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THE EFFECT OF DIFFERENTIAL METHYLATION BY *ESCHERICHIA COLI* OF PLASMID DNA AND PHAGE T7 AND λ DNA ON THE CLEAVAGE BY RESTRICTION ENDONUCLEASE *Mbo*I FROM *MORAXELLA BOVIS*

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Summary

The nucleotide sequence recognized and cleaved by the restriction endonuclease *Mbo*I is 5′↓GATC and is identical to the central tetranucleotide of the restriction sites of *Bam*HI and *Bgl*II. Experiments on the restriction of DNA from *Escherichia coli dam* and *dam*$^+$ confirm the notion that GATC sequences are adenosyl-methylated by the *dam* function of *E. coli* and thereby are made refractory to cleavage by *Mbo*I. On the basis of this observation the degree of *dam* methylation of various DNAs was examined by cleavage with *Mbo*I and other restriction endonucleases. In plasmid DNA essentially all of the GATC sequences are methylated by the *dam* function. The DNA of phage λ is only partially methylated. extended methylation is observed in the DNA of a substitution mutant of λ, λ gal_8bio_{256}, and in the λ derived plasmid, λdv93, which is completely methylated. In contrast, phage T7 DNA is not methylated by *dam*. A suppression of *dam* methylation of T7 DNA appears to act only in *cis* since plasmid DNA replicated in a T7-infected cell is completely methylated. The results are discussed with respect to the participation of the *dam* methylase in different replication systems.

Introduction

The restriction endonuclease *Mbo*I isolated from *Moraxella bovis* recognizes the nucleotide sequence $\begin{matrix}5'\downarrow\text{GATC}\ 3'\\ 3'\ \text{CTAG}\uparrow 5'\end{matrix}$ 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 *Bam*HI isolated from *Bacillus amyloliquefaciens* [2] and *Bgl*II isolated from *Bacillus globigii* [3]. *Bam*HI cleaves the sequence $\begin{matrix}5'C^{\downarrow}GATC\ G3'\\3'G\ CTAG_{\uparrow}C5'\end{matrix}$ and *Bgl*II the sequence $\begin{matrix}5'A^{\downarrow}GATC\ T3'\\3'T\ CTAG_{\uparrow}A5'\end{matrix}$ at the sites indicated by arrows. Therefore, *Mbo*I is expected to cut any *Bam*HI and *Bgl*II site and thus has been proposed to be useful for cloning of *Mbo*I-derived restriction fragments into *Bam*HI and *Bgl*II sites of appropriate vehicles [1,4]. However, when we compared the restriction pattern of *Mbo*I, *Bam*HI and *Bgl*II with the DNA of the composite plasmid pFE42, we observed instead of at least six cuts with *Mbo*I (three *Bam*HI sites and three *Bgl*II 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 *Mbo*I 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 *Mbo*I 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,* $r_K^-m_K^-$), 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* $r_B^-m_B^-$; 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 $3 \cdot 10^8$/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 λdv93 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. λ*c*I857 *Sam*7 was prepared by thermo-induction of *E. coli* strains lysogenic for this phage. λ *cI*857*gal*$_8$*bio*$_{256}$ [10,11] was obtained after infection of cells in liquid culture.

*Restriction endonucleases. Mbo*I was isolated according to Gelinas et al. [1], *Bgl*II according to Pirrotta [3], *Eco*RI according to Greene et al. [12], *Hind*II/III according to Smith and Wilcox [13], and *Sau*3AI according to Sussenbach et al. [14]. *Bam*HI was purchased from Miles Lab. (Elkhart, IN, U.S.A.) and *Pst*I and *Kpn*I were from Biolabs (Beverly, MA, U.S.A.). In some experiments an *Mbo*I preparation from Biolabs was also used.

DNA cleavage by restriction endonucleases. Reaction conditions for restriction with *Eco*RI were 100 mM Tris-HCl, pH 7.5, 50 mM NaCl, and 10 mM $MgCl_2$. Reaction conditions for *Bam*HI, *Bgl*II, *Kpn*I, and *Mbo*I were 6 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, and 6 mM β-mercaptoethanol. In some cases 150 mM NaCl plus bovine serum albumine (100 μg/ml) were included in reactions with *Mbo*I. Reaction mixtures for *Hind*II/III contained 6 mM Tris-HCl, pH 7.5, 6 mM $MgCl_2$, 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 *Sau*3AI were 6 mM Tris-HCl, pH 7.5, 15 mM $MgCl_2$, 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 × 11 cm) and run at 100 V and 40 mA for an appropriate time, generally about 2.5 h. Polyacrylamide gels (4%, 16 × 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 $CaCl_2$ 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 ($13.3 \cdot 10^6$ daltons; Eichenlaub, R., unpublished data) is composed of RSF2124 (ColE1 carrying TnA) [18] and mini-F [8]. This plasmid contains three *Bam*HI and three *Bgl*II restriction sites (Fig. 1; slots C and D). When pFE42 DNA was digested with *Mbo*I, 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 *Bam*HI and the *Bgl*II sites. In fact, significantly more than six *Mbo*I sites might reside in pFE42 DNA due to the smaller recognition sequence of *Mbo*I compared to *Bam*HI and *Bgl*II (see introduction). In order to verify that our preparation of *Mbo*I indeed displayed the published cleavage specificity DNA of phage T7 was also restricted with *Mbo*I (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 *Mbo*I we attempted to localize the *Mbo*I cleavage site on the plasmid DNA. For this purpose double restriction with *Mbo*I plus *Kpn*I was performed. Since this did not produce one specific extra fragment besides the two *Kpn*I fragments it was concluded that the sporadic *Mbo*I 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

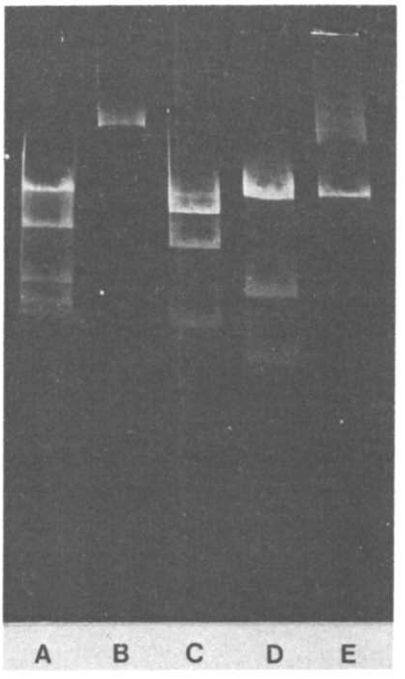

Fig. 1. Agarose gel electrophoresis of T7 DNA and pFE42 DNA treated with various restriction endonucleases. A, T7 DNA plus *Mbo*I; B, pFE42 DNA, no enzyme; C, pFE42 DNA plus *Bam*HI; D, pFE42 DNA plus *Bgl*II; E, pFE42 DNA plus *Mbo*I. The agarose concentration was 0.8%.

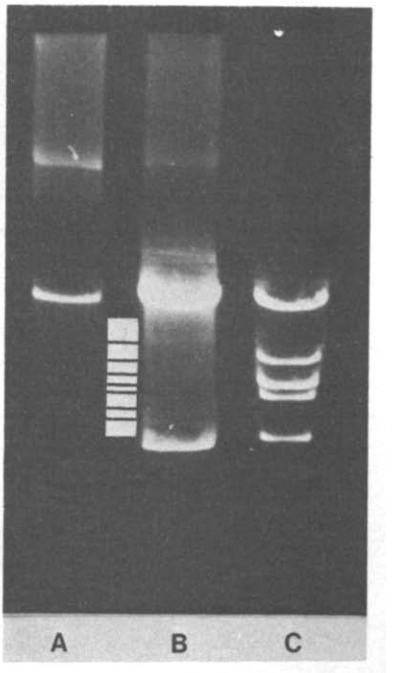

Fig. 2. Agarose gel electrophoresis of pFE42 DNA treated with *Mbo*I (A) and with *Mbo*I plus *Kpn*I (B). λ DNA treated with *Eco*RI (C) served as reference for the molecular weight of the fragments. The agarose concentration was 1.0%.

preparation of *Mbo*I, a notion which is further supported by the ability to ligate the plasmid DNA linearized by *Mbo*I (Dreiseikelmann, B., unpublished results). The same results were obtained with a commercial preparation of *Mbo*I. Thus, the production of a series of discrete fragments upon double restriction with *Mbo*I plus *Kpn*I is consistent with the assumption that *Mbo*I cleaves pFE42 DNA occasionally at one of several sites producing a population of discontinuously permuted linear molecules.

The fact that *Mbo*I cuts the plasmid pFE42 containing three *Bam*HI and three *Bgl*II sites barely once suggested that DNA methylation by the host cell may play a role in the restriction of DNA by *Mbo*I. An influence of the host specific modification system was excluded by the observation that pFE42 DNA isolated from *E. coli* SF8 $r_K^- m_K^-$ and *E. coli met* $r_B^- m_B^-$ was as refractive as wild type DNA to restriction by *Mbo*I. 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 *Mbo*I was dramatically changed (Fig. 3; slot C). *Mbo*I now cleaved pFE42 DNA into more than twenty fragments.

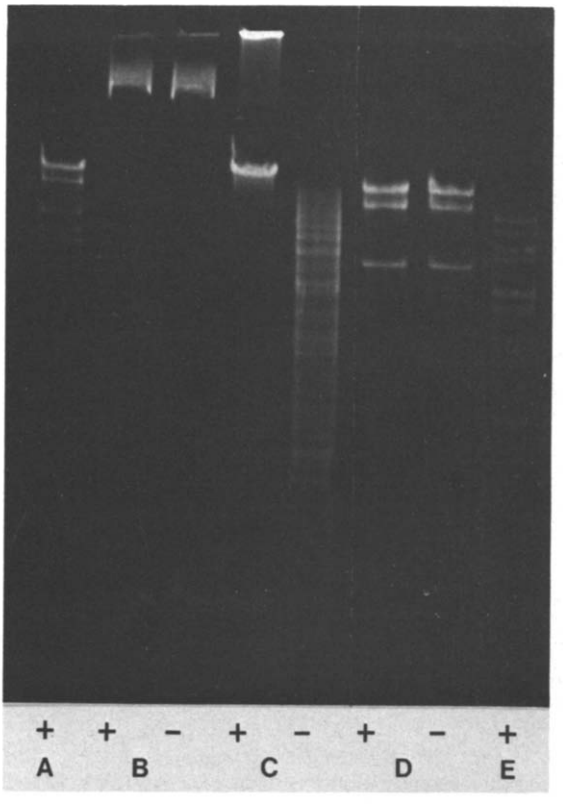

Fig. 3. Agarose gel electrophoresis of DNA replicated in *E. coli* wild type (+) and *E. coli dam* (—) and treated with various restriction endonucleases. A, T7 DNA plus *Mbo*I; B, pFE42 DNA, no enzyme; C, pFE42 DNA plus *Mbo*I; D, pFE42 DNA plus *Bam*HI; E, λ DNA plus *Hind*II/III. The agarose concentration in the gel was 0.8%.

Furthermore it was observed that the sporadic cleavage of *dam*-methylated plasmid DNA by the restriction endonuclease *Mbo*I is depending on the salt concentration. The linearization of pFE42 DNA by *Mbo*I 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 *Mbo*I in methylated DNA cannot be explained by the incomplete methylation of GATC sequences but may rather be due to some intrinsic activity of *Mbo*I which occasionally cleaves the methylated sequence at low salt concentration.

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

2. Restriction of λ DNA

*Mbo*I cleaves λ DNA into more than 50 fragments [1]. We compared the cleavage of λ DNA isolated from *E. coli dam*$^+$ by *Mbo*I and *Sau*3AI, a restriction endonuclease from *Staphylococcus aureus* which also recognizes and cleaves the nucleotide sequence 5'↓GATC [14]. Even with an excess of *Mbo*I only a partial fragmentation of λ DNA was obtained in *dam*$^+$ experiments whereas *Sau*3AI produced more than 50 fragments (Fig. 4; slots A$^+$ and B) in

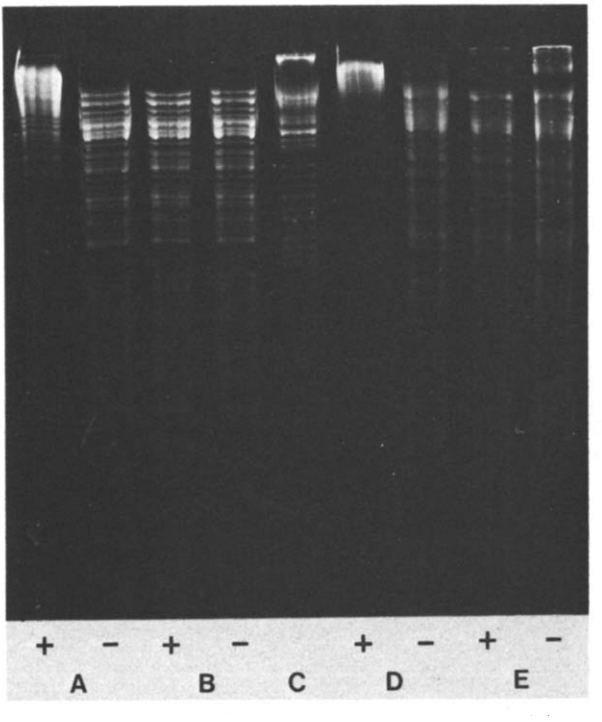

Fig. 4. Polyacrylamide gel electrophoresis of bacteriophage λ DNA replicated in *E. coli* wild type (+) and *E. coli* dam (—) treated with restriction endonucleases *Mbo*I and *Sau*3AI. A, λ$^+$ DNA plus *Mbo*I; B, λ$^+$ DNA plus *Sau*3AI; C, T7 DNA plus *Hind*II/III; D, $^{\lambda}gal_8bio_{256}$ DNA plus *Mbo*I; E, $^{\lambda}gal_8bio_{256}$ DNA plus *Sau*3AI. Polyacrylamide concentration was 4%, *Mbo*I digests were with 150 mM NaCl.

either *dam*$^+$ or *dam* experiments. With λ DNA from a *dam* strain these fragments were also produced by *Mbo*I (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 *Mbo*I, but *Sau*3AI 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 *Mbo*I indicating essentially complete methylation of GATC sequences (Fig. 5). When we tested the methylation of GATC in the DNA of a substition mutant of λ, λ*gal*$_8$*bio*$_{256}$ *cI*857, by restriction with *Mbo*I and *Sau*3AI, 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 *Mbo*I into seven fragments [19]. T7 DNA replicated in a *dam*$^+$ and a *dam* strain of *E. coli* gave identical restriction patterns with *Mbo*I which are indistinguishable from restriction patterns obtained with *Sau*3AI (data not shown). Thus, in T7 DNA the GATC sites are

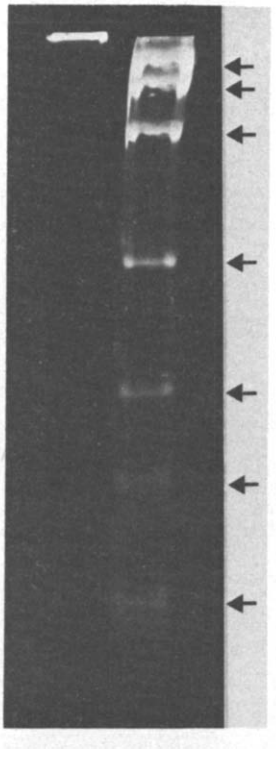

Fig. 5. Polyacrylamide gel electrophoresis of λdv93 DNA from an *E. coli dam*$^+$ strain treated with restriction endonucleases *Mbo*I and *Sau*3AI. A, λdv93 DNA plus *Mbo*I; B, λdv93 DNA plus *Sau*3AI (7 DNA bands). Polyacrylamide concentration was 6.3%.

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 *Mbo*I (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

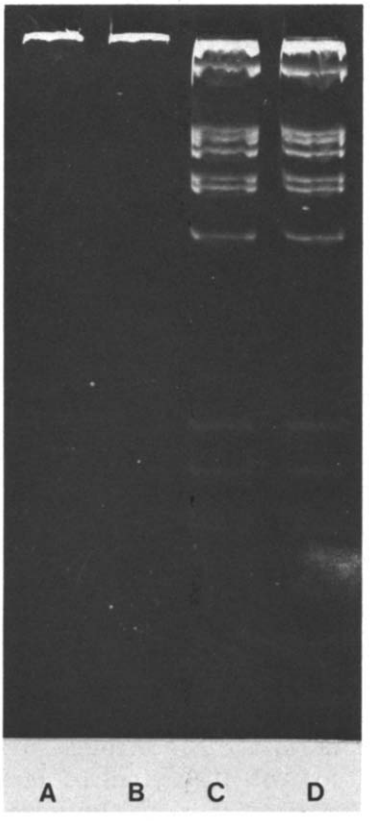

Fig. 6. Polyacrylamide gel electrophoresis of plasmid pBR322 DNA replicated in *E. coli* wild type and in *E. coli* wild type after infection with ultraviolet-inactivated T7 am28am29am233 treated with restriction endonucleases *Mbo*I and *Sau*3AI. A, pBR322 DNA from T7 infected *E. coli* dam^+ plus *Mbo*I; B, pBR322 DNA from normal *E. coli* dam^+; C, pBR322 DNA from T7 infected *E. coli* dam^+ plus *Sau*3AI; D, pBR322 DNA from normal *E. coli* dam^+ plus *Sau*3AI. Polyacrylamide concentration was 6.3%.

cells) chloramphenicol at 250 μg/ml was added to the culture in order to block further protein synthesis and one hour after infection [^{3}H]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 *Mbo*I and *Sau*3AI. As shown in Fig. 6 (slots A and E) pBR322 DNA from the T7 infected cells was as refractive to cleavage by *Mbo*I as DNA from uninfected cells, whereas *Sau*3AI cleaved both plasmid DNAs into discrete fragments (Fig. 6; slots C and D). This result shows that the plasmid DNA is still normally *dam*-methylated after replication in a T7 infected cell. It appears that in contrast to modification-specific methylation a suppression of the *dam* methylase activity does not occur.

Discussion

The restriction endonuclease *Mbo*I which recognizes the sequence 5'GATC [1] cleaves DNA only when it is not methylated by the *dam* function of *E. coli*. This interpretation of our results implies that GATC is one of the sequences which are recognized by the *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 *Mbo*I than DNA from λ grown in *E. coli* K12. It is also consonant with the finding by Pirrotta [3] that the adenine residues in GATC sequences in λ DNA are partially methylated by the *dam* function of *E. coli*. Recently Lacks and Greenberg [20] have made a similar statement on the basis of their finding that the restriction endonuclease *Dpn*II from *Diplococcus pneumoniae* cleaves the nucleotide sequence GATC when it is not methylated by the *dam* function. Thus, *Mbo*I and *Dpn*II are identical in cleavage specificity with respect to recognition sequence and methylation [20,27]. On the other hand, the enzymes *Bam*HI [2] and *Bgl*II [3] having GATC as the central tetranucleotide in their hexanucleotide recognition sequences cleave plasmid DNA irrespective of *dam* methylation (Fig. 1), a result that supports earlier observations obtained with partially methylated DNA [3,28].

Our results with plasmids pFE42, pBR322 and λdv93 show that in plasmid DNA essentially all GATC sites are normally methylated by the *dam* function in *E. coli* K12 and that an enzyme with identical specificity exsists in *E. coli* B. The DNA of phage λ wild type appears to be partially methylated by *dam*, a result which is in accord with published data [3,28]. The almost complete methylation of GATC sequences in λ gal_8bio_{256} DNA remains unexplained but it seems not unreasonable to assume that a gene in λ, lost through the gal_8bio_{256} substitutions and also absent in λdv93, controls the extent of *dam* methylation by the host cell. Further studies will be directed to attribute this control function to a specific gene of phage λ.

The total lack of GATC methylation in T7 DNA contrasts with the partial or complete methylation of λ 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 intracellular 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 transferase 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 replicating 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 phenotype and inviability of *dam* double mutants with *polA* and *recA*, *recB* or *recC* [34,35].

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