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Fragmentation of Bacteriophage S13 Replicative Form DNA by Restriction Endonucleases from *Hemophilus influenzae* and *Hemophilus aegyptius*

FRANK G. GROSVELD, KAIE M. OJAMAA, AND JOHN H. SPENCER

Department of Biochemistry, McGill University, 3655 Drummond Street, Montreal, Quebec, Canada H3G 1Y6

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The restriction enzymes *Hin*d from *Hemophilus influenzae* and Hae_{III} from *Hemophilus aegyptius* cleave bacteriophage S13 replicative form (RF) DNA into 13 and 10 specific fragments, respectively. The sizes of these fragments were estimated by gel electrophoresis, electron microscopy, and pyrimidine isostich analysis. The *Hin*d and Hae_{III} fragments were ordered relative to each other by cross digestion and a physical map of the S13 genome constructed. Comparison of the *Hin*d cleavage patterns of S13 RF DNA and ϕX174 RF DNA showed the majority of the fragments from the two DNAs coincided with each other except for three of the thirteen S13 fragments and three of the thirteen ϕX174 fragments. Comparison of the Hae_{III} patterns of the two DNAs revealed a lack of coincidence of one S13 fragment only and two ϕX174 fragments. From the data obtained by the cleavage of the two DNAs by *Hin*d and Hae_{III}, a correlation between the physical restriction enzyme cleavage maps and the genetic map of the two phages was made. The differences in cleavage of the two DNAs by the restriction enzymes have been explained by changes in two restriction enzyme sites in the AB region and one change of site in the F region of the genetic map of the two bacteriophages.

INTRODUCTION

The DNAs of the small icosahedral bacteriophages ϕX174 and S13 are attractive for sequence determination because the genomes consist of only approximately 5000 nucleotides (Sinsheimer, 1968; Spencer *et al.*, 1972; Berkowitz and Day, 1974). Previous studies in our laboratory described the total pyrimidine-purine oligonucleotide catalog of bacteriophage S13 and S13 replicative form (RF) DNA (Cerny *et al.*, 1969) and the long pyrimidine oligonucleotides have been sequenced recently (Delaney and Spencer, 1973; Harbers *et al.*, 1976). As an extension of these studies, and to obtain larger oligonucleotides containing all four bases, we have utilized the restriction enzymes from *Hemophilus influenzae* (Smith and Wilcox, 1970) and *Hemophilus aegyptius* (Middleton *et al.*, 1972) to obtain specific fragments of bacteriophage S13 RF DNA. Comparison of the physical maps of the restriction fragments and the sizes of the S13 RF fragments with the fragments obtained from ϕX174 DNA (Edgell *et al.*, 1972; Middleton *et al.*, 1972; Lee and Sinsheimer, 1974) using the two enzymes revealed three changes in location of the restriction endonuclease sites between the two DNAs.

Nomenclature. The nomenclature system proposed by Smith and Nathans (1973) for describing the restriction-modification enzymes has been followed. The enzyme preparation from *H. influenzae* strain Rd is designated *Hin*d and comprises both $Hind_{II}$ and $Hind_{III}$ enzymes since separation of these different activities was not attempted. The enzyme preparation from *H. aegyptius* is designated Hae_{III}. The nomenclature system of the DNA fragments departs from that proposed by Smith and Nathans (1973). The fragments have been assigned arabic numerals in or-

der of decreasing size as determined by electrophoretic separation on polyacrylamide gels. Thus S13 *Hind* 2 is the second largest fragment released by endonuclease *Hind* from bacteriophage S13 RF DNA. In the case of two or more fragments of the same apparent size the designations are followed by the letters a, b, c, etc. The nomenclature has been chosen deliberately to avoid confusion between fragments of similar size released from different DNA substrates by various restriction enzymes and between the letters of the genetic map. Fragments obtained by complete digestion with both endonucleases *Hind* and Hae_{III} either concomitantly or serially are designated HH, followed by a number indicating the number of base pairs in the fragment. Thus, S13 HH470 is a fragment of 470 base pairs released from S13 RF I DNA by complete digestion with endonucleases *Hind* plus Hae_{III}. Fragments obtained by partial digestion with either or both endonucleases are designated δ followed by a number indicating the number of base pairs in the fragment. The enzyme (or enzymes) and starting material used are defined separately for each δ fragment.

MATERIALS AND METHODS

Materials. Bacteriophage S13 wild-type was kindly supplied by Drs. I and E. Tessman. Chloramphenicol, ethidium bromide, and bovine serum albumin (Fraction V) were obtained from Sigma Chemical Co. and streptomycin sulphate was obtained from Calbiochem. Optical grade CsCl was from the Harshaw Chemical Co. DEAE Sephadex A-25 was obtained from Pharmacia (Canada) Ltd., and $H_3{}^{32}PO_4$ was obtained from New England Nuclear (Canada) Ltd. Reagents for electrophoresis, acrylamide and bis(*N,N'*-methylenebis (acrylamide)), were obtained from Eastman. The acrylamide was recrystallized from chloroform before use.

Isolation of ^{32}P-labeled replicative form 1 DNA from bacteriophage S13 wild type. Growth was performed as described by Shleser *et al.* (1968). After collection of the cells by centrifugation the superhelical replicative form DNA (RF I) was isolated from the total DNA as described by Schekman *et al.* (1971) except for one modification. The pooled fractions from the ethidium bromide–CsCl gradient centrifugation were extracted with isopropanol three times to remove the ethidium bromide directly, and then concentrated by vacuum dialysis against 10 m*M* Tris–HCl buffer, pH 7.6. This eliminated the necessity for isopropanol precipitation and redissolving of the DNA with the concomitant losses.

Purification of restriction endonuclease Hind. Endonuclease *Hind* was isolated from *H. influenzae* strain Rd (obtained from Dr. D. Denhardt from Dr. N. Axelrod), according to the method of Smith and Wilcox (1970) but modified by replacement of the agarose column step by streptomycin sulphate precipitation as follows: To the clarified sonicate of the cell suspension, 1 ml of 10% streptomycin sulphate, in 50 m*M* Tris–HCl buffer, pH 7.4, containing 2 m*M* dithiothreitol, was added for each 1500 A_{260} units of solution. The solution was stirred for 15 min on ice and the precipitate was removed by centrifugation. Precipitation of the nucleic acids was monitored by measurement of the ratio A_{230}/A_{260} and additional streptomycin sulphate added until the ratio attained 2 to 2.5. The supernatant solution was then diluted according to the original procedure prior to ammonium sulfate precipitation. Enzyme activity from the phosphocellulose column chromatography step was detected by alcohol precipitation of calf thymus DNA. To 20 μl of each column fraction were added 20 μl of a 2 mg/ml calf thymus DNA solution in 10 m*M* Tris–HCl buffer, pH 7.4, containing 6 m*M* $MgCl_2$ and the mixture was incubated at 37° for 30 min. The samples were placed in an ice bath and 50 μl of 0.3% bovine serum albumin followed by 200 μl of cold 95% ethanol was added. Enzyme activity was revealed by a uniform milky solution; a clumped white precipitate indicated that no enzyme was present (D. T. Denhardt, personal communication). The enzyme activity eluted from the phosphocellulose column in a broad peak between 0.315–0.34 *M* KCl. Fractions containing the enzyme were pooled, dialyzed against 20 m*M* Tris–HCl buffer, pH 7.4,

containing 0.2 *M* NaCl in 50% glycerol, and stored at −20°.

Purification of the restriction endonuclease Hae_{III}. Endonuclease Hae_{III} was isolated from *H. aegyptius* (ATCC strain 11116) according to the method of Smith and Wilcox (1970), modified as described for the endonuclease *Hin*d purification. The enzyme activity eluted from the phosphocellulose column between 0.35 and 0.4 *M* KCl.

Digestion of DNA with restriction endonucleases Hind and Hae_{III}. The conditions, amounts of enzyme, and ^{32}P-labeled RF I DNA were used exactly as described by Edgell *et al.* (1972) and Middleton *et al.* (1972).

Gel electrophoresis. Separation of endonuclease *Hin*d and Hae_{III} fragments by acrylamide gel electrophoresis was as described by Edgell *et al.* (1972). The gels were prepared in glass tubes 6 mm in diameter × 15 to 25 cm long. Electrophoresis was for 8 to 10 hr at 4 mA per tube. Gels were sliced into 2-mm fractions using a Gilson aliquogel fractionator and collected in aqueous suspension directly into scintillation vials. In $^{32}P/^{3}H$ double-labeled experiments, the gel fractions were solubilized by incubation in 30% H_2O_2 at 60° overnight, 20 ml Triton X-100 (Sebring *et al.*, 1971) scintillation fluid was added, and the radioactivity was measured in a Beckman LS 250 liquid scintillation spectrometer. Counts were corrected for spill by a dual label correction program using a Beckman tape editor and Olivetti programma 101 fitted to accept punch tape. In experiments in which ^{32}P was the only radioisotope present, radioactivity was measured by Cerenkov radiation in a Beckman LS 250 liquid scintillation spectrometer. For further analysis the peak fractions were pooled and the DNA fragments were extracted from the gel by stirring for 5 hr in 0.56 *M* NaCl solution. The extracted gel was removed by filtration through a fine-grade sintered glass filter and washed with H_2O. This procedure gave a better than 95% yield of ^{32}P-labeled DNA fragments. Extracts of the smaller fragments were concentrated in a Buchler evapo-mix and extracts of the larger fragments were concentrated by lyophilization to avoid further shear degradation.

Slab gel electrophoresis was performed in a water-cooled vertical apparatus. Gels were 4–8% polyacrylamide, 0.3 × 20 × 20 cm in size with a 4-mm 0.6% agarose gel overlay for formation of the pockets (5 × 3 mm). Gels were run for 8 to 10 hr at 75 mA per gel and the separated fragments were located by radioautography.

Pyrimidine isostich analysis. Pyrimidine oligonucleotides were released from DNA restriction enzyme fragments by formic acid/diphenylamine digestion as described by Burton (1967), and the diphenylamine was removed by ether extraction. Chain-length fractionation of the hydrolysate was performed on DEAE Sephadex A-25 at pH 5.5 as described elsewhere (Delaney and Spencer, in preparation). For identification of the long pyrimidine oligonucleotides in each fragment, the hydrolysates were evaporated to dryness, redissolved in water, and subjected to ionophoresis/homochromatography as described by Brownlee and Sanger (1969) and modified by Ling (1972).

Electron microscopy. Bacteriophage S13 RF DNA was used as a standard reference in electron microscopy of DNA *Hin*d fragments prepared by the Kleinschmidt and Zahn technique (1959). Photomicrographs of the DNA molecules were projected and traced and the lengths were measured with a map measurer.

RESULTS

Size of fragments released by Hind and Hae_{III} from S13 RF I DNA. Digestion of ^{32}P-labeled S13 RF I DNA by either endonuclease *Hin*d or Hae_{III} followed by electrophoresis of the respective hydrolysate on a 3% polyacrylamide gel resulted in separation of both the *Hin*d fragments and Hae_{III} fragments each into nine size classes. The results are shown in Fig. 1. The molecular weights of the various *Hin*d and Hae_{III} fragments were calculated by integration of the counts per minute in each size class (peak) and expression of these values as a function of the 5000 base pairs per molecule of S13 RF I DNA (Spencer *et al.*, 1972; Cerny *et al.*, 1969). The results are given in

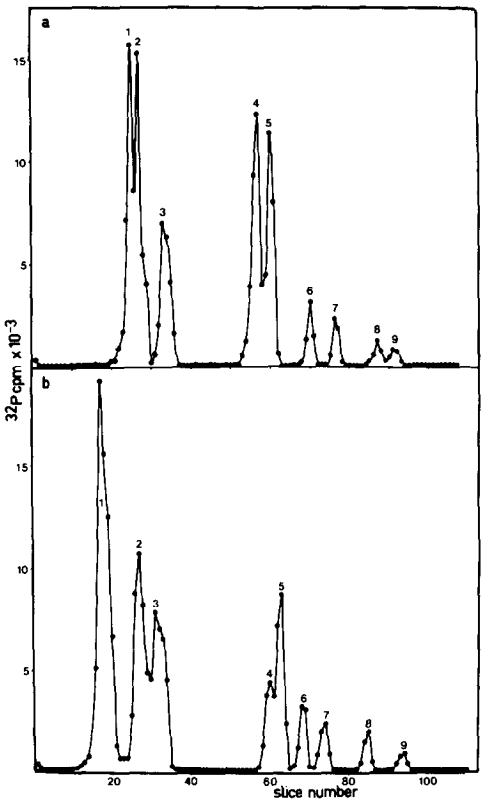


FIG. 1. Polyacrylamide gel electrophoresis of S13 RF I DNA fragments produced by (a) endonuclease *Hin*d and (b) endonuclease Hae_{III}. Twenty micrograms of ^{32}P-labeled S13 RF I DNA, 6 m*M* Tris-HCl (pH 7.6), 6 m*M* $MgCl_2$, 6 m*M* mercaptoethanol, 6 m*M* NaCl, and 50 μl of endonuclease *Hin*d (a) or Hae_{III} (b) in a final volume of 300 μl were incubated at 37° for 16 hr. The solution was evaporated under vacuum to approximately 40 μl; 1 μl of bromophenol blue dye added as a marker and layered on a 6 mm × 25 cm 3% polyacrylamide gel. Electrophoresis was at 4 mA per tube for 10 hr. Slices were 2 mm thick, giving a direct measure of the distance of migration (mobility in mm) at the end of the run.

Tables 1 and 2, respectively. Edgell *et al.* (1972) have shown that a plot of the logarithm of the integrated counts in each peak (the relative masses) versus the electrophoretic mobility of ϕX174 *Hin*d fragments in acrylamide gels provides an estimate of the number of fragments per molecule of ϕX174 RF I DNA in each electrophoretic peak and a confirmation of the size calculation of the fragments. A similar plot of the S13 *Hin*d and Hae_{III} fragments is presented in Fig. 2 which shows that all fragments, except *Hin*d 4 and 5 from Fig. 1a and Hae_{III} 5 from Fig. 1b fit a linear relationship corresponding to one *Hin*d or Hae_{III} fragment per molecule of S13 RF I DNA. Size classes (peaks) *Hin*d 4

TABLE 1

SIZE DETERMINATIONS OF THE FRAGMENTS RELEASED BY ENDONUCLEASE *Hin*d FROM BACTERIOPHAGE S13 RF I DNA

*Hin*d fragments	Number of fragments	Size from integration of dpm in base pairs ± SEM[a]	Size from electron microscopy in base pairs	Size from pyrimidine isostich analysis in base pairs
1	1	1065 ± 13 (7)	1080	
2	1	963 ± 12 (7)	920	
3	1	721 ± 13 (7)		
4	3	314 ± 8 (7)		
5	3	279 ± 6 (7)		
6	1	192 ± 5 (7)		
7	1	143 ± 5 (7)		156, 154
8	1	78 ± 2 (4)		
9	1	59 ± 2 (4)		

[a] The numbers in parentheses refer to the number of determinations used to calculate the standard error of the mean (SEM). The number of base pairs in S13 RF DNA has been calculated as 5000.

TABLE 2

SIZE DETERMINATION OF THE FRAGMENTS RELEASED BY ENDONUCLEASE Hae_{III} FROM BACTERIOPHAGE S13 RF I DNA

Hae_{III} fragments	Number of fragments	Size from integration of dpm in base pairs ± SEM[a]	Size from pyrimidine isostich analysis in base pairs
1	1	1690 ± 77 (5)	
2	1	1025 ± 13 (5)	
3	1	863 ± 24 (5)	
4	1	298 ± 10 (5)	
5	2	270 ± 8 (5)	
6	1	218 ± 7 (5)	
7	1	170 ± 7 (5)	
8	1	107 ± 4 (5)	108
9	1	73 ± 2 (5)	

[a] The numbers in parentheses refer to the number of determinations used to calculate the standard error of the mean (SEM). The number of base pairs in S13 RF DNA has been calculated as 5000.

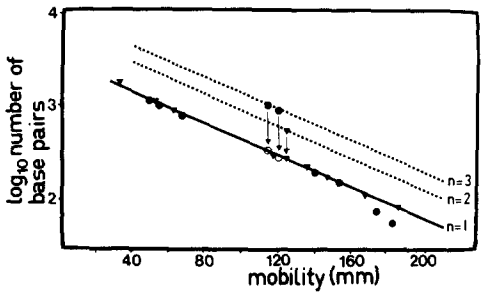


FIG. 2. Relationship between the logarithm of the number of base pairs and electrophoretic mobility of the *Hind* and Hae_{III} fragments of S13 RF I DNA. The number of base pairs was calculated from the integral of the counts in each peak of Fig. 1 and Fig. 3 as a function of the 5000 base pairs per molecule of S13 RF I DNA. The mobility was the distance migrated during the experiments (see legend to Figs. 1 and 3). The parallel lines (-----) were calculated for peaks with two or three times the mass ($n = 2$, $n = 3$) per molecule of S13 RF I DNA. The solid circles are the *Hind* fragments and the solid triangles are the Hae_{III} fragments. The open circles and open triangles are S13 *Hind* 4 and 5 and S13 Hae_{III} 5, respectively, after adjustment (1/3 or 1/2, respectively) of their values to $n = 1$.

and 5 fit a parallel line calculated for fragments three times the unit mass ($n = 3$) and thus contained 3 *Hind* fragments each per molecule of S13 RF I DNA. Size class (peak) Hae_{III} 5 fits a parallel line calculated for $n = 2$ and thus contained 2 Hae_{III} fragments per molecule of S13 RF I DNA.

A second procedure used for determination of the molecular weight of the largest S13 *Hind* fragments was measurement of the length of the two fragments, *Hind* 1 and *Hind* 2 from electron micrographs of mixed samples of peaks 1 and 2 from Fig. 1a. A histogram of the measurement is shown in Fig. 3. The values 1080 and 920 base pairs (Fig. 3) compare favorably with the sizes calculated from the integral of the total radioactivity (Table 1).

A third method used to determine the size of the S13 fragments was pyrimidine isostich analysis (Cerny *et al.*, 1969). S13 *Hind* 7 and Hae_{III} 8 were hydrolyzed with formic acid/diphenylamine and each hydrolysate chromatographed individually on DEAE Sephadex A-25 to separate the pyrimidine oligonucleotides released according to chain length. The distribution of the pyrimidine isostichs is given in Table 3 and calculation of the number of tracts (Cerny *et al.*, 1969) gave the total number of nucleotides in S13 *Hind* 7 and Hae_{III} 8 as 156 and 108, respectively. The results correlate well with the sizes calculated from the integral of the total radioactivity (Tables 1 and 2).

Determination of the relative position of the Hind and Hae_{III} fragments of S13 RF I DNA in a fragment map. The *Hind* and Hae_{III} fragments from S13 RF I DNA were ordered into a physical map by comparison

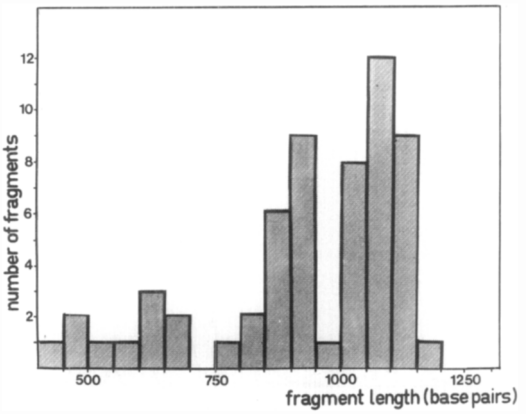


FIG. 3. Histogram of the length measurements of S13 *Hind* 1 and 2 from electron micrographs. The average lengths of the two major size classes were calculated as the mean values of 800 to 950 base pairs (920) and 1000 to 1150 base pairs (1080).

TABLE 3

DISTRIBUTION OF PYRIMIDINE ISOSTICHS IN S13 *Hind* 7 AND S13 Hae_{III} 8

Pyrimidine isostichs	Moles pyrimidine per 100 g atoms DNA phosphorus		Number of Tracts		Number of Nucleotides	
	Hind 7	Hae_{III} 8	*Hind* 7	Hae_{III} 8	*Hind* 7	Hae_{III} 8
1	0.28	0.29	31	32	31	32
2	0.33	0.32	18	13	36	26
3	0.27	0.14	10	5	30	15
4	0.19	0.15	5	4	20	16
5	0.09	0.07	2	2	10	10
6	0.11	NP[a]	2	–	12	–
7	0.05	NP	1	–	7	–
8	NP	NP	–	–	–	–
9	NP	0.08	–	1	–	9
10	0.08	NP	1	–	10	–
Total	1.31	1.05			156	108

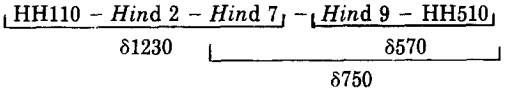
[a] NP, not present.

of the products produced from the fragments by various specific cross-digests with the opposite enzyme. All the fragments and products were separated by polyacrylamide slab gel electrophoresis and the results of the various cross digests are shown in Fig. 4. The size of each product was calculated from the mobility versus size plots as described in Fig. 2 and the results of the size calculations are summarized in Tables 4 and 5.

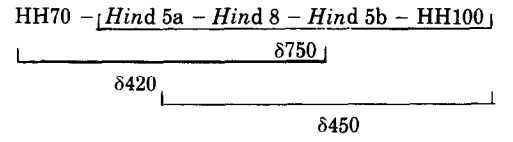
Comparison of separate *Hin*d and Hae_{III} digests with a combined *Hin*d + Hae_{III} total digest of S13 RF I DNA (Fig. 4a) indicated which fragments were to be further examined by cross-digestion to provide overlaps for the ordering of the fragments. The S13 *Hin*d fragments 2, 4a, 5ab, 7, 8, and 9 were not cleaved by endonuclease Hae_{III} (Fig. 4a, Table 4) indicating that these fragments occur within a Hae_{III} fragment. Similarly, S13 Hae_{III} fragments 5, 7, and 9 were not cleaved by endonuclease *Hin*d and therefore occur within a *Hin*d fragment (Fig, 4a, Table 5). To confirm these results each *Hin*d and Hae_{III} fragment was isolated then digested separately with the opposite enzyme. The results showed that S13 *Hin*d fragments 1, 3, 4bc, 5c, and 6 and Hae_{III} fragments 1, 2, 3, 4, 6, and 8 would provide the ordering information on cross digestion (Fig. 4).

The longer S13 Hae_{III} fragments 1 and 3 had more than three total cross digest products with *Hin*d (Fig. 4c). To determine the order of these products partial cross-digests of S13 Hae_{III} 1 and 3 with *Hin*d in addition to complete digests were investigated.

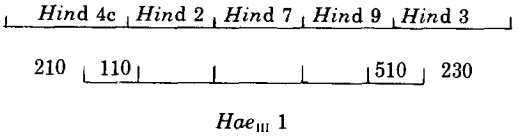
Partial digestion of S13 Hae_{III} 1 by endonuclease *Hin*d (Fig. 4b, Table 5) gave eight products; *Hin*d 2, 7, 9, two products HH510 and HH110 which were also present in a total *Hin*d + Hae_{III} digest of S13 RF I DNA and three products δ1230, δ510, and δ750 which did not correspond to any total *Hin*d + Hae_{III} products and therefore must be partial hydrolysis products of S13 Hae_{III} 1. In order to show the presence of *Hin*d 9 and HH110 in Fig. 4b the radioautograph was overexposed so that δ1230, *Hae* 1 and *Hin*d 2 blurred into each other. When the radioautograph was underexposed the three fragments were clearly delineated as separate bands (not shown). By examination of the size of all eight products from S13 Hae_{III} I (Table 5) one arrangement only is possible. δ1230 is a combination of HH110 + *Hin*d 2 + *Hin*d 7, δ570 a combination of HH510 + *Hin*d 9; and δ750 a combination of HH510 + *Hin*d 9 + *Hin*d 7. Thus the sequence of fragments in S13 Hae_{III} 1 is:



Partial digestion of S13 Hae_{III} 3 with endonuclease *Hin*d (Fig. 4b, Table 5) gave eight products: HH70, HH100, 2 × *Hin*d 5 and *Hin*d 8 which were all present in a total *Hin*d + Hae_{III} digest of S13 RF I DNA and three partial products, δ750, δ950, and δ420. By examination of the size of all eight products one arrangement only is possible. δ750 is a combination of *Hin*d 5a + *Hin*d 5b + *Hin*d 8 + HH100; δ450 is a combination of *Hin*d 8 + *Hin*d 5b + HH100; and δ420 is a combination of HH70 + *Hin*d 5a + *Hin*d 8. The arrangement of the fragments in S13 Hae_{III} is:



The products obtained by complete cross-digestion of *Hin*d fragments with Hae_{III} and the products from Hae_{III} fragments digested to completion with *Hin*d were compared with the products of a complete *Hin*d + Hae_{III} digest of S13 RF I DNA to determine which products were in common with each other. This gave the necessary overlap information. For example, *Hin*d 3 gave a product HH510 and *Hin*d 4c, a product HH110 (Table 4) and identical size products occur with *Hin*d digestion of Hae_{III} 1 (see above). Thus, *Hin*d 3 and *Hin*d 4c overlaps with Hae_{III} 1 can be ordered as follows:



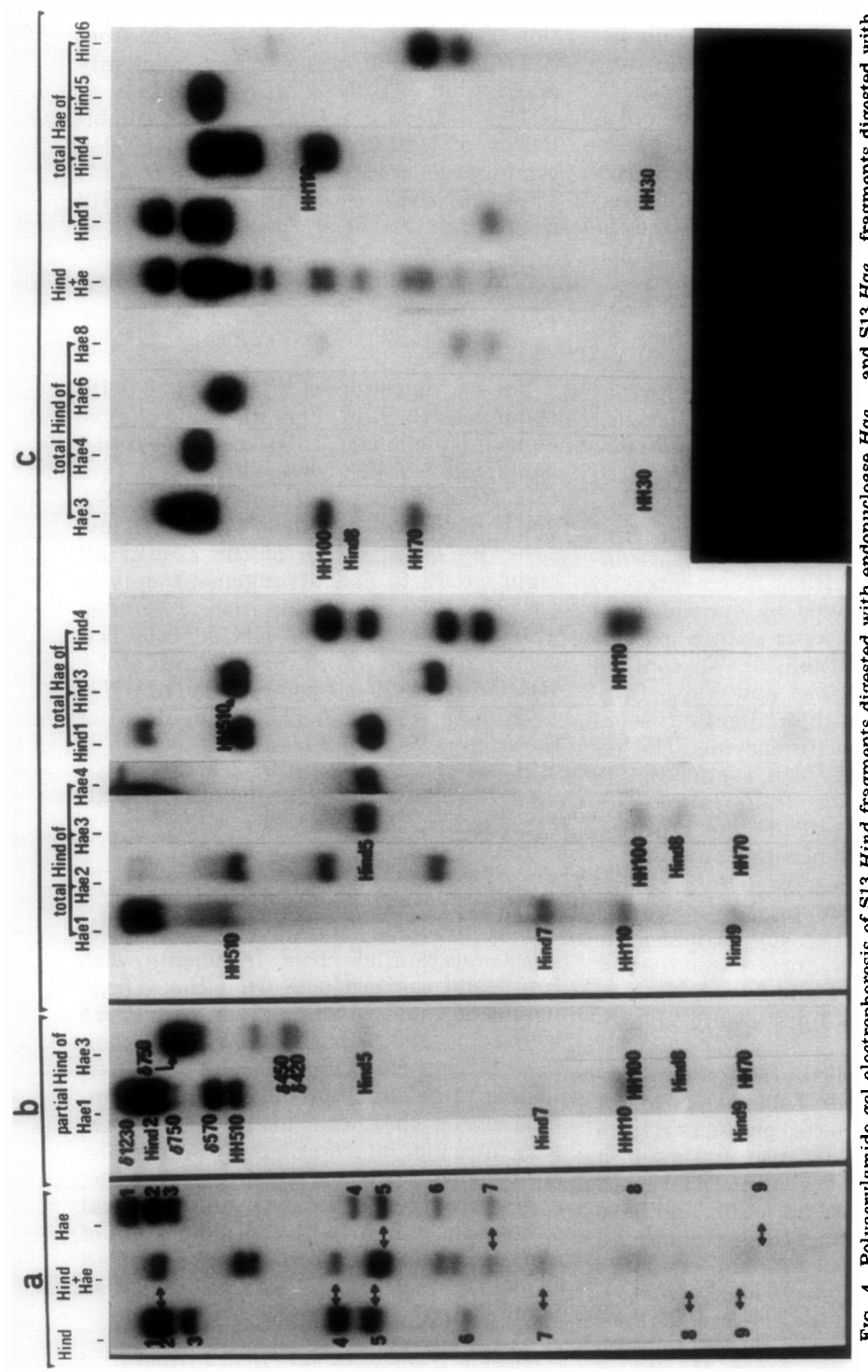


FIG. 4. Polyacrylamide gel electrophoresis of S13 *Hind* fragments digested with endonuclease Hae_{III}, and S13 Hae_{III} fragments digested with endonuclease *Hind*. 10 μl of ^{32}P-labeled S13 *Hind* or Hae_{III} fragment in 6 m*M* Tris–HCl (pH 7.6), 6 m*M* $MgCl_2$, 6 m*M* mercaptoethanol, 6 m*M* NaCl, and 5–10 μl of endonuclease *Hind* or Hae_{III} in a final volume of 40 μl were incubated at 37° for 3 to 12 hr. The solution was evaporated under vacuum to approximately 10 μl; 1 μl of bromophenol blue dye added as marker and layered on a 20 × 20 × 0.3 cm, 4 or 8% polyacrylamide slab gel. Electrophoresis was at 75 mA per gel for 10 hr and the gel analyzed by autoradiography. (a) 4% acrylamide gel of S13 RF I DNA digested with endonucleases *Hind*, *Hind* + Hae_{III} and Hae_{III}, respectively. The patterns show that *Hind* 2, 4a, 5ab, 7, 8, and 9 and Hae_{III} 5, 7, and 9 are not digested by the opposite enzyme (see text). (b) 4% acrylamide gel of S13 Hae_{III} 1 and S13 Hae_{III} 3 after partial digestion by endonuclease *Hind* (see text). (c) 4% (left side) and 8% (right side) polyacrylamide gels of *Hind* fragments and Hae_{III} fragments after total digestion with the opposite enzyme. The *middle gel* (*right side*) is a total digest of S13 RF I DNA by endonucleases *Hind* + Hae_{III} *for reference purposes*. The *shortest cross-*digest product HH20 ran off the end of the gel. Gels (a), (b) and "total *Hae* of *Hind* 1, 3, and 4" in (c) were the same slab gel. The right side of (c) were all from the same gel. The total *Hind* of Hae_{III} 1, 2, 3, and 4 were from the same gel and have been lined up with (a) and (b) for clarity.

TABLE 4

REDIGESTION OF S13 *Hind* FRAGMENTS BY Hae_{III}

Fragment	Size[a]	Product with Hae_{III}[b]	Product size[a]	Pyrimidine oligonucleotide[c]
Hind 1	1065	HH470 (Hae_{III} 2)	470	C_4T_3, C_4T_4, C_4T_5
		2 × Hae_{III} 5	2 × 270	C_6T, C_4T_3
		HH50 (Hae_{III} 8)	50	
			1060	
Hind 2	963	No hydrolysis	963	C_5T, C_3T_5, C_2T_6, C_4T_4, T_7
Hind 3	721	HH510 (Hae_{III} 1)	510	T_6, C_4T_2, C_3T_5
		HH230 (Hae_{III} 2)	230	(C_6T_3), C_4T_7
			740	
Hind 4a	314	No hydrolysis	314	C_4T_4, C_3T_6
b		HH210 (Hae_{III} 6)	210	
		HH100 (Hae_{III} 3)	100	T_6, CT_6
			310	
c		Hae_{III} 7	170	
		HH110 (Hae_{III} 1)	110	C_6T_4
		HH30 (Hae_{III} 4)	30	C_5T
			310	
Hind 5a,b	279	No hydrolysis	279	C_4T_3, C_2T_6, C_3T_8
c		HH270 (Hae_{III} 4)	270	C_4T_4, (C_2T_7)
		HH20 (Hae_{III} 6)	20	(C_5T_4)
			290	
Hind 6	192	Hae_{III} 9	73	C_2T_5
		HH70 (Hae_{III} 3)	70	
		HH57 (Hae_{III} 8)	57	
			200	
Hind 7	192	No hydrolysis	143	C_2T_8
Hind 8	78	No hydrolysis	78	C_5T_6
Hind 9	59	No hydrolysis	59	C_2T_6

[a] Molecular size in base pairs was estimated from relative mobility in polyacrylamide gels.

[b] The origin of fragments is denoted by the originating fragment in parentheses. Only HH products of complete digestion are listed.

[c] Pyrimidine oligonucleotides in parentheses are preliminary assignments.

The final order of all S13 *Hind* and S13 Hae_{III} fragments is given in Fig. 5. To confirm the order all S13 *Hind* and S13 Hae_{III} fragments were subjected to pyrimidine cluster analysis using ionophoresis homochromatography. Since most of the pyrimidine oligonucleotides of S13 which are longer than six nucleotides occur only once or twice per DNA molecule (Cerny *et al.*, 1969), an S13 *Hind* and S13 Hae_{III} fragment containing the same long unique pyrimidine oligonucleotides should be in the same area of the physical map. The results of the analysis of each fragment are given in Tables 4 and 5 and confirm the map shown in Fig. 5.

Relationship of the Hind + Hae_{III} fragment maps of S13 and φX174 DNAs. Bacteriophages S13 and φX174 have identical gene orders in their genetic maps (Jeng *et al.*, 1970). To correlate the restriction enzyme fragment map of S13 DNA with the genetic map, data from φX174 DNA restriction enzyme fragment and genetic

TABLE 5

REDIGESTION OF S13 Hae_{III} FRAGMENTS BY ENDONUCLEASE *Hind*

Fragment	Size[a]	Product with *Hind*[b]	Product size	Pyrimidine Oligonucleotides[c]
Hae_{III} 1	1690	*Hind* 2	963	C_5T, C_3T_5, C_2T_6, C_5T_4, T_7
		HH510 (*Hind* 3)	510	T_6, C_5T_2, C_3T_5
		Hind 7	143	C_2T_8
		HH110 (*Hind* 4c)	110	C_6T_4
		Hind 9	59	C_2T_6
			1785	
Hae_{III} 2	1025	HH470 (*Hind* 1)	470	C_4T_3, C_4T_4, C_4T_5
		Hind 4	314	C_4T_4, C_3T_6
		HH230 (*Hind* 3)	230	C_4T_7, (C_6T_3)
			1014	
Hae_{III} 3	863	2 × *Hind* 5	2 × 279	C_4T_3, C_2T_6, C_3T_8
		HH100 (*Hind* 4b)	100	T_6, CT_6
		Hind 8	78	C_5T_6
		HH70 (*Hind* 6)	70	
			806	
Hae_{III} 4	298	HH270 (*Hind* 5c)	270	C_4T_4, (C_2T_7)
		HH30 (*Hind* 4c)	30	C_5T
			300	
Hae_{III} 5a,b	270	No hydrolysis	270	C_6T, C_4T_3, C_5T_3
Hae_{III} 6	218	HH210 (*Hind* 4b)	210	
		HH20 (*Hind* 5c)	20	(C_4T_4)
			230	
Hae_{III} 7	170	No hydrolysis	170	C_5T_3
Hae_{III} 8	107	HH57 (*Hind* 6)	57	(C_4T_5)
		HH50 (*Hind* 7)	50	
			107	
Hae_{III} 9	73	No hydrolysis	73	C_2T_5

[a] For explanation see footnotes to Table 4.

maps were used. It was necessary to determine first the extent of the differences between the two DNAs. A mixture of ^{32}P-labeled S13 RF I DNA and ^{3}H-labeled ϕX174 RF I DNA (a gift of Drs. K. Bartok and D. T. Denhardt) was hydrolyzed separately with endonucleases *Hind* and Hae_{III} and each hydrolysate was separated on polyacrylamide gels. The individual electrophoretic patterns obtained are shown in Figs. 6a and b. The peak numbers refer to ϕX174 *Hind* and ϕX174 Hae_{III} fragments as reported by Edgell *et al.* (1972), and Middleton *et al.* (1972). The S13 RF I DNA *Hind* hydrolysate contained a fragment larger than any observed in the ϕX174 RF I DNA hydrolysate (S13 *Hind* 1, Fig. 1a) and a smaller fragment (see insert Fig. 6a) which had no corresponding fragment in ϕX174 RF I DNA (S13 *Hind* 8, Fig. 1a). Fragments corresponding to ϕX174 *Hind* 3, 4, and 5 were absent in the S13 RF I hydrolysate. S13 *Hind* 5 comprised three *Hind* fragments (Fig. 2) whereas the corresponding ϕX174 *Hind* 7 contained two *Hind* fragments (Edgell *et al.*, 1972). The S13 RF I DNA Hae_{III} hydrolysate differed from the ϕX174 RF I DNA Hae_{III} hydrolysate by the absence of fragments equivalent to ϕX174 Hae_{III} 1 and ϕX174 Hae_{III} 4

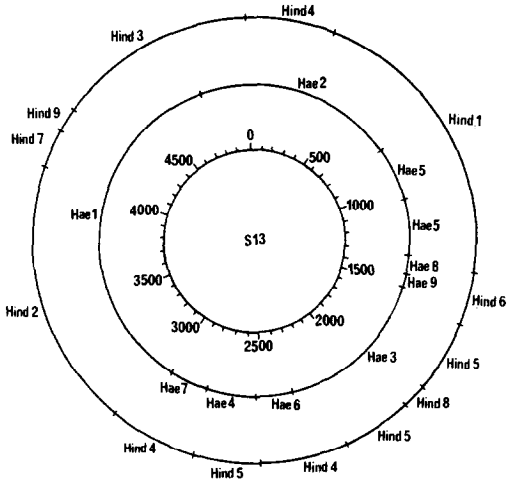


FIG. 5. Physical map of the fragments produced by endonucleases *Hin*d and Hae_{III} from S13 RF I DNA. Inner circle is the S13 DNA molecule divided into 5000 base pair units. The middle circle represents the map of the endonuclease Hae_{III} fragments, the outer circle the map of the endonuclease *Hin*d fragments.

but a larger band S13 Hae_{III} 1, equivalent in size to the sum of ϕX174 Hae_{III} 1 and ϕX174 Hae_{III} 4 was present (Fig. 6b). Correlation of this data with the genetic map is presented in Fig. 7.

DISCUSSION

Endonucleases *Hin*d and Hae_{III} cleave bacteriophage S13 RF I DNA into 13 and 10 fragments, respectively. The sizes of S13 *Hin*d 1 and 2 determined from electron micrographs and S13 *Hin*d 7 and S13 Hae_{III} 8 by pyrimidine isostich analysis correlated very well with sizes calculated from integration of the radioactivity of the fragments separated by gel electrophoresis (Fig. 2, Table 3).

Comparison of the endonuclease *Hin*d products of S13 RF I DNA with those from ϕX174 RF I DNA (Fig. 6a) revealed that the same number of fragments are produced from the two DNAs but only S13 *Hin*d 2, 3, 4, 5, 6, 7, and 9 comigrate with ϕX174 *Hin*d 1, 2, 6, 7, 8, 9, and 10, respectively. Comparison of the endonuclease Hae_{III} products of S13 RF I DNA with those from ϕX174 RF I DNA (Fig. 6b) revealed that one fragment less is produced from S13 RF I DNA and S13 Hae_{III} 2, 3, 4, 5, 6, 7,

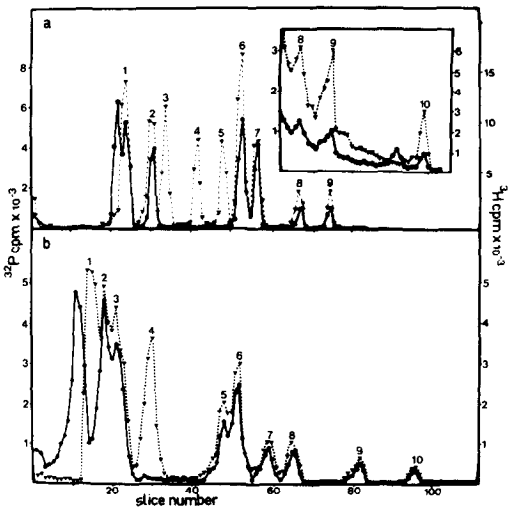


FIG. 6. Polyacrylamide gel electrophoresis of ^{32}P-labeled S13 and ^{3}H-labeled ϕX174 RF I DNA fragments produced by (a) endonuclease *Hin*d and (b) endonuclease Hae_{III}. Twenty micrograms of ^{32}P-labeled S13 RF I DNA and 20 μg (a) or 50 μg (b) of ^{3}H-labeled ϕX174 RF I DNA were treated as described in Fig. 1. In Fig. 5a the larger fragments were separated on a 3% gel the smaller fragments (insert) on a 5% gel. In Fig. 5b the fragments were separated on a 4% gel. Slices were 2 mm thick. Fragment numbers refer to ϕX174 *Hin*d (a) or ϕX174 Hae_{III} (b), respectively. (●–●–●), ^{32}P-labeled S13 RF I DNA. (- - -▼- - -▼- - -), ^{3}H-labeled ϕX174 RF I DNA.

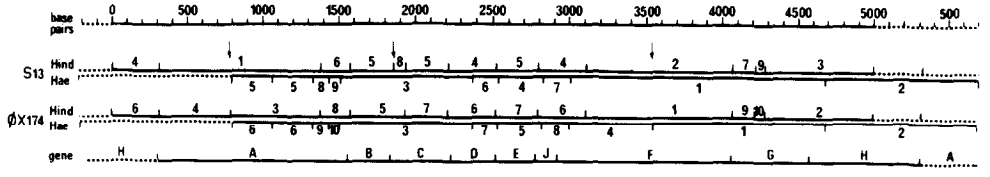


FIG. 7. Comparative map of S13 and ϕX174 *Hin*d and Hae_{III} fragments and correlation with the genetic map. The genetic map is approximate and referred to by the letters A to H (Jeng *et al.*, 1970). The data on ϕX174 DNA are a composite of studies by Chen *et al.* (1973), Lee and Sinsheimer (1974) and Johnson and Sinsheimer (1974). The arrows indicate the regions where differences in the *Hin*d and Hae_{III} sites occur in S13 RF I DNA. The dotted lines are representational of the circularity of the maps.

8, and 9 comigrate with φX174 *Hae*III 2, 3, 5, 6, 7, 8, 9, and 10, respectively. The other fragments produced by the two enzymes are unique to the two different phages.

Examination of the log molecular weight versus mobility plot of the S13 *Hin*d and S13 *Hae*III fragments (Fig. 2) indicates that the smaller *Hin*d fragments do not fit a straight line relationship. This has also been observed with φX174 *Hin*d 8 and 9 in a 5% gel by Johnson and Sinsheimer (1974), who suggested that this may be due to differences in thymine content in the smaller fragments. However, φX174 *Hin*d 8 and 9 comigrate with S13 *Hin*d 6 and 7 in a 3% gel (Fig. 6a) and fit the straight line in Fig. 2 as do the similar sized fragments S13 *Hae*III 6 and 7, whereas S13 *Hin*d 8 and 9 fall below the line and S13 *Hae*III 8 and 9 on the line. The mobility of the smaller fragments is possibly affected by the base composition and nucleotide sequence of the fragments and the gel composition. These data indicate that caution must be exercised in the interpretation of results based on the use of small restriction fragments as size markers in gel electrophoretic separations.

The gene orders of the genetic maps of S13 and φX174 are identical (Jeng *et al.*, 1970) and the physical maps of the φX174 *Hin*d and φX174 *Hae*III fragments have been correlated with the genetic map (Chen *et al.*, 1973; Lee and Sinsheimer, 1974; Johnson and Sinsheimer, 1974). Assuming that the comigrating S13 and φX174 restriction endonuclease fragments correspond to the same parts of the respective DNA molecules, the *Hin*d-*Hae*III fragment map of S13 DNA (Fig. 7), deduced from cross-digests and pyrimidine cluster analyses is identical to the *Hin*d-*Hae*III fragment map of φX174 DNA with three exceptions: In the gene A and gene F region one *Hin*d and one *Hae*III restriction endonuclease site in φX174 DNA have no corresponding site in S13 DNA, while in the gene B region one *Hin*d site in S13 DNA has no corresponding site in φX174 DNA (Fig. 7).

The *Hin*d endonuclease recognizes a sequence of six base pairs (Kelly and Smith, 1970) and *Hae*III endonuclease a sequence of four base pairs (Middleton *et al.*, 1972) and the combined *Hin*d, *Hae*III enzymes from this study recognize 118 base pairs in S13 DNA. Thus, the data show that from 3 to 16 base pairs may be different in S13 DNA compared to φX174 DNA and 102–115 base pairs are identical in the two phage DNAs. Data from our laboratory (Harbers *et al.*, 1976) indicate that the differences in some other sequences of φX174 and S13 are very slight. Recently, Hayashi and Hayashi (1974) have compared the fragments released by the *Hin* H and *Hap* restriction nucleases from φX174 and S13 RF I DNAs and have shown more extensive differences than the ones described here with the *Hin*d and *Hae*III endonucleases. With the *Hap* endonuclease minimally two out of seven sites are different in S13 DNA compared to φX174 DNA and with *Hin* H endonuclease five out of seven sites are changed. The size of the sequence recognition site of *Hap* is four base pairs which means that from two to eight out of 28 base pairs are changed in S13 DNA compared to φX174 DNA. The size of the recognition site of *Hin* H is unknown.

A comparative study of S13 and φX174 DNAs by heteroduplex mapping (Godson, 1973) showed that differences occur in many places in the two phage DNAs with an average 36 out of 100 base pairs mismatched. Only one area of each genome was highly complementary to the other. The distribution of restriction endonuclease sites and long pyrimidine oligonucleotides described in this paper and the *Hin* H-*Hap* physical map described by Hayashi and Hayashi (1974) present defined sequences around the S13 and φX174 genomes with at least one defined sequence per cistron. Also the distribution of the long pyrimidine oligonucleotides indicates that there are no regions in which pyrimidines are clustered. Comparison of the restriction endonuclease defined sequences shows only two differences in the gene A–B region and several changes in the gene F–H regions of the genetic map of S13 compared to that of φX174. The total number of base pair changes revealed by the *Hin*d-*Hae*III restriction sites taken together with the previous study of pyrimi-

dine oligonucleotide sequences (Harbers *et al.*, 1976) of the two DNAs indicate less than a 36% base mismatch between the two genomes. Comparison of longer sequences from the two DNAs is necessary for definitive conclusions on base sequence similarity between the DNAs, since the sequences reported here may be highly conserved. On the other hand, heteroduplex mapping studies allow only a gross comparison between DNA sequences because nonhydrogen-bonded stretches may contain some relatively short homologous base sequences and hydrogen-bonded stretches some mispaired bases.

Note added in proof. S13 RF I DNA is not cleaved by $Hind_{III}$ (Goodchild and Spencer, unpublished results), thus the *Hin*d digests described in this paper are all by $Hind_{II}$.

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