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Production of Specific Fragments of φX174 Replicative Form DNA by a Restriction Enzyme from *Haemophilus parainfluenzae*, Endonuclease HP

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A restriction endonuclease from *Haemophilus parainfluenzae* degrades φX174 replicative form DNA into eight specific fragments, ranging from 1,700 to 150 base pairs and terminated specifically by deoxycytidylic acid.

Several bacterial restriction deoxyriboendonucleases have been described recently which produce unique fragments from high-molecular-weight foreign DNA, are not associated with a known modification activity, and require no special cofactors other than divalent metal ions (3, 6, 8). These enzymes will be very useful for studying DNA structure and function. We are interested specifically in the use of sequence-specific deoxyriboendonucleases for the isolation and nucleotide sequence determination of the initiation sites for DNA replication and transcription, and for the analysis of φX174 replicating intermediates.

Endonuclease HP was isolated from cultures of *Haemophilus parainfluenzae* (strain originally from Sol Goodgal and G. Leidy) by using a modification of the procedure developed by Smith and Wilcox (8) for the purification of endonuclease R from *H. influenzae*. The enzyme eluted from a Biogel column (2.5 by 48 cm) between volumes of 180 and 230 ml. Fractions containing endonuclease activity and $A_{230}/A_{260} > 3$ were pooled. After the ammonium sulfate precipitation (8), the enzyme preparation was desalted by chromatography on Sephadex G-25. The enzyme was then eluted from a phosphocellulose column at 0.2 M KCl.

Contaminating exonuclease activity present at this stage of the purification was removed by chromatography on DEAE-cellulose (Whatman DE52). For this purpose, a column was equilibrated with 0.01 M potassium phosphate buffer, pH 7.4, and the enzyme was applied at 10 mg of protein per ml of column bed volume. Elution was stepwise with three column volumes each of 0.05 M, 0.1 M, and 0.2 M NaCl in 0.01 M potassium phosphate, pH 7.4. Endonuclease HP eluted at 0.05 M NaCl, whereas the exonuclease activity eluted after 0.1 M NaCl. The DEAE fractions were stored at 4 C until used. Endonuclease R was purified as described by Smith and Wilcox (8) and did not include DEAE-cellulose chromatography.

^{3}H-φX174*am3* replicative form (RFI) DNA was isolated from chloramphenicol-treated cells and purified by Biogel chromatography, equilibrium centrifugation in CsCl-ethidium bromide density gradients, and sedimentation in isokinetic neutral sucrose gradients. Details of the isolation procedure will be described elsewhere.

Electrophoresis of DNA fragments was performed in 5% polyacrylamide gels (1 by 20 cm) formed in Plexiglas tubes. The buffer and methods used were those described by Loening (5) plus 0.1% (wt/vol) SDS. Electrophoresis was performed at room temperature at a constant potential of 45 V; running time was 1 h per cm of gel length. To quantitate the radioactivity, gels were frozen and cut into 1-mm segments by using the Mickle gel slicer (Brinkman Instruments). Gel slices were solubilized with toluene-Liquifluor containing 10% NCS (Amersham-Searle), and the radioactivity was measured in a Beckman liquid scintillation counter.

End-group determination was performed as described in the legend to Table 2. Polynucleotide kinase was purified from T4 phage-infected cells by using Richardson's original procedure (7) modified by employing DNA-cellulose and Biogel exclusion chromatography. Details will be published elewhere.

Figure 1 shows the fractionation of an endonuclease HP limit digest of φX174 RF DNA by 5% polyacrylamide gel electrophoresis. Improved separation of fragments P1 and P2 can be obtained by using gels of lower acrylamide concen-

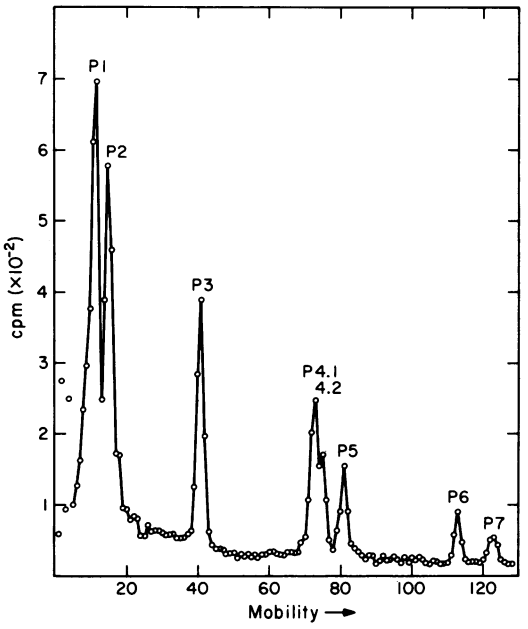

FIG. 1. *Polyacrylamide gel electrophoresis of φX174 RF DNA fragments produced by endonuclease HP. The reaction mixture consisted of 0.1 μg of ^{3}H-RFI, 7mM Tris, 7 mM mercaptoethanol, 50 mM $MgCl_2$, pH 7.4; total volume, 100 μliters. A 20-μliter amount of endonuclease HP was added at zero time and again at 2 h. Digestion was at 37 C for 10 h. To stop the reaction, EDTA was added to 0.01 M. Sucrose and sodium dodecyl sulfate were added to 5 and 0.5% final concentrations, respectively. The mixture was incubated at 37 C for 10 min and layered on a 5% polyacrylamide gel column (1 by 20 cm). Electrophoresis was as described in the text. Mobility is the distance of migration in millimeters at the end of the run.*

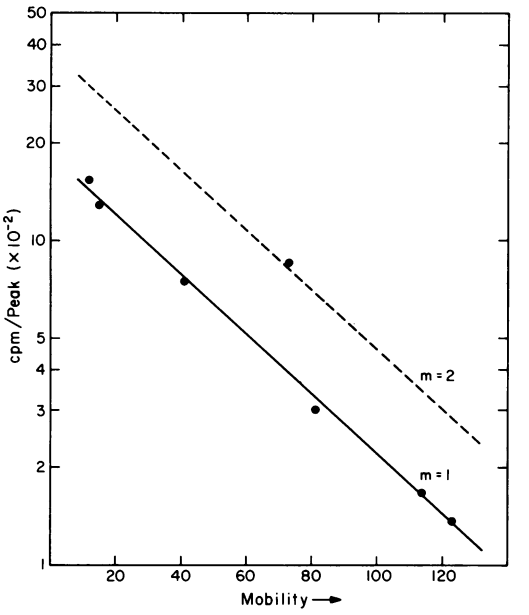

FIG. 2. *Relationship between mass and electrophoretic mobility for φX174 RF DNA fragments produced by cleavage with endonuclease HP. The mass is assumed to be proportional to the total number of counts in each peak and is plotted against the distance migrated during the experiment (mobility). The parallel line (---) is that calculated for a peak with twice the mass; the point on this line represents P4.*

tration. The positions and proportions of the peaks do not change with increasing incubation time or increasing enzyme concentration, indicating that a limit digest of the DNA has been produced.

Figure 2 shows that there is a linear relationship between the logarithm of the integrated counts in each peak (Fig. 1) and the electrophoretic mobility. Fragment P4 contains twice the mass expected, suggesting the presence of two distinct fragments of similar size. A partial separation of these two fragments can be obtained with longer electrophoresis time.

Table 1 shows the size of the endonuclease HP fragments calculated from the fraction of total counts present in each peak. It has been demonstrated that the molecular weights of double-stranded DNA fragments determined by mobility during polyacrylamide gel electrophoresis are in good agreement with values determined by electron microscopy and sedimentation velocity analysis (1, 2). The size and number of fragments produced by endonuclease HP are distinct from those produced by the restriction endonuclease from *H. influenzae*, endonuclease R. We have found that the latter enzyme produces at least 12 specific fragments from φX174

TABLE 1. *Size of φX174 RF DNA fragments produced by cleavage with endonuclease HP*

Fragment no.[a]	Nucleotide pairs[b]
P1	1,670
P2	1,400
P3	825
P4.1, 4.2	470
P5	330
P6	180
P7	150

[a] Fragment numbers correspond to those indicated in Fig. 1.

[b] The number of nucleotide pairs is calculated by assuming that the total is equal to 5,500 and that mass is proportional to the total number of counts in each peak.

TABLE 2. *A comparison of the 5'-terminal nucleotides of φX174 RF DNA fragments produced by endonuclease HP and endonuclease R*[a]

Substrate	Restriction enzyme	Mol % nucleotide at 5' ends			
		A	T	G	C
φX174 RFI	Endo HP	<1	<1	<1	97
φX174 RFI	Endo R	55	6	35	4
T7[b]	Endo R	60	0	40	0
Lambda		44	7	45	4

[a] φX RF DNA (>98% form I) was degraded by endonuclease HP in a reaction mixture consisting of 7 mM Tris (pH 7.5), 7 mM mercaptoethanol, 50 mM $MgCl_2$, 50 mM NaCl, 12 μg of DNA, 125 μliters enzyme (0.1–0.5 units); total volume, 150 μliters. Incubation was at 37 C for 14 h. The reaction with endonuclease R was the same except the $MgCl_2$ concentration was 7 mM. The reaction was stopped by incubation at 0 C with twofold excess EDTA. The reaction mixture was adjusted to 0.1 M Tris (pH 8.0) and 1 unit of alkaline phosphatase (Worthington; purified free from endonuclease activity) per ml in a volume of 200 μliters, and incubated at 37 C for 45 min. The phosphatase was inactivated by addition of ethylene-glycoltetraacetic acid to 6.5 mM followed by incubation for 7 min at 65 C (Theodore Live, personal communication). The reaction volume was adjusted to 300 μliters and 10 mM $MgCl_2$, 20 mM mercaptoethanol, 1.7 mM potassium phosphate, 70 mM Tris (pH 8.0), 15 nmol of $\gamma AT^{32}P$ per ml (20 mCi/μmol, ICN), and 80 units of polynucleotide kinase per ml. The reaction mixture was incubated at 37 C for 1 h and stopped by a final concentration of 50 mM EDTA and pyrophosphate. The labeled DNA was separated from the unreacted ATP by exclusion chromatography on a porous, glass bead column (1 by 38 cm; Sigma G-240-250, approximate exclusion limit 100,000) and precipitated at −20 C for 12 h by two volumes of isopropanol and 0.3 M sodium acetate, pH 5.5. The precipitate was collected by centrifugation and dissolved in 100 μliters of 25 mM Tris (pH 7.5), 10 mM $MgCl_2$, and 200 μg of pancreatic DNase I (Worthington WDPC grade) per ml. Incubation was at 37 C for 30 min. A 10-μliter amount of 1 M glycine (pH 9.5) and 20 μg of venom phosphodiesterase (Worthington, purified by the procedure of Sulkowski and Laskowski [9]) were added, and incubation was continued for 30 min. The reaction was stopped by 50 mM EDTA, and the volume was reduced to 10 μliters. The sample was subjected to electrophoresis on a strip of Whatman 3MM paper for 60 to 80 min at 4 kV with a buffer composed of 5% acetic acid, 0.5% pyridine, 10 mM EDTA, pH 3.5. The paper was dried and cut into 1-cm squares, and the radioactivity was measured in toluene-Liquifluor in a Beckman scintillation counter. Lambda DNA was used as a control in these experiments and was treated identically, except the reaction with endonucleases was omitted. The total incorporation of ^{32}P in the endonuclease HP and endonuclease R reactions was approximately the same, ±10%. Control experiments were performed without endonuclease, and there was no significant incorporation.

[b] From Kelly and Smith (4).

RF DNA which range in size from 1,200 to 100 base pairs, in close agreement with the published data of Edgell et al. (2).

Chemical evidence for a difference in the recognition site for endonuclease HP and endonuclease R is presented in Table 2. The nucleotide sequence of the recognition site for endonuclease R was determined to be G T Py ↓ Pu A C (4) where the center dinucleotide is ambiguous and the arrow indicates the point of cleavage. We find consistent results with φX174 RF DNA as substrate for endonuclease R (Table 2). The enzyme preparation had a very low, but detectable, level of exonuclease contamination which might account for the production of 10% pyrimidines. The data indicate that the recognition site for endonuclease HP is different since only deoxycytidylic acid is found at the 5' ends of fragments produced by this enzyme. The presence of a single, unique nucleotide at the 5' ends of these fragments suggests that the recognition site for endonuclease HP may not contain an ambiguous dinucleotide as does the site for endonuclease R; however, we do not regard this demonstration as rigorous until the terminal nucleotides have been analyzed for each fragment.

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