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NOTES

Quantitation of Dam Methyltransferase in *Escherichia coli*

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An antiserum against *Escherichia coli* Dam methyltransferase has been developed in rabbits and employed to detect and quantitate the enzyme in immunoblots. A wild-type, rapidly growing *E. coli* cell (doubling time = 30 min) was found to contain about 130 molecules of Dam methyltransferase.

Dam methyltransferase of *Escherichia coli* methylates adenines in the sequence 5′-GATC-3′ (18, 23), and the methyl group in the N-6 position serves as a convenient marker for different regulatory purposes. The DNA replication enzymes incorporate unmethylated adenines, and the nascent strand remains unmethylated for several minutes after passage of the replication fork (13). This difference in methylation status can serve to discriminate between the template and the newly replicated strand, a feature exploited by the Dam-directed mismatch repair system (27); a nucleotide mismatch produced at the replication fork can be corrected in the nascent, unmethylated strand, while the correct nucleotide sequence in the old, methylated strand is left unchanged. The success of this repair system is dependent on the level of Dam methyltransferase, since both high and low levels have been shown to cause high mutation rates (4, 17, 19, 26).

Initiation of DNA replication is sensitive to the cellular level of Dam methyltransferase. While in *dam*$^+$ cells all origins are initiated simultaneously (29), initiations appear to be random in time in *dam* mutant cells (7, 9). Also, asynchrony of initiations was observed at high levels of Dam methyltransferase, and proper timing of initiations was found to occur only at intermediate levels (7).

Expression of the *dam* gene is controlled by a complex combination of five promoters and one terminator region (25). The details of the regulatory mechanisms responsible for maintaining an intracellular concentration of Dam methyltransferase which is optimal for mismatch repair and for initiation of DNA replication are not known. In the present work, we have employed immunoblotting of total cell proteins to measure the level of Dam methyltransferase.

The *E. coli* K-12 strains used were AB1157 (3), GM3819 (28), and ALO149 (7). The first two strains are isogenic, except that strain GM3819 carries a deletion of a large part of the *dam* gene. Strain ALO149 is MC1000 (15) carrying a *dam-3* allele and plasmids allowing temperature-inducible overproduction of Dam methyltransferase, as measured by immunoblotting and by enzymatic assays (7). The bacteria were brought to steady-state growth at 37°C (AB1157 and GM3819) or at 42°C (ALO149) in AB medium (16) supplemented with thiamine (0.001%), Casamino Acids (0.5%), and glucose (0.2%). At an optical density at 450 nm of 0.2 to 0.3, the cells were washed and concentrated 50- to 100-fold in 10 mM Tris (pH 8.0)–1 mM EDTA. Total protein in the resuspended pellet was measured (10) with bovine serum albumin (BSA) as a standard. To obtain sharp Dam-specific bands, the cell extracts were boiled for 15 min in sample buffer before being loaded onto 12.5% polyacrylamide gels (24). Repeated boiling did not affect the quality of the bands. Dam methyltransferase, purified as described elsewhere (31), was added as a standard in all gels and contained at least 90% Dam enzyme, as judged by silver staining.

The anti-Dam serum was obtained by injecting a synthetic oligopeptide into New Zealand White rabbits as described previously (14). The sequence of the oligopeptide was identical to residues 92 through 106 of the Dam protein (Tyr-Gln-Phe-Arg-Glu-Glu-Phe-Asn-Lys-Ser-Gln-Asp-Pro-Phe-Arg) and was chosen according to a hydrophilicity analysis (21). Solid-phase peptide synthesis (5) was performed with an Applied Biosystems model A430 synthesizer.

After polyacrylamide gel electrophoresis, the proteins were blotted onto filter paper (Immobilon PVDF; Millipore), which was probed with the rabbit antiserum at a 1:3,000 dilution. A secondary layer of anti-rabbit antibody labeled with alkaline phosphatase was added, and the alkaline phosphatase activity was measured as recommended by the antibody supplier (Promega). The amounts of Dam methyltransferase in extracts of wild-type cells were measured by immunoblotting and compared with known amounts of purified enzyme measured in the same assay system.

The amount of enzyme actually withdrawn from the preparation of purified Dam methyltransferase appeared to vary, possibly because of precipitation of the enzyme. Therefore, in each experiment a sample of purified enzyme was removed and mixed with the sample buffer. Different amounts of this preparation were applied to polyacrylamide gels alongside known amounts of BSA and were also applied to gels for comparison with cell extracts. Gels with samples of BSA and Dam enzyme were silver stained (Sigma), and the bands were quantitated by densitometric scanning (Molecular Dynamics). The actual concentration of Dam methyltransferase was derived from a comparison between the stained bands. The relationship between band intensity and amount applied was approximately linear (Fig. 1). In the example shown in Fig. 1, the Dam concentration was determined to be 6.4 ng/μl. In addition, for the example shown,

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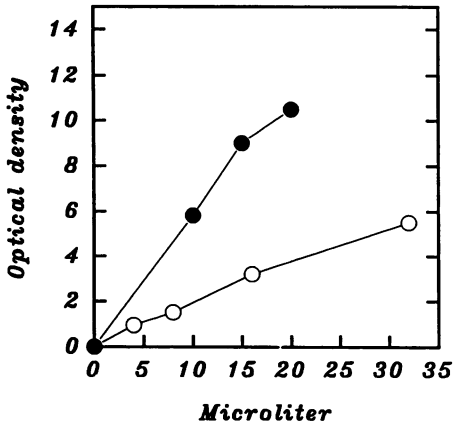

FIG. 1. Measurement of Dam methyltransferase concentration in a purified preparation. Different amounts of Dam methyltransferase (●) and BSA (○) were loaded onto a polyacrylamide gel. After electrophoresis, the gel was silver stained and the bands were quantitated by densitometry. The ordinate is an integral of the optical density in each band, and the units are arbitrary.

the concentration of protein was determined chemically (10) to be 8.0 ng/μl. The calculations below are based on a concentration of 6.4 ng/μl, but it should be pointed out that, given the uncertainties involved in the two assays, the error of our estimate may be as large as 50%.

Different amounts of purified Dam enzyme (6.4 ng/μl) were added to extracts of strain GM3819. In the same gel were run extracts of the Dam-overproducing strain ALO149 and strain AB1157 (Fig. 2). The rabbit anti-Dam antiserum reacted with several different *E. coli* proteins. Major bands were detected at about 60, 48, 44, and 38 kDa in addition to a 32-kDa band. The absence of the 32-kDa band in strain GM3819 (Fig. 2, lane 1) and its strong appearance in small amounts of the Dam overproducer ALO149 (lane 7) demonstrate that the 32-kDa band represents Dam methyltransferase. Interestingly, in addition to the 32-kDa band, the 60-kDa band was much stronger in ALO149 than in AB1157 and GM3819. The identity of the 60-kDa antigen is not known. Presumably, it is a protein induced by the high Dam activity or by the high temperature, since AB1157 and MC1000 (the parent of ALO149) had the same amount of the 60-kDa antigen (data not shown).

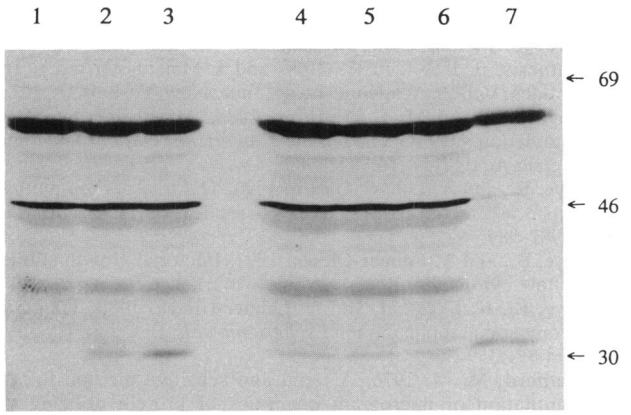

FIG. 2. Immunoblot for quantitation of Dam methyltransferase in different *E. coli* strains. Extracts of strains GM3819 (lanes 1 to 3), AB1157 (lanes 4 to 6), and ALO149 (lane 7) were electrophoresed in a polyacrylamide gel and blotted, and the blot was treated with primary rabbit Dam antiserum and secondary anti-rabbit antibody labeled with alkaline phosphatase. The amounts of extract added were 132 μg (lanes 1 to 3), 220 μg (lane 4), 176 μg (lane 5), 132 μg (lane 6), and 11 μg (lane 7). In addition, 0.5 μl of purified Dam methyltransferase was added in lane 2, and 1.0 μg was added in lane 3. The arrows and numbers at right indicate the positions and sizes of molecular mass markers (in kilodaltons).

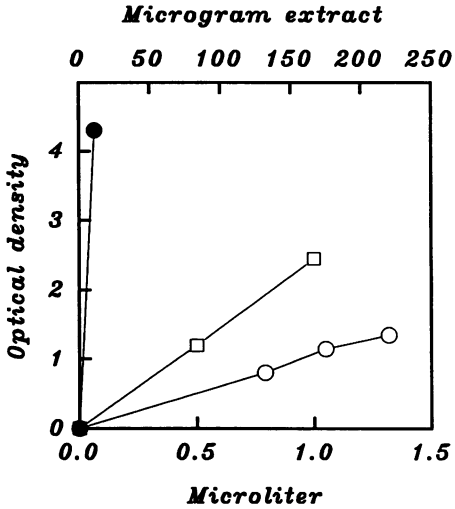

FIG. 3. Quantitation of Dam methyltransferase. The Dam-specific bands in Fig. 2 were quantitated and plotted against micrograms of extract of the wild-type strain AB1157 (○) or the Dam-overproducing strain ALO149 (●) or against microliters of purified Dam methyltransferase added to extracts of the Dam-deficient strain GM3819 (□). The ordinate is an integral of the optical density in each band, and the units are arbitrary.

The Dam-specific bands were quantitated by densitometric scanning. The whole width of each band was considered, so that the wider bands occurring when larger amounts of protein were loaded on the gel constituted no source of error. The results show that band intensities were roughly proportional to the amounts loaded in each well (Fig. 3). It can be seen that extracts of strain AB1157 containing 200 μg of protein had a Dam content corresponding to about 0.5 μl (3.2 ng) of purified Dam protein.

To estimate the number of Dam molecules per cell, the concentration of protein in the cell extracts was measured chemically (10) and the concentration of cells was measured by flow cytometry (8). It was found that 10^9 cells of strain AB1157 contained 446 μg of protein. Parallel measurements of strain B/rA yielded a 30% lower value, 350 μg of protein per 10^9 cells, in agreement with previously published data (12). Also, flow cytometry showed that the amount of light scattered (which is roughly proportional to cell size) by strain AB1157 was on the average 30% larger than for strain B/rA (data not shown). Thus, 4.5×10^8 cells of strain AB1157 contained 200 μg of protein, of which 3.2 ng was Dam methyltransferase (Fig. 3). The 3.2 ng of Dam protein consists of 6×10^{10} molecules, assuming that the molecular mass is as predicted by the *dam* nucleotide sequence, 32 kDa. Therefore, there are approximately 130 Dam molecules per cell. As noted above, the error in this estimate may be as high as 50%. We conclude that a wild-type cell contains between 70 and 250 Dam molecules.

In separate experiments, the cellular Dam contents of different wild-type cells treated as described above were

TABLE 1. Dam methyltransferase contents of *E. coli* strains used in this study

Strain	Temp (°C)	Relative Dam content	Source or reference
AB1157	37	1.0	3
AB1157	30	1.0	3
C600	37	1.0	3
W3110	37	1.0	3
MC1000	37	1.3	15
B/rA	37	1.3	H. E. Kubitschek

measured by immunoblotting. The K-12 strains AB1157, C600, and W3110 contained the same amount of Dam protein, while strains MC1000 and B/rA had a 30% higher Dam content (Table 1). MC1000 is a K-12 strain in most respects but contains some chromosomal segments from a B strain (15), which may explain its higher Dam protein content relative to the other K-12 strains. In addition, it was shown that the levels of Dam methyltransferase in strain AB1157 were the same at 30 and 37°C (Table 1).

The Dam-overproducing strain ALO149 contained about 60-fold more Dam enzyme than the wild type (Fig. 3), i.e., about 8,000 molecules per cell. We have shown earlier (7) that when strain ALO149 is grown at 39°C, it contains 2.5-fold less Dam enzyme than at 42°C and initiations of DNA replication are borderline between synchronous and asynchronous. Thus, a variation from 130 to about 3,000 molecules per cell is of little consequence for initiation. Similarly, a 10- to 15-fold increase in the Dam level does not have any effect on the frequency of spontaneous mutations (2, 26), while a 50-fold increase in the Dam level increases the mutation rate significantly (19). It is not known how much the Dam methyltransferase level can be reduced without affecting initiation of DNA replication or mismatch repair.

Dam methylation must keep up with the rate of DNA replication so that all newly replicated DNA is methylated within a few minutes. The following is a calculation of the number of unmethylated GATC sites created per minute by DNA replication, for comparison with the Dam enzymatic activity. At a doubling time of 30 min, strain AB1157 spends 55 min replicating its DNA (the C period) and waits 23 min from the end of replication to cell division (1). Under these conditions, the average cell contains four origins of replication and six replication forks during the first 8 min of the cell cycle (11). In the remaining 22 min (after initiation of DNA replication), the cells contain eight origins and 14 replication forks. Each of the 14 forks synthesizes DNA at a rate of half a genome, or 4.5×10^6 nucleotides of single-stranded, unmethylated DNA, in 55 min. GATC sequences occur on the average once every 222 nucleotides in *E. coli* (6), so each fork produces about 2×10^4 unmethylated GATC sites in 55 min. In 1 min, the 14 forks produce 5,000 unmethylated GATC sites, which leaves about 39 sites to be methylated per enzyme per min. At saturating DNA concentrations in vitro, the turnover number of Dam methyltransferase has been determined to be 19 methyl transfers per min (20). Comparison with the required 39 transfers per min calculated above indicates that Dam methylation activity is limiting in the cell. This conclusion is consistent with the lag observed between DNA replication and full adenine methylation (13) and with the finding that increased replication rates lead to a higher frequency of unmethylated GATC sites (30). In addition, it may be pointed out that not all of the unmethylated GATC sites are available for methylation at any one time, since they may be protected by other DNA-binding proteins. This effect would further increase the requirement for Dam methyltransferase in order for methylation to keep up with the replication forks. If the level of Dam methyltransferase is increased and is no longer limiting, the period of hemimethylation following in the wake of the replication fork becomes shorter, with profound effects on the timing of initiation of DNA replication (7) and on mismatch repair (19, 26).

Evidence that a wild-type *E. coli* cell contains only 1 to 5 Dam molecules (22) or 20 to 40 Dam molecules (30) has been presented. Both estimates are apparently based on data obtained during methyltransferase purification (20). Since the strain used in those studies is different from the one used here and the growth rate of the cells is not stated, those estimates can not easily be compared with the results presented here. However, our calculations above indicate that one to five molecules per cell cannot methylate all newly synthesized GATC sites.

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