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The binding of two dimers of IciA protein to the *dnaA* promoter 1P element enhances the binding of RNA polymerase to the *dnaA* promoter 1P

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ABSTRACT

Transcription of the *dnaA* gene from the promoter 1P has been shown to be activated *in vitro* and *in vivo* by the binding of IciA protein to two sites on the *dnaA* promoter region [Lee, Y. S., Kim, H., and Hwang, D. S. (1996) *Mol. Microbiol.* 19, 389–396; Lee, Y. S., and Hwang, D. S. (1997) *J. Biol. Chem.* 272, 83–88]. *In vitro* transcription assays using DNA fragments carrying variable combinations of two IciA binding sites revealed that IciA binding site I (IciA I site), which is located upstream of the promoter 1P, is responsible for the transcriptional activation. Binding of one dimeric IciA protein to the IciA I site is followed by binding of the second dimer. Two dimers of IciA protein, rather than one dimer, on the IciA I site appeared to enhance the binding of RNA polymerase to the promoter 1P, resulting in the activation of transcription from the promoter 1P.

INTRODUCTION

Escherichia coli chromosomal DNA replication is initiated by the binding of DnaA proteins to the DnaA boxes (or 9mers) within *oriC* (1,2). The intracellular level of DnaA protein plays an important role in the regulation of initiation of chromosomal DNA replication (3–5). The *dnaA* gene, encoding DnaA protein, has two promoters, 1P and 2P. Binding of DnaA protein to the consensus DnaA box between the two promoters results in the repression of transcription from the two promoters (3,6–10). The autoregulated expression of the *dnaA* gene has been suggested to contribute to the regulation of initiation of chromosomal DNA replication (3–5,11). The binding of DnaA protein to the DnaA box between the two promoters is followed by subsequent oligomerization of DnaA proteins over the two promoter regions (12). The extent of oligomerization of DnaA protein appeared to be proportional to the inhibition of RNA polymerase binding to the promoters and the inhibition of transcription from the promoters. As the intracellular level of DnaA protein is critical for initiation of chromosomal DNA replication (11), *E.coli* might adopt a fine tuning regulatory mechanism, rather than a simple on-off switch, for *dnaA* expression by using the DnaA protein concentration-dependent oligomerization as a controlling mechanism.

IciA protein, a specific inhibitor of initiation of *in vitro oriC* replication, was identified as a sequence-specific binding protein to the AT-rich region of *oriC* (13). IciA protein is a homodimer of 33.4 kDa polypeptides with an elongated shape (14). Primary amino acid sequence analysis of the IciA protein indicated that it has a motif for DNA binding (helix–turn–helix) and has homology to the LysR family proteins which function as transcriptional activators in prokaryotes (15).

The *dnaA* promoter region contains two binding sites for IciA protein, IciA I and IciA II, located upstream of the promoter 1P and downstream of the promoter 2P, respectively (16). Among the two *dnaA* promoters, transcription from the promoter 1P was specifically enhanced by the *in vivo* overproduction of IciA protein or using *in vitro* transcription assays of the *dnaA* gene. IciA function is dominant over that of DnaA protein and the mechanism may involve the ability of IciA protein to counteract the DnaA protein-dependent occlusion of RNA polymerase from the promoter 1P (12).

In this report, we determined the *cis*-element responsible for the IciA-dependent activation of the *dnaA* promoter 1P and the binding mode of IciA protein to the IciA I site.

MATERIALS AND METHODS

Reagents, proteins, bacterial strains and plasmid DNAs

Unless indicated, reagents, proteins, bacterial strains and plasmid DNAs were previously described (12).

To construct plasmid pHJ1, the 473 bp *Hin*fI fragment isolated from plasmid p*dnaA/dnaN* was filled with Klenow fragment and inserted into the *Eco*RV site of vector pBluescript SK(+). Plasmid pYS2 was constructed by insertion of the 404 bp *Bam*HI/*Eco*RI fragment from pBF1509 into the *Bam*HI/*Eco*RI site of vector pBluescript SK(+). To obtain plasmid carrying deletion of IciA I site, PCR (polymerase chain reaction) with a synthetic oligonucleotide corresponding to nucleotide sequence –46 to –28 in Figure 1 and KS primer (Stratagene Corp.) was performed with plasmid pYS2. The PCR-amplified 377 bp fragment was treated with T4 DNA polymerase and inserted into the *Eco*RV site of pBluescript SK(+) to generate plasmid pYSD1. Plasmid pBSHcII was constructed by insertion of the endfilled 430 bp *Hin*dIII/*Hin*cII fragment, isolated from pHJ1, with Klenow fragment into the *Eco*RV site of pBluescript SK(+).

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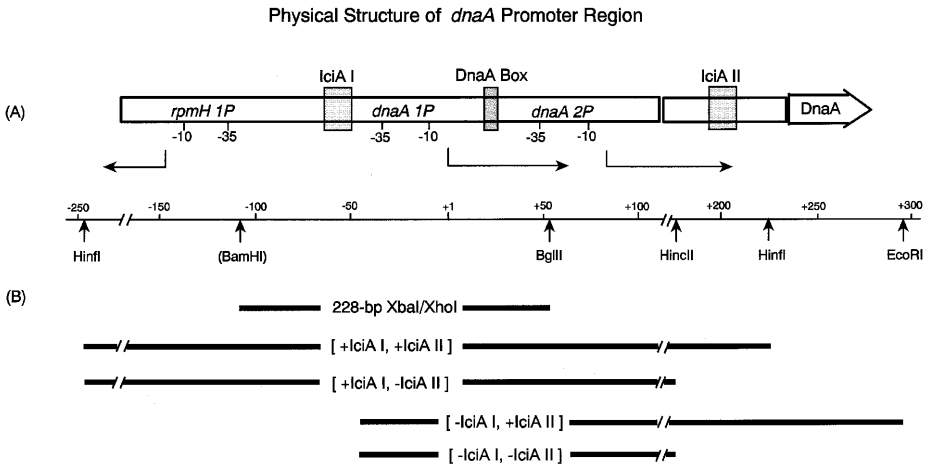

Figure 1. Physical map of the *dnaA* promoter region. (**A**) The two *dnaA* promoters and neighboring regions, as previously determined (22,23), are marked. Numbering of nucleotide sequence is based on the transcription start point of the *dnaA* promoter 1P as position +1 (12). This position corresponds to nucleotide 651 in Hansen *et al.* (22,23). Promoters are as follows: *rpmH* 1P, *rpmH* promoter 1P (–116 to –143); *dnaA* 1P, *dnaA* promoter 1P (–4 to –34); *dnaA* 2P, *dnaA* promoter 2P (+51 to +79). Nucleotide –35 and –10 from the transcription start point of each promoter are indicated. The direction of transcription is indicated by the arrows beneath. DnaA box (+18 to +26) indicates the DnaA protein recognition sequence. IciA I and IciA II are IciA protein binding sites IciA I (–63 to –50 determined by OP-Cu footprinting in Figure 3; 5′-attaaattttccaa-3′) and IciA II (+179 to +224, previously determined by DNase I footprinting (16); 5′-caatcatgaatgtttcagccttagtcattatcgacttttgttcgag-3′). The restriction sites from the left, *Hin*fI, *Bam*HI (from plasmid pBF1509), *Bgl*II, *Hin*cII, *Hin*fI and *Eco*RI, are located at nucleotide sequence –247, –108, +52, +174, +227 and +296, respectively. (**B**) The DNA fragments used for gel-shift and *in vitro* transcription assays are indicated underneath the corresponding regions. (228 bp *Xba*I/*Xho*I): the 228 bp *Xba*I/*Xho*I fragment isolated from plasmid pHJ4; (+IciA I, +IciA II): the 488 bp *Eco*RI/*Hin*dIII fragment from plasmid pHJ1; (+IciA I, –IciA II): the 463 bp *Xho*I/*Eco*RI fragment from plasmid pBSHcII; (–IciA I, +IciA II): the 376 bp *Eco*RV/*Xho*I fragment from plasmid pYSD1; and (–IciA I, –IciA II): the 243 bp *Hin*cII fragment from plasmid pYSD1.

DNA binding assays

Gel-shift and DNase I protection assays were performed as previously described (12).

1,10-phenanthroline-copper (II) (OP-Cu) footprinting was performed as previously described (17) with minor modifications. A gel-shift assay was performed with 21.5 fmol of the 228 bp *Xba*I/*Xho*I fragment from plasmid pHJ4 (Fig. 1) which was ^{32}P-end labeled at the *Xho*I restriction site. After finishing electrophoresis of gel-shift assay, the gel was immersed in 200 ml of 10 mM Tris–HCl (pH 8.0), followed by the addition of 20 ml of OP-Cu (II) solution (2 mM 1,10-phenanthroline and 0.45 mM $CuSO_4$). The cleavage reaction was initiated by adding 20 ml of 58 mM 3-mercaptopropionic acid, followed by incubation for 15 min at room temperature. Then, 20 ml of 28 mM 2,9-dimethyl-1,10-phenanthroline was added to quench the cleavage reaction. The quenched gel was exposed to X-ray film to localize each band. The DNAs in each band were eluted into diffusion buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA and 0.1% SDS) by incubation for 1 h at 50°C, and recovered by phenol/chloroform extraction and ethanol precipitation. The pellet was resuspended in sequencing gel loading buffer and electrophoresed through 5% Long Ranger polyacrylamide sequencing gel containing 7 M urea. The gel was dried and visualized by autoradiography or by scanning of radioactivities in each lane with a FUJIX Bio-Imaging Analyzer (BAS1000).

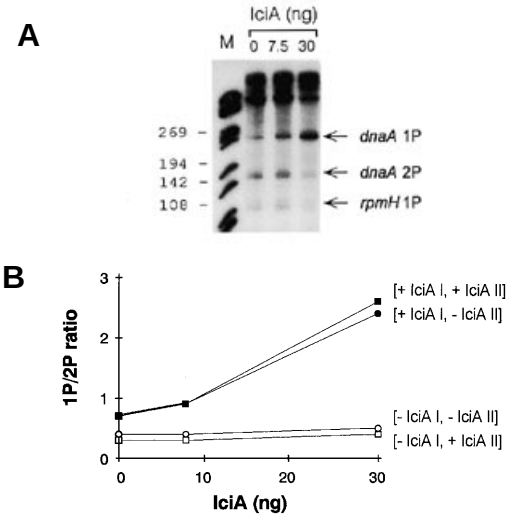

Figure 2. IciA I site activates transcription from the *dnaA* promoter 1P. (**A**) *In vitro* run-off transcription assay with the 488 bp *Eco*RI/*Hin*dIII fragment, which was isolated from plasmid pHJ1, was performed with 184 ng of RNA polymerase and indicated amounts of IciA protein. The transcripts of 238, 156 and 108 nucleotides in length were initiated from *dnaA* promoter 1P, 2P and *rpmH* promoter 1P, respectively. The molecular markers, described as M, are 5′-end-labeled *Hae*III digested plasmid pBSoriC (24). (**B**) Radioactivities in the transcripts of the *dnaA* promoter 1P and 2P were measured as volume in the FUJIX (BAS1000) and divided by the number of uridine residues in each transcript. The ratio of the transcript of the *dnaA* promoter 1P over that of the *dnaA* promoter 2P were plotted against the amounts of IciA protein. The templates used are described in Figure 1.

RESULTS

IciA protein binding to IciA I site is responsible for the activation of transcription from the *dnaA* promoter 1P

IciA protein binds to two sites within the *dnaA* promoter region, one of which is located upstream of *dnaA* promoter 1P (IciA I site) and the other downstream of *dnaA* promoter 2P (IciA II site) (Fig. 1; 16). Of these two *dnaA* promoters, the transcription from

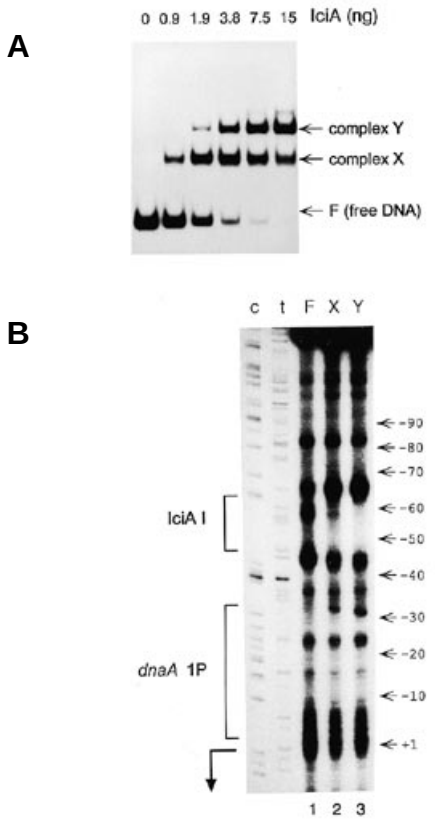

Figure 3. Two dimers of IciA protein bind to the IciA I site. (**A**) Gel-shift assays with the 228 bp *Xba*I/*Xho*I fragment, which was isolated from plasmid pHJ4 and ^{32}P-end-labeled at the *Xho*I restriction site, were performed with the indicated amounts of IciA protein. The two IciA protein–DNA complexes are denoted as X and Y, respectively. F, free DNA. (**B**) Lanes 1 to 3: OP-Cu footprinting analysis of the free DNA (F) and complexes X and Y in (A), which are denoted at the top of each lane, performed as described under 'Materials and Methods'. c and t indicate respective dideoxy-sequencing lanes of plasmid pHJ4.

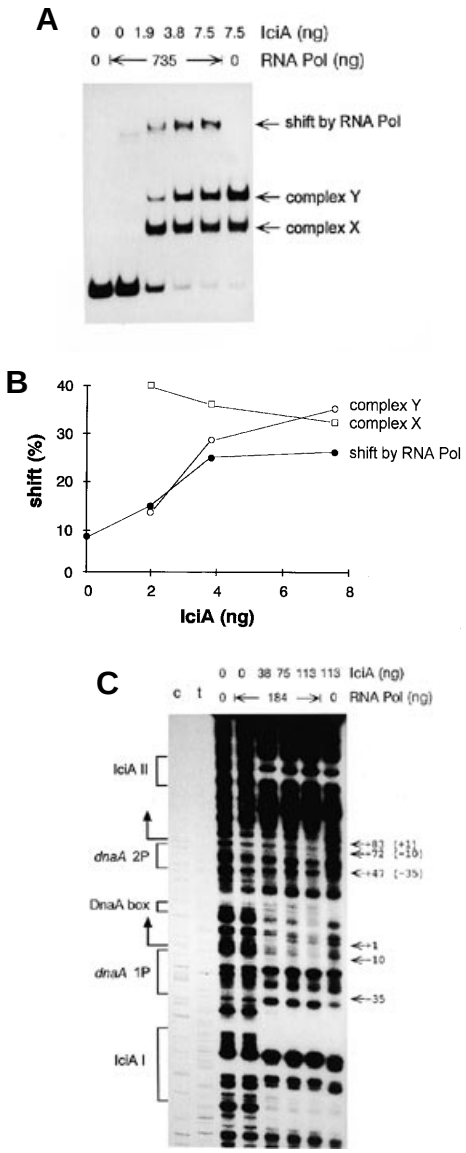

Figure 4. Enhanced binding of RNA polymerase to *dnaA* promoter 1P by IciA protein. Reaction mixture was incubated for 5 min, then RNA polymerase (denoted as RNA pol) was added, followed by further incubation for 10 min. (**A**) With indicated amounts of IciA protein and RNA polymerase, the ^{32}P-end-labeled 228 bp *Xba*I/*Xho*I fragments from plasmid pHJ4 were subjected to gel-shift assay. (**B**) Radioactivities in the bands shifted by RNA polymerase (denoted as shift by RNA Pol) and those by IciA protein (denoted as complex X and Y, respectively) were quantitated using FUJIX (BAS1000), expressed as proportions of radiactivities of the input DNA, and plotted against the amounts of IciA protein. (**C**) DNase I footprinting with the 617 bp *Xba*I/*Xho*I fragment of plasmid pYS1, which was ^{32}P-end-labeled at the XbaI restriction site, was performed as previously described (16). Nucleotides –35 and –10 from the transcription start point of the *dnaA* promoter 2P are parenthesized. c and t indicate respective dideoxy-sequencing lanes of plasmid pHJ4.

the promoter 1P was specifically activated by IciA protein *in vivo* and *in vitro* (16). To determine which of the two IciA protein binding sites is required for this activation, *in vitro* run-off transcription assays were performed with DNA templates possessing variable combinations of IciA binding sites (Fig. 2).

As shown previously (16), IciA protein activated transcription from the *dnaA* promoter 1P in the 488 bp *Eco*RI/*Hin*dIII fragment (+IciA I, +IciA II) of plasmid pHJ1 without affecting transcription from the promoter 2P (Fig. 2A and B). The presence of the IciA I site in the 463 bp *Xho*I/*Eco*RI fragment (+IciA I, –IciA II) of plasmid pBSHcII was sufficient for IciA protein to similarly activate transcription from the promoter 1P (Fig. 2B). However, IciA protein failed to activate transcription when the templates (–IciA I, ±IciA II) lacked the IciA I site. In all of the above transcription assays, transcription from the promoter 2P was not affected by IciA protein. From these results, we conclude that the IciA I site is essential and alone sufficient for the activation of transcription from the *dnaA* promoter 1P by IciA protein.

Two dimers of IciA protein bind to the IciA I site

The stoichiometry and extent of IciA binding to the *dnaA* promoter 1P region was examined in detail to approach how IciA protein on IciA I site activates the transcription. Increasing levels of IciA protein yielded two slowly migrating complexes X and Y (Fig. 3A). At low amounts of IciA protein, the more rapidly migrating complex X was predominant. As the amounts of IciA protein increased, the formation of a more slowly migrating species (complex Y) increased with a concomitant slight decrease in the amounts of complex X.

To analyze the complexes X and Y in more detail, *in situ* footprinting was performed using OP-Cu (II) complex as a chemical cleavage agent (Fig. 3B). In the OP-Cu footprint of the complex X (lane 2), the protection by IciA protein from OP-Cu cleavage resided at the region of nucleotide sequences –50 to –63 with pronounced protection of –50 to –59. Apart from more extensive protection of the region containing nucleotides –59 to –63, the OP-Cu footprint of complex Y (lane 3) was identical to that of complex X and was localized exclusively to a region upstream of the *dnaA* promoter 1P. Given the dimeric nature of IciA protein (13,14), these two complexes presumably reflect the binding of first one and then two dimers of IciA protein.

IciA protein facilitates the binding of RNA polymerase to the dnaA promoter 1P

The mechanism whereby IciA protein activates transcription from the *dnaA* promoter 1P is not known. Several possibilities could include: facilitation or stabilization of RNA polymerase binding to the promoter; or stimulation of subsequent steps of transcription after the stable RNA polymerase–promoter complex formation. According to previous results (16), IciA protein was able to activate *in vitro* transcription from *dnaA* promoter 1P in the presence of limiting amounts of RNA polymerase. When the level of RNA polymerase saturated the reaction, the activation of transcription from promoter 1P by IciA protein was not evident. These results suggest that IciA protein may assist the binding of RNA polymerase to the promoter 1P.

This possibility was investigated using both gel-shift and DNase I protection assays. In the absence of IciA protein, RNA polymerase (735 ng) bound and shifted 8% of the input DNA (Fig. 4A and B). Increasing amounts of IciA protein stimulated the formation of the RNA polymerase–DNA complex. The formation of the complex bound by RNA polymerase was in proportion to the formation of complex Y, in which two dimers of IciA protein were shown to bind to the IciA I site (Fig. 3). This result implies that the binding of two dimers of IciA protein to the IciA I site is required for the activation of RNA polymerase binding to the *dnaA* promoter 1P.

The enhanced binding of RNA polymerase to the *dnaA* promoter 1P by IciA protein was confirmed using a DNase I protection assay (Fig. 4C). At low levels of RNA polymerase (184 ng), RNA polymerase binds more avidly to the promoter 2P than to the promoter 1P (lane 2). Although the binding of RNA polymerase to the promoter 2P was unaffected by IciA protein, IciA protein enhanced the binding of RNA polymerase to promoter 1P (lanes 3–5). Thus, two dimers of IciA protein bound to IciA I site facilitate the binding of RNA polymerase to the *dnaA* promoter 1P resulting in the activation of transcription from the *dnaA* promoter 1P.

DISCUSSION

Physically and functionally heterogeneous complexes containing IciA protein bound to the *dnaA* promoter were isolated and analyzed in order to address the binding mode of IciA protein to DNA containing the IciA I site and how this binding activates transcription from the *dnaA* promoter 1P. We demonstrate in this paper that the mechanism of stimulation of transcription by IciA protein may include: (i) the binding of two dimers of IciA protein to the IciA I site, which is located –50 to –63 from the transcription start site of *dnaA* promoter 1P; and (ii) the stimulation of RNA polymerase binding to the promoter by two IciA protein dimers on the IciA I site. Although the LysR-family proteins such as CatR, OccR, CysB and NodD introduce the bending of DNA (18–21), we could not observe induction of the bending by IciA protein (data not shown). These results suggest that IciA may directly interact with RNA polymerase to stimulate transcription. The presumed interaction enhances the capability of RNA polymerase to be loaded onto the *dnaA* promoter 1P, resulting in the activation of transcription from the promoter.

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