Footprint analysis of the BspRI DNA methyltransferase–DNA interaction

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Received March 26, 1997; Revised and Accepted May 21, 1997 DDBJ/EMBL/GenBank accession no. X15758

ABSTRACT

The interaction between the GGCC-specific BspRI DNA methyltransferase (M. BspRI) and substrate DNA was studied with footprinting techniques using a DNA fragment that was unmodified on both strands. Footprinting with DNase I revealed an ~14 bp protected region. Footprinting with dimethylsulfate detected major groove interactions with the guanine bases of the recognition sequence. Reaction with 1,10-phenanthroline-copper did not show protection, suggesting that minor groove interactions play little role in sequence-specific recognition by M. BspRI. Hydroxyl radical footprinting revealed a protected stretch of 6 nt. The hydroxyl radical footprint of M. BspRI differs markedly from the footprint reported for the HhaI and SssI methyltransferases. The pattern of protection from dimethylsulfate and hydroxyl radicals suggests that the interactions of M. BspRI with DNA are similar to those detected in the co-crystal structure of the HaeIII methyltransferase.

INTRODUCTION

DNA (cytosine-5)-methyltransferases (C5-MTase) catalyze the transfer of a methyl group from S-adenosyl-L-methionine (AdoMet) to the C5 carbon of cytosine in specific sequences (1). C5-MTases share a common architecture. They contain five more and five less conserved amino acid sequence motifs and a variable region. The latter exhibits little sequence homology between the variable regions of C5-MTases that recognize different sequences (1). It has been shown that the sequence conservation in the variable regions of M. HaeIII, M. BspRI, M. BsuRI and M. NgoPII led Verdine and co-workers to propose that the recognition mechanism of the other three GGCC-specific C5-MTases may be similar to that of M. HaeIII (7).

Further information on the interaction between C5-MTases and DNA was provided by footprinting experiments (11,12). One of these studies (12) revealed that M. HhaI and M. SssI, two C5-MTases that recognize the sequence GGCC and CG respectively, display similar specific and non-specific contacts with DNA when bound to their target sequences. A comparison of the footprint phenotype and the co-crystal structure of M. HhaI showed that although there were differences in the backbone contacts identified by the two approaches, the footprint phenotype of M. HhaI was largely consistent with the crystal structure (12). The similarity of the M. HhaI and M. SssI footprint phenotypes suggested that the position of the enzyme with respect to the recognition sequence and the contacts with the DNA backbone might be very similar even among C5-MTases that have different specificity. On the other hand, the differences between the co-crystal structures of M. HhaI and M. HaeIII hinted at a diversity that is perhaps greater than could be expected for this highly homologous class of enzymes. It awaits further studies involving other C5-MTases to better assess the mechanisms by which C5-MTases interact with the substrate DNA.

Here we report footprinting analysis of the GGCC-specific C5-MTase M. BspRI. M. BspRI is part of the BspRI restriction–modification system of Bacillus sphaericus (13). Like M. HaeIII, it methylates the inner cytosine of the recognition sequence. Beyond the general goal of a detailed footprint analysis of a C5-MTase, our specific aim was to test the prediction (7) that monospecific C5-MTases that recognize the sequence GGCC interact with the substrate DNA in a similar fashion.

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MATERIALS AND METHODS

Enzymes and chemicals

M. BspRI was purified from an Escherichia coli strain carrying the bspRII gene. Construction of the overproducer and enzyme purification will be described elsewhere. Restriction endonucleases BamHI and Nhel and E. coli DNA polymerase I large (Klenow) fragment were purchased from Fermentas. Bovine pancreatic DNase I was from Sigma, dimethylsulfate (DMS) from EGA Chemie, 1,10-phenanthroline from Aldrich, poly(dI·dC) from Pharmacia, deoxyadenosine 5′-[α-32P]triphosphate from Izotop Intezet Kft. All other chemicals were analytical grade commercial products.

Radioactive labeling of DNA fragment

The 152 bp BamHI–Nhel fragment of plasmid pBR322 was used for footprinting experiments. To obtain a fragment radioactively labeled at one end, pBR322 plasmid DNA was cleaved with either BamHI or Nhel, then the ends were labeled by a filling-in reaction using E. coli DNA polymerase I large fragment and deoxyadenosine 5′-[α-32P]triphosphate. The DNA was subsequently cut with the other restriction enzyme (Nhel or BamHI) and the 152 bp fragment was purified from agarose gel. The DNA strand corresponding to the Nhel (5′)→BamHI (3′) orientation (14) will be referred to as the A and the complementary strand as the B strand.

Preparation of the enzyme–DNA complex

Binding reactions contained ~6 nM BamHI–Nhel fragment labeled with 32P at either end (0.5–2×10^5 c.p.m.), 0–2 μM M. BspRII, 50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 7 mM β-mercaptoethanol and 250 μg/ml tRNA. The DNA was subsequently cut with the other restriction enzyme (Nhel or BamHI) and the 152 bp fragment was purified from agarose gel. The DNA strand corresponding to the Nhel (5′)→BamHI (3′) orientation (14) will be referred to as the A and the complementary strand as the B strand.

DNase I protection

The reaction mixture was prepared by adding to the enzyme–DNA complex 5 μl of a solution containing 15 mM CaCl2, 60 mM MgCl2 and 5 ng/μl DNase I (15). After incubation at room temperature for 1 min, the reaction was stopped by extracting the sample with 50 μl phenol/chloroform, then the DNA was precipitated twice with ethanol.

DMS protection

The reaction mixture was prepared by adding to the enzyme–DNA complex 1 μl 10% DMS (16). After incubation at room temperature for 3 min, the reaction was terminated by adding 6 μl of a solution containing 1.5 M sodium acetate, pH 7.0, 1 M β-mercaptoethanol and 250 μg/ml tRNA. The DNA was precipitated twice with ethanol, then dissolved in 150 μl 1 M piperidine. Cleavage at the methylated guanines was performed by heating the samples to 90°C for 30 min. To remove the piperidine, the DNA was extracted twice with n-butanol (17) and then lyophilized.

Hydroxyl radical protection

The reaction mixture was prepared by adding to the enzyme–DNA complex 2 μl 75 mM Fe(II)-EDTA, 2 μl 150 mM ascorbic acid and 1 μl 0.3% H2O2. These concentrations were somewhat different from the concentrations typically used in hydroxyl radical footprinting experiments (18,19). A higher than usual concentration of Fe(II)-EDTA was used to compensate for the 0.33% glycerol, which was in the footprinting reaction as a component of the enzyme storage buffer, and we found that H2O2 inhibited DNA binding at concentrations >0.01%. After incubation at room temperature for 3 min, the reaction was stopped and the DNA was precipitated by adding 90 μl of a solution containing 3% glycerol, 0.13 M sodium acetate, pH 7.0, and 92% ethanol. The precipitated DNA was dissolved, extracted with phenol, then precipitated again with ethanol.

1,10-Phenanthroline-copper protection

The reaction mixture was prepared by adding to the enzyme–DNA complex 2.5 μl of a solution containing 1 mM 1,10-phenanthroline/0.25 mM CuSO4 and 2 μl 150 mM ascorbic acid (20). Incubation and further processing was as described for the hydroxyl radical protection.

Electrophoresis of DNA fragments

DNA fragments precipitated after the footprinting reactions were dissolved in 2 μl loading buffer containing 98% formamide, 10 mM EDTA, pH 8.0, 0.025% xylene cyanol, 0.025% bromphenol blue and run in 10% polyacrylamide gels containing 8 M urea. The gel was visualized, scanned and quantified using a phosphorimager (Molecular Dynamics 445 SI). Bands were identified by alignment with co-migrating fragments from Maxam–Gilbert A + G reactions (21).

RESULTS

We found in previous experiments, using a gel retardation assay, that M. BspRII could form a specific recognition complex with DNA fragments containing a BspRII site (not shown). For footprinting experiments reported in this paper we used the Nhel–BamHI fragment of plasmid pBR322. This fragment contains a single BspRII site (14). The fragment labeled with 32P at either end was incubated with 0.2–2 μM M. BspRII and was then used directly for footprinting reactions. We included the AdoMet analog sinefungin in the binding buffer because previous experiments showed that it increased complex stability (unpublished observations).

DNase I protection

DNase I footprinting revealed a protected region that, in addition to the recognition sequence, included several nucleotides in both directions. The protected region was longer on the A strand than on the B strand (Fig. 1). Another difference was that the protected stretch on the A strand was longer in the 3′ direction, whereas on the B strand it was longer in the 5′ direction. On the A strand, 4–5 nt were protected on the 5′- and 4–5 nt on the 3′-side of the recognition sequence. In the cleavage pattern of the A strand there was a very strong band corresponding to the tenth
Figure 1. DNase I protection. The NheI–BamHI fragment labeled either at the BamHI (A) or at the NheI (B) end was incubated with 0–2 µM M.BspRI, then subjected to a footprinting reaction with DNase I. A + G, A + G-specific Maxam–Gilbert reaction. Lines along the gel and the sequence mark the protected region. Bases displaying enhanced reactivity are marked by open arrows. The recognition sequence of M.BspRI is indicated by shading.

Figure 2. DMS protection. The DNA fragment was labeled and incubated with M.BspRI as described in the legend to Figure 1, then treated with DMS. A + G, A + G-specific Maxam–Gilbert reaction. The protected bases are indicated by arrowheads. The open arrow marks the bases displaying enhanced reactivity to DMS. The recognition sequence of M.BspRI is indicated by shading, with the protected guanines in ovals.

Base in the 5′ direction. A less pronounced but still strong band could be seen in an almost equivalent position (the ninth base on the 5′-side of the GGCC sequence) in the cutting pattern of the B strand (Fig. 1). The strong bands were absent in the cleavage pattern of free DNA, suggesting that a conformational change induced by M.BspRI led to preferential cleavage.
Figure 3. Hydroxyl radical protection of the A strand. The DNA fragment was labeled at the BamHI end, incubated with M.BspRI, then subjected to hydroxyl radical footprinting. A + G, A + G-specific Maxam–Gilbert reaction. The protected region is bracketed and indicated by arrows over the sequence. The recognition sequence of M.BspRI is shaded. (Bottom) Line scan of the cleavage patterns (free DNA and complex with 2 µM M.BspRI).

DMS protection

DMS attacks and chemically methylates the N7 atom of guanines in the major groove and the N3 atom of adenines in the minor groove (21). We performed the reaction under conditions where only guanine methylation led to strand breakage (21). The only guanines protected by M.BspRI were those in the recognition sequence (Fig. 2). On the B strand two guanines which are the second and third base on the 5’-side showed enhanced reactivity (Fig. 2).

Hydroxyl radical protection

Hydroxyl radicals attack the DNA backbone at the C4’ atom of the deoxyribose ring (19). M.BspRI protected the 4 nt of the recognition sequence and two adjacent nucleotides on the 3’-side. The protected regions were similar on the two strands (Figs 3 and 4). The boundaries of the footprint can be more clearly seen on the line scans (Figs 3 and 4, bottom panels).

1,10-Phenanthroline-copper protection

The chemical nuclease 1,10-phenanthroline-copper complex binds in the minor groove and induces cleavage of the sugar–phosphate backbone in a sequence-independent manner (20). Using this footprinting reagent, we did not find any protection by M.BspRI (not shown). The failure to obtain a footprint with this reagent suggests that in the minor groove there are probably no specific contacts to the bases of the recognition sequence.

DISCUSSION

The BspRI methyltransferase–DNA interaction has been studied with four footprinting techniques. There is a large battery of experimental evidence supporting the model in which DNA methyltransferases that recognize symmetrical sequences can bind to the substrate DNA in two orientations, each leading to methylation of only one strand (4–9,22). Footprinting studies (12) as well as X-ray structures show that, in a specific binding complex, C5-MTases contact both DNA strands (6–9). In our experiments we used unmethylated DNA, which can support
formation of either complex at a binding site. Therefore, footprints obtained in this study are probably the sum of two footprints resulting from the two binding orientations.

DNase I footprinting revealed 18 and 16 nt protected regions on strands A and B respectively (Fig. 5). The position of the protected regions on the two strands was different relative to the GGCC recognition sequence: on the A strand, it was longer on the 3'-side, whereas on the B strand it was longer on the 5'-side (Fig. 5). We suggest that the reason for this asymmetry must be the GC-rich sequence adjacent to the recognition site. In this sequence, consisting of seven GC base pairs, the incomplete GC-rich sequence adjacent to the recognition site. This BspRI recognition site GCC/GGC occurs twice (Figs 1 and 5). Presumably, M.BspRI can weakly bind to these sites. This is supported by a faint footprint observed at another GCC/GGC site on the same fragment (at position 355 in the pBR322 sequence; not shown). Binding to the incomplete sites is probably much weaker than to the canonical sequence because even a weak binding may modify the footprint obtained from the neighboring GGCC site. If this assumption is correct, then a BspRI methyltransferase molecule bound to a GGCC site covers an ~14 bp stretch of DNA, with the center of the protected region being in the recognition sequence. The protected region is 2 bp shorter than the DNase I footprints of M.MspRI (11) and 4–7 bp shorter than the footprints of M.HhaI and M.SssI (12). It should be mentioned that due to the large size of the probe, DNase I footprints are likely to overestimate the size of the DNA stretch covered by a protein.

The hyper-reactivity to DNase I of a site 9–10 bp away from the recognition site probably reflects a DNA distortion induced by the methyltransferase. A mixture of two M.BspRI makes base-specific contacts to the N7 atom of both guanines in the recognition sequence (7). Residues mediating these contacts (R225 and R227) are conserved in M.BspRI (R298 and R300), thus our finding that the two guanines are protected from DMS is consistent with M.BspRI making the same contacts as M.HaeIII. According to the crystal structure, M.HaeIII interacts with six phosphates of the strand containing the target cytosine and with three phosphates of the complementary strand:

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These interactions were deduced from a complex in which M.HaeIII was in a unique binding orientation that corresponded to methylation of the cytosine (underlined) in the top strand (7). A mixture of two M.HaeIII–DNA complexes representing the two binding orientations would therefore show interactions with seven phosphates (the sum of the contacts in the two complexes) on each strand:

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Our data show that M.BspRI protects 6 nt from hydroxyl radical attack (Fig. 5). Because hydroxyl radicals mainly attack the C4 atom of the deoxyribose ring (19), the cleavage pattern would be consistent with the following phosphate contacts:

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Except for a single missing contact on each strand, this pattern of postulated backbone contacts is the same as the pattern seen in the M.HaeIII co-crystal (7). The small difference may arise from the fact that the footprints of M.BspRI characterize interactions in the initial recognition complex, whereas the X-ray data of M.HaeIII are derived from a complex representing a post-methyl transfer intermediate.

The chemical nucleases 1,10-phenanthroline-copper complex did not yield a footprint with M.BspRI, suggesting a lack of base contacts in the minor groove. Analysis of the M.HaeIII–DNA co-crystal revealed that all but one of the base contacts made by M.HaeIII were in the major groove (7).

In summary, our results lend support to the prediction (7) that monospecific DNA methyltransferases recognizing GGCC interact with their target sequence in a similar fashion. However, in this context it should be noted that the GGCC-specific target-recognizing domains of the phage-encoded multispecific C5-MTases show poor sequence identity with the variable regions of M.HaeIII, M.BspRI, M.BsuRI and M.NgoP11 (23), suggesting that monospecific
and multispecific C5-MTases use different structures for recognition of the GGCC sequence.

ACKNOWLEDGEMENTS

This project was supported by an International Research Scholar’s award from the Howard Hughes Medical Institute and OTKA grant T 016402.

REFERENCES