**Prereplicative Purine Methylation and Postreplicative Demethylation in Each DNA Duplication of the Escherichia coli Replication Cycle**

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*Escherichia coli* plasmid DNA activated for initiation of duplication is in a stable low linking number supercoiled conformation. Low linking number DNA is methylated at the internal purines of a frequent 5′-Pyr-Pyr-Pur-Pur tetramer with a 5′-Pyr-Pyr-3′ axis of symmetry and is cut at the axis of symmetry by pneumococcal restriction enzyme DpnI when methylated in both strands. Purine methylation is of adenine in one strand and guanine in the other. Methylation of one of the two purines is removed during the cell cycle, presumably before the reverse shift to the B-supercoiled conformation. The topological transition was reconstituted in vitro only with DNA unmethylated at purines. Methylation-restriction analyses coupled with the chemical properties of low-linking number DNA and B-DNA respectively, suggest that removal of guanine methylation is essential for the low-linking number to B-DNA transition and hence for the deactivation of replication. Demethylation of methylguanine could explain the presence in *E. coli* of the two-member inducible operon known as *ada*. Characteristics of *ada* suggest a cascade of chemical DNA modifications that reverse prereplicative guanine methylation. Guanine demethylation could provide a model for the pivotal role played by de novo methylation in replication and for the essential role of "repair" enzyme ExoIII in demethylation leading to the reversal of replicative DNA activation and other processes that affect DNA function.

The replication studies reported here and in Ref. 1 deal with the miniplasmid RepFIC, isolated from *Escherichia coli* pathogen 307 (2). The RepFIC origin is prototypic of pathogenic plasmids of *E. coli*, *Salmonella*, and *Shigella*. The four peptides expressed by mini-RepFIC are essential and sufficient for the *in vivo* once/cell cycle activation associated with replication (3, 4). The minimal replicon used in these studies is 3000 base pairs in size and fully regulated, with each plasmid unit essentially replicating once during the cell cycle.

In a previously reported optimal recovery of transformants approximately every plasmid molecule transferred by electroporation initiates replication (1). Such conditions "imprint" the altered L-DNA conformation of plasmid maintenance, characte

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The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) M16167.

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**Experimental Procedures**

*Bacterial Strains and Plasmids—*The following *E. coli* strains were used as hosts: C600 (thi-1 thr-1 leuB6 lacY1 tonA21 supE44 mcrA), JT4000 (Δlon-510), GM3819 (ΔmcrA::Kan), GM31 F− (dcm-6), and
**RESULTS**

**Newly Characterized Methylation of A, G, and C in Replicon DNA**—The replication switch-off associated with methylation of one strand and seen in the transformation of dam− hosts with methylated plasmids (6) is observed with RepFIC only when dam− dcm− hosts are transformed. This suggests that dam-methyltransferase (dam-Mtase) and dcm-methyltransferase (dcm-Mtase) of *E. coli*, contrary to current dogma, methylate a common sequence.

The cutting activity of restriction enzymes is recognized *in vitro* by the electrophoretic separation of resulting double-stranded (ds) fragments. ds fragments are obtained when the cognate restriction sequence has an axis of symmetry (8). The pneumococcal enzyme DpnI makes blunt cuts at 5′-CCWGG with an axis of symmetry through W, whereas it cuts 5′-CCAG/CTGG once at 5′-C↓CAG. Shortening of the cognate EcoRII sequence from pentamer to tetramer increases the number of cognate loci 5-fold from 8 to 39, with 31 of them essential for blocking EcoRII restriction.

Finally, cytosine methylation prevents cutting of all 5′-CCWGG and 5′-CCAG sequences (lane 7), whereas purine methylation does not (lane 9). The results of lane 9 demonstrate a dual pattern of dam-methylation that is explained by susceptibility of each plasmid molecule to EcoRII ds cuts at the four 5′-CCWGG loci in one orientation or alternatively at the four 5′-CCAG loci in the other orientation (the analysis is illustrated in Fig. 2). When one parental strand is methylated at adenine and the other at guanine, semiconservative assortment of parental strands (7) dictates that one offspring inherits methylated adenine and the other inherits methylated guanine. One concludes from the alternative sensitivity of plasmid populations to EcoRII cuts that one-strand methylation of adenines or of guanines is systematically removed after duplica-

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1 The abbreviations used are: Mtase, methyltransferase; ds, double-stranded; MMS, methyl methanesulfonate.
labeled (+ or −). Lane 1, untreated; lane 2, Mval-treated (arrow indicates the diagnostic fragment shown in the map); lane 3, EcoRII-treated; lane 4, untreated; lane 5, EcoRII-treated; lane 6, untreated; lane 7, EcoRII-treated (note lowering of helical density, also seen in lane 3); lane 8, untreated; lane 9, EcoRII-treated. EcoRII treatment was in 10 mM Mg²⁺.

Of helical density, also seen in the map of Fig. 1. A strategy that serendipitously identified the origin (4, 12), controls were carried out by piperidine (1, 9, 11) suggests that methylguanines and not methyladenines are demethylated or replaced in the tetramers. Such a strategy, using sequential primer extensions with Taq polymerase and a radioactive precursor (4). Separation of the extended primers by denaturing PAGE revealed that there was no methylation of purines in the origin region, because the origin has no canonical GATC sequences. The piperidine-treated DNA was used as template in thermally cycled primer extensions by treatment of the low linking number reaction product with a type II topoisomerase. Having obtained evidence for the loss of guanine methylation from DNA raises the possibility that in vivo specific demethylation of L-DNA is essential for the transition.

Fig. 2. Loss of methyl groups from methylguanine after replication. A, methylation motif within the EcoRII cognate sequence. Arrows indicate bases methylated by dam- and dcm-methyltransferase, respectively. B, adjacent-opposite methylation of A (blue) and G (red). C, DNA is duplicated and becomes hemimethylated. D, meA is retained, and meG is corrected. Map and fragment sizes below illustrate the MvaI diagnostic fragment and how the fragment sizes obtained with EcoRIII match those predicted by correction of meG. Red trapezoids, loci that are transmitted in the unmethylated state; blue trapezoids, loci that are alternatively transmitted in the unmethylated state. Red orientation, fragments x2 and x3. Blue orientation, fragments x1 and x5.

The fragments of lane 9 add up to a 6-kb length, whereas the miniplasmid is 5-kb in size. Amplification of the DNA was eliminated by the complete in-lab sequencing of five plasmid preparations originating from five isolated transformants of a Dam− dcm− host and one transformant of a Dam+ Dcm+ host.

Both lane 3 and lane 7 demonstrate that when methylation prevents restriction, EcoRIII shifts the equilibrium between normal density DNA, named H in my previous publication (1), and L-DNA toward L. The transition to L facilitates replication (4). The extreme sensitivity of L-DNA and not H-DNA to piperidine (1, 9, 11) suggests that methylguanines in L-DNA cannot be identified directly, because the marker deoxy-7-methylguanosine is not available. Methylguanine in L-DNA cannot be identified directly, because the marker deoxy-7-methylguanosine is not available.

The analysis illustrated in Fig. 2 identifies ds cuts at all 5′-CCWGG loci except for those designated as 1 and 4 in the map of Fig. 1. A strategy that serendipitously identified the sequence 5′-CCAG and its complement 5′-CTGG for methylation was as follows. In the course of doing in vivo footprinting of the origin (4, 12), controls were carried out by piperidine hydrolysis of isolated plasmid to confirm that there was no methylation of purines in the origin region, because the origin has no canonical GATC sequences. The piperidine-treated DNA was used as template in thermally cycled primer extensions with Taq polymerase and a radioactive precursor (4). Separation of the extended primers by denaturing PAGE revealed that the assumption of no methylation was incorrect, there were nick signals at the two tetramers. Such a strategy, using sequences for location of the nicks (4) and plasmid isolated from a dam− host as control should directly identify all As and Gs that become methylated irrespective of recognition by a methylation-sensitive restriction enzyme. Although straightforward, the experiments are beyond the scope of this work.

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This work with DNA oligonucleotides has given the enzyme its name. ExoIII possesses multiple hydrolytic activities toward the sugar phosphate backbone, all determined with in vitro generated substrates and described in Ref. 8. ExoIII removes from the 3'-end of duplex polymers up to three mispaired nucleotides. In nicked substrates the nick can be enlarged to a gap. In addition ExoIII makes incisions at apurinic and apyrimidinic sites; L-DNA only. Three preparations originated from a Dam+ host and three from a dam- host. The results are shown in Fig. 3. Comparison of each untreated sample (odd-numbered lanes) with the adjacent ExoIII-treated sample shows that ExoIII lowers the electrophoretic mobility of L-DNA and L-DNA only to the lowest in the gel (labeled LL). Dam-methylation does not affect the reaction.

The above results were obtained in 0.66 mM magnesium (Mg2+) buffer. To test whether more Mg2+ was required for an additional reaction, the following experiment, shown in Fig. 4, was done. LL-DNA was prepared by ExoIII treatment of L- and H-DNA mixtures, because the relative insolubility of L-DNA (1) hampers its purification by extraction from gels. The pure material seen in Fig. 3 (lane 3) can only be obtained from cells where the L-conformation has been imprinted, i.e. only from Dam+ cells. The reaction tubes were maintained at 70°C for 20 min (Fig. 4, step I). Mg2+ (lanes 3 and 7) or Mg2+ + ExoIII (lanes 4 and 8, indicated by arrows) was added in a second step. The addition of Mg2+ regardless of the addition of fresh ExoIII resulted in a clear transition of LL-DNA, but only when the DNA was isolated from a dam- host (lanes 7 and 8). ExoIII does not degrade intact circular DNA (8). L-DNA is circular and supercoiled (1). The clarity of the lanes in the position where linear molecules are expected and below the H band, as well as the lack of increase in ethidium-bromide staining material at the bottom of the gel (not shown), confirm that there was no degradation by ExoIII. The transition of LL-DNA in lanes 7 and 8 was most likely to H-DNA, an interpretation that was easier to see with the naked (and protected) eye directly on the UV transilluminator.

The purpose of the treatment at 70°C was inactivation of ExoIII. The L to H transition proceeded in the absence of further addition of ExoIII, suggesting either that the transition was spontaneous or that ExoIII is not inactivated once bound to DNA. The latter seems more likely.

When all possible purines were methylated as they normally are in L-DNA, the addition of Mg2+ resulted in diffuse bands of low helical density (lanes 3 and 4). The diffuse bands of LL-DNA could indicate Mg2+ promoted nicking as is seen with topoisomerases and poor staining caused by inefficient intercalation of ethidium bromide in the nicked structure. Post-replicative specific loss of guanine methylation and the in vitro topological activity of ExoIII can be integrated in a model that is considered in the following "Discussion."

**DISCUSSION**

This work has provided evidence for methylation in both strands of the tetramer sequence motif 5'-CCWG at guanine, adenine, and cytosine in E. coli. The sequence, which contains three G-C pairs, is present 39× in 3000 base pairs. Seventeen of the tetramers are located in the 48% G-C origin sequence. Full methylation optimizes initiation of the duplication process (1) by stabilizing the prereplicative L-DNA conformation. The activated state of replication cannot be removed until methylguanines are replaced or demethylated. L-DNA that is hemi-methylated at de novo purines appears to act as a checkpoint for reversal of specific programmed methylation. As will be discussed, de novo methylated cytosines may be removed as well by an analogous pathway.

ExoIII is here proposed to play a pivotal role in the reversal of DNA modifications that alter DNA function. ExoIII conditional mutants exhibit filamentous growth like E. coli where maintenance of the RepFIC plasmid has been imprinted as L, suggesting that sufficient ExoIII is essential for the L to H transition. This in turn suggests that LL-DNA is an activated intermediate for the alteration or removal of DNA units that interfere with the B-helix. Once interference is removed, the ExoIII reaction is completed. It would be interesting to determine whether combining the ExoIII mutation known as xth with a dam mutation relieves both the filamentous growth phenotype and sensitivity to hydrogen peroxide. Relief of filamentous growth would signify that in the absence of a requirement for demethylation of methylguanine, a type II topoisomerase could promote the L to H transition. Relief of sensitivity to hydrogen peroxide would suggest that the sensitivity is indeed due to the presence of methylguanine in DNA.
Just as mammals methylate cytosine de novo during development (16), *E. coli* methylate adenine and guanine de novo during replication, resulting in changing patterns of methylation during each replication cycle and leading to the conclusion that one common feature of *de novo* DNA methylation is the optimization of DNA replication. The properties of the two major methyltransferases in *E. coli* as well as those of cognate restriction enzymes used in sequence identification are summarized in Table I. Interestingly, the same motif that is methylated by dam-Mtase is methylated by dcm-Mtase, the latter not found in all *E. coli* strains.

In *in vivo* dam-methylated L-DNA appears to be identical to DNA alkylated *in vitro* (1). The major product of *in vitro* ds DNA alkylates is N7-methylguanine (9, 11). The major methylated purine product by a large factor after exposure of *E. coli* to alkylating agents is also N7-methylguanine (17). The indications thus are that methylguanine in L-DNA is modified at N7.

It is likely that methylguanines are demethylated rather than excised for the following reason. *E. coli* adapt rapidly when exposed to mutagenic methylating agents (18). Adaptation is via the inducible protein Ada, specific for removing methyl adducts from the O6 position of methylguanine (18, 19). The O6 position is vital to hydrogen bonding (20), and not surprisingly a system evolved for removing the modification, certain to be mutagenic in view of improper O6-methyl pairing opposite the 4-amino group of cytosine. The question arises why does *E. coli* demethylate a minor product of methylation when N7-methylguanine is the major modification product and is in fact removed. One explanation, the simplest, is that O6-methylguanine is a functional intermediate marked for demethylation.

The methyl group of O6-methylguanine is transferred to cysteine-S of the Ada protein itself (21). Methylated Ada protein is a transcriptional activator of the ada operon (22). The Ada enzyme is also known as O6-methylguanine-DNA methyltransferase (23). The ada operon transcribes one additional protein that has been named AlkB (24, 25). The *in vitro* activity of AlkB is unknown. *In vivo* absence of AlkB sensitizes the cell to killing by the methylating agent MMS (24). Could AlkB take part in the methyl group transfer from N7 to O6 and thus be part of the guanine demethylation reaction?

There is an additional protein in *E. coli* that has the potential to demethylate O6-methylguanine. It is not inducible. Ada and the noninducible DNA methyltransferase remove methyl groups from both O6-methylguanine and O4-thymine, neither one of which is a major product of *in vivo* alkyltion reactions. Ada protein displays greater efficiency in the demethylation of O6-meG and the noninducible enzyme in the demethylation of O4-meT. The noninducible protein is conserved in yeast and man, where it has the same preferential activity toward O4-meT (19). The similarities of the two DNA methyltransferases and the conservation of O4-meT-DNA methyltransferase through the evolutionary ladder suggest a normal biological function for both DNA methyltransferases that is advantageous but does not necessarily reflect a response to mutagen exposure.

7-Methylguanine remains in the DNA helical wall of Z-DNA (26) and presumably in the helical wall of L-DNA (1). Exposure in the wall of the helix could be one recognition basis for base alterations. Methylcytosine on the other hand is frequently found in supercoiled B-DNA. Thus, when methylcytosine is removed it is recognized on a different basis. A strategy used by the scientist in the laboratory to identify methylcytosine in DNA is to convert it to uracil (27, 28). The strategy appears to have its biological counterpart, and furthermore ExoIII is involved in the removal of uracil from DNA. Methylcytosine is altered *in vivo* by hydrolytic deamination to thymine. When dcm-methyltransferase is overproduced in *E. coli*, G-C → T-A transitions are increased (29), suggesting saturation of a system that normally corrects the meC → T transitions. Unambiguous correction of the mismatch at the modified base further suggests that transitions and their reversion are part of DNA metabolism. In fact, the enzyme responsible for meC → T transitions has been described in *E. coli* (30). dcm-methyltransferase deaminates its own product 5-methylcytosine to thymine in the absence of methyl donor S-adenosylmethionine (30, 31). Methyl transfer within T in G-T mismatches from O6 to O4 could provide an intermediate analogous to O-methylguanine, giving rise to uracil when demethylated. Uracil opposite G in DNA is corrected by an active dedicated glycosylase, conserved from bacteria to man, and the correction is dependent on ExoIII in *E. coli* and presumably on the ExoIII homolog HAP1 in man. Thus, the above series of reactions provides a fool-proof system for the unambiguous removal of meC. The methylcytosine removal process ends in mutation, however, when the T-to-U transition followed by U excision does not take place.

It has been suggested that DNA “damage” results from metabolites present in the cell (32, 33). This work provides evidence that methylation of guanine, a modification that optimizes replication, is a normal cellular process that is reversed, deactivating replication. Methylated bases are in fact detected in the DNA of cells that have not been exposed to exogenous mutagens (34, 35). Under the circumstances DNA “lesions” (36) can be explained by the failure to revert programmed DNA alterations. An understanding of mechanisms that regulate DNA function by reversible alterations of the DNA structure or the code itself could lead to new therapeutic approaches for reducing cancer risk, because insufficiencies in DNA metabolism as well as mutagenic agents may result in damaging mutagenesis.

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