# SPECIFIC CLEAVAGE OF COLIPHAGE fd DNA BY FIVE DIFFERENT RESTRICTION ENDONUCLEASES FROM *HAEMOPHILUS* GENUS

M. TAKANAMI
*Institute for Chemical Research, Kyoto University, Uji, Kyoto, Japan*

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## 1. Introduction

It has been shown previously that several species of RNA with unique starting sequences and sizes are transcribed on the doubly closed replicative form (RF-I) DNA of coliphage fd(1). Initiation of these RNA chains was promoted by RNA polymerase holoenzyme, indicating that the sigma factor was involved in the selection of initiation sites [2]. Little information is however available for the structure of the promoter sites on DNA. If unique DNA segments containing such promoter regions could be isolated, analysis of the structure would be correspondingly easier. In an earlier study [3], the action of two different restriction endonucleases from *Haemophilus* genus on fd RF-I DNA was examined; Endonuclease R isolated by Smith and Wilcox [4] cleaved it at one site and endonuclease H-I isolated in our laboratory [3] at three different sites, respectively. One of the fragments produced by cleavage with endonuclease H-I was shown to contain the promoter for an RNA species starting with pppAUG— [5]. In order to cleave these DNA fragments into more smaller pieces, attempts were made to isolate other such enzymes with different cleavage site specificities, and two new enzymes were successfully isolated from *H. gallinarum* and *H. aphirophilus*. These enzymes produced duplex cleavages on fd RF-I DNA at six and thirteen different sites, respectively. It was also shown that endonuclease Z from *H. aegyptium* [6] cleaved this DNA molecule at eleven different sites. By the combination of these five different enzymes from *Haemophilus* genus, therefore, it has become possible to cleave fd RF-I DNA into many smaller fragments.

## 2. Materials and methods

### 2.1. *Strains*

*H. aegyptium* (ATCC 11116), *H. aphirophilus* (ATCC 19415) and *H. gallinarum* (NCTC 3438) were obtained from the Research Institute for Microbial Diseases, Osaka University. The sources of *H. influenzae* H-I and Rd were described previously [3].

### 2.2. *fd DNA*

fd RF-I DNA was prepared from *E. coli* K38 infected with fd, as described previously [7]. For preparation of [$^{32}$P]RF-I DNA, fd was infected in cells growing in a Tris—glucose medium containing $^{32}$P and replicated for several generations before preparing RF-I DNA.

### 2.3. *Preparation of enzymes*

All enzymes were prepared by the procedure essentially identical with that described previously [3]. Cells were grown in brain—heart-infusion supplemented with 10 μg/ml hemin and 2 μg/ml NAD, harvested by centrifugation, washed with saline and stored in a freezer. The frozen cells were suspended in 0.05 M Tris (pH 7.6)—1 mM mercaptoethanol, disrupted by sonication and centrifuged for 30 min at 30 000 rpm. Ammonium sulfate was added to the supernatant to obtain a fraction from 35%—65% saturation. The precipitate was dissolved in 10 mM potassium phosphate buffer (pH 7.6)—1 mM mercaptoethanol, and chromatographed on a phosphocellulose (Whatman P11) column with a linear gradient of KCl. Aliquots of each fraction were incubated with fd RF-I DNA and the infectivity remaining was assayed on



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Table 1
Abbreviations used for enzymes, their sources and number of cleavage sites on fd RF-I DNA.

| Enzyme | Source | Number of cleavage sites |
|---|---|---|
| *Endo* Rd* | *Haemophilus influenzae* Rd | 1 |
| *Endo* H-i | *Haemophilus influenzae* H-I | 3 |
| *Endo* AE** | *Haemophilus aegyptium* | 11 |
| *Endo* AP | *Haemophilus aphirophilus* | 13 |
| *Endo* GA | *Haemophilus gallinarum* | 6 |

* Endonuclease R of Smith and Wilcox [4].
** Endonuclease Z of Middleton et al. [6].

lysozyme-spheroplasts. Fractions degrading DNA were collected, diluted and chromatographed on a DEAE-cellulose (Whatman DE52) column with a linear gradient of KCl. The activity of each fraction was assayed by transfection. The specificity of active fractions was then determined by resolving the hydrolysates of fd RF-I DNA on polyacrylamide gel electrophoresis. Fractions which yielded a characteristic pattern of fragments were collected, concentrated and stored at −20°C in the presence of 50% glycerol. The properties of all enzymes were very similar to those of *Endo* H-I [3], except for the cleavage site specificities. The assay conditions by transfection were described previously [3]. One unit of the enzyme activity was expressed as the activity destroying the infectivity of 0.01 $A_{260}$ unit of fd RF-I DNA within 30 min at 37°C. Strains and abbreviations used for enzymes are listed in table 1.

### 2.4. *Polyacrylamide gel electrophoresis of DNA fragments*

About 40 units of enzymes were added to a reaction mixture (0.3 ml) containing 7 mM Tris (pH 7.6), 7 mM $MgCl_2$, 7 mM mercaptoethanol and 0.03 $A_{260}$ unit of [$^{32}$P]RF-I DNA ($10^4$–$10^5$ cpm). When DNA was digested by a mixture of enzymes, 40 units each of enzymes was added to the reaction mixture. After incubation for 4 hr at 37°C, the reaction was terminated by adding EDTA to 10 mM. The hydrolysates were layered on 5% or 10% gel columns formed in 0.036 M Tris–0.032 M $KH_2PO_4$–0.1 mM EDTA (pH

Table 2
Estimation of chain lengths for fragments produced from fd RF-I DNA by *Haemophilus* restriction endonucleases.

| Fragment No.† | *Endo* H-I | | *Endo* GA | | *Endo* AE | | *Endo* AP | |
|---|---|---|---|---|---|---|---|---|
| | $^{32}$P (%) | Length (μ)‡ | $^{32}$P (%) | Length (μ) | $^{32}$P (%) | Length (μ) | $^{32}$P (%) | Length (μ) |
| 1 | 55.2 | 1.14 ± 0.08 | 30.5 | 0.59 | 32.7 | 0.64 | 26.0 | 0.51 |
| 2 | 20.2 | 0.58 ± 0.07 | 26.0 | 0.51 | 27.8 | 0.54 | | |
| 3 | 10.8 | 0.23 ± 0.05 | 16.1 | 0.31 | 13.0 | 0.25 | } 26.5 | 0.26* |
| 4 | | | | | | | | |
| 5 | | | } 23.4 | 0.23* | 5.6 | 0.11 | } 18.4 | 0.18* |
| 6 | | | | | } 9.3 | 0.09* | | |
| 7 | | | 4.0 | 0.08 | | | } 14.0 | 0.14* |
| 8 | | | | | } 8.0 | 0.05** | 6.0 | 0.12 |
| 9 | | | | | | | 2.6 | 0.05 |
| 10 | | | | | 2.1 | 0.04 | | |
| 11 | | | | | 1.4 | 0.03 | } 4.3 | 0.04*** |
| 12 | | | | | | | 1.3 | 0.03 |
| 13 | | | | | | | 1.0 | 0.02 |

† Numbers correspond to those given in the radioautographs of fig. 1.
‡ Measurements were made by electron microscopy.
* As the percent distribution of label was obtained by a mixture of two bands, the value was divided by 2.
** As the approximate band position corresponded to fragment No. 9 of the *Endo* AP products, the percent distribution of label was divided by 3.
*** As the approximate band position corresponded to fragment No. 10 of the *Endo* AE products, the percent distribution of label was divided by 2.

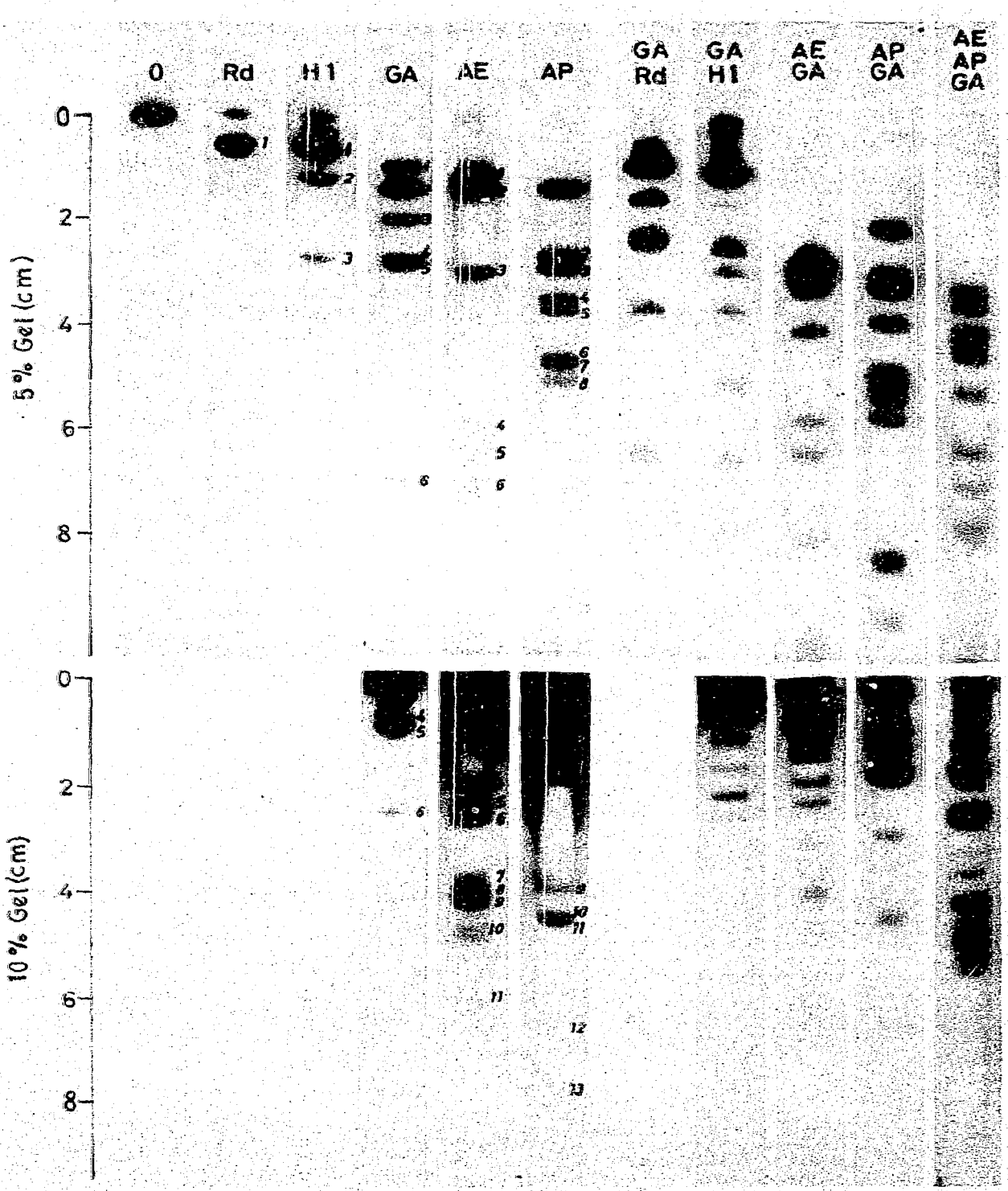


Fig. 1. Polyacrylamide gel electrophoresis of fd RF-I DNA digested by *Haemophilus* restriction endonucleases. [$^{32}$P]RF-I DNA was digested by enzymes indicated in the figure. The hydrolysates were electrophoresed on 5% and 10% gel columns (0.6 cm × 12 cm) for 16 hr at 2 mA/tube and pH 7.8. Gels were covered with thin plastic films and exposed to X-ray films to obtain the radioautographs. O referred to original DNA.

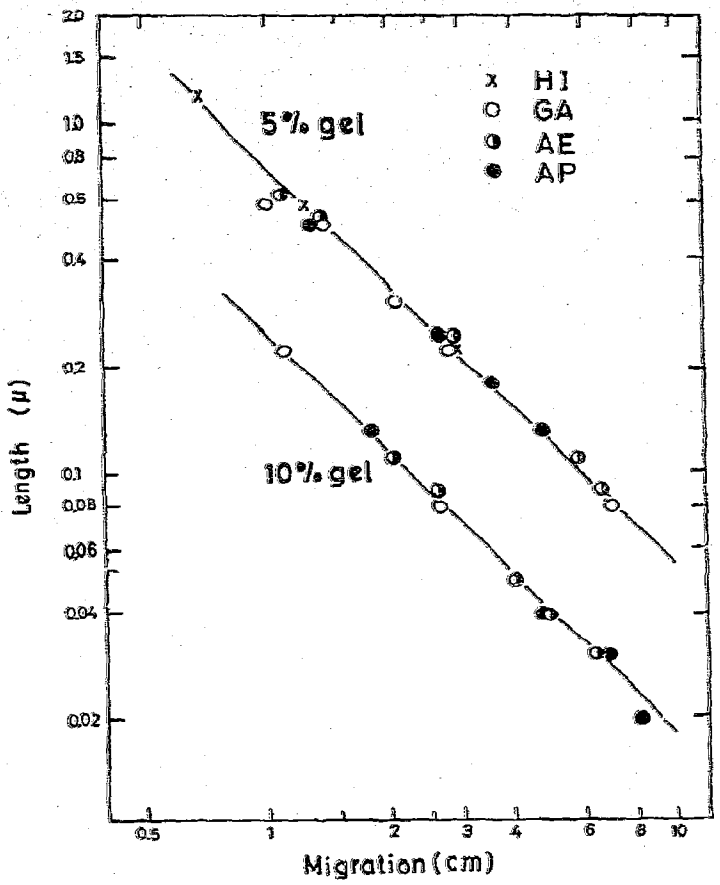


Fig. 2. Plots of chain length versus electrophoretic mobility for fragments produced from fd RF-I DNA by *Haemophilus* restriction endonucleases. [$^{32}$P]RF-I DNA digested by respective enzymes were electrophoresed under very similar conditions, and the migration distances of respective fragments were plotted against their chain lengths estimated by the distribution of label (see table 2).

7.8) and electrophoresed for 16 hr at 2 mA/tube. Gels were covered with thin plastic films and exposed to X-ray films to obtain the radioautographs.

2.5. *Length measurements by electron microscopy*

Specimens were prepared by the protein monolayer technique [8] and the electron micrographs were taken as described previously [9]. Contour lengths were measured with a map measure [9]. Absolute length calibrations were made with a diffraction grating replica.

## 3. Results and discussion

As an incomplete digestion yielded many intermediate products [3], [$^{32}$P]RF-I DNA was exhaustively digested with a large excess of enzyme. The digestion products were resolved by gel electrophoresis and the radioautographs were taken. Each enzyme yielded characteristic patterns of fragments (fig. 1). Gels were sliced and the distribution of $^{32}$P among the bands were determined (table 2).

Three fragments produced by *Endo* H-I were separated by band centrifugation and their lengths were measured by electron microscopy. The mean values obtained for each fragment are given in table 2. The comparison of these values with the percentage of total $^{32}$P in each fragment indicated that three fragments were equimolar with the original RF-I DNA. The sum of the lengths for three fragments was about equal to the contour length of RF-II DNA which was converted from RF-I DNA by introducing a nick [9]. Accordingly, the lengths of fragments produced by other enzymes were estimated by percentage of total radioactivity in a given fragment, assuming the length of original DNA = 1.95 μ (table 2). The values estimated for the *Endo* GA products and three larger fragments of the *Endo* AE products showed good agreement with those measured by electron microscopy (T. Oda and M. Takanami, manuscript in preparation). Under the conditions used for electrophoresis, the plot of chain length versus migration distance in a log–log graphpaper appears to yield a linear line (fig. 2). It would be possible to estimate the approximate length of a fragment with this correlation.

*Endo* GA appears to produce five fragments (fig. 1, GA). As suggested by the distribution of label, however, the fourth band from the top was resolved into two closely spaced bands by further electrophoresis. With *Endo* AE, ten bands can be counted in the radioautograph (fig. 1, AE). However, the distribution of label in the eighth band from the top suggested that this band comprised of two components. Although *Endo* AP yielded twelve discrete bands (fig. 1, AP), the tenth band from the top was shown to contain two fragments by the distribution of label. As reported previously [3], *Endo* Rd cleaved this DNA at one site and *Endo* H-I at three different sites, respectively (fig. 1 Rd and H-I). Number of cleavage sites

Table 3
The action of *Endo* Rd on fragments produced from fd RF-I DNA by other *Haemophilus* enzymes.

| Enzyme | Fragment No.* | Length (μ) | Products by *Endo* Rd (μ)** |
|---|---|---|---|
| *Endo* H-I | 1 | 1.14 | 0.86 + 0.28 |
| *Endo* GA | 1 | 0.59 | 0.44 + 0.15 |
| *Endo* AE | 2 | 0.54 | 0.47 + 0.07 |
| *Endo* AP | 6 | 0.14 | 0.08 + 0.06 |

* Numbers correspond to those given in the radioautographs of fig. 1.
** The approximate sizes of products were estimated by the relative mobilities with the correlation in fig. 2.

on fd RF-I DNA for respective enzymes are summarized in table 1.

Restriction endonucleases are known to cleave DNA by recognizing unique nucleotide sequences with rotational symmetry. Such sequences for *Endo* Rd [10] and RI endonuclease from *E. coli* carrying R factor [11] have been determined. It was also shown that *Endo* AI' cleaved DNA at a specific hexanucleotide sequence (H. Sugisaki and M. Takanami, to be published). It is therefore likely that DNA can be cleaved into more smaller fragments by the combination of these enzymes. This was clearly demonstrated with enzymes used in the present study (fig. 1). *Endo* Rd could cleave specifically one of the fragments produced by other enzymes (table 3). By other combinations, many smaller fragments which were not seen in the digests by each enzyme were yielded. Although exact number of fragments produced were not counted because of overlapping, the patterns were very reproducible. DNA treated by two different enzymes separately gave a pattern identical with that treated by a mixture of these enzymes. After isolating a particular fragment from a digest, therefore, the fragment can be cleaved into smaller pieces by other enzymes. In this manner, it would be possible to obtain specific fragments containing functional sites of DNA such as promoters and those short enough for the determination of nucleotide sequences.

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