Reliable detection of DNA cytosine methylation at CpNpG sites using the engineered restriction enzyme *EcoRII-C*

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Methylation of cytosine to 5-methylcytosine (mC) is the most important epigenetic DNA alteration in eukaryotes. Cytosine methylation is involved in establishing a silenced chromatin stage through interaction with DNA-binding proteins and recruitment of histone deacetylases and other histone-modifying enzymes leading to chromatin remodeling. In this fashion, DNA methylation is connected with processes of normal and pathological gene regulation, DNA replication, virus latency, parental imprinting, embryonic development, carcinogenesis, and genetic diseases in higher organisms (1–4). Analogous to the terms transcriptome and proteome, the neologism methylome has been proposed to describe the complete set of DNA methylation in a cell, which carries information in addition to the naked DNA sequence. The methylome changes over time and, depending on its alterations, is linked to aging, cancer, and polymorphic variation in populations (5).

Until now, most attention has been focused on the appearance and functions of mC in the context of CpG sequences. However, for years it has been known that in genomic DNA of animal cells (e.g., human spleen DNA), methylated cytosine appears as often in CpT or CpA as in CpG sequences (6). When considering more complex sequences as the sites of cytosine methylation, the presence of mC in the CpNpG context, especially in the internal cytosine of the CC(A/T)GG sequence, is particularly important both in plant (7) and mammalian cells (8–11). In its sequence specificity, this methylation corresponds to the prokaryotic Dcm methylation, which has been known for many years (12). In mammalian cells, CpNpG methylation might be catalyzed by the methylases

Dnmt3A and/or Dnmt2 (for a review see Reference 13). It has been shown that the occurrence of CmC(A/T)GG in the DNA of mammalian cells is able to repress promoter activity and to replace transcription factors with new protein complexes (11). Thus, not only CpG methylation but also CC(A/T)GG methylation is involved in epigenetic regulation. Until now, in contrast to CpG, neither the prevalence nor the genomic distribution of CmC(A/T)GG have been systematically addressed.

One of the most important and easiest methods to identify the presence and location of sequence-specific methylation in DNA, and to estimate the proportion of methylated versus nonmethylated sites in bulk DNA, is the

use of methylation-sensitive restriction enzymes (MSREs). In combination with either PCR or Southern blotting analysis, MSRE can be used for sensitive analysis of site-specific methylation in large and complex genomes.

CC(A/T)GG methylation patterns can be determined by comparative DNA cleavage with the methylation-sensitive restriction endonuclease *EcoRII* and a restriction endonuclease not affected by methylation of the internal cytosine, such as *BstNI* or *MvaI*. However, *EcoRII* is a member of the type IIE restriction endonucleases, which require the interaction with two copies of the respective recognition sequence for their activity (14). When the DNA molecule contains only a single CC(A/T)GG site, or the distance between two copies of the site in the DNA molecule is too large to allow functional interaction with the same dimeric *EcoRII* molecule, the sites remain uncleaved even when nonmethylated. This intrinsic cleavage resistance could be erroneously interpreted as proof of CC(A/T)GG methylation, and it cannot be ruled out that publications describing the occurrence of CmC(A/T)GG on the basis of comparative MSRE analysis by *EcoRII* include bias from this source. Unfortunately, the opportunity to create

Figure 1. Detection of internal cytosine methylation in the CC(A/T)GG sequence context by *EcoRII-C* cleavage. The restriction sensitivity of M13 RF DNA carrying two copies of the sequence CC(A/T)GG at a distance of 952 bp (A) or of an isolated M13 RF DNA fragment containing only one CC(A/T)GG site (B) are shown. M13 RF DNA amplified in the Dcm⁻ strain *Escherichia coli* B, and thus not methylated in the CC(A/T)GG sequence (lanes 1–3), as well as M13 RF DNA amplified in *E. coli* JM107 (Dcm⁺) and carrying methylated CmC(A/T)GG sites (lanes 4–7) were used as substrates (A). In addition, 2336-bp fragments of the Dcm⁻ and Dcm⁺ M13 RF DNAs containing only one CC(A/T)GG and CmC(A/T)GG site, respectively, were obtained by *NciI* digestion followed by gel extraction (B). All substrate DNAs were incubated without restriction endonuclease (lanes 1 and 4), with *EcoRII* (lanes 2 and 5), and *EcoRII-C* (lanes 3 and 6), respectively. Cleavage by *BstNI* (not affected by internal cytosine methylation) was used as a control (lane 7). M, Smart ladder (Eurogentec, Seraing, Belgium). Samples were run on 0.8% agarose gels stained with ethidium bromide. (C) Scheme of restriction endonuclease sensitivity of DNA substrates in dependency on the number of CC(A/T)GG sites (depicted as filled rectangles), methylation of the internal cytosine (depicted as lollipops), and the length of the intervening sequence between two sites. Shaded areas mark the correct proof of the methylation state by the use of *EcoRII-C* in contrast to the wild-type enzyme *EcoRII*.

reliable Type IIE restriction endonuclease cleavage patterns by means of co-incubation with site-specific oligonucleotides mimicking the second copy of the recognition sequence *in trans* (14) has not been widely used in the respective investigations. Since there is no other methylation-sensitive restriction endonuclease commercially available that exhibits the same sequence specificity and methylation sensitivity as *EcoRII* at normal incubation temperature, *EcoRII* is unique for purposes of CmC(A/T)GG detection, and no other enzyme can be used instead.

Using protein engineering approaches, we were able to construct a truncated form of the restriction endonuclease *EcoRII*. This recombinant enzyme, *EcoRII-C*, encompasses the C-terminal endonuclease domain (amino acids 173–404) of *EcoRII* that harbors the catalytic site (15,16). In contrast to the original *EcoRII*, the novel enzyme *EcoRII-C* is able to cleave single CC(A/T)GG sites (15). In this study, we sought to establish whether *EcoRII-C* retained the specific methylation sensitivity of the original *EcoRII*, which would allow its use for reliable detection of CmC(A/T)GG.

Phage M13 circular RF DNA carrying two CC(A/T)GG sites separated by 952 bp was replicated in Dcm⁻ and Dcm⁺ cells, respectively, and used as a model substrate for cleavage by *Bst*NI, *EcoRII*, and *EcoRII-C* (Figure 1A). The isoschizomeric enzyme *Bst*NI, able to cleave single CC(A/T)GG sites no matter whether the internal cytosine is methylated or not, was applied as a control (Figure 1A, lane 7). *EcoRII* was unable to attack either the unmethylated (Figure 1, lane 2) or the methylated substrate DNA (Figure 1, lane 5), primarily because of the large distance between the two CC(A/T)GG sites, which prevents the formation of the endonucleolytically active DNA-enzyme complex. However, the novel enzyme *EcoRII-C* was found to be able to discriminate between nonmethylated (Figure 1, lane 3) and methylated DNA (Figure 1, lane 6). Only nonmethylated DNA is cleaved, but methylated DNA remains resistant due to the methylation at the internal cytosine. Use of M13 RF DNA as substrate is diagrammatically shown in the two upper lines of Figure

1C. Here, the two CC(A/T)GG sites are separated by 952 bp of nonspecific DNA and cannot functionally interact.

Accordingly, we demonstrated that a fragment of M13 RF DNA carrying only one CC(A/T)GG site is resistant towards *EcoRII*, both in its nonmethylated as well as methylated state; however, *EcoRII-C* cleaves exclusively the nonmethylated site and thus can detect methylation (Figure 1B). These data correspond to the situation shown in the last two lines of Figure 1C. Only in a substrate with densely spaced methylation sites (e.g., pBR322 DNA) can *EcoRII-C* as well as *EcoRII* be used for methylation detection (compare the central two lines in Figure 1C and data not shown).

In summary, the newly engineered restriction endonuclease *EcoRII-C* represents the perfect methylation-sensitive counterpart to *Bst*NI, allowing reliable detection of CpNpG methylation in the CC(A/T)GG context. Its ease of use should promote the study of the presence, distribution, and functional relevance of CpNpG methylation in epigenome research.

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COMPETING INTERESTS STATEMENT

*The authors declare that the generation of the genetically engineered *EcoRII-C* endonuclease, which was used in this study, is the subject of a patent pending.*

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