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Identification of Hemimethylated DNA Binding Activity in the *seqA* Mutant

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A 245 bp segment of *E. coli* chromosomal replication origin, *oriC*, contains 11 repeats of the GATC sequence in which adenine is methylated by Dam methylase. Newly replicated *oriC* is hemimethylated. The parental strand of the newly replicated *oriC* is methylated, but the nascent strand is not yet methylated until methylated by Dam methylase. The hemimethylated *oriC* plays an important role in the regulation of chromosomal replication. Activity in the *seqA* mutant was identified to bind preferentially to hemimethylated DNA, but not to fully-methylated DNA. This activity may participate in the sequestration of initiation of chromosomal replication.

The *Escherichia coli* genome is circular whose replication starts from an unique site *oriC*, origin of chromosomal replication (Marsh and Worcel, 1977; von Meyenburg et al., 1978). The 245 bp segment of *oriC* contains 11 repeats of the GATC sequence (Oka et al., 1980) in which the N6 position of adenine is methylated by Dam methylase (Geier and Modrich, 1979). When replicated, fully methylated *oriC* becomes the hemimethylated state, in which the parental strand is methylated and a newly synthesized strand is not until methylated by Dam methylase (Campbell and Kleckner, 1990). The hemimethylated *oriC* binds to the outer membrane (Hendrickson et al., 1982; Ogden et al., 1988, Landoulsi et al., 1990). SeqA protein has been reported to be responsible for the sequestration of hemimethylated *oriC* (Lu et al., 1994; Slater et al., 1995). SeqA protein preferentially binds to the hemimethylated *oriC* but neither to the fully methylated nor to the unmethylated. The binding of SeqA protein to the hemimethylated *oriC* results in inhibition of reinitiation of replication at *oriC* as well as the methylation of hemimethylated *oriC*. Also, the *seqA* mutant exhibits asynchronous initiation of chromosomal replication, indicating that the timing of replication initiation is disrupted.

Whereas in other regions of the chromosome the newly replicated strand was immediately methylated by Dam methylase, the methylation of a nascent strand of replicated *oriC* was delayed and existed as the hemimethylated state for 13 min (Lu et al., 1994). However, in the *seqA* mutant, duration of a hemimethylated status of *oriC* was reduced to 5 min. These observations may indicate that an unidentified factor(s) exist and function to sustain the hemimethylated state of *oriC* for 5 min in the *seqA* mutant. Therefore, we attempted to identify novel activity in the *seqA* mutant which appears to bind preferentially to hemimethylated DNA containing a part of *oriC*.

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Materials and Methods

Reagents and buffers

[γ-^{32}P] ATP (5,000 Ci/mmol) was purchased from Amersham. HEPES, acrylamide and other reagents were from Sigma.

The TKE buffer contained 25 mM Tris-HCl (pH 7.2), 1 mM EDTA, 2 mM DTT and 0.1 M KCl. Buffer A contained 25 mM Tris-HCl (pH 7.2), 0.1 mM EDTA, 2 mM DTT and 15% glycerol. Buffer B contained 25 mM Tris-HCl (pH 7.2), 1 mM EDTA and 2 mM DTT.

E. coli strains and plasmids

DH5α (*dam*$^+$) (Sambrook et al., 1989), GM3819 (*dam*$^-$ 16::kan) (Boye and Løbner-Olesen, 1990), NK9050 (*seqA*::tet) (Lu et al., 1994) and *hobH*::kan (Garwood et al., 1996) and plasmid poriB (Kim and Hwang, 1995) were previously described. Fully methylated plasmid was isolated from DH5α (*dam*$^+$) and the unmethylated plasmid was isolated from the GM3819 (*dam*$^-$) cells.

Gel-shift assay

A 126 bp *Bam*HI fragment of plasmid poriB was isolated and used as a DNA probe for the gel-shift assay. End-labeling of the DNA fragment and gel-shift assay were performed as previously described (Lee and Hwang, 1997).

Preparation of crude fractions

E. coli NK9050 (*seqA*::tet) was grown at 37℃ in LB

medium to an OD_{600} of 0.6, harvested by centrifugation and resuspended in the TKE buffer. The cells were broken by passage through a French pressure and centrifuged for 45 min at 30,000 rpm in a Beckman Ti70 rotor. The precipitate was resuspended in the TKE buffer containing 1 M KCl, 10 μg/ml of DNase I, 10 μg/ml of RNase A and 5 mM $MgCl_2$, sonicated for 1 min and incubated for 30 min at 4℃. The suspension was centrifuged for 20 min at 30,000 rpm in a Beckman Ti70 rotor. Polyethyleneimine (pH 7.9) was added to 0.5%, stirred for 30 min at 4℃ and centrifuged for 15 min at 12,000 rpm in a Kontron A8.24 rotor. To the supernatant 0.3 g/ml of ammonium sulfate was added, stirred for 30 min at 4℃ and centrifuged for 20 min at 15,000 rpm in a Kontron A8.24 rotor. The precipitate was resuspended with buffer A containing 0.1 M KCl (fraction I). To the supernatant 0.1 g/ml of ammonium sulfate was added, stirred for 30 min at 4℃ and centrifuged for 20 min at 15,000 rpm in a Kontron A8.24 rotor. The precipitate was resuspened in buffer A containing 0.1 M KCl buffer (fraction II).

Heparin-agarose chromatography

Fraction I was dialyzed against buffer B containing 50 mM KCl for 12 h and applied to a heparin-agarose column (bed volume, 1 ml) equilibrated in buffer B containing 50 mM KCl. The column was washed with 5 ml of buffer A containing 50 mM KCl and eluted with a linear gradient of 10 ml of 50 mM to 1 M KCl in buffer B.

Results

To use as a probe for identifying a novel activity which preferentially binds to hemimethylated DNA, plasmid poriB (Kim and Hwang, 1995), which possesses a middle region of *oriC* containing five GATC sequences in the *Bam*HI site of pBluescript SK(+), was obtained from *dam*$^+$ and *dam*$^-$ strains. The 126-bp *Bam*HI fragments of fully methylated and unmethylated poriB were mixed, denatured and cooled at room temperature. As a result of denaturation and renaturation, 50% of the DNA template was presumably hemimethylated and the other half was a mixture of fully- and unmethylated DNA. This mixture of DNA fragment was used as the hemimethylated probe in the gel-shift assay.

To avoid detection of SeqA protein, fraction I was prepared from *seqA*$^-$ cells. Presence of nonspecific DNA binding activities in fraction I prevented reliable identification of activity for hemimethylated DNA binding (data not shown). Therefore, partial fractionation was performed through heparin-agarose chromatography. The dialyzed ammonium precipitate was applied to a heparin-agarose column and eluted with a linear gradient from 50 mM to 1 M KCl (Fig. 1A). Several distinct binding activities were detected in flow-through and eluted fractions (Fig. 1B).

The binding activities were further analyzed with

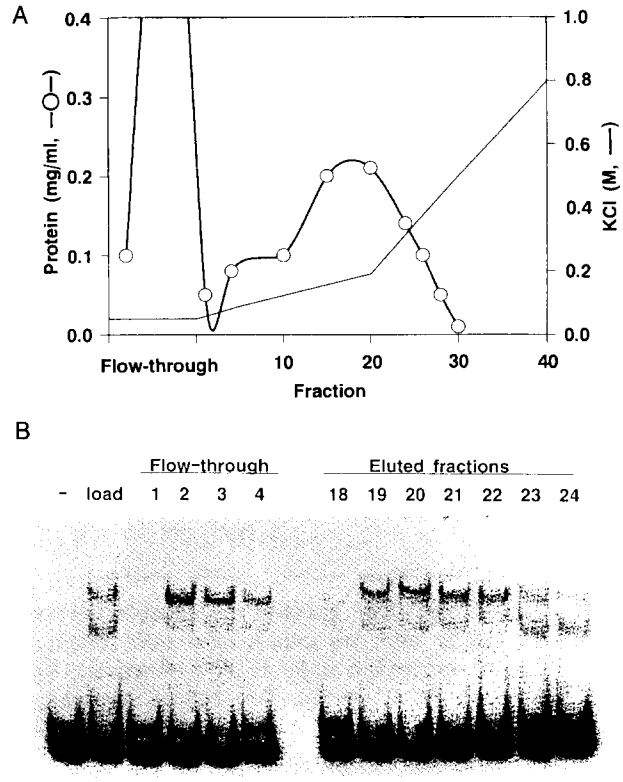
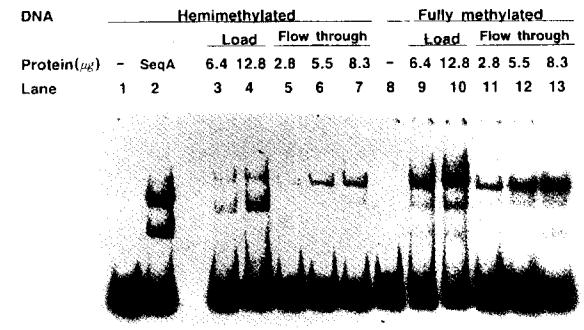


Fig. 1. A, Heparin-agarose chromatography of crude fractions. Fraction I prepared from NK9050 was dialyzed against buffer B containing 50 mM KCl and applied to a heparin-agarose column. The column was washed with 5 ml of buffer A containing 50 mM KCl and eluted with a linear gradient of 10 ml of 50 mM to 1 M KCl in buffer A. Protein concentrations were determined by the method of Bradford (1976). B, Gel-shift assay with fractions of the heparin-agarose column. Gel-shift assay with hemimethylated DNA was performed with 18 μl of each fraction.

hemimethylated and fully-methylated probes (Fig. 2). The load for the heparin-agarose column contained two binding bands with the hemimethylated probe (Fig. 2A, lanes 3 and 4) and two bands with the fully-methylated probe (lanes 9 and 10). The migrations of lower bands appeared to be different. The upper band in the lanes 3, 4, 9 and 10 was also detected in flow-through of the heparin-agarose column (lanes 6, 7, 12 and 13). The weaker signal of the upper band with the hemimethylated probe than with the fully-methylated probe was caused by contamination of fully and unmethylated probes in the hemimethylated probe. The lower band shown with the hemimethylated probe (lanes 3 and 4) appeared to be unique, because the migration of the lower bands with the fully-methylated probe (lanes 9 and 10) were different.

Among the eluted fractions of the heparin-agarose column, fractions 20 and 24 (Fig. 1B) were compared to hemimethylated and fully-methylated probes (Fig. 2B). Fraction 20 contained binding activity (the upper band in lanes 3 to 5 and 9 to 11) to both hemimethylated and fully-methylated probes. However, fraction 24 bound to the hemimethylated probe, but not to the fully-methylated probe.

A



B

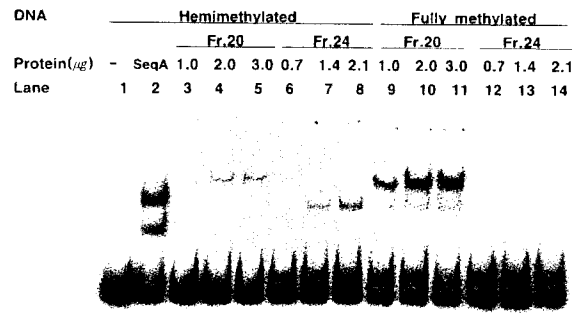


Fig. 2. DNA binding activities dependent on the methylation state. Gel-shift assay was performed with a hemimethylated or fully methylated DNA template. Twenty ng of SeqA protein was used in lane 2 of A and B.

Discussion

Replication of fully methylated *oriC* becomes the hemimethylated state, in which the parental strand is methylated and the newly replicated strand is unmethylated, and this hemimethylated *oriC* is maintained for 13 min until methylated by Dam methylase (Campbell and Kleckner, 1990). It has been reported that SeqA protein is responsible for this sequestration of hemimethylated *oriC* (Lu et al., 1994; Slater et al., 1995). SeqA protein preferentially binds to the hemimethylated *oriC* and inhibits the reinitiation of replication at *oriC* as well as the methylation of hemimethylated *oriC*.

In the *seqA* mutant, remethylation of hemimethylated *oriC* is delayed for 5 min (Lu et al., 1994). On the basis of these results we assumed that unidentified factor(s) exist which can bind preferentially to a hemimethylated *oriC*, independent upon SeqA protein, interrupting the remethylation of hemimethylated *oriC*. Therefore we attempted to identify novel activity in the *seqA*::tet mutant which binds preferentially to hemimethylated DNA.

Among the eluted fractions of the heparin-agarose column, fraction 24 contained novel activity specifically binding to hemimethylated DNA (Fig. 2B) not due to SeqA or HobH protein (Herrick et al., 1994), because this activity was not detected in the *seqA*::tet and *hobH*::kan mutants. Therefore, this hemimethylated *oriC* binding activity may be novel and we are currently continuing to purify this activity.

Acknowledgements

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