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Biological Role of DNA Methylation: Sequence-Specific Single-Strand Breaks Associated with Hypomethylation of GATC Sites in *Escherichia coli* DNA

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The effect of methylation of GATC sites in *Escherichia coli* DNA on the formation of single-strand breaks was studied with *dam*$^+$, *dam* mutant, and Dam-overproducer strains. Single-strand breaks have been observed in *dam* mutant cells predominantly at TpT and, to a lesser extent, at CpC. In *dam* mutant cells harboring pTP166 (a plasmid containing the *dam* gene), no such nicks were observed.

Methylation of GATC sites in *Escherichia coli* DNA by the Dam methylase has been attracting considerable interest over the last decade. Because mutants in the *dam* gene have been constructed (10), the gene has been cloned (1, 3), and the enzyme has been purified to homogeneity (5), the study of its biological role became possible (9). Initially, the attention was drawn to this methylation by the suggestion that it functions by strand discrimination in mismatch repair (15). The interest grew further, as demonstrated by recent studies in which the effect of GATC methylation in *E. coli* on the initiation of DNA replication in vitro (6) and in vivo has been shown (14, 17). Results of other experiments have suggested that the transposition of insertion element IS*10* (16) and the bacteriophage P1 recombinase gene *cre* (19), as well as other genes (18), may be regulated by GATC methylation.

Although, the functions mentioned above seem to be unrelated to each other, a common denominator for all of them could be a nucleolytic activity which is determined by Dam methylation of GATC sites (7). It was therefore of interest to examine the possibility that in the normal *E. coli* cell, in which practically all GATC sites are methylated, a transient hypomethylation may require the formation of specific single-strand breaks, as suggested previously (21). We and others have demonstrated recently (8, 20) that hypomethylated sites may exist transiently in *E. coli* DNA since the *dam* methylase is present in the cell in limiting amounts (20). In this study we demonstrate specific single-strand breaks in a *dam lig* mutant. These breaks do not occur in the same strain that was transformed with a recombinant plasmid containing the *dam* gene (pTP166).

In an attempt to determine whether *E. coli* DNA single-strand breaks are associated with the state of methylation of GATC sites, we used *dam*$^+$, *dam* mutant, and Dam-overproducer strains of *E. coli*. The strains used for this study were *E. coli* GM2161 [F$^-$ *dam-13*::Tn*9* *lig-7*(Ts)] and GM874 [*lig-7*(Ts) *lacYA482*]. *E. coli* GM2161 was transfected with pTP166, a plasmid derived from pBR322 in which the *tac* promoter is 5′ to a 1,140-base-pair (bp) fragment of *E. coli* containing the 854-bp *dam* gene (12). As controls we used GM2161 and GM874 cells transfected with pBR322. GM2161, GM874, and the plasmid pTP166 were all kindly provided by M. G. Marinus. The *dam* mutant strain was practically devoid of Dam methylase activity, whereas GM2161(pTP166), the Dam-overproducer strain, contained 30-fold higher methylase activity than the *dam*$^+$ strain. The extent of methylation of GATC sites in the DNA of the three strains was analyzed by digestion of the DNA with the restriction enzyme *Mbo*I (which cuts only at unmethylated GATC sites) and with its isoschizomer *Dpn*I (which cuts only at methylated GATC sites). This analysis revealed that the *dam*$^+$ and Dam-overproducer strain DNAs were practically fully methylated, while *dam* mutant DNA was readily digested by *Mbo*I and was resistant to *Dpn*I (data not shown). Initially, *lig* mutant (Ts) strains were chosen for this study to enable examination of nicked DNA under ligase-deficient conditions. However, in early experiments an abundance of single-strand breaks was observed in the *lig* mutant strains at the permissive temperature (30°C). The replication load applied by the plasmids introduced into the *lig* mutant cells presented the ligase with excess substrate, thereby allowing the accumulation of single-strand breaks. We therefore performed our experiments at the permissive temperature (30°C). Breaks could not be detected in *lig*$^+$ strains, explaining the failure of previous investigators to detect these *dam* mutant-specific nicks (2, 11). To determine the extent and specificity of the nicks present in the DNA of the three strains used in this study, these DNAs were isolated by the procedures described by Marmur (13) with the following modification. After RNase and proteinase treatments, DNA samples were phenol extracted and 1 volume of chloroform was added. After gentle mixing the mixture was left to settle for 10 h at room temperature. The DNA in the aqueous phase was ethanol precipitated, spooled, air dried, and dissolved in 1 mM EDTA–10 mM Tris hydrochloride (pH 7.4). All manipulations were carried out with caution to prevent DNA breakage.

The nicks in the various DNA samples were end labeled separately for 1 h at 37°C with 10 μCi of each of the four α-^{32}P-labeled deoxyribonucleoside triphosphates (Amersham, 3,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) with 2.5 U of *E. coli* DNA polymerase I (endonuclease free; Boehringer GmbH, Mannheim, Federal Republic of Germany) in a 25-μl mixture containing 50 mM Tris hydrochloride (pH 7.4), 5 mM $MgCl_2$, and 1.4 mM β-mercaptoethanol. The unreacted labeled nucleotide was removed by

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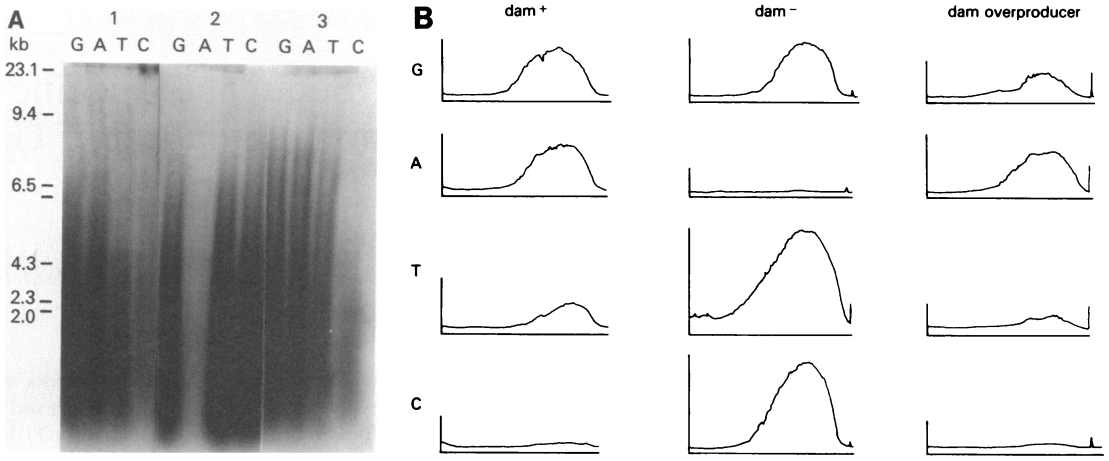

FIG. 1. Specificity of the single-strand breaks in *E. coli* DNA. DNA purified from GM874(pBR322) (*dam*$^+$), GM2161(pBR322) (*dam* mutant) and GM2161(pTp166) (Dam-overproducer) cells was end labeled with each of the four α-^{32}P-labeled deoxyribonucleoside triphosphates as described in the text. The DNA was purified on Sephadex G-50 minicolumns, denatured, and size fractionated by electrophoresis on 1% agarose gels. The gels were dried and autoradiogrammed; and the autoradiograms were scanned by densitometry. (A) Autoradiograms. A, G, C, and T represent labeling with [α-^{32}P]dATP, -dGTP, -dCTP, and -dTTP, respectively. kb, Kilobases. (B) Densitometry. Each lane was scanned with an Helena Quick Scan densitometer.

molecular filtration on Sephadex G-50 (Pharmacia Uppsala, Sweden) minicolumns. The labeled DNA in the effluent was denatured by incubating for 1 h at 37°C in 0.3 M NaOH. The denatured DNA was size fractionated by electrophoresis on neutral 1% agarose gels and autoradiographed.

The DNAs of the *dam*$^+$ and Dam-overproducer strains were predominantly labeled at the nicks by dATP and dGTP, whereas the *dam* mutant strain DNA was heavily labeled by dCTP and TTP (Fig. 1 and Table 1). This difference reflects nuclease activity that is only expressed when the cellular DNA is not methylated at GATC. The similar label obtained with dGTP in all three strains and with dATP in the *dam*$^+$ and Dam-overproducer strains suggests that nicks labeled by these nucleotides are not associated with the Dam methylation. The very low level of labeling obtained consistently with dATP in the *dam* mutant DNA is not understood. The low level of labeling with dATP, however, suggests a bias of the nicking activity against XpA.

To detect the nearest neighbor of the nucleotide added to the nick, the end-labeled DNA was digested to nucleoside 3′-monophosphates, as described previously (4), and the nucleotides were separated by two-dimensional thin-layer chromatography. The chromatograms were autoradiographed, and the intensity of the radioactive spots representing the four nucleoside 3′ monophosphates was determined by densitometry. From the extent of label in these spots and the nucleotide used to end label the nicks, the frequency of nicks at specific dinucleotides could be computed. The frequency of each of the possible 16 dinucleotides was presented as percent of total radioactivity obtained with each labeled DNA (Table 2). The results demonstrate that the frequency of the dinucleotides in *dam*$^+$ and Dam-overproducer DNAs are similar. Note that the normal frequency of a homodinucleotide is somewhat higher than the expected 25% for heterodinucleotide frequency. This is due to the higher label contributed by incorporation of more than one nucleotide into sites that have several of the same nucleotides in a row. It should also be noted that the frequency of TpT is somewhat higher than that of the other homodinucleotides. However, the values obtained for TpT and CpC sequences in *dam* mutant DNA are significantly higher than those observed for TpT and CpC sequences in the *dam*$^+$ strains. Together these results suggest that single-strand breaks are found in *dam* mutant DNAs that are specific for this strain. These breaks occur preferentially at TpT- and, to a lesser extent, at CpC-containing sequences. On introduction of a functional *dam* gene to these cells the nicking pattern of the *dam* mutant DNA is almost indistinguishable from that of the *dam*$^+$ DNA. This result proves unequivocally that the additonal breaks in the *dam* mutant DNA (over the background nicking) are associated with the undermethylation of the GATC sites.

One major consideration in the design of our experiments was to discriminate between *dam*-associated single-strand breaks and other nicks formed by nonspecific nuclease activity or damage to the DNA caused by the extraction procedure. By using careful extraction of DNA, the single strand breaks in the DNA caused by the extraction could be minimized. Previous studies that have addressed the same question presented here have arrived at contradictory conclusions (2, 11). In one of these studies (2) the investigators suggested that the nicks observed in *dam* mutant DNA reflect a mutation which accompanies the *dam* mutation. We

TABLE 1. Relative labeling at the nicks present in DNA from *dam*$^+$, *dam* mutant, and Dam-overproducer cells[a]

Nucleotides[b]	Relative labeling of the following DNAs[c]:		
	dam mutant	*dam*$^+$	Dam overproducer
G	3.5	3.2	2.3
A	<0.1	3.4	3.1
T	7.5	1.8	1.4
C	5.3	1	1

[a] The autoradiographs shown in Fig. 1 were scanned by densitometry (Fig. 1B) and quantitated by estimation of the area under the scan curves.

[b] G, A, T, C represent labeling of the DNA by dGTP, dATP, dTTP, and dCTP, respectively.

[c] The extent of label obtained by dCTP labeling of *dam*$^+$ DNA was taken as 1 and all other scans were calculated relative to it.

TABLE 2. Analysis of the nearest neighbors to the nucleotide used for end labeling of the nicks in *dam* mutant, *dam*$^+$, and Dam-overproducer DNA[a]

Nearest neighbor[b]	Percentage of total counts in the following DNAs:											
	dam mutant				*dam*$^+$				Dam overproducer			
	XpG	XpA	XpT	XpC	XpG	XpA	XpT	XpC	XpG	XpA	XpT	XpC
G	48	12	4	19	39	14	13	23	38	15	16	24
A	4	36	7	5	11	41	11	13	11	40	13	18
T	18	13	83	13	25	19	61	20	25	19	50	19
C	29	34	6	66	25	26	15	44	25	26	21	40

[a] End-labeled DNA samples, as described in Fig. 1, were digested with micrococcal nuclease and spleen phosphodiesterase, and the resulting 3′ nucleoside monophosphates were separated by two-dimensional thin-layer chromatography and autoradiographed as described in the text. The radioactive spots were quantitated by densitometry, and their relative intensities are presented in the table as percentage of total label found in the DNA.

[b] In each dinucleotide A, G, C, and T represent the corresponding labeled nucleotide dATP, dGTP, dCTP, and dTTP that was used for end labeling; X represents the nearest neighbor.

have ruled out this possibility using *dam* mutant cells transfected with the *dam* gene. The extent and specificity of nicks observed in the DNA of this Dam-overproducer strain were not significantly different from those observed in the *dam*$^+$ strain (Fig. 1 and Table 2).

Because the nicks were preferentially at TpT and CpC sequences and were not intimately associated with GATC sites, the question of where these nicks originated was raised. Southern blots of *dam* mutant DNA probed with *oriC* revealed no nicking over the 400-bp region of the *E. coli* origin of replication (data not shown). This result shows that the observed *dam* mutant-associated nicking does not occur in the immediate vicinity of GATC sites. This is in light of the fact that GATC sites are 10-fold overrepresented in this 400-bp region of the *E. coli* origin of replication (6, 14, 17). It cannot be excluded, however, that the nicks originate at GATC sites and are nick-translated in vivo to downstream TpT or CpC sequences that for some reason block further movement of the nicks.

If methylation of GATC sites plays a role in the regulation of the observed nicking phenomenon, how do normal *E. coli* cells control this nicking when they are practically fully methylated at those sites? It was previously demonstrated that transiently undermethylated sites are generated when the replication fork moves along the chromosome while the methylase activity is limiting (8, 20). The transiently hypomethylated sites on a high methylation background may serve as hot spots for the DNA nicking activity. In extreme cases like in *dam rec* double mutants, double-strand breaks were recently observed (22). The single-strand DNA breaks, as demonstrated here, may serve a signal for various DNA-protein interactions (18). Such interactions might be associated with initiation of DNA replication, postreplication mismatch repair, transposition, or other recombination events and gene expression.

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