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Sequences from Polyomavirus and Simian Virus 40 Large T Genes Capable of Immortalizing Primary Rat Embryo Fibroblasts

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We developed a procedure to evaluate quantitatively the capacity of subgenomic fragments from polyomavirus and simian virus 40 (SV40) to promote the establishment of primary cells in culture. The large T antigen from both of these viruses can immortalize primary rat embryo fibroblasts. Both antigens have amino-terminal domains that retain biological activity after deletion of other parts of the polypeptide chain. However, this activity varies considerably among various mutants, presumably because of alterations in the stability or conformation of the truncated polypeptides. The polyomavirus middle T gene alone immortalizes at a low efficiency, which indicates that this oncogene can have both immortalization and transformation potentials depending on the assay system chosen. We generated deletions in the polyomavirus and SV40 large T genes to localize more precisely the functional domains of the proteins involved in the immortalization process. Our results show that the region of the SV40 large T antigen involved in immortalization is localized within the first 137 amino acid residues. This region is encoded by the first large T exon and a small portion from the second exon which includes the SV40 large T nuclear location signal. The polyomavirus sequence involved in immortalization comprises a region from the second large T exon, mapping between nucleotides 1016 and 1213, which shares no homology with SV40 and is thought to be of cellular origin. We suggest that this region of the polyomavirus large T gene functions either as a nuclear location signal or as part of the large T protein sequence involved in DNA binding.

It is now widely accepted that carcinogenesis is a multistep process which requires at least two distinct and additive alterations in the control of cellular growth. The first alteration, cellular immortality, can be induced by a variety of oncogenes, such as polyomavirus large T (35), simian virus 40 (SV40) large T (8, 29), adenovirus E1A (16), the oncogene from avian myelocytomatosis virus (v-*myc*) (27), and the gene for cellular phosphoprotein p53 (10, 17, 28). Cells that have acquired an unlimited growth potential in culture can be fully transformed by transfection with oncogenes belonging to a different complementation group. The latter group includes polyomavirus middle T, adenovirus E1B, and the genes of the *ras* family (10, 36, 48). Similarly, the in vivo steps in polyomavirus-mediated tumorigenesis depend on additional cellular alterations beyond the acquisition of the middle T gene (2). Such alterations can be achieved by either polyomavirus small T or various genes from related DNA tumor viruses that appear to be implicated in immortalization function.

The large T antigens of polyomavirus and SV40 are nuclear phosphoproteins that are 785 and 708 amino acids long, respectively, although direct measurements of their molecular weights have led to higher estimates (46). Both proteins exist as multiple molecular species (14), and their activities have been implicated in several processes that occur after virus infection, including the stimulation of host DNA synthesis, the initiation of viral DNA replication, the repression of early gene expression, the switch from early to late transcription, and the events that lead to integration, excision, and amplification of the virus DNA within the host chromosome (for a review, see reference 38). Although the SV40 protein is sufficient to initiate and maintain transformation of both primary cells and continuous cell lines, its polyomavirus counterpart does not cause tumorigenic transformation. Polyomavirus large T can reduce the serum growth factor requirement of established rodent cell lines and complement the serum-dependent transformed state induced by the expression of middle T antigen (33). Transfection of rodent embryo fibroblasts with polyomavirus large T results in the establishment of permanent cell lines without passage through crisis. These cell lines have the growth properties of nontransformed fibroblasts with relatively low serum requirements and are dependent on continued expression of the large T protein for propagation in culture (35).

It has been shown that the region of the polyomavirus large T protein involved in immortalization is localized within its amino-terminal 334 residues (35). Similarly, the amino-terminal one-half of the SV40 large T protein is sufficient to immortalize primary cells (8) and to complement polyomavirus middle T in the tumorigenic process (2). Thus, both antigens have amino-terminal domains that retain biological activities after deletion of other parts of the polypeptide chain. In this work, we generated deletions in the polyomavirus and SV40 genomes to localize more precisely the functional domain of the large T proteins involved in the immortalization process. One difficulty in evaluating the immortalization potential of a viral genome is that independent immortalizing events cannot be quantitated in assays that involve passing cells each time that they become confluent. In such assays, immortalized clones are pooled and expanded so that established cultures arise from several independent events. To investigate the relationship between immortalization and the functional domains of the large T proteins, we developed an assay in which we used pSV2-neo, a dominant selection marker (42), so that the transfected cultures could be placed under G418 selection to control for gene transfer efficiencies. We show that the SV40 sequences involved in immortalization comprise exon 1, the

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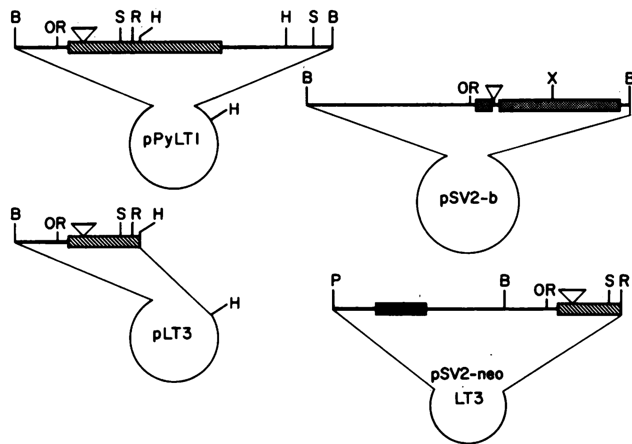


FIG. 1. Structure of recombinant plasmids. pPyLT1 carries the polyomavirus large T gene. The cross-hatched area represents the large T coding sequence. A triangle indicates the position of the deleted intron. pLT3 was derived from pPyLT1 by removing two *Hin*dIII fragments. pSV2-b carries the SV40 large T gene with an *Xho*I linker (X) inserted in the *Hpa*I site at nucleotide 3733. The sequences coding for large T (stippled area) were provided by deletion mutant Δ2005. The deletion in the large T intron between 0.54 and 0.59 map unit is indicated by a triangle. All deletion mutants generated from pPyLT1, pLT3, and pSV2-b were linked to pSV2-neo as shown for pLT3. pLT3 was cleaved with *Bam*HI plus *Eco*RI and inserted between the *Bam*HI and *Eco*RI sites of pSV2-neo. Mutants generated from pPyLT1 and pSV2-b were cleaved with *Bam*HI and inserted into the *Bam*HI site of pSV2-neo. The neo gene in pSV2-neo LT3 is represented by the solid box. Abbreviations: B, *Bam*HI; S, *Sst*I; R, *Eco*RI; H, *Hin*dIII; X, *Xho*I; P, *Pvu*II; OR, origin.

region coding for the amino-terminal portion of large T, together with a small portion of exon 2 which includes the large T nuclear location signal. The polyomavirus sequences involved in immortalization comprise exon 1 and a segment from exon 2 which does not share any sequence homology with SV40.

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 (50). pLT3 was derived from pPyLT1 by removing two *Hin*dIII fragments (polyomavirus nucleotide 1656 to the *Hin*dIII site in pAT153). This plasmid encodes the amino-terminal 366 residues of large T antigen. pMT3 is a recombinant which encodes only the middle T antigen (1); it was obtained by deleting two *Hin*dIII fragments from pPyMT1 (47). pSV2 (Fig. 1) carries the SV40 large T gene. The sequences coding for large T were provided by deletion mutant Δ2005. Because of a deletion in the large T intron between 0.54 and 0.59 map unit, this mutant does not produce the small T antigen (40). pSV2-b was derived from pSV2 by insertion of an 8-base pair (bp) *Xho*I linker into the *Hpa*I site at 0.37 map unit (Fig. 1). pSV2-neo is a plasmid which expresses *neo*, a dominant selection marker (42).

Mutagenesis in the large T genes. Portions of the large T coding sequences were deleted from recombinant plasmids by appropriate digestions with restriction endonucleases. Exonuclease *Bal* 31 was also used to introduce deletions from various sites. The sites were generated as follows. pLT3 was digested to completion by *Sst*I (unique site at nucleotide 1373 [Fig. 1]). pPyLT1 was partially digested with either *Eco*RI (nucleotide 1560) or *Sst*I (nucleotides 1373 and 4341 [Fig. 1]). pSV2-b was cleaved by *Xho*I (*Hpa*I site at nucleotide 3733 [Fig. 1]). The linear full-length plasmids were 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, Inc.). The solution was incubated for 15 s to 4 min at 30°C. Under these conditions, *Bal* 31 removed about 80 bp/min from either end of the molecule. The reaction was terminated by adding 3 μl of 0.2 M EDTA, 3 μl of 0.1 M EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid], and 50 μl of phenol at 0°C. After phenol extraction, the DNA was ethanol precipitated, ligated at 20°C for 2 to 3 h with a 10-fold excess of T4 DNA ligase, and used to transform *Escherichia coli* strain HB101 to ampicillin resistance. Preliminary determinations of the sizes and locations of the deletions were carried out by digestion with *Bam*HI and *Eco*RI (pPyLT1), *Bam*HI and *Sst*I (pLT3 and pPyLT1), or *Bam*HI and *Xho*I (pSV2-b). Polyomavirus recombinants lacking the *Eco*RI site (nucleotide 1560) or the *Sst*I site (nucleotide 1373) were analyzed further by mapping with *Ava*I, *Hin*dIII, *Hpa*II, *Hin*fI, and *Alu*I. SV40 mutants lacking the *Xho*I site were mapped with *Hin*dIII, *Hin*fI, *Xho*II, *Rsa*I, and *Alu*I.

Cells, transfections, and growth assays. Fischer rat embryos that were 15 days old were washed three times in Tris-saline, minced with dissecting scissors, trypsinized for 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 using the calcium phosphate-DNA coprecipitation procedure (3), as modified by Wigler et al. (49). The DNA to be transfected (8 μg) was mixed with high-molecular-weight calf thymus DNA (10 μg) as a carrier. After 20 to 24 h of exposure to DNA, the cells were passaged and plated to 20 to 30% confluence. After 18 h, G418 was added to the medium at a concentration of 400 μg/ml. The medium supplemented with the 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 passaged at least once in medium containing 400 μg of G418 per ml. Most of the cells that underwent senescence did not sustain more than one or 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 that they reached confluence and were maintained in G418-free medium.

RESULTS

Immortalization assay. We devised a simple immortalization assay to detect the ability of recombinant plasmids carrying various portions of the polyomavirus and SV40 genomes to modify the growth of rat embryo fibroblasts. The plasmids were linked to pSV2-neo (Fig. 1) so that the transfected cultures could be placed under G418 selection. Initial experiments were carried out with plasmids pPyLT1 and pLT3, which were known to be active in immortalization (35). pSV2-neo was used as a negative control. Colonies of cells resistant to G418 were first detected after 7 to 10 days

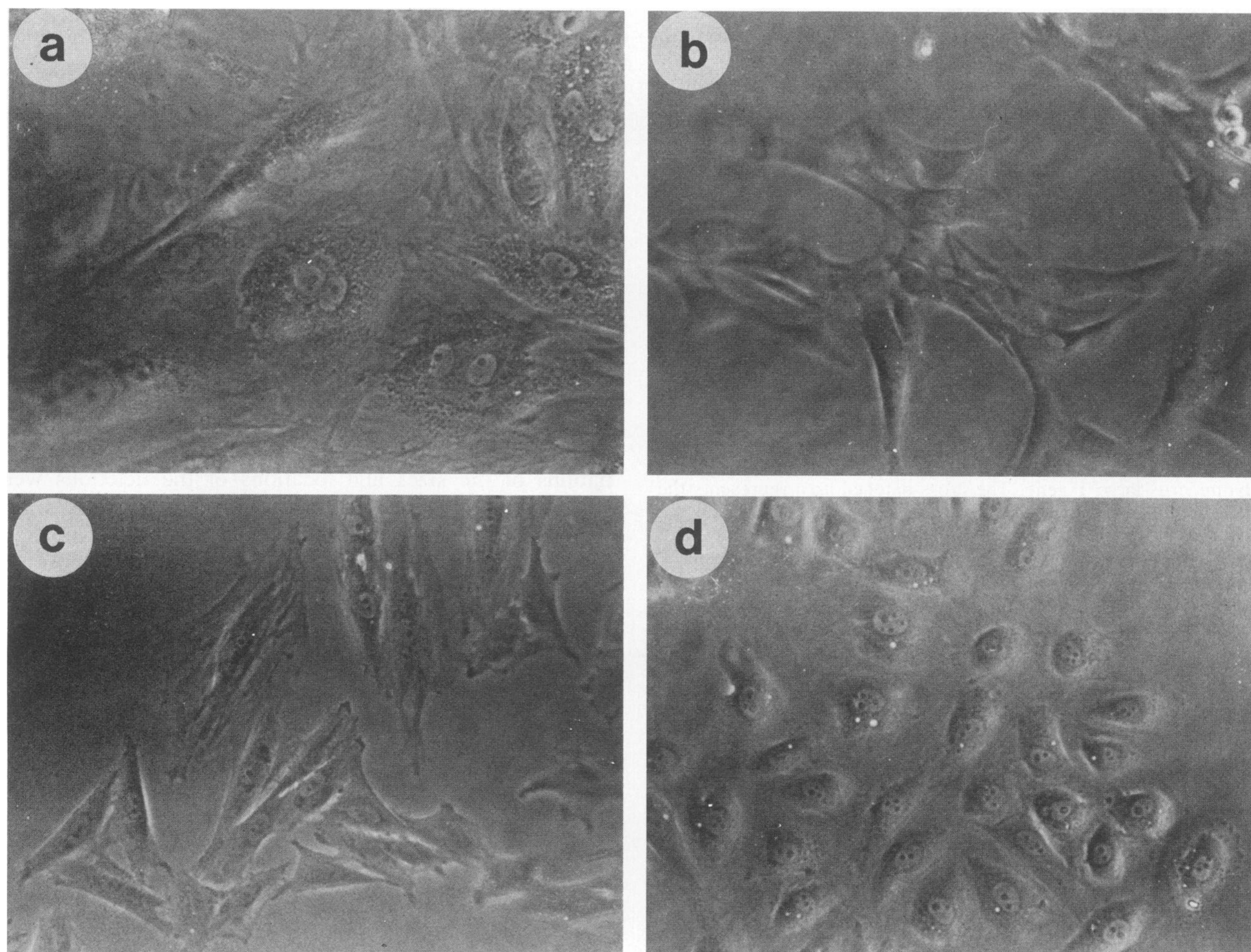


FIG. 2. Morphology of immortalized cell lines. (a) Primary rat embryo fibroblasts transfected with pPyLT1 (not immortalized). (b) Cells immortalized by pSV2 (SV40 large T). (c) Cells immortalized by pLT97. (d) Cells immortalized by LT3. Phase-contrast microscopy. ×425.

of selection. Both pPyLT1 and pLT3 yielded about 50 colonies per transfection, whereas pSV2-neo alone consistently produced fewer colonies (10 to 15 colonies per transfection). At this stage, some of the colonies established with the immortalizing plasmids already exhibited a regular cellular morphology that distinguished them from the other colonies (Fig. 2). Many of the colonies established by transfer of pSV2-neo stopped growing after a few cell divisions and detached from the plastic. This selection was particularly efficient when the medium was replaced frequently. Thus, besides the G418 resistance, the assay selected to some extent for cells that had acquired an increased capacity for growth. This explained the correlation between the presence of an immortalizing gene in the transfection preparation and the number and morphology of G418-resistant colonies that could be isolated. For this reason, colonies were picked after 3 to 4 weeks of selection regardless of their morphology.

When pSV2-neo colonies were transferred to microplates, the cells underwent one or two divisions but most of the time senesced before reaching confluence. Only 1 of 452 colonies established by plasmid pSV2-neo lacking an immortalizing gene could be brought to five passages. This indicated that spontaneous immortalization occurred in the assay with a frequency on the order of 1 in 452 (0.2%). By contrast, transfection with pPyLT1 and pLT3 gave rise to continuous cell lines that could be passaged indefinitely in culture (Fig. 3). Surprisingly, the cell lines established with pLT97, a deletion mutant derived from pPyLT1 (see the legend to Fig. 5 for details), reached the fifth transfer sooner than the other lines. Moreover, transfection with this DNA gave rise to a higher proportion of colonies reaching five transfers in culture. We interpreted these results as indicating that pLT97 immortalized primary rat embryo fibroblasts with an efficiency about twice that of pPyLT1. Similarly, transfection with pPyLT1 and pLT3 led to the establishment of continuous lines at frequencies of 33.1 and 21.3%, respectively. Thus, although pLT3, the large T antigen fragment, was able to immortalize, it did so with a frequency substantially lower than that observed with the intact protein.

In these initial studies, we observed that some cell lines senesced after the third passage or, occasionally, after the fourth passage. However, once cell lines had been passaged five times, they could be considered immortalized. Of 217 lines that reached 5 passages, all could be brought to 10 passages within weeks. Twenty of these lines were picked randomly, and all could be brought to 30 passages. In subsequent experiments, we evaluated immortalization effi-

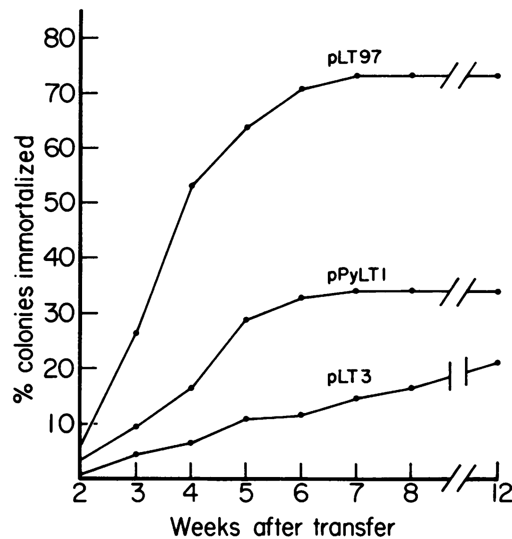


FIG. 3. Efficiency of immortalization. Colonies of cells resistant to G418 were picked 3 to 4 weeks after selection and transferred to 15-mm Linbro microplates. The cells were subcultured at a 1:5 dilution each time that they reached confluence. They were considered immortalized after the fifth passage. The percentage of colonies immortalized by pLT3 was still 21.3% 18 weeks after transfer. Only 1 of 452 colonies established by plasmid pSV2-neo lacking an immortalizing gene could be brought to five passages. Immortalization efficiencies were evaluated by counting the percentage of cell lines reaching five or more passages 7 to 8 weeks after the first transfer.

ciencies by counting the percentage of cell lines reaching five or more passages 7 to 8 weeks after the first transfer, and we ensured that the lines grew to a minimum of 10 passages.

Expression of large T in immortalized cells. As only a fraction of the G418-resistant colonies became immortalized (Fig. 3), we wanted to determine whether establishment of these colonies into cell lines was related to their ability to express the large T antigen. We reasoned that only cells capable of expressing sufficient amounts of large T might escape senescence, while those producing insufficient amounts of the protein would enter crisis before the fifth passage in culture. Alternatively, large T might create a cytopathic effect after its transfer into cells at a high multiplicity (20, 27), which could result in the loss of some cell lines. To evaluate the production of large T antigen at early stages of immortalization, we examined randomly picked colonies by immunofluorescence staining and observed them during the following weeks for their ability to become immortalized. Of 22 G418-resistant colonies established by transfection with pPyLT1, 6 exhibited some nuclear fluorescence characteristic of large T antigen (Fig. 4a through e). These colonies were among the seven colonies that could subsequently be passaged more than five times in culture and were considered immortalized. The cells from the other colonies were less fluorescent (Fig. 4f through j), although some positive nuclei were detected occasionally. These observations indicated that the ability of cells containing the polyomavirus large T gene to become established in culture was probably related to the level of large T expression as early as the first transfer. They also showed that expression of large T did not exert any important toxicity affecting immortalization.

Sequences from the SV40 genome required in immortalization. It has been shown previously that the amino-terminal one-half of the SV40 large T protein is sufficient to transform (7) and to immortalize primary cells (8). To delineate more precisely the functional domain of the protein involved in immortalization, we generated deletions from a single *Xho*I site in Δ2005 (Fig. 1), an SV40 mutant which does not produce the small T antigen (40). The sizes and locations of the deletions were determined by restriction endonuclease mapping, as shown in Fig. 5. This mapping also ensured that the polyadenylation signal was preserved at the 3′ end of the gene. As expected, the deletions around the *Xho*I site at 0.37 map unit did not abolish the immortalization potential of the SV40 large T gene. Less expected, however, was the observation that mutant pSV24 with a large deletion immortalized with a relatively high efficiency. The precise location of the deletion in pSV24 was determined by DNA sequencing, using the Maxam-Gilbert procedure (25). The results showed that this mutant had a deletion of 1,228 bp from nucleotide 4407 to nucleotide 3180 (Fig. 6). The deletion produced a reading frame shift for the truncated large T antigen that resulted in the addition of six amino acid residues to Asp-137. It is interesting that mutant pSV24 kept a short seven-amino acid sequence around Lys-128 (amino acids 126 to 132) that is required for the normal nuclear localization of SV40 large T (19). To determine the importance of this sequence in immortalization, we analyzed the properties of mutant pSV35. This mutant, which was inactive in the immortalization assay (Fig. 5), had a deletion of about 800 bp that removed the *Hin*fI site at position 4459 (six amino acids upstream from Pro-126) but kept the *Hin*fI site at position 4568. This showed that the 3′ border of the minimal SV40 sequence capable of immortalizing primary cells mapped between positions 4568 and 4407.

Expression of a truncated large T polypeptide by pSV24. From the structure of pSV24 we predicted that this mutant encodes a polypeptide of 143 amino acids, consisting of the first 137 residues from large T antigen and a short 6-amino acid sequence unrelated to large T at the carboxy terminus (Fig. 6). To analyze the viral proteins in established cell lines, the cells were labeled with [^{35}S]methionine, and the proteins precipitable by an SV40 anti-T serum were fractionated on sodium dodecyl sulfate-polyacrylamide gels. Figure 7 shows typical results obtained with Δ2005, the intact large T gene, and mutant pSV24. Both the 94,000-dalton large T antigen and the 53,000-dalton nonviral tumor antigen (21, 24) were clearly visible in cells transformed by Δ2005. Cell lines established by pSV24 produced a small polypeptide having a molecular weight of about 19,500. This was slightly more than the expected molecular weight of 16,500 for the pSV24 protein. However, the apparent mobilities of deletion mutants do not always correlate with the sizes of the deletions but appear to reflect further structural changes (14).

Sequences from the polyomavirus genome required in immortalization. To define the sequences from polyomavirus large T required in immortalization, we first generated deletions in pPyLT1 from either the *Eco*RI site at position 1560 or the *Sst*I site at position 1373. Mutants with short deletions, such as pLT97, pLT63, and pLT65, immortalized with varying efficiencies (Fig. 5). One mutant, pLT9, was inactive. This mutant had a deletion of about 690 bp that removed the *Hin*fI site at position 960 and extended from the *Eco*RI site to the vicinity of the large T splicing sites (positions 409 and 795). This suggested that the first exon of large T was not sufficient to immortalize and that some sequences from the second exon were required. To map these sequences further, we constructed mutants pLTav and pLT1016 (Fig. 5). In pLTav, the *Ava*I site at nucleotide 1016

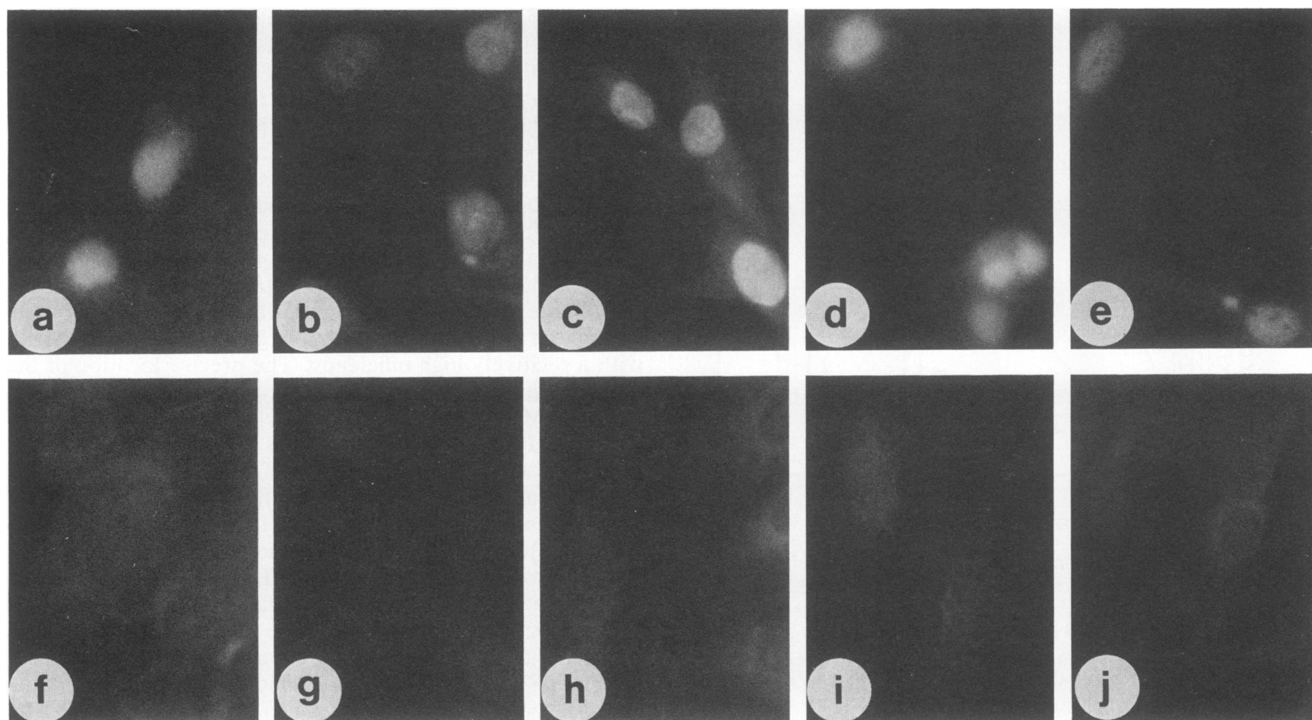
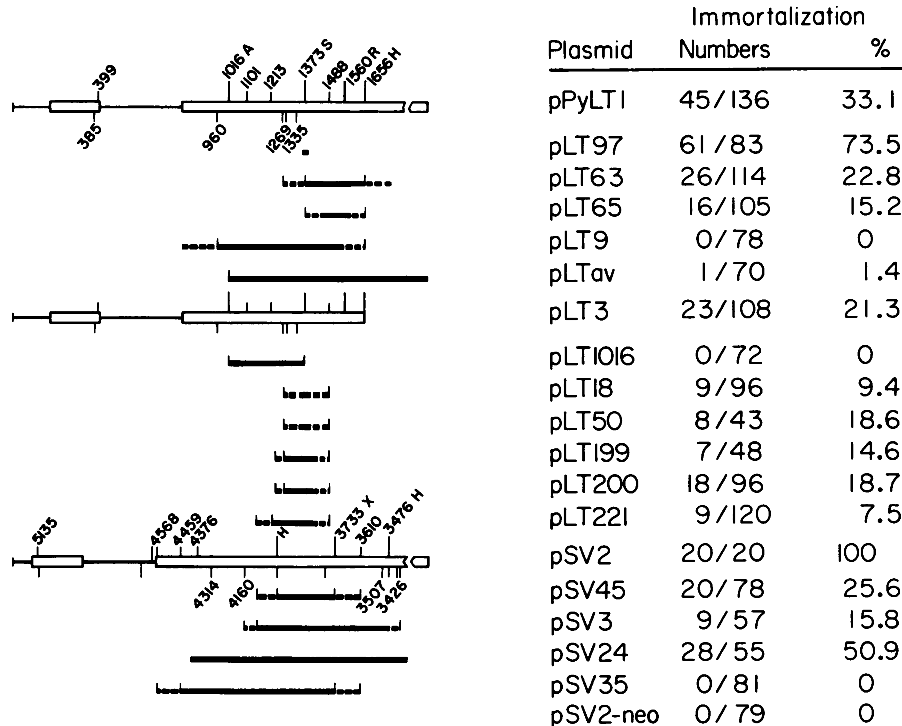


FIG. 4. Immunofluorescence staining for polyomavirus large T antigen. Primary rat embryo fibroblasts were transfected with pPyLT1, and 22 colonies of cells resistant to G418 were picked randomly after 4 weeks of selection. A fraction of each sample (about one-fifth) was seeded onto microscope slides for immunofluorescence staining, and the remaining fraction was grown in Linbro microplates for immortalization assays. After fixation in acetone-methanol (2:1) for 15 min at −20°C, the cells were stained for large T antigen with a rat anti-polyomavirus tumor serum; this was followed by incubation with a fluorescein-conjugated rabbit anti-rat antibody (Miles Laboratories, Inc.). (a through e) Five of the six cell lines positive for large T antigen. (f through j) Representative cell lines (5 of 16 cell lines) negative for large T antigen.

was inactivated by filling the free ends with the Klenow polymerase so that a reading frame shift would occur at this position for the large T antigen. pLT1016 was constructed from pLT3 by deleting the sequences from *Ava*I to *Sst*I (nucleotides 1016 to 1373). Both pLTav and pLT1016 were inactive, indicating that immortalization required some sequences located downstream from the *Ava*I site at nucleotide 1016. This region of the polyomavirus genome contains sequences coding for the unique portion of middle T antigen and therefore does not share any homology with SV40 (41). After the first exon, the next region of homology with SV40 starts at polyomavirus nucleotide 1278 (Gly-241) (Fig. 8). In particular, amino acids 279 through 284 of polyomavirus large T exhibit some homology with the sequence of amino acids around Lys-128 which is required for the normal nuclear localization of SV40 large T (19). As these amino acids are present in pSV24, we wanted to determine whether the corresponding sequence of polyomavirus large T was dispensible in immortalization. As shown in Fig. 5, this sequence was missing in pLT63, as well as in several other mutants generated from the *Sst*I site in pLT3. Surprisingly, several of these mutants, which were active in immortalization, had deletions that extended far beyond the region of homology between polyomavirus and SV40. In particular, mutant pLT221 had a deletion of about 230 bp that removed the *Hpa*II site at position 1213 (Fig. 5). This site is located 22 codons upstream from Gly-241, the 5′ border of the region exhibiting some homology with SV40 (Gly-87 in SV40). Thus, we were able to immortalize with sequences encoding no more than the first 219 residues of the polyomavirus large T antigen.

We made several attempts to identify truncated large T polypeptides in the cell lines by using a polyvalent anti-T serum, as well as a preparation of monoclonal antibodies directed against the amino-terminal region of polyomavirus large T antigen (a gift from B. E. Griffin). Although such species have been described previously in polyomavirus-transformed cells (34), they could not be detected in cell lines established by the deletion mutants. This indicates that the polypeptides either were unstable or were produced in amounts too small to be detected, as commonly observed in polyomavirus transformants (34), or that their continued synthesis was no longer required after establishment of cell lines.

Immortalization by middle T antigen. Up to this point we established that the region of the polyomavirus genome required for immortalization comprises some sequences between nucleotides 1016 (*Ava*I) and 1213 (*Hpa*II). These sequences are part of a region encoding a unique portion of the middle T antigen. They could encode a functional domain of the large T antigen important for immortalization or some amino acid sequence conferring stability or specific structural configurations to other parts of the polypeptide. As both large and middle T antigens have a common amino-terminal end, we wanted to know whether immortalization could be established by a middle T-large T hybrid. Recombinant pmt-avaI was constructed by filling the gaps at the *Ava*I site (nucleotide 1016) in pMT3, a plasmid capable of encoding only the middle T protein. This resulted in a frame shift at nucleotide 1016 that substituted the carboxy-terminal end of middle T antigen with the carboxy-terminal end of large T antigen. This recombinant did not immortalize (data



Plasmid	Immortalization Numbers	%
pPyLT1	45/136	33.1
pLT97	61/83	73.5
pLT63	26/114	22.8
pLT65	16/105	15.2
pLT9	0/78	0
pLTav	1/70	1.4
pLT3	23/108	21.3
pLT1016	0/72	0
pLT18	9/96	9.4
pLT50	8/43	18.6
pLT199	7/48	14.6
pLT200	18/96	18.7
pLT221	9/120	7.5
pSV2	20/20	100
pSV45	20/78	25.6
pSV3	9/57	15.8
pSV24	28/55	50.9
pSV35	0/81	0
pSV2-neo	0/79	0

FIG. 5. Genomes used to delineate the extent of the minimum immortalizing region from the polyomavirus and SV40 large T genes. The structures of different deletion mutants are shown, along with the names of the corresponding plasmids and their abilities to immortalize primary rat embryo fibroblasts. The approximate locations of the deletions were based on estimates of the deletion sizes and the presence or absence of endonuclease sites. Exons are represented by open boxes. For each mutant, the solid portion of the line indicates the region known to be deleted, and the dashed portions indicate the possible extents of the deletions. pLT97 has an in-frame deletion of 30 bp (nucleotides 1367 to 1396). Mutants pLT2 and pLT78, mapping like both pLT18 and pLT50, immortalized with frequencies of 4.2% (4 of 95 colonies) and 2% (2 of 96 colonies), respectively. The single immortalization event obtained with pLTav (1 of 70 colonies) was considered to be spontaneous. Abbreviations: A, *Ava*I; S, *Sst*I; R, *Eco*RI; H, *Hin*dIII; X, *Xho*I linker inserted in to the *Hpa*I site of pSV2.

not shown), indicating the importance of the large T coding sequences upstream from nucleotide 1016. However, in the course of these experiments, we observed that pMT3, containing the middle T gene alone, immortalized cells at a low efficiency (2 of 67 cell lines). Neither of the two cell lines established by pMT3 had the morphology characteristic of transformed cells (Fig. 9), nor were they tumorigenic when they were inoculated into Fischer rats or nude mice (data not shown). One of the cell lines (cell line 2-7) appeared to be flatter, grew slower, and had a lower saturation density than the other (cell line 2-12). They both exhibited some cytoplasmic fluorescence characteristic of middle T antigen (Fig. 9). Interestingly, the fluorescence was more intense in cell line 2-12 than in cell line 2-7, suggesting that the phenotypes which these lines exhibited were related to the level of middle T expression.

DISCUSSION

We used a quantitative assay to evaluate the capacity of viral genomes to immortalize primary rat embryo fibroblasts in culture. Besides its simplicity, this assay has the following advantages compared with other procedures: a low level of spontaneous immortalization, the possibility of analyzing the growth properties of individual clones after gene transfer, and the evaluation of immortalization potentials in terms of quantitative measurements. With the exception of Δ2005, containing the intact large T gene from SV40 which conferred a transformed phenotype to virtually all of the transfected cells, immortalizing genes did not establish all of the colonies isolated by G418 selection. Instead, they immortalized with frequencies ranging from a few percent (e.g., pLT221) to 73.5% (pLT97). Transfection with pPyLT1, containing the intact large T gene from polyomavirus, immortalized with about 30% efficiency. In this case, we found that the colonies that could be rescued from senescence were those that were expressing the large T protein at the time of selection in G418. This suggests that only cells capable of expressing sufficient amounts of large T become immortalized, while those producing insufficient amounts of the protein fail to escape senescence.

Although truncated large T genes are able to immortalize, they do so with frequencies substantially lower than those observed with intact genes. Tevethia (44) observed a similar effect of the amino-terminal one-half of SV40 large T and suggested that the 3′ one-half of the antigen strongly influences the frequency of immortalization. This influence may reflect the presence of a transformation domain in the carboxy terminus of large T or, alternatively, result from an altered conformation or stability of the truncated polypeptide. The second possibility seems more likely for two reasons. First, different deletions in a given region of the gene may affect immortalization to considerably different degrees (e.g., pLT50, pLT18, and pLT78). Second, pSV24, which has a relatively large deletion, immortalizes better than other SV40 mutants, such as pSV3 and pSV45, which have smaller deletions. Therefore, these results support the notion that some sequences may be important in the mutants

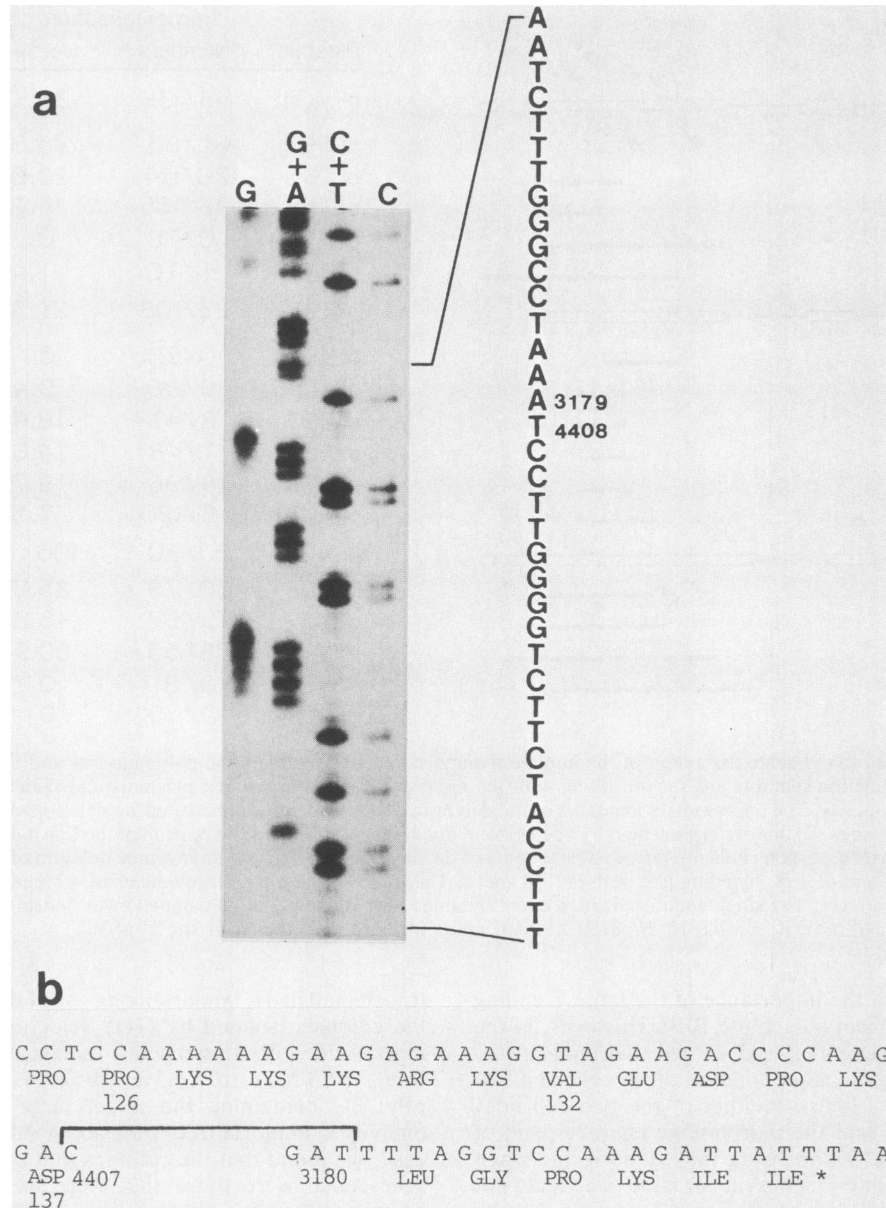


FIG. 6. (a)Autoradiogram of pSV24 DNA subjected to chemical degradation and gel electrophoresis. pSV24 was digested with *Hin*fI, and the fragment spanning the sequences between nucleotides 4459 and 2848 was isolated by agarose gel electrophoresis. After labeling with [α-^{32}P]ddATP and terminal transferase, the fragment was digested wtih *Rsa*I, and the asymmetrically labeled DNAs were separated by polyacrylamide gel electrophoresis and subjected to the chemical degradation procedure of Maxam and Gilbert (25). The products were separated on 8% polyacrylamide gels. (b) Sequence of the region of SV40 DNA (5′ strand) including the deletion found in pSV24 and predicted modifications in the sequence of large T antigen.

either to confer stability or to establish the optimal conformation in the immortalizing domain of the protein.

One of the polyomavirus mutants, pLT97, immortalized more efficiently than intact large T did. In experiments to be described elsewhere (C. Asselin, J. Vass-Marengo, and M. Bastin, J. Virol., in press), we found that unlike intact large T or truncated large T mutants, pLT97 could complement polyomavirus middle T in an in vivo assay for tumorigenicity. We interpret these results as suggesting that the 30-bp deletion in pLT97 activates the immortalization potential of polyomavirus large T and that this is reflected in the ability of the mutant to complement middle T in tumorigenesis. Taken together, these results indicate that both SV40 and polyomavirus large T antigens can immortalize primary cells and that both antigens have amino-terminal domains that retain biological activity after deletions of the other parts of the polypeptide chain. However, this activity varies considerably among the various mutants, presumably because of alterations in the stability or conformation of the truncated polypeptides.

The high immortalization efficiency obtained with pSV24 indicates that the region of the SV40 large T antigen involved in immortalization is localized within the first 137 amino acid residues. This region includes a short sequence of amino acids around Lys-128 which is required for the normal nuclear accumulation of wild-type and deleted forms of the

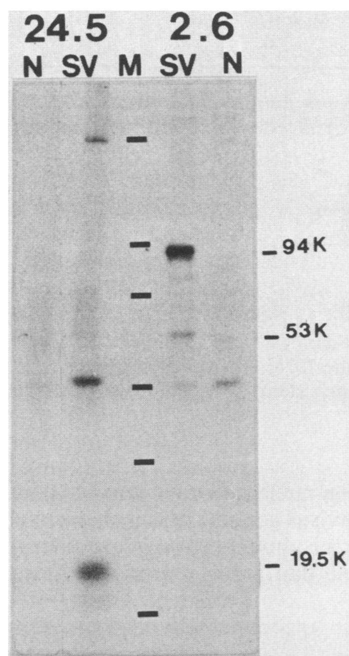


FIG. 7. Viral proteins synthesized by representative cell lines. Cell line 24.5 was obtained by transfecting primary rat embryo fibroblasts with pSV24. Cell line 2.6 was obtained by transfection with pSV2 (intact SV40 large T). The positions of the SV40 large T antigen (94,000 daltons), the nonviral tumor antigen (53,000 daltons), and the protein encoded by pSV24 (19,500 daltons) are indicated. Lanes N, Nonimmune serum; lanes SV, SV40 anti-T serum; lane M, molecular weight markers (from top to bottom molecular weights of 200,000, 97,400, 68,000, 43,000, 25,700, and 18,400). The cells were grown in 60-mm plates and labeled with [^{35}S]methionine (0.1 mCi per plate) for 90 min at 37°C. Proteins were extracted and precipitated with SV40 anti-T protein serum. The proteins were fractionated on 12.5% sodium dodecyl sulfate-polyacrylamide gels.

antigen (18, 19). It is conceivable that immortalization by SV40 requires sequences from the second large T exon to encode the nuclear location signal, although one would expect the nuclear membrane to allow free passage of polypeptides of the size of the polypeptide encoded by pSV24 (9). In experiments not described here, we have found that SV40(cT)-3, a mutant defective in nuclear transport of large T antigen (22), is severely impaired, but not totally inactive, in the ability to immortalize primary rat embryo fibroblasts. It is not clear yet whether the mutant exhibits some residual activity because of the cytoplasmic large T antigen or because, as claimed by Lanford et al. (23), some cells can transport the mutant protein to the nucleus. Interestingly, amino acids 278 through 284 of polyomavirus large T exhibit some homology with the sequence around Lys-128 in SV40. These amino acids are not part of a nuclear location signal (A. Smith, personal communication), and we have shown that they are dispensible in immortalization by polyomavirus large T. Further experiments are in progress to determine whether the polyomavirus large T protein contains any nuclear location signal similar to that of SV40. Whatever the outcome of such experiments, it will be interesting to establish what function of the protein is encoded within the portion of the second exon required for immortalization.

Two biochemical activities of the large T antigens encoded by polyomavirus and SV40 have been demonstrated in vitro. One is the capacity to hydrolyze ATP and ADP and P_i (11, 13, 15, 45), and the other is the capacity to bind DNA (4, 11). Initial attempts to identify the origin-specific DNA-binding domain of SV40 large T antigen have led to conflicting results (5, 6, 31, 32, 37, 39). In particular, it has been difficult to distinguish between a region of the T antigen that may be directly involved in DNA binding and a region that may be conformationally important in inducing an active binding domain elsewhere in the same antigen molecule. According to Morrison et al. (26), an amino-terminal 17,000-molecular-weight fragment of SV40 large T antigen (amino acids 1 to 130) is capable of specifically binding to SV40 DNA. Because the first 82 amino acids are shared between large T and small T antigens and because the latter does not bind DNA, the authors have tentatively concluded that the sequence between Ile-83 and approximately Arg-130 is required, although not necessarily sufficient, for origin-specific binding by the T antigen. As pointed out previously, much of the homology between the polyomavirus and SV40 proteins across the origin-binding domain of SV40 large T is clustered between amino acids 124 to 130, which include the nuclear location signal in SV40 large T antigen, and amino acids 278 to 284 in polyomavirus large T antigen. Because both antigens recognize the same or very closely related DNA sequences, Pomerantz and Hassell (30) have suggested that the protein-DNA contacts are mediated by the side chains of the same or related amino acids, such as the stretch of basic amino acids described in the two proteins. The observation that these amino acids can be dispensible in immortalization mediated by polyomavirus large T suggests that other clusters of amino acids, such as those around Lys-192 or Arg-168, can provide an equivalent function either as a nuclear location signal or as part of the sequence involved in DNA binding. An alternative explanation is that polyomavirus and SV40 immortalize via different mechanisms. Consistent with this possibility is the observation that SV40 large T antigen stabilizes p53 (21), while its polyomavirus counterpart does not. However, is is not known yet whether binding to p53 is important for the oncogenic action of SV40, nor is it understood why small amino-terminal fragments of SV40 large T that are unable to complex p53 nevertheless have the ability to immortalize cells (8).

Of interest is the observation that polyomavirus middle T alone can immortalize primary rat cells at a low efficiency. This is consistent with a recent report showing that high levels of Ha-*ras*-1 expression can immortalize (43), although Ha-*ras*-1 is usually considered to be a late-step or conversion gene (20, 36). Similarly, the adenovirus E1A gene can behave as a second-step conversion gene in immortalized Chinese hamster cells (43), although it is usually considered to be an immortalizing gene (20, 36). Thus, some oncogenes can have both step 1 (immortalization) and step 2 (conversion) transforming potentials depending on the assay system chosen.

Several studies on the structure of the polyomavirus genome have reported some sequence flexibility in the overlap region of middle and large T antigens (14). This may explain the existence of numerous mutations mapping in this part of the genome. In a previous study, we noted that the sequence of the large T polypeptide was not less conserved than that of the middle T polypeptide (12). The reason for this may be that the overlap region encodes parts of two proteins with important viral functions. One is the carboxy-

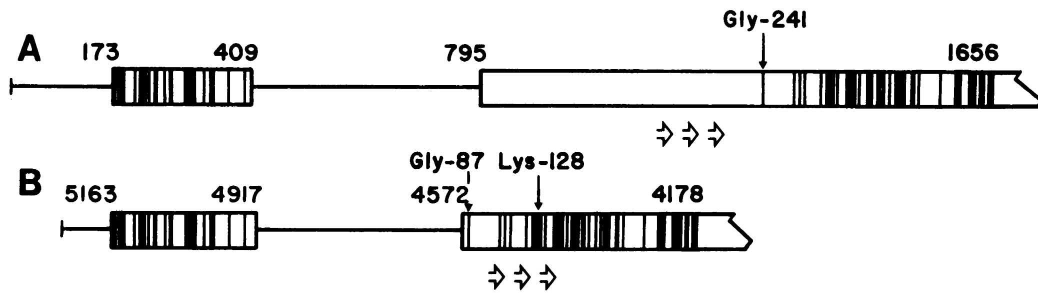


FIG. 8. Amino acid homologies between the amino-terminal fragments of polyomavirus and SV40 large T proteins as predicted from the DNA sequences. (A) 5′ portion of polyomavirus large T. (B) 5′ portion of SV40 large T. Homologies are indicated by vertical lines in the exons up to nucleotide 1656 for polyomavirus and nucleotide 4178 for SV40. Gly-241 (nucleotide 1278) corresponds to the 5′ border of the region exhibiting some homology with SV40 (Gly-87) in the second exon. The arrows indicate the extents of the minimum immortalizing regions in polyomavirus and SV40.

terminal one-half of middle T antigen, and the other is a segment of the large T protein playing a role in the control of cellular growth and tumorigenesis (1, 34). We have now determined that the sequence coding for this portion of large T maps between nucleotides 1016 and 1213, i.e., in a region of the polyomavirus genome which shares no homology with SV40 and which is thought to be of cellular origin (41). It has been suggested that, like a group of retroviruses, poly-

FIG. 9. (a and c) Morphology of cell lines immortalized by polyomavirus middle T antigen. Phase-contrast microscopy. ×430. (b and d) Immunofluorescence staining for middle T antigen. ×1150. Primary rat embryo fibroblasts were transfected with pMT3, the middle T clone, linked to pSV2-neo. Of 67 G418-resistant colonies, 2 were brought over five passages and considered immortalized. (a and b) Cell line 2.7. (c and d) Cell line 2-12. The conditions used for immunofluorescence staining are described in the legend to Fig. 4.

omavirus has acquired its transforming function from its host cells (41) and that the unique sequence of the middle T antigen could be responsible for the particular mode of oncogenic action characterisic of polyomavirus (14). Therefore, it is surprising that the two functions of this virus which cause malignant transformation are encoded by alternative reading frames in a single DNA sequence inherited from the host genome.

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