

Binding of IciA Protein to the *dnaA* Promoter Region

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IciA protein has been shown as an inhibitor for the initiation of *E. coli* chromosomal DNA replication at *oriC*. IciA protein binds the AT-rich region in *oriC* and then blocks the initiation of chromosomal DNA replication. Two binding sites for IciA protein were identified in *dnaA* gene, encoding the initiator for the *E. coli* chromosomal replication, promoter region by gel-shift assay and DNase I footprinting. One, named as IciA site I, is located upstream of the *dnaA* promoter 1P. The other, named as IciA site II, is located downstream of the *dnaA* promoter 2P. The sequence comparison of the regions protected from the DNase I cleavage did not result in a clear consensus sequence for the binding of IciA protein, suggesting that IciA protein may be a member of multimeric complex dsDNA binding proteins. This study provided information about the binding mode of IciA protein. Even though the IciA site II and IciA binding site in *oriC* seem to be composed of two IciA binding units, one binding unit is likely enough to cause the binding of IciA protein to the IciA site I. The binding of IciA protein to the *dnaA* promoter implies that IciA protein may involve not only the control of the initiation of chromosomal DNA replication but also the control of the *dnaA* gene expression.

Key words: IciA protein, *dnaA*, DNA binding proteins

The initiation of the chromosomal DNA replication in *Escherichia coli* begins by the binding of DnaA protein to its four 9-mer (or DnaA box) binding sequences in the replication origin (*oriC*). In the presence of ATP, this binding causes the opening of the AT-rich region containing three repeats of 13-mer and then allows entry of DnaB-DnaC complexes to form a prepriming complex. This leads to the initiation of the replication (1). DnaA protein is encoded by the *dnaA* gene. One DnaA box, to which DnaA protein binds, is located between the two promoters of the *dnaA* gene. The binding of DnaA protein to the DnaA box represses its own expression.

A novel protein with binding specificity to the AT-rich region of *oriC* have been found (2). This 33 kDa protein has been named IciA (inhibitor of chromosomal initiation A) since the binding to the AT-rich region inhibits the opening of this region, leading to the blockage of the initiation of the *oriC* replication (3). The cellular levels of IciA and DnaA proteins appear to remain constant during the rapidly growing phase of *E. coli*. However, in the late logarithmic phase, the amount of IciA protein

increases four times while that of DnaA protein remains relatively constant (4). IciA protein is composed of 297 amino acid and encoded by *iciA* gene (7). IciA protein belongs to LysR family of prokaryotic regulators which act as a transcriptional activator. The shape of IciA is an elongated form with an axial ratio of 8 to 10. Gel-filtration and cross-linking experiments indicated that IciA protein exists as a dimer (4).

We identified two distinct IciA binding sites in the *dnaA* promoter region by gel-shift assay and DNase I footprinting. Although A and T sequences are rich, the two binding sites are different in length and their sequences possess little homology with the AT-rich region of *oriC* to which IciA protein binds. This finding will help to understand the binding mode of IciA protein.

Materials and Methods

Reagents and proteins

Unless indicated, the reagents and proteins were described previously. IciA protein was purified as described previously (4).

Bacterial strains and plasmid DNAs

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E. coli SURE cell (Stratagene Co.) was used as a host for plasmid preparation. Plasmid pBluescript KS (+) was used as a vector for cloning. Unless indicated, DNAs were manipulated as described elsewhere (5).

Gel-shift assay

The DNA fragments were dephosphorylated with calf intestinal alkaline phosphatase and then labeled with [γ - 32 P]ATP and T4 polynucleotide kinase. The labeled DNA fragments (1.5 fmol) were incubated at 32°C for 30 min with the indicated amounts of IciA protein in a gel shift reaction solution containing 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 75 mM KCl, 2 mM DTT, 10% (v/v) glycerol and 2 μ g of poly(dI)-poly(dC). The reaction mixtures (20 μ l) were subjected to 5% polyacrylamide gel electrophoresis at 100 V for 1 to 2 hr in 45 mM Tris-borate (pH 8.3) and 1 mM EDTA. The gel was dried and the DNA bands were detected by autoradiography.

DNase I footprinting

pBluescript plasmids containing desired DNA fragments were digested with *Xho*I, dephosphorylated with calf intestinal alkaline phosphatase and then labeled with [γ - 32 P]ATP and T4 polynucleotide kinase. The labeled plasmids were further digested with *Xba*I and the resulting *Xho*I-*Xba*I fragments were isolated from 1% agarose gel by electroelution and used for DNase I footprinting. The reaction mixture contained 40 mM HEPES-KOH (pH 7.6), 50 mM KCl, 5 mM MgCl₂, 17% glycerol, 2.5 μ g of bovine serum albumin, 50 ng of supercoiled Bluescript plasmid, 12.5 fmol of the unlabeled *Xho*I linearized plasmid, 12.5 fmol of the labeled *Xho*I-*Xba*I fragment and the indicated amounts of IciA protein. The reaction mixture was incubated at 32°C for 30 min and DNase I (20 ng in 1.5 μ l of H₂O) was added, incubated for 30s, and stopped by the addition of 27 μ l of 0.6 M sodium acetate, 0.4% sodium dodecyl sulfate, and 25 mM EDTA. Proteins were removed by phenol-chloroform extraction. DNA was precipitated by ethanol after adding 2 μ g of glycogen. DNA was subjected to electrophoresis through a 5% Long Ranger polyacrylamide sequencing gel containing 7 M urea. The gel was dried and the DNA band were visualized by autoradiography.

Construction of plasmids

Plasmid pBS-AEr contains an insert of 945 bp *Eco*RI fragment possessing the *dnaA* promoter region (Fig.1). The *Eco*RI fragment was obtained by *Eco*RI digestion of M13DJ RF DNA containing the *dnaA* sequence. The *Eco*RI fragment was ligated with pBS, which had been previously digested with *Eco*RI and dephosphorylated with calf intestinal alkaline phosphatase, resulting in the

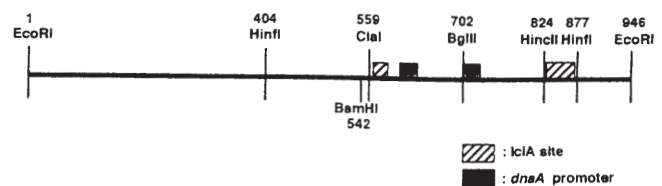


Fig. 1. Physical map of *Eco*RI fragment of *dnaA* promoter region. The *Bam*HI site was generated by subcloning of *dnaA* gene.

construction of pBS-AEr.

Plasmid pBS-AH contains a 473 bp *Hin*II fragment insert (Fig. 1) and pBS-AHr possesses the same insert with opposite orientation. The previously constructed pBS-AEr was digested with *Eco*RI. The *Eco*RI fragment was isolated and further digested with *Hin*II, resulting in the generation of three *Hin*II fragments (473 bp, 403 bp, 69 bp). The 473 bp fragment was isolated using agarose gel electrophoresis and its cohesive ends were filled with Klenow fragment and dNTPs. The blunt-ended fragment was ligated with pBS which had been previously digested with *Eco*RV and dephosphorylated with CIP.

Plasmids pBS-BH and pBS-BHr contain a 282 bp *Bam*HI-*Hinc*II fragment insert (Fig. 1). The *Bam*HI-*Hinc*II fragment was obtained by *Bam*HI and *Hinc*II digestion of pBF1509 plasmid which contains *dnaA* promoter region and one *Bam*HI site at 344 bp from the translation start codon. The *Bam*HI-*Hinc*II fragment was ligated with pBS, which had been previously digested with *Bam*HI and *Eco*RV, resulting in the construction of pBS-BH. The pBS-BH was digested with *Bam*HI and *Hind*III and their cohesive ends were filled with Klenow fragment and dNTPs. The blunt-ended *Bam*HI-*Hind*III fragment was ligated with pBS, which had been previously digested with *Eco*RV and dephosphorylated with CIP, resulting the construction of pBS-BHr which contains the *Bam*HI-*Hinc*II fragment sequence with opposite orientation.

Plasmids pBS-BE and pBS-BEr contain a 244 bp *Bgl*II-*Eco*RI fragment insert (Fig. 1). The previously constructed pBS-AEr was digested with *Bgl*II and *Sma*I. The *Bgl*II-*Sma*I fragment containing the *Bgl*II-*Eco*RI fragment sequence was ligated with pBS which had been previously digested with *Bam*HI and *Eco*RV, resulting in the construction of pBS-BE. The pBS-BE was digested with *Xba*I and its cohesive ends were filled with Klenow fragment and dNTPs. The blunt-ended plasmid was redigested with *Hind*III and its cohesive ends were partially filled with Klenow fragment and dATP and dGTP. The resulting *Xba*I (blunt-end)-*Hind*III (nucleotide CT cohesive end) fragment was ligated with pBS, which had been previously digested with *Xba*I and *Eco*RV and then the *Xba*I cohesive end had been partially filled with

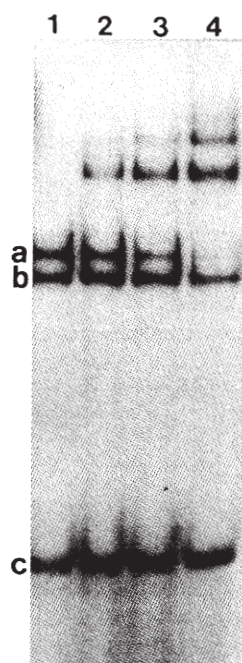


Fig. 2. Gel-shift assay with the 945 bp *EcoRI* fragment digested with *HinfI*. Assay was performed as described in Materials and Methods. The generated three fragments were indicated with alphabet letters; a (473 bp), b (403 bp) and c (69 bp). The amounts of IciA present in the assay were as followed; lane 1: 0 ng, lane 2: 2.3 ng, lane 3: 4.7 ng and lane 4: 9.4 ng.

Klenow fragment and dTTP and dCTP, resulting in the construction of pBS-BER.

These plasmid constructs were used for the following gel-shift assay and DNaseI footprinting experiments.

Results and Discussion

Specific binding of IciA protein to the *dnaA* promoter region revealed by gel-shift assay

In order to search IciA binding sites in the *dnaA* promoter region, the 945 *EcoRI* fragment containing the *dnaA* promoter region was isolated from plasmid *pdnaA/dnaN*. The fragment was further digested with *HinfI* and labeled with ^{32}P . The labeled fragments were used for gel shift assay as described in Materials and Methods. The *HinfI* digestion generated three fragments (473, 403 and 69 bp). As shown in Fig. 2, only 473 bp fragment (indicated by a) was shifted by IciA protein, indicating the presence of IciA binding site(s) in this fragment. This 473 bp sequence contained elements for *dnaA* expression such as two promoters and a DnaA box.

For more detailed analysis of the 473 bp sequence (Fig. 1), this fragment was cloned in plasmid pBluescript as described in Materials and Methods. The 507 bp *XhoI-EcoRI* fragment containing the 473 bp sequence and

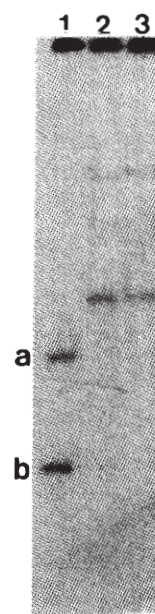


Fig. 3. Gel-shift assay with the 507 bp *XhoI-EcoRI* fragment digested with *BglII*. Assay was performed as described in Materials and Methods. The generated two fragments were indicated with alphabet letters; a (324 bp) and b (183 bp). The amounts of IciA present in the assay were as followed; lane 1: 0 ng, lane 2: 25 ng, lane 3: 50 ng.

an additional 34 bp sequence from the pBluescript multicloning site was isolated from the pBS-AHr. The *XhoI-EcoRI* fragment was further digested with *BglII* and used for gel-shift assay (Fig. 3). The *BglII* digestion generated two fragments, 324 bp *XhoI-BglII* fragment (indicated by a) and 183 bp *BglII-EcoRI* fragment (indicated by b). Both fragments were shifted by IciA protein, suggesting the presence of at least two distinct IciA binding sites in this *XhoI-EcoRI* fragment, which could be separated by *BglII* digestion.

Further analysis was performed to locate the IciA binding site in the 175 bp *BglII-HinfI* fragment (Fig. 1). The 244 bp *BglII-EcoRI* fragment containing the *BglII-HinfI* sequence was cloned in pBluescript plasmid as described in Materials and Methods. The 298 bp *XhoI-XbaI* fragment was isolated from the pBS-BE. This fragment was further digested with *HincII* and *HinfI*. These digestions generated three fragments, 136 bp *XbaI-HincII* fragment (indicated by a in Fig. 4A) containing 122 bp *BglII-HincII* sequence and an additional 14 bp sequence from the multicloning site, 109 bp *HinfI-XhoI* fragment (indicated by b) containing 69 bp *HinfI-EcoRI* sequence and an additional 40 bp sequence from the pBluescript multicloning site, and 53 bp *HincII-HinfI* fragment (indicated by c). As shown in Fig. 4A, no apparent high level of shift was observed. This result suggested that *HinfI* or (and) *HincII* digestions might deteriorate the IciA binding site so that IciA protein could no longer bind.

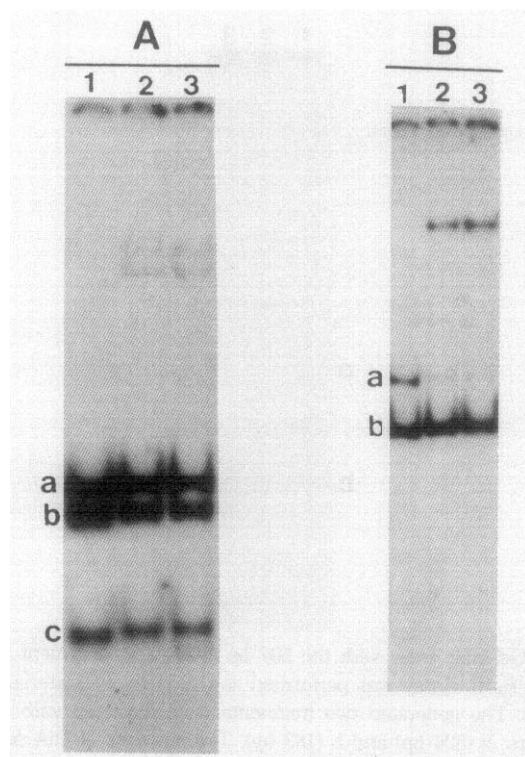


Fig. 4. Gel-shift assay with the 244 bp *Bgl*II-*Eco*RI fragment digested, respectively, with *Hin*II+*Hinc*II (A) and *Hin*II (B). Assay was performed as described in Materials and Methods. The generated fragments were indicated with alphabet letters; a (136 bp), b (109 bp) and c (53 bp) in A and a (189 bp) and b (109 bp) in B. The amounts of IciA present in the assay were as followed; lane 1: 0 ng, lane 2: 25 ng, lane 3: 50 ng.

In order to find out which digestion was responsible for the destruction of IciA binding site, the *Xho*I-*Xba*I fragment was individually digested with *Hin*II and *Hinc*II. The *Hin*II digestion generated two fragments, 189 bp *Xba*I-*Hin*II fragment (indicated by a in Fig. 4B) containing the *Hinc*II-*Hin*II sequence and the *Xba*I-*Hinc*II sequence and 109 bp *Hin*II-*Xho*I fragment (indicated by b). Only the *Xba*I-*Hin*II fragment containing the 175 bp *Bgl*II-*Hin*II sequence was shifted. This result confirmed the previous observation that the *Bgl*II-*Hin*II sequence did contain the IciA binding site. The *Hinc*II digestion also generated two fragments, 162 bp *Xho*I-*Hin*II fragment containing the *Hinc*II-*Hin*II sequence and the *Hin*II-*Xho*I sequence and 136 bp *Xba*I-*Hinc*II fragment. The gel-shift assay revealed that no apparent high level of shift was occurred (data not shown), indicating that the *Hinc*II digestion deteriorated the IciA binding site.

From these gel shift analysis, it was concluded that at least two distinct IciA binding sites existed in *dnaA* promoter region, one in the 175 bp *Bgl*II-*Hin*II sequence which could be destroyed by *Hinc*II digestion and the other in the 324 bp *Xho*I-*Bgl*II fragment.

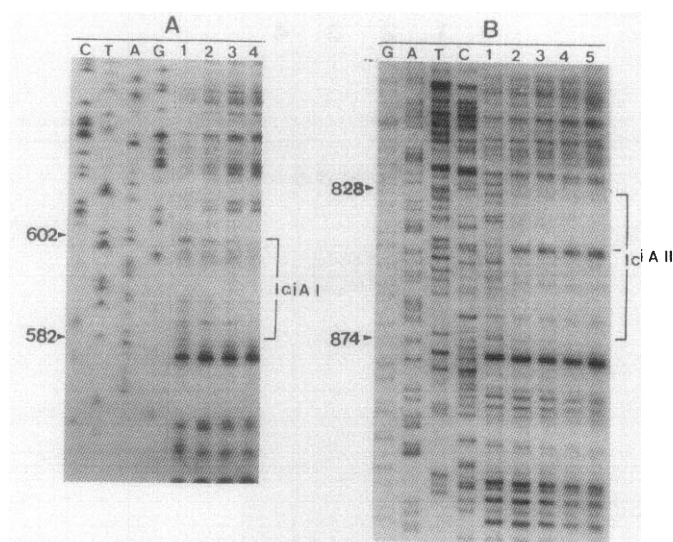


Fig. 5. DNase I footprinting analysis. DNase I footprinting was performed as described in Materials and Methods with pBS-BH (A) and pBS-BE (B). The amounts of IciA protein present in the analysis were as followed; lane 1: 0 ng, lane 2: 50 ng, lane 3: 100 ng and lane 4: 200 ng in A, lane 1: 0 ng, lane 2: 25 ng, lane 3: 50 ng, lane 4: 100 ng and lane 5: 200 ng in B. The protected regions by IciA protein are indicated. The nucleotide sequence numbers beside sequence ladders are based on Fig. 1.

IciA binding site sequences in *dnaA* promoter region revealed by DNase I footprinting

To identify the binding locus of IciA protein to the *dnaA* promoter region, the plasmid pBS-BH containing a 282 bp *Bam*HI-*Hinc*II fragment was constructed as described in Materials and Methods. Using this *Bam*HI-*Hinc*II insert, DNase I footprinting was performed as described in Materials and Methods. From the DNase I footprinting, it was revealed that IciA protein protected about 22 bp sequence near *Cla*I site from DNase I digestion (Fig. 5A), indicating that this binding site was responsible for the shift of the 324 bp *Xho*I-*Bgl*II fragment in Fig. 3. This binding site was named as IciA site I. IciA site I possessed AT-rich sequences. Seventeen bp among 22 bp protected from DNase I cleavage was A and T sequence (77%). Especially, a 10 bp of continuous A and T sequence was observed in the middle of IciA site I.

To identify the other IciA binding site in the 175 bp *Bgl*II-*Hin*II fragment, DNase I footprinting with pBS-BE was performed. The result showed that about 47 bp between *Hinc*II and *Hin*II sites was protected by IciA protein from DNase I digestion (Fig. 5B). This site was named as IciA site II. IciA site II also did not show an apparent homology to the AT-rich sequence containing 13-mers in *oriC* (Fig. 6). IciA site II showed A-T richness (64%) even though it was lower than that of

Fig. 6. DNA sequences protected by IciA protein from DNaseI digestion. IciA site I, site II and *oriC* IciA site sequences are shown and the hypersensitive bases in IciA site II and *oriC* IciA site are underlined. The indicated nucleotide numbers are based on Fig. 1 where left *Eco*RI site is the starting point. The nucleotide sequence of *oriC* is from reference 2. Parts of the *Hinc*II sequence, which are not protected by IciA protein from DNaseI digestion, in the IciA site II are described in small letters.

DnaA protein is not only an initiator protein for chromosomal DNA replication but also an autoregulator for its own gene expression (8). One DnaA box exists between the two *dnaA* promoters and the binding of DnaA protein to the box represses the expression. The presence of the two IciA binding site in *dnaA* promoter region implies the possibility of the involvement of IciA protein in the *dnaA* expression. Actually, the binding of IciA protein specifically activates transcription from the promoter 1P between the two *dnaA* promoters (manuscript in preparation).

This IciA binding study in *dnaA* promoter region provides information about the binding mode of IciA protein to DNA. The length of one IciA binding unit, determined by DNase I protection, is likely from 20 bp to 25 bp. According to this idea, IciA site II and IciA binding site in *oriC* are likely composed of two IciA binding units. The boundary between two binding units may be indicated by a hypersensitivity band in DNaseI footprinting as observed in IciA site II and *oriC* IciA binding site. However, one IciA binding unit may be enough to cause the IciA binding such as IciA site I. Only *HincII* digestion deteriorates the binding of IciA protein to IciA site II even though *Hinfi* site is located to IciA site II as near as *HincII* site does (Fig. 1). This may imply that there is an order of the binding of IciA protein to IciA site II. IciA protein may bind first to the DNA sequences near *HincII* site and then this binding may lead to the further binding of IciA protein to the DNA sequences near *Hinfi* site.

ing proteins. In general, they do not recognize a specific nucleotide sequences but rather the structural properties of the DNA. Usually, the binding induces significant conformational changes in the DNA and the ability of the DNA to accomodate these changes determines the affinity of proteins to the binding sites. When the favorable structures exist with appropriate periodicity on the DNA, the proteins can bind with high affinity. Considering the binding mode of IciA protein revealed by this study, it is conceivable to think that IciA protein is a possible member of the multimeric complex dsDNA binding proteins. The study of conformational changes in DNA upon the binding of IciA protein could help to examine this suggestion. Much more detailed study should be performed to establish a firm knowledge of the binding mode and mechanism of IciA protein to DNA. By introducing systematic changes in IciA binding site sequences and characterizing the differences in the properties of IciA binding to them, a clear understanding of the binding behavior of IciA protein to DNA could be achieved.

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