Viable Deletion Mutants of Simian Virus 40: Selective Isolation by Means of a Restriction Endonuclease from Hemophilus parainfluenzae

(Hpa II/plaque-morphology mutants/polyacrylamide gel electrophoresis/heteroduplex mapping)

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ABSTRACT Resistance of simian virus 40 (SV40) DNA to cleavage by Hemophilus parainfluenzae II (HpaII) restriction endonuclease has been used as a positive, in vitro selection for mutants lacking the one HpaII endonuclease-cleavage site of wild-type SV40 DNA. Each of 18 viable mutants isolated by this procedure multiplies significantly more slowly than wild-type virus and contains a small deletion (80 to 190 base pairs in size) of the region of the genome that includes the HpaII endonuclease-recognition sequence. These well-defined mutants, having a selective disadvantage for growth, would not have been readily obtained by conventional methods used to screen for viral mutants. Therefore, in certain circumstances, restriction endonucleases are effective reagents for the selection of new classes of mutants. Because these small deletions can be visualized in heteroduplexes, these mutants provide internal markers for mapping other alterations or features of the simian virus 40 genome.

Type II restriction endonucleases are enzymes that make double-strand scissions at specific nucleotide sequences in duplex DNA (see ref. 1 for review). This property has been used for the in vitro selection of viral mutants that are resistant to cleavage by a restriction endonuclease because they have deleted the nucleotide sequence recognized by the enzyme (2). In this paper we describe the isolation and physical characterization of a set of naturally arising mutants of simian virus 40 (SV40) that lack the nucleotide sequence recognized by HpaII restriction endonuclease, an enzyme from Hemophilus parainfluenzae that cleaves wild-type SV40 DNA once at a unique site on the genome (3). Each of the mutants described here is viable (although it multiplies more slowly than wild-type SV40) and contains a small (80 to 190 base pairs) deletion of the region that includes the HpaII endonuclease-cleavage site. The isolation of clones of non-viable, HpaII endonuclease-resistant mutants containing large deletions that include this region of the SV40 genome has been reported elsewhere (2).

MATERIALS AND METHODS

Cells, Viruses, and DNAs. Monkey kidney cell lines were obtained and grown as already described (2). Stocks of wild-type (WT800) and mutant virus and DNA were prepared, purified and titered as previously described (2).

Enzymes and Enzyme Reactions. (a) EcoRI restriction endonuclease (4, 5), a gift from M. Thomas and J. Ferguson, was used under conditions previously described (6). (b) H. parainfluenzae I (HpaI) restriction endonuclease, purified by the procedure of Sharp et al. (3), was a gift from J. Carbon. SV40 Form I DNA was treated with the enzyme for 1 hr at 37° in 20 µl reaction mixtures containing 10 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 6 mM KCl, 1 mM dithiothreitol, and 100 µg/ml of gelatin (autodissolved, Difco-Bacto). (c) HpaII restriction endonuclease, prepared from H. parainfluenzae cells by a modification of the second method of Sharp et al. (3), was the enzyme preparation previously described (2). Reactions with HpaII endonuclease were performed for 1-4 hr at 37° in 10 mM Tris-HCl (pH 7.5), 5 mM MgCl2, 0.4 mM dithiothreitol, and 100 µg/ml of gelatin. To insure complete digestion, the endonuclease-resistant DNA was purified by equilibrium centrifugation in CsCl-ethidium bromide (7) and re-incubated with additional HpaII endonuclease. (d) The restriction enzyme preparation obtained from Hemophilus influenzae strain d (8), containing a mixture of the HindIII and HindIII endonucleases (9), was a gift from H. F. Tabak (10). Reactions were performed for 30 min at 37° in 30 µl containing SV40 Form I DNA (approximately 1 µg), 5 mM Tris-HCl (pH 7.4), 7 mM MgCl2, 50 mM NaCl, 1 mM dithiothreitol, and 2 µl of enzyme, and stopped by adding EDTA and sodium dodecyl sulfate to final concentrations of 25 mM and 1%, respectively. Electron Microscopy was performed by the formamide technique of Davis et al. (11).

Polyacrylamide Gel Electrophoresis was performed essentially as described by Tabak et al. (10) with 4% cylindrical gels (0.65 × 15 cm).

RESULTS

Resistance to cleavage by HpaII restriction endonuclease selects for variant SV40 DNA molecules that exhibit altered plaque phenotypes

Serial passaging of SV40 in monkey cells at high multiplicities of infection results in the accumulation of defective virions that contain deletions, substitutions, and additions in their DNA (12, 13). Since these alterations vary greatly in size and are located nearly randomly throughout the viral genome (14, 15), such defective SV40 DNA preparations provide a potential source of mutants. When our wild-type SV40 virus was serially passaged four times in primary African green monkey kidney cells at high input multiplicities of infection, 47% of the SV40 DNA molecules in the resulting preparation were found to be resistant to cleavage by HpaII restriction endonuclease, an enzyme that cleaves wild-type SV40 DNA molecules once at map position 0.735 (see Fig. 3). After sepa-
ration from the cleaved linear DNA by equilibrium centrifugation in CsCl-ethidium bromide, this HpaII endonuclease-resistant DNA preparation produced 10-fold fewer plaques per µg than wild-type SV40 DNA when assayed by the DEAE-dextran method of McCutchan and Pagano (16) because of the defectiveness of the viral genomes (see Table 1 of ref. 2). The residual plaques seen could have been produced by a contaminating trace of wild-type DNA; however, many of them appeared later and grew more slowly in size than ones derived from wild-type molecules.

Sixteen of these plaques were picked. After three serial plaque-purifications of each isolate, virus and viral DNA stocks were prepared from cells infected at low multiplicities of infection. Ten of the virus isolates reproducibly yielded late-appearing, small plaques when assayed on the monkey cell line CV-1P; the remaining six stocks produced plaques that were indistinguishable from those of wild-type virus. Examination of the rate at which the plaques of each of these 10 SV40 variants developed, as judged either, subjectively, by the size of the plaques seen 12 days after infection (Fig. 1) or, quantitatively, by direct measurement of the rate at which the average diameter of the plaques increased (Fig. 2, Table 1), showed that their plaque growth rates span a substantial range. Even in a single cycle of growth, replication of the mutant DNAs occurs more slowly than that of wild-type DNA (J. E. Mertz, S. P. Goff, and P. Berg, unpublished).

As anticipated, the DNA isolated from each of the 10 plaque-morphology mutants hereafter referred to as pm(dI)-

**TABLE 1. Rate of growth of the plaques of HpaII endonuclease-resistant mutants:**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>9.9</th>
<th>10.8</th>
<th>11.8</th>
<th>12.8</th>
<th>15.1</th>
<th>17.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT800</td>
<td>1.4</td>
<td>2.4</td>
<td>3.2</td>
<td>3.9</td>
<td>5.6</td>
<td>ND</td>
</tr>
<tr>
<td>pm(dI)801</td>
<td>0.9</td>
<td>1.8</td>
<td>2.6</td>
<td>3.7</td>
<td>4.4</td>
<td>ND</td>
</tr>
<tr>
<td>pm(dI)802</td>
<td>0.5</td>
<td>0.9</td>
<td>1.3</td>
<td>1.9</td>
<td>2.9</td>
<td>4.3</td>
</tr>
<tr>
<td>pm(dI)803</td>
<td>0.3</td>
<td>0.9</td>
<td>1.8</td>
<td>2.6</td>
<td>3.8</td>
<td>5.6</td>
</tr>
<tr>
<td>pm(dI)804</td>
<td>0.2</td>
<td>0.9</td>
<td>1.3</td>
<td>1.9</td>
<td>3.3</td>
<td>4.4</td>
</tr>
<tr>
<td>pm(dI)805</td>
<td>0.3</td>
<td>1.0</td>
<td>1.3</td>
<td>1.9</td>
<td>3.1</td>
<td>4.3</td>
</tr>
<tr>
<td>pm(dI)806</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>0.3</td>
<td>0.9</td>
<td>1.9</td>
<td>2.5</td>
</tr>
<tr>
<td>pm(dI)807</td>
<td>0.3</td>
<td>1.2</td>
<td>2.4</td>
<td>2.9</td>
<td>3.5</td>
<td>5.4</td>
</tr>
<tr>
<td>pm(dI)808</td>
<td>0.5</td>
<td>1.4</td>
<td>2.4</td>
<td>3.2</td>
<td>4.2</td>
<td>5.6</td>
</tr>
<tr>
<td>pm(dI)809</td>
<td>0.5</td>
<td>1.2</td>
<td>2.0</td>
<td>2.6</td>
<td>3.7</td>
<td>ND</td>
</tr>
<tr>
<td>pm(dI)810</td>
<td>&lt;0.1</td>
<td>0.4</td>
<td>1.2</td>
<td>2.2</td>
<td>3.4</td>
<td>4.1</td>
</tr>
</tbody>
</table>

ND, not determined.

* Determined as described in the legend to Fig. 2.
801 through pm(dl)810, where "pm" = plaque-morphology and "dl" = deletion (17) was completely resistant (>99%) to cleavage by HpaII endonuclease, whereas the DNA from each of the six isolates that produced wild-type plaques was almost completely cleaved (>98%) by the enzyme to unit length linear SV40 DNA. Thus, resistance to cleavage by HpaII endonuclease serves as an efficient, positive, in vitro selection for the isolation of viable, SV40 plaque-morphology mutants whose DNAs lack that restriction enzyme-cleavage site. [Non-viable, defective mutants with extensive deletions of the region of the genome that includes the HpaII endonuclease-cleavage site have also been isolated by this procedure, but these mutants can only grow when complemented by a suitable helper virus (2).]

The HpaII endonuclease-resistant plaque morphology mutants have deleted a small region of the SV40 genome that includes the HpaII endonuclease-cleavage site

Is the DNA of each of these plaque-morphology mutants resistant to cleavage by HpaII endonuclease because of base pair changes in the nucleotide sequence recognized by the enzyme or because of more extensive alterations (e.g., deletions) that modify or eliminate the restriction site? An examination of the contour lengths of pm(dl)810 and WT800 DNA by electron microscopy (with bacteriophage PM2 DNA serving as an internal length standard) revealed that pm(dl)810 DNA is 96.7 ± 0.2% [mean ± standard error (SEM); standard deviation (s) = 3%] the length of wild-type DNA, suggesting that a small region of the viral genome had been deleted from this mutant.

To obtain a more accurate estimate of the size and location of the molecular change in each mutant's genome, we compared the electrophoretic mobilities of the DNA fragments produced by cleavage of the mutant and wild-type DNAs with HindII+III restriction endonuclease. These two enzymes together cleave wild-type SV40 DNA into eleven fragments labeled Hind-A through K in order of their size (see Fig. 3). Since the HpaII endonuclease-cleavage site is contained within Hind fragment C (Fig. 3), a mutant with a deletion of this region should yield a faster migrating Hind-C fragment. On the other hand, base changes should alter the HindII+III fragment pattern only if they create new, or eliminate old, HindII+III endonuclease-cleavage sites. Coelectrophoresis of the HindII+III endonuclease digest products of wild-type and each of the 10 mutant DNAs revealed no significant differences in the mobility or relative quantity of 10 of the 11 HindII+III endonuclease-generated fragments. However, instead of the normal Hind-C fragment, each of the mutant DNAs showed one new, faster migrating fragment (designated Hind-C') (Fig. 4; Table 2). Therefore, each mutant has an alteration in the HindII+III DNA fragment that contains the HpaII endonuclease-cleavage site. Furthermore, the faster migration rate of these new fragments, combined with the fact that the relative electrophoretic mobility of DNA is generally related inversely to molecular length, suggests that the alteration in each mutant is a deletion within the Hind-C fragment that results in a shorter Hind fragment C.

The sizes of the DNA fragments produced by cleavage of the mutant DNAs with HpaI restriction endonuclease, an enzyme that cleaves wild-type SV40 DNA at three sites (see Fig. 3), were also examined. As expected, the HpaI-A and C fragments were unaltered in their electrophoretic mobility in 1.3% agarose gels, but the third fragment resulting from each mutant migrated more rapidly than HpaI-B, the fragment that contains the Hind-C segment (Fig. 3) (data not shown).

* We have assumed that the WT800 Hind fragments A through K designated in Fig. 4 correspond to the analogous Hind A through K fragments mapped by Danna et al. (9) for SV40 strain 776; in actuality, the WT800 Hind fragments A, B and C migrate significantly more slowly and Hind-F more rapidly than their counterparts in strain 776 (Merz and Berg, unpublished).

† This correlation may not be strictly valid, since differences in G+C content affect the mobility of DNA in polyacrylamide gels (22). Note that the mobility of the Hind-C and F fragments, containing the highest and one of the lowest G+C contents, respectively (23), deviates significantly from the curve relating mobility to molecular length (Fig. 5); in fact, Hind-F migrates more slowly than Hind-G even though Hind-F is the smaller of the two fragments.
By assuming that the mobility of the Hind-C' fragment of each mutant reflects its size and that the difference in the relative mobilities of Hind-C and Hind-C' measures the difference in their size, one can calculate the approximate size of the deletion in the genome of each mutant (Fig. 5; Table 2): each of the plaque-morphology mutants lacks a small region (80 to 190 base pairs) of DNA contained in the wild-type Hind-C and HpaI-B fragments.

The precise map position of these small deletions can be determined by heteroduplex analysis.

For mapping accurately the location of these deletions, approximately equal quantities of EcoRI restriction endonuclease-generated linear DNA molecules of mutant pm(dl)810 and WT800 were mixed together, denatured, renatured, and examined by electron microscopy. Forty percent of the duplexes seen contained one small discontinuity in their structure (Fig. 6A). These discontinuities probably represent deletion loops in the heteroduplexes because most of them (42 out of the 48 that were measured) occur at a unique position 0.245 ± 0.001 (mean ± SEM; \( \sigma = 0.007 \)) SV40 fractional length from the nearer EcoRI endonuclease-generated end (Fig. 6B). These data, together with the estimate of the size of the DNA segment deleted from the Hind-C fragment (see above; Table 2), indicate that the deletion in mutant pm(dl)810 begins at approximately 0.720, extends to 0.755 on the SV40 map, and includes the HpaII endonuclease-cleavage site at 0.735 (Fig. 3). Therefore, this mutant is resistant to cleavage by HpaII endonuclease because it lacks the region of wild-type SV40 DNA containing that restriction endonuclease-recognition sequence.

In a similar manner, the DNA segment missing in mutant pm(dl)806 has been located at 0.723 to 0.758 (SEM = 0.001, \( \sigma = 0.007 \)) on the SV40 map. Our inability to detect any specific discontinuities in heteroduplexes formed between the DNAs of these two mutants by conventional electron microscopic techniques is consistent with the extensive overlap in the map positions of the deletions present in pm(dl)806 and pm(dl)810. Furthermore, that the only region of non-homology in heteroduplexes formed between pm(dl)806 and WT800 DNA occurs at this map position has been shown using S1 enzyme (18), a single-strand-specific nuclease that can make double-strand scissions at mismatches present in duplexed DNA molecules (19). Therefore, the peculiar phenotype of this mutant must result from this one mapped alteration in the structure of its DNA.

**DISCUSSION**

This paper describes the isolation of 10 plaque-morphology, deletion mutants of SV40 by a positive selection procedure: the *in vitro* resistance of mutant DNA to cleavage by HpaII restriction endonuclease. Viable plaque-morphology mutants of SV40 have previously been described (see refs. 20 and 21, for examples), but these are the first such mutants isolated using a positive selection and for which the alteration in the genome has been well defined. Recently, SV40 mutants containing an insertion of poly(dA)·poly(dT) at the HpaII endonuclease-cleavage site have been "constructed" *in vitro* (15); these mutants behave physiologically similar to the ones described in this paper. Hopefully, procedures similar to these will greatly facilitate the isolation of mutants that could not otherwise be readily obtained.

The nature of these mutants' physiological defect(s) is, as yet, unknown. The fact that there is not a positive correlation between the size of the deleted region and the defectiveness of the mutant as measured by plaque growth rate [e.g., compare mutants pm(dl)806 and pm(dl)808 (Tables 1 and 2)] indicates that the exact location, rather than absolute size,
must be cautious and take care to distinguish them from similar-appearing artifacts of electron microscopy (e.g., cytochrome c aggregates).

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Fig. 6. (A) Electron micrograph of heteroduplexes formed between EcoRI endonuclease-cleaved WT800 and mutant pm(dl)810 DNA. Approximately equal amounts of EcoRI endonuclease-cut linear SV40 DNA from WT800 and pm(dl)810 were mixed together, denatured in alkali, annealed at room temperature in 47% formamide, and spread for electron microscopy from 40% formamide (11). Grids were examined as previously described (6). The schematic diagram depicts the presumed structure of the heteroduplexes. The arrows point to the putative deletion loops. The black bar at the top indicates the size of unit length duplexed SV40 DNA. (B) Histogram of the measured distances from the nearer EcoRI endonuclease-generated ends to the putative deletion loops. Double-stranded DNA molecules resulting from the experiment described in (A) were photographed at random. Lengths 'a' and 'b' were measured for all duplexed molecules that contained discontinuities similar to those indicated by arrows in panel A. The fractional length of the beginning of each putative deletion loop to the nearer of the two ends was calculated as equal to a/((a+b)/0.965), assuming that the length of pm(dl)810 DNA, a+b, equals 0.965 SV40 fractional length.

of the deletion may be the crucial factor determining a given mutant's specific phenotype.

These mutants are particularly useful because they provide an easily recognizable marker for heteroduplex analysis of SV40 DNA. Since the only structural anomaly reproducibly seen by electron microscopy in heteroduplexes between pm(dl)808 and WT800 or SV40 strain SV-S (20) DNA occurs at the HpaII endonuclease-cleavage site (Mertz and Berg, unpublished), this feature and the EcoRI endonuclease-generated ends together can serve as markers for unambiguously mapping other alterations in the SV40 genome (e.g., the location of deletions in uncloned populations of defective SV40 genomes). Because these deletion loops are so small, one