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María Belén Jerez* and Maximiliano Juri Ayub*

Instituto Multidisciplinario de Investigaciones Biológicas de San Luis, IMIBIO-SL-CONICET and Facultad de Química, Bioquímica y Farmacia, Universidad Nacional de San Luis, San Luis Argentina.

* Corresponding Authors. E-mail: jerezbel@gmail.com; mjuriayub@hotmail.com

Graphical Abstract:

Abstract: Cytosine methylation of DNA in mammals has been associated with both physiological and pathological changes in gene-expression. DNA treatment with methylation sensitive and/or dependent restriction enzymes, followed by PCR amplification is a widely used approach to test CpG methylation. Recently, restriction endonuclease MspJI has been proposed as a promising tool for epigenetic analyses. In this paper, we have tested MspJI as a tool for detecting CpG methylation on mammalian genomic DNA. For this experiment mouse genomic sequences harboring or lacking CpG sites were selected. The extent of degradation was evaluated by PCR using primers flanking the chosen genomic regions. Digestion of mouse genomic DNA, in combination with end-point and real-time PCR reactions, revealed that MspJI treatment reduced the amplification of genomic regions either containing or lacking CpG sites were selected. The extent of degradation was evaluated by PCR using primers flanking the chosen genomic regions. Digestion of mouse genomic DNA, in combination with end-point and real-time PCR reactions, revealed that MspJI treatment reduced the amplification of genomic regions either containing or lacking CpG motifs. In addition, treatment of bona fide non-methylated (in vitro amplified) DNA samples definitely demonstrated that MspJI shows significant activity against non-methylated DNA. These results show that star activity can be an important concern when using MspJI, even under standard conditions. Therefore, we conclude that (in contrast to classical restriction enzymes), careful case by case evaluation of reaction conditions is mandatory for optimizing the usefulness of MspJI in epigenetic studies.

Keywords: DNA methylation; restriction enzyme; star activity; epigenetic; cytosine; MsPJI; Real time PCR
Introduction

Methylation of DNA is associated with transcriptional repression, as well as other highly specialized processes such as genomic imprinting and X chromosome inactivation [1]. There is also growing evidence linking DNA methylation to pathological processes such as cancer development [2]. These findings have raised interest about methodologies addressing DNA methylation states. In particular, cytosine methylation at CpG sites on gene promoters of animal genomes is a widely explored feature, since 5-methylcytosine (5mC) has been described as a major regulator of gene expression [3].

Basically, two general approaches are currently used for detecting 5mC, namely bisulfite conversion and restriction with methylation sensitive and/or dependent enzymes. A comprehensive study comparing the relative merits of these strategies has been recently performed [4]. Bisulfite treatment followed by sequencing is the ‘gold standard’ for methylation quantification. However, this method is often time-consuming and demands lots of resources, impairing its wide usage in many non-specialized laboratories, in particular for exploratory or preliminary analyses. Therefore, restriction-based approaches remain as a valuable alternative to analyze CpG methylation at the single-locus level. One of these strategies involves the treatment of genomic DNA with methylation-dependent endonucleases. Then, the methylation level of the selected genomic region is evaluated by PCR (preferably qPCR), using primers flanking the DNA region under study (Figure 1) [5].

One of the drawbacks of the restriction-based methods is that they are able to detect a subset of 5mCpG sites; namely those where the surrounding bases yield an adequate restriction site. Therefore, the discovery of new methylation-dependent enzymes may allow expansion of the toolkit and the coverage of different methylation sites. Recently, several new promising methyl-dependent enzymes have been discovered: MspJI, FspEI, LpnPI, AspBHI, RlaI, and SgrTI [6]. In particular, MspJI has been characterized and described as specifically recognizing the 5mCNNR sequence and cleaving both strands by a few bases on the 3’ side of methylated cytosine, being inactive on non-methylated DNA. For this reason, it represents a powerful tool for epigenetic studies, as it is able to detect around 50% of methylated CpG sites: 5mCGNA and 5mCGNG [6]. Digestion of Arabidopsis thaliana genomic DNA with MspJI followed by size fractionation of digested product has been reported to be useful for generating a methylated cytosine enriched sequencing library [7]. More recently, MspJI has been used in combination with 5-methyl-dCTP to achieve DNA fragmentation prior to running samples in short-read DNA sequencing platforms [8].

Materials and Methods

All experiments shown in this paper have been performed at least three times.

DNA isolation

DNA was purified from different sources using the Wizard® Genomic DNA Purification Kit (Promega), following manufacturer’s instructions. Integrity was confirmed by agarose electrophoresis and quantification was spectrophotometrically performed by measuring absorbance at 260 nm.
Digestion with MspJI

Genomic DNA from different sources (1 µg) was digested according to the manufacturer’s instructions, using 4 units of MspJI (NEB) in the presence of 1 µL of double-stranded DNA activator, in a final volume of 30 µL. All reactions were incubated at 37°C for 4 or 8 h. Control and MspJI treated samples were incubated at 65 °C for 20 min in order to inactivate the enzyme. After that, samples were always kept on ice or stored at -20 °C. To exclude any artifacts, control reactions included all the components except MspJI, and were processed in parallel with MspJI treated samples. Digestion products were visualized by gel electrophoresis. It should be noted that in order to avoid artifacts, DNA and enzyme concentrations, temperature and incubation times were kept in the range suggested by the manufacturer (0.5-1 µg DNA; 2.5-5 U MspJI, incubation at 37ºC for 4-16 h). Digestion products were analyzed by agarose electrophoresis and/or used directly as templates for end-point or real-time PCR reactions.

End-point PCR

PCR reactions were performed in 20 µL volume using TaqUBA DNA polymerase (gifted by Dr. Mauro Morgenfeld) essentially as previously described [9]. Reaction buffer included Tris-HCl (pH 9.0) 10 mM, KCl 50 mM, Triton X-100 0.1 %, MgCl2 1.5 mM). We used 50 ng of genomic DNA as a template, which was previously incubated at 37°C in the presence or in the absence of MspJI, as described above. The following oligonucleotides were designed to amplify a 1,799 bp fragment from the IL-12b promoter gene: IL-12bFw_1: 5’-TCGGCCCCATATTGCTTTGT-3’ and IL-12bRev_1: 5’-ACAGCTCTAGATGCAGGGA-3’ (Figure 2). Cycling conditions were as follows: 1 step at 94°C for 30 s; 35 cycles of 94 °C for 30s, 63 °C for 45 s, 72 °C for 2 m; followed by a final elongation step of 72 °C for 10 m. Amplicons were visualized by electrophoresis on 1.5 % agarose gel.

Real-time PCR

PCR amplification mixtures contained 50 ng of genomic DNA, FastStart Universal SYBR Green Master (Rox) (Lifesciences, Roche), 500 nM primers and UltraPure™ DNase/RNase-Free Distilled Water (Life technologies). Reactions were run on an ABI PRISM 7500 (Applied Biosystems) instrument. Cycling conditions comprised 2 m at 50 °C, 10 m polymerase activation at 95 °C and 40 cycles of 95°C for 15 s and 60 °C for 60 s. The following primers were used to amplify a 333 bp sub-fragment from the IL-12b promoter: IL-12bFw_2: 5’-AAGTTGTGTGGCTGGGAAG-3’ and IL-12bRev_2: 5’-GTTGATGTATCCTCCCTTCCCTC-3’. In addition, as negative control, a 95 bp CpG-lacking fragment of the beta-actin gene was amplified using the following oligonucleotides: Actin-Fw: 5’-CTTGATCTTCATGGTGTCTTAGGAG-3’ and Actin-Rev: 5’-CAGTGTCTGTCTGGGTGTACC-3’ (Figure 2).

Results and Discussion

The mouse genome harbours a high percentage of cytosine methylation (7.6 %) [10]. As expected, a smeared pattern was observed after digestion of mouse DNA with MspJI under standard conditions (Figure 3, left panel). As a negative control, genomic DNA from the parasite Giardia lamblia, which was available at our lab, was used. It is known that cytosine methylation is very low or even null in all the studied eukaryotic microorganisms [11,12,13,14,15]. In addition, it has been reported that the genome of G. lamblia is completely devoid of cytosine methyl transferase encoding genes [16]. We have further confirmed this by searching on the G. lamblia genomic databases for genes encoding the corresponding protein domain (InterPro: IPR001525/PFAM: PF00145). As expected, smearing of G. lamblia DNA treated with MspJI was negligible (Figure 3, right panel). These results are consistent with a specific digestion of methylated DNA by MspJI, according to previous reports [6,7,8,17].

Figure 2. Scheme depicting the relevant features of target amplified sequences. Left: a 1,799 bp fragment belonging to the IL-12b promoter (including 40 CpG sites) was used for end-point PCR. A sub-fragment of 333 bp was used for qPCR amplification. Right: A 95 bp fragment belonging to the beta actin gene, lacking CpG sites was used as a negative control in qPCR. Green and yellow circles are CpG sites which can be or not interrogated by the MspJI specificity, respectively. These sites are represented schematically on the figure and their number does not match with the actual number on these genome regions.
However, careful observation of Figure 3 right panel shows that the band size of the digested sample (lane 7) is slightly lower compared to the non-treated DNA (lane 6). This fact is compatible with both a low proportion of methylated DNA and/or non-specific activity of MspJI on unmethylated DNA.

Next, we used MspJI-digested mouse DNA as template to amplify a 1,799 bp fragment from the IL-12b gene (Gene ID: AH004859) (nt -1693 to +106, according to the transcription initiation site), containing 40 CpG sites (see Figure 2). Out of these 40 CpG sites, 27 are overlapped with MspJI recognition sites on one or both strands, and their methylation could be addressed (through digestion) with this enzyme. Notably, MspJI treatment completely prevented the amplification of the IL-12b promoter, suggesting that this region was densely methylated at CpG sites (Figure 4).

This result prompted us to set up a quantitative amplification analysis by using qPCR instead of end-point PCR. We selected two different PCR targets (see Figure 2): a 333 bp sub-region of the IL-12b promoter (nt -1136 to -801, according to the transcription initiation site) including 19 CpG sites (13 overlapping with MspJI restriction sites), and a 95 bp fragment belonging to the beta-actin gene (Gene ID: NC_000071.6, nt +999 to +1093 according to the transcription initiation site) lacking CpG sites. Surprisingly, in both cases MspJI treatment produced a 2-3 cycle shift of the Cq value, when amplifying both regions containing or lacking of CpG sites (Figure 5A). The identity of amplified fragments was confirmed by thermal dissociation (Figure 5B).

The Cq value shift for the 95 bp fragment lacking CpG sites strongly suggests that unmethylated DNA is digested by MspJI. However, as an alternative hypothesis it can be postulated that methylated cytosines at sequences other than CpG were present in the genomic samples, recognized and cleaved by MspJI. In fact, non-CpG methylation has been reported in stem cells and, more recently, in differentiated mammalian cells [18]. Therefore, in order to definitively evaluate the specificity of the assay using a bona fide non-methylated sample, we replaced genomic DNA by an aliquot of the 1,799 bp PCR product. Interestingly, MspJI degraded this unmethylated DNA (Figure 6A) and, consequently, prevented further PCR amplification (Figure 6B). This experiment definitely confirmed that MspJI degraded non-methylated DNA at a significant extent. These results demonstrate that although MspJI selectively degrades methylated DNA [6,7,8,17], residual activity on unmethylated DNA should be considered and case by case evaluation and optimization is needed to improve its usefulness for epigenetic analyses based on methylation-dependent restriction coupled to qPCR strategies.
Figure 5. Effect of MspJI treatment on the real time amplification of genomic templates. Top panel: real-time amplification of the 333 bp IL-12b promoter fragment harboring 13 MspJI-detectable CpG sites. Bottom panel: real-time amplification of the 95 bp beta-actin fragment lacking CpG sites. A. Amplification plots for genomic samples treated with (D) or without (ND) MspJI. Curves are representative of 12 independent experiments performed in duplicates. Fluorescence values are shown on a logarithmic scale in order to better detect Cq differences among samples. The identity of amplified fragments was confirmed by thermal dissociation (B).

Figure 6. Degradation of bona fide non-methylated DNA by MspJI. A. An aliquot of the 1799bp PCR product was incubated in the absence (lane 2) or in the presence (lane 3) of MspJI and reactions were analyzed by agarose electrophoresis. B. Digestion products (2 ng) of (A) were used as templates for PCR amplification and products were analyzed by agarose electrophoresis. A negative PCR control was run in parallel (lane 4) with controls.

In summary, we have demonstrated that MspJI displays significant activity against non-methylated DNA. Therefore, cautions should be taken when using this enzyme for epigenetic studies. Our results indicate that further characterization experiments on MspJI would be helpful in order to improve the performance of this enzyme as an epigenetic tool. It would be relevant to address whether non-specific activity is sequence-independent, or unmethylated target sequences (CNNR) are preferentially cleaved. Providing a better understanding of these issues would allow developing genetic engineered variants of higher selectivity MspJI. This would, in turn, yield High Fidelity variants for this enzyme, a strategy that has shown to be successful with classical restriction enzymes.

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