA only at 59.5 m.u.), it was possible to determine which of the two SstI sites was affected in each of the mutants (results not shown). pPdl9 lacked an SstI site at 81.3 m.u., whereas pPdl1 and -15 lacked the SstI site at 96.5 m.u. In each case the deletions were small because cleavage of their DNAs with other restriction endonucleases yielded fragments which comigrated with those of pPBR2 DNA (i.e., PvuII; Fig. 3c). In addition to these three mutants, one mutant was isolated by treating SstI partially digested pPH1-8 DNA with the Bal 31 nuclease. This mutant, pPdl17, was resistant to SstI cleavage at 81.3 m.u. and carried a small deletion at this site (Fig. 2c and 3c).

PstI. PstI hydrolyzed pPBR2 DNA once (Fig. 1b). Four deletion mutants were isolated which lacked the PstI site at 79.7 m.u., within coding sequences for small and middle T-antigens, by treating PstI-linearized pPBR2 DNA with the
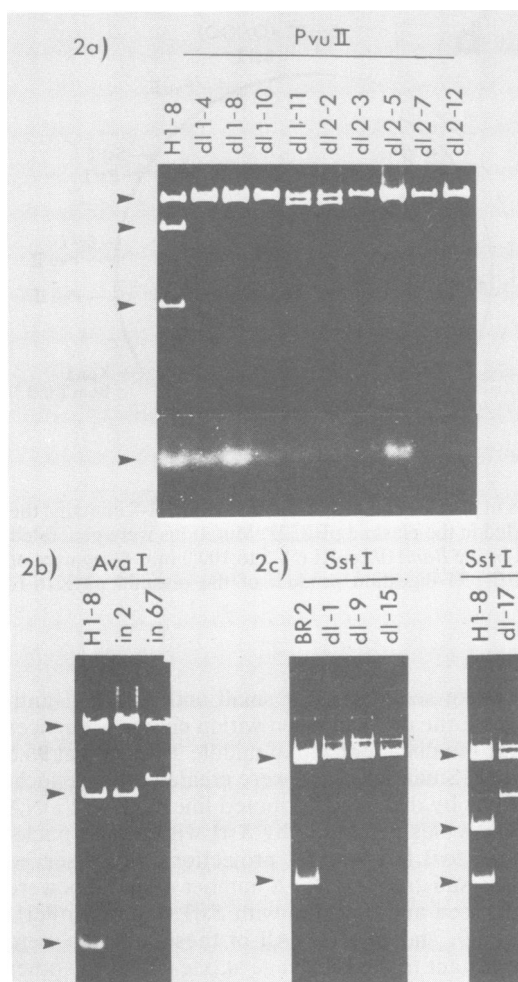
FIG. 2. Analysis of mutated, recombinant plasmids by restriction endonuclease digestion and agarose gel electrophoresis. Individual drug-resistant colonies were isolated after in vitro mutagenesis and transfection. Plasmid DNA was prepared from these colonies and monitored for the loss of a restriction endonuclease cleavage site. (a) In the first lane, arrows point to the four products obtained when the parental plasmid pPH1-8 is cleaved with PvuII. DNAs lacking the site at 92.1 m.u. lose fragments 2 and 3 and gain a new fragment which migrates close to or with fragment 1. The bottom portion of the gel is derived from a different exposure in order to clearly show the smallest fragments. (b) Arrows indicate the three fragments obtained when pH1-8 is cleaved with AvaI. DNAs lacking the AvaI site at 82.9 m.u. (pPin2) lose fragment 3, retain fragment 2, and have a slower-migrating fragment 1. When the AvaI site at 89.7 m.u. is mutated, fragment 3 is lost, fragment 1 is retained, and here fragment 2 migrates more slowly. (c) pPBR2 is cleaved twice by SstI, and arrows indicate the position of the digest products (left panel). When the SstI site at 81.3 m.u. (pPdl1) or the SstI site at 96.5 m.u. (pPdl9 and pPdl15) is lost, digestion of these mutant DNAs with SstI produces full-length linears. When pPH1-8 is digested with SstI, three fragments are obtained (right-

Bal 31 nuclease. These mutants, pPdl3, -4, -10, and -11, carried deletions which varied in size, none being greater than 100 bp in length (Fig. 3d).

Transforming activity of mutant, recombinant plasmid DNAs. To accurately measure the specific transforming activity of various mutated DNA molecules by comparison with wild-type DNA, we optimized the conditions for transformation of Rat-1 cells. By transfecting Rat-1 cells at low cell density (2×10^5 to 3×10^5 cells/10-cm-diameter petri dish), using the protocol described by Wigler et al. (42), we routinely obtained transformation frequencies between 3,000 and 10,000 foci/μg of DNA per 2×10^5 to 3×10^5 Rat-1 cells initially inoculated (Fig. 4). The assay was linear and directly proportional to DNA concentration between 5 and 20 ng of DNA. Within this range of DNA concentrations, the specific transforming activity of the DNA remained constant. Foci were visible 1 week after transfection and could be readily scored 10 to 14 days later (Fig. 4). As the DNA concentration was increased beyond 50 ng/2×10^5 to 3×10^5 cells, the total number of foci increased until a plateau was reached at 500 to 1,000 ng of DNA/2×10^5 to 3×10^5 cells. However, within this range of DNA concentrations the specific transforming activity of the DNA continuously declined and finally reached a level of about 500 to 1,000 foci/ng of DNA per 2×10^5 to 3×10^5 cells inoculated (data not shown). Within an experiment, the precision of the transformation assay was very good. However, the specific transforming activity of wild-type or mutant DNA could vary over a threefold range from one experiment to the next. For this reason, comparisons between mutant and wild-type DNA were made in a single experiment over a range of DNA concentrations. The capacity of 19 mutant, recombinant plasmid DNAs to transform Rat-1 cells was measured. Each DNA sample was tested in triplicate at 32.5 and 38.5°C on at least two separate occasions by transfecting with 500 to 1,000 ng of supercoiled, recombinant plasmid DNA per dish (2×10^5 to 3×10^5 cells). Foci were scored 2 weeks posttransfection for positive samples, whereas negative samples were incubated up to 6 weeks before the cells were fixed and stained (Table 1). Mutations which affected the coding sequences for small and middle T-antigens, regardless of their location (79.7, 81.3, or 82.9 m.u.) or nature (inser-

most panel). pPdl17 has lost the SstI site at 81.3 m.u. When digested with SstI, pPdl17 DNA yields two fragments, one that comigrates with fragment I of the pPH1-8 digest and one that migrates more slowly than fragment 2 and at the same position as a partial digest product of pPH1-8 DNA.
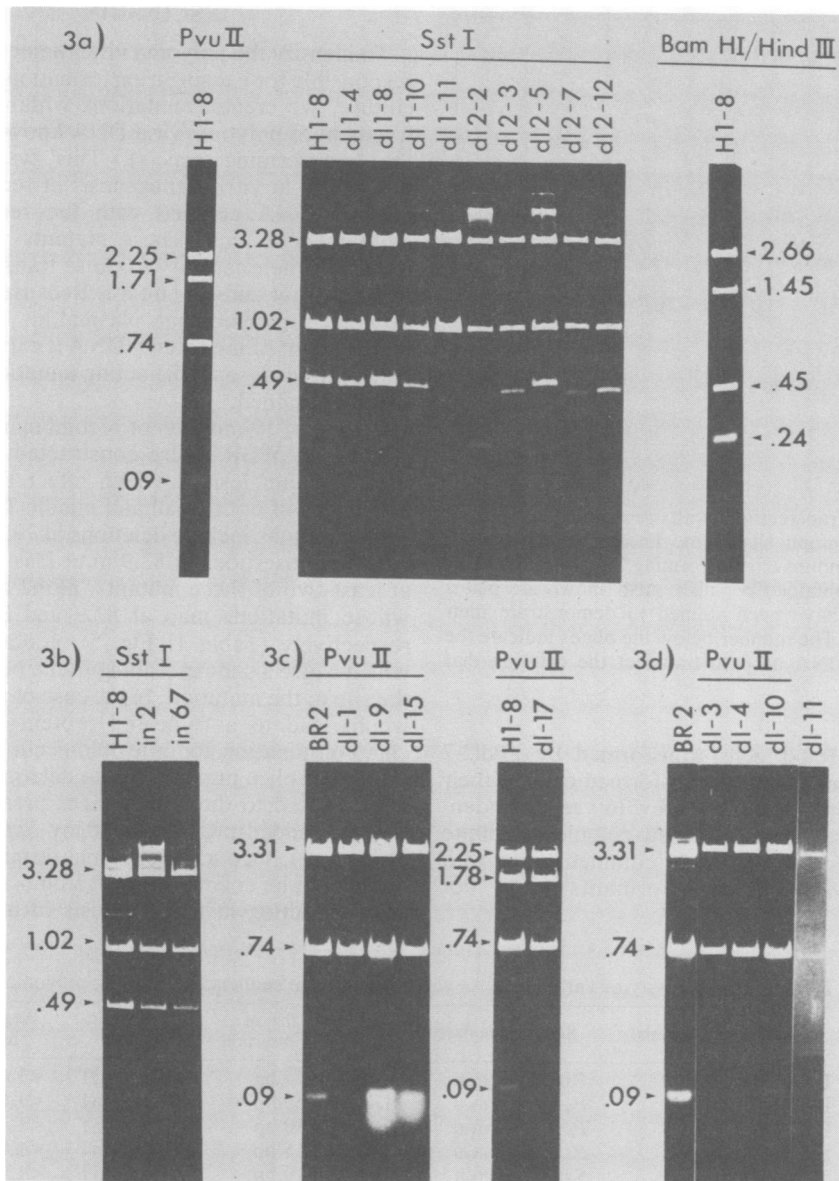
FIG. 3. Estimation of the size of deletions or insertions by restriction endonuclease digestion and gel electrophoresis. Mutant DNAs were cleaved with various restriction endonucleases, and the products were separated through 1.4% agarose gels along with molecular weight markers in order to determine the approximate size of the lesions. Molecular sizes are indicated in megadaltons. The restriction endonuclease used is indicated above each gel. The upper portion of each gel is a longer exposure of the negative of the gel; this was done to clearly display all fragments.

tions or deletions), completely abolished the transforming activity of the DNA. Similarly, the majority (8 of 10) of the DNAs which carried mutations affecting the coding sequences of middle T-antigen but not small T-antigen were also incapable of transforming Rat-1 cells. These included mutations at 89.7, 92.1, and 96.5 m.u. By contrast, two mutants, both of which

mapped at 92.1 m.u. (pPdl1-4 and pPdl2-7), transformed Rat-1 cells. By measuring the transforming activity of these DNAs over a range of DNA concentrations and comparing them with pPH1-8 DNA (the parental plasmid), we calculated that pPdl1-4 transformed cells at 100% the frequency of pPH1-8 DNA, whereas pPdl2-7 did so at 10% the frequency of the wild-type plasmid
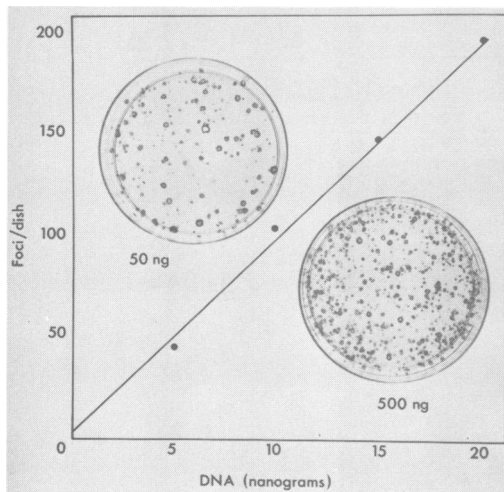
FIG. 4. Transfection with recombinant plasmid DNA. The graph shows the linear relationship between the amount of transforming DNA and the number of foci obtained per dish. Also shown are plates whose foci have been stained to demonstrate their appearance. The number below the plates indicate the amount of DNA used to transfect the cells on that plate.

(pPH1-8). Rat-1 cells transformed by pPdl2-7 DNA resembled fully transformed cells in their growth properties. They grew to very high densities and efficiently formed colonies in dilute agar suspension at a rate commensurate with that of wild-type transformants (data not shown).

DISCUSSION

To identify the polyoma viral tumor antigen(s) responsible for causing transformation of cells in culture, we created mutations within a cloned fragment of polyoma viral DNA known to carry the transforming gene(s). This was accomplished by in vitro mutagenesis of recombinant plasmid DNA coupled with the recovery of mutants in E. coli. These mutants were then tested for their capacity to cause transformation of normal rat cells in culture. Because propagation of a recombinant plasmid in E. coli is independent of the foreign DNA it carries, totally defective cis- or trans-acting mutations can be readily obtained.

A total of 19 mutants of recombinant plasmid pPH1-8 or pPBR2 were constructed. Seven of these contain lesions which affect the coding sequences of both small and middle T-antigens. The mutations include deletions at 79.7 and 81.3 m.u. and insertions at 82.9 m.u. It is likely that at least two of these mutants, pPin2 and pPdl9, whose mutations map at 82.9 and 81.3 m.u., respectively (Table 1; Fig. 5), contain lesions which cause a shift in reading frame beginning at the site of the mutation. In the case of pPin2, this would lead to a truncated protein because a chain termination codon would occur before the proximal splice junction. If the deletion in pPdl9 were limited to those sequences present in the single-stranded projection left by SstI cleavage (81.3 m.u.), then a translational termination codon would be encountered 10 amino acids later, again resulting in the synthesis of a truncated

TABLE 1. Transforming activity of various DNAs with mutations in sequences encoding polyoma T-antigens

DNA source	Mode of mutagenesis	Site mutagenized	Approx size of deletion or insertion	T-antigen affected[a]	Avg no. of foci/ μg of DNA
pPdl3	Bal 31 nuclease	PstI; 79.7 m.u.	Deletion 15 bp	sT and mT	<1.0
pPdl4	Bal 31 nuclease	PstI; 79.7 m.u.	Deletion 60 bp	sT and mT	<1.0
pPdl10	Bal 31 nuclease	PstI; 79.7 m.u.	Deletion 90 bp	sT and mT	<1.0
pPdl11	Bal 31 nuclease	PstI; 79.7 m.u.	Deletion 15 bp	sT and mT	<1.0
pPdl9	S1 nuclease	SstI; 81.3 m.u.	Deletion 10 bp	sT and mT	<1.0
pPdl17	Bal 31 nuclease	SstI; 81.3 m.u.	Deletion 10 bp	sT and mT	<1.0
pPin2	Reverse transcriptase	AvaI; 82.9 m.u.	Insertion 4 bp	sT and mT	<1.0
pPin67	Reverse transcriptase	AvaI; 89.7 m.u.	Insertion 4 bp	mT	<1.0
pPdl1-4	Bal 31 nuclease	PvuII; 92.1 m.u.	Deletion 45 bp	mT	~3,000 (100%)
pPdl1-8	Bal 31 nuclease	PvuII; 92.1 m.u.	Deletion 30 bp	mT	<1.0
pPdl1-10	Bal 31 nuclease*	PvuII; 92.4 m.u.	Deletion 90 bp	mT	<1.0
pPdl1-11	Bal 31 nuclease	PvuII; 92.1 m.u.	Deletion >400 bp	mT	<1.0
pPdl2-2	Bal 31 nuclease	PvuII; 92.1 m.u.	Deletion 350 bp	mT	<1.0
pPdl2-3	Bal 31 nuclease	PvuII; 92.1 m.u.	Deletion 100 bp	mT	<1.0
pPdl2-5	Bal 31 nuclease	PvuII; 92.1 m.u.	Deletion 45 bp	mT	<1.0
pPdl2-7	Bal 31 nuclease	PvuII; 92.1 m.u.	Deletion 150 bp	mT	~300 (10%)
pPdl2-12	Bal 31 nuclease	PvuII; 92.1 m.u.	Deletion 120 bp	mT	<1.0
pPdl1	S1 nuclease	SstI; 96.5 m.u.	Deletion 10 bp	mT	<1.0
pPdl15	S1 nuclease	SstI; 96.5 m.u.	Deletion 10 bp	mT	<1.0
pPBR2	—			—	3,000–8,000
pPH1-8	—			—	3,000–8,000

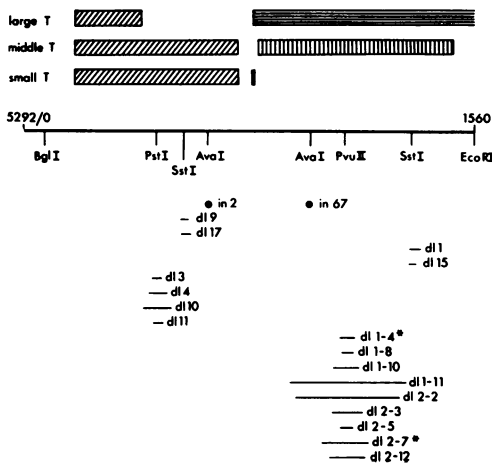[a] sT, Small T-antigen; mT, middle T-antigen.

FIG. 5. Schematic summary of mutations affecting the coding sequences of the polyoma viral T-antigens. The figure shows the coding sequences for large, middle, and small T-antigens. Note that the amino-terminal portion of the three T-antigens (before the proximal splice) as well as the distal portion of small T-antigen are in the same reading frame. The distal portion of middle and large T-antigens (after the distal splice) are in the two remaining reading frames and are different from each other. Nucleotide numbers are given (39). Shown as well is the approximate position and size of the lesions we have generated in the T-antigen-coding sequences. Symbols: ●, insertion; −, deletion. The asterisk indicates lesions which do not abolish the transforming capacity of the DNA.

protein. The consequences of the deletions at 79.7 and 81.3 m.u., generated by the Bal 31 nuclease, cannot be inferred from the methods used in their construction.

The remaining 12 mutants carry mutations which affect the coding sequences of middle T-antigen but not small T-antigen. These mutations map at 89.7 m.u. (represented by pPin67), 92.1 m.u. (represented by nine mutants), and 96.5 m.u. (represented by pPdl1 and pPdl15). Of these mutants, two, pPin67 (89.7 m.u.) and pPdl15 (96.5 m.u.), may cause a change in reading frame beginning at the site of the mutation. In mutant pPin67-transfected cells, this would lead to the synthesis of an altered form of middle T-antigen that would carry a long stretch of foreign protein at its carboxy terminus. A chain termination codon would not occur until pBR322 plasmid sequences were encountered. The mutation in pPdl15 (Table 1; Fig. 5) may also cause a change in reading frame. This mutant, like pPdl9, was constructed by removing the 3' projections left by SstI cleavage of pPH1-8 DNA with the S1 nuclease. If only single-stranded sequences were removed, then this mutation would cause a shift in reading

frame resulting in the generation of a termination codon some 21 bp later. A truncated middle T-antigen which contained 7 novel amino acids and lacked 44 amino acids from its carboxy terminus would result. Because none of these mutations has been sequenced, these interpretations remain speculative.

Nine mutants have been constructed with deletions spanning 92.1 m.u. One of these, pPdl1-4, transforms cells at frequencies comparable to those of the parental plasmid, pPH1-8. The remaining mutants, with a single exception (pPdl2-7), do not transform cells at all. Mutant pPdl2-7 transforms cells at 10% the frequency of the parent plasmid, pPH1-8.

Viral mutants analogous to some of those which we have described here have been previously isolated. Those recombinant plasmid mutants which carry mutations that affect the primary structure of both small and middle T-antigens resemble in phenotype (transformation defective) the hr-t viral mutants described by Benjamin and his colleagues (4, 40). Moreover, the hr-t region and the region defined by our mutants (79.7 to 82.9 m.u.) overlap (5, 18).

Another class of viable deletion mutants with lesions that affect the coding sequences of middle but not small T-antigen have been described (3, 16, 27). PPdl1-4 resembles the viral mutants dl23 (16) and dl45 (3). These mutants transform cells at frequencies comparable to those of wild-type virus, and their mutations (deletions) map in similar locations (near 90 m.u.). A second group of mutants in this class, represented by viral mutants dl1013 and dl1015 (27), resemble pPdl2-7 in their phenotype (reduced capacity to transform cells in culture, typically 5 to 20% the frequency of wild-type virus or DNA). Mutant dl1013 carries a deletion within the region of 91.6 to 93.4 m.u., whereas dl1015 carries a deletion which maps somewhere between 93.4 and 98.5 m.u. By comparison, pPdl2-7 bears a deletion of 150 bp which spans the PvuII site at 92.1 m.u.

Several conclusions can be gleaned from these results. First, truncated forms of large T-antigen are not sufficient to cause transformation of rat cells, because mutations that map within a region known to be absent in large T-antigen mRNA (79.7 to 82.9 m.u.) abolish the transforming activity of the DNA (Fig. 5). Moreover, it is also known that complete large T-antigen by itself is incapable of conferring the transformed phenotype to untransformed cells (26, 35). Second, small T-antigen alone is also insufficient to establish the transformed state. Rat-1 cells transfected with recombinant plasmids that carry the coding sequences for small T-antigen and all the regulatory sequences thought to be required for its expression fail to become transformed as measured by the focus assay. More-

over, we have recently observed that Rat-1 cells that harbor and express the coding sequences for small T-antigen resemble untransformed cells in their growth properties (A. M. Mes, B. Pomerantz, and J. A. Hassell, unpublished data). Finally, our results are compatible with the hypothesis that middle T-antigen is the transforming protein of polyoma virus. Most mutations in middle T-antigen-coding sequences either abolish or reduce the transforming activity of the DNA. The exceptions (pPdl1-4 and viral mutants dl45 and dl23) may delete amino acids that are dispensable for the functioning of middle T-antigen in transformation. Whether middle T-antigen by itself is sufficient to cause transformation or whether it is required in conjunction with small T-antigen remains to be determined.

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