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Mutation in the Polyomavirus Genome That Activates the Properties of Large T Associated with Neoplastic Transformation

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We have constructed a polyomavirus mutant genome which exhibits an increased immortalization potential when transfected into primary rat embryo fibroblasts. The mutation is a 30-base-pair deletion (nucleotides 1367 through 1396) that inactivates the transforming potential of middle T but activates some of the properties of large T associated with neoplastic transformation. Unlike the wild-type large T, the mutant large T can fully complement polyoma middle T in the tumorigenic process in vivo as well as in the transformation of primary cells in vitro. The activity of the mutant can be explained by its inability to replicate in cells and, hence, its inability to exert a cytopathic effect after gene transfer at high multiplicity. A recombinant which encodes the middle and small T antigens, but not the large T antigen, can also elicit a fully transformed phenotype when introduced into primary rat fibroblasts. These results confirm previous observations from this laboratory indicating that two, and not three, viral gene functions are required for polyomavirus-mediated oncogenic transformation.

The early region of the polyomavirus genome encodes three distinct proteins, the large T (*plt*), middle T (*pmt*), and small T (*pst*) antigens (see reference 31 for a review). Of these three proteins, the *pmt* antigen plays a central role in both oncogenic transformation in vitro (32) and tumorigenesis in vivo (3, 5). *pmt* alone is capable of transforming established cells in culture (32), but, unlike the wild-type viral genome, it cannot efficiently transform primary cells (24) nor induce tumors when inoculated into newborn rats (2). Thus, functions other than those expressed by the *pmt* antigen may be required for the elaboration of all the properties associated with neoplastic transformation. The role exerted by the other two viral proteins is still a matter of controversy. Since the *ras* oncogene can transform primary rodent cells after cotransfer with polyoma *plt*, adenovirus E1A, or the *myc* oncogene (14, 26), one would expect simultaneous transfer of both the *pmt* and *plt* genes to result in the establishment of the fully transformed phenotype in vitro as well as in vivo. In agreement with this scheme, Rassoulzadegan et al. (25) have observed that cell lines established by transfer of *plt* into primary fibroblasts can be subsequently transformed by transfer of the *pmt* gene. However, when both genes were simultaneously transferred into cells, no stable transformant could be isolated by focus formation. Combinations of *pmt* and *pst* or *plt* and *pst* were similarly inefficient. Cuzin et al. (8) have reported that transformation of the fully normal rat embryo fibroblasts can only be achieved by the wild-type genome or by a combination of modified genomes that allow simultaneous synthesis of the three early proteins.

By contrast, studies from this laboratory have shown that the *pst* protein can complement *pmt* in the absence of *plt* for tumor induction in animals (2). Similar complementary functions can also be exerted by the simian virus 40 (SV40) large T antigen as well as by one or several products of the adenovirus E1A region (3). Thus, as for the in vitro transformation of primary cells, the in vivo steps in polyomavirus-mediated tumorigenesis depend on additional cellular alterations beyond the acquisition of the polyomavirus transforming gene. As reported by this laboratory, such alterations can be achieved by either polyoma *pst* antigen or other early gene products from related DNA tumor viruses. Some of these genes, such as SV40 large T (1, 6, 7, 23) and E1A (11), appear to be associated with functions that confer on primary cells the ability to grow indefinitely in culture. However, although the polyoma *plt* gene is capable of immortalizing primary cells (25), the combination of the *plt* and *pmt* genes fails to induce tumors in newborn rats (3). It seems, therefore, that certain experimental conditions do not allow simultaneous expression of both genes in vivo or that *plt* is not sufficient to complement *pmt* in the tumorigenic process.

In this work, we have attempted to mutagenize the polyoma large T gene in such a way as to enable it to complement *pmt* in tumorigenesis. We describe the construction of a *plt* deletion mutant which exhibits an increased immortalization potential, and we show that the mutation enables *plt* to fully complement *pmt* not only in the tumorigenic process in vivo but also in the transformation of primary rat fibroblasts in vitro. These results demonstrate that two, and not three, viral gene functions are required for polyomavirus-mediated malignant transformation.

MATERIALS AND METHODS

Plasmids. pPyLT1 (Fig. 1) carries the polyomavirus large T gene. This plasmid was constructed by deleting the large T protein intron from the polyomavirus genome (35). pMT3 is a recombinant encoding only the middle T antigen (2). It was obtained by deleting two *Hin*dIII fragments from pPyMT1 (32). bc1051 is a modified polyoma genome expressing only the middle and small T antigens (21). pPB21 carries the polyomavirus genome (A2 strain) in the *Bam*HI site of pBR322 (9). pSV2-neo is a plasmid expressing *neo*, a dominant selective marker (28). pneo-LT1 (Fig. 1) was constructed by inserting the polyoma large T gene into the *Bam*HI site of pSV2-neo. pMLT1 (Fig. 1) is a hybrid plasmid encoding independently the polyomavirus large and middle T antigens.

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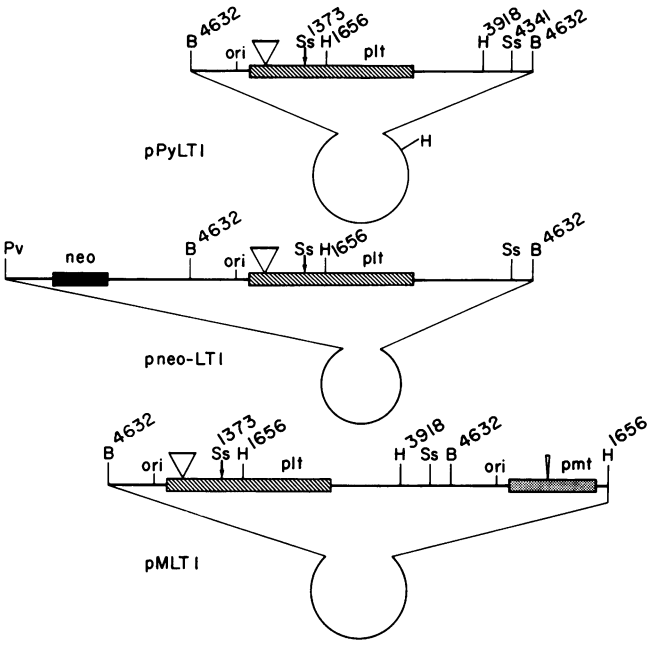


FIG. 1. Structure of recombinant plasmids. pPyLT1 carries the polyomavirus large T gene (*plt*) inserted in the *Bam*HI site of pAT153. The hatched area represents the large T coding sequence. The position of the deleted intron is indicated. The arrow shows the position of the 30-bp deletion in pLT97. pLT3 was derived from pPyLT1 by removing two *Hin*dIII fragments (polyoma nucleotide 1656 to the *Hin*dIII site in pAT153). This plasmid encodes the N-terminal portion of large T antigen (366 residues). pneo-LT1 carries both polyoma large T (*plt*) and *neo*. This plasmid was constructed by inserting *plt* into the *Bam*HI site of pSV2-neo (28). To link pLT3 to the *neo* gene, the plasmid was cleaved by *Bam*HI plus *Eco*RI (nucleotide 1560) and inserted between the *Bam*HI and *Eco*RI sites of pSV2-neo. pMLT1 is a hybrid plasmid encoding independently polyoma large T antigen (▨) and middle T antigen (▩). The position of the deleted intron is indicated on both genes. The arrow shows the position of the 30-bp deletion in the large T gene. All plasmids confer ampicillin resistance. Abbreviations: B, *Bam*HI; Ss, *Sst*I; H, *Hin*dIII; Pv, *Pvu*II; ori, origin.

Mutagenesis in the polyomavirus large T gene. pPyLT1 DNA was partially digested with *Sst*I (pPyLT1 contains two *Sst*I sites at nucleotides 1373 and 4341; Fig. 1), and the linear full-length plasmid was isolated by agarose gel electrophoresis. About 2 μg of DNA was added to a preheated (30°C) reaction mixture (50 μl) containing 20 mM Tris hydrochloride (pH 8.0), 600 mM NaCl, 12.5 mM $CaCl_2$, 12.5 mM $MgCl_2$ and 1 U of BAL 31 (Bethesda Research Laboratories). The solution was incubated for 15 s to 4 min at 30°C. Under these conditions, BAL 31 removed about 80 base pairs (bp) per min from either end of the molecule. The reaction was terminated by addition of 3 μl of EDTA (0.2 M), 3 μl of EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,-*N*′-tetraacetic acid; 0.1 M], and 50 μl of phenol at 0°C. After phenol extraction, the DNA was precipitated with ethanol, ligated at 20°C for 2 to 3 h with T4 DNA ligase (10-fold excess), and used to transform *Escherichia coli* HB101 to ampicillin resistance. A preliminary determination of the sizes and locations of the deletions was carried out by digestion with *Bam*HI and *Sst*I. Recombinants lacking the *Sst*I site at nucleotide 1373 were analyzed further by mapping with *Hpa*II, *Hin*fI, and *Alu*I.

Cells, transfections, and growth assays. Fischer rat embryos (15 days old) were washed three times in Tris saline, minced with dissecting scissors, trypsinized (20 min at 37°C), and sieved through a gauze mesh to isolate single cells. The cells were collected in 30 to 50 ml of fetal calf serum, centrifuged at 1,800 rpm for 10 min, and plated at a density of 3×10^6 cells per 60-mm dish in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. The medium was changed after 16 to 20 h of culture at 37°C (30 to 50% confluence), and 4 h later, the cells were transfected by the calcium phosphate-DNA coprecipitation procedure (4) with modifications (33). The DNA to be transfected (8 μg) was mixed with high-molecular-weight calf thymus DNA (10 μg) as carrier. After 20 h of exposure to DNA, the cells were passaged and replated at 20 to 30% confluence. After 18 h, G418 was added to the medium at a concentration of 400 μg/ml. The medium plus drug was changed every 5 days. Colonies were first detected after 7 to 10 days in the selective medium, and 2 to 3 weeks later, independent colonies were picked, transferred to 15-mm Linbro microplates, and grown at least once in medium containing G418 (400 μg/ml). Most of the cells that underwent senescence did not sustain more than one to two divisions after this first passage. The cell lines that were either immortalized or oncogenically transformed were subcultured at a 1:5 dilution each time they reached confluence and maintained in G418-free medium.

Tumorigenicity assays. Recombinant DNAs were injected subcutaneously (2 μg of DNA in 50 μl of phosphate-buffered saline) into the neck of 1-day-old Fischer rats as described previously (5). To evaluate the tumorigenicity of transformed cell lines, cells were harvested by trypsinization, and the indicated number of washed cells was injected into young adult Fischer rats and nude mice as described previously (2).

DNA replication assay. CsCl gradient-purified, supercoiled plasmid DNAs were transfected into mouse 3T6 cells by a modification of the DEAE-dextran transfection technique (17, 30). At 72 h posttransfection, low-molecular-weight plasmid DNA was isolated by the Hirt extraction procedure (10). Portions of the DNA samples were sequentially digested with *Bcl*I and *Dpn*I restriction endonucleases, and subjected to electrophoresis through 1.0% (wt/vol) agarose gels. The DNA fragments were transferred to nitrocellulose filters (27) and hybridized to a [^{32}P]-labeled RNA probe as previously described (8a).

RESULTS

Isolation of *plt* mutants. We wished to determine whether the failure of pPyLT1 to complement *pmt* in tumorigenesis was due to a lack of activity of the *plt* antigen or to a cytopathic effect exerted by the protein, as had previously been observed in transformation of primary embryo fibroblasts (14, 18). We postulated that to complement *pmt* in an in vivo assay, *plt* should lose its toxicity while retaining most of its immortalizing activity. Initial experiments were carried out with a cloned fragment of the *plt* gene, termed pLT3, that allowed synthesis of the N-proximal half of the *plt* antigen implicated in alteration of serum dependency and immortalization (25). Although a similar construction had been shown to complement EJ *ras* in the transformation of primary cells (14), injection of both pLT3 and pMT3 (truncated *plt* plus *pmt*) failed to induce tumors in newborn rats (Table 1). We then attempted to determine whether pLT3 had retained all of the immortalizing potential of pPyLT1, the intact *plt* gene. To this end, we used a simple immortalization assay to detect the ability of recombinant plasmids to modify the growth of rat embryo fibroblasts. The plasmids were linked

to pSV2-neo (Fig. 1) and introduced into primary cells by the calcium phosphate transfection procedure (33). Transfection with pneo-LT1 and pneo-LT3 gave rise to continuous cell lines with frequencies of 33.1 and 21.3%, respectively (Table 2). Thus, although the truncated *plt* antigen was able to immortalize primary cells, it did so with a frequency significantly lower ($P \simeq 0.056$) than that observed with the intact protein. For this reason, we attempted to generate deletions in *plt* that did not reduce its immortalizing potential. Mutagenesis with *BAL* 31 yielded recombinants with deletions of various lengths around the *Sst*I site at nucleotide 1373. Details on these mutants and their biological properties have been presented elsewhere (1). One of the mutants, designated pLT97, immortalized with high efficiency (Table 2). The colonies obtained with this mutant reached the fifth transfer in culture sooner than the others. However, like other established cell lines, they appeared normal with a flat, regular morphology and exhibited contact inhibition. Several representative cell lines were examined by immunofluorescence staining with a polyvalent antitumor serum. The cell lines established by transfection with either pneo-LT1 or pneo-LT97 exhibited a strong nuclear fluorescence characteristic of *plt* antigen (data not shown). Those immortalized with pneo-LT3 showed virtually no fluorescence (not shown).

Complementation assays. To determine the activity of pLT97 in tumorigenesis, we constructed plasmid pMLT97 (Fig. 1), which independently encoded *pmt* and the *plt* deletion mutant. Surprisingly, injection of this plasmid into newborn rats induced more tumors than did wild-type genomic DNAs that encoded the three polyoma early proteins (Table 1). Thus, the 30-bp deletion not only conferred to *plt* a greater ability to immortalize primary embryo fibroblasts but also enabled the gene to complement *pmt* in the tumorigenic process. This result prompted us to examine the properties of the *plt* mutant in a transformation assay of primary cells. pMLT97 was introduced into primary rat embryo fibroblasts together with the *neo* marker, and the cells were selected for growth in G418. Of the resultant G418-resistant colonies, about 5% resembled oncogenically transformed cells. The cells overgrew the flat monolayer and reached high densities in the plates to produce dense foci characteristic of transformed cells (Fig. 2). When 2×10^5 cells derived from two such foci were inoculated into nude mice and Fischer rats, they did not produce tumors (Table 3). This may indicate that cells transfected with the combination of *pmt* plus *plt* exhibited only a partially transformed phenotype. It could also be that the tumorigenicity defect was a result of the selection procedure. Two different observations support this hypothesis. First, transfection of primary cells with wild-type polyomavirus DNA in the

TABLE 1. Tumorigenicity of recombinant plasmids

Plasmid	Coding capacity	Incidence of tumors in newborn rats[a] (%)
pMT3	Middle T	0/58 (0)
pMLT1	Middle T, large T	0/39 (0)
pMLT3	Middle T, larget T (N terminus)	0/24 (0)
pMLT97	Middle T, large T (mutant)	5/7 (71.4)
bc1051	Middle T, small T	8/20 (40.0)
pPB21	Middle T, small T, large T	18/37 (48.6)
pPB97	Small T, middle T, large T (mutants)	0/16 (0)

[a] Fischer rats (1 day old) were inoculated in the neck with 2 μg of DNA in 50 μl of phosphate-buffered saline. The DNAs were linearized as follows: pMT3 and bc1051 were cleaved by *Hin*dIII; pMLT1 and pMLT3 were cleaved by *Sal*I; pMLT97 was cleaved by *Cla*I; pPB97 was cleaved by BamHI; and pPB21 was cleaved by *Hin*dIII, *Bam*HI, or *Hin*dIII plus *Bam*HI. Animals were observed for 4 months for the development of tumors. Incidence of tumors is shown as the number of rats in which tumors were found/the number of rats that were tested.

TABLE 2. Properties of recombinant plasmids

Plasmids	Coding capacity	No. of established lines/no. of colonies (%)
pSV2-neo		0/11, 0/15, 0/11, 0/9, 0/12, 0/11, 0/10 (0)
pneo-MT3	Middle T	2/67 (2.9)
pneo-LT1	Large T	20/37, 6/33, 14/44, 5/22 (33.1)
pneo-LT3	Large T (N terminus)	10/46, 7/28, 6/34 (21.3)
pneo-LT97	Large T (mutant)	26/39, 18/20, 17/24 (73.5)
pMLT1 plus pSV2-neo	Middle T, large T	0, 0, 0/1 (0)
pMLT97 plus pSV2-neo	Middle T, large T (mutant)	8/16, 16/41, 10/29 (39.5)
bc1051 plus pSV2-neo	Middle T, small T	6/8, 2/5 (61.5)
pPB21 plus pSV2-neo	Middle T, small T, large T	3/5, 3/4 (66.6)

[a] Nine foci were observed in confluent cultures transfected with pMLT97 in the absence of neo selection. Some foci were also observed in cultures transfected with pMLT1 and bc1051; however, these could not be established in culture.

[b] Colonies of cells resistant to G418 were picked 3 to 4 weeks after selection and transferred into Linbro microplates. The cells were subcultured at a 1:5 dilution each time they reached confluence. Immortalization efficiencies were evaluated by counting the percentage of cell lines reaching five or more passages 7 to 8 weeks after the first transfer. A zero indicates that no G418-resistant colony was obtained in the experiment.

TABLE 3. Phenotypic properties of cell lines[a]

DNA transfected	Cell line	Tumor incidence		Growth in agar[b]	Cellular morphology
		Nude mice	Fischer rats		
pneo-MT3	2-7	0/4		−	Flat
	2-12	0/4		−	Flat
pneo-LT97	LT97neo-1	0/4		−	Flat[c]
pMLT97 plus pSV2-neo	MLT97-7neo	0/4	0/12	−	Flat
	MLT97-3neo	0/4	0/12	+	Transformed
pMLT97	MLT97-12	4/4[d]		+ + +	Transformed
	MLT97-13			+ +	Transformed
bc1051 plus pSV2-neo	1051-10neo		0/4	−	Flat
	1051-11neo	0/4	0/10	−	Flat
	1051-22neo	12/12	1/12	+	Transformed
	1051-7neo	4/4[d]	4/10	+	Transformed

[a] The cell lines were isolated as indicated in footnote *b* of Table 2. Unless otherwise indicated, 2×10^5 cells were injected subcutaneously as a suspension in 50-100 μl of phosphate-buffered saline. Tumors appeared within 3 weeks in nude mice and within 2 months in Fischer rats.

[b] Cells (10^5) were grown in Dulbecco modified Eagle medium containing 10% fetal calf serum and 0.34% (wt/vol) agarose (Difco). Microscopically visible colonies were counted after 14 days at 37°C. Symbols: −, no growth; +, 1 to 10^3 colonies; + +, 10^3 to 10^4 colonies; + + +, more than 10^4 colonies.

[c] All cell lines established by pneo-LT97 were flat.

[d] Only 5×10^4 cells were injected.

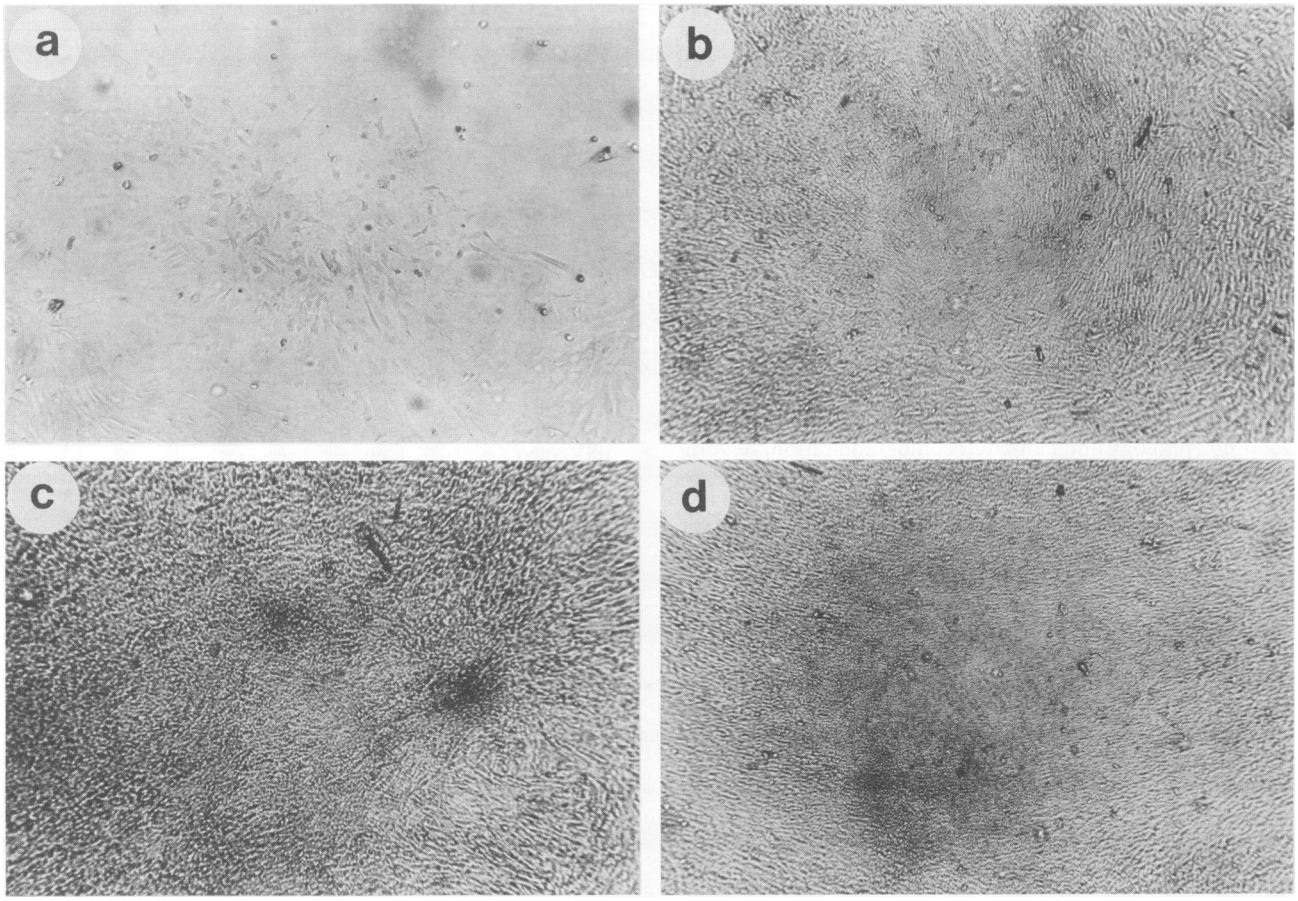


FIG. 2. Morphology of primary rat embryo fibroblasts transfected with recombinant plasmids, as observed through an inverted microscope. (a) pSV2-neo. Colony of G418-resistant cells with normal morphology. The cells stopped growing after a few divisions. (b) pMLT1. Rare colony of G418-resistant cells with altered morphology. The cells could not be established into cell lines. (c) bc1051. (d) pMLT97. About 5% of the G418-resistant colonies had an altered morphology. Supercoiled plasmid DNA (6 μg) was cotransfected with 2 μg of pSV2-neo. Magnification, ×100.

presence of pSV2-neo yielded several G418-resistant colonies (three of four colonies) that exhibited the same partially transformed phenotype (data not shown). Second, introduction of pMLT97 into primary rat embryo fibroblasts without a selection marker produced foci of oncogenically transformed cells (Table 2), some of which grew rapidly as did tumors upon transplantation into nude mice (Table 3) or Fischer rats (data not shown). Transfection of pMLT1 with pSV2-neo also led to the formation of dense colonies (Fig. 2). However, in this case, the total number of colonies was greatly reduced (1 versus 86), presumably because of a toxic effect of the large T antigen. This toxicity of large T has been observed in several instances (14, 18) and could very well explain why simultaneous transfer of both *pmt* and *plt* genes failed to induce tumors in vivo (Table 1) as well as transformation of primary cells in vitro (25) (Table 2).

Cuzin et al. (8) reported that bc1051, a recombinant encoding only the middle and small T proteins (21), did not transform rat embryo fibroblasts. This result seemed paradoxical because experiments from this laboratory had shown that the same recombinant induced tumors in newborn rats with virtually the efficiency of wild-type genomic DNA (2). We found, in agreement with Cuzin et al. (8), that no stably transformed line could be isolated by focus formation after transfer of bc1051 into primary rat embryo fibroblasts (Table 2). However, under the conditions of transfection with the *neo* marker, bc1051 produced dense colonies of transformed cells (Fig. 2). Most of the cell lines derived from these colonies exhibited a partially transformed phenotype. The cells appeared large and flat and resembled immortalized cells. By contrast, some cell lines displayed an irregular growth pattern in culture and had a morphology characteristic of fully transformed cells. Unlike the flat cells, these cells grew in soft agar and were tumorigenic when inoculated into nude mice or Fischer rats (Table 3).

Replication of plasmid DNAs. It was shown by Land et al. (14) that the *plt* gene strongly inhibited establishment of cotransfected genes, which might be due to the presence of the polyoma replication origin together with the gene for the entire large T antigen. This might allow the large T antigen to trigger repeated rounds of viral DNA replication and, in turn, create a cytopathic effect. We wished to see whether the capability of pLT97 to complement *pmt* in both transformation and tumorigenesis could be explained by the inability of the mutant protein to trigger viral DNA replication. To this end, we transfected permissive mouse 3T6 cells by a modification of the DEAE-dextran procedure (17, 30) and assessed the capacity of both pPyLT1 and its deletion mutant pLT97 to replicate in these cells. The low-molecular-weight DNA was extracted 72 h after transfection, and the state of methylation of the recombinant DNA was measured as an indirect assay of replication by digestion with *Dpn*I and

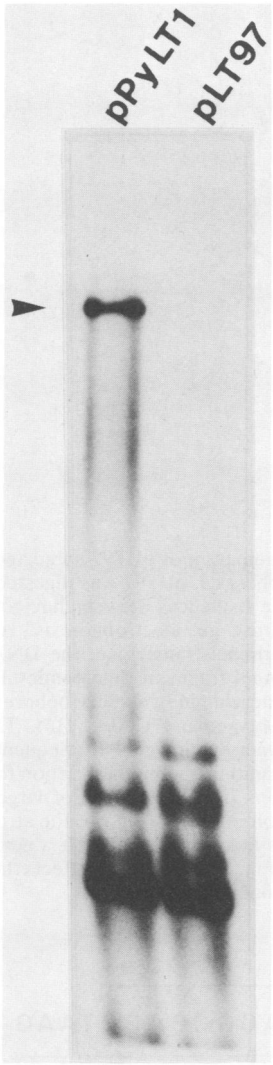


FIG. 3. Replication of pPyLT1 and its deletion mutant pLT97 in mouse 3T6 cells. Plasmid DNA (1 μg) was transfected into ca. 5 × 10^5 cells in 60-mm petri dishes by the DEAE-dextran procedure. Replication of plasmid DNAs was measured by isolating low-molecular-weight DNA and cleaving it with *Bcl*I and *Dpn*I as described in the text. The replicated DNA is resistant to *Dpn*I cleavage and appears as a band which comigrates with a linear marker of pPyLT1. The position of the marker is shown by an arrowhead. The input unreplicated DNA appears as a smear of low-molecular-weight fragments at the bottom of the autoradiogram.

*Bcl*I followed by Southern blotting hybridization (19, 20). In this assay, the replicated *Dpn*I-resistant DNA appeared as a single band that comigrated with linear recombinant DNA (*Bcl*I cleaves the large T plasmid only once). Any unreplicated DNA appeared as numerous fragments of low-molecular-weight at the bottom of the autoradiogram because the large T plasmids contained many sites of cleavage for *Dpn*I. The results of the analysis are shown in Fig. 3. Unlike the control plasmid pPyLT1, the deletion mutant pLT97 was not able to replicate in permissive cells.

Transfer of the mutation into the *pmt* gene. The precise location of the deletion in pLT97 was determined by DNA sequencing. We found that the *plt* gene had an in-frame deletion of 30 bp between nucleotides 1366 and 1397 (Fig. 4a). This deletion removed 10 amino acids from the *plt* protein. However, in a polyoma genome encoding the three early proteins, the same deletion would affect the structure of the *pmt* protein as well. To determine what effect the mutation would have on middle T, we transferred the former into two different plasmids carrying the *pmt* gene and evaluated the transformation properties of the mutant genomes. In a first construction, pLT97 was cleaved by *Ava*I and *Hin*dIII, and the *Ava*I-*Hin*dIII fragment spanning the deletion was substituted for the corresponding fragment in pMT3, a recombinant capable of encoding exclusively the *pmt* protein (32). The resulting plasmid, pMT97 (Fig. 5), contained the *pmt* antigen-coding sequence with a 30-bp deletion affecting the protein as illustrated in Fig. 4b. Although pMT3, the wild-type *pmt* gene, is able to transform FR3T3 cells in culture (2), its deletion mutant pMT97 was inactive (data not shown). By exchanging appropriate restriction fragments (Fig. 5), we also transferred the mutation into pPB21, a plasmid carrying the entire polyoma genome (9) and capable of expressing the three polyoma early proteins. The deletion in pPB21 affected both the middle and large T antigens (Fig. 4b) but not the small T antigen. Mutant pPB97 can therefore be considered as a *mlt* mutant, i.e., a mutant affected in both *pmt* and *plt* gene functions. Although pPB21 was highly tumorigenic in newborn rats, inoculation of its deletion mutant pPB97 failed to induce tumors (Table 1). Taken together, these results indicate that the 30-bp deletion severely impaired or inactivated the transforming and tumorigenic potential of the *pmt* gene.

DISCUSSION

We have shown that only two of the three viral gene functions are required for the polyomavirus-mediated transformation of primary cells. One is conferred by the *pmt* antigen, the transforming protein, whereas the second one can be conferred by either small T or large T. This is in agreement with previous studies from this laboratory showing that *pmt* requires the presence of either polyoma small T or an immortalizing gene from a related DNA tumor virus to induce tumors in newborn rats (3). These results can be interpreted by a multistep process of carcinogenesis: a first step in which cells are prevented from senescing (immortalization), and a second one involving the acquisition of phenotypes characteristic of malignant cells (completion). Thus, some viral and cellular transforming genes can be classified operationally into two complementation groups, each group defining a different step required for transformation of primary cells in vitro (14, 22, 26), as well as induction of tumors in vivo (3). Although polyoma small T is operationally very similar to the immortalizing genes, it cannot be implicated in establishment and immortalization functions (25). This suggests that all the genes that complement the transforming functions of *pmt* do not create identical changes in cellular behavior but may, on the contrary, interact with different cellular targets. Polyomavirus has, in fact, developed three distinct oncogenes, and it has been reported that a function of the small T protein is necessary in addition to and in conjunction with the functions of the large and middle T proteins for transformation of primary cells (8). The results presented here show that this is not the case.

Transfection of the polyoma genomes with the *neo* marker yielded cell lines that displayed only a partially transformed phenotype. For several reasons, we believe that this is due to the *neo* selection rather than to the lack of oncogenic potential of the combination of genes transfected. First, both combinations of *pmt* plus *pst* and *pmt* plus pLT97 are

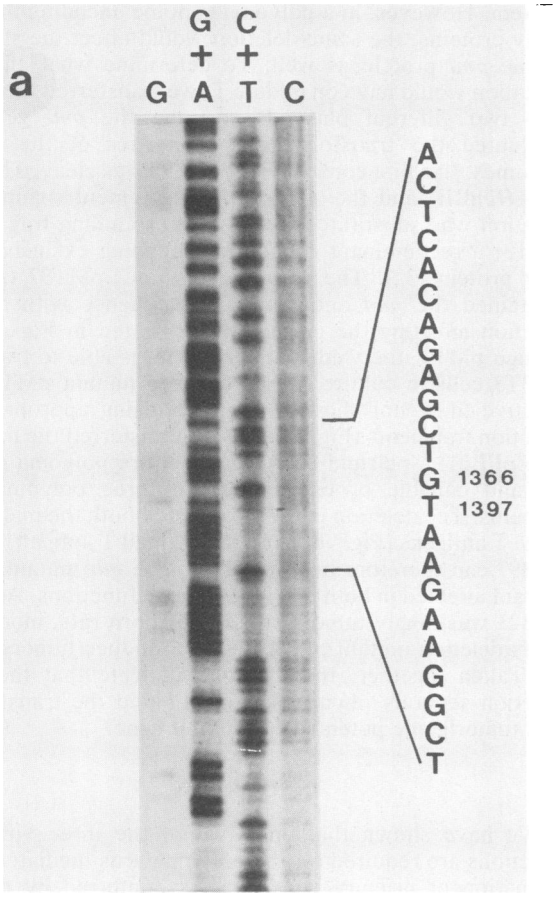
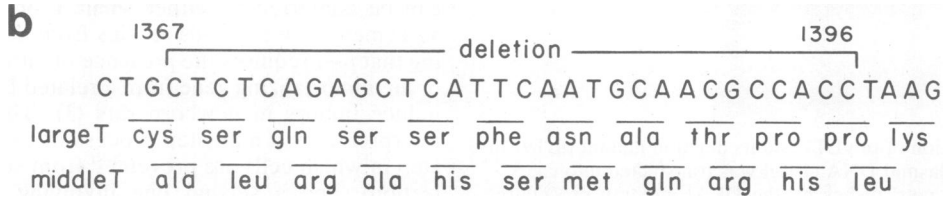


FIG. 4. (a) Autoradiogram of pLT97 subjected to chemical degradation and electrophoresis. pLT97 was digested by *Acc*I, and the fragment spanning the sequences between nucleotides 368 and 1500 was isolated by agarose gel electrophoresis. After labeling with [α-^{32}P]ddATP and terminal transferase, the DNA was cleaved by *Ava*I, and the *Ava*I-*Acc*I fragment (nucleotides 1016 through 1500) was isolated by polyacrylamide gel electrophoresis and sequenced by the method of Maxam and Gilbert (16). The products were separated on 8% polyacrylamide gels. (b) Sequence of the region of polyoma DNA (5′ strand) including the deletion found in pLT97 and predicted modifications in the sequence of large T antigen. After transfer of the deletion in pMT3 (Fig. 5), the structure of middle T antigen is affected as indicated. In pPB97, a genome encoding the three early proteins (Fig. 5), the deletion affects the structure of both middle and large T antigens.



tumorigenic when inoculated into newborn rats, which can be considered the most stringent and rigorous criterion of oncogenic transformation (5). Second, foci of oncogenically transformed cells, obtained by transfecting primary rat embryo fibroblasts with pMLT97 in the absence of *neo* selection, are highly tumorigenic when transplanted into animals. Third, transfection of primary cells with wild-type genomic DNA in the presence of pSV2-neo yields G418-resistant colonies that usually exhibit the same partially transformed phenotype. Thus, cells selected for G418 resistance exhibit a phenotype indistinguishable from that of immortalized cell lines on the basis of several criteria. Such a phenotype can be conferred by the combination of *pmt* plus *pst* and, to some extend, by *pmt* alone (1). These genes, however, are not usually considered to be immortalizing genes. Similar results have been obtained with the Ha-*ras*-1 oncogene (29). Furthermore, the adenovirus E1a gene, which is usually considered to be an immortalizing gene (14, 26), has been shown to behave as a second-step conversion gene in immortalized Chinese hamster cells (29). These results show that some oncogenes can have both step 1 (immortalization) and step 2 (conversion) transforming potential, depending on the assay system chosen. They also suggest that classification of oncogenes into different complementation groups on the basis of their transformation properties may be more artificial than real.

As to the role of large T, our work shows that this oncogene is sufficient to complement *pmt* in transformation, even when both genes are transferred simultaneously into cells. However, the full-sized *plt* appears to be very inefficient in the process. Land et al. (14) could not study the activity of the intact protein because of a toxic effect after gene transfer at high multiplicity by the calcium phosphate transfection method. This toxicity was not observed when we studied the effect of large T in immortalization, but it was apparent when pMLT1 (*pmt* plus *plt*) was cotransfected with pSV2-neo in transformation assays. It is interesting to note that tumors induced by inoculating polyomavirus or its DNA into animals invariably contain deletions or rearrangements in the distal portion of the polyoma early region (5, 12). Loss or interruption of viral sequences coding for the C-terminal portion of the large T antigen is well documented (12, 15), and it has been postulated that a selection operates in vivo against cells producing a functional large T antigen and

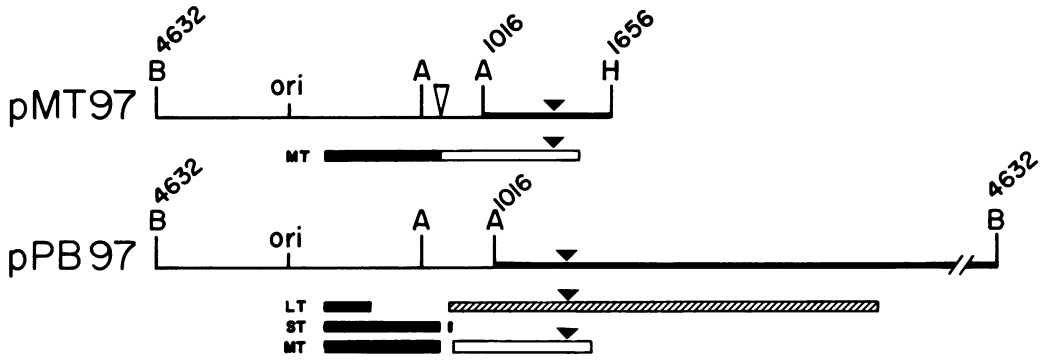


FIG. 5. Diagram showing how the 30 bp deletion was introduced into pMT3, a recombinant encoding exclusively the middle T antigen, and pPB21, a recombinant encoding the three early proteins. pMT97 was obtained by replacing the *Ava*I-*Hin*dIII fragment of pMT3 (nucleotides 1016 through 1656) by the corresponding fragment from pLT97 (thick line on the map). The *Bam*HI-*Hin*dIII fragment is inserted between the *Bam*HI and *Hin*dIII sites of pAT153. The plasmid encodes a middle T antigen with a deletion of 10 amino acids near the C terminus. pPB97 was obtained by replacing the *Ava*I-*Hin*dIII fragment of pPB21 (nucleotides 1016 through 4632) with the corresponding fragment from pLT97 (thick line on the map). The genome is cloned in the *Bam*HI site of pBR322. It encodes the wild-type small T antigen, the middle T antigen from pMT97, and the large T antigen from pLT97. The coding regions for the T antigens are indicated. Shown are the sequences translated in frame 1 (■), frame 2 (▨), and frame 3 (□). The 30-bp deletion is represented by an arrowhead. Abbreviations: B, *Bam*HI; A, *Ava*I; H, *Hin*dIII; ori, origin; MT, middle T; ST, small T; LT, large T.

consequently free viral genomes (15). One would expect the tumors to produce, in addition to the middle T antigen, a modified large T antigen equivalent to mutant pLT97 or, more likely, the small T antigen. The latter, which is sufficient to complement middle T in tumorigenesis, is indeed found in tumors induced by polyomavirus DNA (2, 5). Truncated forms of large T antigen have been found in transformed cells (13) but not in tumors (12), and it is not clear whether they have any role to play in transformation (34). The large T fragments arise very likely as a consequence of deletions and rearrangements affecting the distal half of the early gene region which precludes the synthesis of large T antigen (5, 12) but not that of the middle and small T antigens. The deletion in pLT97 may have occurred in a very particular region of the large T gene; i.e., removal of a few amino acids from some parts of the protein may result in an activation of its biological properties. Although the activity of the mutant is likely due to its inability to replicate and, hence, its inability to exert a toxic effect, its efficiency in both immortalization and complementation of *pmt* is reminiscent of proto-oncogene activation. However, because the deletion occurs in the region coding for the C terminus of middle T antigen, it inactivates the transforming and tumorigenic functions of the genome. For this reason, mutations analogous to pPB97 are unlikely to be generated in vivo.

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