
Site-dependent cleavage of pBR322 DNA by restriction endonuclease *HinfI*

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Received 17 February 1983; Revised and Accepted 9 May 1983

ABSTRACT

Cleavage of pBR322 DNA I by the restriction endonuclease *HinfI* is preferentially inhibited at specific *HinfI* cleavage sites. These sites in pBR322 DNA I have been identified and ordered with respect to the frequency with which they are cleaved. The *HinfI* site most resistant to cleavage in pBR322 DNA I is unique in that runs of G-C base pairs are immediately adjacent on both sides. Two differently permuted linear (DNA III) species were produced by cleavage with two different restriction endonucleases, *PstI* and *AvaI*. Only one of these linear molecules, that produced by *PstI*, exhibits the same preferential cleavage pattern as DNA I. The second linear species, that arising from *AvaI* digestion, shows pronounced relative inhibition of cleavage at the *HinfI* sites nearest the ends of the molecule (100 and 120 base pairs away, respectively). This result suggests that proximity to the termini of a linear DNA molecule might also influence preferential cleavage. The possibility of formation of stem-loop structures does not appear to influence preferential cleavage by *HinfI*.

INTRODUCTION

Type II restriction endonucleases recognize a specific duplex deoxynucleotide sequence, usually 4-6 bp in length, and introduce double-strand scissions either within or adjacent to this sequence. In spite of the fact that the nucleotide sequences of all recognition sites are usually identical for a given enzyme, several restriction endonucleases have been shown to cleave at some recognition sites within a given DNA molecule more readily than at others (1-6). We term this phenomenon preferential cleavage.

As we have previously reported (6), when non-limit digests of *PstI* and covalently closed circular supercoiled pSM1 plasmid (7-9) DNA I are examined by gel electrophoresis, it is clear that some *PstI* sites are cleaved more readily than others. Specifically, cleavage at one of the four *PstI* sites of pSM1 DNA is relatively strongly inhibited. This particular *PstI* site is unique in two respects: it lies quite close to a possible stem-loop structure, and the adjacent nucleotide sequence is

especially rich in G-C base pairs. Four different structural forms of pSM1 DNA (superhelical, closed relaxed, nicked and linear), were examined and all showed the same pattern of preferential cleavage (6).

The investigation of factors that could contribute to site-dependent preferential cleavage continues in the present study. We find that the DNA of a second plasmid, pBR322 (10-13), exhibits a comparable pattern of preferential cleavage when digested with the restriction endonuclease HinfI. The last six partial digestion products remaining before digestion is complete were characterized. The HinfI fragments which compose these six partials have been determined, and the regions adjoining the HinfI sites contained therein were examined for structural features which might be expected to influence preferential cleavage.

MATERIALS AND METHODS

Bacterial strains and plasmids. Escherichia coli K-12 strains C600 (F^- thr leu lacY tonA supE44 ΔtrpE5) and RR1 (F^- pro leu thi lacY str^r_k⁻m_k⁻), each carrying plasmid pBR322 (10-13), were obtained from D.R. Helinski and S.J. Keller, respectively.

Reagents and enzymes. Acrylamide, N,N'-methylene-bis-acrylamide, N,N,N',N'-tetramethylethylene diamine (TEMED), and ammonium persulfate were electrophoresis purity reagents obtained from Biorad Laboratories. Ethidium bromide (EtdBr) and Triton X-100 were supplied by Calbiochem and Biorad, respectively. Restriction endonucleases HinfI, PstI, AvaI and HindIII were obtained from Bethesda Research Laboratories and restriction endonuclease EcoRI from Miles Laboratories and were used according to the manufacturer's instructions. Bacteriophage λ DNA was obtained from Bethesda Research Laboratories. Chloramphenicol was purchased from Sigma Chemical Company.

Preparation of plasmid DNA. Cultures containing plasmid pBR322 were amplified using chloramphenicol (250 μg/ml), and plasmid DNA was purified by the cleared lysis method (14) followed by two sequential dye-CsCl density gradients. EtdBr was removed by extraction with isopropanol equilibrated with CsCl-saturated buffer (50 mM Tris, pH 8.0, 5 mM EDTA and 50 mM NaCl). CsCl was subsequently removed by dialysis against TE buffer composed of 10 mM Tris (pH 8.0) and 1 mM EDTA.

Cleavage of DNA with HinfI. To demonstrate preferential cleavage of pBR322 DNA by HinfI, the enzyme was diluted appropriately with the HinfI assay buffer (6 mM Tris, pH 7.5, 6 mM MgCl₂, 100 mM NaCl, 6 mM 2-mercaptoethanol)

recommended by Bethesda Research Laboratories. Digests were incubated at 37°C for one hr, and incubation was terminated by heating at 65°C for 5-10 min before adding one-quarter volume of tracking dye solution (0.03% bromphenol blue in 50% glycerol). Digests were typically 30-50 μ l in volume and contained 0.35 μ g pBR322 DNA.

Electrophoresis. The standard E buffer used for electrophoresis has been described previously (7). The bis-acrylamide/acrylamide ratio used for gel preparation was 1:20. Slab gels of 4% polyacrylamide were 16 cm X 14 cm X 0.2 cm in size and were allowed to polymerize at room temperature for approximately one hr before samples were loaded. Electrophoresis was carried out at 90-100 V for 3-4 hr or until the tracking dye had migrated three quarters of the length of the gel. The gel was removed from the apparatus and stained with EtdBr (0.5 μ g/ml) in E buffer for approximately 30 min prior to photography under short wave ultraviolet illumination. For identification of partial digestion products, DNA bands were excised from the gel with a razor blade, and the DNA was electroeluted from the gel and treated as described previously (6). The resulting DNA pellet was dried under vacuum and resuspended in HinfI assay buffer using two cycles of freezing and thawing. A 3- to 5-fold excess of HinfI was used for these final digestions.

Determination of mole fractions of partial digestion products. Fixed amounts (usually 0.35 μ g) of pBR322 DNA's I and III were digested at 37° C for one hr using 1.0, 0.8, 0.6, 0.4, 0.3 or 0.25 units HinfI/ μ g DNA. After staining with EtdBr (0.5 μ g/ml), gels were photographed with Kodak Royal Pan film. Negatives were traced with a Joyce-Loebl microdensitometer, and mole fractions of different partial digestion products were determined from these tracings as previously described (6). At least two digests containing each partial digestion product were used for these determinations.

Computer Search. A computer program (15) designed to predict the optimum local DNA structure was used to search two regions of pBR322 DNA for stem-loop structures. The maximum allowed size of a loop was 50 nucleotides, and the regions of pBR322 searched were nucleotides 2250-2700 and nucleotides 3000-3450 according to the designation of Sutcliffe (13) .

RESULTS

Preferential cleavage of pBR322 DNA by HinfI.

The entire nucleotide sequence of the plasmid pBR322 (4362 bp) has

been previously determined (11-13). pBR322 DNA contains ten cleavage sites (13) for the restriction endonuclease *Hinf*I (Hutchison, C.A., Barrell, B.G., Middleton, J.H., Stankus, P.V., Edgell, M.H., Murray, K. and Morrison, A., unpublished observations), which recognizes the sequence 5'-GANTC-3', N being any deoxyribonucleotide (Fig. 1, Table 1). *Hinf*I is a type II restriction endonuclease whose recognition sequence is not uniquely specified.

To determine whether *Hinf*I shows preferential cleavage with pBR322 plasmid DNA, as has been observed with *Pst*I and *pSM1* plasmid DNA (6), purified pBR322 DNA I was digested with limiting amounts of *Hinf*I. The digestion products were then examined after fractionation by electrophoresis in a 4% polyacrylamide gel (Fig. 2). The result of nearly complete digestion of pBR322 DNA I by *Hinf*I (enzyme/DNA of 0.8 units/ μ g DNA) is shown in Fig. 2, lane b. The 10 bands characteristic of limit digestion of pBR322 DNA by *Hinf*I (13) are readily apparent. In addition, a faint band of partially-digested DNA that is larger than *Hinf*I fragment 1 is present. At lower enzyme/DNA ratios, additional partial digestion products appear. Two

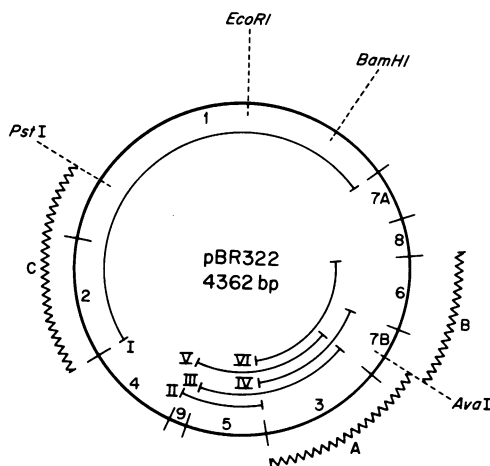


Figure 1. Diagrammatic representation of the restriction endonuclease cleavage sites of pBR322. The *Hinf*I cleavage sites delineated by Sutcliffe are shown as solid lines and are numbered using the original designation (13). The unique cleavage sites for *Pst*I, *Eco*RI, *Bam*HI and *Ava*I are shown as dotted lines. The partial digestion products resulting from *Hinf*I cleavage of pBR322 DNA I are drawn as curves inside the circle and are numbered I-VI, as explained in the text. Partial digestion products resulting from *Hinf*I cleavage of pBR322 linear DNA's generated by *Ava*I and *Pst*I are shown as sawtooth lines outside the circle and are designated A, B and C (see text).

prominent partial digestion products are visible at an enzyme/DNA ratio of 0.6 units/ μ g DNA. These two products (partial I and partial II) are approximately 2180 and 427 bp in size, respectively, (Fig. 2, lane c). At the same HinfI/DNA ratio, three additional partial digestion products (partials III, IV and V) of approximate sizes 850, 730 and 960 bp are also present, although in relatively smaller amounts than the first two products (Fig. 2, lane c). At a still lower ratio of HinfI to DNA (0.4 units/ μ g DNA), several more partial digestion products are visible, the most prominent of which is about 1050 bp in size and is designated partial VI

Table I. HinfI Sites of pBR322 and Adjacent Nucleotides

Location of <u>HinfI</u> site ^a	Nucleotide sequence ^b	Coupled <u>HinfI</u> Fragments ^c	Partial Designation
631	CCTAATGCAGGAGTCGCATAAGGGA GGATTACGTCCTCAGCGGTATTCCC	1,7A	
851	GCGGTATTCGGAATCTTGCACGCC CGCCATAAGCCTTAGAACGTGCGGG	7A,8	
1005	TCCCCATTATGATTCTTCTCGCTTC AGGGGTAATACTAAGAAGAGCGAAG	8,6	
1303	GCATGGATTGGATTCACCACTCAA CGTACCTAACCTAAGTGGTGAGGTT	6,7B	VI
1524	TTAGCAGAATGAATCACCGATACGC AATCGTCTTACTTAGTGGCTATGCG	7B,3	IV,VI
2030	AGACATCTGTGAATCGCTTCACGAC TCTGTAGACACTTAGCGAAGTGCTG	3,5	III,V
2374	CTCGCTCACTGACTCGCTGGGCTCG GAGCGAGTGACTGAGCGACGCGAGC	5,9	II,V
2449	GTTATCCACAGAATCAGGGGATAAC CAATAGGTGTCTTAGTCCCCTATTG	9,4	
2845	CTATCGTCTTGAGTCCAACCCGGTA GATAGCAGAACTCAGGTTGGCCAT	4,2	
3361	ATAGTTGCCTGACTCCCCGTCGTGT TATCAACGGACTGAGGGGCAGCACA	2,1	I

^a Location of the 5' nucleotide clockwise from the unique EcoRI site, according to Sutcliffe (13).

^b The recognition sequence for HinfI is underlined.

^c The HinfI fragments designated are those joined to the left and right of the restriction sequence as written.

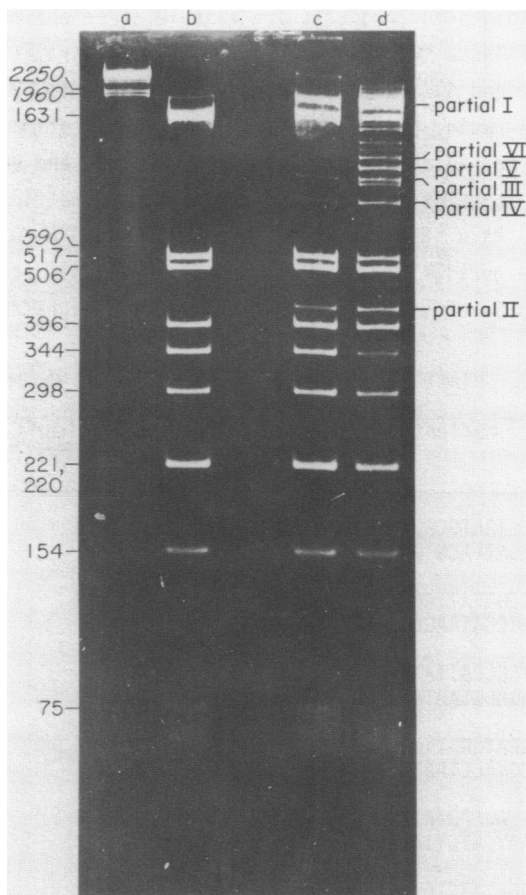


Figure 2. 4% polyacrylamide gel showing HinfI partial digestion products. Electrophoresis in slab gels was performed as described in Materials and Methods. The DNA fragments visualized in different lanes are: a) HindIII digest of bacteriophage λ DNA with molecular sizes designated by the italicized numbers to the left; b-d), pBR322 DNA I subsequent to cleavage with 0.8, 0.6, and 0.4 units HinfI/ μ g DNA, respectively. The sizes of pBR322 HinfI fragments in bp are designated by non-italicized numbers to the left. The partial digestion products are labeled I-VI. The fragments referred to as 7A, B in the text comigrate at approximately 220 bp.

(Fig. 2, lane c). Because partial digestion products appear in a fixed order after treatment with limiting HinfI, we conclude that HinfI exhibits preferential site-dependent cleavage with pBR322 DNA I as substrate.

Determination of fragments contained within partial digestion products I-VI.

The fragment compositions of the first six partial digestion products

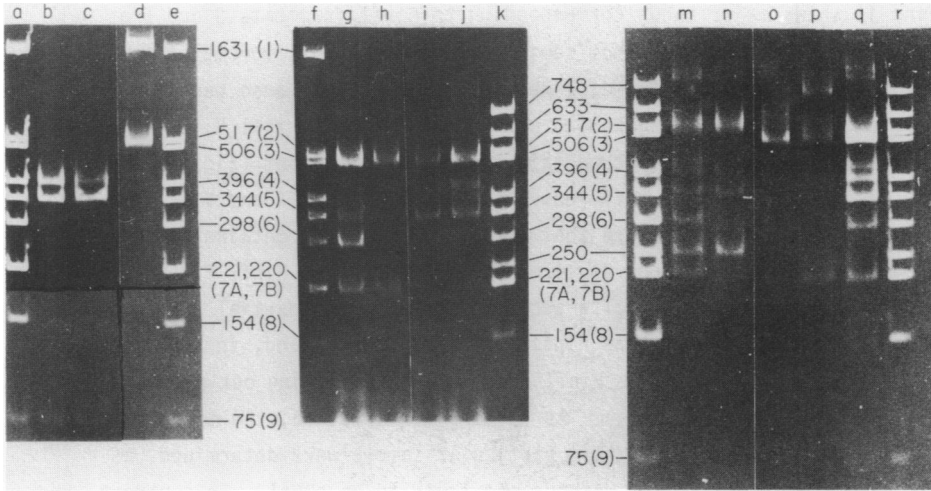


Figure 3. Products of *Hinf*I cleavage of partial digestion products I-VI after electrophoresis in a 4% polyacrylamide gel. Single digests of pBR322 DNA I with *Hinf*I are shown in lanes a, e and f and triple digests with *Hinf*I, *Pst*I and *Eco*RI are shown in lanes k, l and r, respectively. The sizes of the fragments in bp are indicated, and the fragment designations as given by Sutcliffe (13) are in parentheses. *Pst*I and *Eco*RI both cleave within *Hinf*I fragment 1, producing fragments of 748, 633, and 250 bp (13). Partial I DNA is in lane d; partial II, lanes b and c; partial III, lane i; partial IV, lanes h and p; partial V, lanes j and q; partial VI, lane g; partial A, lane o; partial B, lane m; and partial c, lane n.

were determined. DNA bands corresponding to these six partials were eluted from polyacrylamide gels, cleaved with excess *Hinf*I, and subjected to electrophoresis in 4% polyacrylamide gels. After complete digestion of partial I DNA with *Hinf*I, two bands corresponding to *Hinf*I fragments 1 and 2 are visible (Fig. 3, lane d). Digests of two different preparations of partial II DNA showed bands corresponding to *Hinf*I fragments 5 and 9 and, in one of the preparations, fragment 4 (Fig. 3, lanes b and c). *Hinf*I fragment 4 is quite likely a contaminant in this latter preparation (lane b), since fragment 4 and partial II are of comparable size and complete physical separation is difficult (Fig. 1). Partial III and IV give rise to *Hinf*I fragments 3 and 5 or 3 and 7, respectively (Fig. 3, lanes i or h and p). It was assumed that the particular fragment 7 seen in partial IV digests is fragment 7B and not fragment 7A, because fragment 7B is adjacent to fragment 3 while fragment 7A is not (13, Fig. 1).

An extensively digested preparation of purified partial V (Fig. 3,

lane j) shows major bands corresponding to HinfI fragments 3 and 5 as well as a third band that does not comigrate with any HinfI monomer fragment and which probably results from incomplete digestion. Because partials III and V both contain HinfI fragments 3 and 5 and because partial V is larger than partial III, partial V must contain a third DNA fragment (Fig. 2, lanes c and d). Since the size difference between partials III and V is less than 100 bp (Fig. 2), we surmised that partial V probably contains HinfI fragment 9, which is 75 bp in size and is adjacent to HinfI fragment 5 (13, Fig. 1). HinfI digestion of a second preparation of partial V produced major bands the same size as HinfI fragments 3 and 5 and, in addition, a faint band the same size as HinfI fragment 9, confirming our previous assignments (Fig. 3, lane q). As further confirmation, the mole fractions of fragments 3, 5 and 9 in this particular sample were determined as described in Materials and Methods and found to be equal. In contrast, the mole fractions of the minor bands are less than those of fragments 3, 5 or 9. Minor bands the same size as HinfI fragments 4, 6 and 7 in this preparation of partial V probably arise from contamination with other partial digestion products, which migrate quite close to partial V in gels (Fig. 2, lane d). Finally, if we apply the same reasoning to fragment 7 of partial VI as used above for partial IV, partial VI is composed of HinfI fragments 3, 6 and 7B (Fig. 3, lane g). In summary, the HinfI partial digestion products I-VI are composed of the pBR322 HinfI DNA fragments 1 + 2, 9 + 5, 3 + 5, 3 + 7B, 3 + 5 + 9 and 3 + 6 + 7B, respectively (Figs. 1,3; Table 1). Therefore, the first four partial digestion products contain one residual HinfI cleavage site and the last two each contain two HinfI sites.

Ordering of partial digestion products.

The order of appearance of these partial digestion products was determined by calculating their respective mole fractions using microdensitometer tracings of several gels containing digests in which the HinfI/DNA ratios had been varied systematically (See Materials and Methods). The partial digestion product present at the highest level was assumed to contain the HinfI site most refractory to HinfI cleavage; the partial digestion product present at the next highest level, to contain the HinfI site second most resistant to HinfI cleavage, etc. This order of appearance of partial digestion products is listed in Table 1 and is the same as the numerical designation of different partials, with partial I appearing first, and partial II, second, etc. Because of the large number of possible partial digestion products (100), we were unable to order other

HinfI cleavage sites with respect to their frequencies of scission. However, all remaining HinfI sites are cut more readily than any of the sites contained within the six partial digestion products described here. Preferential cleavage of pBR322 linear DNA.

Next, the effects of DNA tertiary structural type upon preferential cleavage of pBR322 DNA were examined. Two permuted pBR322 linear molecules were prepared by digestion of pBR322 DNA I with two different restriction endonucleases, AvaI (15) and PstI (16,17). The choice of these specific endonucleases was based upon three considerations: 1) each endonuclease cleaves pBR322 DNA only once (13, Fig. 1); 2) the PstI site lies closest (250 bp) to the junction most refractory to HinfI cleavage [that between HinfI fragments 1 and 2(13)]; and 3) the cleavage site for AvaI is the farthest (2174 bp) unique cutting site from the junction of fragments 1 and 2 (13, Fig. 1).

The pattern of DNA bands that results from cleavage of PstI-generated linear pBR322 DNA molecules with limiting amounts of HinfI is shown in Fig. 4. Because the DNA has been cleaved first with PstI, subsequent digestion with HinfI resulted in the production of two new fragments that were 1381 and 250 bp in size (Fig. 4, lane h). These new fragments are produced from HinfI fragment 1 (1631 bp), which contains the PstI cleavage site (13, Fig. 1). As a further consequence of PstI cleavage prior to HinfI cleavage, partial I (2148 bp), composed of HinfI fragments 1 and 2, is absent. Instead, the two most prominent partial digestion products are partial II, previously observed in digests of pBR322 DNA I, and a new species, which was designated partial C, approximately 770 bp in size (Fig. 4, lane j).

This new partial (C) is close to the size expected (767 bp) if cleavage is inhibited at the HinfI site between fragments 2 (517 bp) and the adjacent 250 bp portion of PstI-cleaved HinfI fragment 1. Indeed, when partial C DNA was electroeluted from gels and cut with excess HinfI, it was found to consist of HinfI fragment 2 and a new fragment of approximately 250 bp (Fig. 3, lane n). This latter band comigrates with a fragment of the same size produced by limit digestion of pBR322 DNA I with HinfI, EcoRI and PstI and presumably is the region of DNA between the junction of HinfI fragments 1 and 2 and the PstI site (Fig. 1). Therefore, the two sites at which HinfI cleavage is most inhibited when PstI-generated pBR322 DNA III is the substrate are the same sites that are most inhibited when pBR322 DNA I is the substrate: the HinfI sites at the junctions of fragments 2 and 1 and fragments 5 and 9.

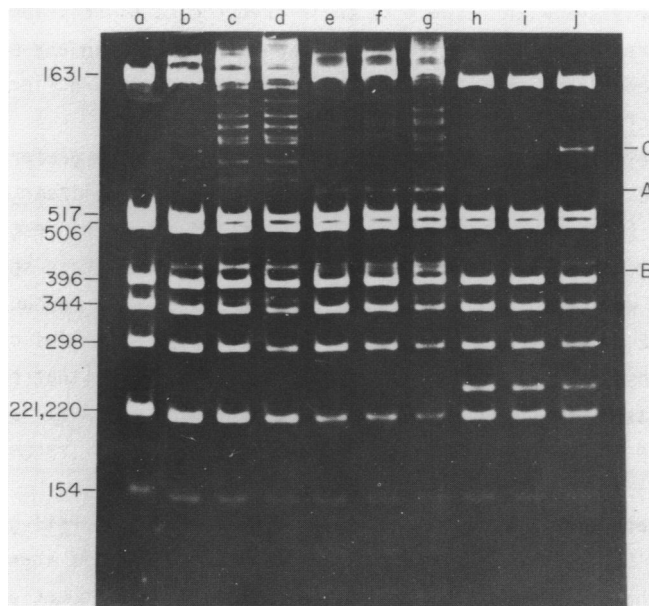


Figure 4. Cleavage of pBR322 DNA I and two differently permuted DNA III molecules with *HinfI*. DNA I is shown in lanes a-d following incubation with 0.8, 0.5, 0.4, and 0.3 units *HinfI*/μg DNA. *AvaI*- and *PstI*-generated DNA III's subsequently digested with *HinfI* are visible in lanes e-g and h-j, respectively. The *HinfI*/DNA ratios used in these latter digests were 0.5, 0.4, and 0.3 units *HinfI*/μg DNA from left to right for each DNA III. The sizes in bp of pBR322 *HinfI* fragments are shown to the left. The partials (A, B, and C) produced by cleavage of DNA III are labeled to the right.

The partial digestion products resulting from the use of *AvaI*-generated pBR322 DNA III as substrate for *HinfI* were then examined. *AvaI* cleaves pBR322 DNA within *HinfI* fragment 7B, producing fragments 120 and 100 bp in size from fragment 7B (13, Fig. 1). After digestion of *AvaI*-cleaved DNA with limiting *HinfI*, two new prominent partials are visible (Fig. 4, lanes f and g). These new bands are approximately 590 bp (partial A) and 414 bp (partial B) in size. The 590 bp fragment is nearly the size expected (606 bp) if the *HinfI* site between fragment 3 (506 bp) and the shorter (100 bp) part of *AvaI*-cleaved fragment 7B is not cut.

After elution of the DNA in the partial A fragment and digestion with *HinfI*, a band the size of fragment 3 (506 bp) is observed. However, the expected band of 100 bp in size is not visible, presumably because the amount of DNA eluted from the partial A band was inadequate for its

detection (Fig. 3, lane o). Nevertheless, the conclusion that partial A is composed of HinfI fragment 3 and the 100 bp portion of fragment 7B seems to be correct, considering the size of partial A and the demonstration that it does contain fragment 3.

The smaller new prominent partial, partial B, is nearly the size expected (418 bp, 13) if the HinfI site between fragment 6 (298 bp, 13) and the larger portion of AvaI-cut fragment 7B (120 bp, 13) was not cleaved. Elution and recutting of partial B with HinfI resulted in major bands that comigrate with HinfI fragments 2,5,6 and 7 (Fig. 3 lane m), of which only fragment 6 was expected. The putative presence of fragment 5 in this preparation could be due to contamination of partial B with partial II (419 bp), since these latter two partial digestion products nearly comigrate in the gel (Fig. 2). The apparent contamination of this sample with either HinfI fragment 2 or 7 is, however, difficult to rationalize. Failure to observe a fragment 120 bp in size was presumably due either to the small amount of partial B DNA present in the sample or to incomplete digestion. On the basis of size alone, we conclude that partial 7B is composed of HinfI fragment 6 and the larger portion of AvaI-cut fragment 7B. Therefore, in contrast to results obtained with PstI-generated DNA III as substrate, when AvaI-generated DNA III is cut with limiting HinfI, cleavage at the two HinfI sites nearest the ends of this linear DNA molecule appear to be preferentially inhibited.

DISCUSSION

We have examined preferential cleavage of pBR322 plasmid DNA by the restriction endonuclease HinfI. The last six partial digestion products which remain before digestion is complete were characterized as to their fragment composition and ordered according to the frequency with which they were cleaved by HinfI. Two differently permuted pBR322 DNA linear molecules were produced with different restriction enzymes, and the cleavage patterns were compared with that obtained with pBR322 DNA I.

The cleavage pattern of the pBR322 linear DNA species generated by AvaI showed marked inhibition of cleavage at sites near the linear termini. In contrast, both the PstI-generated linear species from pBR322 and the EcoRI-generated linear species produced from plasmid pSM1 (6) showed the same preferential cleavage patterns as their respective DNA I species. However, the HinfI sites in pBR322 lie relatively close to the AvaI site (100 and 120 bp away). In comparison the HinfI sites in pBR322 lie 250 and

1381 bp away from the PstI site (13) and the PstI sites in pSM1 lie 380 and 1220 bp away from the EcoRI site (18). It has been reported (19) that both the association and dissociation rates of EcoRI with a series of DNA fragments containing the EcoRI scission site are dependent on fragment length over a range of 34-6,200 bp, although the equilibrium binding constant is independent of length. It was also found that the kinetic constants varied by a factor of 8, while the DNA fragment length increased by a factor of nearly 200. It is unclear whether these effects are dependent upon the absolute fragment length or upon the attendant variation of the distance of the scission site from the fragment termini.

Endonuclease EcoRI is also known to cleave different sites within the same linear DNA molecule at very different rates (2). Because there did not seem to be any correspondence between the location of a site within a DNA molecule and the frequency of cleavage at that site, Thomas and Davis suggested that the local nucleotide sequence could be the dominant factor determining the rates of site specific cleavage (2). This idea was reinforced by an observation of Rubin and Modrich: following single strand cleavage, the dissociation rate of EcoRI from the single EcoRI site in SV40 DNA is greater than from the EcoRI site in ColE1 DNA (4). These investigators suggested that different nucleotide sequences surrounding the EcoRI sites of these two DNA substrates are responsible for the differences in dissociation rates. The EcoRI site of SV40 DNA is the poorer substrate and, in fact, there is a run of either three (20,21) or four (22) G-C base pairs adjacent to the SV40 EcoRI site and a run of only two G-C base pairs adjacent to the ColE1 EcoRI site (22).

Nath and Azzolina (5) observed that sites near the termini of bacteriophage lambda linear DNA are cleaved faster than sites near the middle of the molecule by the endonucleases BamHI and HindIII as well as by EcoRI. However, these results are probably of limited generality because they are not supported by the EcoRI cleavage results with P4 DNA reported by Davis and collaborators (1) or by our observation that the sites near the ends of one pBR322 linear molecule were preferentially inhibited rather than preferentially cleaved (see Results).

Two of the six partial digestion products (partials V and VI) characterized in this work each contains three HinfI pBR322 fragments instead of two (Figs. 1 and 2). Each of these partials also contains at least one dimeric partial digestion product (partial V contains partials II and III and partial VI contains partial IV). It is interesting that the

order of appearance in the presence of limiting HinfI of the trimeric partial digestion products (partial V before partial VI) is the same as the order of appearance of the dimeric partials they contain (partials II and III appearing before partial IV). This suggests that the pattern of preferential cleavage is not influenced by the length of the DNA molecule used as substrate. With the exception of partial I, all other partial digestion products are clustered in one 1071 bp section of the pBR322 genome, from nucleotides 1303 to 2374. This is the same region of pBR322 in which the AvaI site is located (Fig. 1, 13), and this region of the pBR322 genome is also close to the replication origin of the plasmid and is replicated first (11,23). The structural basis for this clustering is unknown.

The molecular origins of preferential cleavage have yet to be elucidated. In a previous study, the site most resistant to cleavage by PstI in plasmid pSM1 was found to be very close to a possible stem-loop structure and also to have adjacent sequences that are rich in G-C base pairs (6). Accordingly, the HinfI sites of pBR322 DNA were examined for the presence of special duplex structures and for the composition of adjacent nucleotide sequences. A computer search (15) was conducted of the pBR322 nucleotide sequences around the two HinfI sites most resistant to cleavage in an attempt to identify possible special duplex structures such as loops, hairpins and cruciforms. The nucleotide sequences searched included nucleotides 3000-3450 of the pBR322 sequence, which encompass the junction of HinfI fragments 2 and 1 (partial I), and nucleotides 2250-2700, which encompass the junctions of HinfI fragments 5 and 9 (partial II) and fragments 9 and 4 (not identified as being part of any partial digestion product). The 2/1 junction is part of a possible stem-loop structure of moderate stability and is surrounded by other possible stem loop structures of approximately the same stability (Table 2). However, these stem-loop structures do not appear to be of sufficient stability to inhibit HinfI cleavage because no stem-loop structures that are closer than 130 bp to this junction were identified experimentally by either Lilley (24,25) or Panayotatos and Wells (26).

At the site second most resistant to HinfI cleavage, the 5/9 junction, the HinfI cleavage site is part of a putative stem-loop structure considerably more stable than those near the 2/1 junction. However, neither Lilley nor Panayotatos and Wells identified any SI-sensitive stem-loop structures in this part of the pBR322 genome (24-26); and a

Table 2. Regions Containing Possible Stem-loop Structures in pBR322 DNA.

Location ^a	ΔG (kcal/mole)	Structural feature included
Search region: nucleotides 2250-2700		
2260 - 2300	-9.4	
2300 - 2319	-9.7	
2321 - 2371	-15.9	
2371 - 2412	-29.8	Junction, fragments 9/5 (partial I)
2419 - 2469	-11.5	Junction, fragments 4/9
2486 - 2535	-27.5	
2548 - 2598	- 8.4	
2603 - 2651	-17.0	
2652 - 2696	-11.2	
Search region 2, nucleotides 3000-3450		
3018 - 3047	-9.6	
3047 - 3088	-29.5	Major S1-sensitive site (24,25)
3090 - 3106	-4.3	
3106 - 3145	-14.8	Subminor S1-sensitive site (24,25)
3145 - 3181	-8.2	
3183 - 3232	-13.0	Minor S1-sensitive site (24,25)
3237 - 3255	-0.2	
3264 - 3306	-9.0	
3314 - 3362	-13.3	Junction, fragments 2/1 (partial I)
3364 - 3399	-16.7	
3408 - 3447	-11.3	

^a Location of the 5' nucleotide clockwise from the EcoRI site, according to Sutcliffe¹³.

computer search conducted by Lilley did not identify any significantly stable stem-loop structures within this region (24). On the basis of our own data and in agreement with that of Lilley and Panayotatos and Wells (26), we therefore conclude that stem-loop structures play little or no part in the inhibition of cleavage of pBR322 DNA by HinfI. An alternative possibility is that the cleavage rate at the 9/5 junction is influenced by its relative proximity to the 4/9 junction terminus, 75 bp away.

It is probably noteworthy that the two sites in pBR322 which appear most resistant to cleavage with HinfI have adjoining sequences that are relatively rich in G-C base pairs (Table 1). This is also true of the site

Table 3

Nucleotide Frequencies Adjacent to pR322 <u>Hinf</u> Sites																				
A. Nucleotide frequencies^a adjacent to all ten <u>Hinf</u>I sites^b																				
	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	+1	+2	+3	+4	+5	+6	+7	+8	+9	+10
A	20	0	50	20	20	30	10	40	30	10	30	10	20	10	30	30	10	10	30	30
T	20	50	20	20	30	40	30	30	20	60	20	20	20	30	10	20	10	30	20	10
C	30	40	20	20	40	10	30	30	40	0	20	60	40	20	50	30	40	30	20	50
G	30	10	10	40	10	20	30	0	10	30	30	10	20	40	10	20	40	30	20	10
B. Nucleotide frequencies adjacent to <u>Hinf</u>I sites within partials I-IV																				
	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	+1	+2	+3	+4	+5	+6	+7	+8	+9	+10
A	50	0	75	0	25	25	0	75	25	0	25	0	0	0	25	25	25	0	25	0
T	25	75	0	0	25	75	0	0	0	100	0	0	50	25	25	25	0	50	0	25
C	25	0	25	25	50	0	50	25	50	0	25	100	50	0	50	25	50	25	25	50
G	0	25	0	75	0	0	50	0	25	0	50	0	0	75	0	25	25	25	50	25
C. Nucleotide frequencies adjacent to remaining six <u>Hinf</u>I sites																				
	-10	-9	-8	-7	-6	-5	-4	-3	-2	-1	+1	+2	+3	+4	+5	+6	+7	+8	+9	+10
A	0	0	33	33	17	33	17	17	33	17	33	17	33	17	33	33	0	17	33	50
T	17	33	33	33	33	17	50	50	33	33	33	33	0	33	0	17	17	17	33	0
C	33	67	17	17	33	17	17	33	33	0	17	33	33	33	50	33	33	33	17	50
G	50	0	17	17	17	33	17	0	0	50	17	17	33	17	17	17	50	33	17	0

^b Nucleotide frequencies are expressed as the nearest integral percentage.

^b The sites on the 5' side of the recognition sequence are negative, and those on the 3' side are positive.

in pSM1 DNA most resistant to PstI (6). The ordering of the remaining HinfI sites of pBR322 that are refractory to cleavage appears, however, to correlate less well with overall base composition of surrounding nucleotide sequences and is possibly complicated by the variability of the central base pair in the HinfI recognition sequence. In addition, there appears to be no correlation at all between the presence or absence of runs of G-C base pairs to the 5' side of the cleavage sites and the frequency of cleavage by HinfI. Upon close examination of the adjoining sequences, however, the sequences most resistant to scission, those within partials I and II, contain the longest runs of G-C base pairs immediately adjacent to the 3' end of the recognition site. For example, the most resistant site (that within partial I) contains a run of 5 G-C's to the 3' side of the recognition sequence. The next most resistant site, that within partial II, contains a run of 3 G-C base pairs at this location, with 7 G-C base pairs occurring within 8 base pairs of the cleavage site (Table 1). The

currently available data, therefore, suggests that preferential cleavage at some sites is associated with G-C runs to the 3' side of the recognition site, with susceptibility to cleavage decreasing as the number of G-C base pairs increases.

Analysis of the frequency of occurrence of each nucleotide in the ten positions both to the 3' and 5' sides of the ten HinfI restriction sequences is presented in Table 3. The data have been grouped into three categories: A) all ten sites considered together, B) the four sites most resistant to scission, and C) the six sites least resistant to scission. Examination of the nucleotide frequency distribution in Group A suggests little obvious departure from the expected average value of 25%. In the case of Group B, however, the observed frequencies appear to depart significantly from the expected mean value of 25%. In particular, the first position (-1) to the 5' side of the restriction site is invariably occupied by thymine, a base which is then excluded from the next three positions in the sequence (-2,-3 and -4). Thymine is also excluded from the first two positions at the 3' side (+1 and +2), and cytosine appears to be highly preferred in the second position at the 3' side (+2). Finally, guanine is excluded from positions +2 and +3 and adenine from positions +2, +3, and +4. Although the number of samples used in this analysis is insufficient for application of statistical tests, the observed distribution appears to deviate significantly from that expected by chance. For instance, the chance expectation of finding thymine exclusively in the first 5' position (-1) is 1/64. The frequency of distribution of nucleotides in Group C shows no particular bias.

On the basis of the above considerations, we suggest that resistance to enzymatic scission by HinfI is associated with the occurrence of certain bases in positions immediately proximal to the cutting sequence, especially at the 5' side. In particular, the high apparent selectivity for thymine in the -1 position and its subsequent exclusion from the next three sites (-2,-3 and -4) strikes us as especially significant. Such a proximity effect was found in the case of eucaryotic topoisomerase I from HeLa cells, wherein certain bases were excluded with high probability from regions surrounding the binding site (27). The nature and extent of interaction of restriction enzymes with DNA might also be expected to depend upon the occurrence of specific bases in surrounding regions. Such adjacent base specificity might influence either the binding of the enzyme to DNA or the specific rate of cleavage at the scission site or both.

There exists a precedent for the dependence of the rate of endonucleolytic scission upon local DNA structure. In the case of DNase I, Klug and co-workers (28) have shown that the rate constant for scission varies by at least two orders of magnitude among the various bonds of a defined dodecamer. In this case, the apparent sequence dependence of the scission rate was attributed to local duplex structures that are known to accompany these particular sequence differences. In the case of restriction enzymes, in contrast to the case of DNase I, the first step in the recognition and scission process must be specific binding of the enzyme in the vicinity of the recognition sequence itself. This event might well also depend on local variations in DNA structure associated not only with the sequences of bases in the recognition site but also with those sequences immediately adjacent (29). The result of this more complex problem must await studies of the effect of restriction enzymes upon various synthetic oligonucleotides containing a given recognition site in the middle of this sequence.

ACKNOWLEDGMENTS

This work was supported by National Needs Postdoctoral Fellowship SPI-7913986 from the National Science Foundation to K.A.A. and by Public Health Service Grant GM-21176 from the Institute of General Medical Sciences to W.R.B.

We are indebted to Ann Jacobson for making her modified computer program available to us and for her assistance in its use. We are also grateful to Daniel B. Davison and William Wishart for assistance with computer programs.

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