Interaction of SeqA and Dam Methylase on the Hemimethylated Origin of Escherichia coli Chromosomal DNA Replication*  

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Preferential binding of SeqA protein to hemimethylated oriC, the origin of Escherichia coli chromosomal replication, delays methylation by Dam methylase. Because the SeqA-oriC interaction appears to be essential in timing of chromosomal replication initiation, the biochemical functions of SeqA protein and Dam methylase at the 13-mer L, M, and R region containing 4 GATC sequences at the left end of oriC were examined.

We found that SeqA protein preferentially bound hemimethylated 13-mers but not fully nor unmethylated 13-mers. Regardless of strand methylation, the binding of SeqA protein to the hemimethylated GATC sequence of 13-mer L was followed by additional binding to other hemimethylated GATC sequences of 13-mer M and R. On the other hand, Dam methylase did not discriminate binding of 13-mers in different methylation patterns and was not specific to GATC sequences. The binding specificity and higher affinity of SeqA protein over Dam methylase to the hemimethylated 13-mers along with the reported cellular abundance of this protein explains the dominant action of SeqA protein over Dam methylase to the newly replicated oriC for the sequestration of chromosomal replication.

Furthermore, SeqA protein bound to hemimethylated 13-mers was not dissociated by Dam methylase, and most SeqA protein spontaneously dissociated 10 min after binding. Also, SeqA protein delayed the in vitro methylation of hemimethylated 13-mers by Dam methylase. These in vitro results suggest that the intrinsic binding instability of SeqA protein results in release of sequestrated hemimethylated oriC.

In the Escherichia coli chromosome, the adenine residues of GATC sequences are recognized and methylated by Dam methylase (1–3). The protein transfers a methyl group from a methyl donor, S-adenosylmethionine, to the 6-amino group of adenine residues in hemimethylated and unmethylated GATC sequences (4). Until methylated by Dam methylase, the newly replicated GATC sequences exist in a hemimethylated state (4). Until methylated by Dam methylase, the newly replicated GATC sequences (4). Under the reported cellular abundance of this protein explains the dominant action of SeqA protein over Dam methylase to the hemimethylated 13-mers along with the binding of SeqA protein to the hemimethylated GATC sequence of 13-mer L was followed by additional binding to other hemimethylated GATC sequences of 13-mer M and R. On the other hand, Dam methylase did not discriminate binding of 13-mers in different methylation patterns and was not specific to GATC sequences. The binding specificity and higher affinity of SeqA protein over Dam methylase to the hemimethylated 13-mers along with the reported cellular abundance of this protein explains the dominant action of SeqA protein over Dam methylase to the newly replicated oriC for the sequestration of chromosomal replication.

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Regulation of E. coli chromosomal replication is strict and occurs mainly at the origin of E. coli chromosomal replication, oriC (16, 17). Within the 245 base pairs of oriC are 11 repeats of GATC sequences (18); fully methylated oriC is active for initiation of replication, whereas hemimethylated oriC is inactive for initiation of replication (6, 8, 20). In E. coli with a doubling time of 30 min, methylation of oriC by Dam methylase is delayed for 13 min after replication (21). In seqA mutants, methylation occurs after only 5 min. These results indicate that the 8-min difference is in part caused by sequestration of oriC by SeqA protein encoded by seqA gene. SeqA preferentially binds to hemimethylated plasmid DNA and GM3819(dam16) (22, 25); seqA null mutant exhibits asynchronous and increased chromosomal initiation, indicating that SeqA protein is a negative modulator of E. coli chromosomal replication (19, 21, 34). Also, SeqA protein was independently identified as a protein that bound to the hemimethylated bacteriophage P1 origin (22).

To understand the in vivo role of SeqA and Dam proteins on hemimethylated oriC, we studied biochemical functions of the two proteins on the three AT-rich 13-mer sequences termed L, M, and R containing four GATC sites at the left end of oriC (23). In addition, footprints demonstrate that SeqA protein binds hemimethylated GATC sequences.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Sources were as follows: [γ-32P]ATP (5000 Ci/mmol), Amersham Pharmacia Biotech; polysty-d(C) and Sephadex-G50, Amersham Pharmacia Biotech; long range polyacrylamide, FMC Corp. Bioproducts; T4 polynucleotide kinase, New England Biolabs Inc.; restriction and cloning enzymes, Promega; and nitrocellulose filter (HAWP, 0.45 μm), Millipore. Unless otherwise indicated, other reagents were purchased from Sigma.

**Bacterial Strains and Plasmid DNAs**—The E. coli strain DH5α (24) for fully methylated plasmid DNA and GM3819(dam16:Km) (7) for unmethylated plasmid DNA were used for isolation of plasmid DNA. E. coli MC1061 (24) and BL21 (24) were used for the overproduction of Dam and SeqA proteins, respectively. The plasmid pSS1 (25), pBMA1 (26), pBAD18 (27), and pBlueScript SK+(Stratagene) were previously described. To construct pBAD18-Dam, the coding region of Dam was obtained by polymerase chain reaction and inserted into the EcoRI and HindIII sites of vector pBAD18.

**Proteins**—SeqA protein was purified from BL21(pLys, pSS1) as described previously (22, 25) with modifications. Following a 2-h induction after the addition of isopropyl-1-thio-galactopyranoside to 0.5 mM at A600 = 0.4, cells were harvested by centrifugation, resuspended in suspension buffer (25 mM HEPES-KOH (pH 7.5), 1 mM EDTA, 10% sucrose, and 1 mM DTT), frozen in liquid nitrogen, and stored at 80 °C. On thawing, cells were autolysed by T7 lysozyme expressed from plasmid plys. Lysis was augmented by the addition of 1/30 volume of lysis salts (2.5 mM KCl, 0.2 mM EDTA, 0.2 mM spermidine, and 10 mM

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1 The abbreviations used are: DTT, dithiothreitol; OP-Cu(II), 1,10-phenanthroline-copper (II).
DTT). The autolysed cells were centrifuged for 45 min at 45,000 rpm in a Beckman 70Ti rotor. The supernatant was precipitated by the addition of 0.35% ammonium sulfate followed by centrifugation for 15 min in a Rontron A8.24 rotor and resuspended in buffer A (50 mM Tris (pH 7.5), 5 mM EDTA, and 1 mM DTT). The resuspended material was dialyzed against buffer A containing 50 mM KCl and loaded onto a heparin-agarose column equilibrated with buffer A containing 50 mM KCl. SeqA protein was eluted by a linear gradient of 10 column volumes of 50 mM to 1 M ammonium sulfate. Near homogeneous and concentrated (1.38 mg/ml) SeqA protein was obtained by elution with buffer D containing 1 M ammonium sulfate.

Dam methylase was purified from CM1061 (pBAD18-Dam) as described previously (28) except the blue-Sepharose column was omitted.

**Gel-shift Assay**—To obtain differently methylated duplex 13-mers (Fig. 1), synthesized oligonucleotides containing 13-mer regions were mixed with appropriate combinations, [32P]-labeled with T4 polynucleotide kinase and [γ-32P]ATP, heated, and annealed by slow cooling. The annealed 13-mers were separated from ATP by passing through Sephadex G-50 column.

Gel-shift assays were performed as described previously (26) with minor modifications. 20 μl of binding mixture contained 1 μg of poly(dI-dC) and 1.5 fmol of [32P]-labeled hemimethylated 13-mers, unless indicated, in binding buffer (10 mM Tris-HCl (pH 7.6), 50 mM KCl, 1 mM EDTA, 1 mM DTT, and 10% glycerol). The indicated amounts of proteins were added and incubated for 20 min at 32 °C. The subsequent steps were performed as described previously (29).

**Filter Binding Assay**—Filter binding assays were performed as described previously (30) with minor modifications. The indicated amounts of proteins were added to 20 μl of the binding mixture described under “Gel-shift Assay” and incubated for 20 min at 32 °C. The reactions were then filtered through nitrocellulose. Filters were washed with 200 μl of binding buffer, dried, and quantitated using a liquid scintillation counter.

1,10-Phenanthroline-Copper Ion Nuclease in Situ Footprinting—1,10-Phenanthroline-copper (II) (OP-Cu(II)) nuclease footprinting was performed as described previously (31) with minor modifications. The EcoRI/HindIII fragment of plasmid pBMA1 from *E. coli* (DH5α (dam−) or GM38519dam16Km−) (7) were dephosphorylated with calf intestinal alkaline phosphatase and [32P] end-labeled either at the EcoRI or HindIII restriction site as described above. Unmethylated and methylated fragments were then mixed, and hemimethylated fragments were generated by heat denaturation and renaturation. A gel-shift assay described above was performed with 20 fmol of the fragment. After electrophoresis, the gel was immersed in 200 μl of 10 mM Tris-HCl (pH 8.0), followed by addition of 20 μl of OP-Cu(II) solution (2 mM 1,10-phenanthroline and 0.45 mM CuSO₄). The cleavage reaction was initiated by the addition of 20 μl of 38 mM 3-mercaptopropionic acid, followed by incubation for 8 min at room temperature. 20 μl of 28 mM 2,9-dimethyl-1,10-phenanthroline was added to quench the cleavage reaction. Subsequent steps were performed as described previously (29).

**In Vitro Methylation Assay**—The Dam methylation assay was performed as described previously (32). 500 fmol of indicated 13-mers was added to 20 μl of Dam assay mixture (0.1 mM Tris-HCl (pH 8.0), 10 mM EDTA, 2.5 mM DTT, and 1.6 μM S-adenosyl-[methyl-3H]-methionine. Dam methylase was then added to the mixture, incubated at 37 °C, and filtered through Whatman DE81 paper. Filters were washed with 0.4 mM ammonium bicarbonate, further washed with cold ethanol, dried, and quantitated using a liquid scintillation counter.

**RESULTS**

Preferential Binding of SeqA Protein to the Hemimethylated 13-mers—Because there are 11 unique GATC sequences within oriC (18), it was first necessary to decrease the ambiguity of analyzing SeqA protein binding to these regions. This was accomplished by synthesizing un-, hemi-, and fully methylated 13-mer oligonucleotides containing 4 GATC sequences. Synthesized oligonucleotides were annealed to each other and used for this study unless otherwise indicated (Fig. 1). 20 μl of binding reaction mixture contained 0.08 ng (1.5 fmol) of [32P]-labeled 13-mers and 1 μg of poly(dI-dC) as competitor DNA to prevent nonspecific binding.

In nitrocellulose filter binding assays, SeqA protein preferentially bound to hemimethylated 13-mers compared with fully or unmethylated 13-mers (Fig. 2A). Approximately 32 ng of SeqA protein was required to saturate the binding. Such preference of SeqA protein to hemimethylated 13-mers was also observed in gel-shift experiments (Fig. 2B). SeqA protein formed two distinct complexes with hemimethylated 13-mers, whereas fully or unmethylated 13-mers did not form any complex. Of the complexes, the fast migrating complex was formed at lower SeqA concentration, whereas the slow migrating complex was at higher concentration. Both bands were supershifted by the addition of SeqA antiserum, indicating that these complexes were in fact formed by binding of SeqA protein (Fig. 2C).

The binding affinities of SeqA protein for differently methylated 13-mers were compared by challenging the binding of SeqA to labeled hemimethylated 13-mers with unlabeled and differently methylated 13-mers using filter binding assays (Fig. 3A). Binding of SeqA protein to hemimethylated 13-mers was reduced by competition with hemimethylated but not by fully or unmethylated 13-mers. These results also confirm the preferential binding of SeqA protein to hemimethylated 13-mers. The fast migrating complex, which was formed at lower SeqA concentrations (Fig. 2B), persisted at higher concentrations of unlabeled hemimethylated 13-mers compared with the slow migrating complex formed at higher SeqA concentrations (Fig. 3B). This result indicates that SeqA protein possesses different affinities for each binding site of hemimethylated 13-mers.

Localization of the Tight Binding Site of SeqA Protein on the Hemimethylated 13-mers—To localize SeqA binding sites on the hemimethylated 13-mers containing four GATC sequences, in situ footprinting was performed using the OP-Cu(II) complex as a chemical DNA cleavage agent (31). A gel-shift assay was performed with the hemimethylated 13-mers in which adenine residues of GATC sequence were methylated on either the top (Fig. 4A) or bottom strand (Fig. 4B). The 5’-ends of methylated strands were [32P]-labeled. After treatment of the gel with OP-Cu(II), the DNA in each band was isolated and subjected to electrophoresis through a denaturing sequencing gel. Regardless of top or bottom strand methylation, the protected region in the fast migrating complex localized to the GATC sequence of the 13-mer L and its close sequences. In the slow migrating complex, additional protected regions appeared at the GATC sequences of the 13-mer M and R. These footprinting results imply that SeqA protein binds first to the hemimethylated GATC sequence of the 13-mer L and subsequently binds to the remaining hemimethylated GATC sequences.

**Dissociation of SeqA Protein from the Hemimethylated 13-mers Is Not Affected by Dam Methylase**—Methylation of hemimethylated oriC by Dam methylase is delayed 8 min longer in

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**Fig. 1. 13-mer region of oriC.** The 13-mer region of oriC studied contains 13-mer L, M, R, and four GATC sequences (23, 28). Oligonucleotides for the top and bottom strands were synthesized in forms of adenomethylated and unmethylated GATCs. For the adenomethylated residune in GATC sequences, N6-methyl deoxyadenosine β-cyanoethylphosphoramide (Amersham Pharmacia Biotech) was used for oligonucleotide synthesis. The oligonucleotides were annealed to generate duplex DNA containing fully-, hemi- and unmethylated 13-mers.
wild type cells than in seqA mutants (21). To assess the contribution of SeqA protein to this delay, the binding stability of SeqA protein to hemimethylated 13-mers was determined.

After incubation of SeqA protein with 32P-labeled hemimethylated 13-mers without poly(dI-dC), a large excess of unlabeled hemimethylated 13-mers was added. At the indicated time, the residual complex was separated through filter binding (Fig. 5A) or gel-shift assays (Fig. 5, B and C). In filter binding assays, the half-life of SeqA protein bound to hemimethylated 13-mers was determined to be 4 min (Fig. 5A). In gel-shift experiments in which the complexes were separated (Fig. 5, B and C), the half-life of the fast migrating complex was determined to be 4 min, whereas the half-life of the slow migrating complex was 1.5 min. After 10 min, most of the bound SeqA protein was dissociated from the hemimethylated 13-mers.

Binding specificity of Dam methylase to un-, hemi-, and fully methylated 13-mers was also determined using filter binding assays (Fig. 2B).
assays (Fig. 6A). In contrast to SeqA protein, Dam methylase did not discriminate between the methylation status of 13-mers and bound equally to hemi-, fully, and unmethylated 13-mers. Also, the differently methylated 13-mers yielded identical patterns of shifted bands by Dam methylase in gel-shift assays (data not shown). Binding of Dam methylase was found to be nonspecific because Dam methylase did not discriminate between the number or existence of GATC sequences.

Because SeqA and Dam methylase both act on the same
substrate, the possible dissociation of SeqA protein bound to hemimethylated 13-mers by Dam methylase was examined (Fig. 6, B and C). Incubation of 17 ng (39 nM) of SeqA protein with hemimethylated 13-mers and S-adenosylmethionine was followed by the addition of 250 ng (400 nM) of Dam methylase (Fig. 6B, left panel) or a large excess of unlabeled hemimethylated 13-mers (Fig. 6B, right panel). Re-binding of dissociated SeqA protein to the labeled probe was inhibited by methylation of the SeqA binding sites by Dam methylase. Re-binding could also be inhibited by the addition of a large excess hemimethylated 13-mers.

Migration of the fast migrating SeqA-13mer complex was close to one of the bands shifted by Dam methylase. Therefore, the slow migrating SeqA-13 mer complex was quantitated (Fig. 6C). The stability of the slow migrating complex in the presence of Dam methylase was similar to the stability obtained in the presence of unlabeled hemimethylated 13-mers. These results indicate that Dam methylase does not affect the stability of SeqA protein bound to hemimethylated 13-mers, although both SeqA and Dam methylase act on the same hemimethylated GATC sequence. Because the presence of a large excess amount of poly(dI-dC) in SeqA binding assays reduced the stability of both fast and slow migrating complexes in Fig. 6B, the half-life of the fast migrating complex determined in Fig. 6B was different from that in Fig. 5C.

**Delay of Dam Methylation on Hemimethylated GATC Sequences by SeqA Protein**—Dam methylase catalyzes the conversion of unmethylated GATC to hemimethylated GATC and of hemi- to fully methylated GATC. Therefore, the effect of SeqA protein on Dam methylation in the 13-mer region of oriC was examined. Un- or hemimethylated 13-mers were incubated with or without SeqA protein, followed by the addition of Dam methylase and S-adenosyl-[methyl-3H]methionine (Fig. 7B). At the indicated time, incorporation of the [3H]methyl group into DNA was determined. In the absence of SeqA protein, the incorporation of the methyl group onto hemimethylated 13-mers was half that of unmethylated 13-mers. This is presumably because the available Dam methylase sites on the hemimethylated DNA were half the available sites on the unmethylated DNA. In the presence of SeqA protein, Dam methylation of unmethylated 13-mers was somewhat inhibited (Fig. 7A). This inhibition could be caused by the binding of SeqA protein to the intermediate hemimethylated GATC sequences produced by Dam methylase. When the hemimethylated 13-mers were bound by SeqA protein, Dam methylation was delayed (Fig. 7B). Because Dam methylase was unable to dissociate SeqA protein bound to hemimethylated 13-mers (Fig. 6B), the lag before methylation might be attributed to the time required for dissociation of SeqA protein because of intrinsic binding stability of the protein.

**DISCUSSION**

During replication of the *E. coli* chromosome, newly synthesized strands exist as unmethylated species until methylated by Dam methylase (21). In most regions of the chromosome, nascent strands are methylated immediately, but methylation of the nascent oriC strand, which contains 11 GATC sequences, is delayed for about 13 min, resulting in a hemimethylated state of oriC (21). Duration of this hemimethylated oriC is reduced to 5 min in seqA mutants (21). These results, along with studies demonstrating the preferential binding of SeqA protein to hemimethylated oriC (25), imply that SeqA protein is responsible for the maintenance of hemimethylated oriC.

Because oriC contains 11 repeats of GATC sequence (18), the complexity of binding kinetic analysis and localization of SeqA protein to hemimethylated oriC were reduced by using the 13-mer region of oriC containing only 4 GATC sequences (Fig. 1). Consistent with the results reported with oriC region (25) and bacteriophage P1 DNA (22), SeqA protein specifically bound to hemimethylated 13-mers (Fig. 2). Two differently migrating complexes formed by SeqA and hemimethylated 13-mers were separated in gel-shift assays, and their formation and stability were analyzed (Figs. 2–5). The fast migrating complex, which was produced at lower concentrations of SeqA protein, was formed by the binding of SeqA protein to the hemimethylated GATC sequence of 13-mer L and its close sequences. At higher concentrations of SeqA protein, additional binding to unoccupied hemimethylated GATC sites of the fast migrating complex by SeqA protein produced the slow migrating complex. Regardless of top or bottom strand methylation, binding modes of SeqA protein appeared to be similar in the OP-Cu(II) footprinting. Measurement of complex stability indicated that binding of SeqA protein to the GATC sequence of 13-mer L is tighter and more stable than to other GATC sequences in hemimethylated 13-mers. These results suggest that the flanking sequences as well as hemimethylation of GATC duplex DNA is a determinant for the binding of SeqA protein. It remains to be determined whether additional binding of SeqA protein is cooperative. Currently, we are analyzing whether SeqA protein preferentially binds certain sites in hemimethylated oriC and dnaA promoter region.

It has been suggested that Dam methylase binds nonspecifically to DNA and diffuses along the duplex DNA until a GATC sequence is encountered (32). Dam methylase then transfers methyl group with equal efficiency to both hemi- and unmethylated DNA (33). Our studies, using differently methylated 13-

![Fig. 7. Delay of Dam methylation by SeqA protein.](https://example.com/fig7.png)
mers (Fig. 6), showed that binding of Dam methylase to DNA is nonspecific and does not exhibit methylation specificity.

The binding of SeqA protein will compete with methylation by the Dam protein to the hemimethylated GATC sequences on newly replicated oriC. A single E. coli contains approximately 1,000 molecules of SeqA protein (25), whereas there are about 130 molecules of Dam methylase (5). Compared with Dam methylase, not only the abundance of SeqA protein but also the specific binding and higher affinity of SeqA protein to hemi-methylase, not only the abundance of SeqA protein but also the

oriC

seqA

Figure 5, which roughly matches with the 8-min delay of Dam methylation in

vitro

after 10 min with a half-life of 4 min (Fig. 5), which suggests that the spontaneous dissociation of SeqA protein will be followed by Dam methylation.

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