We have used the Koppes and Nordström (Cell 44:117-124, 1986) CsCl density transfer approach for analysis of DNA from exponentially growing, isogenic Escherichia coli dam+ and dam mutant cells to show that timing between DNA replication initiation events is precise in the dam+ cells but is essentially random in the dam cells. Thus, methylation of one or more GATC sites, such as those found in unusual abundance within the origin, oriC, is required for precise timing between rounds of DNA replication, and precise timing between initiation events is not required for cell viability. Both the dam-3 point mutant and the Δ(dam)100 complete deletion mutant were examined. The results were independent of the mismatch repair system; E. coli mutHLS cells showed precise timing, whereas timing in the isogenic E. coli mutHLSΔ(dam)100 double mutant was random. The mechanism is thus different from the role of Dam methylation in mismatch repair and probably involves conversion of hemimethylated GATC sites present in daughter origins just after initiation to a fully methylated state.

The bacterial origins of DNA replication have been isolated from several enteric bacteria and shown to function as origins in Escherichia coli via E. coli trans-acting factors (D. W. Smith and J. W. Zyskind, in K. W. Adolph, ed., Chromosomes: Eukaryotic, Prokaryotic, and Viral, vol. III, in press). Comparison of the nucleotide sequences of these origins and their ability to function as origins in E. coli provides the rationale for a consensus sequence of the enteric origin of DNA replication, oriC (29). A dominant feature of these origins is the presence of between 9 and 14 GATC sites within each of the six minimal 245-base-pair (bp) origins, plus additional GATC sites immediately outside the minimal origin. Eight of the GATC sites in the minimal origin are positionally conserved, strongly suggesting that these sites are important for origin function.

The dam gene of E. coli encodes a DNA adenine methylase which specifically catalyzes conversion of the adenine residues in GATC sites of double-stranded DNA to 6-methyladenine (5, 11). Dam-catalyzed methylation of GATC sites provides the discriminatory signal whereby the MutHLS-dependent mismatch repair system distinguishes between the correct and incorrect bases in a mismatch introduced during replication (14, 17, 23, 24). Dam methylation also affects expression of some E. coli and phage genes (15), including decreased expression of the E. coli dnaA (3, 10) and mioC (8) genes in dam mutant cells. Transposition frequencies of transposons Tn10 (25) and Tn5 (13, 28) are greatly increased when specific GATC sites are hemimethylated, a state that exists just after replication. GATC sites in oriC function in initiation via methylation of one or more of these sites. E. coli dam mutants are transformed very inefficiently by oriC plasmids (16, 27). Hemimethylated oriC plasmids are incapable of replicating in E. coli dam mutants (26), suggesting that hemimethylated daughter origins of an initiation event must become fully methylated prior to the next initiation event. Furthermore, oriC DNA binds specifically to an outer membrane fraction (19) only when it is in a hemimethylated state (22), leading Schaechter and co-workers to propose a role of oriC GATC methylation state in segregation of daughter chromosomes (22). Here we provide evidence that GATC methylation is important for proper timing of DNA replication initiation events. Specifically, such initiation timing was found to be random during the cell cycle in E. coli dam mutants.

The approach taken, shown schematically in Fig. 1, is similar to that of Koppes and Nordström (9), a modification of the Nagata and Meselson (20) CsCl experiments, which demonstrated sequential replication of the E. coli chromo-

FIG. 1. Schematic rationale of the CsCl experiment. See text for explanation. ssDNA, Single-stranded DNA; cts, counts.
some. Cells are grown exponentially for several generations in heavy medium, pulse-labeled with $^3$H]thymidine, and sampled periodically during growth in light medium. Since chain elongation proceeds sequentially, if initiation timing is precise, the $^3$H]DNA will shift from HH (heavy label in both strands) density to HL (heavy label in only one strand) density within a short time centered at one generation time. If timing is imprecise (time between initiation events is not specific), then some $^3$H]DNA would shift prematurely, and some would shift later than after one generation time.

Strains used were E. coli LE392 (F’ metB1 trpR55 lacY1 galK2 galT22 supE44 supF58 hsdR514 [18]), DS1310 (dam-3 [27]), and DS1355 [Δ(dam)100 kan; Bakker and Smith, manuscript in preparation], isogenic derivatives of LE392 constructed by P1 transduction, and NR3939 [mutH101 thi ara trpE9777 Δ(pro-lac) (F’ pro+ lacIq lacPL8)] (6) and DS1360 [Δ(dam)100 mutH101 kan], an isogenic derivative of NR3939 constructed by P1 transduction. The Δ(dam)100 mutation is a complete deletion of the dam gene, with a kan fragment substituted for a PvuII fragment containing the dam gene (6a); cells harboring this mutation are kanamycin resistant. In the experimental procedure, cells were streaked for single colonies from frozen stocks on agar plates containing minimal MOPS (morpholinepropanesulfonic acid) medium (21) supplemented with 100 μg of methionine per ml and 0.01% casamino acids. Heavy supplemented MOPS medium containing 2 mM $^{13}$C]glucose (ICN) and 1.3 mM $^{15}$NH$_4$Cl (ICN) was inoculated from a single colony, grown overnight at 37°C, diluted 1:200 into 25 ml of fresh heavy medium, and grown to an OD$_{450}$ of 0.1 (about 5 x 10$^7$ cells per ml). Generation times in this heavy medium were 55 to 60 min for LE392, DS1310, and DS1355, 30 min for NR3939, and 37 min for DS1360 (data not shown). Exponentially growing cultures, grown for >8 generations in heavy medium, were pulse-labeled for 7 min with $^3$H]thymidine (50 μCi, 3,000 μCi/mmol; NEN Du Pont) and diluted into 60 ml

FIG. 2. CsCl gradient profiles. (A, C, E) DS1310 dam-3; (B, D, F) LE392 dam$^+$. (A and B) 0.3 generation time; (C and D) 0.9 generation time; (E and F) 1.4 generation times. Positions of HH and HL DNA bands are indicated.
FIG. 3. Time of transfer of \(^{3}H\) radioactivity out of HH DNA band (fraction of DNA unreplicated). Results are plotted on a semilogarithmic plot. The exponential curve expected for random initiation timing and conservation of total amount of DNA per cell is shown as a dashed line. (A) Symbols: \(\circ\), LE392 \(\text{dam}^{+}\); \(\bullet\), DS1310 \(\text{dam}-3\); \(\bigtriangleup\), DS1355 \(\Delta\text{dam}\)100. (B) Symbols: \(\circ\), NR3939 \(\text{mutH}\)100; \(\bullet\), DS1360 \(\text{mutH}\)100 \(\Delta\text{dam}\)100.

of light medium warmed to 37°C and containing nonradioactive thymidine (100 \(\mu\)g/ml) and uridine (200 \(\mu\)g/ml). No lag in growth rate or in rate of DNA synthesis was observed due to these treatments. Periodically, 10-ml samples were removed, cells were lyzed, and DNA was prepared for CsCl equilibrium sedimentation as described previously (30). To confirm the positions of double-heavy HH and light-density LL (light label in both strands) DNA bands, \(^{34}C\)-labeled DNA prepared from LE392 grown in heavy MOPS medium or in light MOPS medium was used as density marker DNA. Centrifugation was for \(>36\) h at 35,000 rpm and 15°C in a Beckman VTi65 rotor. Approximately 100-\(\mu\)l fractions were collected into microtiter dishes, 20-\(\mu\)l portions of each fraction were spotted onto Whatman GF/C filters, and the filters were dried and counted in a Beckman liquid scintillation counter. Correction was made for \(^{14}C\) counts appearing in the \(^{3}H\) channel when needed.

Representative CsCl gradient profiles are shown in Fig. 2 for LE392 and DS1310 \(\text{dam}-3\), and conversion of \(^{3}H\) radioactivity from the HH peak into the hybrid-density HL peak as a function of time of growth in light medium is shown in Fig. 3A for LE392 and its \(\text{dam}\) mutant derivatives. The \(^{3}H\) radioactivity in LE392 \(\text{dam}^{+}\) DNA was converted to HL density in a relatively short time. The midpoint of this transition was at one generation time (Fig. 2B, D, and F; Fig. 3A), demonstrating that initiation timing in this strain is precise, as is typical of wild-type \(E.\ coli\) K-12 bacterial strains (9). In contrast, some transfer of \(^{3}H\) to HL density was seen in DS1310 \(\text{dam}-3\) DNA even after 0.3 generation time of growth in the light medium (Fig. 2A). Furthermore, less conversion of \(^{3}H\) to HL density at 0.9 generation time was seen for DS1310 \(\text{dam}-3\) (Fig. 2C) than for LE392 \(\text{dam}^{+}\) (Fig. 2D), and considerable \(^{3}H\) remained (about 20\%) at HH density even after growth in light medium for 1.4 generation times (Fig. 2E). Indeed, as a function of time of growth in light medium, \(^{3}H\)DNA from either DS1310 \(\text{dam}-3\) or DS1355 \(\Delta\text{dam}\)100 was converted to HL density in an exponential manner (Fig. 3A), as predicted if timing of initiation is completely random (equal probability per unit time for an initiation event at any origin in the exponentially growing population). The slope of this exponential curve was close to that expected for random initiation timing and conservation of total amount of DNA per cell (Fig. 3A, dashed line).

Some \(^{3}H\) counts appeared at light LL density after growth in light medium for 1 generation time or longer. These could be due to residual incorporation of \(^{3}H\)thymidine despite the presence of nonradioactive thymidine and uridine or to repair processes with excision of \(^{3}H\)-labeled nucleotides and subsequent reincorporation. The latter is likely to be more important in the mismatch repair-defective \(\text{dam}\) mutants, although little difference was seen between DS1310 \(\text{dam}-3\) DNA (Fig. 2E) and LE392 DNA (Fig. 2F) at 1.4 generation time.

To determine whether mismatch repair is significant in these experiments, timing in the \(\text{mutH}\) strain NR3939 and an isogenic \(\Delta\text{dam}\)100 derivative of NR3939, DS1360, was examined. The results (Fig. 3B) were nearly identical to those for LE392 and its \(\text{dam}\) mutant derivatives. The \(^{3}H\) radioactivity in NR3939 \(\text{mutH dam}\)1 DNA was converted to HL density in a short time, about 1 generation time, whereas for DS1360 \(\text{mutH dam}\)100 was converted to HL density in an exponential manner (Fig. 3B). Timing in the \(\text{mutH}\) strain, deficient in the GATC-dependent MutHLS mismatch repair system (12, 17), was precise, whereas timing in a \(\text{dam}\) mutant derivative of this strain was essentially random. Thus, the observed timing of initiation properties is independent of the MutHLS mismatch repair system, and this provides further evidence that the role of Dam methylation in initiation is different from its role in mismatch repair, as suggested previously (27).

These experiments provide a clear and definitive demonstration that precise timing between rounds of DNA replication in \(E.\ coli\) K-12 requires methylation of one or more GATC sites, most probably one or more of those found in...
oriC. Although all GATC sites within oriC are methylated in wild-type cells (16, 27), some sites are methylated more rapidly than others after an initiation event (22). Although the mechanism by which timing is rendered precise by GATC methylation events is unknown, such a mechanism is probably associated with conversion of one or more hemi-methylated GATC sites, resulting from replication through such sites after an initiation event, to a completely methylated state. Such methylation, however, may not be sufficient for precise timing; other events may also be required. E. coli dam mutants most likely initiate from oriC, although this has not been shown definitively. Constitutive stable DNA replication (7) does not occur in a dam-3 mutant (16), and hence the initiation sites used in stable DNA replication (4) are probably not used by dam mutants. Furthermore, Δ(dam)100 derivatives of EC560 dnaA508 and EC558 dnaA46 remain temperature sensitive for growth, indicating that initiation in dam mutants remains DNA dependent (data not shown).

Flow cytometry studies showed that rifampin-treated DS1310 dam-3 cells contain a broad distribution of numbers of chromosomes, with an average of 12 per cell (2). Although these findings were interpreted to indicate lack of precision in timing between initiation events, they could also be the result of other defects in the initiation process or of improper segregation of daughter chromosomes. The CsCl method used here examines timing of replication as the difference in time between successive replication events in different parts of the bacterial chromosome as found in different cells of the exponentially growing population at the time of the 3H pulse. It thus examines only timing effects, under balanced growth conditions.

These results provide further evidence that precise timing between rounds of DNA replication is not required for E. coli cell viability, as also shown by Koppes and Nordström (9). The function of Dam methylation in important but nonessential steps in initiation (Smith and Zyskind, in press) provides a rationale for viability of DS1335 Δ(dam)100 cells, cells completely lacking the dam gene. Dam-catalyzed methylation of GATC sites is found only in the enteric bacteria and a few other genera (1). One may then ask whether bacteria lacking Dam-mediated GATC methylation use an alternative mechanism to effect precise timing between initiation events or whether initiation timing is imprecise in most of the bacterial world.

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