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Mutation Causing Premature Termination of the Polyoma Virus Medium T Antigen Blocks Cell Transformation

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We used site-specific mutagenesis to introduce a termination codon, TGA, into the reading frame for the polyoma virus medium T antigen. We induced this mutation in a region of the polyoma genome in which the overlapping coding regions for the large and medium T antigens are translated in different reading frames. Therefore, the mutation terminated translation of the medium T antigen, but it caused only a single amino acid substitution in the large T antigen and did not affect the small T antigen. Cells infected by the mutant virus produced normal-size small and large T antigens. The infected cells produced a 28,000-dalton fragment of the 48,000-dalton medium T antigen, whose size and tryptic peptide map were consistent with its being a truncated N-terminal fragment terminating at the new termination codon of the mutant. Immunoprecipitates of mutant-infected cell extracts did not show medium-T-antigen-associated protein kinase activity. The mutant virus replicated normally in mouse 3T6 cells and induced cellular DNA synthesis in resting mouse 3T3 cells, but it failed to transform rat or hamster cells, as judged by focus formation and growth in agar. The mutant complemented a *tsA* mutant which affects the large T antigen for transformation, implying that the mutant defect for transformation was in the medium T antigen. These results imply that the small T antigen and the large T antigen together are insufficient to cause transformation and support the conclusion that the medium T antigen is essential for cell transformation by polyoma virus.

The early region of the polyoma virus genome encodes three proteins, which are called the large, medium (or middle), and small T antigens (14, 29, 31). The coding regions and amino acid sequences of these proteins have been deduced from the nucleotide sequence of polyoma virus DNA and from analyses of the viral mRNA's and proteins. The three T antigens share common N-terminal 79-amino acid regions encoded between 74 and 79 map units on the physical map of the polyoma genome. The small and medium T antigens share an additional 112 amino acids encoded between 79 and 85 map units. These sequences are not present in the large T antigen because they occur in an intervening sequence which is absent from the large-T-antigen mRNA. The coding region for the small T antigen terminates at about 86 map units. The coding regions for the large and medium T antigens overlap in the region between 86 and 99 map units, but they are translated in different reading frames. The medium T antigen terminates at 99 map units. The large T antigen contains additional unique sequences encoded between 99 and 26 map units.

This overlapping organization of the coding regions for the three T antigens, with each protein having both unique and shared amino acid sequences, has complicated efforts to separate the functions of the proteins by genetic methods. Nevertheless, several kinds of mutations have been used to investigate which T antigens are necessary for cell transformation. The *tsA* mutations, which map in the unique C-terminal region of the large T antigen, block initiation of cell transformation at the nonpermissive temperature (6). However, the large T antigen is often truncated or missing in transformed cells, suggesting that the C-terminal portion of the large T antigen is not required for maintenance of the transformed phenotype (4, 12, 13, 15, 18). The C-terminal portion of the large T antigen may not be required even for initiation under some conditions, since transformation can be accomplished by cloned fragments of viral DNA lacking the coding regions for the C-terminal portion of large T antigen (9, 22, 23). The host range nontransforming (hr-t) mutations (1), which map in the intervening sequence of the large T antigen, affect the small and medium T antigens coordinately. These mutations block cell transformation, suggesting that either the small T antigen or medium T antigen or both are required for transformation. Some viable deletion mutations in the coding regions shared by the medium and large T anti-

gens decrease the frequency of cell transformation and produce an altered transformed phenotype (8, 15, 20). These viable deletions most likely affect cell transformation because of a defect in the medium T antigen rather than in the large T antigen (15).

The involvement of the T antigens in transformation has also been investigated by constructing cells that express only one of these proteins. Cells containing hr-t mutant genomes, which express only the large T antigen, are not transformed, showing that the large T antigen by itself is not sufficient for transformation (19). Recently, a polyoma virus genome that expresses only the medium T antigen was constructed (30). This genome transforms rat cells in culture, and the transformed cells form tumors in animals (30). Therefore, the medium T antigen is sufficient to establish and maintain the major phenotypic properties of transformed cells. Whether the small T antigen plays some role in transformation (alone or in combination with the medium T antigen) is not yet clear.

To characterize the functions of each of the T antigens more precisely, it would be desirable to have mutations that affect each of the proteins specifically. An approach to this can be made by the use of site-specific mutagenesis (26) directed at nucleotide sequences in which alterations might be expected to have differential effects on the proteins. For example, in the shared coding regions for the medium and large T antigens, which are translated in different reading frames, single base changes can affect the two proteins differently, depending upon the codon change in each reading frame. In this paper we describe the use of site-specific mutagenesis to introduce a termination codon in the reading frame of the medium T antigen, causing the production of a truncated medium T antigen that was about two-thirds normal size without any detectable effect on the function of large T antigen. Viral genomes containing this mutation replicated normally but failed to cause cell transformation.

MATERIALS AND METHODS

Cell culture and virus infection. Cells were grown in Dulbecco modified Eagle medium supplemented with 5 or 10% calf serum. We used the wild-type large-plaque strain WS of polyoma virus (7). For lytic infections, cultures of mouse 3T6 cells were infected at a multiplicity of approximately 10 PFU/cell. The virus was allowed to adsorb for 60 min. The cultures were then covered with fresh medium and incubated under the conditions described below. Cell transformation of rat F2408 and 3Y1 cells was carried out by infecting cell cultures as described above for lytic infections. After virus adsorption, the infected cultures were incubated overnight at 37°C. The cultures were then trypsinized and reseeded at a concentration of either 10^4 or 10^5 cells per 5-cm dish. Incubation was continued for 2 to 4 weeks. Cell transformation of hamster BHK cells was carried out by infecting the cells in a suspension (approximately 5×10^6 cells per ml) with a virus suspension (10^8 PFU/ml) for 1 h at 0°C, with periodic agitation. After adsorption, the cells were suspended in 0.34% agar at a concentration of 5×10^3 or 2×10^4 cells per 5-cm dish. Incubation was continued for 2 to 4 weeks at 37°C.

Site-directed mutagenesis of viral DNA. We used the nucleotide numbering system of Friedmann et al. (2, 7). Restriction enzyme fragments of purified polyoma DNA consisting of a portion of the early region between the *Hae*II site (nucleotide 111) and the *Eco*RI site (nucleotide 1,575) were cloned in a plasmid pBR322 vector containing the plasmid origin of replication and the Amp^r gene. To do this, pBR322 DNA was cut singly and randomly with *Hae*II by using ethidium bromide to inhibit additional digestion, as described by Parker et al. (24). The resulting partially digested molecules were then cut with *Eco*RI and fractionated on an agarose gel. Fragments containing an intact plasmid replication origin and an Amp^r gene were identified with appropriate markers, and the DNA was purified as described below. After ligation to the viral insertion, the resulting plasmids were susceptible to cleavage with *Ava*I only in viral sequences. Two plasmids were constructed; one contained both viral *Ava*I sites (nucleotides 672 and 1,031), and the other was made by using a spontaneous viral mutant containing the *Ava*I site at nucleotide 1,031 but lacking the *Ava*I site at nucleotide 672. DNA from the first plasmid was randomly nicked at a single site per molecule by using the method of Parker et al. (24); this produced circular form II molecules containing both *Ava*I sites. DNA from the second plasmid was digested with *Ava*I and then with nuclease S1; this produced linear molecules that lacked the *Ava*I site at nucleotide 672 and the four nucleotides of the staggered ends produced by *Ava*I digestion of the site at nucleotide 1,031.

The linear and circular viral DNAs were isolated by electrophoresis in agarose gels, as follows. The DNA was electrophoresed through 4-mm 1.5% vertical agarose gels in 0.8× Tris-phosphate buffer (37 mM Tris base, 30 mM NaH_3PO_4, 1 mM EDTA, pH 7.2) at 100 to 200 V. After staining with ethidium bromide and visualization with UV light, bands were cut from the gel and dissolved in three times the volume of a gel piece containing saturated NaI (454 g of NaI dissolved in 250 ml of 20 mM Tris-chloride [pH 7.5]–1 mM EDTA). A 50% ceramic silica slurry (glass powder previously washed in boiling nitric acid) was added (1 μl/μg of DNA, although not less than 5 μl), and the mixture was shaken at 4°C for 20 min to 16 h. The DNA bound to the glass was precipitated with a microfuge and washed twice in 60% saturated NaI and twice in 50% ethanol–100 mM NaCl–10 mM Tris (pH 7.5)–1 mM EDTA. The DNA was eluted in either 10 mM Tris (pH 7.5)–1 mM EDTA (twice, 20 μl) or 200 mM NaCl–20 mM Tris (pH 9.0)–0.1% sodium dodecyl sulfate (twice 50 μl; followed by ethanol precipitation). The sodium dodecyl sulfate elution removed 95% of the bound radioactive DNA from the glass powder; other methods were less effective. DNA purified from agarose in this manner could be ligated, treated with kinase, or cut with restriction endonucleases without further treatment.

Approximately 5 μg of linear DNA fragments was mixed with approximately 0.5 μg of randomly nicked circular DNA in 200 μl of 50 mM NaCl–10 mM Tris-chloride (pH 7.5)–1 mM EDTA, boiled for 1 min, and allowed to reanneal for 16 h at 45°C. This procedure produced circular heteroduplex molecules that were resistant to *Ava*I and had a single base pair mismatch at nucleotide 672 and a single-stranded region four bases long at nucleotides 1,032 through 1,035. The circular *Ava*I-resistant molecules were isolated by electrophoresis in agarose gels, as described above.

The single-stranded regions of the heteroduplex molecules were mutagenized with bisulfite by the method described by Shortle and Nathans (26). DNA and 10 μg of carrier tRNA in 100 μl of 10 mM Tris (pH 7.5)–1 mM EDTA were mixed with 300 μl of 4 M sodium bisulfite (pH 6.0) (360 mg of $NaHSO_3$ and 150 mg of Na_2SO_3 dissolved in 1 ml of water) and 5 μl of 50 mM hydroquinone and then overlaid with paraffin oil and incubated in the dark for 4 to 24 h at 37°C. The bisulfite reagent is highly specific for single-stranded DNA, and under these conditions the half-life of conversion of deoxycytosine residues was estimated empirically to be 8 h. The bisulfite reaction was terminated by dialysis in (i) 50 mM NaCl–10 mM Tris (pH 7.5)–1 mM EDTA–0.01 mM hydroquinone (2 liters, 6 h at 4°C), (ii) 50 mM NaCl–10 mM Tris (pH 7.5)–1 mM EDTA (2 liters, 16 h, 4°C), (iii) 50 mM NaCl–200 mM Tris (pH 11) (200 ml, 16 to 24 h, 37°C), and (iv) 50 mM NaCl–10 mM Tris (pH 7.5)–1 mM EDTA (2 liters, 16 h, 4°C), followed by ethanol precipitation.

The mutagenized DNA was transfected directly into *Escherichia coli*, and individual ampicillin-resistant colonies were screened to identify plasmids resistant to *Ava*I by restriction analysis and sequence determination. Molecules containing the desired mutation were removed from the plasmid and introduced into a wild-type viral genome in the following way. *Sst*I fragments 1 (nucleotides 1,388 to 4,366) and 2 (nucleotides 4,366 to 589) and *Hgi*AI fragment 6 (nucleotides 584 to 740) were isolated from wild-type polyoma DNA, and *Hgi*AI fragment 4 (nucleotides 740 to 1,388, including the mutation) was isolated from the plasmid DNA. The fragments were pooled, ligated en masse, and used to infect mouse 3T6 cells. Infectious virus was plaque purified three times, and the viral DNA was tested to verify that it was resistant to *Ava*I cleavage at nucleotide 1,031.

T-antigen and DNA nucleotide sequence analysis. Extracts of infected cells were precipitated with rat anti-tumor serum and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as described previously (12).

An analysis of the methionine-containing tryptic peptides was also performed essentially as described previously (12). Detection by fluorography was enhanced by using 2-methylnaphthalene.

DNA nucleotide sequence analysis was performed by the method of Maxam and Gilbert (21).

RESULTS

Site-directed mutagenesis to produce a termination codon in the reading frame for medium T antigen. Initially, we selected the *Ava*I restriction enzyme cutting site located at about 90 map units (nucleotide 1,031 in the numbering system of Friedmann et al. [7], which we used) in the common coding region for the medium and large T antigens as a target for mutagenesis. (The coding regions of the T antigens are shown in Fig. 1.) Cleavage of polyoma DNA by *Ava*I at this site left single-stranded ends four nucleotides long, which were potential targets for mutagenesis by bisulfite. Treatment of single-stranded DNA by bisulfite produces cytosine-to-thymine transitions (16). Such a transition at nucleotide 1,033 would have introduced a termination codon into the reading frame for the medium T antigen. Our attempts to mutagenize the single-stranded ends of the linear DNA produced by cleavage with *Ava*I were unsuccessful, apparently because the mutagenized DNA could not be ligated in a form that preserved the integrity of the staggered ends. (The procedure produced deletions of various sizes around the *Ava*I site; the properties of the resulting deletion mutants will be described elsewhere.) Therefore, we devised a method of creating a circular viral DNA molecule containing a small single-stranded region around the *Ava*I site, which

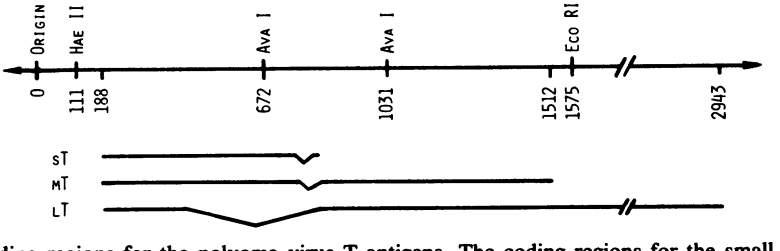

FIG. 1. Coding regions for the polyoma virus T antigens. The coding regions for the small (sT), medium (MT), and large (LT) T antigens are diagrammed beneath a linear representation of the early region of the polyoma virus genome. (On the circular genetic map of the polyoma virus genome the early region extends clockwise from the origin of replication at about 71 map units.) Intervening sequences in the three T antigens are shown by diagonal lines. The initiation codon for all three T antigens begins at nucleotide 188. The medium T antigen terminates at nucleotide 1,512. The *Ava*I cutting site used in mutagenesis is at nucleotide 1,031.

could be mutagenized by bisulfite. This procedure is summarized in Fig. 2 (see above).

We began with two plasmids; one of these plasmids contained a portion of the early region of polyoma virus DNA between the *Hae*II site at nucleotide 111 and the *Eco*RI site at nucleotide 1,575, including two *Aval* cleavage sites at nucleotides 672 and 1,031 and the other was identical, except that it lacked the *Aval* cleavage site at nucleotide 672 because of a cytosine-to-adenosine transversion at that position. We digested these molecules with *Aval* and then with nuclease S1, which produced full-length linear molecules that were shortened by four bases at the *Aval* site (nucleotides 1,032 to 1,035) because of removal of the staggered ends by the S1 treatment. We hybridized these shortened molecules to full-length circular plasmid molecules, and

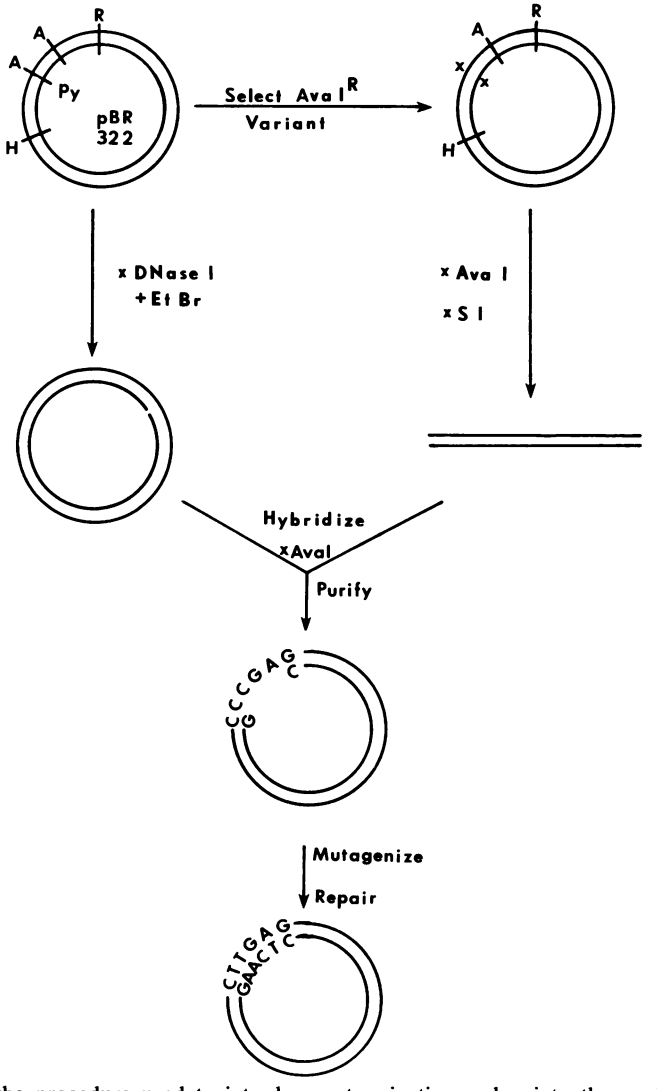

FIG. 2. Outline of the procedure used to introduce a termination codon into the reading frame for the polyoma virus medium T antigen. A portion of the early region of the polyoma virus genome between the *Hae*II (H) and *Eco*RI (R) restriction enzyme sites was cloned in plasmid pBR322. We prepared heteroduplex molecules that contained gaps at the *Aval* (A) site at nucleotides 1,032 to 1,035 by annealing full-length circular molecules to linear molecules which were cleaved with *Aval* and trimmed with nuclease S1 to remove the single-stranded ends at the staggered cutting sites. We mutagenized the single-stranded regions of the gapped molecules to cause cytosine-to-thymine transitions, thereby producing a TGA codon in the reading frame for the medium T antigen. Then we reinserted the mutagenized DNA into polyoma virus DNA molecules and selected infectious viral genomes containing the mutations. EtBr, Ethidium bromide.

this procedure produced heteroduplexes with four-base gaps in one strand at nucleotides 1,032 to 1,035. We mutagenized these single-stranded regions with bisulfite, transfected *E. coli* with the mutagenized DNA, and selected mutagenized plasmids which were resistant to *AvaI* digestion. After we identified an appropriate mutated molecule by nucleotide sequencing (see below), we reintroduced the mutated region into a polyoma genome and characterized the resulting virus after serial plaque purification.

The mutant virus produced had the following two changes: a cytosine-to-thymine transition at nucleotide 1,032, which had no effect on the amino acid sequence of the medium T antigen, and a cytosine-to-thymine transition at nucleotide 1,033, which produced an opal termination codon (TGA) at nucleotides 1,033 to 1,035 in the reading frame for the medium T antigen and a proline-to-leucine change in the amino acid sequence of the large T antigen. We designated this mutant MOP 1033 to indicate the location and nature of the termination codon (base 1,033, opal). Figure 3 shows the nucleotide sequence of the mutant DNA around the site of the mutation, and Fig. 4 summarizes the nucleotide sequences and inferred amino acid sequences of the mutant and wild-type viruses.

Mutant MOP 1033 carried out an apparently normal cycle of lytic infection in mouse 3T6 cells. The titers of infectious virus and the morphologies and sizes of the plaques produced after infection of 3T6 cells were not detectably different from those of the wild-type virus. Infection of resting mouse 3T3 cells with the mutant virus resulted in the initiation of cellular DNA synthesis, as measured by incorporation of tritiated thymidine (data not shown).

Wild-type and mutant T antigens. We analyzed the T antigens that were synthesized in 3T6 cells infected with wild-type polyoma virus and with mutant MOP 1033 by using immunoprecipitation and polyacrylamide gel electrophoresis, as described above. Figure 5 shows the [^{35}S]methionine-labeled proteins that were precipitated from the infected cell extracts. Normal-size large and small T antigens were present in both extracts. The cells infected with mutant MOP 1033 lacked a normal-size medium T antigen. (A preliminary analysis by tryptic peptide mapping indicated that the faint band that migrated slightly faster than the wild-type medium T antigen was a cellular protein which was not related to the medium T antigen. We could not rule out the possibility that an undetectable amount of authentic medium T antigen was produced by some mechanism which bypassed the termination codon at a low frequency, but it seemed

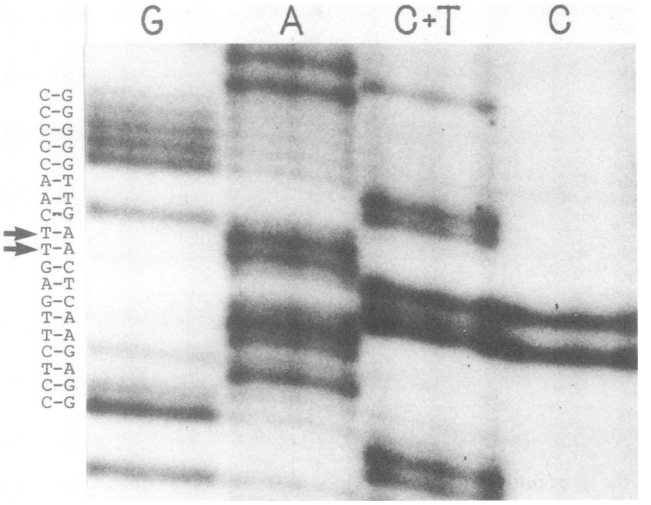

FIG. 3. Nucleotide sequence analysis of MOP 1033 mutant DNA in the vicinity of the mutation. A portion of the Maxam-Gilbert sequencing gel is shown. Reading 3′ to 5′ (top to bottom), the sequence of nucleotides 1,031 to 1,039 in the DNA strand complementary to the mRNA was 3′-GAACTCAAG-5′. This coded for an mRNA sequence of 5′-CUUGAGUUC-3′, compared with the wild-type sequence 5′-CCCGAGUUC-3′ (2). The nucleotides changed in the mutant DNA are indicated by arrows.

WT— ˇTAˇTCˇCˇCCˇAˇACˇCˇGˇAˇGTˇTˇCTˇC

MT—Tyr Pro Pro Thr Arg Val Leu—

LT——Ser Pro Asn Pro Ser Ser——

Mop— ˇTAˇTCˇCˇCCˇAˇACTˇTˇGˇAˇGTˇTˇCTˇC

MT—Tyr Pro Pro Thr Term

LT——Ser Pro Asn Leu Ser Ser——

FIG. 4. Nucleotide and amino acid sequences of wild-type (WT) and mutant T antigens in the region of the MOP 1033 mutation. The nucleotide sequence of the wild-type DNA has been reported previously (2); the MOP 1033 mutant sequence was derived from Fig. 3. The carets above the lines designate codons for the medium T antigens (MT), and those below the lines designate codons for the large T antigens (LT).

unlikely that this amount of the protein would be biologically significant [see below].) The mutant-infected cell extracts contained a new immunoprecipitable protein, which had a molecular weight of approximately 28,000 (28K); this protein was not present in the wild-type extracts. The apparent molecular weight of this protein was close to that of a protein which would have been produced by termination of translation of the medium T antigen at the termination codon in mutant MOP 1033 (30K) (11).

To confirm the relationship of the 28K protein to the medium T antigen, we analyzed the tryptic peptides of these two proteins radiolabeled with [^{35}S]methionine by using two-dimensional thin-layer chromatography and electrophoresis. For this comparison we used the viable deletion mutant dl8 (8, 28), which produces a shortened medium T antigen compared with the wild type; this antigen is more easily resolved from contaminating proteins in preparative gels than the wild-type antigen. Figure 6 shows a comparison of the tryptic peptides of the two proteins. The designation system used for the peptides was the same as the system used previously for the wild-type T antigens (12). Using different lines of evidence, we previously assigned tentative positions for some of the T-antigen peptides in the coding regions of the polyoma genome (11). These positions (in nucleotides) are indicated below.

Peptide 1 (nucleotides 188 to 196) is the N-terminal peptide that is shared by the small, medium, and large T antigens. Peptide 4 (nucleotides 275 to 192) is also shared by all three T antigens. Peptides C (nucleotides 398 to 430), B (nucleotides 506 to 535), and A (nucleotides 677 to 825) are shared by the small and medium T antigens, but are absent from the large T antigen. All of these peptides were present in both dl8 medium T antigen and mutant MOP 1033 28K fragment preparations, as expected if the 28K fragment had an intact N-terminal region. Peptides U (nucleotides 850 to 960) and Y (nucleotides 970 to 1,011) are unique to the medium T antigen and were present in the mutant 28K fragment, suggesting that the mutant coding region was intact, at least up to nucleotide 1,011. Peptide Y was missing from the dl8 preparation. This was expected, because the dl8 deletion removes nucleotides 1,006

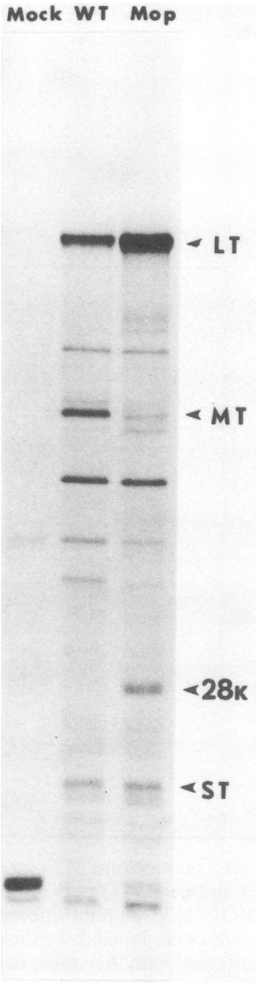

FIG. 5. Wild-type (WT) polyoma virus and mutant MOP 1033 T antigens. We infected mouse 3T6 cells with wild-type polyoma virus or with the MOP 1033 mutant at a multiplicity of 10 PFU/cell. We radiolabeled the cultures with [^{35}S]methionine for 2 h at 28 to 30 h after infection. We analyzed extracts of the infected cells by precipitation with anti-tumor serum, followed by polyacrylamide gel electrophoresis. LT, MT, and ST, Large, medium, and small T antigens, respectively. The position of the 28K medium T fragment produced in the MOP 1033 mutant-infected cells is also indicated.

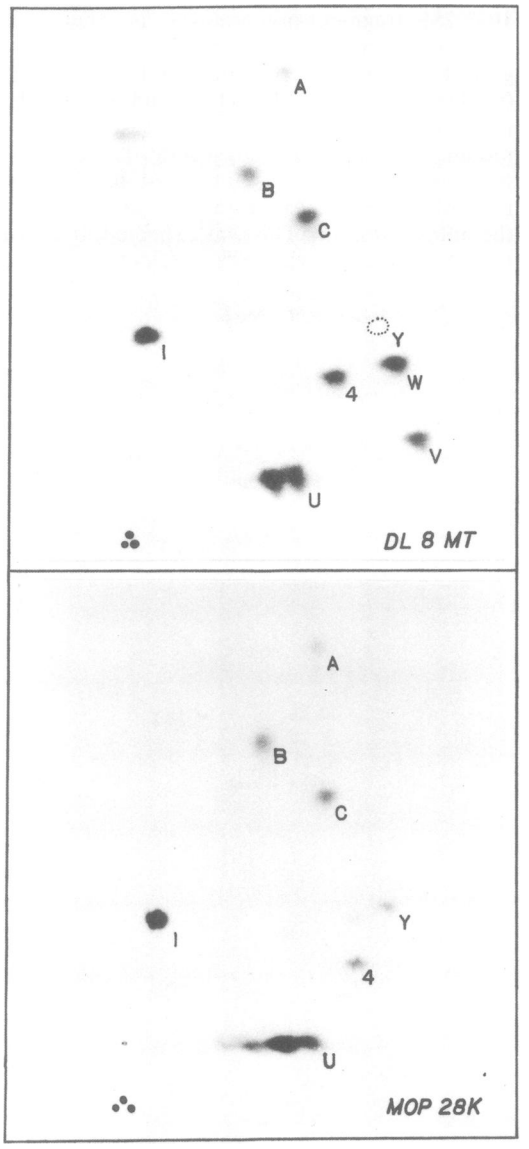

FIG. 6. Tryptic peptide analysis of the polyoma virus medium T antigen (MT) and the 28K fragment produced in MOP 1033 mutant-infected cells. We isolated [^{35}S]methionine-labeled medium T antigen from 3T6 cells infected with the viable deletion mutant dl8 and the 28K protein from cells infected with the MOP 1033 mutant. We analyzed the tryptic digests of these proteins by two-dimensional electrophoresis and chromatography, as described previously (12).

through 1,095, including the codon for the methionine residue of peptide Y. The peptide difference between dl8 and the wild type was also demonstrated by Ito et al., who used a different separation system (15). (The position which wild-type peptide Y would have occupied is shown by the dotted lines in the dl8 map.) The identity of peptides W and V was established previously by using synthetic peptides predicted from the DNA nucleotide sequence (11). Peptides W (nucleotides 1,360 to 1,365) and V (nucleotides 1,390 to 1,407) were present on the dl8 map but missing from the mutant MOP 1033 28K fragment map, as expected if the 28K fragment lacked the C-terminal region encoded beyond nucleotide 1,033.

In summary, the tryptic peptide patterns were consistent with the interpretation that the MOP 1033 28K protein was an N-terminal fragment of the medium T antigen, terminating at nucleotide 1033.

The medium T antigen of polyoma virus is associated with a protein kinase activity in immunoprecipitates (3, 25, 27). This activity phosphorylates a tyrosine residue on the medium T antigen (3). The large T antigen is also phosphorylated under these conditions, but to a lesser extent than the medium T antigen. We tested whether immunoprecipitates of extracts of MOP 1033 mutant-infected 3T6 cells showed a similar activity. Figure 7 shows the proteins that were phosphorylated in vitro during incubation of immunoprecipitates with [γ-^{32}P]ATP. As reported previously, the wild-type medium T antigen was phosphorylated under these conditions. By contrast, there was very little radiolabeling of material in the region of the normal medium T antigen in the mutant-infected extract and no detectable radiolabeling of the 28K fragment. These observations suggested that the 28K protein lacked the medium-T-antigen-associated kinase activity or was not capable of being phosphorylated by it.

Cell transformation by wild-type and mutant viruses. To test the ability of mutant MOP 1033 to carry out cell transformation, we infected two lines of rat cells (3Y1 [17] and F2408 [5]) and hamster BHK cells with wild-type polyoma virus or with the MOP 1033 mutant and tested for transformation by focus formation in liquid culture or by colony formation in agar, as described above. The results of one experiment are shown in Fig. 8. Infection of rat F2408 or 3Y1 cells with wild-type virus at a multiplicity of approximately 5 PFU/cell yielded transformed foci at a frequency of about 10^{-3}. By contrast, infection with the MOP 1033 mutant did not produce any transformed foci. Increasing the mutiplicity fivefold and doubling the time of incubation after infection did not result in the appearance of any transformed foci after infection with the mutant virus. Similar results were observed with BHK cells seeded in soft agar; no transformed colonies appeared after infection with the mutant virus under conditions in which the wild-type produced transformed colonies at a frequency of 10^{-3} to 10^{-4}. We concluded that

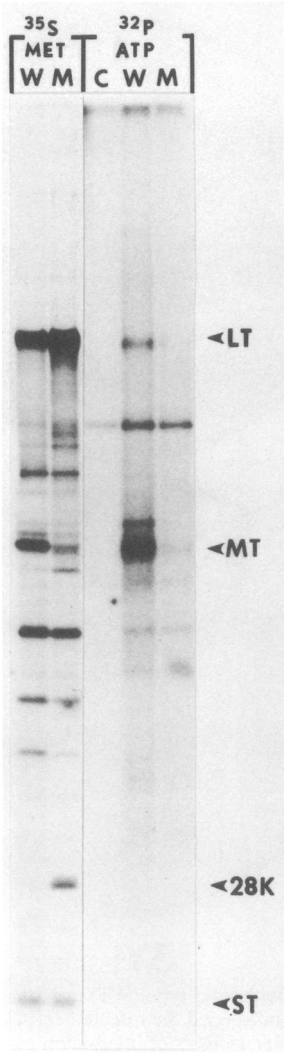

FIG. 7. Protein kinase activity in immunoprecipitates of wild-type virus-infected and MOP 1033 mutant-infected 3T6 cells. Mock-infected (lane C), wild-type virus-infected (lanes W), and mutant-infected (lanes M) mouse 3T6 cells were radiolabeled with [^{35}S]methonine (MET), extracted, and analyzed as described in the legend to Fig. 5. Nonradioactive cell extracts were immunoprecipitated in parallel and were incubated with [γ-^{32}P]ATP to assay protein kinase activity, as described previously (3). The positions of the large, medium, and small T antigens (LT, MT, and ST, respectively) and the 28K fragment are indicated.

the mutant was at least several-hundred-fold less efficient in transformation than wild-type polyoma virus.

To test whether the defect in transformation was caused by the premature termination of the medium T antigen or by the proline-to-leucine change in the large T antigen, we studied the ability of the MOP 1033 mutant to complement *tsA* mutants for transformation after a mixed infection. The *tsA* mutations affect the large T antigen. Therefore, complementation between the MOP 1033 mutant and *tsA* mutants would have suggested that the MOP 1033 mutant synthesized a nondefective large T antigen. The results of such a complementation experiment are shown in Table 1. A mixed infection with the MOP 1033 mutant and a *tsA* mutant (*tsA*25E) resulted in transformation at the nonpermissive temperature. Although it is possible that there could have been complementation between two defective forms of the large T antigen, the most plausible interpretation of these results is that the MOP 1033 mutant synthesized a nondefective large T antigen, compensating for the *tsA* mutant defect, whereas the *tsA* mutant synthesized a nondefective medium T antigen, compensating for the MOP 1033 mutant defect.

DISCUSSION

We used the technique of site-directed mutagenesis with bisulfite to introduce a termination codon into the reading frame for the polyoma medium T antigen. Cells infected with a virus containing this mutation produce the large and small T antigens but fail to produce a full-length medium T antigen. Instead, a new 28K protein is produced. This protein has the size and tryptic peptide pattern expected for an N-terminal fragment of the medium T antigen, terminating at the new termination codon in the mutant.

The MOP 1033 mutant is defective in cell transformation, as assayed either by focus formation or by growth in agar. This mutant complements a *tsA* mutant which carries a mutation in the large T antigen for transformation. These results strongly imply that the medium T antigen is essential for transformation and that the small and the large T antigens together are not sufficient to cause transformation. The medium T antigen alone is also sufficient for transformation, as shown by the ability of altered viral genomes which express only the medium T antigen to transform (30).

Involvement of the medium T antigen in transformation has been suggested previously by the properties of the hr-t mutants, whose mutations affect both the small and medium T antigens, and by the properties of some viable deletion mutants, whose mutations affect both the medium and large T antigens. The following two kinds of effects of viral mutations on transformation can be considered: effects on transformation frequency and effects on expression of the transformed phenotype. Some of the viable deletion mutations affect the frequency of transformation (20), and at least one affects the phenotype of transformed cells (15). In the case of these mutants it is possible that the effects on

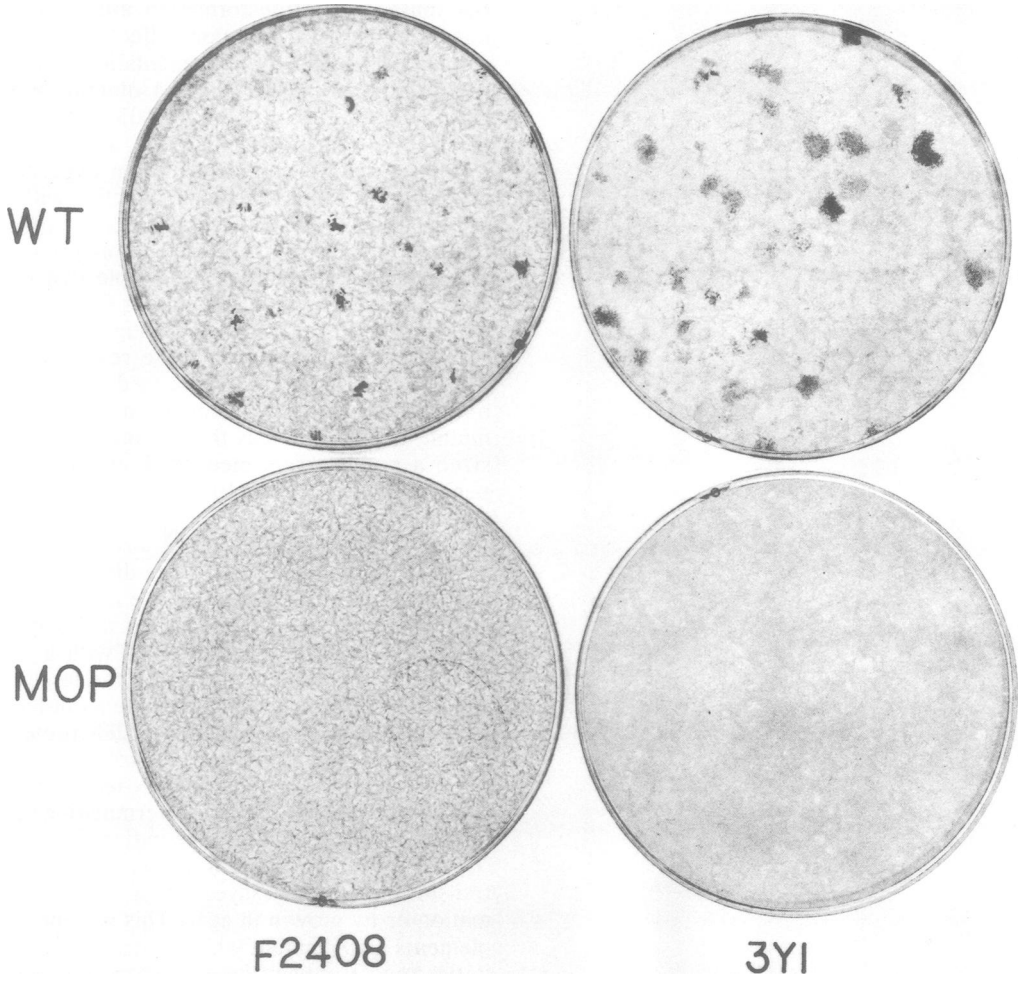

FIG. 8. Focus formation after infection of rat F2408 or 3Y1 cells with wild-type (WT) polyoma virus or the MOP 1033 mutant. We infected cell cultures as described in the text and seeded the infected cells at a density of about 10^4 cells per 5-cm dish. The cultures were fixed and stained after 15 days of incubation at 37°C.

transformation could result from changes in either the medium T antigen or the large T antigen. An effect on transformation frequency alone is perhaps more likely to be caused by a defect in the large T antigen. For example, *tsA* mutant-infected cells which are incubated at temperatures intermediate between the permissive and nonpermissive temperatures for transformation show changes in the frequency of transformation, but not in the sizes of transformed foci or colonies in agar, suggesting that a partially functional large T antigen decreases the frequency of transformation but has no effect on the growth properties of the transformants produced (Eckhart, unpublished data).

The MOP 1033 mutation has a more pronounced effect on transformation than the viable deletion mutations described so far, perhaps because it deletes a larger portion of the medium T antigen, possibly including sequences that are essential for its function in transformation. Among the sequences deleted are some which apparently affect the medium-T-antigen-associated protein kinase activity and a hydrophobic region near the C terminus that may be involved in membrane association.

The MOP 1033 mutant carries out apparently normal lytic infections of mouse 3T6 cells. This implies that an intact medium T antigen is not necessary for lytic infection, as has been suggested previously by the properties of the hr-t mutants (some of which fail to produce a medium T antigen) and of the viable deletion mutants, which shorten the medium and large T antigens coordinately. Both kinds of mutants are nondefective in at least some cell types, although their growth is not always optimal. It is possible that a medium-T-antigen function is

TABLE 1. Complementation for transformation between the MOP 1033 mutant and the *tsA*25E mutant[a]

Infection	Temp of incubation (°C)	No. of foci per 4×10^4 cells seeded[b]
Mock	37	0
Wild type	37	100–200
MOP 1033	37	0
*tsA*25E	32	50, 60
	39	0
MOP 1033 + tsA25E	32	21, 15
	39	18, 25

[a] Subconfluent cultures of rat 3Y1 cells were infected with wild-type or mutant virus suspensions at a total multiplicity of approximately 50 PFU/cell. After adsorption, the cultures were incubated for 4 h at 37°C. Each culture was then trypsinized and reseeded at a density of 4×10^4 cells per dish containing Dulbecco modified Eagle medium supplemented with 5% calf serum. Transformed foci were counted after incubation for 14 days (for the 37 and 39°C cultures) or after incubation for 10 days at 32°C, followed by incubation for 4 days at 37°C (for the 32°C cultures).

[b] Two numbers indicate numbers of foci in two dishes.

dispensable for lytic infection under the conditions used in our experiments or that a function resides in the N-terminal portion of the molecule and is retained in the 28K truncated fragment. Studies of the host range of the MOP 1033 mutant should help clarify whether the host range properties of the hr-t mutants result from changes in the small T antigen or the medium T antigen.

The possible involvement of the small and medium T antigens in lytic infections is of interest for two reasons. First, in view of the economy of organization of the genetic information in polyoma virus (the use of overlapping coding regions and alternative reading frames), it would be surprising if this virus retained coding capacities for proteins that are not advantageous for its life cycle. (In fact, there is evidence that the hr-t mutants multiply relatively poorly in animals compared with the wild type [T. Benjamin, personal communication].) Second, there is a link between lytic infection and transformation, as exemplified by the hr-t mutants. These mutants were selected initially for their ability to grow better on some host cells than on others (1). The basis of the host range effect has not been defined, but operationally this effect can be used to select mutations, all of which map in the intervening sequence for the large T antigen, affect the small and medium T antigens coordinately, and completely block cell transformation. That a host range effect for growth can be used to select such a specific class of mutations is very intriguing. Perhaps both the small and medium T antigens play a role in lytic infections, and perhaps only mutations affecting both can exhibit a strong host dependence for growth.

Taken together, the properties of the mutants with mutations that affect the medium T antigen and the transforming ability of the genomes that express only the medium T antigen provide strong evidence that this protein is both necessary and sufficient to bring about the major phenotypic changes that are associated with transformation. Whether the other T antigens have some more subtle role in transformation is not yet clear. This may be revealed by further characterization of mutations that affect each of the T antigens and by studies of cells in which the proteins are expressed in various combinations. For example, since the mutation of the MOP 1033 mutant deletes about 40% of the medium T antigen but does not affect the small T antigen, it should be interesting to study the effect of this mutation on virus-induced changes in cytoskeletal organization and mitogenic stimulation, which on the basis of present evidence cannot be ascribed conclusively to either the small T antigen or the medium T antigen. Mutagenesis of other regions of these molecules to introduce defined changes in the amino acid sequence should further clarify the functions of the T antigens in cell transformation.

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