Proc. Natl. Acad. Sci. USA
Vol. 81, pp. 3278–3282, June 1984
Biochemistry

DNA methylation pattern is determined by the intracellular level of the methylase

(bacteriophage λ DNA/pBR322/*Escherichia coli dam* or *mec* methylation)

MOSHE SZYF*, KADMIELLA AVRAHAM-HAETZNI*, ARIEH REIFMAN*, JOSEPH SHLOMAI†, FEIGE KAPLAN*, AMOS OPPENHEIM‡, AND AHARON RAZIN*

Departments of *Cellular Biochemistry, ‡Molecular Genetics, and †Parasitology, The Hebrew University–Hadassah Medical School, Jerusalem, Israel 91 010

Communicated by Robert L. Sinsheimer, January 23, 1984

ABSTRACT **Extrachromosomal plasmid DNA is transiently undermethylated in *Escherichia coli* during amplification in the presence of chloramphenicol. In addition, undermethylation of phage λ DNA was observed after thermal induction of a λcI857 lysogen while the integrated λ phage DNA was found to be fully methylated. These methylation pattern changes occur under conditions (extensive replication) in which the intracellular methylase level becomes limiting. In an *E. coli* strain that harbors a plasmid that carries the *dam* methylase gene and therefore overproduces *dam* methylase, there is no undermethylation of *dam* sites in either of the extrachromosomal DNAs. The sites that are methylated by the *mec* methylase in both plasmid and λ phage DNAs were undermethylated in the *dam* overproducer as well. These results indicate that the intracellular level of the *E. coli* methylase determines the DNA methylation pattern.**

Partial undermethylation of CpG-containing sequences in eukaryotic DNA forms a discrete tissue-specific pattern of methylation. In vertebrate this DNA methylation pattern shows a remarkable correlation with the activity of tissue-specific genes (1). Several studies in mammalian cells clearly demonstrate that an established pattern can be clonally inherited for many cell generations (2, 3). However, the mechanism by which such a methylation pattern is formed is still obscure. It was previously suggested that in *Escherichia coli* DNA, as in eukaryotes, few unmethylated G-A-T-C sites on a high methylation background may serve as hot spots to nuclease activity, which might play a role in biological processes such as genetic recombination, replication, and mismatch repair (4–7). Therefore, it is possible that a methylation pattern of prokaryotic DNA may also be of biological significance.

Although *E. coli* DNA is methylated essentially in all G-A-T-C sites (adenine residue methylated by the *dam* methylase) and C-C-$^{A}_{T}$-G-G sites [inner cytosine residue methylated by the *mec* methylase (8–10)], the DNA of the bacteriophage λ that propagates in these cells is only partially methylated (11). This phenomenon prompted us to study the pattern of methylation of the λ DNA and to examine the possibility that the rate of replication and intracellular level of the methylase plays a role in forming this pattern. In the present study, we have analyzed the changes in the pattern of methylation of λ DNA and the DNA of the plasmid pBR322 during their replication. The effect of the intracellular level of the *dam* methylase on these methylation patterns was examined by analysis of the methylation patterns in a *dam* overproducer strain (12). The results clearly demonstrate that the methylase level in *E. coli* cells is limiting; therefore, the methylase level affects the methylation patterns of actively replicating extrachromosomal DNA.

MATERIALS AND METHODS

Bacterial Strains. The following bacterial strains were used in this study: *E. coli* JC5183 cells harboring the pBR322 plasmid; *E. coli* JC4583/pGG503 (*dam* methylase overproducer) harboring the plasmid pGG503 composed of a *Pst* I fragment containing the *dam* gene inserted into a *Pst* I site of pBR322 (12); λ lysogen λcI857cro$_{27}$P$_3$ and λcI857susO$_{29}$ (prophages incapable of DNA replication); SA500su$^-$ host lysogenic for λgtλ*csus*EsusWsusS prophage (defective in their packaging function) and λcI857Sam7/pGG503 (*dam* methylase overproducer).

RESULTS

As a first step towards the elucidation of the mechanisms underlying λ DNA hypomethylation, we analyzed the pattern of methylation of mature λ phage DNA (isolated from a heat-inducible lysogen of *E. coli* λcI857S7). The large number of methylatable sites in bacteriophage λ DNA (116 G-A-T-C sites and 71 C-C-$^{A}_{T}$-G-G sites) makes the analysis of the state of methylation of all of these sites in the entire λ genome very complicated. The λ DNA was therefore cleaved by *Eco*RI and the analysis was performed separately on the individual *Eco*RI fragments (13). Two different approaches were taken to analyze the distribution of 5-methylcytosine residues along the λ genome. The first approach was to analyze the extent of methylation of the internal cytosine in the C-C-$^{A}_{T}$-G-G sites. Each fragment was cleaved with the restriction enzyme *Bst*NI (which cleaves in $\begin{matrix}\text{C-C} \downarrow \text{A-G-G} \\ \text{G-G-T} \uparrow \text{C-C}\end{matrix}$ at the arrow, whether or not the internal cytosine residue is methylated) and the resulting sticky ends were filled with [α-^{32}P]dTTP by using *E. coli* DNA polymerase I large fragment. The labeled DNA was subjected to nearest neighbor analysis and the extent of methylation of the internal cytosine residue was calculated (14). The extent of methylation of C-C-T-G-G sequences was 32%, 59%, 43%, and 25% for *Eco*RI fragments 1, 2, 3 + 3a, and 4, respectively (see *Eco*RI restriction map in Fig. 1). These results imply that the distribution of methylated C-C-T-G-G sequences is not uniform throughout the λ phage genome. The second approach was to digest each λ *Eco*RI fragment with *Bst*NI and in parallel with *Eco*RII (which cleaves ↓C-C-$^{A}_{T}$-G-G sites at the arrow exclusively when the inner cytosine residue is not methylated on both strands). The *Bst*NI fragments were labeled as described above. The *Eco*RII fragments were labeled by filling the sticky ends with [α-^{32}P]dCTP. The labeled *Bst*NI and *Eco*RII fragments were size fractionated by gel electrophoresis and the gel was autoradiographed (Fig. 1). The *Bst*NI fragments (lanes 2, 4, 6, and 8) represent the distribution of all C-C-$^{A}_{T}$-G-G sites, whereas the parallel

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: kb, kilobase(s); CAM, chloramphenicol.

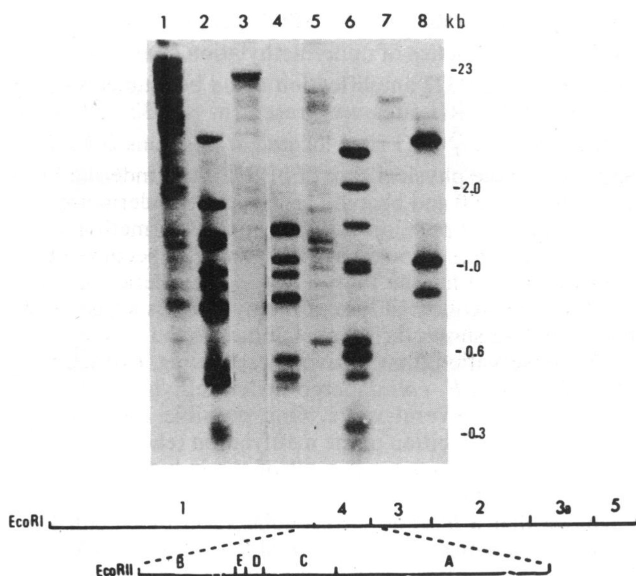

FIG. 1. The pattern of methylation of λ phage DNA. Mature λ phage DNA has been digested with *Eco*RI. Isolated *Eco*RI fragments were treated with *Bst*NI or *Eco*RII. The resulting sticky ends were ^{32}P-labeled by end filling (see text). The labeled DNA was size fractionated by gel electrophoresis. The gels were dried and autoradiographed. Each pair of lanes represents analysis of another *Eco*RI fragment (from left to right, RI1, RI2, RI3 + 3a, and RI4; see map). *Eco*RII digests, odd-numbered lanes; *Bst*NI digests, even-numbered lanes. A restriction map of the entire genome cut with *Eco*RI is shown; the expanded band is a restriction map of RI4 with *Eco*RII. kb, Kilobases.

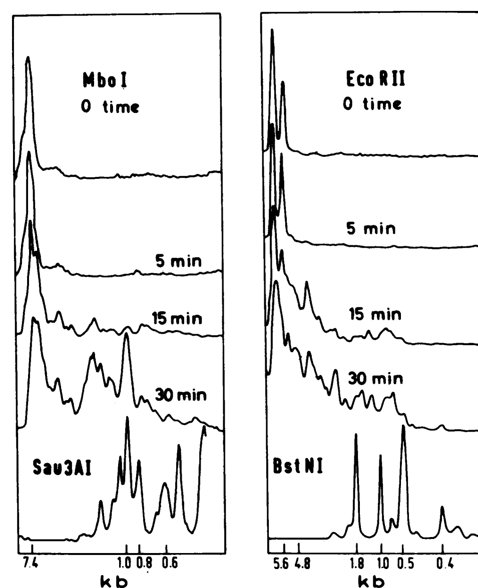

FIG. 2. Analysis of the extent of methylation of replicating λ phage DNA. *E. coli* cells lysogenic for λcI857Sam7 prophage were grown in rich medium (L broth) at 30°C to mid-logarithmic phase. At this stage the incubation temperature was shifted to 42°C. Aliquots were removed from the culture at 0, 5, 15, and 30 min after the temperature shift. Cellular DNA was purified and digested with *Eco*RI together with one of the restriction enzymes *Sau*3A1, *Mbo* I, *Bst*NI, and *Eco*RII. DNA fragments were fractionated by 1% agarose gel electrophoresis, blotted to nitrocellulose sheets, and hybridized to ^{32}P-labeled λRI-2 fragment for the *Mbo* I digest and λRI-3 fragment for the *Eco*RII digest. The autoradiograms were scanned by using a Helena Laboratories (Beaumont, TX) Quick Scan. The range was adjusted to accommodate the peak of undigested DNA. Note that at time 0, in the *Eco*RII panel the smaller band reflects contamination of the λRI-3 probe with λRI-4.

*Eco*RII fragments (lanes 1, 3, 5, and 7) monitor nonmethylated sites.

A similar analysis has been performed with respect to G-A-T-C sites. As these sites are more abundant than C-C-$\frac{A}{T}$-G-G sequences, the pattern is more complex and a detailed analysis was not attempted. The overall picture, however, is of a more pronounced undermethylation of the G-A-T-C sites (data not shown). The fact that methylated sites are found along the entire λ genome makes the possibility very unlikely that the mature λ DNA is undermethylated because the DNA is packaged into the coat before a processive methylase methylates the entire genome.

The hypomethylation of mature λ phage DNA may have resulted by inhibition of the methylation reaction caused by some properties of the λ phage, or, alternately, it may reflect the high rate of replication of λ DNA. To test these possibilities the following experiment was devised. Cells lysogenic for λcI857Sam7 prophage were induced to undergo a lytic cycle by a temperature shift to 42°C. The cells in which the λ DNA was replicating were harvested at various time intervals after induction and the DNA was purified. The DNAs were digested with the two isoschizomer pairs *Mbo* I/*Sau*3A1 and *Eco*RII/*Bst*NI. The digested samples were electrophoresed, blotted, and hybridized to a ^{32}P-labeled λ DNA probe and the autoradiograms were scanned. The results of such an experiment are presented in Fig. 2. At time 0, when the λ genome is integrated into the cell chromosome, both G-A-T-C and C-C-$\frac{A}{T}$-G-G sites were highly methylated, as judged by the resistance of the DNA to *Mbo* I and *Eco*RII treatment. Five minutes after the temperature shift, the λ DNA was already excised and replicating (see Fig. 4*A*); however, it was still methylated. Pronounced undermethylation was observed around 15–30 min after induction. In a more detailed kinetic experiment, hypomethylation of λ DNA was first observed between 7 and 9 min after induction. However, extensive undermethylation was detected 3 hr after induction in a λ strain defective in its lysis function (λcI857Sam7) (data not shown).

To study the rate of replication of λ DNA after induction, aliquots were removed from cell cultures at various time periods after induction, dot blotted as described (15), and hybridized to a ^{32}P-labeled λ DNA probe. The λ DNA copy number per cell was calculated by determining the intensity of radioactive spots relative to the intensity of the 0 time spot (1 λ copy of prophage DNA) (see Fig. 4*A*). This analysis demonstrated that λ DNA proliferation is initiated immediately after induction and proceeds at a rapid rate thereafter, reaching a level of 15–20 copies per cell at 30 min and 200–300 copies per cell at 3 hr. A comparison of the kinetics of undermethylation with the parallel kinetics of replication reveals that λ DNA is fully methylated as long as the copy number of λ DNA remains low (7 min). Amplification of λ DNA is accompanied by substantial undermethylation of the DNA.

Control experiments were carried out by using the strains lysogenic for λ mutants incapable of DNA replication (λcI857cro$_{27}$P$_3$ and λcI857susO$_{29}$). Methylation of these λ DNAs was analyzed as described above at 0, 15, and 30 min after induction. The λ DNAs were fully methylated even at 30 min after induction. It has been argued before that undermethylation may have resulted from rapid packaging of the DNA into phage particles (11). To test this possibility, we analyzed the extent of methylation of the DNA of λ mutants defective in their packaging function. After induction of SA500su$^-$ host lysogenic for λgtλ*csus*EsusWsusS prophage, λ DNA was found to be undermethylated to a similar extent as the wild-type control (data not shown).

It has been shown previously that plasmids prepared from an overnight *E. coli* culture are methylated in essentially all C-C-$\frac{A}{T}$-G-G and G-A-T-C sites (8). As plasmid replication is completed long before the isolation of the DNA, it is possible that plasmid DNA undergoes undermethylation during its active replication, as demonstrated above for λ DNA. To test this possibility, *E. coli* cells harboring pBR322 were harvested at various time intervals after the addition of chloramphenicol (CAM) and plasmid DNA was isolated. Plasmid DNA samples were digested with *Mbo* I, *Sau*3A1, *Bst*NI, and *Eco*RII. The restricted DNA samples were electrophoresed, blotted, and hybridized to a ^{32}P-labeled pBR322 probe. The autoradiograms of this blot were scanned and the results presented in Fig. 3. These analyses revealed that actively replicating pBR322 molecules are undermethylated at G-A-T-C sites and at C-C-$\frac{A}{T}$-G-G sites. This undermethylation, which was almost undetected before the addition of CAM, gradually increased, reaching its peak 3 hr after CAM addition. From this point on, unmethylated sites were methylated *de novo*, resulting in almost 100% methylation by 16 hr. It was of interest to determine whether the changes in the methylation pattern during plasmid DNA replication correlate with the rate of replication. To this end, the rate of pBR322 amplification was analyzed by dot blotting DNA that was isolated from *E. coli* cells harboring the plasmid at various time periods after the addition of CAM and hybridization with a ^{32}P-labeled pBR322 probe. The relative pBR322 copy number was determined by measuring the intensity of the radioactive spots relative to the intensity of the 0 time spot (≈50 copies per cell) (Fig. 4*B*). pBR322 DNA was maximally undermethylated at 2–3 hr (Fig. 3), coinciding with the peak of the rate of amplification (Fig. 4*B*). The decrease in the rate of amplification was accompanied by an almost full restoration of all methyl groups 16 hr after CAM addition. The kinetics of undermethylation of all C-C-$\frac{A}{T}$-G-G sites during pBR322 amplification could be studied in detail as only six *Eco*RII sites are present in pBR322 DNA. Although the C-C-$\frac{A}{T}$-G-G sites located at positions 2636, 2626, and 2502 on the physical map of pBR322 are undermethylated even at time 0 and become prominently undermethylated at 2 hr, the site at position 1059 becomes undermethylated by 1 hr and the sites at positions 1442 and 131 become undermethylated by 3 hr (see Figs. 3 and 6). This series of events resulting in a sequential loss of methyl groups suggests that the methylase shows different affinities to the various sites.

The observation that actively replicating extrachromosomal DNAs in *E. coli* undergo undermethylation could be interpreted in several ways. One possible interpretation could involve inhibition of the methylation reaction or depletion of the methylase as a result of the induction (CAM or temperature shift). However, a more likely explanation might be that a limiting intracellular level of *E. coli* DNA methylases on one hand and a rapid formation of methylatable sites on the other hand may result in undermethylation. Based on data reported for the *dam* enzyme in the overpro-

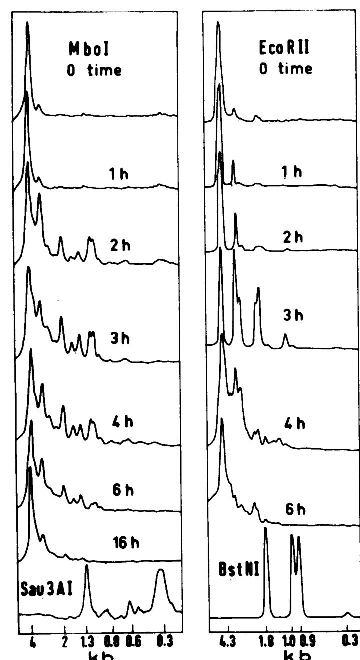

FIG. 3. Analysis of the extent of methylation of replicating pBR322 DNA. *E. coli* cells (5183 cells) harboring the pBR322 plasmid were grown and treated with CAM (100 μg/ml). Aliquots were removed at 0, 1, 2, 3, 4, 6, and 16 hr after addition of CAM. Total DNA was isolated from the cells and restricted with *Eco*RI to linearize the plasmid and with *Mbo* I, *Sau*3A1, *Eco*RII, and *Bst*NI. The restricted DNA samples were electrophoresed, blotted, and hybridized to a ^{32}P-labeled pBR322 probe. Autoradiograms were scanned as described in the legend to Fig. 2. The 4.3-kb band represents uncut linearized pBR322 DNA.

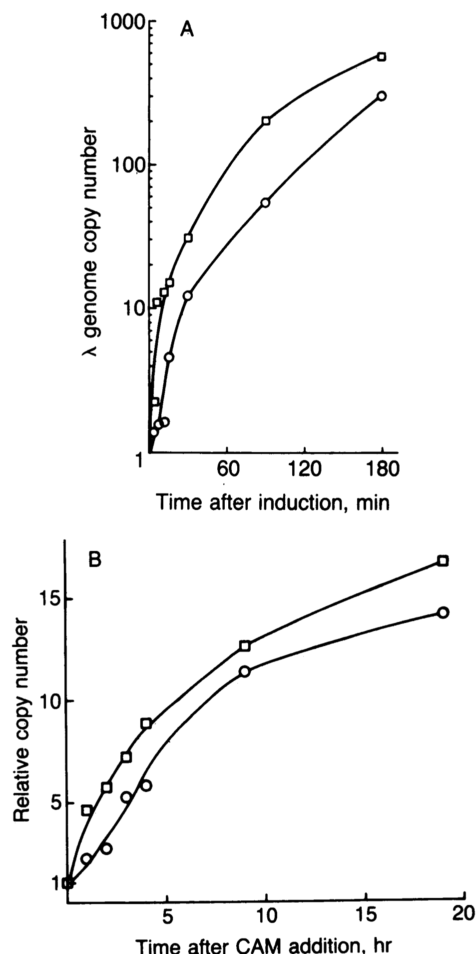

FIG. 4. The kinetics of λ phage and pBR322 DNA replication. (*A*) *E. coli* cells lysogenic for λcI857Sam7 (○) and the same cells transformed with pGG503 (□) were grown and induced by a temperature shift to 42°C, as described in the legend to Fig. 2. Aliquots of 10 μl containing ≈10^6 cells were removed at 0, 5, 7, 9, 15, 30, 90, and 180 min after induction. The λ copy number per cell was determined. (*B*) *E. coli* cells 5183/pBR322 (○) and JC4583/pGG503 (□) were grown to mid-logarithmic phase and treated with CAM as in Fig. 3. Aliquots of 10 μl containing 5 × 10^6 cells were removed at 0, 1, 2, 3, 4, 5, 9, and 16 hr after addition of CAM. The relative pBR322 copy number per cell was determined.

Table 1. Methylase activity in wild-type and *dam* overproducer *E. coli* strains

Methylation	Wild type (5183) Without CAM	Wild type (5183) With CAM	Overproducer (JC4583/pGG503) Without CAM	Overproducer (JC4583/pGG503) With CAM	Wild type (λcI857Sam7) 30°C	Wild type (λcI857Sam7) 42°C	Overproducer (λcI857Sam7/pGG503) 30°C	Overproducer (λcI857Sam7/pGG503) 42°C
mec	73	55	48	12	73	70	98	82
dam	34	39	498	212	27	28	594	374

Methylase specific activity, units/mg of protein.

E. coli 5183 cells harboring the pBR322 plasmid or *E. coli* JC4583/pGG503 cells (*dam* methylase overproducer) were grown to mid-logarithmic phase and treated with CAM. Cells were harvested before and 3 hr after the addition of CAM and were saved for the preparation of methylase. *E. coli* cells lysogenic for λcI857Sam7 prophage and the same cells transformed with the plasmid pGG503 were grown at 30°C and induced to the lytic phase by a temperature shift (30°C → 42°C). Cells were harvested before and 30 min after the temperature shift. Methylase activities (*dam* and *mec*) in these crude extracts were assayed as described (14) by using calf thymus DNA digested with *Bst*NI as substrate for the *dam* methylation reaction and DNA cleaved with *Mbo* I as substrate for the *mec* methylation reaction. One unit of methylase activity = 1 nmol of CH_3 incorporated per hr under standard assay conditions.

ducer strain (JC4583/pGG503) (12), 450 methylase molecules were estimated in such an overproducer cell. As this *E. coli* strain overproduces the enzyme 10- to 20-fold, as compared with wild-type *E. coli* (see Table 1; ref. 13), the number of *dam* methylase molecules per *E. coli* wild-type cell must be in the range of 20–40. Given the number of methylase molecules per cell, the *in vivo* rate of replication fork movement (16), the frequency of G-A-T-C sites in *E. coli* DNA (8), and the turnover number of the enzyme (12), a methylation quotient could be calculated. The methylation quotient Q designates the ratio of methylation capacity (number of methylase molecules times turnover number) over newly replicated methylatable sites. By this calculation, a methylation quotient of about 1 is obtained.§ This calculated methylation quotient indicates that the intracellular level of the *dam* methylase is limiting.

The argument that the low methylase activity in *E. coli* cells results in the demethylation of λ and pBR322 DNA gained further support from kinetic experiments in a *dam* overproducer strain. The λ lysogen (cI857Sam7) has been transformed with the pGG503 plasmid. The methylation of G-A-T-C sites in λ DNA after a temperature shift and in pGG503 DNA after addition of CAM has been analyzed as described above. Undermethylation of these sites was observed neither in the λ DNA nor in pGG503 DNA (Fig. 5). As an internal control, the methylation of C-C-$\frac{A}{T}$-G-G sites has been analyzed in the two DNAs. The λ DNA and pBR322 DNA were not affected by the presence of pGG503 in the cells and underwent demethylation as expected (Fig. 5).

The *dam* methylase and *mec* methylase activities in the crude extracts were estimated by eliminating the C-C-$\frac{A}{T}$-G-G sites in the substrate (treatment of the calf thymus DNA with *Bst*NI) and eliminating the G-A-T-C sites (*Mbo* I digestion of the substrate DNA). The results of these methylase assays are summarized in Table 1. Two conclusions based on these results can be made. (*i*) The temperature shift or CAM treatment has almost no effect on the methylase activities. (*ii*) The level of *dam* methylase activity in the overproducer cells is 10- to 20-fold higher than the activity in the wild-type *E. coli*, whereas, as expected, no change in the *mec* methylase activity is observed.

A kinetic analysis of the rate of replication of λ phage and pBR322 DNA in wild-type and overproducer cells revealed that the overproducer has no effect on the rate of replication as well (Fig. 4). This observation ruled out the possibility that a decrease in the rate of replication contributed to the lack of hypomethylation in the overproducer.

§$Q = \frac{NTG}{B} = \frac{(20\text{–}40) \cdot 20 \cdot 250}{2 \times 10^5} = 0.5\text{–}1.0$, where N = number of molecules in a single cell = 20–40 (see above); B = rate of replication (base per min) = 2×10^5; G = frequency of G-A-T-C sites^{-1} = 250; T = turnover number of enzyme = 20 CH_3 per min per molecule.

DISCUSSION

In the present study we tested the possibility that the pattern of DNA methylation is determined by an interplay between the rate of DNA replication and the intracellular level of the DNA methylase. Two *E. coli* extrachromosomal DNAs were analyzed: bacteriophage λ and pBR322 DNA. Although these DNAs differed from each other in many respects, both were extensively undermethylated during active replication. The kinetics of the loss of methyl groups in these DNAs correlated with the rate of DNA replication. As λ phage is packaged into a protein coat; the mature λ phage DNA remains undermethylated, whereas pBR322 molecules may undergo methylation after replication has been completed. The requirement for replication in the undermethylation process was clearly demonstrated by using replication-defective λ mutants in which loss of methyl groups was not observed.

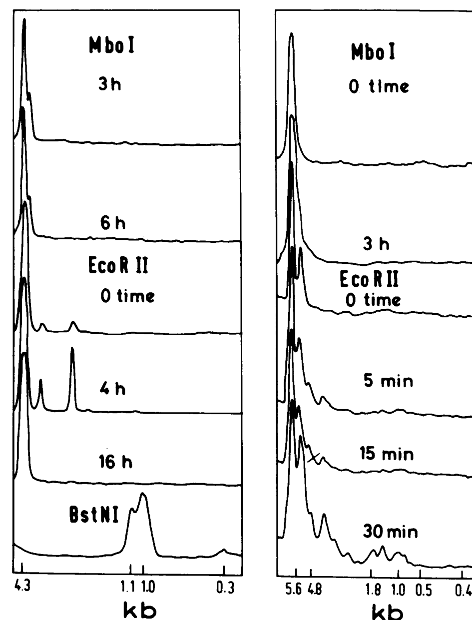

FIG. 5. Analysis of the extent of methylation of replicating λ phage and pBR322 DNA in a *dam* overproducer *E. coli* strain. (*Left*) *E. coli* cells JC4583/pGG503 were grown to mid-logarithmic phase. Aliquots were removed at 0, 3, 4, 6, and 16 hr after the addition of CAM (100 μg/ml). The state of methylation of G-A-T-C and C-C-$\frac{A}{T}$-G-G sites in pGG503 was determined. The scans of the respective autoradiograms are presented (for details, see legend to Fig. 3). (*Right*) *E. coli* cells lysogenic for λcI857Sam7 were transformed with the plasmid pGG503 (kindly provided by P. Modrich). These λ lysogen *dam* overproducer cells were induced by a temperature shift to 42°C. Aliquots were removed at 0, 5, 15, 30, and 180 min after induction. The state of methylation at G-A-T-C and C-C-$\frac{A}{T}$-G-G sites in DNA was determined as described above. The scans of the autoradiograms are presented (for details, see legend to Fig. 2).

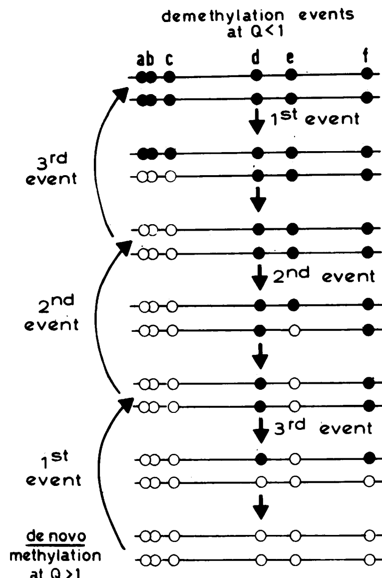

FIG. 6. Sequential changes in a methylation pattern. At a methylation quotient $Q < 1$ (for definition of Q, see text), sequential hypomethylation takes place. The first hypomethylation event results in loss of methyl groups in the sites with lowest affinity for the enzyme: a, b, and c (●, methylated site; ○, unmethylated site). The second event occurs on site e (intermediate affinity) and the last event occurs on sites d and f, which represent sites of highest affinity for the methylase. When $Q > 1$, *de novo* methylation takes place in an opposite order: first at sites d and f, followed by methylation of site e, and lastly a third *de novo* methylation occurs at sites a, b, and c. This scheme is based on experimental data obtained for changes in methylation of the *Eco*RII sites of pBR322, as presented in Fig. 3. For simplicity, only molecules that underwent changes in their methylation pattern are presented in the scheme. It should be noted that every hypomethylation event requires two replication cycles.

An attractive explanation for the observed replication-dependent loss of methyl groups is that the intracellular level of DNA methylase is limiting. The data presented here indicate that one of the *E. coli* DNA methylases, the *dam* gene product, is indeed limiting. It is just sufficient to adequately methylate methylatable sites emerging during replication of the cell chromosome, whereas rapidly replicating extrachromosomal DNA becomes undermethylated. In addition, in a *dam* overproducer strain no detectable hypomethylation of extrachromosomal DNA has been observed. A careful examination of the methylation pattern of the extrachromosomal DNA during its replication reveals that the methylation changes are site specific (Fig. 6). This might add to our understanding of the biological role of DNA methylation in *E. coli*. It has been suggested that transiently unmethylated sites exist during DNA replication (4). These sites may serve as hot spots for nuclease activity, which may, among other functions, direct the mismatch repair system (6), as recently demonstrated (17). The present study indicates that unmethylated sites may exist if the intracellular level of the methylase is not in excess. In a methylase overproducer cell no such sites exist, resulting in a hypermutable phenotype (18).

When the value of the methylatable quotient Q is <1, sequential loss of methyl groups occurs (for experimental data, see Figs. 2 and 3). In the case of $Q > 1$, *de novo* methylation will replenish the methyl groups in the inverse sequence of events that took place during their loss (see Fig. 3, 4–16 hr). In view of the results described and summarized in Fig. 6, we propose that the sequence of events leading to the formation of a methylation pattern is determined by the affinity of the methylase to the various sites and that a major factor in the regulation of the processes involved in the establishment of a pattern may be the intracellular level of the DNA methylase.

Comparable evidence for limiting levels of methylase in eukaryotic systems is not available as yet; however, some reported observations are supportive of such a possibility. In all cases studied, extrachromosomal viral genomes are completely unmethylated, whereas the integrated viral genomes are methylated (19). The rRNA genes of *Xenopus* are amplified 10^5-fold at early stages of development and are completely unmethylated, whereas the original unamplified genomic gene is heavily methylated (20). It is possible that the increase in DNA replication during the process of amplification is not compensated by an adequate methylase level and the result is undermethylation. Such a loss of methyl groups has been observed when a methylated *aprt* gene was introduced into L cells and underwent amplification in response to selection for the $Aprt^+$ phenotype (21). 5-Azacytidine is known to inhibit the methylase and to cause undermethylation (22). The fact that a relatively limited inhibition of the methylase can cause a decrease in the extent of methylation of the cellular DNA strongly suggests that the methylase is limiting. In addition, a loss of methyl groups also has been observed during mouse embryogenesis in rapidly replicating cells (23). If, indeed, the eukaryotic DNA methylase is limiting it might play a significant role in establishing the methylation pattern of eukaryotic DNA that is believed to be involved in gene activity.

We are grateful to Dr. P. Modrich for the JC4583/pGG503 overproducer strain and to Dr. Yaakov Pollack for critical reading of the manuscript. We thank Mr. Y. Akabi for technical assistance and Caroline Gopin for her help in preparing the manuscript. This study was supported by National Institutes of Health Grant GM 20483.

1. Razin, A. & Riggs, A. D. (1980) *Science* **210**, 604–610.
2. Pollack, Y., Stein, R., Razin, A. & Cedar, H. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 6463–6467.
3. Wigler, M., Levy, D. & Perucho, M. (1981) *Cell* **24**, 33–40.
4. Szyf, M., Gruenbaum, Y., Ureili-Shoval, S. & Razin, A. (1982) *Nucleic Acids Res.* **22**, 7247–7259.
5. Marinus, M. G. & Morris, N. P. (1975) *Mutat. Res.* **28**, 15–26.
6. Wagner, R. W. & Meselson, M. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 4135–4139.
7. Marinus, M. G. (1982) in *Chromosome Damage and Repair*, eds. Seeberg, E. & Kleppe, R. (Plenum, New York), pp. 469–473.
8. Razin, A., Urieli, S., Gruenbaum, Y., Pollack, Y. & Glaser, G. (1980) *Nucleic Acids Res.* **8**, 1783–1792.
9. Geier, G. E. & Modrich, P. (1979) *J. Biol. Chem.* **254**, 1408–1413.
10. Hattman, S. (1977) *J. Bacteriol.* **129**, 1330–1334.
11. Hattman, S., Schlagman, S. & Cousens, L. (1973) *J. Bacteriol.* **115**, 1103–1107.
12. Herman, G. E. & Modrich, P. (1982) *J. Biol. Chem.* **257**, 2605–2612.
13. Vogelstein, B. & Gillespie, D. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 615–619.
14. Urieli-Shoval, S., Gruenbaum, Y. & Razin, A. (1983) *J. Bacteriol.* **153**, 274–280.
15. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), pp. 326–328.
16. Kornberg, A. (1980) *DNA Replication* (Freeman, San Francisco), p. 361.
17. Lien Lu, A., Clark, S. & Modrich, P. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 4639–4643.
18. Herman, G. E. & Modrich, P. (1981) *J. Bacteriol.* **145**, 644–646.
19. Doerfler, W. (1981) *J. Gen. Virol.* **57**, 1–20.
20. Bird, A. P. & Southern, E. (1978) *J. Mol. Biol.* **118**, 27–47.
21. Stein, R., Razin, A. & Cedar, H. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 3418–3422.
22. Jones, P. A. & Taylor, S. M. (1980) *Cell* **20**, 85–93.
23. Razin, A., Webb, C., Szyf, M., Yisraeli, J., Rosenthal, A., Naveh-Many, T., Sciaky-Gallili, N. & Cedar, H. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 2275–2279.