Cleavage of SV40 DNA by restriction endonucleases has provided specific DNA fragments which are proving useful in analyzing the structure and function of the viral genome (1-4). Danna and Nathans (1) have previously reported that restriction endonuclease from H. influenzae produces 11 fragments from SV40 DNA which are separable by gel electrophoresis. To determine the order of these fragments in the DNA molecule and to simplify the task of nucleotide sequence analysis of SV40 DNA, it would be helpful to have overlapping sets of such fragments, produced by different restriction endonucleases. To this end, we have used an enzyme from H. parainfluenzae, described by Gromkova and Goodgal (5), to cleave SV40 DNA, and here report that the major products of this digestion are three large fragments which are about 40, 30, and 20%, respectively, of the length of SV40 DNA.

Small plaque SV40, isolated from strain 776 by K. Takemoto and plaque purified, was grown in monolayers of BSC-1 cells in minimal Eagle's medium containing 10% fetal calf serum, penicillin, and streptomycin. Viral DNA labeled with or [32P]thymidine was prepared by the method of Hirt (6) followed by isolation of covalently closed form I DNA by equilibrium centrifugation in CsCl-ethidium bromide and sedimentation through neutral sucrose gradients, as detailed elsewhere (7). Restriction endonuclease from H. influenzae was prepared as described by Smith and Wilcox (7). Restriction endonuclease from H. parainfluenzae was prepared from a strain provided by Dr. S. Goodgal, to whom we are grateful for advice on the growth of this organism. H. parainfluenzae DNA was grown to late log phase in BBL brain-heart infusion broth supplemented with 2 μg/ml of NAD, and restriction enzyme was prepared from the packed frozen cells by the method developed by Smith and Wilcox for the purification and assay of the H. influenzae restriction enzyme (7). However, in the case of the H. parainfluenzae enzyme, most of the enzyme precipitated from the Biogel eluate between 37 and 45% saturation with ammonium sulfate. After application of the ammonium sulfate fraction to a phosphocellulose column and stepwise elution with 0.1 M, 0.2 M, and 0.3 M KCl, enzyme was found primarily in the 0.3 M KCl fraction. The eluate was then concentrated using a Diaflo pressure device and stored in 25% glycerol at 4°C; it has retained activity for 12 months. The enzyme was active on many bacterial DNAs, including that of H. influenzae, but not on the DNA of H. parainfluenzae. DNA from M. lysodeikticus was used as substrate during enzyme purification.

Digestion of SV40 DNA by restriction endonuclease from H. parainfluenzae was carried out as described earlier (1). Digestion of SV40 DNA by the H. parainfluenzae enzyme was carried out at 30°C for 90 min in 13 mM Tris-Cl pH 7.4, 20 mM KCl, 5 mM MgCl2, 13 mM 8-mercaptoethanol, 3% bovine serum albumin and 0.024 units of enzyme (63 μg protein) per μg of DNA. (A unit of enzyme is as defined by Smith and Wilcox (9), but with M. lysodeikticus DNA as substrate.) The ratio of DNA to enzyme appreciably influenced the extent of the reaction and was optimized to the ratio just given. Also critical were the salt concentration (Tris-Cl and KCl) and the Mg2+ concentration. Purified enzyme still had slight exonuclease activity under optimal conditions for endonucleolytic cleavage of SV40 DNA. However, as shown below, distinct digestion products could be readily isolated by gel electrophoresis. Further purification of the enzyme is underway.

Electrophoretic separation of digestion products of [32P]SV40 DNA was performed.
FIG. 1. Autoradiogram of electrophoretically separated fragments of SV40 DNA produced by restriction endonucleases from *H. parainfluenzae* (Hpa-A to D, center column) and *H. influenzae* (Hin-A to K, right and left columns). Origin is at the top. The actual distance of fragment Hpa-A from the origin was 28 mm. The gel contained 4% acrylamide with 5% cross linking.

in slabs of 3 or 4% polyacrylamide gel (5% cross linking) measuring 15 × 40 × 0.16 cm as described earlier (8), after which the slabs were dried (9) and applied to X-ray film for autoradiography.

Digestion of [³²P]SV40 DNA by restriction enzyme from *H. parainfluenzae* resulted in the appearance of four main DNA fragments separable by electrophoresis, designated Hpa-A, Hpa-B, Hpa-C, and Hpa-D (Fig. 1). In addition, a small amount of another fragment is often found between Hpa-B and Hpa-C (Fig. 1), which we believe is derived from a minor population of SV40 DNA molecules present in some preparations of DNA. Prolonged incubation or addition of more enzyme did not affect this digest pattern, and we, therefore, conclude that the fragments detected are limit products. Also
shown in Fig. 1 are reference digests of SV40 DNA made with the H. influenzae restriction enzyme (I).

Molecular weights of the Hpa fragments have been estimated by the relative amount of DNA present in each fragment (32P radioactivity), by electron microscopic measurements of length relative to open circular SV40 DNA molecules, and by electrophoretic mobility. For 32P measurement each fragment was first localized by radioautography of the wet gel slab and the dissolved fragments directly counted. For length measurements, fragments Hpa-A, B, and C were separated in 3% polyacrylamide gels, localized by radioautography, and eluted in 0.015 M NaCl, 0.0015 M Na citrate, pH 7.0. Each fragment was then mixed with open circular SV40 DNA II and samples mounted for electron microscopy. The results of length measurements are presented in Fig. 2. The size of the Hpa fragments could also be estimated by electrophoretic mobility relative to H. influenzae fragments of known molecular weight (I). All of the molecular weight estimates obtained by the various methods are summarized in Table 1.

Fragment Hpa-A is about 40% of the length of SV40 DNA, Hpa-B is about 30%, and Hpa-C is about 20%. Since the values obtained by length measurements and relative yield are similar, these fragments are each equimolar with the starting DNA. In the case of Hpa-D, the amount of DNA present appears to exceed even a rough estimate of molecular weight based on electrophoretic mobility relative to H. influenzae fragments (I). Therefore, it is likely that Hpa-D is multiple.

It is clear from the results presented that the number of sites in SV40 DNA susceptible to the H. parainfluenzae restriction enzyme is fewer than the number susceptible to the H. influenzae enzyme. The two sets of fragments thus have extensive overlaps and have, therefore, proved useful in mapping the enzyme cleavage sites (Danna, Sack, and Nathans, submitted for publication). In addition, the large fragments of SV40 DNA produced by incomplete or complete digestion with the H. parainfluenzae enzyme should contain intact genes or operons and may, therefore, show biological activity, thus helping to localize SV40 genes.

TABLE 1

<table>
<thead>
<tr>
<th>Hpa fragment</th>
<th>Electron microscopy*</th>
<th>32P Distribution*</th>
<th>Electrophoretic mobility*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>36.4 ± 5.3</td>
<td>39.2</td>
<td>(01)</td>
</tr>
<tr>
<td>B</td>
<td>28.9 ± 5.2</td>
<td>32.8</td>
<td>(01)</td>
</tr>
<tr>
<td>C</td>
<td>18.5 ± 3.2</td>
<td>21.1</td>
<td>21</td>
</tr>
<tr>
<td>D</td>
<td>6.9</td>
<td>(&lt;4)</td>
<td></td>
</tr>
</tbody>
</table>

* Electron microscopic length measurements are taken from Fig. 2 and are percentage of the length of open circular SV40 DNA ± 1 standard deviation.

To estimate the molecular weights by distribution of 32P in the various fragments, 0.35 μg of [32P]SV40 DNA 1 containing 53,000 cpm was digested to completion in 35 μl of the standard reaction mixture and subjected to electrophoresis in the same slab gel after division into three equal portions. After electrophoresis (150 V [30 mA] for 18 hr at room temperature) the wet gel was covered with Saran Wrap and exposed to film for 24 hr; the film was then developed and used to locate the radioactive areas on the wet gel. Fragments of the gel containing the radioactivity were then cut out and placed in scintillation vials containing 0.2 ml of 30% H2O2 and dissolved at 75°C. After dissolution of the gel fragments, 5 ml of Triton-toluene fluor was added and the samples were counted. The results are expressed as percentage of total radioactivity present in each fragment. Each value is the average of three electrophorograms.

To estimate molecular weight from electrophoretic mobility, H. influenzae fragments were used as standards (I). Since no standards were available above 22.5% or below 4% of the length of SV40 DNA, only the value for fragment Hpa C could be accurately determined by this method.

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