# Structure subtraction as an approach to investigation of the mechanism of restriction enzyme action

V.V. Zinoviev, J.A. Gorbunov, M.M. Baclanov, S.G. Popov and E.G. Malygin

*All-Union Scientific Research Institute of Molecular Biology, Koltsovo, Novosibirsk Region, 633159, USSR*

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Endonuclease *Bam*HI cleaves the phosphodiester bonds between the guanine residues within the duplex DNA sequence G↓GATCC. The substrate characteristics of oligonucleotides, containing some defects in the sequence recognized by endonuclease (nick, absence of some internucleotide phosphate or nucleotide, partially single-stranded form of the recognition site) were investigated. The results suggest that the specificity of synthetic oligonucleotide cleavage is strongly dependent on the ribosophosphate backbone intactness inside the recognition site. *Bam*HI was found not to hydrolyse the phosphodiester bonds outside the double helix. Also *Bam*HI forms a productive complex with the non-symmetrical substrate, having half the recognition sites, of a single strand.

*Restriction endonuclease* *Oligonucleotides* *Specificity* *Recognition site*

## 1. INTRODUCTION

Site-specific endonuclease *Bam*HI cleaves the phosphodiester bonds between the guanine residues within the duplex DNA sequence G↓GATCC [1]. In [2] we reported, that the self-complementary oligodeoxyribonucleotide, d(5′-TCCAGATCTGGA), flanked with the halfsize recognition sequence 5′-TCC from its 5′-side and 5′-GGA from its 3′-side is cleaved with *Bam*HI. It was of interest to examine the substrate characteristics of oligonucleotides, containing some defects in the sequence recognized by endonuclease. For example, it could be a nick, absence of some internucleotide phosphate or nucleotide, partially single-stranded form of the recognition site. The substrates deprived of some primary structure elements might be obtained directly by chemical synthesis. Such a structure subtraction method permits one to estimate the significance of the individual substrate elements for the specific interaction with restriction endonucleases. A similar method was used for investigation of tRNA–aminoacyl-tRNA synthetase interaction using the partially hydrolysed products of tRNAs (dissected molecules approach) [3]. We have examined this possibility for the endonuclease *Bam*HI.

The results suggest that the specificity of synthetic oligonucleotides cleavage is strongly dependent on the ribosophosphate backbone intactness inside the recognition site. Endonuclease *Bam*HI was found not to hydrolyse the phosphodiester bonds outside the double helix. Our experiments have also shown the endonuclease *Bam*HI to form the productive complex with non-symmetrical substrate, having the half of the recognition site as a single strand.

## 2. MATERIALS AND METHODS

Endonuclease *Bam*HI from *Bacillus amyloliquefaciens* was prepared as in [4]. Oligodeoxyribonucleotides were synthesised by the phosphotriester method [5]. Oligonucleotides were $^{32}$P-labeled with polynucleotide kinase at the 5′-termini [6]. The mixtures of 5′-[$^{32}$P]oligonucleotides with non-labeled complementary oligonucleotides (1.5–3 pmol/$\mu$l) were digested with *Bam*HI (1–2 units/$\mu$l) for 2 h at 30°C in 6 mM Tris–HCl (pH 7.4), 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol and 50 mM NaCl. Reaction was stopped by heating at 65°C for 5 min. After digestion the samples were separated in 23% polyacrylamide gels as in [7]. The $^{32}$P-labeled products were



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visualized by autoradiography, the radioactivities of the corresponding slices were counted for determining of the hydrolysis degree. Sequence analyses of the oligodeoxyribonucleotides and *Bam*HI digestion products were performed as in [8].

## 3. RESULTS AND DISCUSSION

To produce a number of alternatives of substrates for endonuclease *Bam*HI we have synthesised the oligonucleotides CACTTTA-GGATCCATTTCAC (I), CCTAAACTG (II), TCCTAAACTG (III), GTGAAATGGA (IV), containing full or partial recognition sequences for endonuclease (underlined). Oligonucleotides II and III are complementary to 5′-moiety of 20-mer I, oligonucleotide IV is complementary to 3′-moiety of I. Preformation of the complexes between different oligonucleotides permits one to obtain the substrates, lacking some internucleotide phosphate or a total nucleotide (one or several) in different positions of the recognition site. Results of the *Bam*HI action on this oligonucleotide complexes are summarised in table 1.

The combination of oligonucleotides used in the reaction mixtures are indicated in the left column of table 1. One of the oligonucleotides (I or IV) was $^{32}$P-labeled for quantitation of the hydrolysis

Table 1

*Bam*HI action on the oligonucleotide complexes

| No. | Oligonucleotide combination | [$^{32}$P]oligonucleotide contents (%) after hydrolysis | Recognition site structure[a] |
|---|---|---|---|
| 1. | $^{32}$P-TCCAGATCTGGA | 18.0 | ↓ ↓ ↓<br>-G-G-A pT-C-C-<br>-C-C-Tp A-G-G-<br>↑ ↑ ↑ |
| 2. | $^{32}$P-(I + III + IV)<br>I + III + $^{32}$P-IV | 16.0<br>28.0 | ↓ I<br>-G-G-A-T-V-C-<br>-C-C-T A-G-G-<br>III IV |
| 3. | $^{32}$P-(I + II + IV)<br>I + II + $^{32}$P-(IV) | 96.0<br>23.0 | I<br>-G-G-A-T-C-C-<br>-C-C A-G-G-<br>II IV |
| 4. | $^{32}$P-(I + IV)<br>I + $^{32}$P-IV | 95.0<br>34.0 | I<br>-G-G-A-T-C-C-<br>A-G-G-<br>IV |
| 5. | $^{32}$P-(I + II) | 99.0 | I<br>-G-G-A-T-C-C-<br>-C-C<br>II |
| 6. | $^{32}$P-(I + III) | 94.0 | I<br>-G-G-A-T-C-C-<br>-C-C-T<br>III |
| 7. | $^{32}$P-I | 97.0 | I<br>-G-G-A-T-C-C- |
| 8. | $^{32}$P-IV | 100.0 | IV<br>-G-G-A |

[a] Arrows indicate the cleavage points

degrees. The hypothetical site structures implicated by the sequences of the oligonucleotides used are shown in the right column of the table 1.

Results of the 4–10 parallel experiments are used for calculation of the oligonucleotide hydrolysis degrees (middle column of table 1). Previously obtained data concerning the hydrolysis of the self-complementary dodecanucleotide are also represented here [2].

In the case of the self-complementary oligonucleotide TCCAGATCTGGA there are two nicks positioned on the symmetry axis of the recognition site (N 1). Phosphodiester bond is absent between A and T in one chain of the complex I + III + IV (N 2). Yet the formal structure of both complexes is similar to the native one. This proposition seems to be correct because endonuclease *Bam*HI attacks both chains of this substrate. The appearance of the defect zone is accompanied by the loss of enzyme action on the 20-mer I, but the significant hydrolysis of decanucleotide IV is conserved (N 3). The complete elimination of oligonucleotide which is complementary to 5′-moiety of 20-mer does not change the cleavage pattern (N 4). The base-pairing of 20-mer 3′-moiety is not possible in the complexes I + II (N 5) and I + III (N 6). In this case hydrolysis is practically absent.

These hydrolysis experiments suggest several implications for the mechanism of *Bam*HI action. The 20-mer containing complete sequence recognition GGATCC is cleaved always between the guanosine residues. Decanucleotide IV containing on the 3′-end only 5′-GGA moiety of the site is hydrolyzed preferentially with the liberation of 3′-external adenosine residue the extent of the correct cleavages between guanosine residues being 10–20% (N 2,3). This ambiguity of the cleavage position is observed also when the enzyme binds with the composite recognition site formed by the ends of two duplex molecules (N 1).

The obligate condition for the cleavage of the phosphodiester bonds between nucleotide residues is their participation in double helix. Indeed, if it is not the case (N 4) the cleavage of the corresponding bonds is absent. Thus, unlike endonucleases *Hae*III, *Hha*I, *Sfa*I, *Mbo*I and *Hin*fI [9] endonuclease *Bam*HI forms the productive complex only with double-stranded DNA. It should be noted that single-stranded oligonucleotides I and IV are not hydrolyzed by the endonuclease (N 7,8).

A particularly striking feature is the different action of enzyme on the complexes 'I + III' (N 6) and 'I + IV' (N 4). In both complexes a half of the recognition site exists in a single stranded form. In the first one it is 5′-TCC moiety and endonuclease *Bam*HI does not digest this complex. In the second one the 5′-GGA moiety is single stranded and this does not hinder the enzymatic cleavage of the double-stranded part of the substrate. It seems likely that both 5′-GGA sequences are necessary for the productive enzyme–substrate complex formation. Also the complex 'I + IV' is the most prominent example of the non-symmetrical substrate which is still cleaved by the endonuclease *Bam*HI.

In conclusion, the usefulness of the approach exploited for investigation of the restriction endonuclease mechanism of interaction with DNA should be marked. In contrast to the chemical modification method [10] no additional chemical group is introduced and therefore the interaction of the substrate with the enzyme is not disturbed. The proposed structure subtraction approach permits us to examine systematically a number of substrate variants, lacking some elements of the native structure and to estimate their significance in the forming of productive enzyme–substrate complexes. Such experiments are under way in our laboratory.

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