THE EFFECT OF DIFFERENTIAL METHYLATION BY \textit{ESCHERICHIA COLI} OF PLASMID DNA AND PHAGE T7 AND $\lambda$ DNA ON THE CLEAVAGE BY RESTRICTION ENDONUCLEASE \textit{MboI} FROM \textit{MORAXELLA BOVIS}

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Summary

The nucleotide sequence recognized and cleaved by the restriction endonuclease \textit{MboI} is $5'\text{GATC} 3'$ and is identical to the central tetranucleotide of the restriction sites of \textit{BamHI} and \textit{BglII}. Experiments on the restriction of DNA from \textit{Escherichia coli dam} and \textit{dam} $^+$ confirm the notion that GATC sequences are adenosyl-methylated by the \textit{dam} function of \textit{E. coli} and thereby are made refractory to cleavage by \textit{MboI}. On the basis of this observation the degree of \textit{dam} methylation of various DNAs was examined by cleavage with \textit{MboI} and other restriction endonucleases. In plasmid DNA essentially all of the GATC sequences are methylated by the \textit{dam} function. The DNA of phage $\lambda$ is only partially methylated. extended methylation is observed in the DNA of a substitution mutant of $\lambda$, $\lambda$ \textit{gal$5$bio$256$}, and in the $\lambda$ derived plasmid, $\lambda$dv$93$, which is completely methylated. In contrast, phage T7 DNA is not methylated by \textit{dam}. A suppression of \textit{dam} methylation of T7 DNA appears to act only in \textit{cis} since plasmid DNA replicated in a T7-infected cell is completely methylated. The results are discussed with respect to the participation of the \textit{dam} methylase in different replication systems.

Introduction

The restriction endonuclease \textit{MboI} isolated from \textit{Moraxella bovis} recognizes the nucleotide sequence $5'\text{GATC} 3'$, and cleaves at the sites marked by the arrows [1]. The enzyme produces 'sticky ends' of the sequence GATC at the
cleavage site and thus should be suitable for molecular cloning of DNA fragments. The sequence GATC represents the central tetranucleotide of the recognition sequence of other restriction endonucleases, among them the enzymes BamHI isolated from Bacillus amyloliquefaciens [2] and BglII isolated from Bacillus globigii [3]. BamHI cleaves the sequence 5'C-GATC-G3' and BglII the sequence 3'T-CTAG-A5' at the sites indicated by arrows. Therefore, MboI is expected to cut any BamHI and BglII site and thus has been proposed to be useful for cloning of MboI-derived restriction fragments into BamHI and BglII sites of appropriate vehicles [1,4]. However, when we compared the restriction pattern of MboI, BamHI and BglII with the DNA of the composite plasmid pFE42, we observed instead of at least six cuts with MboI (three BamHI sites and three BglII sites) barely one in the plasmid DNA. Since this result suggested that modification of the DNA by methylation was involved, we examined the restriction of plasmid and phage DNAs by MboI and other restriction endonucleases with respect to DNA methylation conferred by the host cell, in particular by the deoxyadenosine methylase coded by the dam gene [5]. The results indicate that in plasmid DNA the MboI restriction sites are methylated by the dam function and thereby made refractory to cleavage. In phage DNA the adenosyl-methylation of GATC sequences appears to vary depending on the respective phage and also on its particular genetic constitution. The results are discussed with respect to the participation of the dam methylase in different replication systems.

Materials and Methods

**Escherichia coli strains.** Plasmids were generally maintained and isolated from C600 (thr, leu, thi, tonA). Other strains were SF8 (recB21, recC22, lop-11, tonA1, thr-1, leu-6, thi-1, lacY1, supE44, rKmK), AB1157 dam-4 (argE3, his-4, thr-1, leu-6, proA2, thi-1, strA31, lacY1, gal, ara, xyl, mtl, strA; from B. Glickman) and WA 834 (met rKm; from W. Arber).

**Isolation of plasmid DNA.** The plasmids pFE42 and pBR322 as all ColE1-containing chimeras can be amplified by chloramphenicol [6]. A 500 ml culture of a plasmid-containing strain was aerated in tryptone-yeast extract medium at 37°C. At a titer of 3 · 10⁸/ml chloramphenicol (Sigma) was added to a final concentration of 250 µg/ml. The culture was then aerated for another 16 h at 37°C. A Triton-lysate was prepared as described [7] with some small modifications. The cells were harvested by centrifugation and resuspended in 8 ml TES (100 mM Tris, pH 7.5, 50 mM NaCl, 5 mM EDTA) containing 25% sucrose. Spheroplasts were prepared by addition of 2 ml of lysozyme (Serva, 10 mg/ml) and 4 ml of 0.25 M EDTA. After 15–20 min at 0°C the spheroplasts were lysed by addition of the same volume of 'lytic mix' consisting of 0.4% Triton X-100 (Serva) in 50 mM Tris-HCl, pH 7.5, 62.5 mM EDTA [8]. After 20 min at 0°C the lysate was centrifuged for 20 min at 35 000 × g. The supernatant was subjected to CsCl-gradient centrifugation in the presence of ethidium-bromide as described [8]. The supercoiled DNA was collected from the gradients and the centrifugation was repeated. Ethidium
bromide was removed by extraction three times with isopropanol saturated with CsCl. The plasmid DNA was then dialysed against 10 mM Tris-HCl, pH 7.5, 1 mM EDTA. DNA of the plasmid λadv93 was a gift of M. Lusky from the laboratory of Dr. G. Hobom.

Isolation of phage DNA. DNA from a T7' strain (F.W. Studier) was isolated according to Seroka and Wackernagel [9]. λ DNA was isolated after phenol extraction of phage purified by CsCl gradient centrifugation. λcI857Sam7 was prepared by thermo-induction of E. coli strains lysogenic for this phage. λcI857gal4bio256 [10,11] was obtained after infection of cells in liquid culture.

Restriction endonucleases. MboI was isolated according to Gelinas et al. [1], BglII according to Pirrotta [3], EcoRI according to Greene et al. [12], HindII/III according to Smith and Wilcox [13], and Sau3AI according to Sussenbach et al. [14]. BamHI was purchased from Miles Lab. (Elkhart, IN, U.S.A.) and PstI and KpnI were from Biolabs (Beverly, MA, U.S.A.). In some experiments an MboI preparation from Biolabs was also used.

DNA cleavage by restriction endonucleases. Reaction conditions for restriction with EcoRI were 100 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 10 mM MgCl2. Reaction conditions for BamHI, BglII, KpnI, and MboI were 6 mM Tris-HCl, pH 7.5, 6 mM MgCl2, and 6 mM β-mercaptoethanol. In some cases 150 mM NaCl plus bovine serum albumine (100 μg/ml) were included in reactions with MboI. Reaction mixtures for HindII/III contained 6 mM Tris-HCl, pH 7.5, 6 mM MgCl2, 1 mM dithiothreitol and gelatine (0.1 mg/ml). Reaction mixtures were incubated at 37°C for appropriate times, mostly two to three h. Reaction conditions for Sau3AI were 6 mM Tris-HCl, pH 7.5, 15 mM MgCl2, 60 mM NaCl, and 6 mM β-mercaptoethanol. The incubation was at 30°C.

Electrophoresis. Agarose gel electrophoresis was performed as described [12] using 5-mm slab gels (16 x 11 cm) and run at 100 V and 40 mA for an appropriate time, generally about 2.5 h. Polyacrylamide gels (4%, 16 x 11 cm) of 1.5 mm width were prepared according to Loening [15] and run at 100 V and 40 mA for 5 h in a Tris-Phosphate buffer [16].

Visualization of DNA fragments. Gels were stained with ethidium bromide and photographed on a Chromato-Vue transilluminator, model C-62 (Ultra-Violet Products Inc., San Gabriel, CA, U.S.A.). Photographs were taken on Polaroid Land film type 665 or on Ilford film Pan F 50ASA.

Transformation. Transformation was carried out as previously described [17] except that a concentration of 80 mM CaCl2 was used. Plasmid DNA (between 0.5 and 2 μg) was diluted in 10 mM Tris-HCl, pH 7.5, to a volume of 0.1 ml. The transformants were selected for their resistance to penicillin by plating samples of the transformation mixture on complete agar which contained 200 μg/ml penicillin (Serva).

Results

1. Restriction of pFE42 DNA

Plasmid pFE42 (13.3 x 106 daltons; Eichenlaub, R., unpublished data) is composed of RSF2124 (ColE1 carrying TnA) [18] and mini-F [8]. This plasmid contains three BamHI and three BglII restriction sites (Fig. 1; slots C and D). When pFE42 DNA was digested with MboI, only one cut was intro-
duced into a fraction of the DNA molecules (Fig. 1; slot E), even with excess of enzyme. This was surprising, since at least six cuts per molecule were expected corresponding to the BamHI and BglII sites. In fact, significantly more than six MboI sites might reside in pFE42 DNA due to the smaller recognition sequence of MboI compared to BamHI and BglII (see introduction). In order to verify that our preparation of MboI indeed displayed the published cleavage specificity DNA of phage T7 was also restricted with MboI (Fig. 1; slot A). The restriction pattern obtained was identical to that reported by McDonell et al. [19] except that the two small fragments of 408 and 120 base pairs are not visible on the gel shown in Fig. 1.

Although only a fraction of the pFE42 DNA molecules was linearized by MboI we attempted to localize the MboI cleavage site on the plasmid DNA. For this purpose double restriction with MboI plus KpnI was performed. Since this did not produce one specific extra fragment besides the two KpnI fragments it was concluded that the sporadic MboI cleavages cannot be attributed to one specific site on the DNA. However, doubly restricted DNA produced a set of discrete bands of smaller molecular weights on the gel (Fig. 2; slot B). This observation argues against a contaminating unspecific endonuclease in our

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Fig. 1. Agarose gel electrophoresis of T7 DNA and pFE42 DNA treated with various restriction endonucleases. A, T7 DNA plus MboI; B, pFE42 DNA, no enzyme; C, pFE42 DNA plus BamHI; D, pFE42 DNA plus BglII; E, pFE42 DNA plus MboI. The agarose concentration was 0.8%.

Fig. 2. Agarose gel electrophoresis of pFE42 DNA treated with MboI (A) and with MboI plus KpnI (B). λ DNA treated with EcoRI (C) served as reference for the molecular weight of the fragments. The agarose concentration was 1.0%.
preparation of MboI, a notion which is further supported by the ability to ligate the plasmid DNA linearized by MboI (Dreiseikelmann, B., unpublished results). The same results were obtained with a commercial preparation of MboI. Thus, the production of a series of discrete fragments upon double restriction with MboI plus KpnI is consistent with the assumption that MboI cleaves pFE42 DNA occasionally at one of several sites producing a population of discontinuously permuted linear molecules.

The fact that MboI cuts the plasmid pFE42 containing three BamHI and three BglII sites barely once suggested that DNA methylation by the host cell may play a role in the restriction of DNA by MboI. An influence of the host specific modification system was excluded by the observation that pFE42 DNA isolated from E. coli SF8 rKmK and E. coli met rBmB was as refractive as wild type DNA to restriction by MboI. However, when pFE42 DNA was isolated from E. coli dam-4 which is defective for the major adenine methylase (Ref. 5; Glickman, B., personal communication), the restriction pattern for MboI was dramatically changed (Fig. 3; slot C). MboI now cleaved pFE42 DNA into more than twenty fragments.

Fig. 3. Agarose gel electrophoresis of DNA replicated in E. coli wild type (+) and E. coli dam (−) and treated with various restriction endonucleases. A, T7 DNA plus MboI; B, pFE42 DNA, no enzyme; C, pFE42 DNA plus MboI; D, pFE42 DNA plus BamHI; E, λ DNA plus HindII/III. The agarose concentration in the gel was 0.8%.
Furthermore it was observed that the sporadic cleavage of dam-methylated plasmid DNA by the restriction endonuclease MboI is depending on the salt concentration. The linearization of pFE42 DNA by MboI is abolished in the presence of 100 mM NaCl, a condition which does not interfere with the cleavage of T7 DNA. Therefore it seems that the few cuts produced by MboI in methylated DNA cannot be explained by the incomplete methylation of GATC sequences but may rather be due to some intrinsic activity of MboI which occasionally cleaves the methylated sequence at low salt concentration.

These results support the notion made on the basis of experiments with MboI and other restriction endonucleases [1,3,20] that MboI favourably cleaves unmethylated recognition sequences and that the dam gene product modifies all MboI sites in plasmid DNA.

2. Restriction of λ DNA

MboI cleaves λ DNA into more than 50 fragments [1]. We compared the cleavage of λ DNA isolated from E. coli dam⁺ by MboI and Sau3AI, a restriction endonuclease from Staphylococcus aureus which also recognizes and cleaves the nucleotide sequence 5’GATC [14]. Even with an excess of MboI only a partial fragmentation of λ DNA was obtained in dam⁺ experiments whereas Sau3AI produced more than 50 fragments (Fig. 4; slots A⁺ and B) in

Fig. 4. Polyacrylamide gel electrophoresis of bacteriophage λ DNA replicated in E. coli wild type (+) and E. coli dam (−) treated with restriction endonucleases MboI and Sau3AI. A, λ⁺ DNA plus MboI; B, λ⁺ DNA plus Sau3AI; C, T7 DNA plus HindII/III; D, λgal8bio256 DNA plus MboI; E, λgal8bio256 DNA plus Sau3AI. Polyacrylamide concentration was 4%; MboI digestes were with 150 mM NaCl.
either dam⁺ or dam experiments. With λ DNA from a dam strain these fragments were also produced by MboI (Fig. 4; slots A⁻ and B⁻). These data are consistent with the finding by fingerprint analysis of cohesive ends with the sequence GATC that in λ DNA the adenine in GATC is only approx. 50% methylated [3]. Thus, adenine-methylated GATC sequences are probably not cleaved by MboI, but Sau3AI cleaves GATC irrespective of methylation. Interestingly, the DNA of a plasmid derived from λ, λdv93, which consists of a 4.7% fragment of λ ranging from 78.2% to 82.9% of the total length of DNA [21], is not cleaved by MboI indicating essentially complete methylation of GATC sequences (Fig. 5). When we tested the methylation of GATC in the DNA of a substitution mutant of λ, λgal6bio256 cI857, by restriction with MboI and Sau3AI, the DNA appeared to be almost completely methylated (Fig. 4; slots D and E).

3. Restriction of T7 DNA

The DNA of T7 is cleaved by MboI into seven fragments [19]. T7 DNA replicated in a dam⁺ and a dam strain of E. coli gave identical restriction patterns with MboI which are indistinguishable from restriction patterns obtained with Sau3AI (data not shown). Thus, in T7 DNA the GATC sites are

Fig. 5. Polyacrylamide gel electrophoresis of λdv93 DNA from an E. coli dam⁺ strain treated with restriction endonucleases MboI and Sau3AI. A, λdv93 DNA plus MboI; B, λdv93 DNA plus Sau3AI (7 DNA bands). Polyacrylamide concentration was 6.3%.
presumably not methylated by the host cell. The mechanism which prevents dam methylation of T7 DNA could be analogous or identical to the process which suppresses host specific modification of T7 DNA and which involves the function of gene 0.3 [22,23]. However, DNA of a deletion mutant of T7, D104 am 193LG3, in which one deletion extends over the complete gene 0.3 [24], is still devoid of methylated GATC sequences as evidence by the unimpaired cleavage by MboI (data not shown). Nevertheless, a suppressive mechanism controlled by genes not deleted in the T7 mutant could still be operative. It has recently been demonstrated by conjugation experiments that infection of a cell receiving an F'factor by ultraviolet-inactivated T7 results in a suppression of the host specific restriction of the F'DNA [25]. Therefore, a trans-test for possible suppression of dam* activity was performed. Growing cells of E. coli W3550 su− carrying the plasmid pBR322 were infected at a multiplicity of 10 with T7 am28am29am233 defective for DNA polymerase, endonuclease and exonuclease [26]. The phage was irradiated with ultraviolet light prior to infection to give a survival of 10−5; 15 min after infection at 37°C (control: uninfected

Fig. 6. Polyacrylamide gel electrophoresis of plasmid pBR322 DNA replicated in E. coli wild type and in E. coli wild type after infection with ultraviolet-inactivated T7 am28am29am233 treated with restriction endonucleases MboI and Sau3AI. A, pBR322 DNA from T7 infected E. coli dam* plus MboI; B, pBR322 DNA from normal E. coli dam*; C, pBR322 DNA from T7 infected E. coli dam* plus Sau3AI; D, pBR322 DNA from normal E. coli dam* plus Sau3AI. Polyacrylamide concentration was 6.3%.
cells) chloramphenicol at 250 \( \mu \text{g/ml} \) was added to the culture in order to block further protein synthesis and one hour after infection \([^{3}\text{H}]\text{thymidine} \) was added. Aeration was continued for 14 h to amplify the plasmid. Supercoiled pBR322 DNA was then isolated. The radioactivity in this DNA showed that plasmid DNA had replicated in the infected cells. A comparison of label incorporated into pBR322 supercoils in infected and uninfected cells (control) indicated that at least 80% of the supercoil DNA in infected cells was newly synthesized. The DNA was digested with \( \text{MboI} \) and \( \text{Sau3AI} \). As shown in Fig. 6 (slots A and E) pBR322 DNA from the \( T7 \) infected cells was as refractive to cleavage by \( \text{MboI} \) as DNA from uninfected cells, whereas \( \text{Sau3AI} \) cleaved both plasmid DNAs into discrete fragments (Fig. 6; slots C and D). This result shows that the plasmid DNA is still normally \( \text{dam} \)-methylated after replication in a \( T7 \) infected cell. It appears that in contrast to modification-specific methylation a suppression of the \( \text{dam} \) methylase activity does not occur.

**Discussion**

The restriction endonuclease \( \text{MboI} \) which recognizes the sequence 5’|GATC\] cleaves DNA only when it is not methylated by the \( \text{dam} \) function of \( E. \text{coli} \). This interpretation of our results implies that GATC is one of the sequences which are recognized by the \( \text{dam} \) enzyme and which are specifically methylated at the internal adenine. This conclusion is in accord with the observation by Gelinas et al. \([1]\) that adenovirus-2 DNA is a better substrate for \( \text{MboI} \) than DNA from \( \lambda \) grown in \( E. \text{coli} \) K12. It is also consonant with the finding by Pirrotta \([3]\) that the adenine residues in GATC sequences in \( \lambda \) DNA are partially methylated by the \( \text{dam} \) function of \( E. \text{coli} \). Recently Lacks and Greenberg \([20]\) have made a similar statement on the basis of their finding that the restriction endonuclease \( \text{DpnII} \) from \( \text{Diplococcus pneumoniae} \) cleaves the nucleotide sequence GATC when it is not methylated by the \( \text{dam} \) function. Thus, \( \text{MboI} \) and \( \text{DpnII} \) are identical in cleavage specificity with respect to recognition sequence and methylation \([20,27]\). On the other hand, the enzymes \( \text{BamHI} \) \([2]\) and \( \text{BglII} \) \([3]\) having GATC as the central tetranucleotide in their hexanucleotide recognition sequences cleave plasmid DNA irrespective of \( \text{dam} \) methylation (Fig. 1), a result that supports earlier observations obtained with partially methylated DNA \([3,28]\).

Our results with plasmids pFE42, pBR322 and \( \lambda \text{dv93} \) show that in plasmid DNA essentially all GATC sites are normally methylated by the \( \text{dam} \) function in \( E. \text{coli} \) K12 and that an enzyme with identical specificity exists in \( E. \text{coli} \) B. The DNA of phage \( \lambda \) wild type appears to be partially methylated by \( \text{dam} \), a result which is in accord with published data \([3,28]\). The almost complete methylation of GATC sequences in \( \lambda \text{gal}_{s}\text{bio}_{256} \) DNA remains unexplained but it seems not unreasonable to assume that a gene in \( \lambda \), lost through the \( \text{gal}_{s}\text{bio}_{256} \) substitutions and also absent in \( \text{dv93} \), controls the extent of \( \text{dam} \) methylation by the host cell. Further studies will be directed to attribute this control function to a specific gene of phage \( \lambda \).

The total lack of GATC methylation in \( T7 \) DNA contrasts with the partial or complete methylation of \( \lambda \) DNA and plasmid DNA, respectively. By coincidence, this order in methylation density may be correlated to the mode of
DNA replication in that plasmid DNA replicates only as a circular molecule, λ DNA replicates as a circle early and as a linear molecule late during intra-
cellular development [29,30] and T7 DNA replicates exclusively via linear
intermediates [31–33]. However, a correlation appears not very plausible if
one considers that the individual nucleotide sequences recognized by a transfer-
ase should not differ in a circular and a linear DNA molecule. One could also
argue that phage DNA may not allow for extensive methylation before it is
packaged into phage particles. However, the observed difference in methylation
density between T7 and λ and even between different λ variants cannot be
explained easily. An alternate interpretation relates dam methylation to the
type of replication system that is active in the replication of the different
DNAs. The plasmid DNA in our experiments, either ColE1, mini-F or λdv is
replicated by the enzymes which also replicate the chromosomal DNA. The
virulent bacteriophage T7, on the other hand, provides its own replication
apparatus. An intermediate case is the temperate phage λ which depends largely
on host functions for replication. Our tentative interpretation would then be
that the dam methylase is an integral component of the E. coli replication
system so that DNA replicated by this system is concomitantly being dam-
methylated. The T7 replication apparatus apparently does not require or allow
the participation of the dam product. This notion is in agreement with our
observation that plasmid DNA is being normally dam-methylated when repli-
cating in a T7-infected cell. A necessity of the dam function for normal E. coli
replication is evident from the manyfold physiological alterations observed in
dam-deficient mutants of E. coli, such as increased spontaneous mutability,
filamentation, high spontaneous induction of prophage λ, accumulation of
single-strand interruptions in chromosomal DNA, hyper-recombination pheno-
type and inviability of dam double mutants with polA and recA, recB or recC
[34,35].

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