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RESTRICTION OF PM2 SUPERCOILED DNA BY Hap II ENDO-
NUCLEASE**

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Restriction of the covalently closed circular DNA from phage PM2 (CCC PM2 DNA) by Hap II endonuclease was studied under varying enzyme concentration. At low Hap II concentration, accumulation of the intermediate product, OC DNA, was observed at the early stages of the reaction. The resulting final mixture of restriction products consists of OC and L DNA, and their relative content depends on the concentration of the enzyme used. The affinity of the enzyme for the intact recognition site of the substrate in different conformational forms does not seem to be affected. Basically identical results were obtained with the two different CCC DNA used: PM2 and SV40 DNA.

In spite of the widespread use of restriction enzymes of type II for the in vitro DNA recombination, very little is known about the mechanism of DNA recognition and the consecutive steps of restriction. Endonuclease Eco B of type I restricts DNA substrate in two steps, i.e. each strand separately (Adler & Nathans, 1973). This does not hold for the same process catalysed by endonucleases of type II such as Eco RI (Modrich & Zabel, 1976; Ruben et al., 1977; Stepień et al., 1979). The available data on DNA restriction by Sal I and Bam HI point to the simultaneous action of these enzymes on both DNA strands (Halford et al., 1979). If the restriction of both DNA chains does not occur at the same time, it is important to know whether the enzyme dissociates or not from the recognition site when the first chain has been split (Rubin & Modrich, 1978).

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We present here data on the restriction of the phage PM2 circular supercoiled DNA (mol. wt. 6.2 x 10^6; Espejo et al., 1969) by Hap II restriction endonuclease isolated from Haemophilus aphrophilus cells. The Hap II endonuclease recognizes CCGG (Sugisaki & Takanami, 1973), an unique sequence in PM2 DNA. Thus, scission of a single PM2 DNA chain would lead to an open circular relaxed PM2 DNA, whereas the full-length linear PM2 DNA would be the product of scission of both chains. All three structural forms can be easily separated by physical methods such as ultracentrifugation or electrophoresis, and quantitatively determined. In this respect our model resembles the previously used Col E1 and Eco RI (Modrich & Zabel, 1976; Stepień et al., 1979), SV40 and Eco RI (Rubin & Modrich, 1978; Ruben et al., 1977) or SV40 and Hpa II (Ruben et al., 1977) systems.

EXPERIMENTAL

Materials

Bacterial and bacteriophage strains. Alteromonas espejiana BAL 31 and PM2 phage were a gift from Dr. R. R. Schäfer (Biozentrum, Basel, Switzerland); the bacteria were grown and infected by PM2 phage as described by Espejo & Canelo (1968) and Hinne et al. (1974). The Haemophilus aphrophilus strain was kindly supplied by Dr. R.J. Roberts (CSH Laboratory, U.S.A.); the bacteria were cultivated according to Sugisaki & Takanami (1973), and collected at the late logarithmic phase.

DNA preparations. PM2 DNA (CCC and OC forms) was isolated from the phage derived from marine bacterium Alteromonas espejiana BAL 31 according to Marcoli et al., (1979). The purified unlabelled CCC DNA preparations contained 90% of CCC and 10% of OC DNA. CCC PM2 [^H]DNA was purified according to Streek et al., (1974). The specific activity of the preparation used was 1.3 x 10^6 c.p.m. per 1 μg DNA and the labelled DNA was composed of 80% CCC and 20% OC PM2 DNA.

SV40 DNA was a commercial product of BRL (Richmond, Calif., U.S.A.) and was kindly offered by Dr. N. Pieniążek of the University of Warsaw. Plasmid pBR 322 DNA purified as earlier described (Rempoła & Fikus, 1979).

Hap II restriction endonuclease. The general procedure for purification of restriction endonucleases of type II published by Bickle et al., (1977) was applied with some modification for purification of the enzyme. From the crude extract (2.5 g protein) derived from 40 g of Haemophilus aphrophilus cells, nucleic acids were precipitated with polyethyleneimine and

Abbreviations used: CCC, covalently closed circular DNA; OC, open circular DNA; L, linear DNA; DAPI, 4,6-diamidine-2-phenylindole; PEI, polyethyleneimine. Restriction enzymes are named according to Smith & Nathans (1973).
the enzyme (1.5 g protein) was salted out with ammonium sulphate at 0.7 saturation. One gram of protein was then applied on Bio-Gel 1.5m coupled with heparin (heparose) and eluted with a NaCl gradient. The active fractions eluted at 0.2 - 0.3 m-NaCl (16 mg protein, 19 000 units of Hap II endonuclease) were then chromatographed on DE 52 cellulose, and the pooled active fractions eluted at 0.07 - 0.1 m-NaCl (0.38 mg protein, 7000 units of Hap II) were then run on the second heparose column. The final preparation of Hap II (4.5 ml) contained 2200 units of the enzyme; one unit is defined as that amount of the enzyme which digests 1 µg of CCC PM2 to the linear product at 37 C in 1 h.

The enzyme was stored at -20 C in Tris/HCl, pH 7.5 (20 mm), EDTA (0.5 mm), β-mercaptoethanol (7 mm) in 50% glycerol, and it was diluted, if necessary, with the same buffer.

Chemicals. DAPI was a commercial product of Serva (Heidelberg, F.R.G.). Tris and agarose type II were purchased from Sigma (St. Louis, Mo., U.S.A.), Bio-Gel from Bio-Rad Laboratories (Richmond, Calif., U.S.A.); DE 52 cellulose was from Whatman Biochemicals (Clifton, N.J., U.S.A.). Heparose was prepared according to Miller-Andersson et al., (1974) with a large excess of BrCN. [3H]Thymidine was produced by UVVVR, (Prague, Czechoslovakia). All other chemicals were of analytical grade; distilled water was used for all solutions.

Analytical methods

The enzyme assay. The standard reaction mixture for DNA digestion by Hap II endonuclease consisted of: 1 µg of PM2 DNA (6 µg/ml) and 0.25 unit of Hap II in Tris/HCl buffer, pH 7.6 (10 mm) containing MgCl₂ (7 mm) and β-mercaptoethanol (7 mm). The mixture was incubated at 37 C for the time period indicated. Restriction was stopped by heating at 65 C for 10 min.

Electrophoresis. Different structural forms of PM2 DNA were separated on 1%, agarose gels with or without DAPI, as previously described (Stepień et al., 1979; Naimski et al., 1980). Hap II-digests of pBR 322 were analysed on 7.5%, polyacrylamide gels, according to Maniatis et al. (1975).

Ultracentrifugation. To the linear PM2 [3H]DNA (1 µg), 300 µl of 0.3 m-NaOH was added, the mixture was applied on alkaline sucrose gradients with a shelf of 60% sucrose, and centrifuged in a Beckman SW 50.1 rotor at 50 000 r.p.m. for 4 h at 15 C. Fractions were collected on Whatman 3 MM filters and counted.

Protein and DNA determination. In calculating DNA concentration it was assumed that A₂₆₀ of the DNA solution of 50 µg per ml is 1.0.
The relative content of CCC, OC, and L forms of PM2 DNA was determined, after electrophoretic separation, by radiometric and/or fluorimetric methods (Naimski et al., 1980).

Protein was determined according to Lowry et al. (1951).

RESULTS

Our final Hap II preparation did not contain any unspecific endonuclease that could interfere with its restriction activity. This was proved by electrophoretic analysis of the 3-h digests of pBR 322 DNA with a tenfold excess of the enzyme. The electrophoretic pattern of the digests remained unchanged. Another proof was obtained by preparative ultracentrifugation in alkaline sucrose gradients (Fig. 1) of the final digestion product of CCC PM2 DNA with Hap II endonuclease: solely linear molecules of unit length were found even when an excess of the enzyme and prolonged time of incubation were applied. This excludes a random cleavage of PM2 DNA by unspecific endonucleases during incubation.

The Hap II endonuclease was thermally stable under our incubation conditions; 10 min incubation at 65°C abolished the restriction activity.

![Graph showing ultracentrifugation of CCC DNA digests.](image-url)

**Fig. 1.** Ultracentrifugation of the CCC DNA digests. Increasing amounts of Hap II endonuclease were used: 1 μg of CCC PM2 [^3H]DNA was digested under standard conditions with 1.25 (1), 2.5 (2), 5 (3) and 5 (4) units of Hap II endonuclease, respectively, at 37°C. Samples no. 1-3 were digested for 30 min, sample no. 4 for 60 min. The alkaline gradients were applied as described under Methods.
Fig. 2. Time-course of the restriction of CCC PM2 DNA by Hap II endonuclease at low enzyme concentration. (○), CCC DNA; (●), OC DNA; (×), L DNA. The relative content of particular DNA forms was measured by the fluorimetric method. The same results were obtained by the radiometric method.

The enzyme was not adsorbed on glass surfaces, as shown by its incubation with porous glass beads.

The time course of the formation of restriction products of CCC PM2 DNA by Hap II endonuclease at low concentration is shown in Fig. 2. Transient accumulation of the intermediate product, OC DNA, was observed, but even after prolonged incubation the digestion of DNA was incomplete, yielding 30 - 40% of the OC form and 70 - 60% of the L form of PM2 DNA. The same was found when the substrate PM2 DNA was additionally deproteinized before digestion with the phenol/chloroform/isoamyl alcohol mixture (1:1:0.05, by vol.).

Digestion of the CCC PM2 DNA by Hap II endonuclease was complete when one unit of the enzyme per 1 µg of DNA was added to the incubation mixture. When the enzyme concentration was decreased by dilution, the relative content of final products did not change within a broad range of the enzyme concentration (Table 1).

This unexpected result could be explained by the preferential recognition of the supercoiled substrate over the relaxed one, by Hap II endonuclease.

Table 1

<table>
<thead>
<tr>
<th>DNA forms (%)</th>
<th>Hap II units</th>
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<tbody>
<tr>
<td></td>
<td>1.0 0.25 0.05 0.025 0.017 0.01 0.006 0.003 0.0025 0.00125 0.0001 0.00005</td>
</tr>
<tr>
<td>OC</td>
<td>35.0 35.5 36.6 31.1 34.2 35.1 37.2 37.5 40.1 60.1 77.8</td>
</tr>
<tr>
<td>L</td>
<td>100 65.0 64.5 63.4 68.9 65.8 64.9 62.8 62.5 59.9 35.7 15.1</td>
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In order to check this assumption, the randomly nicked OC PM2 [\(^3^H\)]DNA was incubated with Hap II (Fig. 3). At low enzyme concentration the OC PM2 DNA, similarly as CCC DNA was not completely digested to the final, linear product and the same kind of "equilibrium" of the OC and L forms was reached within a time comparable to that required for CCC DNA restriction.

![Graph showing restriction of OC PM2 DNA by Hap II endonuclease](image)

Fig. 3. Restriction of randomly nicked OC PM2 [\(^3^H\)]DNA by Hap II endonuclease. Hap II, 0.25 (O), 0.5 (x) and 1.0 (□) unit, was used under standard conditions. The results obtained by the fluorimetric method are given. The control OC PM2 [\(^3^H\)]DNA was kept for 2 h at 37 C and contained 8.4% of linear DNA. The same results were obtained by the radiometric method.

A new portion of unlabelled CCC PM2 DNA was added at this equilibrium state to see whether the enzyme would be still active towards the CCC DNA added. It was found that the enzyme was able to recognize and split the CCC PM2 DNA added but the OC [\(^3^H\)]DNA produced by Hap II in the first stage was not further restricted (Table 2).

Although restriction enzymes of type II recognize only short nucleotide sequences, it is known that the affinity of a given enzyme for the recognition sequence can be modified by the surrounding sequences (Thomas & Davis, 1975; Rubin & Modrich, 1978). Therefore CCC SV40 DNA was used as a substrate for Hap II endonuclease. The SV40 DNA has, like PM2 DNA, an unique CCGG sequence recognized by Hap II, but the surrounding nucleotide sequences and the supercoiling density of the two DNA substrates, are different. Since digestion of SV40 DNA followed the same general pattern as restriction of PM2 DNA, the final results of restriction of supercoiled DNA at low Hap II concentration seem to depend mainly on the catalytical properties of the enzyme itself.

If the non-digested OC DNA were an artefact, one could assume that linear DNA with both free ends is kept in circular structure by undissociated
Table 2
Restriction of two consecutive portions of CCC PM2 DNA by Hap II endonuclease.

CCC PM2 [3H]DNA (1.2 μg) was incubated with 0.25 unit of the enzyme at 37°C. After 2-h incubation unlabelled CCC PM2 DNA (1.2 μg) was added and restriction was followed for the next 2 h. The relative content of the substrate and products was measured by both radiometric (R) and fluorimetric (F) methods and expressed as % of total DNA. In brackets, relative amounts of DNA forms are calculated for unlabelled DNA. For these calculations we corrected the total DNA in a given fraction, measured by the fluorimetric method for [3H]DNA present in the same fraction and measured by the radiometric method.

<table>
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<tr>
<th>Time of incubation (min)</th>
<th>OC</th>
<th></th>
<th>L</th>
<th></th>
<th>CCC</th>
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</tr>
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<tbody>
<tr>
<td></td>
<td>F</td>
<td>R</td>
<td>F</td>
<td>R</td>
<td>F</td>
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<tr>
<td>5</td>
<td>42.9</td>
<td>41</td>
<td>5.4</td>
<td>3</td>
<td>51.7</td>
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<td>10</td>
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<td>8.1</td>
<td>6</td>
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<td>30</td>
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</tr>
<tr>
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<td>47</td>
<td>30.1</td>
<td>53</td>
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</tr>
<tr>
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<td>44</td>
<td>57.0</td>
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</table>

Hap II enzyme, or a covalently closed circular relaxed DNA is ligated either by an unknown ligase present in our enzyme preparation or by the restriction enzyme itself. To examine in more detail the possibility of artefact formation, CCC PM2 DNA (2 μg) was incubated with 0.5 unit of Hap II under standard conditions for 2 h until the equilibrium state was reached; then the incubation mixture was divided into two portions, one of which was deproteinized with the phenol/chloroform/isoamyl alcohol mixture. Both samples were subjected to electrophoresis and the relative content of the OC and L products was determined. It was found that the control sample contained 37.2%, of the OC DNA, whereas the deproteinized sample 38.1%.

To examine the possible backward ligation, an analogous experiment was performed. The final restriction mixture of the OC and L forms was similarly divided and run on agarose gels in the presence and absence of 0.5 μg of ethidium bromide. In the ethidium bromide gel the OC product of PM2 DNA restriction did not change its position in relation to CCC and L DNA. This proves that this product is a true open circular PM2 DNA, since the covalently closed circular relaxed DNA undergoes supercoiling in the gel and migrates as CCC DNA (Keller, 1975).
DISCUSSION

Many of the restriction enzymes of type II recognize palindromic nucleotide sequences, with a symmetry axis, in the DNA substrate. Most of them do not split phosphodiester bonds within the same complementary base-pair. As a result, the restriction products have “sticky ends”:

\[
\begin{align*}
3' & \text{C C G G G 5'} \quad \text{Hap II} \quad 3' \text{C G G 5'} \\
5' & \text{G G C C 3'} \quad 5' \text{G G C}^+ \quad \text{C 3'}
\end{align*}
\]

This suggests the coordinated action of at least two enzyme molecules at the recognition site, as was shown for Eco RI (Modrich & Zabel, 1976) and Hpa II (Hines & Agarval, 1979). However, when the space-filling model of a double-stranded DNA was constructed it could be seen that, in the case of the six-nucleotide-recognition site, the two phosphodiester bonds to be split lie in close proximity on the same side of the DNA double helix, thus permitting a single enzyme molecule to cover the restriction site (G. Maas, personal information). Moreover, it was shown that some restriction enzymes such as Bsp I (Koncz et al., 1978) Pal I (Balesi & Rushizky, 1979) Hha II (Mann & Smith, 1979) and Bgl I (Lee & Chirikjian, 1979) are probably active as monomers, whereas the active form of Bam HI is presumably a multimer (Smith & Chirikjian, 1979).

Splitting of both DNA chains in an either one- or two-step process clearly depends on the incubation conditions, the substrate DNA and the enzyme studied (Modrich & Zabel, 1976; Ruben et al., 1977; Rubin & Modrich, 1978; Halford et al., 1979).

In this paper we have shown that restriction of the two different supercoiled DNAs (PM2 and SV40) by Hap II endonuclease at low concentration leads not only to an accumulation of the intermediate product (OC DNA) but also to splitting of this intermediate to the final, linear product only when more enzyme was added. Thus, the final distribution of restriction products depends exclusively on concentration of the enzyme in the incubation mixture.

As shown by Baumstark et al. (1979), the Hpa II endonuclease, an isoschisomomer of Hap II, exhibits preferential affinity to one of the two DNA strands, and this could explain the transient accumulation (up to 80%) of the OC DNA intermediate during the early stages of catalysis under our standard conditions.

The results presented suggest that Hap II endonuclease probably acts on DNA in different aggregation forms. It seems probable that for splitting of the single DNA chain only one molecule of the enzyme is necessary, whereas two simultaneous cuts are possible only when the dimeric form of the enzyme binds to the recognition site. It seems also that the enzyme has a lower affinity for the recognition site when one
chain is already split. To digest such an intermediate form more enzyme or higher aggregates are required. In our experimental conditions this aggregation state is reached with one unit of the enzyme per one µg of CCC DNA.

At present, with the data available, it seems impossible to definitely confirm the above assumptions, and the problem requires further studies, which are under way in our laboratory.

We are indebted to Professor Dr. K. L. Wierzchowski for his constant interest in this study and helpful advice, to Dr. T. Bickle for his helpful comments, to Dr. A. Bierzyński for many discussions, and to Mrs. H. Kozłowska for her skillful technical assistance.

REFERENCES


RESTRYKCJA SUPERSPIRALNEGO DNA FAGA PM2 PRZEZ ENDONUKLEAZĘ Hap II

Streszczenie
Badano warunki restrykcji superspiralnego DNA faga PM2 przez endonukleazę restrykcyjną Hap II. W niskich stężeniach enzymu w czasie restrykcji obserwuje się gromadzenie produktu pośredniego i zrelaksowanego, kołowego DNA. Otrzymana mieszanina produktów restrykcji składa się z OC i L DNA, a ich wzajemny stosunek zależy od użytego stężenia enzymu. Powinowactwo enzymu do nie zmienionego miejsca rozpoznania w różnych formach konformacji substratu wydaje się być nie zmienione. Podobne wyniki uzyskano trawiąc superspiralny DNA wirusa SV40 endonukleazą Hap II.

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