tour length of the circle assuming it had the same mass per length as the arms.

The studies on the polysaccharide side chains, although incomplete, show that they are probably fairly large. A theoretical calculation based on the elution properties of polyacrylamide gel columns and the elution properties of carbohydrates relative to protein, leads to an estimate of 20,000–40,000 daltons for the molecular weight of the polysaccharide unit. If a subunit is 2×10^5 daltons and is half polysaccharide, there would be only three or four polysaccharide molecules per subunit. In summary, these results suggest that the aggregation factor is a 20×10^6 dalton complex of smaller glycoprotein molecules arranged in fibers with an open, sunburst structure. The relationship of these physical properties of the factor and its various parts to its function as a material which makes cells aggregate remains to be elucidated.

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Detection of Two Restriction Endonuclease Activities in *Haemophilus parainfluenzae* Using Analytical Agarose–Ethidium Bromide Electrophoresis†

Phillip A. Sharp,* Bill Sugden, and Joe Sambrook

ABSTRACT: A rapid assay for restriction enzymes has been developed using electrophoresis of DNA through 1.4% agarose gels in the presence of 0.5 μg/ml of ethidium bromide. The method eliminates lengthy staining and destaining procedures and resolves species of DNA which are less than 7×10^6 daltons. As little as 0.05 μg of DNA can easily be detected by direct examination of the gels in ultraviolet light. Using this technique, we have identified two different restricting activities in extracts of *Haemophilus parainfluenzae*. The two activities have different chromatographic properties on phosphocellulose and Bio-Gel A-0.5m, and they attack SV40 DNA at different sites. One activity (*Hpa* II) cleaves SV40 DNA at a single position situated 0.38 fractional genome length from the insertion point of SV40 sequences into the adenovirus SV40 hybrid $Ad2^{++}ND_1$. The other activity (*Hpa* I) cleaves SV40 DNA at three sites which appear to coincide with 3 of the 11 cleavage points attacked by a restriction system isolated from *H. influenzae* strain *Rd*.

Restriction enzymes have been isolated from a variety of strains of *Escherichia coli* and from various species of *Haemophilus* (see review by Meselson *et al.*, 1972; Yoshimori, 1971; Gromkova and Goodgal, 1972; Sack and Nathans, 1973). All of them are highly specific endonucleases which produce double-strand cleavages of native unmodified DNA. At least three of these enzymes (*Haemophilus influenzae* (Kelly and Smith, 1970), *E. coli* $R \cdot R_I$ (Hedgpeth *et al.*, 1972; Mertz and Davis, 1972), and *E. coli* $R \cdot R_{II}$) (H. W. Boyer, personal communication) attack base sequences that possess twofold rotational symmetry about an axis perpendicular to the axis of the DNA duplex; in other words, palindromic sequences of the type

$$\begin{array}{cccccc} A & B & C & C' & B' & A' \\ A' & B' & C' & C & B & A \end{array}$$

Because different enzymes attack different palindromes, each of them generates a characteristic set of cleavage products when reacted with DNA. For any particular enzyme the number of fragments obtained is a measure of the number of palindromic sites in the DNA specific to the enzyme, and the size of the fragments reflects the distribution of the sites along the DNA.

The two principal methods which have been used to analyze the fragments of DNA produced by restriction enzymes are velocity sedimentation and electrophoresis through poly-

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acrylamide gels. Both have unsatisfactory features; velocity centrifugation through sucrose gradients is not only laborious but also imprecise, because fragments of DNA of similar size are not resolved. Polyacrylamide gel electrophoresis gives a more accurate picture of the number and size of the DNA fragments but is sensitive to the ionic strength of the loading buffer and requires either lengthy staining and destaining, tedious slicing of gels, or time-consuming autoradiographic procedures. In this paper, we report a method to assay restriction enzymes using electrophoresis through agarose gels in the presence of ethidium bromide. The technique is both rapid and precise and requires small amounts of unlabeled DNA. We have used the method to examine *H. parainfluenzae* for restriction endonucleases.

Gromkova and Goodgal (1972) isolated from *H. parainfluenzae* a restriction activity which was subsequently shown by Sack and Nathans (1973) to cleave closed circular SV40 DNA into four fragments. During purification of this enzyme, we noticed that *H. parainfluenzae* contained two different endonuclease activities, one of which cleaved SV40 DNA once, the other three times. This paper describes the partial purification of these two enzymes. For convenience, we call the three-cleavage enzyme *Hpa* I and the single-cleavage enzyme *Hpa* II, following the convention proposed by H. O. Smith and D. Nathans (personal communication).

Materials and Methods

Bacterial Strains and Culture Conditions. Haemophilus parainfluenzae (obtained from Dr. Jane Setlow, Oak Ridge National Laboratory) was grown in brain-heart infusion broth supplemented with 2 μg/ml of NAD. *E. coli* RY-13 carrying resistance transfer factor R_I (Yoshimori, 1971) was grown in Luria broth. For enzyme preparations the bacteria were grown to midlogarithmic phase in a New Brunswick fermenter. The cells were collected by centrifugation at 0° and stored as a pellet at −70°.

Isolation of Restricting Endonuclease $R \cdot R_I$. The enzyme was partially purified from *E. coli* RY-13 as described elsewhere (Mulder and Delius, 1972).

Assays for Restriction Activity. Component I SV40 DNA or unit length linear SV40 DNA obtained by digestion of component I DNA with endonulease $R \cdot R_I$ (Mulder and Delius, 1972) was incubated with *Hpa* I or *Hpa* II restriction enzymes for 1–4 hr at 37° in 100-μl reaction mixtures consisting of 0.01 M Tris (pH 7.4), 0.010 M $MgCl_2$, 0.006 M KCl, and 0.001 M dithiothreitol. Generally incubation of 1 μg of SV40 DNA with 5 μl of the purified enzyme preparations resulted in complete digestion as determined by electrophoresis of the DNA products (see below). All reactions were terminated by addition of EDTA to 0.05 M.

Gel Electrophoresis. (i) Agarose gels were prepared by dissolving agarose (1.4% w/v) (Sigma electrophoresis grade) in a hot solution of 0.04 M Tris (pH 7.9)–0.005 M sodium acetate–0.001 M EDTA (pH 7.7) (E buffer) (Hayward and Smith, 1972). After cooling the homogeneous solution to 50°, ethidium bromide solution (10 mg/ml) was added to give a final dye concentration of 0.5 μg/ml. After mixing, the melted agarose was poured into glass tubes (10 × 0.6 cm i.d.). After gelatinization at room temperature, the top few millimeters of the gels were cut off with a razor blade to ensure a flat gel surface. The base of the glass tubes contained a slight restriction which served to prevent the gels sliding out. The gels were run in a 24-hole vertical tube apparatus in E buffer containing 0.5 μg/ml of ethidium bromide/ml. The samples were adjusted to 8% sucrose–0.025% Bromophenol Blue before loading on the gels. Electrophoresis was carred out for 2 hr at 5 mA/gel at room temperature. After the run, the gels were blown gently out of the tubes and examined by direct illumination from a short wave uv light (UV-Products, San Gabriel, Calif.). The DNA was seen as fluorescent bands and control experiments showed that as little as 0.05 μg of DNA was easily visible. The gels were photographed using Polaroid type 55 P/N film and a Kodak no. 23A red filter. (ii) Electrophoresis through 2.2% acrylamide–0.7% agarose gels (Peacock and Dingman, 1968) was performed as described by Pettersson *et al.* (1973).

Cell Lines and Viruses. SV40, strain 777 (Gerber, 1962), was grown from a four-times plaque-purified stock in the BS-C-1 line of African green monkey kidney cells, as previously described (Sambrook *et al.*, 1972).

Adenovirus 2 was grown in suspension cultures of the KB line of human cells and purified as described elsewhere (Pettersson *et al.*, 1967).

The adenovirus 2 SV40 hybrid $Ad2^{+}ND_1$ (Levin *et al.*, 1971) obtained from Dr. A. Lewis, N. I. H., was grown in KB cells and purified by the method as described previously for adenovirus 2 (Pettersson *et al.*, 1967).

Preparation of ^{32}P-Labeled SV40 DNA. Procedures for infection of cells with SV40 and for labeling and purification of covalently closed SV40 DNA have been published (Sambrook *et al.*, 1972). In brief, BS-C-1 cells were infected at a multiplicity of 5 plaque-forming units/cell, labeled with [^{32}P]orthophosphate and lysed by the method of Hirt (1967). Viral DNA was purified from the supernatant fluid by equilibrium centrifugation in CsCl–ethidium bromide. The final specific activity of [^{32}P]SV40 DNA was 5×10^8 cpm/μg.

Isolation of Restricting Endonucleases from H. parainfluenzae. Two methods of isolating restricting endonucleases were used. The first method adapted from that of Smith and Wilcox (1970) permitted separation of the two restriction activities with one chromatographic step. Frozen *H. parainfluenzae* (10 g) were suspended in 17 ml of 0.01 M Tris (pH 7.4)–0.01 M β-mercaptoethanol in a stainless steel beaker which was immersed in an ice bath. The cell suspension was sonicated for ten periods of 30 sec at maximum output (approximately 60 W, Heat Systems Model W185) during which the temperature of the suspension remained below 4°. Cell debris was removed by centrifugation in a Spinco angle 50 titanium rotor at 40,000 rpm for 60 min at 0°. The supernatant (19 ml) was adjusted to 1 M by addition of solid NaCl, and was layered on an agarose A-0.5 m column (2.6 × 55 cm, Bio-Rad), equilibrated in 1.0 M NaCl, 0.02 M Tris (pH 7.4), and 0.01 M β-mercaptoethanol having a flow rate of 15 ml/hr. Only 2 μl of 6-ml fractions were assayed to minimize salt concentrations in the assay and were sufficient to give greater than 75% conversion of SV40 I to cut DNA in the peak fractions. *Hpa* II eluted at 0.65–0.7 column volume, an exonucleolytic activity at 0.85–0.9 column volume, and *Hpa* I at 0.9–0.95 column volume.

After chromatography on A-0.5 m *Hpa* II was free from detectable *Hpa* I and exonucleolytic activities. It was concentrated by dialysis against 0.01 M Tris (7.4), 0.01 M $MgCl_2$, 0.01 M β-mercaptoethanol, 0.0001 M EDTA, and 50% glycerol. The restriction activity was stored in this buffer at −20°. After agarose chromatography, *Hpa* I was contaminated both with exonucleolytic activity and with *Hpa* II. *Hpa* I was further purified by chromatography on phosphocellulose. Fractions containing *Hpa* I activity were pooled and dialyzed against 100 volumes of 0.01 M KPO_4

(pH 7.6), 0.01 M β-mercaptoethanol, 0.0001 M EDTA, and 10% glycerol with one buffer change for 24 hr. The dialyzed material was passed onto a phosphocellulose column (1.2 × 15 cm) equilibrated with the dialysis buffer, at a rate of 1.2 ml/hr, washed with three to four volumes of dialysis buffer at 4 ml/hr, and developed with a linear 100-ml gradient of 0–0.6 M KCl in that buffer at 4 ml/hr. The *Hpa* I activity which eluted between 0.30 and 0.35 M KCl was stored at 0°.

A second method of extracting endonucleases gave rise only to *Hpa* II activity. *H. parainfluenzae* suspended in 0.01 M potassium phosphate (pH 7.4) and 0.001 M dithiothreitol (buffer A) were disrupted by passage at 0° through a French press at 12,000 psi. The viscous solution was centrifuged for 60 min in a Spinco angle 50 titanium rotor at 40,000 rpm. The pellet was discarded and the optical density of the supernatant at 260 nm was determined. Nucleic acids were removed by the slow addition of 1 ml of a fresh solution of 10% streptomycin sulfate for every 1500 OD_{260} units in the supernatant. The solution was stirred for 1 hr and then centrifuged for 15 min at 15,000 rpm in a Sorvall GSA rotor. The supernatant was adjusted to 50% saturation by addition of solid ammonium sulfate and stirred for 1 hr. After centrifugation for 30 min at 15,000 rpm in a Sorvall GSA rotor, the supernatant was adjusted to 70% saturation by addition of more solid ammonium sulfate. The protein precipitate was collected by centrifugation and dissolved in 10 ml of buffer A. Excess ammonium sulfate was removed by passing the yellow solution through a 2 × 50 cm column of Sephadex G-75 equilibrated in buffer A. The enzyme activity eluted in the void volume. The solution was passed onto a 2 × 20 cm column of phosphocellulose (Whatman P11) equilibrated with buffer A. The column was washed with four column volumes of buffer A and then with three column volumes of buffer A containing 0.15 M KCl. A linear gradient (220 ml) of 0.15–0.60 M KCl in buffer A was applied to the column and 2-ml fractions were collected. Fractions containing restriction activity against SV40 DNA eluted at about 0.45 M KCl and were pooled and concentrated by dialysis against Aquacide II (Calbiochem). The enzyme was pure enough for most purposes at this stage and was stored in 50% glycerol at −20°. Some preparations were purified further as follows. The active fractions from the phosphocellulose column were dialyzed against buffer A and applied to a 1 × 10 cm column of carboxymethylcellulose equilibrated in buffer A. The column was washed with three volumes of buffer A and the enzyme was eluted by addition of 1 volume of 0.25 M KCl in buffer A. The enzyme was concentrated by dialysis against Aquacide II and was stored in glycerol at −20°.

Electron Microscopy. Molecular lengths of duplex DNA were determined by measurement of molecules spread by the aqueous basic protein film technique. The DNA preparations were diluted into a solution of 0.5 M ammonium acetate (pH 7.0) containing 100 μg of cytochrome *c*/ml and spread on the surface of 0.25 M ammonium acetate. Films were picked up on palladium-coated grids and the DNA was stained and shadowed as described by Davis *et al.* (1971).

In order to examine heteroduplexes formed between $Ad2^{++}ND_1$ DNA and linear SV40 DNA, mixtures of the DNAs were denatured in 0.1 M NaOH, neutralized, and then dialyzed for 2 hr at 25° in 50% formamide buffered with 0.1 M Tris (pH 8.5)–0.01 M EDTA. The total concentration of the DNA during the dialysis procedure was 5 μg/ml. The reannealed DNA molecules were mounted for observation with the electron microscope using the formamide basic protein technique described by Davis *et al.* (1971). The general principles of length measurements of DNA and the standard deviations of such measurements using internal standards have been discussed by Davis *et al.* (1971).

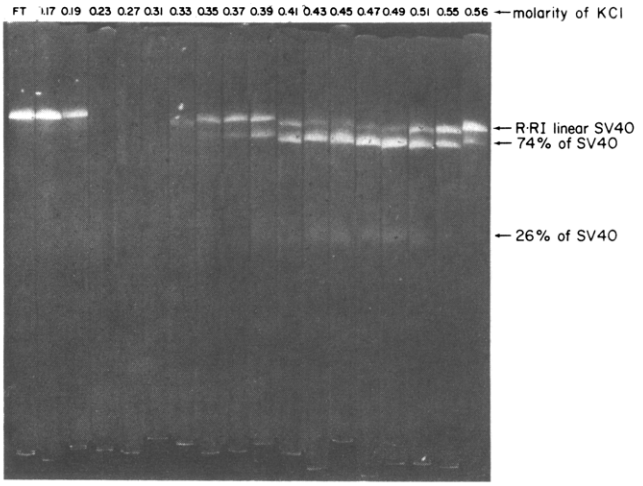

PLATE 1: Ethidium bromide–1.4% agarose gel electrophoresis assay of phosphocellulose column fractions of French press disrupted *H. parainfluenzae* extracts. The column was developed and assayed as described in Materials and Methods. The gels were 10 cm in length and were electrophoresed for 2 hr at 5 mA/tube. The peak of *Hpa* II activity eluted between 0.39 and 0.5 M KCl.

Bacteriophage DNAs. T7 DNA was a gift of Dr. Hajo Delius. φR DNA was purified from infected cells (a gift of Dr. Walter Keller) by extraction using the method of Hirt (1967) followed by equilibrium centrifugation of the supernatant in CsCl–ethidium bromide.

Results

Identification of Two Restriction Activities in H. parainfluenzae. Cells of *H. parainfluenzae* were disrupted by passage through a French pressure cell and the extracted protein was subjected to chromatography on phosphocellulose as described in Materials and Methods. Aliquots of each fraction eluting from the column were assayed for restriction activity against SV40 DNA which had been converted to the linear form by the action of endonuclease $R \cdot R_I$ (SV40-R_I linear DNA). The pattern of cleavage of the DNA was determined by electrophoresis through 1.4% agarose gels in the presence of ethidium bromide. The results obtained with representative fractions eluting from the column are shown in Plate 1. No restriction activity was detected in the protein which appeared in the flow through from the column: an exonuclease activity eluted between 0.23 and 0.31 M KCl and a restriction activity that cleaved SV40-R_I linear DNA into two fragments eluted between 0.39 and 0.55 M KCl. The active fractions were pooled and purified as described in Materials and Methods.

For comparison, when the same batch of *H. parainfluenzae* cells was broken by sonication, a very different pattern of restriction activity was observed eluting from the phosphocellulose column developed in buffer A (Plate 2). In addition to the single-cleavage activity which eluted at high salt, there was a large amount of restriction activity which eluted between 0.30 and 0.39 M KCl and which cleaved SV40-R_I linear DNA in at least three places. Smith and Nathans (personal communication) have devised a nomenclature for restriction activities and in accord with this system we call the enzyme from *H. parainfluenzae* which produces multiple cleavages in SV40 DNA *Hpa* I and the single-cut activity *Hpa* II.

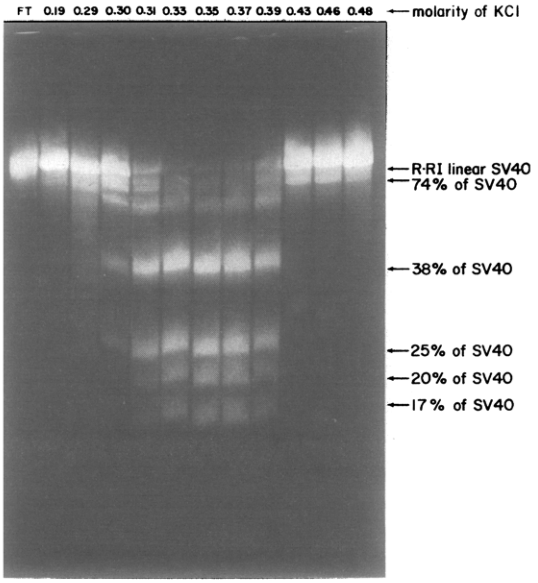

PLATE 2: Ethidium bromide–1.4% agarose gel electrophoresis assay of phosphocellulose column fractions of sonicated extracts of *H. parainfluenzae*. The column was developed and assayed as described in Materials and Methods. The gels were 10 cm in length and were electrophoresed for 2 hr at 5 mA/tube. The peak of *Hpa* I activity eluted between 0.31 and 0.39 M KCl and that of *Hpa* II eluted between 0.43 and 0.46 M KCl.

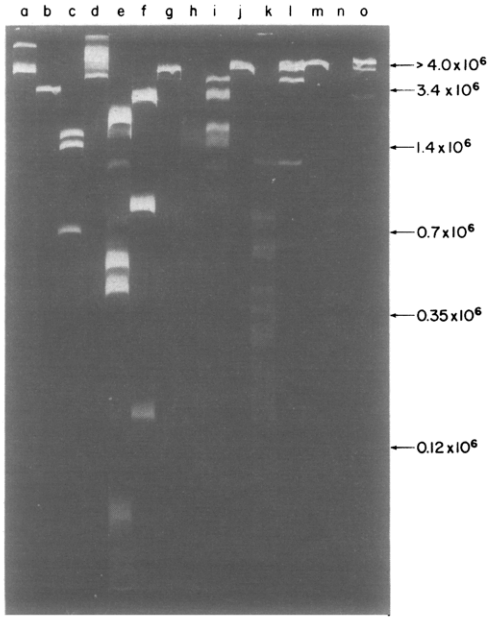

PLATE 3: Products of digestion of various DNAs by *Hpa* I and *Hpa* II. The DNAs (1–5 μg) were digested with *Hpa* I or *Hpa* II as described in Materials and Methods, and the reaction mixtures were applied directly to 2.2% acrylamide 0.7% agarose gels. Electrophoresis was carried out for 6 hr at 4 V cm^{-1}. The gels were stained by immersion for 30 min in E buffer containing 0.5 μg of ethidium bromide/ml, and photographed as described in Materials and Methods. The molecular weights shown were estimated using the calibration of electrophoretic mobility of DNAs of known sizes through 2.2% acrylamide–0.7% agarose gels (Pettersson *et al.*, 1973): (a) SV40 DNA, (b) SV40 DNA-*Hpa* II; (c) SV40 DNA-*Hpa* I; (d) φR RF DNA; (e) φR RF DNA-*Hpa* II; (f) φR RF DNA-*Hpa* I; (g) T7 DNA; (h) T7 DNA-*Hpa* II; (i) T7 DNA-*Hpa* I; (j) Adenovirus 2 DNA; (k) Adenovirus 2 DNA-*Hpa* II; (l) Adenovirus 2 DNA-*Hpa* I; (m) Adenovirus 3 DNA; (n) Adenovirus 3 DNA-*Hpa* II; (o) Adenovirus 3 DNA-*Hpa* I. The DNA frays produced by *Hpa* I cleavage are limit digestion products. The faint bands visible in some DNA preparations treated by *Hpa* II may be partial digestion products (*e.g.*, φR RF DNA, gel e).

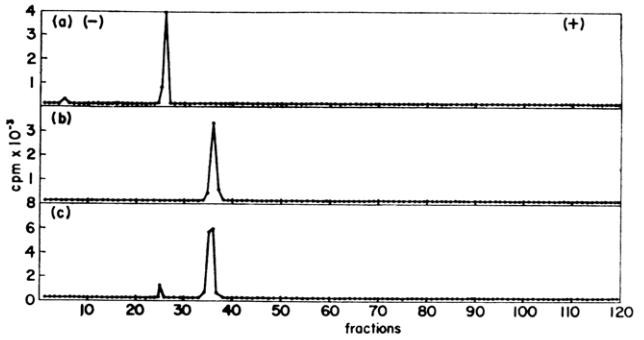

FIGURE 1: Conversion of SV40 covalently closed, circular to linear DNA by R·R_I and *Hpa* II restriction endonucleases. DNA samples were digested under standard conditions, extracted with chloroform–isoamyl alcohol, and precipitated with ethanol at −20°. The samples were electrophoresed in 2.2% acrylamide–0.7% agarose at 50 V for 16 hr. The gels were sliced into 1-mm segments and counted in "Aquasol" (New England Nuclear). Nicked circular SV40 DNA migrated 5 mm; superhelical SV40 DNA migrated 26 mm; and linear SV40 DNA migrated 36 mm under these conditions (without ethidium bromide in the running buffer): (a) undigested SV40 DNA; (b) R·R_I digested SV40 DNA; (c) *Hpa* II digested SV 40 DNA.

Hpa I was purified from sonicated extracts of *H. parainfluenzae* by chromatography on agarose A-0.5m followed by fractionation on phosphocellulose as described in Materials and Methods. *Hpa* II was purified either from French press extracts of *H. parainfluenzae* by chromatography on phosphocellulose or by sequential chromatography of sonicated extracts on agarose A-0.5m and phosphocellulose. Preparations of *Hpa* II made by each of these methods were indistinguishable from one another in their restriction activity. The final preparations of *Hpa* I and *Hpa* II were free of detectable exonuclease as assayed by liberation of acid-soluble radioactivity from ^{32}P-labeled adenovirus 2 DNA, and the evidence given below will show that there was no contamination of *Hpa* I by *Hpa* II or *vice versa*. Five microliters of the final enzyme preparations was sufficient to cleave 1 μg of DNA in 2 hr at 37°.

In addition to their behavior on phosphocellulose, *Hpa* I and *Hpa* II are different from one another in several other respects. *Hpa* I is considerably smaller than *Hpa* II as determined by chromatography on agarose A-0.5m and on Sephadex G-100. When a mixture of *Hpa* I and *Hpa* II was chromatographed on Sephadex G-100 (1.6 × 80 cm) in 1 M NaCl–0.02 M Tris (pH 7.4), and 0.01 M β-mercaptoethanol, flow rate 2 ml/hr, *Hpa* II eluted at 0.39 column volume and *Hpa* I at 0.43 column volume. Hemoglobin eluted from this column at 0.36 column volume and cytochrome *c* at 0.46 column volume. By this determination, then, *Hpa* I has a molecular weight slightly smaller than that of hemoglobin (65,000), while *Hpa* II has a molecular weight slightly greater than that of cytochrome *c* (13,370).

Hpa I and *Hpa* II produce different patterns of cleavage when tested against DNAs isolated from different sources. Plate 3 shows the results of electrophoresis through 2.2% acrylamide–0.7% agarose gels of the products obtained after incubation of various DNAs with *Hpa* I and *Hpa* II restriction endonucleases, as described in Materials and Methods. In all cases except SV40 DNA, the number of cleavages produced by *Hpa* II was greater than that produced by *Hpa* I. Thus, there seems little doubt that *Hpa* I and *Hpa* II are distinct restriction enzymes, each with its own specificity. Both *Hpa* I and *Hpa* II require Mg^{2+} for activity but no other

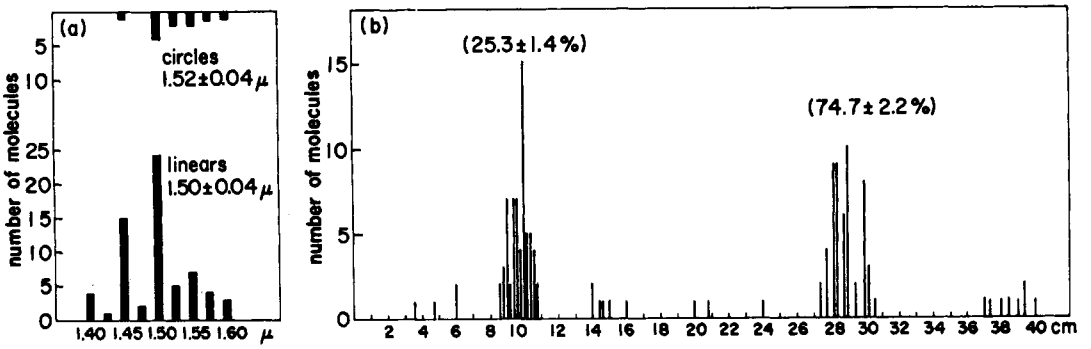

FIGURE 2: Length distributions of SV40 open circular and $R \cdot R_I$ restricted linear DNAs cleaved by *Hpa* II endonuclease. (a) Superhelical SV40 DNA digested with *Hpa* II endonuclease was mixed with undigested SV40 DNA and mounted for observation with the electron microscope by the aqueous basic protein film technique. The length histograms are given in microns, μ. The circular SV40 and the *Hpa* II cleaved linear SV40 DNAs have the same length within experimental error of 1.50 and 1.52 $\pm$ 0.04 μ, respectively. (b) $R \cdot R_I$ linear SV40 digested with *Hpa* II was spread by the aqueous technique. The lengths of the measured magnified molecules are given in centimeters. $R \cdot R_I$ linear SV40 measured at an equal magnification on another grid gave a mean length of 38.0 cm. The two peaks represent 25.3 $\pm$ 1.4 and 74.7 $\pm$ 2.2% of intact SV40. The intact undigested DNA forms a small peak around 38.5 cm on the histogram.

cofactor. Their optimum pH is around 7.4. *Hpa* II is inhibited by salt concentrations greater than 0.05 M KCl; *Hpa* I is not affected by salt concentrations as high as 0.1 M KCl.

Thanks to elegant studies by Nathans and his colleagues, a great deal is already known of the pattern of cleavage of SV40 DNA by restriction enzymes isolated from various species of *Haemophilus*. We have compared the sites at which *Hpa* I and *Hpa* II cleave SV40 DNA with those already mapped by the Nathans group.

Site of Cleavage of Hpa II on SV40 DNA. When closed circular SV40 DNA was digested with *Hpa* II, over 98% of the DNA was converted to linear molecules. The product of the digestion is unit length as judged by the electrophoretic mobility of the DNA through polyacrylamide–agarose gels and by electron microscopy (Figures 1 and 2a). These results mean that *Hpa* II introduces one cleavage into closed circular SV40 DNA.

Digestion of ^{32}P-labeled R_I-linear SV40 DNA with *Hpa* II resulted in the appearance of two DNA fragments (Figure 2b). The molecular weights of these fragments were estimated by the relative amount of radioactivity present in the two peaks (73 and 27%) (Figure 3) and by electron microscopic measurements of their lengths relative to unit length SV40 DNA molecules (74.7 $\pm$ 2.2 and 25.3 $\pm$ 1.4%). The larger fragment is therefore about 74% and the smaller about 26% of the length of R_I linear SV40 DNA. Because endonuclease $R \cdot R_I$ cleaves SV40 DNA at a unique site (Morrow and Berg, 1972; Mulder and Delius, 1972; Fareed *et al.*, 1972), these results mean that *Hpa* II attacks SV40 DNA at only one position. In order to determine whether this site is located clockwise or counterclockwise from the position of the $R \cdot R_I$ cleavage, heteroduplexes between $Ad2^{++}ND_1$ and *Hpa* II-cleaved SV40 DNAs were prepared and mounted for electron microscopic observation by the formamide–basic protein technique. Single strand can be distinguished from duplex DNA because of its irregular contour and kinky appearance (Westmoreland *et al.*, 1969; Davis *et al.*, 1971). However, both types of DNA are extended sufficiently to allow length measurements. Plates 4a and 5a show heteroduplexes formed between $Ad2^{++}ND_1$ DNA and R_I-linear SV40 DNA and between $Ad2^{++}ND_1$ DNA and *Hpa* II-linear SV40 DNA. Intact single strands of $Ad2^{++}ND_1$ DNA appear as single-strand circles due to the presence of terminal inverted complementary base sequences (Garon *et al.*, 1972; Wolfson and Dressler, 1972). The majority of the heteroduplexes observed were formed between circular single-strand $Ad2^{++}ND_1$ DNA and linear SV40 DNA (Plate 6). Therefore, in order to determine the orientation of the shorter arm of single-stranded SV40 DNA, the only heteroduplexes measured were those formed between linear partial length $Ad2^{++}ND_1$ DNA and SV40 linear DNA molecules. The shorter arm of single-stranded SV40 DNA in R_I- and *Hpa* II-SV40/$Ad2^{++}ND_1$ heteroduplexes was extended toward the nearest end of $Ad2^{++}ND_1$ DNA. The homology in the R_I-SV40/$Ad2^{++}ND_1$ heteroduplex accounts for 16% of the SV40 genome. The mean lengths of the single-strand arms of SV40 DNA, and that of the duplex portion of the heteroduplex shown on Plates 4b and 5b, were determined by averaging measurements from more than 10 heteroduplexes. If the R_I cleavage site is assigned the position 0, and the segment of SV40 contained in $Ad2^{++}ND_1$ is assigned the coordinates 12 $\leftrightarrow$ 28, then the cleavage site of *Hpa* II is unambiguously placed at position 74 on the circular genome of SV40. This assignment of the SV40 segment in $Ad2^{++}ND_1$ relative to the endonuclease $R \cdot R_I$ cleavage site is in agreement within experimental error with

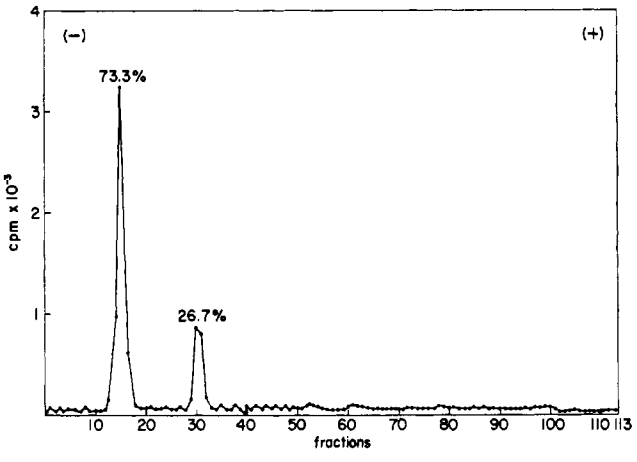

FIGURE 3: Digestion of $R \cdot R_I$ restricted SV40 linear DNA by *Hpa* II enzyme. $R \cdot R_I$ SV40 linear DNA (5000 cpm) was digested with *Hpa* II under standard conditions, extracted with chloroform–isoamyl alcohol, and precipitated with ethanol at −20°. The digested DNA was electrophoresed in 2.2% acrylamide–0.7% agarose gels without ethidium bromide at 50 V for 6 hr. The gels were sliced into 1-mm segments and counted in Aquasol (New England Nuclear). The percentages given above each peak represented the percentage of radioactivity migrating in that peak.

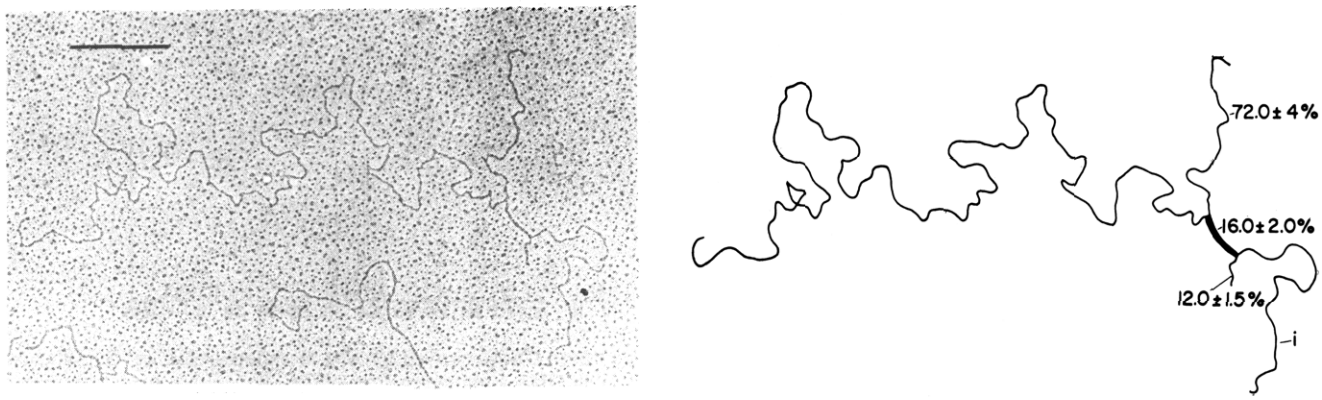

PLATE 4: (a, left) Heteroduplex of $R \cdot R_I$ linear SV40/Ad2^{++}ND$_1$ DNA mounted by the formamide basic protein technique. The bar represents a length of 0.5 μ. (b, right) Schematic of the heteroduplex of $R \cdot R_I$ linear SV40/Ad^{++}ND$_1$ DNA. The duplex region is represented by the heavy line from which four light lines representing single-strand DNA extend. The percentages in fractional lengths of SV40 given beside each segment of the heteroduplex is the average of a number of measurements from similar heteroduplexes and then standard deviation. The single-strand end of Ad2^{++}ND$_1$ denoted by i is 14% of Ad2^{++}ND$_1$ in length.

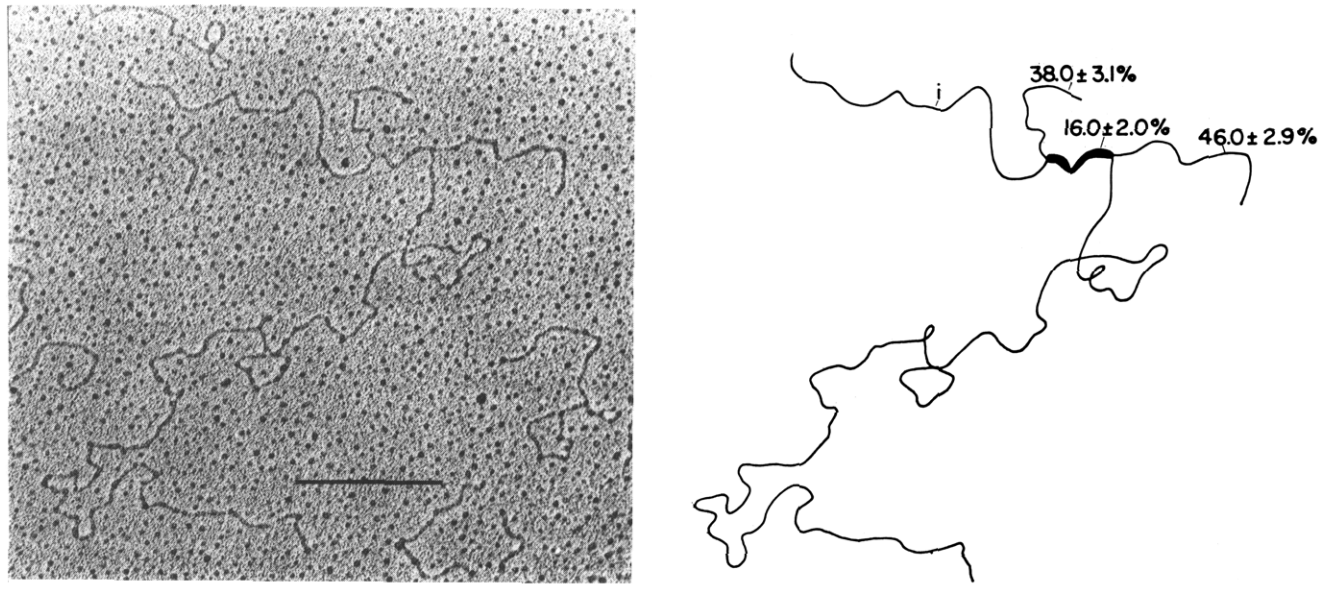

PLATE 5 (a, left) Heteroduplex of *Hpa* II cleaved superhelical SV40 and Ad2^{++}ND$_1$ DNA mounted by the formamide basic protein technique. The bar represents a length of 0.5 μ. (b, right) Schematic of the heteroduplex shown in 5a. See legend to Plate 4b and text for details.

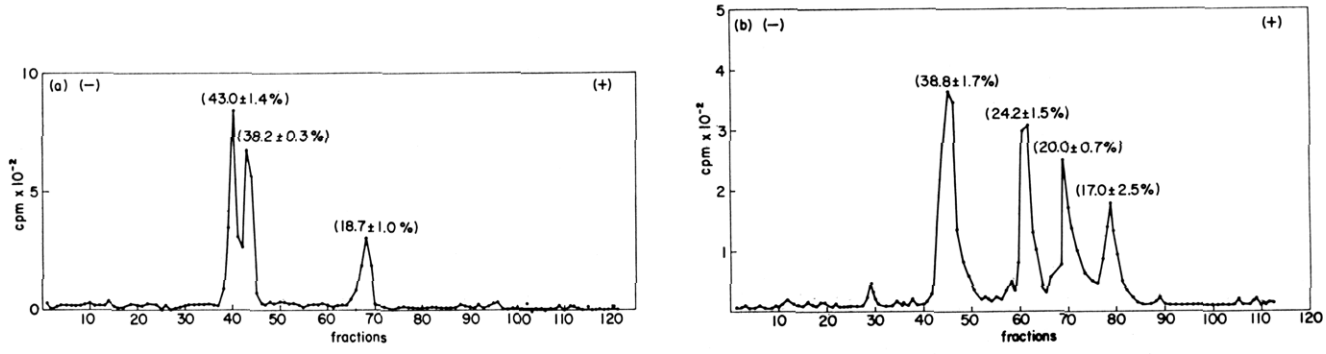

FIGURE 4: Digestion of SV40 superhelical DNA and $R \cdot R_I$ restricted SV40 DNA with *Hpa* I. SV40 superhelical DNA and $R \cdot R_I$ restricted SV40 DNA were digested under standard conditions and treated as described in legend to Figure 3. The percentage and its standard deviation given above each peak represent the average of the percentage of radioactivity migrating in that peak in two or more similar gels: (a) *Hpa* I digestion of SV40 superhelical DNA; (b) *Hpa* I digestion of $R \cdot R_I$ restricted SV40 linear DNA.

the previously published results of Morrow and Berg (1972).

Cleavage of SV40 DNA by Hpa I. Digestion of ^{32}P-labeled component I SV40 DNA by *Hpa* I generated three DNA fragments (Figure 4a). Neither prolonged incubation nor addition of more enzyme affected this pattern and we therefore con-

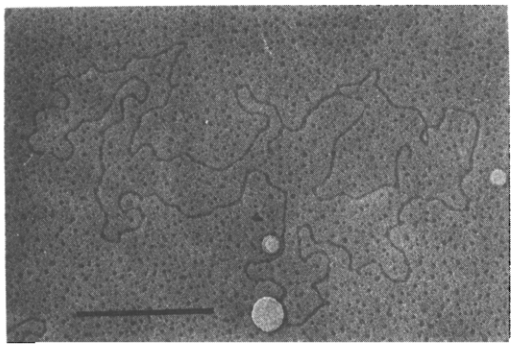

PLATE 6: Heteroduplex of an intact single strand of $Ad2^{++}ND_1$ and $R \cdot R_I$ linear SV40 DNA. The single strand of $Ad2^{++}ND_1$ forms a circle due to the inverted complementary sequences at its ends.

clude that the fragments detected are limit products. The approximate molecular weights of each of these fragments have been estimated by the relative amount of radioactivity in each fragment, their electrophoretic mobility through 2.2% acrylamide 0.7% agarose gels (Figure 4a) and by electron microscopic measurements of their lengths (Table I). The fractional length determined by the electron microscope of each of the fragments is approximately 10% smaller than the percentage of ^{32}P counts per minute migrating in the peak corresponding to the fragment. A similar result was observed for *H. parainfluenzae* (Sachs and Nathans, 1973) and for *Haemophilus influenzae* (Danna and Nathans, 1971) digestion products of SV40 DNA. In the subsequent discussion, the fractional length of each fragment based on the distribution of ^{32}P counts per minute is used. Thus, the two large fragments are 43 and 38% of the length of SV40 DNA and the small fragment is 19% of the length of SV40 DNA.

By similar methods, it was further established that digestion of ^{32}P-labeled R_I-linear SV40 DNA by *Hpa* I generated 4 DNA fragments whose sizes correspond to 38.8, 24.2, 20.0, and 17.0% of the SV40 genome (Figure 4b and Table I).

The two gels whose radioactivity profiles are shown in Figure 4a,b were run in parallel so that the relative mobilities of the DNA fragments produced by digestion of component I SV40 DNA and R_I-linear DNA could be compared. A photograph of the gels after staining in ethidium bromide is shown in Plate 7. Both from the relative mobilities and from the size of the fragments it is clear that the largest fragment (43%) obtained by digestion of component I SV40 DNA by *Hpa* I is cleaved by endonuclease $R \cdot R_I$ into a 26 and 17% fragment. Plate 7 also shows the position of DNA fragments obtained by digestion of component I SV40 DNA by *Hpa* I alone and by a mixture of *Hpa* I and *Hpa* II after electrophoresis through 2.2% acrylamide 0.7% agarose gels. It is clear that the site attacked by *Hpa* II lies within the 38% fragment that is obtained by digestion of SV40 DNA by *Hpa* I. The *Hpa* II site must be situated close to one end of this fragment because the increase in electrophoretic mobility of the fragment after treatment with the enzyme is not great. In all probability, *Hpa* II removes from the 38% fragment a small segment of DNA perhaps 2–3% of the length of total SV40 DNA. This small segment would have migrated off the end of the gel shown in Plate 7.

We have not mapped the position of the cleavage sites of *Hpa* I on SV40 DNA. But our data on the size of the DNA products are in excellent agreement with those published by Sack and Nathans (1973) for the major products of *Hpa* digestion of SV40 DNA, and are consistent with the major map positions assigned by Danna *et al.* (1973).

TABLE I: Fractional Lengths of SV40 *Hpa* II DNA Fragments.

% SV40 by Length[a]	% cpm[b]
Hpa II Digestion of Superhelical SV40 DNA	
38.1 ± 2.0[c]	43.0
34.3 ± 1.0	38.2
17.2 ± 1.0	18.7
Hpa II Digestion of $R \cdot R_I$ Linear SV40 DNA	
36.0 ± 1.3[c]	38.8
22.3 ± 1.2	24.2
17.7 ± 1.0	20.0
15.8 ± 0.6	17.0

[a] Lengths determined by electron microscopy as described in Materials and Methods. The specific fragments were recovered from agarose–acrylamide gels and mounted by the aqueous technique with SV40 circular DNA as an internal standard. [b] Cpm migrating in peaks during electrophoresis. [c] 95% confidence level (Davis *et al.*, 1971).

Discussion

The technique of electrophoresis of DNA fragments through agarose gels in the presence of low concentrations of ethidium bromide has several advantages over the methods previously employed to assay for restricting endonucleases. It requires small quantities of unlabeled DNA, is rapid and cheap and gives a good idea of the size and number of specific cleavage products. Furthermore, agarose gels are relatively insensitive to the ionic strength of the sample buffer so that the method can be used to screen fractions eluting from chromatography columns.

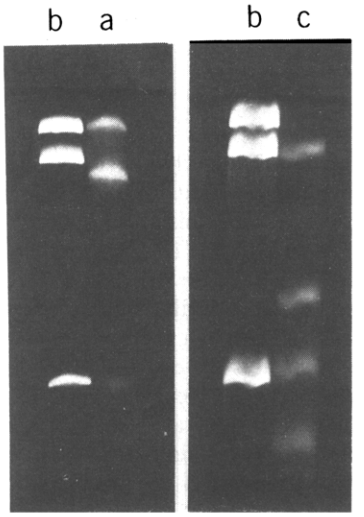

PLATE 7: Comparison of *Hpa* I and *Hpa* II digested SV40 DNA samples. SV40 DNA samples were digested under standard conditions, extracted with chloroform–isoamyl alcohol, and precipitated with ethanol at −20°. The digested DNA was electrophoresed in 2.2% acrylamide–0.7% agarose gels without ethidium bromide at 50 V for 10 hr, and stained before being photographed by setting in 0.5 μg/ml of ethidium bromide in E buffer for 0.5 hr: (a) SV40 superhelical DNA digested with both *Hpa* I and *Hpa* II endonuclease; (b) SV40 superhelical DNA digested with *Hpa* I; (c) $R \cdot R_I$ restricted SV40 DNA digested with *Hpa* I.

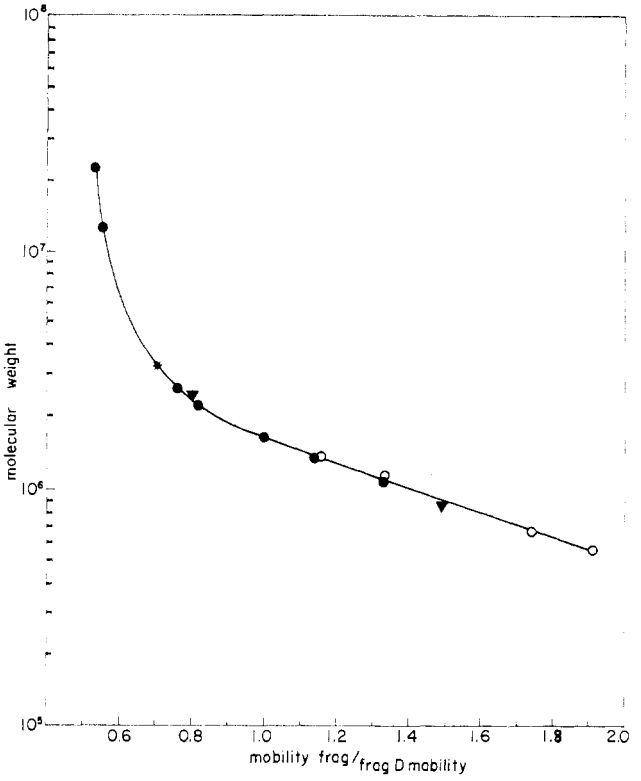

FIGURE 5: Comparison of the relative mobility to molecular weight of different DNA samples electrophoresed in 1.4% agarose gels in the presence of ethidium bromide. Relative mobilities are expressed as the ratios of the distance migrated by the sample DNA to the distance migrated by $R \cdot R_I$ restricted Ad2 DNA fragment D which has a molecular weight of 1.7 ± 0.1 million. In order of decreasing molecular weight the migrated DNA samples are Ad2 DNA 23×10^6; $R \cdot R_I$ fragment A of Ad2 13.6×10^6; $R \cdot R_I$ linear SV40 3.4×10^6; $R \cdot R_I$ fragment B of Ad2 2.7×10^6; *Hpa* II fragment of $R \cdot R_I$ linear SV40 2.55×10^6; $R \cdot R_I$ fragment C of Ad2 2.3×10^6; $R \cdot R_I$ fragment D of Ad2 1.7×10^6; *Hpa* I fragment of SV40 1.5×10^6; $R \cdot R_I$ fragment E of Ad2 1.4×10^6; *Hpa* fragment of SV40 1.3×10^6; $R \cdot R_I$ fragment F of Ad2 1.1×10^6; *Hpa* II fragment of $R \cdot R_I$ linear SV40 0.85×10^6; *Hpa* I fragment of SV40 0.68×10^6; *Hpa* I fragment of $R \cdot R_I$ linear SV40 0.58×10^6. The molecular weights of the $R \cdot R_I$ fragments of Ad2 are taken from Pettersson *et al.* (1973).

The central feature of the technique is the staining of DNA in the gels with low concentrations of ethidium bromide. In the experiments described in this paper, we have incorporated ethidium bromide in the gels and the running buffer. It is equally satisfactory to perform the electrophoresis in the absence of the dye and to stain the DNA subsequently by immersing the gels for 30 min in a solution of 0.5 μg of ethidium bromide/ml. In both cases the dye bound to the DNA in the gels gives a high fluorescent yield on exposure to uv light so that bands containing as little as 0.05 μg of DNA can easily be seen. Thus the lengthy staining and destaining or autoradiographic procedures which are an integral part of most of the techniques that are used for electrophoresis of DNA are eliminated.

Electrophoresis through 1.4% agarose gels resolves fragments of DNA whose size is less than 6×10^6 daltons (see Figure 5). By changing the gel matrix and running time, the range of molecular weights of DNA that are resolved by the gels can be extended or reduced at will. In our hands the technique has proved to be a very useful and flexible tool for assaying restriction enzymes.

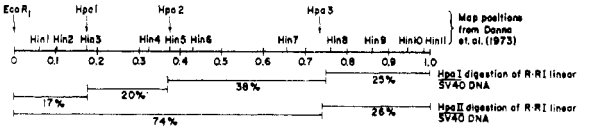

FIGURE 6: Diagram of SV40 DNA showing the cleavage positions assigned by Danna *et al.* (1973) for *H. influenzae* and *H. parainfluenzae* restriction activities, and the size of the digestion products of *Hpa* I and *Hpa* II. For convenience, the map shows SV40 DNA as a linear molecule generated by cleavage with endonuclease $R \cdot R_I$.

Two different restriction activities have been identified in *H. parainfluenzae*. In order to eliminate the possibility that the two activities resulted from a mixed population of bacteria, we have examined restriction activity in extracts of bacteria grown from individual colonies. Both restriction activities were always present. The pattern of restriction activity varies with the method used to break open the bacteria. Both activities are present in sonicated extracts, but only one enzyme is recovered if the cells are broken in a French press. We do not know the mechanism by which this selective extraction occurs. After passage through the French press, the cell lysate is extremely viscous and it is possible that *Hpa* I remains attached to the nucleic acid and is pelleted during the centrifugation step. Attempts were made to recover restriction activity from the pellet by sonication. Although activity of both *Hpa* I and *Hpa* II was detectable, the yields were very poor.

Danna *et al.* (1973) reported that digestion of SV40 DNA with *H. parainfluenzae* restriction activity generated 4 DNA fragments which correspond to 40, 34, 20, and 3–5% of the total viral genome. Two of the cleavage sites mapped at or very close to positions (*Hin* 3 and *Hin* 5) which are attacked by the *H. influenzae* restriction system. A third site was located within fragment *Hin* C, *i.e.*, at a site not attacked by *H. influenzae*. The fourth cleavage site was tentatively identified as *Hin* 8—again a position which is cleaved by the *H. influenzae* restriction system. The paradox posed by these data—how is it that a restriction activity which presumably is specific, can attack SV40 DNA at sites which consist of at least two different base sequences—is now resolved. Our data suggest that Danna *et al.* (1973) were working with a mixture of *Hpa* I and *Hpa* II.

Hpa I cleaves closed circular SV40 DNA at three places. The resulting fragments are 43, 38, and 19% of the length of SV40 DNA. Because the products of *Hpa* I digestion of $R \cdot R_I$ linear SV40 DNA correspond to 38, 26, 19, and 17% of the length of SV40 DNA, it is clear that the site for endonuclease $R \cdot R_I$ cleavage is located within the largest fragment obtained after digestion of component I SV40 DNA with *Hpa* I. These data are shown in Figure 6 together with the map positions of *H. influenzae* and *H. parainfluenzae* cleavage established by Danna *et al.* (1973).

The single site which is attacked by *Hpa* II is located 0.38 fractional genome length from the insertion site of SV40 sequences into the DNA of the adenovirus-SV40 hybrid $Ad2^{++}$-ND_1 and 0.26 fractional length from the R_I cleavage site. This places the site of *Hpa* II cleavage within fragment *Hin* C and corresponds to the only site of *H. parainfluenzae* cleavage which is not coincident with an *H. influenzae* site (Danna *et al.*, 1973). Consistent with these map positions is the observation that simultaneous digestion of SV40 DNA with a mixture of *Hpa* I and *Hpa* II yields a fragment which is approximately 35% of the length of SV40 DNA and which is derived from the 38% fragment shown in Plate 7. All our data on the

length of the *Hpa* I fragments are in good agreement with those already published by Sack and Nathans (1973) and are consistent with the map assignments given to the three major *Hpa* cleavage sites by Danna *et al.* (1973) (and see Figure 6). In all likelihood then, *Hpa* I cleaves SV40 DNA at positions *Hin* 3,5,8. This result means that *H. influenzae* serotype *d* and *H. parainfluenzae* contain a common restriction activity which attacks SV40 DNA at three sites.

Danna and Nathans (1971) have shown that SV40 DNA is cleaved at 11 positions by a restriction activity isolated from *H. influenzae*, and T. N. H. Lee, K. J. Danna, H. O. Smith, and D. Nathans (unpublished results) have been able to divide this activity into two fractions which cleave SV40 DNA at 5 and 6 sites, respectively. Our results suggest that one of these fractions still must be a mixture of the *Hpa* I cleavage restriction endonucleases and at least one other enzyme. In fact, the situation may be even more complicated for there is no evidence that *Hpa* I itself is not a mixture. Similarly, *Hpa* II could conceivably be a mixture of restriction activities, one of which cleaves SV40 DNA once and the other, not at all. Nevertheless, the restriction activities isolated from *H. parainfluenzae* are extremely useful because one preparation of bacteria yields two endonuclease activities, one of which (*Hpa* I) produces only a limited number of cleavages in large DNAs; the other (*Hpa* II) yields products of lower molecular weight which are useful for fine structure mapping (Allet, 1973).

Acknowledgments

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