CLEAVAGE SITE SPECIFICITY OF AN ENDONUCLEASE PREPARED FROM *HEAMOPHILUS INFLUENZAE* STRAIN H-I

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1. Introduction

Restriction enzymes which degrade foreign DNA at a limited number of sites have been prepared from several bacterial strains [1–4]. Kelly and Smith explored the specificity of a restriction enzyme prepared from *Haemophilus influenzae* Rd (abbreviated as *Endo Rd*) and showed that this enzyme attacked double-stranded DNA at a specific hexanucleotide sequence [5]. It is therefore possible to cleave DNA molecules at sites with such a sequence [6, 7]. In order to obtain unique fragments from the double closed replicative form DNA (RF-I DNA) of coliphage fd, we examined the action of *Endo Rd* on this DNA molecule. The enzyme however cleaved it only at one site. So we looked for other such enzymes with different cleavage site specificities and found that *H. influenzae* strain H-I contained an endonuclease (abbreviated as *Endo H-I*) which split RF-I DNA at three different sites. The DNA molecule was therefore cleaved into four unique fragments by the combination of these two enzymes. Analysis of the 5′-terminal nucleotides at the cleaved sites suggested that *Endo H-I* attacked DNA at a different nucleotide sequence from that reported for *Endo Rd* by Kelly and Smith [5].

2. Materials and methods

2.1. *Strains and fd DNA*

H. influenzae H-I was a strain isolated at Research Institute for Microbial Diseases, Osaka University. *H. influenzae* Rd was obtained from Dr. J.K. Setlow via Dr. S. Kondo of Osaka University. RF-I DNA and single-stranded DNA(SS DNA) of phage fd were prepared as described previously [8]. For preparation of [^{3}H] RF-I DNA, fd was infected in *E. coli* cells growing in a Tris-glucose medium containing [^{3}H]thymidine and replicated for several generations, before preparing RF-I DNA.

2.2. *Assay of the enzyme activity by transfection*

The enzyme fraction was added to a reaction mixture (0.3 ml) containing 7 mM Tris (pH 7.6), 7 mM $MgCl_2$, 1 mM mercaptoethanol and 0.01 A_{260} unit of RF-I DNA or 0.001 A_{260} unit of SS DNA. After incubation for 30 min at 37°, the mixture was directly added to an assay mixture containing lysozyme-spheroplasts, and infectious DNA was assayed [8].

One unit of the enzyme activity was defined as the activity which digested 0.01 A_{260} unit of RF-I DNA within 30 min at 37°.

2.3. *Polyacrylamide gel electrophoresis of DNA fragments*

0.3 A_{260} unit of RF-I DNA was incubated for 2 to 6 hr at 37° with 10 units of enzyme in 0.2 ml of 7 mM Tris (pH 7.6)–7 mM $MgCl_2$–1 mM mercaptoethanol. The hydrolysate was layered on a 3% gel column (0.6 cm × 12 cm) formed in 0.036 M Tris–0.032 M KH_2PO_4–0.1 mM EDTA (pH 7.8), and electrophoresed for 18 hr at 2 mA/tube. The gel was stained with 0.4% acridine orange, washed with 0.2 N acetic acid, and the color density was traced by a densitometer.

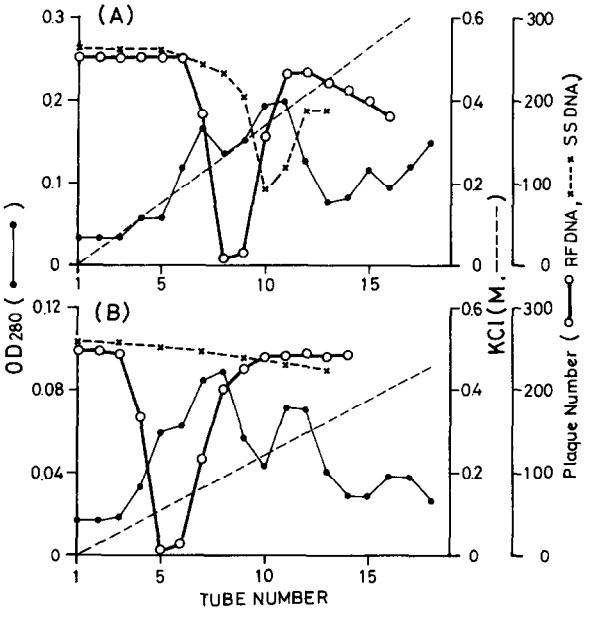

Fig. 1. Purification of *Endo H-I* by chromatography on phospho- and DEAE-cellulose columns. (A) The 35–50% ammonium sulfate fraction was loaded on a Whatman P 11 column and eluted with a linear gradient of KCl. Aliquots of fractions were incubated with RF-I DNA or SS DNA and the plaque-forming abilities were assayed on lysozyme-spheroplasts. (B) The 7 to 9th tubes in (A) were diluted, loaded on Whatman DE 52 column and eluted with a linear gradient of KCl.

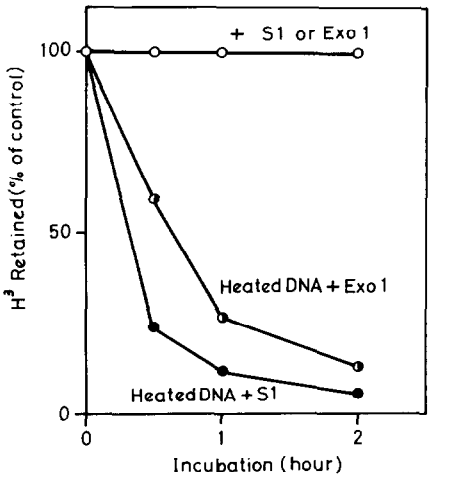

Fig. 2. Effects of exonuclease I and nuclease S1 on the digestion products. The hydrolysate of [^{3}H] RF-I DNA by *Endo H-I* (8,200 cpm/0.02 A_{260} unit/tube) was incubated at 37° with exonuclease I (2 units/tube in 70 mM glycine buffer, pH 9.5–7 mM $MgCl_2$–0.1 mM mercaptoethanol) or nuclease S1 (10 μg/tube in 0.1 M acetate buffer, pH 4.5–0.1 mM $ZnCl_2$), before and after heat denaturation. At intervals, 5% TCA was added. The precipitate was collected on a nitrocellulose filter and the radioactivity was determined.

2.4. *Purification of Endo H-I*

For this purpose, the plaque-forming abilities of RF-I DNA and SS DNA were assayed on lysozyme-spheroplasts and the fraction which preferentially destroyed the infectivity of RF-I DNA was prepared.

H. influenzae H-I was grown in brain heart infusion, supplemented with 10 μg/ml hemin and 2 μg/ml NAD, harvested by centrifugation and washed once with saline. The cells (about 6 g) were suspended in 0.05 M Tris (pH 7.6)–1 mM mercaptoethanol, disrupted by sonication and centrifuged for 30 min at 39,000 rpm. Ammonium sulfate was added to the supernatant to obtain a precipitate from 35% to 50% saturation. The precipitate was dissolved in 20 ml of 10 mM potassium phosphate buffer (pH 7.5)–1 mM mercaptoethanol (buffer A), and dialyzed against the same buffer. The dialyzed fraction was loaded onto a phosphocellulose column (Whatman P 11, 2 cm × 12 cm). After washing the column with buffer A, adsorbed proteins were eluted with a linear gradient from buffer A to buffer A + 0.8 M KCl (120 ml each). Fractions of 10 ml were collected. 50 μl each of the fractions was incubated with either RF-I DNA or SS DNA, and the infectivity remaining was assayed. The bulk of the activity degrading RF-I DNA was eluted at about 0.3 M KCl (fig. 1A). The activity peak region was diluted with five vol of buffer A, and loaded on a DEAE-cellulose column (Whatman DE 52, 1 cm × 10 cm). After washing the column with buffer A, adsorbed proteins were eluted with a linear gradient from buffer A to buffer A + 0.5 M KCl (60 ml each). Fractions of 5 ml were collected and the activity was assayed. The active fraction was eluted at about 0.12 M KCl (fig. 1B). The fraction was concentrated by dialyzing against buffer A containing 50% glycerol and stored at −20°. The activity was stable for at least 6 months.

2.5. *Other enzymes*

Endo Rd was prepared from *H. influenzae* Rd by the method of Smith and Wilcox [2], and purified further by DEAE-cellulose column chromatography. The enzyme activity was assayed by transfection and

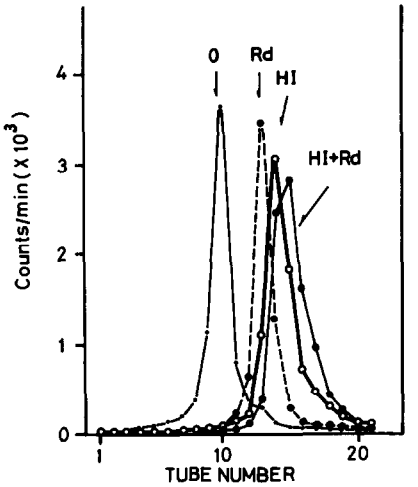

Fig. 3. Band centrifugation analysis of the digestion products. [^{3}H]RF-I DNA (8,200 cpm/0.02 A_{260} unit) was incubated for 2 hr at 37° with 6 units of enzyme. The hydrolysates were layered on 8–24% sucrose density-gradients formed in 5 mM phosphate buffer (pH 7.5)–0.1 mM EDTA and centrifuged for 5 hr at 39,000 rpm and 5°.

expressed by units defined in this paper. Exonuclease I was prepared by the method of Lehman and Nussbaum [9]. Nuclease S1 was a gift from Dr. T. Ando [10]. Polynucleotide kinase was prepared as described previously [11].

3. Results and discussion

Incubation of [^{3}H]RF-I DNA with the purified *Endo H-I* fraction produced little radioactivity in the trichloroacetic acid-soluble fraction, indicating that the fraction did not contain an exonuclease activity. The hydrolysate of [^{3}H]RF-I DNA by *Endo H-I* was not attacked by either exonuclease I or nuclease S1 (fig. 2). When the hydrolysate was heated for 10 min at 95°, rapidly cooled and then treated with these nucleases, the bulk of radioactivity was released into the trichloroacetic acid-soluble fraction. As these nucleases preferentially attack single-stranded DNA, the result suggests that, like *Endo Rd*, *Endo H-I* produces duplex cleavages in double-stranded DNA. *Endo H-I* required Mg ions on its action, the optimal concentration being around 5 to 10 mM.

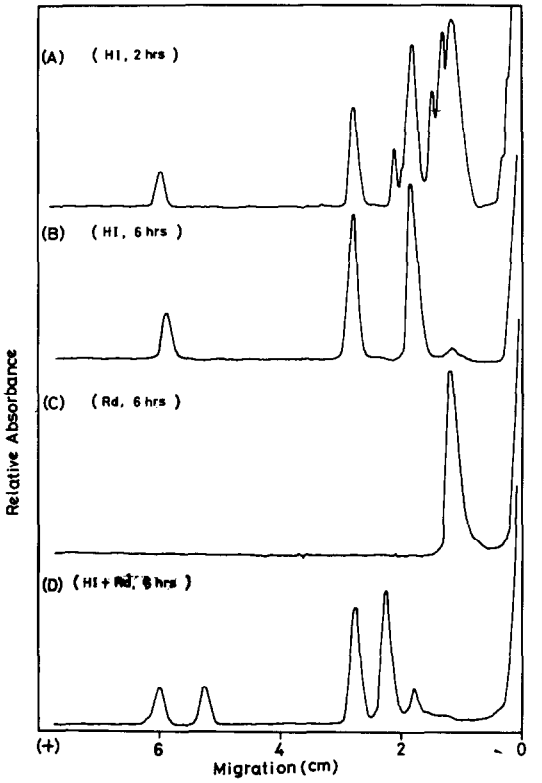

Fig. 4. Polyacrylamide gel electrophoresis of the digestion products. The hydrolysates of RF-I DNA were prepared as in the text. The incubation period is given in parentheses. The hydrolysates were layered on 3% gel columns and electrophoresed. Gels were stained with acridine orange and the color density was traced.

[^{3}H]RF-I DNA was incubated with an excess of either *Endo H-I* or *Endo Rd* and the hydrolysates were analyzed by band centrifugation (fig. 3). Under the conditions, RF-I DNA with a twisted circular form was sedimented at a rate of about 21 S and RF-II DNA with an open circular form at about 16 S [8]. Treatment of RF-I DNA with *Endo Rd* reduced the peak position in the vicinity of RF-II DNA. DNA treated with *Endo H-I* moved slightly slower than the peak yielded by *Endo Rd*. When RF-I DNA was digested by the combination of these two enzymes, the peak position further shifted to a lighter region.

The hydrolysates of RF-I DNA were resolved by polyacrylamide gel electrophoresis. Incubation of DNA with *Endo H-I* for a short period produced seven discrete peaks (fig. 4A). Under the conditions

Table 1
Analysis of the 5′-terminal nucleotides.

Nucleotides	Mole %
dCMP	43.9
dAMP	9.8
dTMP	40.3
dGMP	6.0

DNA fragments produced by *Endo H-I* were labelled with ^{32}P using polynucleotide kinase, hydrolyzed to mononucleotides, and the distribution of ^{32}P was determined.

for electrophoresis, RF-I DNA did not penetrate into the gel and RF-II DNA migrated in the vicinity of the slowest peak in fig. 4A. On increasing the incubation period, three major peaks were obtained (fig. 4B), and the pattern was not changed by further incubation. Therefore, the additional peaks observed in fig. 4A were assumed to be intermediates of digestion. The molecular weights of three fragments were estimated from the relative mobilities of CP virus RNA containing ten fragments with different molecular weights (double-stranded RNA from cytoplasmic polyhedrosis virus of silk worm, a gift from Dr. K. Miura) [12]. The molecular weights were roughly 2.2×10^6, 1.2×10^6 and 0.2×10^6 daltons, respectively. As RF-I DNA has a molecular weight of about 3.6×10^6 daltons [13], the sum of three fragments are roughly equal to the original DNA molecule.

In contrast to the pattern with *Endo H-I, Endo Rd* produced only a single peak with a slow mobility, and no smaller fragment was obtained even after the incubation was prolonged (fig. 4C). When RF-I DNA was treated by the combination of two enzymes, four peaks were obtained; among three fragments produced by *Endo H-I*, the species of 2.2×10^6 daltons was cleaved into two fragments of 1.8×10^6 and 0.4×10^6 daltons (fig. 4D). In accordance with observations by band centrifugation, the results clearly indicate that *Endo Rd* split RF-I DNA at a specific but different site from the cleavage sites by *Endo H-I.*

The hydrolysate of RF-I DNA by *Endo H-I* was denatured by heating and incubated with [γ^{32}P]ATP in the polynucleotide kinase reaction, using the conditions of Richardson [14]. However, little radioactivity was found in the DNA fraction. When the fragments were treated with *E. coli* alkaline phosphatase and then re-phosphorylated by polynucleotide kinase, they received ^{32}P at their 5′-termini (about 40 pmoles/1 A_{260} unit of fragments), indicating that the 5′-termini at the cleaved sites had been phosphorylated. The labelled fragments were hydrolyzed with pancreatic DNAase and snake venom phosphodiesterase, and the resulting nucleotides were resolved by Dowex I column chromatography [14]. ^{32}P was mostly found in C and T (table 1). According to Kelly and Smith, the species of 5′-terminal nucleotides produced by *Endo Rd* were A and G [5]. The results suggest that these two enzymes cleaved DNA by recognizing different nucleotide sequences. Analysis of the sequences adjacent to the 5′-termini are now in progress.

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