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**Methylation of
DNA**

Edited by T. A. Trautner

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Edited by Thomas A. Trautner

With 22 Figures



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Preface

Postreplicative methylation of bacterial DNA has long been known to be the molecular basis of "modification," which protects DNA against destruction by restriction endonucleases. More recently, another function of DNA methylation was found in *Escherichia coli*, where methylation is involved during DNA replication in the recognition of old and newly synthesized strands. The intensive search for new restriction enzymes during the 1970s yielded an enormous arsenal of such enzymes and revealed the ubiquitous distribution of restriction/modification systems in the bacterial kingdom without providing much information on the corresponding modification methyltransferases. However, it is obvious that DNA methyltransferases represent an ideal class of enzymes to those interested in protein/DNA interactions; these enzymes are at least as interesting as the restriction enzymes, with which they share the capacity to recognize and interact with specific sequences of DNA.

In recent years the interest in DNA methylation has been greatly stimulated by two discoveries: the correlation between gene expression and hypomethylation in eukaryotes and the convertability of DNA into its Z form through cytosine methylation. In fact, studies on DNA methylation are now being intensively performed in many laboratories. A description of the state of the art of DNA methylation has been the topic of two congresses: The Cologne Spring Meeting in 1981 organized by WALTER DOERFLER and an EMBO Workshop at Nethybridge in 1982 organized by ROGER ADAMS.

Due to the expansion of this field, it is no longer feasible for a review book to be encyclopedic in dealing with DNA methylation. However, this volume does contain a number of topical reviews which will, hopefully, be of broad interest. I wish to thank the contributors of the volume for their cooperation and the members of the staff of Springer-Verlag for their patient and meticulous editing of articles. I am grateful to my colleague

DR. URSULA GÜNTHER for many suggestions and discussions during the editing of this volume.

Berlin, January 1984

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B. SURI, V. NAGARAJA, and T.A. BICKLE

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1 Introduction

As first proposed by ARBER (1965), DNA restriction/modification systems (R/M systems) are mediated by endonucleases and DNA methylases that recognize the same DNA sequences. The endonuclease recognizes its specific sequence as a signal to cleave the DNA unless the sequence has been previously methylated by the modification enzyme. Chromosomal DNA from cells harboring the R/M system is normally methylated, and is thus not a substrate for the restriction enzyme. Foreign DNA lacking the specific methylation pattern and introduced into the cell by phage infection, conjugation, or transformation is the only known natural substrate for restriction. R/M systems can therefore be considered primitive prokaryotic analogues of the eukaryotic immune system.

A vast body of literature on the genetics and biochemistry of R/M systems has accumulated since they were first investigated 20 years ago (ARBER and DUSSOIX 1962), and it is now clear that R/M systems can be conveniently classified into three types (BOYER 1971; KAUC and PIEKAROWICZ 1978; NATHANS and SMITH 1975).

The most complicated of these are the type-I systems, which are mediated by complex, multifunctional enzymes and which were the first proteins shown to recognize specific DNA sequences. The restriction enzymes *EcoK* and *EcoB* from the *Escherichia coli* strains K12 and B are the two prototypes, and are still the only ones to have been studied in detail.

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The enzymes contain three nonidentical subunits coded by three contiguous genes on the *E. coli* chromosome. They require Mg^{+2} , ATP, and S-adenosylmethionine (AdoMet) for enzymatic activity and can function as restriction endonucleases (cleaving the DNA far from their recognition sequence), ATPases, or, as detailed later, modification methylases.

The type-II R/M systems include all those enzymes that have simple cofactor requirements (Mg^{+2} for restriction, AdoMet for modification) and simple subunit structures, and in which the restriction enzymes cut the DNA at, or close to, the sequences that they recognize. In the cases that have been investigated, separate enzymes catalyze restriction and modification. The type-II restriction enzymes are those that have found such wide application in recent years in molecular genetics.

Type-III R/M systems have been characterized more recently than the others, and represent an intermediate level of complexity. The restriction enzymes contain two nonidentical subunits and require ATP and Mg^{+2} for activity. AdoMet is not required for the endonuclease activity, as it is by type-I enzymes, although it stimulates the reaction. In the presence of both AdoMet and ATP, restriction and modification methylation are competing reactions. A separate modification enzyme which consists of the smaller of the two subunits of the restriction enzyme has also been isolated. Detailed reviews on restriction enzymes have recently been published (BICKLE 1982; MODRICH 1979; NATHANS and SMITH 1975; YUAN 1981; MODRICH and ROBERTS 1982). Here we will restrict ourselves to modification methylases, with emphasis on type-I systems.

2 Various Modification Methylases

Modification methylases methylate specific residues within their recognition sequences at either the 6N position of adenine or the 5C position of cytosine, depending on the system. They use AdoMet as the methyl donor and, once methylated, the DNA is resistant to cleavage by the corresponding restriction enzyme.

Although type-I restriction enzymes can modify appropriate substrate DNAs, separate modification methylases can be isolated. The modification enzymes from *E. coli* B (LAUTENBERGER and LINN 1972a) and *E. coli* K12 (this paper) have been characterized. They both contain two nonidentical subunits which are the same as two of the three subunits of the corresponding restriction enzymes.

The few type-II modification enzymes that have been characterized have proved to be relatively simple enzymes. One of them is discussed in detail elsewhere in this volume (U. GÜNTHER and T.A. TRAUTNER). In general, they require only a substrate DNA and AdoMet for activity (DUGAICZYK et al. 1974; RUBIN and MODRICH 1977). Although both the restriction enzymes and the modification methylases recognize the same DNA sequences, they seem to be physically and genetically unrelated.

A modification enzyme lacking endonuclease activity was isolated from the type-III *EcoP1* system as early as 1972 (BROCKES et al. 1972). Detailed studies on the type-III modification methylases, however, have only been done recently in this laboratory (HADI et al. 1983; IIDA et al. 1983). The modification methylase consists of one of the two subunits of the restriction enzyme, this subunit being responsible for recognition of the specific DNA sequences in both the restriction and the modification reactions. Unlike most modification methylases, this one requires Mg^{+2} as well as AdoMet for methylation. In contrast to modification by the restriction enzyme, ATP does not stimulate the reaction.

3 Features of Modification Sequences

A general feature of most recognition sequences for restriction and modification enzymes is that they have methylatable residues in both strands of the DNA. This is extremely important physiologically because DNA in which only one strand carries the specific methylation (hemimethylated DNA) is resistant to cleavage by the corresponding restriction enzyme. Since hemimethylated DNA is the normal product of DNA replication or repair, this feature of the reaction provides the mechanism whereby cells avoid restricting their own chromosomal DNA. For the type-I enzymes *EcoK* and *EcoB*, hemimethylated DNA is by far the preferred substrate for methylation, the reaction rate with this substrate being more than 100 times faster than with completely nonmodified DNA (Vovis et al. 1974; BURCKHARDT et al. 1981). In contrast, the type-II *EcoRI* methylase shows no preference for hemimethylated over nonmethylated sites (RUBIN and MODRICH 1977).

Some interesting exceptions can be found to the rule that fully modified R/M recognition sites are methylated in both strands of the DNA. All three of the type-III enzymes known at present methylate adenosyl residues in one strand of the DNA only. Two of the recognition sequences have no adenosyl residues in the other strand, and while the other – that of *HinIII* – has adenosyl residues in both strands, only one strand is methylated (BÄCHI et al. 1979; HADI et al. 1979; PIEKAROWICZ et al. 1981). In cells carrying these R/M systems, DNA replication generates one daughter DNA molecule containing the parental modification and a second daughter with the corresponding recognition site completely unmodified. These unmodified sites ought to be targets for restriction and we do not yet understand how cells containing type-III R/M systems avoid restricting their own chromosomal DNA.

Some of the type-II R/M recognition sequences are asymmetric and are most likely only methylatable in one strand. *MboII* is one such example. The recognition sequence of this enzyme is 5'-GAAGA-3' in one strand and 5'-TCTTC-3' in the other: one strand contains no cytosines and the other no adenosines (BROWN et al. 1980). Unless the *MboII*

modification methylase is capable of methylating both adenosyl and cytosyl residues, a property that has not yet been found for any DNA methylase, only one strand can be methylated in modified DNA (BÄCHI et al. 1979).

4 Type-I Modification Methylases

4.1 Genetic Organization

The type-I R/M systems of *E. coli* K12 and B are fully specified by three contiguous genes mapping at 98.5 min on the *E. coli* K12 chromosome (SAIN and MURRAY 1980; BACHMANN and LOW 1980). Genetic, physical, and immunochemical studies have revealed that these two systems are allelic and that they also share homology with several R/M systems from different *Salmonella* species (BOYER and ROULLAND-DUSSOIX 1969; BULLAS et al. 1980; HUBACEK and GLOVER 1970; MURRAY et al. 1982). Recent studies have shown that another system which, in physiological studies, behaves as though it were type I (LARK and ARBER 1970) – the *EcoA* system of *E. coli* 15T⁻ (ARBER and WAUTERS-WILLEMS 1970) – in fact shows no homology on the DNA level with the classical type-I systems. Moreover, antibodies prepared against *EcoK* do not cross-react with extracts from cells expressing *EcoA* (MURRAY et al. 1982).

The three genes involved in the *E. coli* K12 and B R/M systems are called *hsdR*, *hsdM*, and *hsdS* (*hsd*, for “host specificity for DNA”). Strains carrying mutations in the *hsdR* gene are defective in restriction, those with mutated *hsdS* genes lack both restriction and modification, and *hsdM* mutants can be isolated only in strains that already carry a mutation in one of the other two genes. These phenotypes, together with the results of complementation analysis, led to the suggestion that all three gene products are necessary for restriction, while the *hsdM* and *hsdS* gene products suffice for modification (BOYER and ROULLAND-DUSSOIX 1969; HUBACEK and GLOVER 1970). The *hsdS* gene product would be responsible for recognizing the specific sequences in DNA in both restriction and modification, the *hsdM* gene product would catalyze modification, and both *hsdM* and *hsdR* would be required for restriction.

The *hsd* locus of *E. coli* K12 has been cloned in bacteriophage λ and a deletion analysis of this cloned DNA has revealed the gene order to be *hsdR*-*hsdM*-*hsdS*. All three genes are transcribed in the same direction but from two promoters, one of them upstream of *hsdR* and the other between *hsdR* and *hsdM* (SAIN and MURRAY 1980). This organization of the *hsd* locus into two transcriptional units allows the genes which code for the subunits of the modification enzyme to be transcribed independently of the *hsdR* gene, and this may be of considerable physiological importance.

4.2 Enzyme Structure

Before the discovery that the type-I restriction enzymes, which contain the products of all three *hsd* genes, had modification activity (VOVIS et al. 1974), it was predicted that the type-I modification methylases ought to contain the products of the *hsdS* and *hsdM* genes only. Such an enzyme was isolated 10 years ago from *E. coli* B (LAUTENBERGER and LINN 1972a). The enzyme contained two nonidentical subunits with molecular weights of 60000 and 55000 which comigrated on polyacrylamide-SDS gels with the two smaller subunits of the *EcoB* restriction enzyme (LAUTENBERGER and LINN 1972b). The stoichiometry of the subunits in the enzyme was variable and changed upon storage. However, freshly purified enzyme had equimolar amounts of the two subunits.

Quite recently we isolated a modification methylase from an *E. coli* K12 strain in which the *hsd* genes are transcribed from the strong bacteriophage λ promoter, P_L (MURRAY et al. 1982). This enzyme also contains the *hsdM* and *S* gene products in equimolar amounts. Its reaction characteristics will be described in the next section.

4.3 Kinetics of Methylation

We have compared the methylation properties of the *EcoK* restriction enzyme with those of the modification methylase described in the preceding section. With unmodified DNA, the restriction enzyme is considerably less effective than the methylase and is partially inhibited by the presence of ATP. The methylase is unaffected by the presence of ATP (Fig. 1). For both enzymes the overall reaction is of the first order, indicating that the rate-limiting step follows the formation of the enzyme-DNA complex. The first-order rate constants were $6 \times 10^{-5} \text{ s}^{-1}$ for the methylase and $2 \times 10^{-5} \text{ s}^{-1}$ for the restriction enzyme (without ATP). The apparent inhibition by ATP of the reaction with the restriction enzyme may be due to the fact that ATP stimulates a conformational change in the enzyme, which was shown to result in the release of AdoMet bound to it (BICKLE et al. 1978). Results similar to these have been obtained for *EcoB* and the B-specific methylase (HABERMAN et al. 1972; LAUTENBERGER and LINN 1972a).

The kinetics of modification of hemimethylated pBR322 DNA (prepared by hybridizing modified with nonmodified DNA) is shown in Fig. 2. The reaction is now much faster with both enzymes; (the time scale of Fig. 2 is minutes, whereas that of Fig. 1 is hours). For the restriction enzyme, the results are similar to those previously reported for *EcoB* and *EcoK* (VOVIS et al. 1974; BURCKHARDT et al. 1981). The principal difference is that the stimulation of the reaction by ATP and Mg^{+2} reported earlier is now seen to be composed of about a 1.6-fold stimulation by Mg^{+2} and a further twofold stimulation by ATP. The modification enzyme is slightly more efficient than the restriction enzyme and is unaffected by the presence of ATP. For both enzymes, the reaction is again

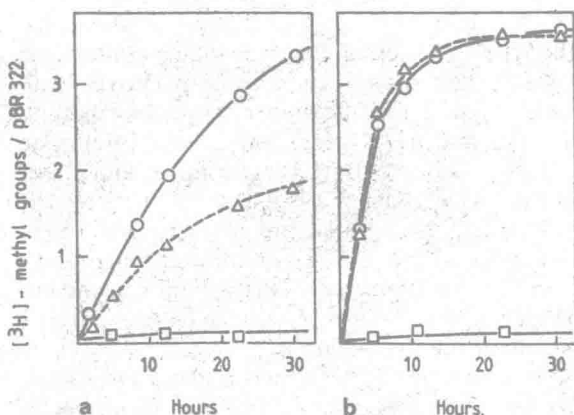


Fig. 1a, b. Methylation of unmodified DNA, a by the *EcoK* restriction enzyme, b by the modification enzyme. The reactions contained 100 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid, pH 6.7; 0.25 mM EDTA; 14 mM 2-mercaptoethanol; and 3.6 μ M ³H-AdoMet (Amersham, 74 Ci/mmol). When present, Mg²⁺ was at a concentration of 6.6 mM and ATP at 1 mM. The DNA was pBR322, linearized by cleavage with *SalI*, and was used at a concentration of 7.7 μ g/ml. The plasmid pBR322 has two *EcoK* recognition sites. Incubations were at 37° C. Samples were removed at the indicated times, the reaction stopped by the addition of phenol, and the DNA separated from low-molecular-weight radioactive material by gel filtration through small Bio-Gel A 0.5 M columns. The DNA-containing fractions were counted in Instagel (Packard) with an efficiency of 50%. □—□, modified DNA, +ATP; Δ—Δ, nonmodified DNA, +ATP; ○—○, nonmodified DNA, -ATP

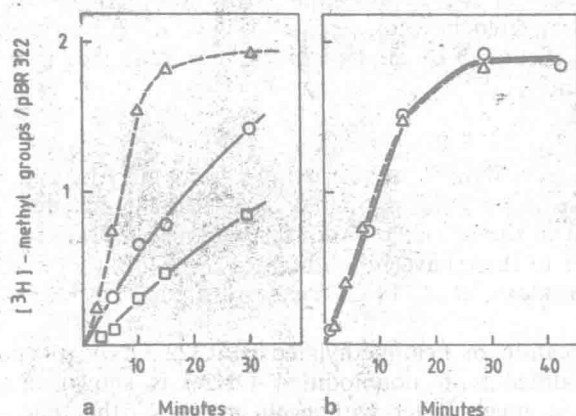


Fig. 2a, b. Methylation of heteroduplex DNA, a by the *EcoK* restriction enzyme; b by the modification enzyme. The reaction conditions were the same as those described in Fig. 1, except that the reaction mixtures were preheated to 37° for 10 min before adding enzyme, and the DNA concentration was 15 μ g/ml. The DNA was prepared by heating equal amounts of modified and nonmodified *SalI*-linearized pBR322 DNA in a heat-sealed glass capillary at 100° C to melt the DNA; then reannealing was done by allowing the temperature to drop to 55° C over 90 min. The DNA should contain 50% heteroduplex molecules and 25% each of homoduplex modified and nonmodified molecules. The latter are essentially not methylated in the 40 min that this experiment takes (see Fig. 1). Δ—Δ, +ATP, +MgCl₂; ○—○, -ATP, +MgCl₂; □—□, -ATP, -MgCl₂

of the first order and the first-order rate constants are $2 \times 10^{-3} \text{ s}^{-1}$ for the methylase and $3 \times 10^{-3} \text{ s}^{-1}$ for the restriction enzyme in the presence of both Mg^{+2} and ATP.

A comparison of the rate constants for the modification of nonmodified versus hemimethylated DNA shows that the restriction enzyme methylates the latter some 150 times faster than the former while the corresponding figure for the methylase is 35 times. Under all incubation conditions the methylase is more efficient than the restriction enzyme, especially with nonmodified DNA.

5 Physiological Implications

The most obvious question that arises from these studies on type-I R/M systems is: Why should there be a separate modification methylase when the restriction enzyme present in the same cells is itself an efficient modification methylase? One trivial possibility would be that the methylases are artifacts of the purification procedure. If this were so, it is difficult to see why the *hsdS* and *hsdM* genes, which are the structural genes for the subunits of the methylase, should be organized as a single transcriptional unit, while the *hsdR* gene is transcribed from a separate promoter (SAIN and MURRAY 1980). This arrangement could allow an independent regulation of the production of the restriction and modification enzymes; whether such a regulation occurs is not known.

It might be argued that most bacterial cells are never challenged by foreign DNA, and thus never use the endonuclease activity of their restriction enzymes. A relatively low level of restriction activity may therefore suffice to provide protection to the cells. On the other hand, modification activity is continually required, because DNA replication and repair are constantly generating hemimethylated DNA that must be modified before the next round of replication creates unmodified sites that would be a target for restriction. The relative amounts of restriction and modification enzyme have never been quantitated in *E. coli* strains K12 or B. The availability of antibodies means that such a quantitation is now feasible, and it would be interesting to see whether there is an excess of the modification enzyme.

Very recently, we have purified the enzymes involved in the *EcoA* restriction-modification system. As expected from the earlier studies (MURRAY et al. 1982) described above, these enzymes show some interesting differences from the classic type-I enzymes. For the present argument the most important difference is that the basic enzyme is a modification methylase containing two subunits of approximately the same molecular weights as the classic type-I *hsdS* and *hsdM* gene products. A protein of about the same molecular weight as a classic *hsdR* subunit can be purified separately. This protein has no detectable enzymatic activity by itself; however, when added to the modification methylase in the presence of a substrate DNA the methylase is converted to a restriction endonuclease

(SURI and BICKLE, unpublished results). Thus, cells carrying the *EcoA* restriction-modification system always contain a modification methylase and probably assemble an active restriction endonuclease only when the cell is challenged by foreign DNA.

The concentrations of the different cofactors, in particular that of ATP, may play a crucial role in regulating restriction and modification *in vivo*. The restriction enzyme shows an absolute requirement for ATP in the restriction reaction and is stimulated by it in the modification reaction (with hemimethylated substrates). The modification enzyme, on the other hand, is unaffected by the presence of ATP. This may ensure that newly replicated or repaired DNA is efficiently methylated even when ATP levels in the cell are low. It is worth noting that one condition in which ATP concentrations are expected to be low is following restriction, when the restriction enzyme has transformed itself into a potent ATP hydrolase.

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