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The Hemimethylated Replication Origin of *Escherichia coli* Can Be Initiated In Vitro

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Unmethylated, fully methylated, and hemimethylated *oriC*-containing plasmids were assayed as substrates for DNA replication in vitro by using a system reconstituted with pure proteins. In contrast to the in vivo situation, all three substrates were initiated efficiently; the fully methylated plasmid was about twice as active as the other two.

The minimal chromosomal origin of replication in *Escherichia coli*, the 245-bp *oriC* sequence, contains 11 GATC sequences (18, 25). GATC is the recognition sequence for Dam methyltransferase (10, 14), which methylates the adenine in the N-6 position. As *oriC* is replicated, each fully methylated parental strand becomes paired with a newly synthesized and therefore unmethylated strand to yield a hemimethylated duplex, whereupon Dam methyltransferase methylates the unmethylated GATC sites in the nascent strand.

Evidence is mounting from in vivo experiments that GATC methylation is a regulatory event in the initiation of DNA replication. (i) Plasmids dependent upon *oriC* for replication (minichromosomes) transform *dam* mutants poorly (19, 24), and hemimethylated minichromosomes accumulate after transformation (22). (ii) Hemimethylated but not fully methylated *oriC* can be found sequestered in the membrane (20). In cells growing rapidly, methylation of *oriC* after passage of the replication fork takes 8 to 10 min (6, 20), a lag that may provide an eclipse period during which *oriC* is not available for reinitiation. (iii) In a *dam* mutant cell, all origins present are not initiated simultaneously (4), in contrast to the synchrony of initiation observed in wild-type cells (23). Synchronous initiation requires a specific level of Dam methyltransferase activity (3), consistent with a model in which the rate of methylation of hemimethylated sites in *oriC* is critical for the timing of initiation.

In vitro experiments have failed to demonstrate any major deficiencies of undermethylated *oriC* as a substrate in initiation, although unmethylated minichromosomes are somewhat poorer substrates for replication than are fully methylated ones (12, 19, 24). Recently, hemimethylated plasmids were shown to be replicated in a crude enzyme system at about half the rate of fully methylated ones (15). In the present work we investigated the significance of the methylation status of *oriC* in an in vitro replication assay employing pure protein components.

The bacterial strains used for propagating the *oriC*-containing plasmid pTB101 (2) were AB1157 *dam*$^+$ (1) and GM2927 *dam-13*::Tn*9* (17). In cells carrying the latter mutation, no evidence of 6-methyl-adenine could be found in the DNA (21). Restriction enzymes were from Pharmacia, Dam methyltransferase was from New England BioLabs, and *E. coli* DNA ligase was from Toyobo Biochemicals. Enzymes were used according to the suppliers' instructions.

Unmethylated or fully methylated pTB101 plasmids were propagated in strains GM2927 and AB1157, respectively. The methylated plasmids were digested with *Hin*cII; the unmethylated plasmids were digested with *Eco*RV. These restriction enzymes each cleave the plasmid at one site in the linker region. The digested linear plasmids were denatured, mixed, and renatured essentially as described earlier (11). The renatured plasmids were of three kinds: fully methylated and unmethylated linear molecules and hemimethylated molecules in which the single-stranded overhang allowed the formation of circular plasmids. These circles were closed covalently by treatment with *E. coli* DNA ligase, which is unable to ligate the blunt-ended linear molecules. Covalently closed circles were purified by equilibrium density gradient centrifugation. Insignificant degradation of the purified, hemimethylated circles was observed after digestion with the restriction enzymes *Dpn*I and *Mbo*I, which cleave fully methylated and unmethylated DNA, respectively.

oriC-containing plasmids differing in their methylation status were tested as substrates for the initiation of DNA replication in the standard reconstituted DNA replication assay (2, 13), with the following modifications: the standard reaction (25 μl) contained 170 ng of DnaA protein, 21 ng of HU protein, and no RNA polymerase. DNA gyrase, which allows rapid supercoiling of the relaxed hemimethylated templates, is a standard ingredient in the assay. The radioactively labeled precursor was [α-^{32}P]dCTP (Amersham, England). All constitutents were mixed at 0°C and incubated at 37°C for a predetermined period of time. Total nucleotide incorporation (picomoles of DNA synthesized) was measured by liquid scintillation counting after precipitation with trichloroacetic acid and filtration onto Whatman GF/C filters.

After a brief lag, replication of minichromosomes is complete within a few minutes in the assay reconstituted with pure proteins (2, 13). Replication of unmethylated and hemimethylated plasmids was efficient and at about half the rate of fully methylated plasmids (Fig. 1). Hemimethylated plasmids methylated by Dam methyltransferase to full resistance to *Mbo*I cleavage were replicated as efficiently as those purified from a *dam*$^+$ strain (Fig. 1). The relative template efficiencies of the plasmids in the three states of methylation proved to be similar at all DnaA concentrations (Fig. 2).

Thus, initiation of replication of hemimethylated and unmethylated *oriC* in a reconstituted pure protein system is efficient and about half that of a fully methylated template. It may be inferred that proteins necessary for initiation (DnaA,

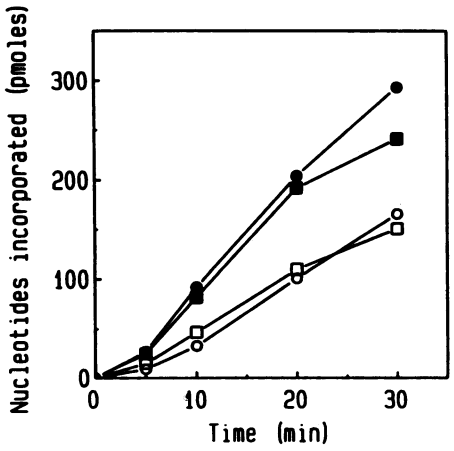

FIG. 1. Replication of minichromosome pTB101 in different states of methylation. Replication was in the standard reconstituted assay with DNA templates that were unmethylated (○), fully methylated (●), hemimethylated (□), and fully methylated in vitro from the hemimethylated state by *dam* methyltransferase (■).

DnaB, DnaC, primase) are not appreciably sensitive to the methylation status of the *oriC* region. This result is in agreement with replication assays in cruder systems employing unmethylated plasmids (12, 19, 24) and hemimethylated plasmids (15). The lack of initiation of hemimethylated origins in vivo implies that *E. coli* contains a factor, absent from our replication system, that inhibits initiation of hemimethylated *oriC*. In accordance with this, recent evidence shows that the outer membrane of *E. coli* contains a factor that inhibits specifically the initiation of hemimethylated *oriC* (16). This factor may sequester hemimethylated origins in a membrane-bound state (20) that protects them from Dam methyltransferase action (6, 20). In vivo, unmethylated minichromosomes are replicated better than hemimethylated ones (21), in contrast to the results presented here, further arguing that the in vivo and in vitro effects of methylation are distinct.

Base pairing of 6-methyl-adenine with thymine occurs only in the energetically unfavorable *trans* configuration (8). The lowered thermal stability of fully methylated DNA compared with that of partly methylated or unmethylated DNA (7, 9) has been demonstrated directly for *oriC* (26). The initial unwinding of *oriC* occurs within a segment containing three consecutive repeats of a 13-mer nucleotide sequence (5), each with the methylatable GATC site. Facilitation of unwinding mediated by the methylation of these GATC sites may be the basis for the enhanced efficiency of initiation of fully methylated *oriC* compared with those of the unmethylated and hemimethylated forms.

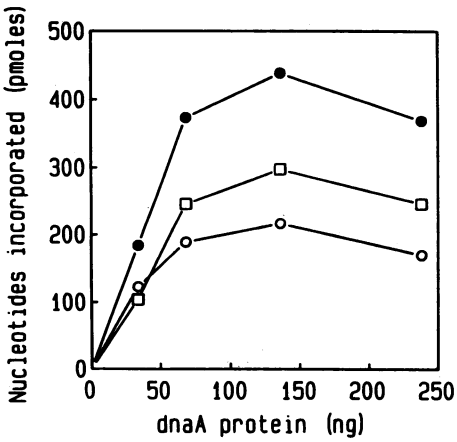

FIG. 2. Dependence on DnaA protein for replication activity. Replication was in the standard reconstituted assay with different amounts of DnaA protein and plasmid pTB101 that was unmethylated (○), fully methylated (●), and hemimethylated (□). The reaction time was 30 min.

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