Volume 14 Number 17 1986 Nucleic Acids Research

Restriction endonucleases from *Herpetosiphon giganteus*: an example of the evolution of DNA recognition specificity?

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Received 2 July 1986; Accepted 6 August 1986

ABSTRACT

We describe the partial purification and characterisation of five Type II restriction endonucleases from two strains of Herpetosiphon giganteus. One of the activities, HgiJII, was the first enzyme found that cleaves DNA at the family of related sequences 5'-G-R-G-C-Y/C-3'. This enzyme may be related to the enzyme HgiAI from a different strain of the same species, and which cleaves at the sites 5'-G-W-G-C-W/C-3'. We have shown that DNAs from the strains producing HgiAI and HgiJII are resistant to both of these restriction endonucleases.

The remaining four enzymes described here share recognition and cleavage specificities with other restriction endonucleases. The evolution of Type II restriction-modification systems and their role in vivo are discussed.

INTRODUCTION

Restriction endonucleases occur in a large number of prokaryote species. The latest published compilation of restriction endonuclease activities (1) contains 515 such enzymes from 397 bacterial strains, representing a minimum of 116 different cleavage specificities. Many of these enzymes are 'isoschizomers', enzymes which have identical recognition sites, but such enzymes are not necessarily closely-related proteins. The involvement of Type II restriction endonucleases in biological restriction has been demonstrated for only a few enzymes (2).

In nearly all the strains containing one or more restriction endonucleases, the DNA of the organism is protected against each endonuclease by a corresponding methylase activity (3), which modifies the same sequences in DNA that are recognised by the endonuclease. These methylases have been less well studied than the endonucleases; presumably because they have been of less practical value. Restriction endonucleases are widely used in the analysis of DNA and in the construction of novel DNA molecules. They also find use as model systems for the study of DNA-protein interactions.

In spite of the large number of restriction endonucleases now known

(1), new enzymes with novel recognition and cleavage specificities are still required in order to increase the range of DNA manipulations that can be performed. Novel activities may also provide new insight into the mechanisms of protein-DNA interactions. We have screened some bacteria for the presence of restriction endonucleases, and we report here the restriction endonuclease complement of two strains of Herpetosiphon giganteus.

MATERIALS AND METHODS

Preparation of restriction endonucleases: Herpetosiphon giganteus strains HP1049 and HFS101 (gifts of Drs J.H. Parish and H.A. Foster respectively) were grown at 30°C with shaking in 0.3% Bacto-casitone, 0.1% yeast extract until stationary phase was reached. The cells were harvested and stored at -20°C. Cells (10g) were resuspended in 20ml PCI buffer (10%(v/v) glycerol, 10mM 2-mercaptoethanol, 0.1mM EDTA, 10mM potassium phosphate, pH 7.4, 0.02mM phenylmethylsulphonyl fluoride, 0.05mM benzamidine) and then sonicated for 12 x 30s at 80W output from a 3mm probe, while maintaining the temperature below 4°C. The sonicate was centrifuged at 100,000g for 90 min, and the supernatant was applied to a column (5cm x 1cm) of phosphocellulose P-11. The column was washed with PCI buffer and eluted with a 100ml linear gradient of 0 - 1.0M NaCl in PCI buffer. Fractions were assayed for specific endonuclease activity, and active fractions were pooled according to their fragmentation patterns on bacteriophage λ DNA. After dialysis against PCI buffer, the endonuclease activities were further purified on a DEAE-cellulose column (Whatman DE52; 15cm x 1.5cm) and then, for HgiHII and HgiHIII, on a Heparin-sepharose column (Pharmacia; 4cm x 1cm). A summary of the elution positions of the various endonuclease activities is given in Table 1. Active fractions from the final column were dialysed against 50%(v/v)glycerol in PC buffer (as PCI buffer without the protease inhibitors) and stored at -20°C.

Preparation of cell-free extract for modification methylase assays: A small quantity of H. giganteus cells (approx. 1.0g wet weight) was sonicated in 2 ml of PCI buffer. The extract was kept at 0°C and was used within a few hours.

DNA and DNA modifying enzymes: DNA from bacteriophages λ cI857Sam7, replicative form (RFI) DNA from bacteriophages M13, M13mp7 and ØX174am3cs70, and the plasmids pBR322, pAT153 and pAO3 were prepared as described elsewhere (7). SV40 DNA was purchased from Gibco-BRL, Paisley, Scotland. Chromosomal

DNA from H. giganteus strains HP1023, and HFS101 was prepared by resuspending 1g freshly-harvested cells in 20ml 1% NaCl, 1% tri-isopropyl-naphthalene sulphonate, 6% p-aminosalicylate. The suspension was incubated at 0°C for 30 min, then extracted with an equal volume of 1:1 phenol-chloroform by occasional gentle agitation for 45 min. at 0°C. The aqueous phase was extracted once more with phenol-chloroform, then three times with chloroform. After ethanol precipitation and dialysis against TE buffer (10mM Tris-HCl, pH 8.0, 0.1mM EDTA), the nucleic acids were incubated with DNase-free RNase A, extracted with phenol and precipitated with ethanol. The preparation was resuspended and dialysed against TE buffer, and stored at 4°C.

HgiAI and CauI were gifts of Dr J. Littlechild and S.P. Bennett, respectively. Other restriction endonucleases, DNA polymerase and DNA ligase were purchased from New England Biolabs Inc, Beverly, MA, USA; or from Boehringer Corporation (London) Ltd, Lewes, Sussex.

Restriction endonuclease digests: The standard digestion procedure was to incubate 0.25-1.0 ug of DNA in 20ul of buffer at 37°C. The standard buffer used prior to determining the optimal conditions was buffer R (10mM $MgCl_2$, 10mM 2-mercaptoethanol, 50mM NaCl, 10mM Tris-Cl, pH7.5). The eventual buffer and digestion conditions used are given in RESULTS. The products of digestion were analysed by electrophoresis in 1% agarose or 5% polyacrylamide gels. In both cases the electrophoresis buffer was 90mM Tris-borate, 2.5mM EDTA, pH 8.3. With agarose gels 0.5 ug/ml ethidium bromide was present in the gel during electrophoresis, and with polyacrylamide the gel was stained in 0.5 ug/ml ethidium bromide in water after electophoresis. The ethidium-stained DNA was visualised on a 300nm transilluminator and was photographed on polaroid film.

Determination of restriction endonuclease recognition and cleavage sites: The general protocol was to determine the minimum number of cleavage sites on DNAs of known sequence and to compare these data with computer-generated tables (8). (This gives the minimum number as sites occurring close together on the molecule may not be identified in restriction digests.) A suitable site was identified for each enzyme and was cloned into a derivative of bacteriophage M13mp7. This was then used as a template in the cleavage site-location method of Brown & Smith (9), as described previously (7,10). The major exception to this was HgiHII, where ØX174 DNA was used as template for the cleavage site-location. The exact experimental routes required to determine the recognition and cleavage specificities for each of the enzymes described here are given in RESULTS.

RESULTS

Purification of restriction endonucleases: H. giganteus HP1049 was found to contain three restriction endonuclease activities, which were named HgiHI, HgiHII and HgiHIII. HgiHI was partially purified, free of contaminating nuclease activities, after phosphocellulose and DEAE-cellulose chromatography. Only partial separation of HgiHII and HgiHIII was obtained on the chromatography media used (Table 1). This was sufficient to characterise these activities, and further purification was not attempted. The yield of HgiHI was approx. 500 units/g wet weight cells; the yields of HgiHII and HgiHIII were not determined. H. giganteus HFS101 contained two activities, HgiJI and HgiJII, which were separated from contaminating nuclease activities by phosphocellulose chromatography (Table 1). HgiJII was sufficiently free of contaminating non-specific nucleases to be used after one column, HgiJI was further purified on DEAE-cellulose. The presence of protease inhibitors was essential to the isolation of active HgiJI enzyme, the yield of which was about 300 units/g wet weight. HgiJI activity was rapidly lost during storage. The yield of HgiJII was about 250 units/g wet weight. The H. giganteus strains grew to about 0.5-1.0 g(wet weight)/l under the growth conditions used here.

Determination of the recognition and cleavage specificity of the restriction endonucleases. The specificities of the five enzymes were determined by different routes.

HgiHI:

Optimal HgiHI activity was obtained in buffer R. The enzyme was inhibited by NaCl concentrations above 150mM. DNAs of known nucleotide

Table 1. Chromatography of Herpetosiphon restriction endonucleases.

	Concentration of KCl (M) for elution from column.		
Enzyme	Phosphocellulose P11	DEAE-cellulose DE52	Heparin-sepharose
HgiHI	0.10-0.25	0.35-0.45	-
HgiHII & HgiHIII	0.25-0.35	0.25-0.3	0.15-0.35
HgiJI	0.05-0.15	0.05-0.15	-
HgiJII	0.20-0.30	-	-

sequence were digested with HgiHI, and the minimum numbers of sites identified were three in ØX174 RFI DNA, five in M13mp7 RFI DNA and seven in plasmid pBR322. One of the sites on ØX174 DNA was mapped to within 20 nucleotides of position 2470 (sequence numeration as in 11). Reference to computer-generated tables (8) suggested that hexanucleotide sequences of the family 5'-G-G-Y-R-C-C-3' occur at about the same frequency as HgiHI sites in the tested DNAs, and one of these sites is at position 2478 in ØX174 DNA. Sites of this family occur three times in ØX174 DNA, six times in M13mp7 DNA and nine times in pBR322 DNA; the discrepencies between the predicted and observed numbers being due to sites being close together on M13mp7 and pBR322 DNAs. Thus, this was proposed to be the recognition sequence of HgiHI.

The cleavage specificity of HgiHI was determined by methods previously described (9), using a recombinant M13mp7 DNA. An autoradiograph of the site-location experiment is shown in Figure 1. Cleavage occurs in the sequence:

$$\begin{array}{l} 5'\text{-}G\text{-}G\text{-}C\text{-}A\text{-}C\text{-}C\text{-}3' \\ 3'\text{-}C\text{-}C\text{-}G\text{-}T\text{-}G\text{-}G\text{-}5' \end{array}$$

Thus, HgiHI cleaves DNA at the sequence 5'-G-G-Y-R-C-C-3' to generate fragments with non-identical 5'-tetranucleotide extensions. The sequence 5'-G-G-T-A-C-C-3' did not occur in any of the DNA molecules tested, but this was confirmed as a HgiHI cleavage site by digestion of SV40 DNA with HgiHI.

The sequence 5'-G-G-C-G-C-C-3' is not cleaved in M13mp7 RFI DNA (position 6001; 13), whereas this sequence is cleaved in pBR322 DNA (positions 413, 434, 547 and 1204; 14). The site in M13mp7 overlaps a site for the dcm methylase (5'-C-^{m}C-W-G-G-3'; 15), indicating that methylation of the last residue in one strand of the HgiHI site prevents cleavage of the site.

HgiHII and HgiHIII:

These two activities were not completely separated in these experiments. Optimal conditions for digestion could not easily be obtained, and digests were routinely performed in buffer R. Various DNA molecules of known sequence were digested with this mixed preparation. Plasmid pAO3 (16) was shown to have two cleavage sites, which mapped in close proximity to the two AvaII sites in this DNA. Further digestion of this cleaved pAO3 DNA with AvaII did not cause new bands to appear, indicating that one activity in the preparation had a recognition specificity identical with AvaII or of which AvaII sites constitute a subset. This activity was designated HgiHIII.

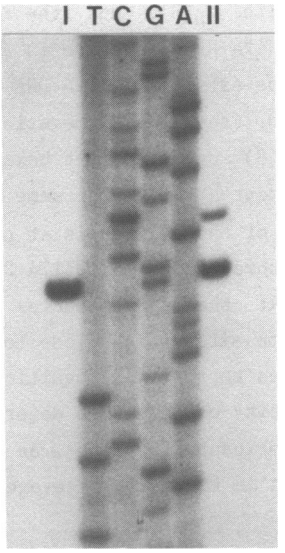

Figure 1. Location of the HgiHI cleavage site. The autoradiograph is of a M13 cloning/chain-termination DNA sequencing experiment through the HgiHI site at position 1271 of transposon Tn501 (12). The fragment in channel I locates the phosphodiester bond cleaved in the newly-synthesised DNA strand, and the additional, longer fragment in channel II locates the bond in the template strand (see ref 9). The smaller fragment persists in channel II due to either incomplete action of T4 DNA polymerase on the fragment terminus or, more probably, to incomplete cleavage with HgiHI.

In limited digests of ØX174 DNA, cleavage at a single site was obtained, and this mapped to the position of the single AvaII site in this DNA (11). In more extensive digests of ØX174 DNA a second enzyme activity was detected that cleaved the DNA at least six times. This activity was designated HgiHII. Comparison of the number and sizes of the DNA fragments with computer-generated tables (8) indicated that HgiHII has the same recognition specificity as AcyI. In double digests of ØX174 DNA, cleavage sites for HgiHII mapped to those fragments in which AcyI sites occur. Plasmid pAO3 has no AcyI sites, hence only HgiHIII activity is detected on this DNA. The cleavage patterns of the mixed preparation on DNAs of known sequence are those expected for a mixture of two enzymes, with recognition specificities identical to AvaII and AcyI respectively.

The cleavage specificity of HgiHIII was determined using the site at position 798 of pBR322 DNA (14), cloned in M13mp7 as a Sau3AI fragment (data

not shown). The cleavage specificity of HgiHIII was identical with that of AvaII:

```
    ↓
5'-G-G-A-C-C-3'
3'-C-C-T-G-G-5'
        ↑
```

The cleavage specificity of HgiHII was determined using HaeIII fragments Z5 and Z7 (11) as primers on ØX174 non-viral strand DNA as template (data not shown). The cleavage of the two sequences by HgiHII was consistent with the cleavage specificity being identical with that of AcyI:

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    ↓
5'-G-R-C-G-Y-C-3'
3'-C-Y-G-C-R-G-5'
        ↑
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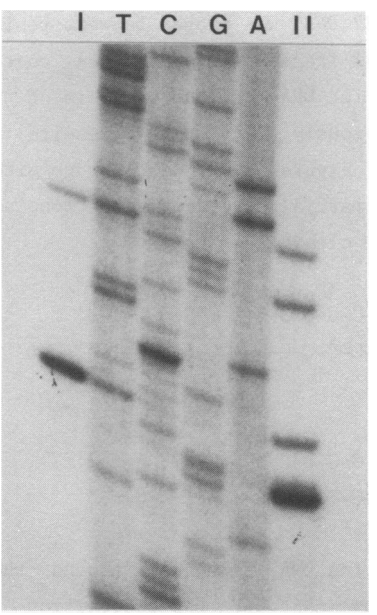

Figure 2. Location of the HgiJII cleavage site. The autoradiograph is of a M13 cloning/chain-termination DNA sequencing experiment through a pair of HgiJII sites at positions 471 and 485 of pBR322 (14). Partial digestion conditions were used to generate the fragments in channels I and II, so that both cleavage sites could be identified. Not all the fragments have been converted to the shorter forms in channel II, presumably due to the DNA being nicked and not cleaved in both strands under these conditions. The fragments in channel I locate the phosphodiester bonds cleaved in the newly-synthesised DNA strand, and the new fragments in channel II locate the bonds cleaved in the template strand (see ref 9).

HgiJI:

HgiJI activity was unstable after purification by the protocol described here. Digests were performed at 37°C in buffer R, and the number and size of DNA fragments generated by HgiJI on plasmids pAO3 and pBR322 and the approximate locations of these sites were determined. These data were compared with those in the tables of Fuchs et al. (8), and indicated that the recognition site of HgiJI was identical with that of AvaII (and HgiHIII). The cleavage specificity was determined as described for HgiHIII, and was shown to be identical with it.

HgiJII:

Optimal HgiJII activity was obtained in buffer R at 37°C. Digestion of several fully-sequenced DNAs showed that ØX174 DNA and pAO3 DNA contained no HgiJII sites, and that pBR322 and SV40 DNA contained at least one cleavage site. The site on pBR322 DNA was mapped between positions 467 and 485 (14). The 458-nucleotide Sau3AI fragment of pBR322 DNA containing the HgiJII site was cloned in M13mp7 for DNA sequence analysis of the cleavage site by methods described previously (9). The results of this site-location experiment are shown in Figure 2. Two cleavage sites can be identified in this sequence under the partial digestion conditions used in this experiment. The sequences around the cleavage sites are:

```
                    ↓
5'-G-A-T-C-G-G-G-C-T-C-G-C-C-A-3'
3'-C-T-A-G-C-C-C-G-A-G-C-G-G-T-5'
          ↑
```

```
                    ↓
5'-C-T-T-C-G-G-G-C-T-C-A-T-G-A-3'
3'-G-A-A-G-C-C-C-G-A-G-T-A-C-T-5'
          ↑
```

Computer comparison of the DNA sequences around these and other sites of HgiJII cleavage are consistent with the recognition and cleavage specificity of HgiJII being:

```
          ↓
5'-G-R-G-C-Y-C-3'
3'-C-Y-C-G-R-G-5'
    ↑
```

Cross-protection of DNA against HgiAI and HgiJII. Total DNA preparations from H. giganteus strains HP1023 and HFS101 were incubated with purified HgiAI or HgiJII. The results of this experiment are shown in Figure 3. Total DNA from

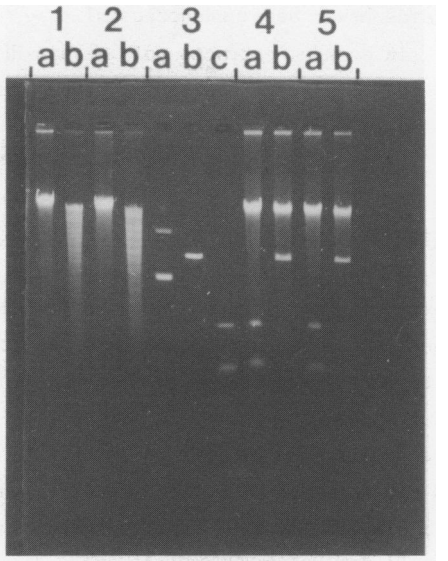

Figure 3. Resistance of Herpetosiphon giganteus DNA to cleavage by HgiAI and HgiJII. The following samples were analysed by electrophoresis on 1% agarose: 1. H. giganteus HFS101 DNA (a) uncut, (b) cut with HindIII; 2. H. giganteus HP1023 DNA (a) uncut, (b) cut with HindIII; 3. plasmid pAT153 DNA (a) uncut, (b) cut with HgiJII, (c) cut with HgiAI; 4. H. giganteus HFS101 DNA and pAT153 DNA cut with (a) HgiAI and (b) HgiJII; 5. H. giganteus HP1023 DNA and pAT153 DNA cut with (a) HgiAI and (b) HgiJII

each strain is protected against both HgiAI and HgiJII, under digestion conditions in which the internal control, plasmid pAT153, is digested.

We have shown that the recognition sequences for HgiAI and HgiJII do occur in H. giganteus HP1023 DNA by cloning an unidentified 4.6 kb fragment from a partial Sau3AI digest of this DNA in E. coli, using the vector pBR322. The DNA fragment was confirmed to be from H. giganteus as it hybridised to total DNA from that strain, but not to E. coli chromosomal DNA. Following replication in E. coli K-12, the DNA fragment could be cleaved by both HgiAI and HgiJII, each having several sites. Thus, we assume that H. giganteus DNA is protected against both HgiAI and HgiJII by modification of the sites.

We have also found (data not shown) that DNA from H. giganteus strain HFS101, but not that from strain HP1023, is protected against CauI, which has the same specificity as HgiJI. This is presumably due to the HgiJI modification activity.

Attempts to purify the activities responsible for the modification of

DNA in these two strains have been unsuccessful. We have detected some modification activity in crude extracts of strain HP1023, which protects against HgiAI, but the activity has not been further purified or characterised.

DISCUSSION

Restriction-modification systems in Herpetosiphon giganteus

Seventeen type II restriction endonucleases have been isolated from eight strains of Herpetosiphon giganteus strains (Table 2; 4-6, and this paper). These represent seven known recognition specificities, of which five are found in other bacterial genera (1). The specificities of the enzymes HgiFI and HgiKI are not known.

Although the recognition specificities of HgiHI and HgiCI are the same, their reported cleavage specificities are different. HgiCI is reported to cleave to give a six base-pair 5'-terminal extension (5), whereas HgiHI gives a four base-pair 5'-extension. However, the cleavage specificity of HgiCI may be in error, as the autoradiograph presented by Krüger et al (5) is difficult to interpret, and these authors do not discuss the relative electrophoretic mobilities of the restriction fragment (with a presumptive 3'-hydroxyl terminus) and the fragments in the chemical sequencing channels (3'-phosphate termini). These differences in charge can cause misalignment of the cleavage site against the sequence, as discussed previously (9). Such misalignment is not a problem with the chain-termination method used here. In the absence of further evidence, we cannot assume that HgiCI cleaves in a manner different from HgiHI and BanI (1).

In the eight H. giganteus strains examined, type II restriction endonucleases occur in different combinations and permutations (Table 2). If we assume that the type II restriction endonucleases described so far (and the corresponding modification activities) represent the major or total contribution to biological restriction of transforming DNA, this could have interesting biological consequences for the transfer of genetic information between strains of this species. Thus, H. giganteus Hpg5, which contains only the HgiBI system, may be transformable by DNA from strains Hpg9, Hpg24, HP1049 and HFS101 (all of which contain isoschizomers of HgiBI; Table 2). Systems for genetic exchange have not been characterised in Herpetosiphon (H.A. Foster, personal communication), so this prediction cannot yet be tested.

We have proposed that in genera in which there are many type II

restriction-modification systems in different combinations and permutations, these systems act as an "index of relatedness" between strains (18). Biological restriction is not 100% efficient, and restriction ratios for type II systems have been found to vary from about 10^{-1} (19) to 10^{-4}(20) (the number of target sites on the transforming DNA contribute to this difference). Therefore, the frequency of gene transfer between related strains of the same genus would depend, in part, on the number and the nature of differences between the restriction-modification profiles of the donor and recipient strains. Thus, for Herpetosiphon (Table 2), strain Hpg5 (which produces HgiBI) would be more efficiently transformed by DNA from strain HP1049 (which produces HgiHI, HgiHII, and HgiHIII) than it would by DNA from strain Hpa1 (which produces HgiGI), yet Hpa1 and Hpg5 may be transformed by HP1049 at similar efficiencies. Transformation of HP1049 (which has three known R-M systems) by DNA from Hpa1 may be less efficient than transformation

Table 2. Type II restriction endonucleases in Herpetosiphon

Herpetosiphon strain	Restriction enzymes	Isoschizomers in Herpetosiphon	Sequence of site	Reference
HP1023	HgiAI	-	GWGCW/C	(4)
Hpg5	HgiBI	CII, EI, HIII, JI	G/GWCC	(5)
Hpg9	HgiCI	HI	/GGYRCC	(5)
	HgiCII	BI, EI, HIII, JI	G/GWCC	
	HgiCIII	DII	G/TCGAC	
Hpa2	HgiDI	GI, HII	GR/CGYC	(5)
	HgiDII	CIII	G/TCGAC	
Hpg24	HgiEI	BI, CII, HIII, JI	G/GWCC	(5)
	HgiEII	-	ACC------GGT	
Hpg14	HgiFI	?	?	(6)
Hpa1	HgiGI	DI, HII	GR/CGYC	(5)
HP1049	HgiHI	CI	G/GYRCC	This paper
	HgiHII	DI, GI	GR/CGYC	
	HgiHIII	BI, CII, EI, JI	G/GWCC	
HFS101	HgiJI	BI, CII, EI, HIII	G/GWCC	This paper
	HgiJII	-	GRGCY/C	
Hpg32	HgiKI	?	?	(6)

of Hpa2 (which has two known R-M systems) by the same DNA, even though all three strains have one R-M system of identical specificity.

However, our data on cross-protection of DNA against the HgiAI and HgiJII restriction endonucleases show that a simple examination of the restriction endonuclease complement of the strains is insufficient to determine the contribution made by type II restriction-modification systems to constraints on gene transfer within a genus. Such an analysis requires much more data than are currently available.

The evolutionary relationship of H. giganteus restriction-modification systems.

Kröger et al (5) propose a complex scheme in which the evolutionary relationship between the restriction endonucleases of Herpetosiphon giganteus is reflected in the relatedness of their recognition specificities. They have tabulated the enzymes to give maximum overlap between recognition and cleavage specificities, and suggest that this may represent the evolutionary relationship. However, this interesting proposal does not consider the contribution of evolution outside the genus (e.g. isoschizomers). In the absence of other supporting evidence, we must take issue with the suggestion of Kröger et al that HgiEII (Table 2) has evolved from an enzyme similar to HgiCII by virtue of changes in subunit interactions. These authors imply major changes in the interaction of identical subunits, with very small changes in the specificity of recognition of each half of the site by each subunit. This proposal does not fit with accepted ideas of the evolution of multi-subunit proteins, as subunit contacts are among the most highly conserved regions of a polypeptide (21). However, as discussed below, our data are compatible with the proposal that HgiAI and HgiJII activities may have evolved within a Herpetosiphon strain.

Isoschizomers are now known for all the enzymes reported here (1). The relationship between these isoschizomers is not known, but the possibility of some of the enzymes being encoded by mobile genes cannot be discounted. Thus, the evolution of the recognition specificity of these enzymes must not be limited to consideration of Herpetosiphon; the relationship between the enzymes may be due to the selection of mobile genes rather than due to their divergent evolution. In this regard, enzymes of the same specificity as HgiHIII and HgiJI (the AvaII family) are found in at least ten different genera, covering a wide taxonomic range; these genera are Achromobacter, Anabaena, Bacillus, Caryophanon, Chloroflexus, Escherichia, Fremyella, Herpetosiphon, Nostoc and Salmonella (1). Studies on members of

the HaeIII family of isoschizomers (22) show that these enzymes have different mechanisms, and are likely to be unrelated proteins.

Are the restriction endonucleases HgiAI and HgiJII related?

The restriction endonucleases HgiAI and HgiJII share four out of six positions in their recognition sites and cut the DNA in the same relative positions to leave 3'-tetranucleotide terminal extensions. The hexanucleotide recognition sites of these enzymes differ only in the patterns of degeneracy at the second and fifth positions. This raises the interesting possibility that these two enzymes bear a close evolutionary relationship. (Such evolutionary considerations must take into account the fact that enzymes of the same specificity as HgiJII have now been found in Bacillus and Escherichia, neither of which are closely related to one another or to Herpetosiphon. It is possible that the HgiJII restriction-modification system is encoded by mobile genes, or that enzymes of identical specificity may arise by convergent evolution; 22.)

In principle, a relatively small number of differences in the interactions of the endonuclease subunits with DNA would account for the differences in recognition specificity between HgiAI and HgiJII. Those DNA-protein contacts involved in recognition of the non-degenerate positions of the recognition sites would be the same; only those involved in recognition of the second and fifth positions of the site need be different. The recognition of purine and pyrimidine degeneracies in HgiJII (Table 2) can be explained by a hydrogen bond donor on the protein, which interacts with the N7 of the purine in the major groove (23). The A-T/T-A degeneracy in the HgiAI site (Table 2) may be recognised solely in the minor groove, by hydrogen bonds to the N3 of adenine and the O2 of thymine, together with steric exclusion of the N2 of guanine to prevent recognition of G-C base pairs (23). This difference of major against minor groove interaction is a large one in terms of the mechanisms of DNA-protein interaction and any evolutionary scheme must allow for intermediate stages in conversion from one specificity to the other (such as an activity that lacks base-pair specificity at the second and fifth positions of the site), or for a common ancestral enzyme of lower specificity. However, the DNA recognition specificity of a restriction endonuclease is constrained by the specificity of the protective mechanism (e.g. modification methylase). For the specificity of one of the restriction endonucleases to have evolved from the other, or for them both to have evolved from a common ancestral enzyme, the chromosomal DNA of one or more of the strains must have been protected

against both parental and daughter endonucleases.

Our experiments show that the total DNAs from the strains containing HgiAI and HgiJII are resistant to cleavage by both enzymes. There are several possible explanations for this. The first, trivial explanation is that DNA from neither strain contains sites for these enzymes. We have eliminated this possibility for strain HP1023 by cloning DNA from this strain in E. coli and showing that susceptible sites for both enzymes are then present. A second explanation is that both strains contain both restriction-modification systems. Although this cannot formally be eliminated as an explanation, we have been unable to detect the presence of the other endonuclease in either strain. Alternatively, both M.HgiAI and M.HgiJII modification enzymes may be present in both strains, even though only one of the endonucleases is present. A third possibility is that the single modification enzymes M.HgiAI and M.HgiJII have a more degenerate specificity than their corresponding endonucleases, and modify several different sequences which include both HgiJII and HgiAI sites. (A methylase recognising both HgiAI and HgiJII sites need have no discriminatory recognition of the degenerate base pairs in these sites.)

In order to test between these possibilities, we need to purify the modification activities from both these strains and to perform modification and challenge experiments in vitro. We have not yet succeeded in purifying these activities.

ACKNOWLEDGEMENTS: We thank Drs J.H. Parish and H.A. Foster for strains, and S.P. Bennett and Dr J.A. Littlechild for enzymes. S.P. Bennett, Dr H.A. Foster, Dr S.E. Halford, Dr P.A. Lund and Dr R. Mullings provided useful discussion. This work was supported by the Medical Research Council and the Royal Society, of which NLB is a EPA Cephalosporin Fund Senior Research Fellow. DJ is supported by an SERC studentship.

Abbreviations:
PMSF = phenylmethylsulphonyl fluoride. Degenerate nucleotide sequences are named in accordance with IUB proposals (Nucleic Acids Res. 13, 3021-3030, 1985). R = A or G; Y = C or T; W = A or T; S = G or C.

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