Accurate Scanning of the BssHII Endonuclease in Search for Its DNA Cleavage Site*

(Received for publication, November 2, 1995)

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A facilitated diffusion mechanism has been proposed to account for the kinetic efficiency with which restriction endonucleases are able to locate DNA recognition sites. Such a mechanism involves the initial formation of a non-specific complex upon collision of the protein with the DNA, with the subsequent diffusion of the protein along the DNA helix until either a recognition site is located or the protein dissociates into solution. Protein translocation may be facilitated by either sliding along the DNA, hopping to nearby sites, or intersegment transfer over larger distances. Previous analyses of the manner in which restriction enzymes cleave DNA substrates did rule out the latter mechanism. To discriminate between protein sliding or scanning and protein hopping, we designed a unique DNA template with three overlapping, mutually exclusive recognition sites for the BssHII endonuclease. Analysis of the cleavage pattern demonstrated efficient usage of both external sites, whereas the centrally located site was not efficiently cleaved. These results confirm that linear diffusion of the BssHII enzyme occurs by scanning along the DNA. Furthermore, the scanning enzyme was found to stop and cleave at the first site encountered. Thus, a sliding restriction endonuclease recognizes cleavage sites with high fidelity, without skipping of potential sites.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The 11.6-kilobase plAI plasmid (34) contains a single BssHII site (GCCGCG or GC3) that was used to construct the GC5 derivative. In short, the DNA was linearized with BssHII, and sticky ends were filled in with Klenow enzyme in the presence of dNTPs, followed by religation of the blunt ends. The GC5 mutation was verified by sequence analysis. Both GC3 and GC5 forms were subcloned as XbaI-ClaI fragment in the Bluescript KS vector, yielding the 3.8-kilobase plasmid Blue-SL43 (15).

BssHII Endonuclease Cleavage Reactions—BssHII, HindIII, XhoI, and T4 polynucleotide kinase were obtained from commercial sources (BssHII from Boehringer Mannheim, 10 kilounits/ml). Linearized plasmids (approximately 100 ng) were 5’-end labeled with T4 polynucleotide kinase and [γ-32P]ATP (typically 3000 Ci/mmol). Standard BssHII cleavage reactions were performed in buffer A (33 mM Tris-acetate, pH 7.9, 10 mM magnesium acetate, 66 mM potassium acetate, 0.5 mM dithiothreitol) at 50°C for 1 h with 32P-labeled DNA and 2 units of endonuclease. Reactions were terminated by addition of 0.5 volume of formamide-loading buffer, heated for 5 min at 85°C, and separated by electrophoresis on 6% polyacrylamide-urea gels.

RESULTS AND DISCUSSION

We designed a DNA sequence containing three identical but overlapping recognition sites for a restriction endonuclease. The rationale of this design is that cleavage of one site will destroy the other two recognition sites, which allows one to viewed in Ref. 3), RNA polymerase (4, 5), and Micrococcus luteus UV endonuclease (6).

Despite their abundant use in molecular biology, only a few restriction endonucleases have been studied seriously as DNA-binding enzymes. The best understood of the type II restriction enzymes is the EcoRI endonuclease (7–10). Diffusion mechanisms along the DNA helix contour have been proposed to account for the kinetic efficiency with which this protein is able to locate its recognition and cleavage sites. Similar diffusion/sliding/scanning mechanisms have been proposed for the HindII and BamHI endonucleases, as well as for the BamH methylase enzyme (10, 11).

Knowledge on the DNA search mechanism of restriction enzymes is based primarily on kinetic studies, combined with a detailed analysis of the cleavage pattern with circular and linear DNA templates containing one or more cleavage sites. These experiments demonstrated that the EcoRI enzyme has more leeway to find the cleavage sites if the sites are centrally located on a linear DNA rather than near an end (9, 10). This finding is consistent with the involvement of bidirectional diffusion, facilitated by a non-specific endonuclease-DNA interaction, in the path by which the EcoRI protein locates substrate sites. Furthermore, this endonuclease was demonstrated to act processively over distances up to several hundred base pairs. Thus, evidence for the involvement of positionally facilitated diffusion (sliding) during DNA search by EcoRI endonuclease appears quite compelling, but a contribution of positionally uncorrelated mechanisms (e.g. intersegment transfer) cannot be ruled out completely. Likewise, kinetic analyses of the lac repressor-operator system demonstrated that sliding was the dominant mechanism under the experimental conditions used (12), but different experimental approaches have led to the suggestion that intersegment transfer may also be involved (13). We have investigated some aspects of the DNA site selection mechanism for the BssHII endonuclease using a novel approach with a unique DNA template containing multiple, mutually exclusive recognition sites.

* This work was supported by the Dutch AIDS Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Printed in U.S.A.
measure the cleavage efficiency of individual sites in a non-kinetic type of experiment. In order to create an overlap of three identical restriction sites, one is restricted to palindromes consisting of dinucleotide repeats. For 6-mer recognition sites there are only four candidate sequences: ATATAT, TATATA, CGCGCG, and GCGCGC. No restriction enzymes with specificity for the former three sites are currently known. This leaves GCGCGC or GC3, recognized by the *BssHII* enzyme from *Bacillus stearothermophilus* H3, as a unique restriction site to perform this type of analysis. Fig. 1 shows the schematic of a multimerized GC5 sequence containing three overlapping *BssHII* sites numbered I, II, and III. Products of all three possible cleavage reactions are shown to indicate that no complete *BssHII* site remains after the first cleavage. The lower panel shows schematics of the two DNA plasmids used in this study. The Bluescript-derived vector Blue-5’LTR (left) is shown in the GC5 form (triple *BssHII* sites boxed), but a GC3 variant with one *BssHII* site was used as control plasmid. The unique HindIII and XhoI sites and all *BssHII* sites are indicated. The pLAI vector (right) is shown as GC5 variant, but a GC3 plasmid was used as control. Fragment sizes are expressed as center-to-center distances between restriction endonuclease recognition sequences.

![Schematic of the triple *BssHII* site construction (GC5) and the two test plasmids used in this study.](image)

**Fig. 1.** Schematic of the triple *BssHII* site construction (GC5) and the two test plasmids used in this study. The top panel shows the GC5 sequence with three overlapping *BssHII* sites (I, II, and III). Products of all three possible cleavage reactions are shown to indicate that no complete *BssHII* site remains after the first cleavage. The lower panel shows schematics of the two DNA plasmids used in this study. The Bluescript-derived vector Blue-5’LTR (left) is shown in the GC5 form (triple *BssHII* sites boxed), but a GC3 variant with one *BssHII* site was used as control plasmid. The unique HindIII and XhoI sites and all *BssHII* sites are indicated. The pLAI vector (right) is shown as GC5 variant, but a GC3 plasmid was used as control. Fragment sizes are expressed as center-to-center distances between restriction endonuclease recognition sequences.

If the *BssHII* enzyme locates cleavage sites by tediously testing all possible 6-mers in the target DNA (trial-and-error), the three sites should be recognized with approximately equal efficiency. In contrast, if the *BssHII* enzyme transfers along the DNA in a positionally correlated manner, the enzyme should sample and cleave the external sites I and III before the internal site II is being recognized. Because usage of one site will preempt any further cleavage, the latter mechanism predicts that the external sites I and III will be preferentially cleaved.

Initial tests were performed with a Bluescript-derived test plasmid (Fig. 1, Blue-5’LTR) containing either one or three *BssHII* sites (GC3 and GC5). The GC3 and GC5 plasmid was first linearized with either HindIII or XhoI, 32P-end labeled, and subsequently treated with *BssHII*. The cleavage products were separated on a denaturing polyacrylamide gel (Fig. 2a). The HindIII-labeled GC3 control sample produces two *BssHII*–HindIII fragments of 180 and 694 bp (lane 2). In GC5, the smallest of the two fragments is heterogeneous in size due to cleavage at one of the three *BssHII* sites (lane 4). The fragment of 180, 182, and 184 bp correspond to cleavage at *BssHII* site I, II, or III, respectively. In the XhoI-labeled experiment, GC3 produces 52- and 134-bp fragments (lane 6), the 52-bp fragment can be seen with shorter electrophoresis times, see e.g. Fig. 2b, lanes 1–5. The GC3 construct produces a mixture of 134-, 136-, and 138-bp fragments (Fig. 2a, lane 12) that correspond to *BssHII* cleavage at site III, II, or I, respectively.

1 The abbreviation used is: bp, base pair(s).
The individual bands were quantitated in order to calculate the relative efficiency of BssHII cleavage. The results are summarized in Fig. 3 and demonstrate that the internal site II is not efficiently recognized/cleaved by the BssHII enzyme. These results were obtained under standard BssHII digestion conditions at 50°C, but similar digestion patterns were observed in reactions at 37°C (data not shown).

The major BssHII cleavage product with the XhoI-linearized plasmid is the smallest 134-bp fragment. Although this is thought to reflect efficient recognition of site III, we cannot formally exclude that it is in fact the result of additional BssHII activity after initial cleavage at sites I and/or II (nibbling of the end). This hypothetical possibility seems unlikely because the same site III is preferred in the HindIII experiment, where it produces the largest (184 bp) of three possible fragments. To further rule out this scenario, we analyzed DNA samples at early and late time points after addition of the BssHII enzyme (Fig. 2b, lanes 1-5). It is obvious that the ratio of cleavage sites I:II:III cleavage remains constant over the whole incubation period. Similar results were obtained in a kinetic analysis of the HindIII-linearized plasmid (lanes 6-10).

Restriction enzymes have been reported to display a substantial preference for more centrally located recognition sites on linearized DNA templates (9, 10). This effect can be understood in terms of the initial contact between the enzyme and flanking, nonspecific DNA segments. It is clear that this process is restricted for cleavage sites located close to an end of linear DNA. Because the GC5 motif is asymmetrically located on the linearized Blue-5LTR plasmid, one expects the more centrally located sites to be favored by virtue of the lengthy DNA flanks. For instance, the XhoI-treated DNA has a short 134-bp arm for nonspecific enzyme binding to site III, whereas site I is flanked by 3710 bp (Fig. 1). Likewise, there is pronounced asymmetry in the HindIII-cleaved template, with 3664 and 180 bp flanking sites III and I, respectively. Consistent with the idea of a positional effect, we did repeatedly observe a somewhat higher cleavage efficiency of site I versus site III in the XhoI samples compared with the HindIII samples (Fig. 3).

However, two experimental conditions are likely to restrict the magnitude of this effect in our assays. First, length dependence
Served for a distinct group of restriction endonucleases (same plasmid substrate. Most dramatic effects have been observed because it is in a “bad” sequence context. Certain restriction enzymes show preferential cleavage of some sites in the same plasmid substrate. Most dramatic effects have been observed for a distinct group of restriction endonucleases (NarI, Nad, and SacII) that like BssHII recognize sites composed entirely of G and C bases. This group of restriction enzymes was demonstrated to require the simultaneous interaction with two copies of the recognition sequence before cleavage occurs (16, 17). However, other GC hexamer-recognizing enzymes (SmaI, ApaI) do not exhibit such marked site preference. Kinetic analysis of BssHII cleavage of the three non-overlapping sites in plasmid Blue-5-LTR GC3 indicates that BssHII does not exhibit a marked site preference (see e.g. Fig. 2b and data not shown). Furthermore, the unique BssHII site in pLAI-GC3 is efficiently cleaved (not shown), suggesting that BssHII does not belong to the group of restriction endonucleases that require at least two simultaneously bound substrate sites for their activation.

A second remark concerns the accuracy of DNA site selection during the scanning process. If scanning of the BssHII enzyme was inaccurate with respect to recognition of the GCGCGC site, the enzyme would frequently slip over the first site encountered (that is the external site I or III), and this would increase the frequency with which internal site II is reached and cleaved. Apparently, the search capacity of the sliding enzyme is extremely accurate such that the majority of enzyme will recognize, stop, and cleave at the first site encountered (either site I or III). In other words, the BssHII enzyme does read each 6-mer it passes with high fidelity. This finding is striking if we realize that the sliding rate of a restriction enzyme has been reported to be approximately $7.3 \times 10^{6}$ base pairs/s (9). In order for the enzyme to accurately read all 6-mers along the way, this would translate into $7.3 \times 10^{6}$ discrete diffusion steps/s.

Acknowledgments—We thank Koen Verhoef for helpful comments and Wim van Est for photography work.

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J. Biol. Chem. 1996, 271:1837-1840.
doi: 10.1074/jbc.271.4.1837

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