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Deletion Mutants of Polyoma Virus Defining a Nonessential Region Between the Origin of Replication and the Initiation Codon for Early Proteins

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Mutants of polyoma virus with deletions as large as 90 base pairs were isolated by selecting spontaneously arising genomes resistant to endonuclease *Hae*II or by treating *Hae*II- or *Bgl*I- cleaved linear DNAs with S1 nuclease and exonuclease III. All of the mutants were viable and, therefore, defined a nonessential region in the polyoma genome between the origin of DNA replication and the initiation codon for translation of early proteins. Several mutants with large deletions had altered growth properties, giving smaller plaques and lower virus yields than the parental wild-type virus. These viruses may lack sites that are important for DNA replication or for transcription and translation of early mRNA's. All of the mutants tested could transform BHK-21 cells to anchorage independence.

Recent analyses of the function of the genomes of polyoma and simian virus 40 (SV40) viruses have depended greatly upon the use of mutants (see references 8 and 14 for recent reviews). In particular, mutagen-induced and biochemically constructed mutants with deletions in the early region of the viral genome have helped define sequences important for DNA replication and cell transformation (5, 6, 9, 10, 12, 23, 30, 32, 33, 35). In SV40, 35 to 40 base pairs separate the initiation codon for early protein synthesis from the 85-base pair sequence known to include the origin for DNA replication (11, 28, 29, 35). In polyoma virus, the region containing the origin of DNA replication is not as well defined (7, 18, 24), but there is considerable homology between the polyoma sequences near the *Hpa*II-3/5 junction and those SV40 sequences that include the SV40 origin of replication (15, 34). If this conserved region in the polyoma genome demarcates the polyoma origin of replication, then in polyoma virus, the sequence separating the origin of DNA replication from the initiation codon for early protein synthesis (15) is three times larger than in SV40.

In this report, we describe the isolation of polyoma virus mutants with deletions in the sequence between the putative origin of DNA replication and the initiation codon for early protein synthesis. All of the mutants are viable and, therefore, define a nonessential region. Some of the mutants, however, grow poorly and must have deletions extending to the boundaries of the nonessential region. These deletion mutants may be useful in defining one boundary of the region essential for the origin of replication and in determining regulatory sites near the 5' end of early mRNA's.

MATERIALS AND METHODS

Cells and media. Secondary whole mouse embryo (WME) cells were used to prepare virus stocks and viral DNAs and in plaque assays. BHK-21/13 cells (from G. diMayorca) were used in transformation assays.

The media used in most experiments have been previously described (2). In transformation assays, the base layer medium was AutoPow minimal essential medium (Flow Laboratories) supplemented with $NaHCO_3$ (1.5 mg/ml), glutamine (0.6 mg/ml), penicillin (100 U/ml), streptomycin (40 μg/ml), nystatin (50 U/ml), and calf serum (10%, vol/vol). Agar base layer medium also contained agar (5 mg/ml) and tryptose phosphate broth (5%, vol/vol); agarose base layer medium contained agarose (5 mg/ml), dextran sulfate (10 μg/ml), and adenosine (10 μM).

The procedures for preparing virus stocks, infecting with viral DNA, and performing plaque assays have been described (13). Viral DNAs were purified by a modification of the Hirt procedure (13, 21).

Enzymes, digestion conditions, and gel electrophoresis. S1 nuclease and endonucleases *Alu*I and *Hpa*II were prepared according to published procedures (4, 31, 36). Endonucleases *Bgl*I, *Hae*II, and *Hph*I and exonuclease III were purchased from New England BioLabs, and endonuclease *Hae*III was purchased from Bethesda Research Laboratories. Endonuclease digestions were incubated at 37°C for 1 to 3 h in reaction buffers specified in the published purification procedures or by the suppliers.

Digestion products of endonuclease *Bgl*I or *Hae*II were separated by electrophoresis through 1% agarose gels. Polyacrylamide gels (4 or 6%) were used to frac-

tionate digests with endonuclease *Alu*I, *Hae*III, *Hpa*II, or *Hph*I. Electrophoresis and autoradiography were performed as previously described (2).

Transformation assay. The ability of polyoma virus to transform BHK-21/13 cells to anchorage independence was determined by suspending infected cells in a layer of 0.33% agar or agarose medium over a layer of base medium containing 0.5% agar or agarose (25, 27). After 21 to 28 days at 37°C, macroscopically visible colonies were scored as transformed.

Isolation of mutants. Several procedures were used to isolate mutants with deletions in the region between the origin of DNA replication and the initiation codon for early protein synthesis. Since the single *Hae*II site and the single *Bgl*I site in polyoma DNA are in this region (15, 17; M. M. Bendig and W. R. Folk, unpublished data), the first step in each procedure was to exhaustively digest form I (FI) DNA from unmutagenized Pasadena large-plaque polyoma virus with either *Hae*II or *Bgl*I. The digested DNAs were fractionated by electrophoresis through agarose gels and then stained with ethidium bromide. The region of the gel where resistant FI DNA would migrate and the visible band of linear (FIII) DNA were excised, and the DNA was extracted (38). WME cells were infected directly with the *Hae*II-resistant FI DNA. DNA was prepared from the resulting virus stocks, and the *Hae*II digestion and gel electrophoresis steps were repeated. Bands of *Hae*II-resistant FI and FII DNA were now visible. Virus stocks were prepared from these resistant DNAs; from the resulting virus stocks, individual plaques were picked and tested for their resistance to *Hae*II.

The *Hae*II- or *Bgl*I-generated FIII DNAs were further treated with exonucleases before infection of cells. In one procedure, 2 μg of *Hae*II-generated FIII DNA was incubated with S1 nuclease for 10 min at 37°C (5.5 U of enzyme in 50 mM NaCl–14 mM sodium acetate–1 mM zinc acetate–5% glycerol, pH 4.6, with 15 μg of boiled salmon sperm DNA). Cells were infected with the S1 nuclease-treated FIII DNA, and DNA was prepared from the resulting virus stocks. This DNA was subsequently digested with *Hae*II and electrophoresed as before. Virus stocks were prepared from the resistant FI and FII DNAs; from these stocks, individual plaques were picked and tested for *Hae*II resistance. (G. LePlatte provided mutants by the two preceding procedures.)

In another procedure, *Hae*II- or *Bgl*I-generated FIII DNAs were first digested with S1 nuclease for 30 min at 25°C (14 U of enzyme/2.5 μg of DNA in 280 mM NaCl–25 mM sodium acetate–4.5 mM zinc acetate, pH 4.6) and then digested with either of two amounts of exonuclease III for 0, 30, 60, or 90 min at 37°C (0.4 or 4.0 U of enzyme/1.2 μg of DNA in 60 mM Tris-hydrochloride [pH 8.0]–0.66 mM $MgCl_2$–1.0 mM 2-mercaptoethanol). WME cells were infected in a plaque assay with the digested DNAs. The S1 nuclease-exonuclease III-treated DNAs were reduced in infectivity by 2- to 20-fold, and the reduction in infectivity correlated with the extent of exonuclease treatment. Well-isolated plaques were picked and tested for deletions in *Hpa*II fragment 5. *Hpa*II-5 contains the *Hae*II site (Fig. 1), and mutants deleted at this site have smaller *Hpa*II-5 fragments that migrate ahead of wild-type *Hpa*II-5 in 4% polyacrylamide gels. All mutants were plaque purified at least once more before being further characterized.

RESULTS

In all, 67 mutants were isolated with deletions between the putative origin of DNA replication and the initiation codon for early protein synthesis. Of the 26 plaques picked that were derived from spontaneously arising *Hae*II-resistant FI DNA, 3 yielded *Hae*II-resistant mutants. Of the 36 plaques picked that were derived from S1 nuclease-digested, *Hae*II-generated FIII DNA, 14 yielded *Hae*II-resistant mutants. Digestion of the *Hae*II- or *Bgl*I-generated FIII DNAs with both S1 nuclease and exonuclease III resulted in the highest frequency of mutants. Of the 67 plaques picked, 50 had detectable deletions in *Hpa*II-5, the *Hpa*II fragment which contains the *Hae*II site (Fig. 1).

All 67 mutants had *Hpa*II-5 fragments that were smaller than the parental *Hpa*II-5 fragment and are, therefore, thought to be resistant to *Hae*II as the result of deletions which remove the *Hae*II site. The sizes of their deletions were estimated by measuring the electrophoretic mobility of either, or both, *Hpa*II fragment 5 and *Alu*I fragment 10 (the *Alu*I fragment which contains the *Hae*II site; Fig. 1). Figure 2 shows the altered mobilities of *Hpa*II-5 and *Alu*I-10 from three mutants with representative deletions. Based on the altered mobility of *Alu*I-10, mutant 3-22 appeared to have a deletion of 19 base pairs, the smallest observed. Mutant 3-22 was isolated

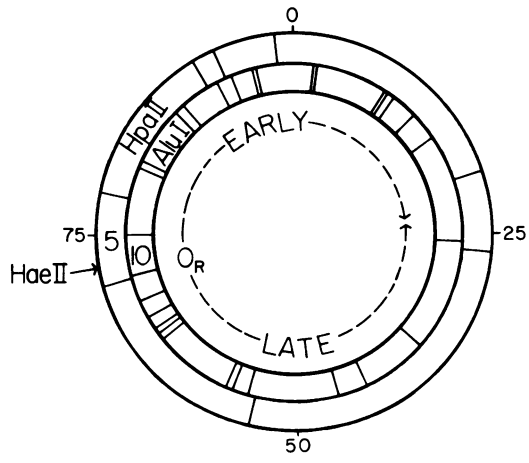

FIG. 1. *Map of the polyoma genome. The location of the cleavage sites of endonucleases HaeII and HpaII are from Griffin (16), and the cleavage sites of AluI are from Berkner and Folk (3). The origin of replication and the early and late regions of the genome are also indicated (22, 24).*

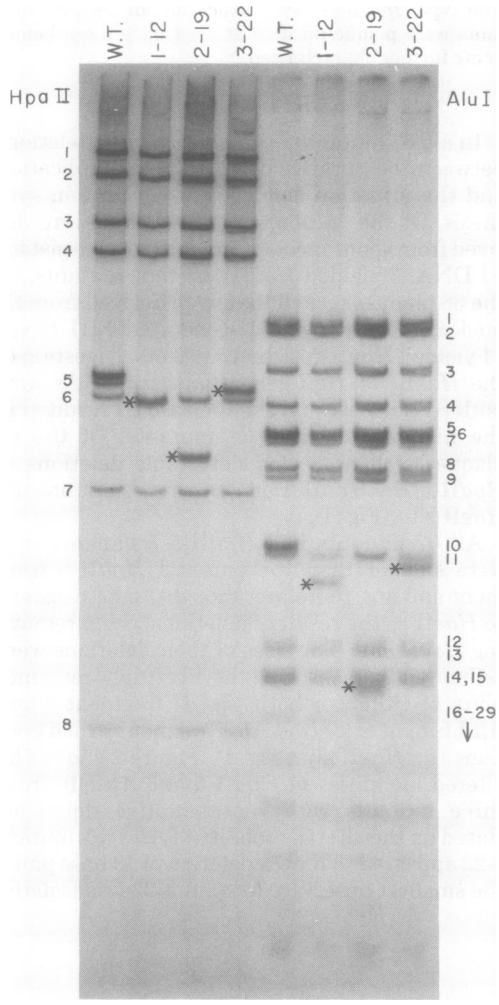

FIG. 2. *Autoradiogram showing the altered mobilities of the HpaII-5 and AluI-10 fragments from three deletion mutants. The first four slots are HpaII digests of wild type, mutant 1-12, mutant 2-19, and mutant 3-22. The second four slots are AluI digests of wild type, mutant 1-12, mutant 2-19, and mutant 3-22. The positions of wild-type HpaII-5 and AluI-10 fragments are indicated along the sides. Altered HpaII-5 and AluI-10 fragments from mutant DNAs are indicated with asterisks.*

from spontaneously arising *Hae*II-resistant FI DNA. Mutant 1-12 had a deletion of approximately 29 base pairs, and mutant 2-19 had one of the largest deletions, approximately 89 base pairs. Mutants 1-12 and 2-19 were isolated from S1 nuclease-treated FIII DNAs.

Of the 50 independent isolates from the S1 nuclease-exonuclease III isolation procedure, the mutant with the largest deletion (mutant 17) lacked approximately 92 base pairs. Since the S1 nuclease-exonuclease treatment resulted in a significant reduction in DNA infectivity, it is likely that the deletions in these mutants define the extent of the nonessential region surrounding the *Hae*II site.

To better determine the location and extent of the deletion, [^{32}P]DNAs from representative mutants were digested with a battery of restriction endonucleases (*Alu*I, *Bgl*I, *Hae*II, *Hae*III, *Hpa*II, *Hph*I). Figure 3 illustrates the results obtained with a few of the mutants. Mutant 3-22 was deleted for approximately 19 base pairs between nucleotides 89 and 122 of the polyoma DNA sequence of Friedmann et al. (15); it lacked the *Hae*II and *Bgl*I sites. Mutant 1-12 was deleted for approximately 29 base pairs between nucleotides 92 and 132. Its deletion included the *Hae*II, *Bgl*I, and *Hae*III sites. Mutant 2-19 also lacked these same three sites, but it was deleted for approximately 89 base pairs between nucleotides 32 and 179. Mutant 17 was similar to mutant 2-19 except that its deletion was slightly larger, approximately 92 base pairs. Mutant 75 was deleted for approximately 58 base pairs between nucleotides 103 and 175 and lacked the *Hae*II, *Bgl*I, *Hae*III, and *Hph*I sites. This mutant was the only one lacking the *Hph*I site, so its deletion must extend to within 11 base pairs of the AUG codon that initiates translation of early proteins (15). In wild-type polyoma, the *Hae*II and *Hph*I sites are separated by 72 nucleotides (15). Since mutant 75 lacked both of these sites, the estimate of its deletion size may be low or it may have DNA sequence rearrangements. As well as *Hae*II-resistant mutants, Fig. 3 shows a deletion mutant, mutant 43, that is sensitive to *Hae*II but resistant to *Bgl*I. Mutant 43 was isolated from S1 nuclease-exonuclease III-treated, *Bgl*I-generated FIII linear DNA. It was deleted for approximately 56 base pairs between nucleotides 103 and 164.

In addition to deletion mutants of the types described above, several mutants with insertions as well as deletions at, or near, the *Hae*II site were isolated. Some of these mutants may contain duplications of the origin of replication, but at present we do not have sufficient information to precisely define the nature of their alterations.

All isolation procedures were carried out at 33°C in order to avoid selecting against possible temperature-sensitive mutants. Virus stocks of mutants with the larger deletions were plaque assayed at 33 and 37°C, and in most cases no significant difference in plaquing efficiency at the two temperatures was observed. Growth curves at 33 and 39.5°C for wild-type virus and for mutants 1-12, 2-19, and 3-22 showed no significant differences between wild-type virus and any of the mutants at either temperature (Fig. 4A, B; data for mutant 1-12 not shown). Mutants

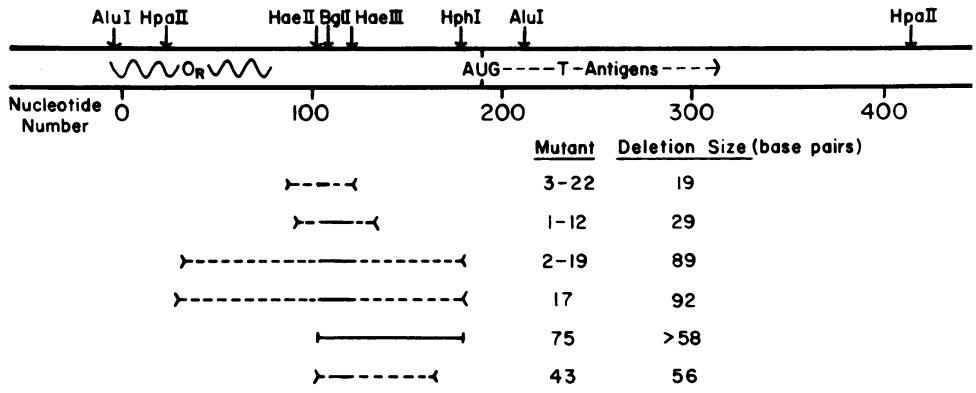

FIG. 3. *Map of the deletions in a few representative mutants. Based on the estimates of the sizes of the deletions and the presence or absence of restriction endonuclease sites, the approximate location of the deletions on the polyoma DNA sequence of Friedmann et al. (15) can be determined. For each mutant, the solid portion of the line indicates the region known to be deleted, and the dashed portions indicate the possible extent of each deletion. The wavy line labeled O_R indicates the polyoma sequence that is homologous to the SV40 sequence known to contain the SV40 origin of DNA replication (6, 15, 19, 23, 34, 35).*

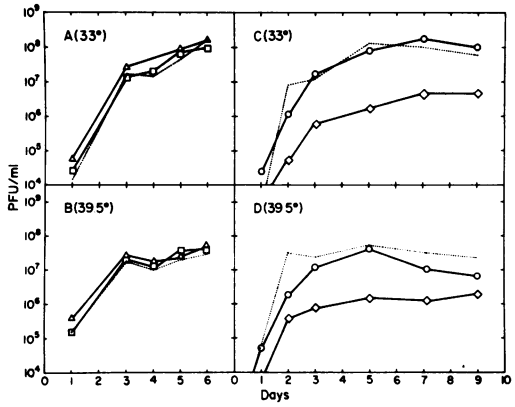

FIG. 4. *Growth curves at 33 and 39.5°C. WME cells in 60-mm plates were infected at a multiplicity of approximately 0.1 PFU/cell. After adsorption for 1 h at 33°C, the cells were washed twice with Tris saline buffer (13), 8 ml of medium was added per plate, and half of the plates were incubated at 33°C and half at 39.5°C. At the indicated times, identically infected plates incubated at either temperature were harvested by scraping the cells into the media. The virus stocks were frozen and thawed three times and plaque assayed at 33°C. (A and C) Growth curves at 33°C; (B and D) growth curves at 39.5°C. Symbols: - - -, wild type; □, mutant 2-19; △, mutant 3-22; ○, mutant 17; ◇, mutant 75.*

17 and 75, however, had altered growth properties. Both gave smaller plaques than wild-type virus, and plaques of mutant 75 were less distinct than those of the wild type. Mutant 17 grew as well as wild-type virus at 33°C but appeared to be slightly thermosensitive at 39.5°C (Fig. 4C, D). Mutant 75 showed a significant reduction in virus yield at both temperatures (Fig. 4C, D).

Five representative mutants were tested for their ability to transform BHK-21/13 cells to anchorage independence. The mutants tested included mutant 17, with its deletion of approximately 92 base pairs, and mutant 75, with its deletion that extends to within 11 base pairs of the AUG codon. All five mutants were able to transform BHK-21/13 cells (Table 1).

DISCUSSION

The polyoma virus origin of DNA replication is located near, or at, the *Hpa*II-3/5 junction (7, 18, 24). This region of the polyoma genome shares considerable sequence homology with the putative SV40 origin of DNA replication (15, 34). The boundaries of a region of SV40 essential for DNA replication have been defined by analysis of deletion mutants which eliminate sequences on either side of the origin of replication (6, 19, 23, 35). The polyoma sequence homologous to this 85-nucleotide sequence of SV40 is indicated in Fig. 3.

Our results indicate that between the polyoma virus origin of replication and the initiation codon for early protein synthesis, there are approximately 100 base pairs of nonessential sequence. Deletion mutants in this region have been independently isolated by several other laboratories (14, 26, 37). In SV40, it appears that a much smaller region separates the origin of DNA replication from the initiation codon for early protein synthesis (11, 29). Viable mutants of SV40 with deletions in this region also have been isolated (D. DiMaio and D. Nathans, personal communication).

To determine the extent of the nonessential region in polyoma virus, we attempted to gen-

TABLE 1. *Transformation of BHK-21/13 cells*

Expt	Multiplicity of infection	Transformed colonies: Medium A[a] (per 4×10^5 to 6×10^5 infected cells)	Transformed colonies: Medium B[b] Per 4×10^5 to 6×10^5 infected cells	Transformed colonies: Medium B[b] Per 4×10^4 to 6×10^4 infected cells
1				
Mock	0	0	—[c]	—
Wild type	127	256	—	—
Mutant 1-12	118	418	—	—
Mutant 2-19	145	307	—	—
Mutant 3-22	136	264	—	—
2				
Mock	0	0	—	—
Wild type	67	26	—	—
Mutant 1-12	87	27	—	—
Mutant 2-19	73	19	—	—
Mutant 3-22	73	41	—	—
3				
Mock	0	0	0	0
Wild type	21	219	>500	61
Mutant 17	12	201	280	9
4				
Mock	0	0	0	0
Wild type	80	363	>700	94
Mutant 17	90	186	696	32
Mutant 43	75	112	543	13
5				
Mock	0	0	0	0
Wild type	70	252	>500	210
Mutant 75	26	25	421	19

[a] Medium A contained 0.33% agarose, 10% calf serum, 10 μg of dextran sulfate per ml, and 10 μM adenosine.

[b] Medium B contained 0.33% agar, 10% calf serum, and 5% tryptose phosphate broth.

[c] —, Not determined.

erate mutants with the largest deletions possible by treating the *Hae*II- or *Bgl*I-generated FIII DNAs with S1 nuclease and exonuclease III. Approximately 50 deletion mutants were independently isolated and, by examining the mutants with the larger deletions, it may be possible to define the limits of the nonessential region. Mutant 75 is deleted for an *Hph*I site that is 6 to 11 base pairs from the AUG codon, and this deletion may define one limit. The other limit may be defined by the deletion in mutant 17 which extends at least 16 base pairs beyond the *Hae*II site in the direction of the *Hpa*II-3/5 junction. This limit lies within approximately 10 base pairs of the region with considerable homology to the SV40 sequence containing the origin of DNA replication (Fig. 3) (35). The precise boundaries of the polyoma deletions and their effect upon DNA replication are being determined. Preliminary experiments indicate that viral DNA replication is impaired in mutants 17 and 75.

It may be predicted that between the origin of DNA replication and the AUG codon are located sites for ribosome binding and possibly sites for posttranscriptional processing and modification of early transcripts. Although recent comparative studies of the 5′ noncoding regions of eucaryotic mRNA's determined that AUG is the only universally conserved sequence (1), several eucaryotic mRNA's contain in their 5′ noncoding region a sequence with partial complementarity to a sequence at the 3′ end of 18S rRNA (3′ GAAGGC 5′) (20). In polyoma virus, a sequence complementary to 18S rRNA (5′ CTTCCG 3′) is found 24 base pairs from the 3′ side of the AUG codon. This sequence is probably deleted in mutant 75 and thus may account for some of the altered growth properties of this mutant. Experiments are in progress to test this possibility.

ACKNOWLEDGMENTS

Geoffrey LePlatte contributed significantly to this work by showing that *Hae*II-resistant mutants can be isolated relatively easily. We also thank him, Patricia Vollmer, and Roseann White for preparing the secondary whole mouse embryo cells used in this work.

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