NUCLEOTIDE SEQUENCES AT THE CLEAVAGE SITES OF TWO RESTRICTION ENDONUCLEASES FROM HEMOPHILUS PARAINFLUENZAE

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SUMMARY: The nucleotide sequences at the cleavage sites of two restriction endonucleases from Hemophilus parainfluenzae, Hpa I and Hpa II, have been determined. Terminal labeling at both the 3'- and 5'- termini was used to show that the Hpa I enzyme cleaves the sequence

5'. . .N - G - T ↓ p A - A - C - N. . .3'
3'. . .N - C - A - A p ↑ T - T - G - N. . .5'

and the sequence cleaved by the Hpa II enzyme is

5'. . .N - C ↓ p C - G - G - N. . .3'
3'. . .N - G - G - C p ↑ C - N. . .5'

where the arrows indicate the points of strand scission.

INTRODUCTION. Hemophilus parainfluenzae contains two restriction endonucleases, designated Hpa I and Hpa II*, which have different specificities (1,2,3). Hpa I makes three double-strand cleavages and Hpa II one double-strand scission in simian virus 40 (SV40) DNA. For all other DNA's tested Hpa II makes more breaks than Hpa I (2). We have made use of these differences to determine the nucleotide sequences at the cleavage sites of the two endonucleases.

EXPERIMENTAL PROCEDURES. The restriction enzymes of H. parainfluenzae were prepared from sonicated cells according to the procedure of Smith and Wilcox (4) or by the method of Sharp et al. (2). For most of the studies reported here, the enzyme solution was a mixture of the Hpa I and Hpa II activities and a contaminating exonuclease (2). The fact that it was possible to obtain unambiguous results from 5'-

*Abbreviations. The abbreviations for restriction endonucleases are those proposed by Smith and Nathans (3). The symbols A, C, G, T, and N without prefixes represent deoxynucleosides.

labeling experiments (see below) indicates that the contaminant was an exonuclease that worked in the 3'- to 5'- direction. In the experiments designed to determine the nucleotides at the 3'- ends of the cleavage sites, purified preparations of Hpa I and Hpa II free of exonuclease and nonspecific endonuclease activities were used (2).

The basic strategy employed for the nucleotide sequence analyses of the cleavage sites of the Hpa enzymes was similar to the schemes used in analyses of other restricition specificities (5,6,7). The specificity of Hpa II was investigated by digesting bacteriophage λ DNA (5) to completion with excess enzyme in 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, and 10 mM β-mercaptoethanol (3-4 hours, 37°C). Hpa I was studied by hydrolyzing SV40 DNA (6) in the same buffer also containing 0.1 M KCl to inhibit Hpa II (2). Samples were dialyzed overnight against 50 mM Tris-HCl, pH 8.5, 5 mM EDTA and then treated with bacterial alkaline phosphatase (Worthington BAPF; 1-2 units/ml, 1 hour, 37°C). The digestion mixtures were extracted with phenol, dialyzed against 0.15 M NaCl-1 mM EDTA, and precipitated from 75% ethanol. Digests were assayed for completeness by electrophoresis in 1.2% Agarose gels (2).

RESULTS. The phosphorylation of the Hpa cleavage products with or without prior treatment with bacterial alkaline phosphatase was examined by means of the polynucleotide kinase reaction (5). As controls, intact λ and SV40 DNA's, also with and without phosphatase treatment, were labeled by the kinase reaction. Total acid-insoluble ^{32}P-radioactivity was measured (6). Four-fold increases in precipitable counts were observed when Hpa-generated fragments were treated with phosphatase prior to labeling (data not shown). This and the observation that terminal deoxynucleotidyl transferase (8) catalyzed nucleotide polymerizations equally well on the 3'- termini of Hpa fragments regardless of prior dephosphorylation (data not shown) indicate that both Hpa endonucleases produce 5'-phosphates and 3'- hydroxyls at the cleavage sites, as do other restriction enzymes.

Fragments terminally labeled with ^{32}P at the 5'- ends were completely digested to mononucleoside 5'-monophosphates. After electrophoretic separation the radioactivity in each mononucleotide was measured (9). The results of this analysis (Table I) show clearly that all Hpa I fragments have pA at their 5'- ends while Hpa II

TABLE I. 5'-Terminal Residues of Hpa I and Hpa II Cleavage Products.

Enzyme	DNA	Mole Percent Nucleotides at 5'-Termini			
		pC	pA	pG	pT
Hpa I	SV40	1.2	96.7	0.8	1.3
Hpa II	λ	86.5	6.5	3.2	3.8

Dephosphorylated DNA fragments produced by Hpa I and Hpa II digestions were treated for 3-4 hours at 37°C with 30 units per ml of polynucleotide kinase (P-L Biochemicals) in 0.07 M Tris-HCl, pH 7.4, 0.01M $MgCl_2$, 0.014M β-mercaptoethanol, containing 0.1 mM [γ-^{32}P]rATP at about 15 m C i/μ mole (12). EDTA and sodium phosphate (to 20 mM each) were added to stop the reactions and the mixtures were passed through a 1.2 x 40 cm column of Sephadex G-75 in 0.1 M NaCl to separate the ^{32}P-labeled fragments from unreacted ATP. The first peak eluted from the column was collected and the DNA precipitated with ethanol. The terminally labeled fragments were reduced to small 5'- phosphorylated oligomers with pancreatic DNase (Worthington; 0.1 mg/ml in 0.01 M Tris-HCl, pH 7.5, 0.01 M $MgCl_2$, for 2 hours at 37°C). One-tenth of the DNase digest was hydrolyzed completely to mononucleoside 5'- monophosphates by addition of snake venom phosphodiesterase (Worthington) to 0.1 mg/ml (3 hours at 37°C). The resultant mononucleotides, along with 5'- mononucleotide markers, were fractionated by electrophoresis at pH 3.5 on Whatman 540 paper (9) and located by radioautography. The radioactive spots coincided with the ultraviolet markers and were measured for ^{32}P-activity by liquid scintillation counting. Counting times were adjusted to give better than 5% accuracy for each mononucleotide.

fragments have pC. Restriction endonuclease-digested DNA fragments were labeled at their 3'-termini by polymerization of a single [α-^{32}P] deoxynucleoside triphosphate using terminal deoxynucleotidyl transferase (8). The products, fragments with [α-^{32}P]- labeled deoxynucleotide homopolymers linked to their 3'-ends, were

TABLE II. 3'-Terminal Residues of Hpa I and Hpa II Cleavage Products.

Enzyme	DNA	[α-^{32}P] dNTP Polymerized	Mole % Nucleotides in 3'-Polymers			
			Cp	Ap	Gp	Tp
Hpa I	SV40	dATP	0.3	96.9	0.2	2.6
		dGTP	0.6	1.0	90.8	7.6
Hpa II	λ	dGTP	4.7	0.7	93.4	1.2
		dTTP	2.5	0.2	0.7	96.6

Hpa digestion products, without prior phosphatase treatment, were denatured for 10 minutes at room temperature with the addition of 0.1 volume of 1N NaOH. 2.5 volumes of water were added and the solution was neutralized with 0.3 volumes of 2 M Tris, pH 7.2, and made 0.2 M in sodium cacodylate and 2 mM in β-mercaptoethanol. Separate reactions were carried out with three [α-^{32}P] deoxynucleoside triphosphates: 0.1 mM dGTP (10 mCi/μmole), 0.1 mM dTTP (20 mCi/μmole), and 1 mM dATP (1 mCi/μ mole) (New England Nuclear). Divalent cation concentrations were 7 mM $MgCl_2$ for dATP and dGTP reactions and 3 mM $CoCl_2$ with dTTP. In addition, 40 mM KCl was included in the dTTP reaction (8). Polymerizations were for 4 hours at 37^{o}C with 100 units/ml of terminal deoxynucleotidyl transferase (P-L Biochemicals). Reactions were stopped and labeled fragments freed of unreacted materials as described for the 5'- labeled products (Table I). The 3'- terminally labeled compounds were digested to mononucleoside 3'- monophosphates for nearest neighbor analysis by consecutive 2 hour reactions at 37^{o}C with micrococcal nuclease (1 mg/ml) and spleen phosphodiesterase (7 units per ml) both from Worthington, and both in 0.1 M Tris-HCl, pH 8.5, 0.01 M $CaCl_2$. The mononucleotides were coelectrophoresed with appropriate 3'- mononucleotide markers at pH 3.5 on Whatman 3MM paper (9) and located radioautographically. Quantitation was by liquid scintillation counting.

digested to mononucleoside 3'- monophosphates (Table II). The majority of the radioactivity (90.8% - 96.9%) appeared in the same mononucleotide as the input [α-^{32}P] deoxynucleoside triphosphate. This is the product of the digestion of the homopolymer. The other labeled mononucleotide resulted from a transfer of ^{32}P from the 5'- position of the [α-^{32}P] deoxynucleoside triphosphate to the 3'- terminus of the DNA fragment. The results (Table II) show that pT-OH occupies the 3'- termini of Hpa I

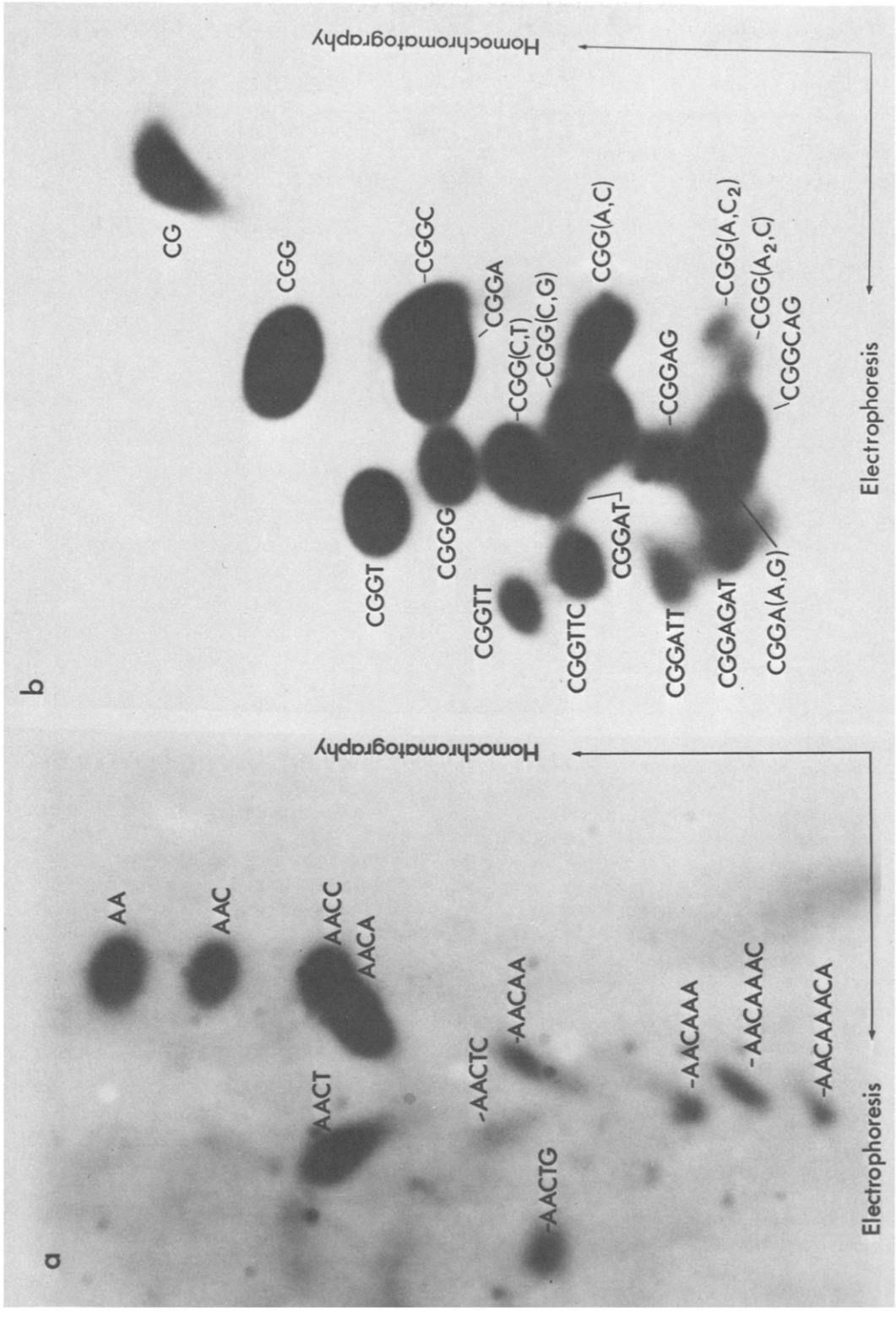
TABLE III. Mobilities of Oligonucleotides Arising from the 5'-Termini of Hpa I and Hpa II Digestion Products.

Enzyme	DNA	Sequence	Mobility Relative to Xylene Cyanol DEAE-cellulose pH 1.9	DEAE-cellulose pH 3.5	AE-cellulose pH 3.5
Hpa I	SV40	pA	2.4	1.8	-
		pApA	2.2	1.0	0.51
		pApApC	2.1	0.7	0.32
Hpa II	λ	pC	2.6	1.9	-
		pCpG	2.0	1.2	0.58
		pCpGpG	0.8	0.4	0.16

Pancreatic DNase digestion products (Table I) were subjected to one-dimensional electrophoresis on either DEAE-cellulose paper (Whatman DE-81) at pH 1.9 (9) or on AE-cellulose paper (Whatman AE-81; gift from K. Murray) at pH 3.5 (10). Other portions of the DNase digest were separated in two dimensions by electrophoresis on cellulose acetate (7M urea, pH 3.5) and DE-81 paper in 7% formic acid (9), and by cellulose acetate electrophoresis and homochromatography (9). All oligomers were further analyzed by means of partial digestion with snake venom phosphodiesterase. The one-dimensional fractionations of the partial digestion products were used to confirm the identities of the compounds listed in this table (10).

breaks and that pC-OH is the 3'- terminal nucleotide on Hpa II fragments.

To determine the complete sequences of the cleavage sites, DNA fragments produced by restriction endonuclease digestion and bearing [5'-^{32}P] phosphate groups, were hydrolyzed to small oligonucleotides with pancreatic DNase. The oligomers were fractionated on a variety of one- and two-dimensional systems and the labeled products were located by radioautography (9). Comparison with published fraction-


a
AA
AAC
AACC
AACA
AACT
-AACTC
-AACAA
-AACTG
-AACAAA
-AACAAAC
-AACAAACA
Homochromatography
Electrophoresis
b
CG
CGG
-CGGC
CGGT
CGGG
CGGA
-CGG(C,T)
-CGG(C,G)
CGGTT
CGGTTC
CGGAT
CGG(A,C)
-CGGAG
CGGATT
CGGAGAT
-CGG(A,C2)
-CGG(A2,C)
CGGA(A,G)
CGGCAG
Homochromatography
Electrophoresis

ation patterns (10) and previous work in our laboratory (5,6) led to the identifications of the sequences at the 5'- ends of the Hpa fragments (Table III, Fig. 1). The electrophoretic mobilities listed in Table III were taken from one-dimensional separations of pancreatic DNase digestion products and from the fractionations of the products of partial digestions with snake venom phosphodiesterase (10) which were carried out on all DNase end-products to determine or confirm their identities. Two-dimensional electrophoretic and homochromatographic (9) analyses were also performed on the DNase digestion products. The use of homochromatographic fractionation (Fig. 1) is advantageous in that the positions of products in a series having a common terminus is indicative of their sequences (11). Further analysis was, of course, necessary to confirm the sequences, and those shown in Fig. 1 were deduced from snake venom phosphodiesterase partial digestion products of each oligonucleotide (10).

As seen in Fig. 1, the uniqueness of the sequences at the cleavage sites of the two Hpa endonucleases terminates at the third nucleotide from the 5'- end; the sequences become degenerate thereafter. Combining all results the structures of the restriction sites diagrammed in the "Summary" were deduced.

DISCUSSION. The nucleotide sequences at the cleavage sites of the restriction en-

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Fig. 1. Homochromatographic fractionations of the products of pancreatic DNase digestions of [5'-^{32}P]-labeled Hpa I fragments from SV40 DNA (a) and Hpa II fragments from bacteriophage λ DNA (b). The first dimension was electrophoresis on cellulose acetate in 7M urea at pH 3.5, and the second dimension was thin-layer homochromatography in "homomixture C" made with a 3% solution of 15-minute hydrolyzed RNA (BDH) (9). The sequences of molecules larger than trimers were deduced both from their positions on the fractionation patterns (11) and the results of partial digestion with snake venom phosphodiesterase. The partial hydrolyses were carried out at room temperature with 0.01 mg/ml of the phosphodiesterase in 0.02 M Tris-HCl, pH 8.5, 0.01 M $MgCl_2$ for 60-90 minutes. Sample aliquots were taken at zero time (control) and every 15 minutes thereafter. The partial digestion products were separated electrophoretically on DE-81 paper at either pH 3.5 or pH 1.9. Identification of the products was made on the basis of absolute and relative (M-values) electrophoretic mobilities (10). All of the sequences have [5'-^{32}P] phosphate groups. Symbols for all phosphates have been omitted from the figure.

donucleases from H. parainfluenzae have been analyzed by 5'- end labeling procedures and a novel use of a 3'- end labeling scheme (8,13). The combined data unequivocally establish the sequences. These are recognizable as being the same as those cleaved by the enzymes from two other strains of Hemophilus. The Hpa I sequence is the same as one of the specificities of Hind II from H. influenzae (3,14) and the sequence cleaved by Hpa II is identical to the one split by the H. aphirophilus restriction endonuclease (15). Mapping studies of restriction enzyme cleavage sites on SV40 DNA have placed two of the Hpa I breaks (Hpa 1 and Hpa 2) at positions coincident with two of the Hind II splits (Hin 3 and Hin 5), and have localized the third Hpa I scission close to the Hin 8 cleavage point (2,16). The identity of the two sequences proves that Hpa 1 and Hpa 2 are identical to Hin 3 and Hin 5, respectively, and, moreover, establishes Hpa 3 as the same site as Hin 8. This also means that the sequences at sites Hin 1 and Hin 2 are probably the double-stranded equivalents of the sequence . . .N-G-T-C-G-A-C-N. . . (3,14). An earlier finding that H. parainfluenzae restriction enzyme generates fragments from the replicative form of ϕX174 DNA bearing 5'- deoxycytidylate residues (17) is verified by the sequence specificity of Hpa II and shows that Hpa II makes the majority of breaks in that particular substrate.

The double-strand scission produced by Hpa I is an "even" break while that made by Hpa II is a "staggered" break (14). Nonetheless, both of the double-stranded structures at the cleavage sites contain centrally-located dyad axes of symmetry. The possible significance of this symmetry has previously been discussed (14, 18).

Results essentially identical to those reported here have been obtained by K. Murray using different methods (personal communication).

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