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Escherichia coli Cells Lacking Methylation-Blocking Factor (Leucine-Responsive Regulatory Protein) Have Precise Timing of Initiation of DNA Replication in the Cell Cycle

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A protein that is required for specific methylation inhibition of two GATC sites in the *papBA* pilin promoter region, known as methylation-blocking factor (Mbf) and recently shown to be identical to the leucine-responsive regulatory protein (Lrp), is not responsible for the delayed methylation at *oriC* implicated in an eclipse period following initiation of DNA replication. Cells containing a transposon mutation within the *mbf* (*lrp*) gene initiate DNA replication at the correct time during the cell cycle, whereas cells with increased amounts of the Dam methyltransferase initiate DNA replication randomly throughout the cell cycle.

Initiation of DNA replication at *oriC* in the *Escherichia coli* chromosome is tightly controlled by mechanisms that we are beginning to understand. Experimental evidence suggests that there are two modes of control; one acts to prevent reinitiation, and the other is required to begin the process of initiation. The first mode of control functions immediately after an initiation event has occurred. Delayed adenine methylation of individual GATC sites within *oriC* and within the *dnaA* gene promoter region has been implicated in the mechanism underlying the eclipse period during which new rounds of initiation are prevented. The other level of control involves the intracellular concentration of DnaA protein; initiation appears to occur when a critical concentration of this protein has accumulated. In this report, we focus on the first mode of control, which involves adenine methylation of GATC sites by the Dam methyltransferase, the product of the *E. coli dam* gene.

The observation that the origins of replication of enteric bacteria contain a high frequency of GATC sites, eight of which are positionally conserved among six bacterial species (28), suggested that methylation of these sites by the Dam methyltransferase serves a function in the initiation process of DNA replication (30). There are several lines of evidence supporting such a role for Dam methyltransferase. The first type of evidence is the effect of *dam* mutations on replication of plasmids dependent on the *oriC* origin. *E. coli dam* mutants are transformed inefficiently by fully methylated *oriC* plasmids (20, 26). Finding that unmethylated *oriC* plasmids replicate efficiently in *dam* mutants, that hemimethylated daughter molecules accumulate in *dam* mutant strains after transformation with fully methylated plasmids, and that hemimethylated plasmids transform *dam* mutant bacteria poorly, Russell and Zinder (25) concluded that hemimethylated *oriC* replicons cannot undergo initiation. Although unmethylated, fully methylated, and hemimethylated *oriC* templates are replicated in cell-free systems (4, 16), replication of hemimethylated *oriC* templates is inhibited by an outer membrane fraction (16).

The second type of evidence is provided by examination of the effects of Dam methyltransferase on timing of initiation of DNA replication in the cell cycle. Initiation of DNA replication occurs at a specific time in the cell cycle, a time that is dependent on the growth rate of the cell; the period of time between initiation events in a single cell is equal to the cell-doubling time (12). Because *dam* mutants (2, 19), including insertions (18) and a complete deletion of the *dam* gene (1), are viable, GATC methylation events are not essential for the initiation process. However, after it was demonstrated that proper timing of initiation is not required for cell viability (15), the possibility that Dam methyltransferase was required for precise timing in the cell cycle was examined. Cells carrying the *dam-3* mutation have the asynchrony phenotype (6), and results of density shift experiments indicate that *dam* mutants initiate successive rounds of replication at random intervals rather than at a precise interval equal to the doubling time (1). Even though timing is random, the origin of replication used in *dam* mutants is *oriC*, and fork velocity is similar in *dam* mutant and dam^+ cells as indicated by marker frequency analysis (17).

It has been suggested that the function of Dam methyltransferase in the cell other than its role in DNA repair (21) is to participate in maintaining the eclipse period after an initiation event has occurred (25). During the eclipse period, new initiation events would be prevented by maintaining *oriC* DNA in a hemimethylated state. Full methylation of *oriC* could be precluded by binding of a protein to specific *oriC* GATC sites. In fact, hemimethylated *oriC* DNA binds specifically to a membrane fraction, and certain GATC sites in the origin remain hemimethylated for up to 10 min after initiation of replication (24). This work has been extended by Campbell and Kleckner (9), who have shown that two GATC sites in *oriC* as well as one in the *dnaA* promoter region remain in a hemimethylated state after initiation of replication for several minutes longer than GATC sites located outside of these regions. Campbell and Kleckner (9) also found that the period of hemimethylation in the *dnaA*

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promoter region correlated with a 90% reduction in transcription from the two *dnaA* promoters.

Phasing of gene pilus expression by uropathogenic *E. coli* involves oscillation between transcription and lack of transcription from the *papBA* promoter, the phase-on and phase-off states, respectively, and is controlled by differential methylation of two GATC sites, GATC-1028 and GATC-1130, near the *papBA* pilin promoter (3, 7). The GATC-1130 site is unmethylated in phase-off cells, and the GATC-1028 site is unmethylated in phase-on cells. *E. coli dam* mutant strains fail to transcribe *pap* genes, and overexpression of the Dam methyltransferase prevents transition from phase off to phase on (3). A recently discovered protein called methylation-blocking factor (Mbf) functions to protect the GATC-1028 and GATC-1130 sites in the *papBA* pilin promoter from methylation (7). This is the first protein to have been shown to regulate gene expression by binding to GATC sites. Pap pilin expression is greatly reduced in cells containing an mTn*10* transposon insertion in the *mbf* gene, and the GATC-1028 and GATC-1130 sites are fully methylated in this mutant. The Mbf protein recently has been shown to be the global regulatory protein, leucine-responsive regulatory protein (Lrp), which regulates many *E. coli* genes (8).

The Mbf (Lrp) protein is an obvious candidate for the cause of delayed methylation at *oriC* and the *dnaA* promoter region. If delayed methylation at *oriC* and the *dnaA* promoter was mediated by the Mbf (Lrp) protein, then in the absence of this protein the eclipse period would be eliminated. Mutants lacking a protein required for delayed methylation would have the same phenotype as that observed for cells containing excess Dam methyltransferase; both conditions would lead to premature methylation of GATC sites. Here we show that cells that overexpress Dam methyltransferase initiate DNA replication randomly throughout the cell cycle; however, timing is precise in the absence of the Mbf (Lrp) protein.

Overexpression of Dam methyltransferase causes random timing of initiation. We reasoned that methylation of critical GATC sites in newly replicated *oriC* DNA would occur immediately after replication in a mutant defective in a protein responsible for the delayed methylation of these GATC sites and predicted that the phenotype of this mutant would be identical to the phenotype of cells with increased concentrations of Dam methyltransferase. Boye and Løbner-Olesen (5) have shown previously that cells containing a very high concentration of Dam methyltransferase frequently had other than 2^n (n = 0, 1, 2, 3, or 4) number of chromosomes, indicating a defect in DNA replication or in partitioning or segregation of daughter chromosomes. Here we show definitively that this defect occurs in the initiation step of DNA replication. To demonstrate this, we examined whether initiation of DNA replication was properly timed in cells with increased expression of the *dam* gene, using density transfer analysis of pulse-labeled DNA (1, 15, 22). The rationale for the experimental approach is accurately depicted in Fig. 1. Cells are grown exponentially for several generations in heavy (^{13}C, ^{15}N) medium, pulse-labeled with [^{3}H]thymidine, transferred to light (^{12}C, ^{14}N) medium, and sampled for transfer of ^{3}H from HH (heavy label in both strands) to HL (heavy label in only one strand) DNA as determined in a CsCl density gradient. When timing of initiation is normal, the [^{3}H]DNA will shift from HH density to HL density after precisely one generation time. When timing is abnormal, a different pattern of time of transfer will appear. The hypothetical case of abnormal timing shown in Fig. 1 is that of random timing: a second initiation event is equally likely to occur at any time after a first initiation event, with the requirement that total DNA content of the culture doubles each generation time. The fraction of ^{3}H remaining in the HH peak is equal to $1/e^n$, where n is equal to the number of generations after the cells are pulse-labeled with [^{3}H]thymidine and shifted to growth in light medium.

In the experiments for which the results are shown in Fig. 2, single colonies were inoculated into heavy MOPS (morpholinepropanesulfonic acid) medium (23) supplemented with 100 μg of methionine per ml, 0.01% Casamino Acids, 2 mM [^{13}C]glucose (NEN DuPont), and 1.3 mM $^{15}NH_4Cl$ (ICN). After growth overnight, cells were diluted 100-fold into 13 ml of the same medium. When growth reached an optical density at 450 nm of 0.1, cells were pulse-labeled for 8 min with [^{3}H]thymidine (50 μCi at 3,000 μCi/mmol; NEN DuPont) and then diluted into 30 ml of light MOPS medium warmed to 37°C. Light MOPS medium contained nonradioactive thymidine (100 μg/ml), uridine (200 μg/ml), 37 mM [^{12}C]glucose, 33 mM $^{14}NH_4Cl$, methionine (100 μg/ml), and 0.01% Casamino Acids. All cultures were grown at 37°C with shaking. Ampicillin (50 μg/ml) was included in culture media used for growth of DS1310 *dam-3* (pAB20). For induction of *dam* gene expression, IPTG (isopropyl-β-D-thiogalactopyranoside; 1 mM) was added to the medium into which the overnight culture of DS1310 *dam-3* (pAB20) was diluted and was also present in the light-medium chase. Periodically, 10 ml of cells was removed and lysed, and DNA was separated by CsCl equilibrium sedimentation as previously described (29). A ^{14}C-labeled control DNA prepared from strain MG1655 grown in light MOPS medium containing [^{14}C]thymidine was used as density marker DNA. Gradients were centrifuged for >36 h at 35,000 rpm and 15°C in a Beckman VTi65 rotor. Two drop fractions were collected, and 20 μl of each fraction was counted in Cytoscint (ICN).

Timing of initiation in cells with increased Dam methyltransferase is shown to be random in Fig. 2A. The strain of *E. coli* used in these studies was DS1310 F$^-$ *dam-3 rpsL metB1 trpR55 lacY1 galK2 galT22 supE44 supF58 hsdR514* (26). The plasmid pAB20 (*Ptac dam$^+$ bla lacI^q*) was constructed by ligating the 1.3-kb *Pvu*II fragment of pPJ39 (14) into the *Eco*RI-*Hin*dIII fragment of pJF118EH (10). The ends of pJF118EH were filled in with the large fragment of DNA polymerase I prior to ligation. Plasmid pJF118EH contains the *lac* repressor gene, *lacI^q*, and the *tac* promoter which is repressed by *lac* repressor. Expression of the *tac* promoter can be induced with IPTG. The 1.3-kb *Pvu*II fragment contains the complete reading frame of the *dam* gene, and the orientation of the fragment is such that transcription of the *dam* gene is directed by the *tac* promoter. As shown in Fig. 2A, timing in strain DS1310 occurs randomly, as previously demonstrated (1). Interestingly, DS1310 cells containing the plasmid pAB20 have precise timing; the amount of Dam methyltransferase in these cells must approach that in *dam$^+$* cells. When IPTG is added to DS1310 cells containing pAB20 to induce expression of the *dam* gene, however, timing again becomes random.

The *mbf* (*lrp*) gene is not required for precise timing of initiation. Because the Mbf (Lrp) protein binds to certain GATC sequences and prevents their methylation, it could also bind transiently to the GATC sequences in *oriC* and the *dnaA* promoter region, thereby preventing methylation after initiation. To test this hypothesis, we first moved the *mbf-7*::mTn*10* insertion mutation in strain DL845 (7) by P1 bacteriophage transduction (27) to strain MG1655 (11). This *mbf-7*::mTn*10* derivative of strain MG1655 is designated ALS845. As can be seen in Fig. 2B, timing is precise in

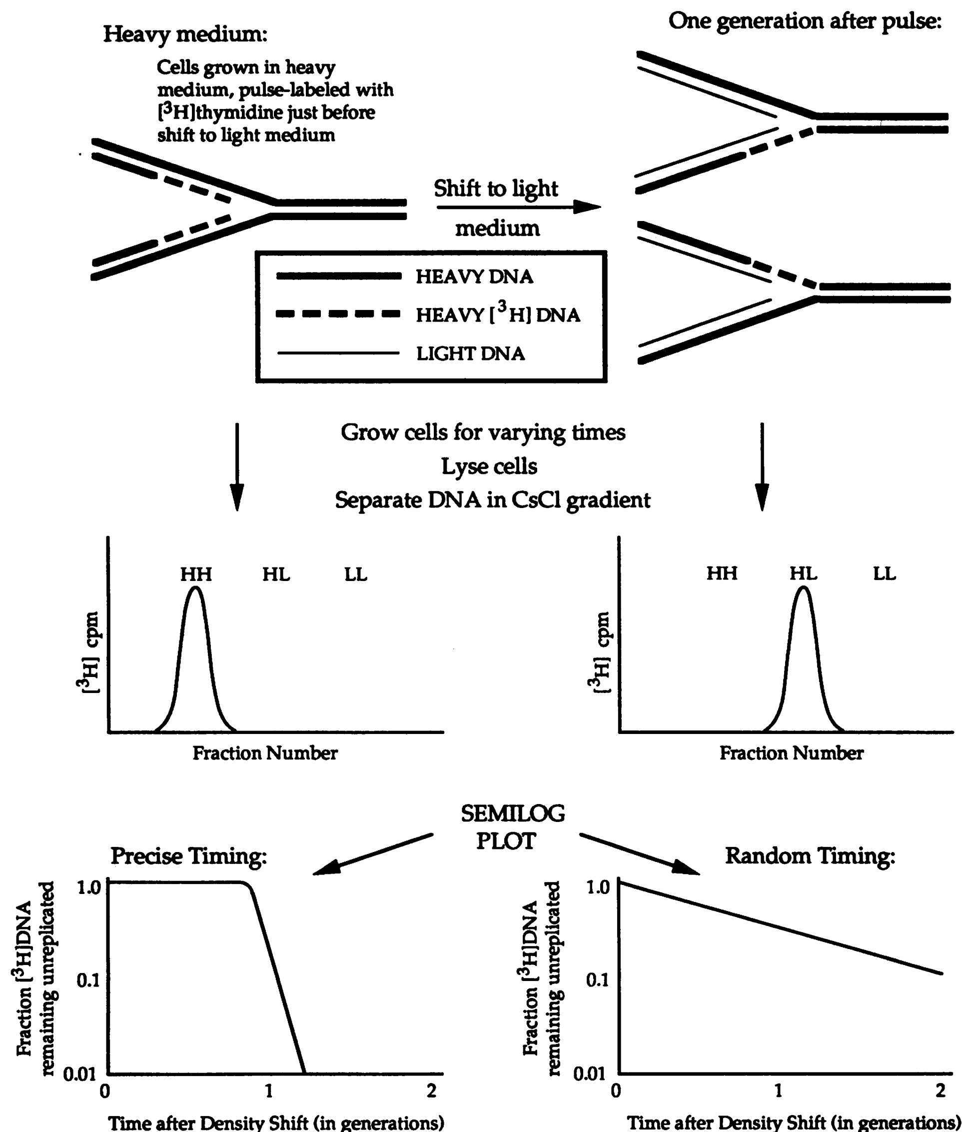

FIG. 1. Rationale for the density transfer experiment to measure timing of initiation of DNA replication during the cell cycle. See the text for details.

strains MG1655 and ALS845. This result rules out the possibility that the Mbf (Lrp) protein is involved in timing of initiation, unlike the Dam methyltransferase, which is required for precise timing.

Strain ALS845 was examined for possible effects of the *mbf-7*::mTn*10* mutation on the number and size of nucleoids in individual cells by the method of Hiraga et al. (13). Cells were grown to mid-log phase in L broth and were spread on a microscope slide, fixed, and stained with DAPI (4′,6-diaminodino-2-phenylindole). No differences between strains MG1655 and ALS845 in the size of cells and the number and size of nucleoids were observed. Less than 0.03% of the cells in strains MG1655 and ALS845 contained no DNA, whereas 1.6% of the cells in strain DS1310 *dam-3* were anucleate.

If the eclipse period following initiation requires that critical GATC sites remain hemimethylated for a period of time after replication, then premature methylation of these sites would result in premature initiation. We demonstrate that premature methylation caused by increasing the intracellular concentration of Dam methyltransferase results in random timing; all origins in these cells have the same probability of initiating DNA replication at any time in the cell cycle. We also demonstrate that an important global regulator, the Mbf (Lrp) protein, which could have played a major role in initiation of DNA replication, is, in fact, not involved in initiation. The possibility that the Mbf (Lrp) protein either regulates expression of a gene involved in precise timing of initiation of DNA replication or prolongs the hemimethylated state of GATC sites in *oriC* was eliminated because a mutation in the *mbf* (*lrp*) gene has no effect on timing of initiation (Fig. 2B). An outer membrane fraction that binds only to hemimethylated *oriC* (24) and inhibits DNA replication of hemimethylated *oriC* templates (16) may contain a protein that causes delayed methylation of these GATC sites. The phenotype of a mutant deficient in a protein that delays methylation at GATC sites in *oriC* would include a deficiency in precise timing of initiation such as is observed

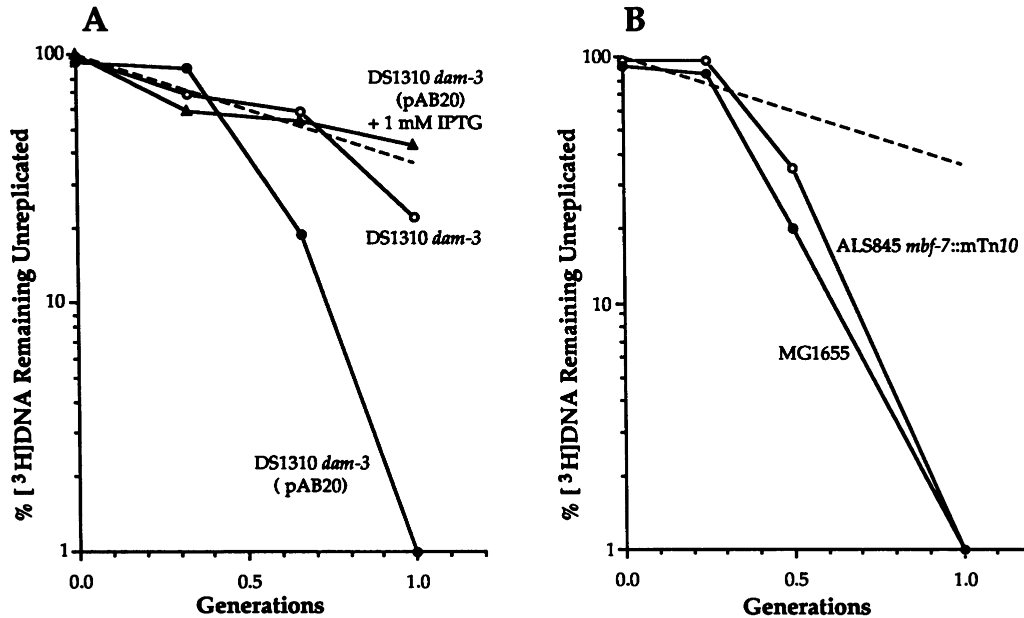

FIG. 2. Time (plotted as fraction of generation time) of transfer of ^{3}H radioactivity from the HH-DNA band into the HL-DNA band (plotted as percent [^{3}H]DNA remaining unreplicated). The rate of transfer expected for random initiation timing is shown as a dashed line. (A) Effects of *dam* overexpression on timing. Strains used were DS1310 *dam-3* (○) and DS1310 *dam-3* (pAB20) (●). Strain DS1310 *dam-3* (pAB20) also was grown in the presence of 1 mM IPTG (▲). (B) Effects of *mbf-7*::mTn*10* on timing. Strains used were MG1655 (●) and ALS845 *mbf-7*::mTn*10* (○).

for *dam* overexpression. Because cells that initiate DNA replication from *oriC* in a random fashion are viable, it should be possible to recover such a mutant after transposon insertion mutagenesis.

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