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# Location of the Bases Modified by M.*Bco*KIA and M.*Bco*KIB Methylases in the Sequence 5′-CTCTTC-3′/5′-GAAGAG-3′

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**Abstract**—The strain *Bacillus coagulans* K contains two DNA-methyltransferases, M.*Bco*KIA and M.*Bco*KIB, which recognize the sequence 5′-CTCTTC-3′/5′-GAAGAG-3′ and possess N4-methylcytosine and N6-methyladenine specificities, respectively. A special construct containing the recognition site of *Bco*KI and sites of four IIS restriction endonucleases (IIS restriction endonuclease cassette) was designed to locate the nucleotides modified by the methylases. The modified bases were determined as: 5′-m$^4$CTCTTC-3′/5′-GAAGAm$^6$G-3′.

*Key words*: M.*Bco*KIA, M.*Bco*KIB, DNA-methyltransferase, IIS restriction endonuclease

The important characteristic of the DNA-methyltransferases of restriction-modification systems, in addition to the recognition sequence and specificity (N6-methyladenine, N4-methylcytosine, or C5-methylcytosine), is the location of modified bases within the recognition sequence. The location of the methylated bases is easily determined when methylase specificity is known and when each strand of the recognition sequence contains a single base to be modified. When this is not the case, the problem of identifying the position of the methylated bases arises. Different methods have been developed for locating methylated nucleotides, most of them being for cytosine location [1-3]. Here we report the location of methylated nucleotides modified by M.*Bco*KIA and M.*Bco*KIB using a new method based on the property of IIS restriction endonucleases to cleave DNA at a fixed distance from their recognition sites.

The *Bco*KI restriction-modification system recognizes the nonpalindromic sequence 5′-CTCTTC-3′/5′-GAAGAG-3′ and is a type IIS system. Type IIS systems generally contain two DNA-methyltransferases, each methylating one of the strands of the site. However, there are systems containing one methylase that methylates both strands. Thus, each of the nonhomologous restriction-modification systems *Alw*26I, *Eco*31I, and *Esp*3I contains one methylase that modifies cytosine in one strand and adenine in the complementary strand [3]. In the case of the *Fok*I system, which also contains one methylase, the enzyme consists of two domains, and the methylation of adenines is realized by different domains [4]. Finally, the unique restriction-modification system *Bst*F5I is known, which contains four methylases. One pair of the methylases modifies the top strand of the recognition sequence, and the other pair, the bottom strand. It is supposed that one methylase from the pair serves for the methylation of hemimethylated DNA and/or single-stranded DNA [5].

The system of restriction-modification *Bco*KI contains two methylases. M.*Bco*KIA modifies cytosine in the recognition sequence, and M.*Bco*KIB modifies adenine at the N4 position [6]. Here we report the location of the bases modified by these methylases in the recognition sequence.

## MATERIALS AND METHODS

DNA-methyltransferases M.*Bco*KIA and M.*Bco*KIB [6] and restriction endonucleases *Bco*KI, *Bbv*II, *Bsp*KT5, and *Ssp*D5I [7-10] were isolated and characterized by us earlier. *Bam*HI and *Xba*I were purchased from Fermentas (Lithuania). [$^3$H]S-Adenosyl-L-methionine (AdoMet) (15 Ci/mmol) was from Amersham (UK). The QIAquick

*Abbreviations*: 5mC) C5-methylcytosine; N4mC) N4-methylcytosine; N6mA) N6-methyladenine; AdoMet) S-adenosyl-L-methionine.

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Gel Extraction Kit was from Qiagen (USA). Nusieve agarose 1 : 3 was from FMS (USA). The following oligodeoxyribonucleotides were synthesized by Syntol (Russia):

IIS1 – (5′-GATCCTGAAGCGGGTGAAGACGAAG-AGACCT-3′),

IIS2 – (5′-CTAGAGGTCTCTTCGTCTTCACCCGC-TTCAG-3′),

NM1 – (5′-ACCACCCTGGCGCCCAATACGC-3′),

NM2 – (5′-GCTATTACGCCACGTGGCGAAAG-3′).

**Construction of recombinant DNA for locating methylated bases.** The annealing of two oligonucleotides IIS1 and IIS2 (31-mer each) forms a DNA duplex with 5′-single-strand extensions of four bases that match the termini from *Bam*HI and *Xba*I digestion (figure, panel (b)). The oligoduplex was cloned into *Bam*HI and *Xba*I cleaved vector M13tg131. The validity of the recombinant M13tg131(*Bco*K) DNA was confirmed by DNA sequencing. M13tg131(*Bco*K) was used as a template for PCR synthesis of a 410 bp fragment with asymmetric location of the inserted duplex within the fragment (figure, panel (c)). The fragment was purified from a 1.2% agarose gel using the QIAquick Gel Extraction Kit following the routine supplier protocols.

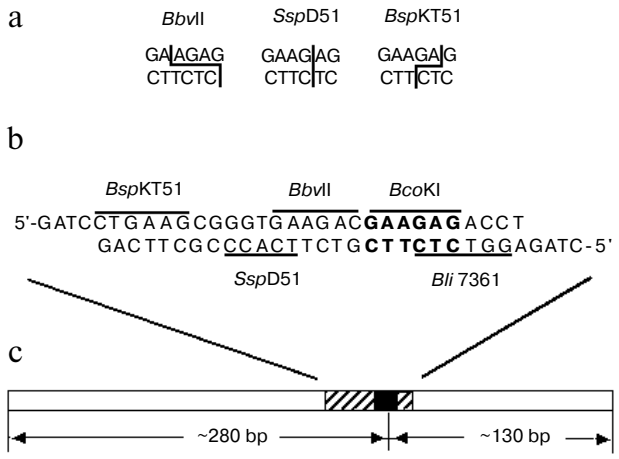
**Methylation of 410 bp DNA fragment.** Methylation was performed in reactions containing 50 mM Tris-HCl, pH 8.0, 5 mM 2-mercaptoethanol, 4 mM EDTA, 5 μM [$^3$H]AdoMet, and 1 μg fragment. To the reaction volume (50 μl), 1 unit of the corresponding methylase was added, and the reaction mixture was incubated at 48°C for 4-16 h. The methylase was then inactivated by phenol–chloroform extraction. An aliquot of the reaction mixture was used to verify completeness of methylation by testing resistance of the methylated fragment to digestion by *Bco*KI endonuclease. Another aliquot was counted for its $^3$H-radioactivity on a Beckman LS1801 (USA) scintillation counter.

**Digestion of $^3$H-labeled fragment.** The methylated fragment was separately digested with IIS restriction endonucleases *Bbv*II, *Bsp*KT5, and *Ssp*D5I. The cleavage products were separated by 3% Nusieve agarose gel electrophoresis. Ethidium bromide-stained fragments were excised, extracted from the gel by the QIAquick Gel Extraction Kit, and counted for their $^3$H-radioactivity in a scintillation fluid.

## RESULTS AND DISCUSSION

The methyltransferases M.*Bco*KIA and M.*Bco*KIB and cognate restriction endonuclease *Bco*KI, an isoschizomer of *Ksp*632I [10], recognize the asymmetric site 5′-CTCTTC-3′/5′-GAAGAG-3′ and R.*Bco*KI cuts DNA at a distance of one and four nucleotides distal to the sequence. There are three cytosines in the top-strand and three adenines in the bottom-strand of the recognition sequence. To determine the position of methylated nucleotides yielded by M.*Bco*KIA and M.*Bco*KIB, we designed a synthetic oligonucleotide duplex (figure, panel (b)). The duplex contains the recognition site of *Bco*KI and sites of type IIS restriction endonucleases *Bbv*II, *Bsp*KT5I, *Ssp*D5I, and *Bli*736I (IIS restriction endonucleases cassette). *Bbv*II recognizes the sequence 5′-GAA-GAC-3′ (2/6). *Bsp*KT5I, an isoschizomer of *Eco*57I, recognizes 5′-CTGAAG-3′ (16/14), *Ssp*D5I, a neoschizomer of *Hph*I, recognizes the sequence 5′-GGTGA-3′ (8/8), and *Bli*736I, an isoschizomer of *Bsa*I, recognizes 5′-GGTCTC-3′ (1/5). The number and location of the sites with respect to the site under study are such that the restriction endonucleases cleave the DNA between all the neighboring bases that could be methylated in the *Bco*KI site (A or C) (figure, panel (a)). The digestion of the 410 bp fragment containing the IIS restriction endonucleases cassette (figure, panel (c)) with these enzymes produces two fragments, ~280 and ~130 bp. Distribution of the $^3$H-activity between the two fragments allows the determination of the nucleotide modified with the corresponding methylase.

It is known that some restriction endonucleases of IIS type can cut DNA at different distances from their recognition sites, depending on the sequence between the recognition and cleavage sites [11]. So the cleavage points




a) Cleavage points of IIS restriction endonucleases within the *Bco*KI site. b) Oligonucleotide duplex containing sites of IIS restriction endonucleases (IIS restriction endonuclease cassette). c) Scheme illustrating the oligoduplex location within the 410 bp fragment and the fragments produced by IIS restriction endonucleases. The shaded box represents the oligoduplex, and the black box is the *Bco*KI site

Identification of M.*Bco*KIA and M.*Bco*KIB modified bases

| Restriction endonucleases | $^3$H-radioactivity, cpm | |
|---|---|---|
| | ~280 bp fragment | ~130 bp fragment |
| M.*Bco*KIA | | |
| *Ssp*D5I | 150 | 27,300 |
| *Bsp*KT5I | 180 | 24,700 |
| *Bbv*II | 25,500 | 230 |
| M.*Bco*KIB | | |
| *Ssp*D5I | 220 | 18,400 |
| *Bbv*II | 170 | 20,100 |
| *Bsp*KT5I | 19,500 | 490 |

Note: In the absence of the restriction endonuclease, for the 410-bp fragment radioactivity is equal to 31,200 and 25,800 cpm in case of M.*Bco*KIA and M.*Bco*KIB, respectively.

produced by the enzymes were verified by sequencing of recombinant M13tg131(*Bco*K) DNA according to Brown and Smith [12].

Distribution of the tritium radioactivity between the two restriction fragments is summarized in the table. It is seen that in the case of M.*Bco*KIA cleavage of the 410 bp fragment with *Bsp*KT5I and *Ssp*D5I produces a $^3$H-labeled ~130 bp fragment, whereas cleavage with *Bbv*II produces a $^3$H-labeled ~280 bp fragment. This proves that M.*Bco*KIA methylates the first cytosine in the sequence 5′-CTCTTC-3′. In the case of M.*Bco*KIB, digestion of the 410 bp fragment with *Bbv*II and *Ssp*D5I produces a $^3$H-labeled ~130 bp fragment, whereas cleavage with *Bsp*KT5I produces a $^3$H-labeled ~280 bp fragment. These results prove that the last adenine is methylated in the sequence 5′-GAAGAG-3′. Thus, the bases modified by the methyltransferases M.*Bco*KIA and M.*Bco*KIB were determined as:

5′-m$^4$CTCTTC-3′

3′-Gm$^6$AGAAG-5′.

There are some restriction-modification systems that recognize the site 5′-CTCTTC-3′ [13]. However, the *Bco*KI system is the first one in which both specificity and methylated bases are determined for two methylases.

The recombinant M13tg131(*Bco*K) DNA can easily be used for insertion of any oligonucleotide duplex with a desirable site. For this purpose the recombinant DNA should be cleaved with *Bli*736I and *Xba*I, and the duplex should have protruding ACGA at one end and four bases that match the termini from *Xba*I digestion at the other end. The IIS restriction endonuclease cassette can be used not only for locating methylated bases but also for IIS restriction enzyme footprinting, as has been already proposed for *Eco*57I.

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