IciA Protein, a Specific Inhibitor of Initiation of Escherichia coli Chromosomal Replication*

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Specific binding of IciA protein to the 13-mers in the origin of a minichromosome (oriC) inhibits initiation of replication in vitro by blocking the opening of this region effected by the initiator DnaA protein (Hwang, D. S., and Kornberg, A. (1990) Cell 63, 325–331). Isolation of the iciA gene (Thony, B., Hwang, D. S., Fradkin, L., and Kornberg, A. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 4066–4070) has made possible the construction of an IciA-overproducing strain, which in turn has simplified the isolation of a large quantity of the protein, indistinguishable from that of the wild-type strain. Based on sedimentation and gel filtration, the IciA protein is an elongated dimer of a 33.4-kDa subunit. The specific binding of IciA protein to the 13-mers was stable for 2 h at 30 °C. The amounts of IciA protein, detected by immunoassays, increased 4-fold compared with levels (about 100 dimers) in log-phase cells whereas levels of DnaA protein decreased upon entry of cells into the stationary phase.

Initiation of replication of the Escherichia coli chromosome at its unique origin (oriC) is a regulated, key stage in the cell cycle (1, 2). Plasmids that use the minimal, 245-bp origin for their autonomous replication conform to the genetic and physiological controls of the chromosomal replication process and may be regarded as minichromosomes (3–6). The replication of such oriC plasmids, reconstituted with purified proteins (7), has afforded biochemical insights into some of the many parameters that govern the switch that operates chromosome initiation. Among these are the status of the key initiator DnaA protein (8–11), transcriptional activation (12, 13), specificity factors which include proteins HU (12–14), IHF (13), topoisomerase I (15), and RNase H (16), and the topological state of the DNA (12, 13). It might have been supposed and it is now known that a negative effector exists to shut off initiation. The IciA protein binds specifically to the oriC region that contains three 13-mers in tandem (17), the opening of which is the main event at an early stage (17). The iciA gene is located at 62.8 min on the circular E. coli chromosome (19). The encoded IciA protein of 33.4 kDa contains a helix-turn-helix motif which may be involved in the DNA binding and also possesses sequence homologies to the large LysR family of transcriptional regulators (20).

We report here the large overproduction of IciA protein which has enabled us to obtain by a simplified procedure quantities of the protein adequate for biochemical studies. Some features of the IciA protein and its cellular abundance are also described.

EXPERIMENTAL PROCEDURES

Reagents and Proteins—Unless indicated, the reagents and proteins were as described previously (17). Buffer A was 50 mM HEPES/KOH (pH 7.6), 1 mM EDTA, and 2 mM dithiothreitol. Buffer B was Buffer A plus 250 mM KC1 and 20 mM spermidine-HC1. Buffer C was 25 mM HEPES/KOH (pH 7.6), 0.1 mM EDTA, 2 mM dithiothreitol, and 15% glycerol. Buffer D was Buffer C plus 50 mM KC1. Buffer E was Buffer C plus 150 mM KC1.

Bacterial Strains and Plasmids—The E. coli strains were: TG-1 supE, hsdD5, thi, Δ(lac-proAB) F′ [traD36, provB'), lacI, lacZAM15; MC1061 araD139, Δ[ara,leu]7697, Δ[lacX74, galU, galK, hsdR, rpsL, and W3110 F' lacI'. TG-1 served as host for M13 derivatives. MC1061 was the host for plasmid pING I (21) and its derivatives. Plasmid pBSoriC was described previously (22). The plasmid pBMA1 contains the 91-bp BamH1 fragment harboring the 13-mers on the BamH1 restriction site of pUC18. pBT6 was obtained by insertion of the 1.8-kilobase SalI fragment containing iciA (19) into the SalI site of the M13mp19. Construction of IciA-overproducing Plasmids—An XhoI restriction site and translation stop codons were introduced by a synthetic oligonucleotide containing the mutated sequences and 12-nucleotide flanking sequences at both sides of the mutated region (Fig. 1). The oligonucleotide phosphorylated with T4 polynucleotide kinase was annealed to pBT6 single-stranded DNA containing the iciA gene. Mutagenesis was performed by using the oligonucleotide-directed mutagenesis system (Version 2, Amersham Corp.). The nucleotide sequence between the mutated XhoI restriction site and the bazHI restriction site (Fig. 1) and that of bazHI (position 715 in Ref. 19) was confirmed by dideoxy sequencing. The 410 bp of the Hpa1/BazHI fragment (position 305–715 in Ref. 19) containing the mutated sequences replaced the wild-type restriction fragment on pBT6 Rfl DNA to avoid additional mutations at the region unsequenced. The resulting M13 derivative is M13RSI. To construct M13 ISD2, containing the modified Shine-Dalgarno sequence (23), a synthetic oligonucleotide containing the modified sequences was inserted using the same strategy as described above. Then, insertions of 1.2-kilobase XhoI/EcoRI fragments from the M13 RSI and M13 ISD2 Rfl DNAs into the SalI/EcoRI sites of plasmid pING I (21) yielded pIC1 containing the iciA natural Shine-Dalgarno sequence and pICSI containing the modified sequence.

Purification of IciA Protein—The IciA-overproducing strain, MC1061 (pICSI), was grown in a Chemap 300-liter fermentor at 32 °C in 200 liter of LB medium containing 40 μg/ml ampicillin to an OD at 600 nm of 0.45. Overproduction of IciA protein was achieved in 2.5 h with the addition of 1.5 kg of L-(+)-arabinose (Sigma). The cells were collected in a Sharples centrifuge, resuspended to an OD at 600 nm of 550 in Buffer B followed by lyophilization. Half of the frozen cells (100 g) were thawed at 8 °C, diluted to 300 ml with...
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Buffer A, lysed, and centrifuged as described previously (17). To the supernatant (Fraction I, 230 ml) 55.2 g of ammonium sulfate was added with stirring and then centrifuged. To remove the contaminating nucleic acids, the precipitate was resuspended in 30 ml of Buffer B, precipitated by the addition of 6.3 g of ammonium sulfate, and centrifuged. The precipitate was resuspended in Buffer A (Fraction II, 24 ml). Of Fraction II, 7 ml was diluted with Buffer B to a conductivity equivalent to Buffer D and applied to a phosphocellulose column (P-11, Whatman; bed volume, 100 ml) equilibrated in Buffer D. The column was washed with 250 ml of Buffer D; the bound IciA protein was step eluted with 300 ml of Buffer E. The active fractions measured by gel-shift assays (see below) were pooled (Fraction III, 7 ml). Of Fraction III, 3.5 ml was diluted with Buffer C to a conductivity equivalent to Buffer D, applied to a Mono Q HR 16/10 column (fast protein liquid chromatography, Pharmacia LKB Biotechnology Inc.) equilibrated with Buffer D, washed with 40 ml of Buffer D, eluted with a linear gradient of 150 ml of 0.05-0.6 M KCl in Buffer C, and collected as 4-ml fractions. IciA protein was eluted at about 0.17 M KCl (Fraction IV, 4 ml).

Gel-shift Assay—The assay was performed with 1.5 fmol of the 638-bp C1 fragment isolated from pSBorC cleaved into three by Sau96I restriction and 32P labeled, as described previously (17). Among the three fragments, the 198 bp of the L-ori fragment contains the three 13-mers of oriC. The fragment shifted by IciA protein was cut out from the dried gel and the radioactivity measured in a liquid scintillation counter. One unit of IciA activity shifts one-fourth of the L-ori fragment input.

RESULTS

Overproduction of IciA Protein—Cells harboring the iciA gene on the multicopy number plasmid pUC18 exhibited a pronounced lag before achieving exponential growth (19). To circumvent abnormal growth responses that might affect overproduction, the expression of iciA was placed under control of the inducible araB promoter on a pLING1 plasmid (Fig. 1). This construction (pIC1) also contained two stop codons to prevent the formation of an AraB'-IciA fusion protein. An extract from cells harboring pIC1 with the natural iciA Shine-Dalgarno sequence possessed 174-fold more IciA activity than found in cells with the vector (pING1) (Table I). Modification of the Shine-Dalgarno sequence of pISC1 increased overproduction of IciA another 3-fold (Table I). Approximately 12% of the total protein in the soluble extract (Fraction I) from cells containing pISC1 was IciA protein.

Isolation of IciA Protein—The numerous steps needed to obtain homogeneous IciA protein from a nonoverproducing strain yielded only 100 µg of the protein from 700 g of cell paste (OD at 600 nm = 600) (17). Overproduction of the protein from induced cells containing pISC1 enabled isolation of 274 mg from 160 g of cell paste; more than 2 mg of homogeneous IciA protein was obtained per liter of culture (Table II). A simple step elution from a phosphocellulose column yielded a nearly pure Fraction III (Fig. 2), and a Mono Q column removed minor contaminants.

The IciA protein obtained previously (17) was shown to bind specifically to the 13-mers in oriC and to block initiation of oriC plasmid replication at the stage that preceded opening of the 13-mers. IciA protein from the overproducing strain (Fraction IV) behaved similarly in the binding of the 13-mers (Fig. 3A) and inhibition of oriC plasmid replication (Fig. 3B).

Specificity and Stability of IciA Binding to the 13-mers—IciA protein bound the L-ori fragment (198 bp) containing the 13-mers, thereby shifting the mobility of the L-ori fragment (Fig. 4A) (17). A 2.8-kilobase plasmid (pBMA1) containing only the 13-mers inserted into a pUC18 plasmid was able to compete with the L-ori fragment for binding to the IciA protein (Fig. 4B). Present with 1.5 fmol of the L-ori fragment, 2.7 fmol (5 ng) of pBMA1 reduced the binding by 24%; 5.4 fmol (10 ng) of pBMA1 reduced it by 50%.

### Table I

**Overproduction of IciA**

The indicated plasmid-containing strains of MC1061 were grown at 32 °C in 1 liter of LB medium containing 40 µg/ml ampicillin. At an OD (595 nm) of 0.5, L-(+)-arabino was added to 0.8% (w/v). The culture was grown for 2.75 h and harvested by centrifugation at 6,000 rpm for 15 min in a Sorval GS rotor. The pellets were resuspended in 3 ml of Buffer A and frozen in liquid nitrogen. The thawed cell paste was lysed as described previously (17) and cleared by centrifugation at 12,000 rpm for 2 min in a Beckman Ti-50 rotor. The supernatant was precipitated by addition of 0.24 g of ammonium sulfate/ml of supernatant. The precipitate was collected by centrifugation at 12,000 K rpm for 20 min in a Sorval SS34 rotor and resuspended in 150 µl of Buffer A. The 13-mer binding activity of IciA protein was measured by gel shift (see "Experimental Procedures").

| Plasmid       | Specific activity × 10^{-9} | units/mg |
|---------------|-----------------------------|----------|
| pING1 (vector)| 1.4                         | 0.61     |
| pIC1          | 244                         | 2.26     |
| pISC1         | 792                         | 3.20     |

### Table II

**Purification of IciA**

IciA protein was purified from MC1061 (pISC1) induced with arabinose (see "Experimental Procedures"). The values indicated in Fractions II, III, and IV are adjusted to correct for the fact that only part of the fractions were used.

| Fraction     | Volume | Protein Activity | Specific activity | Yield | Purification |
|--------------|--------|------------------|-------------------|-------|-------------|
|              | ml     | units × 10^{-4}  | units × 10^{-9}/mg| %     | -fold       |
| I. Lysate    | 230    | 6,440            | 1,803             | 100   | 1           |
| II. Ammonium sulfate | 24     | 1,776            | 1,136             | 64    | 2.3         |
| III. Phosphocellulose | 102    | 438              | 867               | 48    | 7.1         |
| IV. Mono Q   | 27     | 274              | 611               | 34    | 8           |
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contrast, 10 ng of pUC18 (5.6 pmol) did not reduce the binding at all, nor did comparable amounts of another DNA (M13mp18 Rp) affect it significantly. The binding activity of IciA protein was sensitive to N-ethylmaleimide which alkylates sulfhydryl groups on protein. Exposure to 50 mM N-ethylmaleimide for 15 min at 37 °C destroyed more than 95% of the activity.

After incubation of IciA protein (27 fmol) with the labeled L-ori fragment (1.5 fmol) in the gel-shift assay, 54 fmol of unlabeled pBMA1 was added to measure dissociation of IciA protein from the L-ori fragment. The IciA-L-ori fragment complex was relatively stable for about 2 h at 30 °C (Fig. 5) and then dissociated with a half-life of about 1.5 h. Incubation of IciA protein under comparable conditions without any DNA present did not alter the 13-mer binding activity of IciA protein.

IciA Protein Is a Dimer—IciA protein migrated as a 33-kDa polypeptide in SDS-polyacrylamide gel electrophoresis (17) (Fig. 2), consistent with the calculated value of 33.4 kDa for the 297 amino acids deduced from the nucleotide sequence in the open reading frame of iciA (19). However, the native size, as judged by gel filtration, coincided with that of bovine serum albumin (68.2 kDa) (17). Upon sedimentation in an 18–36% (v/v) glycerol gradient, the peak of IciA activity migrated slightly slower than the peak of bovine serum albumin (Fig. 6A), with a sedimentation coefficient of 4.9 S (Fig. 6B). The Stokes radius of IciA protein upon gel filtration was estimated to be 39 Å, with a frictional coefficient 1.46 (Fig. 6C), indicating an elongated shape with an axial ratio of 8–10 (24, 25). Dimethyl suberimidate, a cross-linking agent (26), generated

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\text{Stability (percent)} = 100 - \frac{\text{radioactivity at indicated time}}{\text{radioactivity before addition of pBMA1 DNA}}
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FIG. 3. IciA proteins from over-producing and wild-type are equally active. A, binding of the IciA proteins to the L-ori fragment containing the 13-mer region was measured in the gel-shift assay (see “Experimental Procedures”). B, inhibition of oriC plasmid replication of pBSoriC (83 fmol of plasmid/200 ng of DNA) by the IciA proteins was performed as described previously (17).
Fig. 6. Sedimentation of IciA protein. A, IciA protein (10 μg in 0.1 ml of Buffer E) was loaded onto a 18–38% (v/v) glycerol gradient in Buffer E. Two different mixtures (0.1 ml of each) containing marker proteins, catalase (kat, 238 kDa), aldolase (aldo, 158 kDa), bovine serum albumin (BSA, 66 kDa), ovalbumin (oval, 45 kDa), and lysozyme (lys, 14 kDa), each at 200 μg, were prepared in Buffer E and loaded onto separate gradients. The gradients were centrifuged for 24 h at 50,000 rpm at 4°C in a Beckman SW 50.1 rotor. Fractions (100 μl each) were collected from the bottom. Those containing IciA protein were diluted 10-fold in Buffer E, and 0.5 μl samples were measured in the gel-shift assay. The marker proteins were detected by Bradford assay (33) and by SDS-polyacrylamide gel electrophoresis. B, sedimentation coefficients were determined based on the values obtained above (24). C, Stokes radii were determined from Superose-12 gel filtration fast protein liquid chromatography (24); the marker proteins were as above.

Identification of idiA gene and its nucleotide sequences (19) enabled us to construct IciA-overproducing strains from which the protein could be isolated readily and in quantity. By gel-shift assay and DNase I footprinting, the IciA protein was found to bind the three 13-mers in the E. coli chromosomal origin (oriC) (17). This binding blocks the initiation of replication which depends on the opening of the 13-mer region (17). Mutations in each of three 13-mers altered the binding affinities of the IciA protein and affected the inhibition of cellular abundance of IciA and DnaA proteins in cultural growth phases. A, growth curve. E. coli, W3110, was grown in LB medium containing 0.2% glucose at 37°C. B, Western blot analysis. At the indicated OD (at 600 nm), cells were collected by centrifugation at 8,000 rpm in a Sorvall SS34 rotor for 10 min, washed with cold TE buffer (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA), collected by centrifugation, and resuspended in TE buffer to an OD (at 600 nm) of 26.6. Resuspended cells (15 μl) were mixed with 15 μl of SDS-polyacrylamide gel electrophoresis sample buffer (10 mM Tris-HCl (pH 6.8), 1% SDS, 0.1 M Tris(hydroxymethyl)aminomethane, 10% glycerol, and 0.005% bromophenol blue) and boiled for 2 min followed by electrophoresis through a 10% SDS-polyacrylamide gel with known amounts of IciA and DnaA proteins as standards. IciA and DnaA proteins were visualized with rabbit anti-IciA and anti-DnaA sera, horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad), and ECL detection reagents (Amersham Corp.). The contents of IciA and DnaA proteins were determined by comparison with known amounts of the proteins using the GS300 scanning densitometer (Hoefer Scientific Instruments Corp.). C, the cellular abundance of the proteins was calculated, assuming that an OD value of 1 at 595 nm represents 8 x 10^10 cells/ml (31).
replication in vitro in a corresponding way. The protein failed to bind pUC18 or M13mp18 R which do not possess the 13-mer sequences (Fig. 4). These findings prove that the specific binding of IciA protein to the 13-mers is required to block initiation.

The binding of DnaA protein to the four 9-mers in oriC triggers the initiation of chromosomal replication by the opening of the 13-mer region (17, 22). Although the level of DnaA protein appeared to be constant during the rapid growth phase of the culture (Fig. 7 and Refs. 28–30), its activity might still be modulated by its state of aggregation (10, 11), complexing with ATP or ADP (8, 9), and interactions with membranes (29, 32).

Cells in which the level of IciA protein was elevated exhibited a pronounced lag of growth upon transfer to fresh medium (19). This and the 4-fold increase of IciA as cells approach the stationary phase (Fig. 7) support a role for IciA protein as an inhibitor of chromosomal initiation as observed in in vitro (17).

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