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iciA, an *Escherichia coli* gene encoding a specific inhibitor of chromosomal initiation of replication *in vitro*

(13-mer binding protein/*oriC* replication/LysR family)

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ABSTRACT **The gene encoding the protein that binds the three 13-mers in the origin (*oriC*) of *Escherichia coli* to block initiation of replication *in vitro* has been cloned, sequenced, and overexpressed. The gene possesses an open reading frame for 297 amino acids (mass of 33,471 Da). The protein has a motif for DNA-binding (helix-turn-helix) and has homology to a diverse set of prokaryotic regulatory proteins, known as the LysR family. The protein, previously referred to as the 33-kDa protein, has been named IciA (for inhibitor of chromosome initiation). The *iciA* gene is at 62.8 min on the chromosomal map. Cells with enhanced levels of the protein grow at a normal rate but generally exhibit a pronounced lag upon transfer to a fresh medium.**

Chromosomal replication in *Escherichia coli* is regulated at initiation (1). The timing of this event may be influenced by many factors, including the DnaA initiator protein (2–5), nearby transcription at *oriC* (6, 7), the membrane attachment of DNA (8), and the methylation of DNA (9–12). Initiation is localized at *oriC*, a 245-base-pair (bp) sequence with highly conserved and biologically essential regions (13, 14). Among them are the four binding sites for the DnaA protein (15) and an A+T-rich region containing three 13-mers (Fig. 1) that are opened by DnaA protein (16), the dominant positive effector in this early stage of chromosome replication. No specific negatively acting protein had been described until the discovery of a 33-kDa protein that binds to the 13-mers to block initiation (17). In a replication assay reconstituted with purified proteins and an *oriC*-containing plasmid, binding by this protein prevents the opening of the A+T-rich region by the DnaA protein. Once the 13-mers are opened, the inhibitory protein has no effect on the subsequent stages of replication *in vitro* (17).

In this report, we describe the cloning and sequencing of the gene for the 33-kDa protein, renamed IciA, for inhibitor of chromosome initiation.* Elevated levels of the protein in cells delay their resumption of growth upon transfer to fresh medium.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Phages. The *Escherichia coli* strains used were as follows: C600 (*supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21*); DH5α [*supE44 ΔlacU169 (φ80 lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1*]; TG-1 (*supE hsdΔ5 thi Δ(lac-proAB)* F'[*traD36 proAB*$^+$ *lacI*^{q} *lacZΔM15*]; W3110 (F^-, λ^-). *E. coli* C600 served as host for λ phages [EMBL4 derivatives of the Kohara collection (18)] and was grown in NCZYM plus 0.2% maltose (19). Amplification and DNA preparation of λ phages were performed according to standard methods (19). All other strains were grown in L broth/0.2% glucose. Strain DH5α was the host for pUC18 and pUC19 derivatives and was used for plasmid-DNA preparations. Strain TG-1 was the recipient for M13-phage derivatives mp18 and mp19 and was used to isolate single-stranded DNA. Strain W3110 was used to isolate chromosomal DNA and to prepare fraction II. Growth rates were determined by measurements of OD at 600 nm.

Protein Sequencing and PCR with Deduced Oligonucleotides. Purified IciA protein (17) (10 μg; 300 pmol) was subjected to N-terminal amino acid sequencing, done by Alan Smith (Stanford University). Based on a sequence of 41 amino acids, two degenerate oligonucleotide primers were designed: 5'-ATGAA(A,G)CG(A,T,C,G)CC(A,T,C,G)-GA(T,G)TA-3' [nucleotides (nt) 650–666, amino acids 1–6]; and 3'-AA(A,G)CT(T,C)(T,G)CICGICG(A,T,C,G)GT-(T,C)TT-5' (nt 710–729, amino acids 21–27). The oligonucleotides, synthesized in a DNA synthesizer (Applied Biosystems) at the Protein and Nucleic Acid (PAN) facilities of Stanford University, were used in a PCR with 120 ng of high-molecular-weight, genomic-DNA template from *E. coli* W3110 under standard conditions (Cetus) plus 6 mM $MgCl_2$ in a TwinBlock System thermocycler (Ericomp, San Diego). An initial denaturation step for 7 min at 94°C was followed by 35 repeats of 2-min denaturations at 94°C, 30 sec of hybridizing at 37°C, and 1.5 min of extension at 72°C. After removing the mineral oil by phenol extraction, the product was phosphorylated by T4 polynucleotide kinase and resolved on a 4% agarose gel. An 80-bp DNA fragment was isolated and cloned into the *Sma* I restriction site of pUC18.

Screening of the Kohara Phage Library of *E. coli*. The cloned 80-bp PCR product was used as a probe to screen the Kohara λ-phage library ["mini"-set collection (18)]. The isolated DNA fragment was labeled with [^{32}P]dCTP (Amersham) by using the random-priming method (20). The individual λ phages were transferred to nylon filters (Colony/PlaqueScreen, DuPont) and hybridized at 65°C under standard conditions (19). From 476 screened colonies, a single phage was hybridized (IA2 in mini-set no. 471). DNA was isolated from this λ phage to subclone several restriction fragments into pUC18/19 vectors.

DNA Sequence Analysis. DNA sequencing was performed by the dideoxynucleotide termination method (19) using the Sequenase system (United States Biochemical). The 1.8-kilobase (kb) *Sal* I fragment cloned in both orientations into M13mp18 was sequenced, starting from both ends with the universal primer (Pharmacia) and subsequently designed specific primers (synthesized by Pam Patek, Stanford University). At every ≈250 bp, another oligonucleotide was designed and used for a primer-extension reaction; the DNA sequence of both strands was thus determined. For computer analysis of the DNA and protein sequences, the University of

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Abbreviation: nt, nucleotides.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M62865).

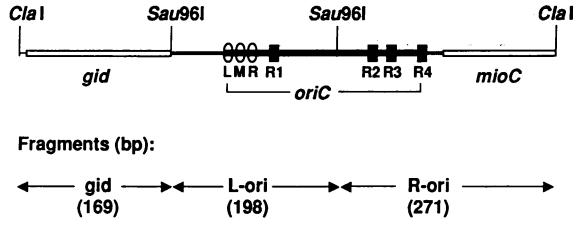

FIG. 1. Physical map of the *Cla* I restriction fragment from pBSoriC (16) that spans the *oriC* and neighboring regions. The extent of the minimal *oriC* region is indicated by the bracket. L, M, and R are the left, middle, and right 13-mers, respectively. R1, R2, R3, and R4 are the 9-mer DnaA protein-binding sites. The open bars at the left and right of *oriC* represent partial *gidA* and *mioC* genes, respectively. The 638-bp *Cla* I fragment was cleaved by *Sau*96I into the gid, L-ori, and R-ori fragments.

Wisconsin Genetics Computer Group software package was used.

Preparation of Fraction II and the Gel-Shift Assay. *E. coli* W3110 was grown in 1 liter of L broth to OD_{600} = 0.8, collected by centrifugation, and lysed as described (17). Protein was precipitated by adding 0.28 g of ammonium sulfate per ml of fraction I and centrifuged for 20 min at 18,000 rpm in Sorvall SS34 rotor. The pellet was resuspended in 400 μl of buffer containing 25 mM Hepes·KOH (pH 7.6), 15% (vol/vol) glycerol, 0.1 mM EDTA, and 2 mM dithiothreitol and dialyzed in the same buffer to a conductivity equivalent to 0.2 M KCl (fraction II). Gel-shift assay was done as before (17).

RESULTS

Cloning of the *iciA* Gene. The purified IciA protein (17), subjected to N-terminal amino acid sequencing, yielded a stretch of 41 amino acids starting with methionine. Two degenerate oligonucleotides were designed to amplify the intervening sequence using standard PCR with genomic DNA from *E. coli* W3110. An amplified DNA fragment with the expected length of 80 bp, as judged by agarose gel electrophoresis, was obtained. This fragment was subsequently cloned and sequenced. The sequence revealed an open reading frame coding for the N-terminal 27 amino acids of the IciA protein.

The cloned PCR product was used to probe genomic DNA by Southern hybridizations and to screen the Kohara λ phage library of *E. coli* (6) (data not shown). Although the fragment sizes mapped with the 80-bp fragment probe did not completely match to any region of the published linkage map (Fig. 2), a single hybridizing phage was found in the Kohara library. From this one candidate (λ phage IA2), DNA was isolated and used to generate different subclones in pUC18 (data not shown). Sequence analysis of the subcloned 1.8-kb *Sal* I fragment using the degenerate oligonucleotides revealed an open reading frame coding for the N-terminal 41 amino acids of the IciA protein (Figs. 2 and 3). Complete sequence analysis and overexpression (see below) verified that the cloned gene encoded the 13-mer binding protein (17).

Location of the *iciA* Gene. The *iciA* gene was located near the kilobase coordinate 3074 corresponding to 62.8 min on the *E. coli* chromosomal map. Partial DNA-sequence analysis confirmed its location near the *serA* gene about 1.3 kb upstream of the *iciA* start codon (Fig. 2; refs. 21, 22). The *serA* gene, which is transcribed counterclockwise in the genomic map coordinates, encodes the 3-phospho-D-glycerate dehydrogenase that catalyzes the first step in L-serine biosynthesis. The DNA restriction pattern of λ clone IA2 revealed two *Kpn* I sites and one *Bam*HI site in addition to those in the published Kohara map of the *E. coli* genome (18; Fig. 2). Southern hybridizations using λ-DNA IA2 as a

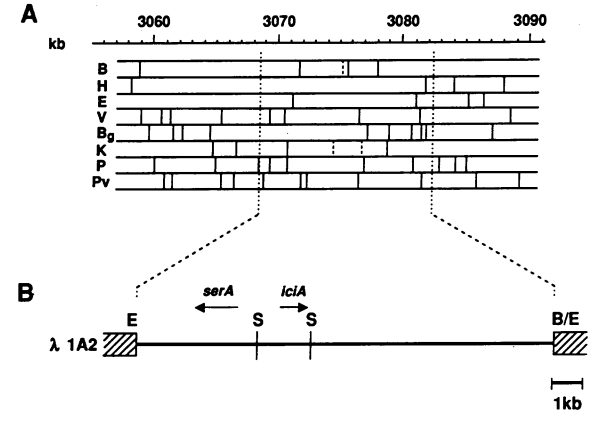

FIG. 2. Physical map of the 62.8-min chromosomal region and location of λ phage IA2 and the *iciA* gene. (*A*) Chromosomal segments produced by the eight restriction enzymes used to map the chromosome (18). On top are the kilobase coordinates. The three dashed vertical lines indicate previously unidentified restriction sites. (*B*) Physical map of λ phage IA2 (mini-set no. 471) from the Kohara collection (18) and location of the sequenced *Sal* I fragment that includes coding region for the *iciA* gene. The *serA* gene maps 1.3 kb upstream from the *iciA* gene and is transcribed in the opposite direction. B, *Bam*HI; H, *Hin*dIII; E, *Eco*RI; V, *Eco*RV; Bg, *Bgl* I; K, *Kpn* I; P, *Pst* I; Pv, *Pvu* I; and S, *Sal* I.

probe confirmed the sizes of these fragments in the genomic DNA of *E. coli* W3110.

Sequence Analysis of the *iciA* Gene. The sequence of the 1815-bp *Sal* I fragment (Fig. 3) reveals an open reading frame extending from the N-terminal 41 amino acids of the IciA protein for 297 amino acids (nt 650–1540) to encode a polypeptide of 33,471 Da. This size is consistent with the 33-kDa value determined for the purified protein by SDS/PAGE (17). Sequences conforming to the consensus for *E. coli* promoters (−35 and −10) are not present upstream of the start codon for the *iciA* gene. Nevertheless, signals to expressing the *iciA* gene must be located on the *Sal* I fragment, as judged by the overexpression of the gene from a high-copy-number plasmid bearing this fragment (see below). An inverted repeat with potential for involvement in transcriptional termination is present between nt 1557 and 1577. The calculated free energy value for this hairpin structure is −11.4 kcal/mol [Genofit (Geneva) PC/Gene program package].

Homology of IciA Protein to Prokaryotic Transcriptional Regulators. The IciA protein, when compared with those in the Swiss-Prot data bank, shared extensive sequence homology with several bacterial proteins. The homology was generally restricted to the N-terminal third or half of the sequences (data not shown); only three proteins showed more overall identities (Fig. 4). All of these (12 or more) proteins belong to the LysR family of prokaryotic regulators and behave as transcriptional activators (for review, see ref. 23). They also negatively regulate their own expression and possess a potential helix-turn-helix DNA-binding motif in their N-terminal domain.

Considerable homology exists between IciA and the AmpR, MetR, and NahR proteins (Fig. 4). The AmpR protein of *Citrobacter freundii* is responsible for the induction of the *ampC* β-lactamase gene (24), the MetR gene product of *Salmonella typhimurium* regulates the methionine biosynthesis genes *metE* and *metH* (25, 26), and the NahR protein of *Pseudomonas putida* activates transcription of the *nah* and *sal* operons responsible for the metabolism of napthalene and salicylate, respectively (27, 28). The amino acids that might form the helix-turn-helix motif are among the most conserved of the residues. Although each of these proteins are known to bind their regulatory DNA sites, the amino acids that form

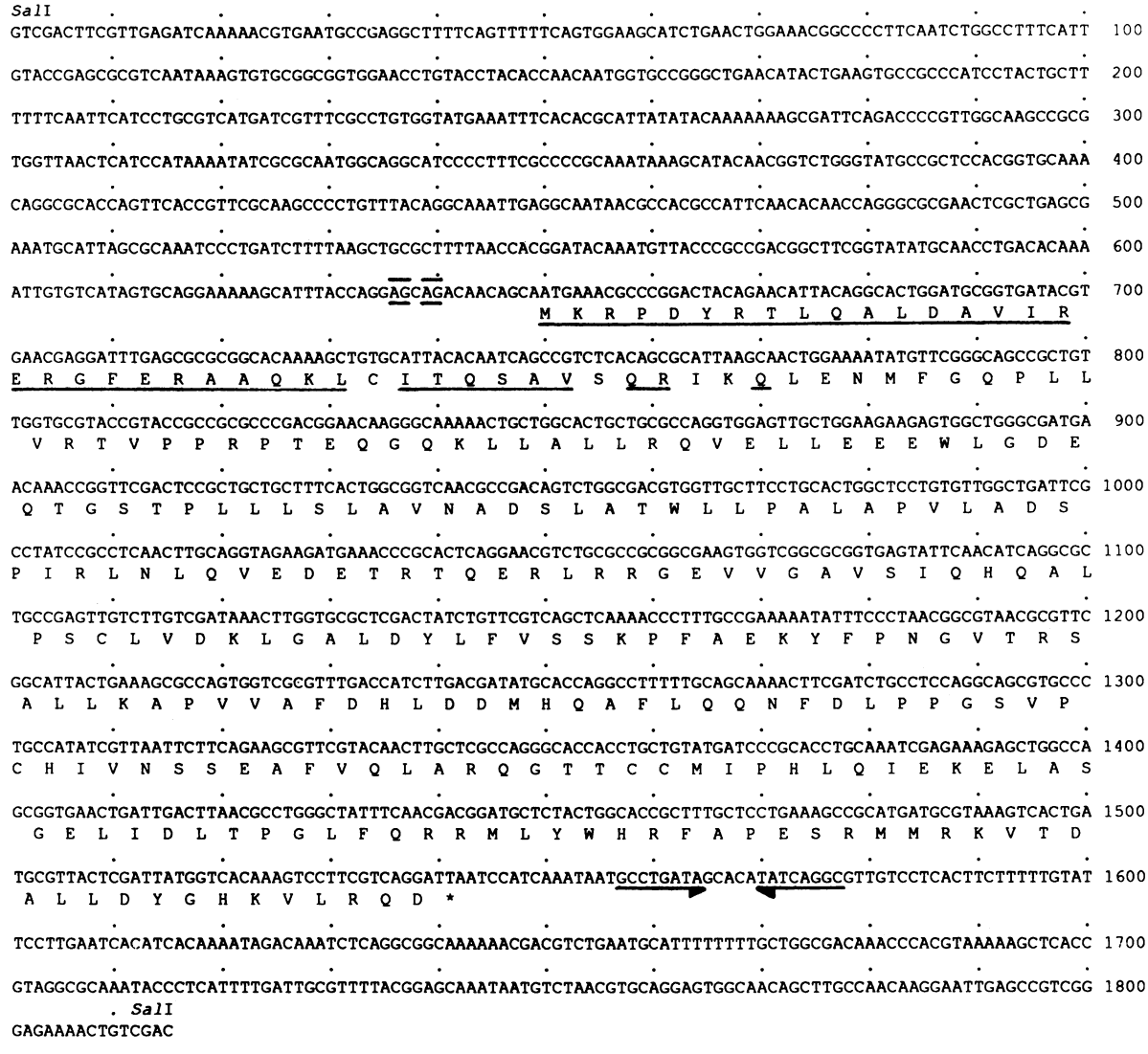

FIG. 3. Nucleotide sequence of the 1815-bp *Sal* I fragment encoding the *iciA* gene. The deduced amino acids agree with N-terminal sequence analysis of the purified IciA protein and are underlined. An inverted repeat with the potential to form a stem-loop structure for transcriptional termination is shown by the two arrows between nt 1557 and 1577. Indicated at nt 636–640 is the putative Shine–Dalgarno sequence.

the proposed helix-turn-helix DNA-binding motif have yet to be shown directly involved in the protein–DNA interaction.

Among the LysR family some of the many leucine residues present (15% of the total amino acids in IciA) seem to be highly conserved throughout the whole length of the proteins (Fig. 4) and might be responsible for some of their common functional features.

Expression of the *iciA* Gene. The IciA protein had been purified and characterized by specific binding to the three 13-mer in the *oriC* region (17). As proof that the cloned gene is encoding the 13-mer binding protein, the gene was overexpressed, and crude extracts were examined for enhanced binding activity. To achieve overexpression, several restriction fragments (data not shown) were cloned in the high-copy-number plasmid pUC18 and transformed into *E. coli* (strain W3110). Late-logarithmic-phase cells grown in rich medium (OD_{600} = 0.8) were the source of extracts assayed for binding the L-ori fragment that contains the 13-mers (Fig. 1); a specific shift in mobility of this fragment in electrophoresis through a 5% polyacrylamide gel is observed and coincided with the same shift using the previously purified IciA protein (Fig. 5). An extract from cells harboring the 1.8-kb *Sal* I restriction fragment in pUC18 showed a 33-fold specific activity increase in shifting the L-ori fragment (Fig. 5, lanes 8–10), compared to an extract from cells bearing only the pUC18 vector (lanes 5–7 in Fig. 5; see also Table 1). About the same increase in binding activity was observed in strains bearing the larger 6.2-kb *Pst* I fragment (Fig. 2) cloned in pUC18 (data not shown). Inasmuch as the reading frame of the *iciA* gene is in the direction opposite to that of the *lacZ* gene, expression of *iciA* is independent of *lac* promoter activity. These results establish that the 1.8-kb *Sal* I fragment is sufficient for expression of the *iciA* gene from its promoter.

E. coli harboring the *iciA* gene on a pUC18 plasmid and overexpressing the protein, as shown above, apparently grew at the same rate as isogenic wild-type cells (doubling time with pUC18, 25 min; doubling time with pUC18-iciA, 26.5 min; Fig. 6). However, in 25 of 38 experiments, these cells lagged in growth. They attained an OD_{600} value of 0.5 from 1–4 hr later (average of 2.2 hr). In the other 13 cases, the delay was 1 hr or less. Lag times were independent of whether the inoculations into fresh LB medium were made from stationary or logarithmic-phase cultures at OD_{600} = 0.5.

DISCUSSION

The IciA protein blocks initiation of *E. coli* chromosomal replication *in vitro* by binding the 13-mers of the origin (*oriC*) sequence and preventing the action of the DnaA initiator protein (17). Amino acid sequencing of the N-terminal part of

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                                     Helix Turn Helix
Ec IciA   1  MKRP  DYRTLQALDAVIRERGFERAAQKLCITQSAVSQRIKQLENMFGQPLLVRTVPP RPTEQGQKLL
Cf AmpR   1  MTRSYIPLNSLRAFEAAARHLSFTRAAIELNVTHSAISQHVKSLEQQLNCQLFVRGSRGLMLTTEGESLL
St MetR   1  MI     EIKHLKTLQALRNSGSLAAAAAVLHQTQSALSHQFSDLEQRLGFRLFVRKSQPLRFTPQGEVLL
Pp NahR   1  MELRDLDLNLLVVFNQLLVDRRVSITAENLGLTQPAVSNALKRLRTSLQDPLFVRTHQGMEPTPYAAHLA

IciA     68  ALLRQVELLEEEWLGDEQTGSTPLLLS  LAVNADSLATWLLPALAPVLADSPIRLNLQVEDETRTQERL
AmpR     71  PVLNDSFDRMAGMLDRFATKQTQEKLK  IGVVGTFAIGCLFPLLSDFKRSYP HIDLHISTH   NNRV
MetR     67  QLANQVLPQISRAL QACNEPQQTRLR  IAIECHSCIQWLTPALENFRASWP QVEMDFTSGVTFDPQP
NahR     71  EPVTSAMHALRNALQHHESFDPLTSERTFTLAMTDIGEIYFMPRLMDVLAHQAPNCVISTVRDSSMSLMQ

IciA    136  RRGEVVGAVSIQHQALPSCLVDKLGALDYLFVS SKPFAEKYFPNGV TR SALLKAPVVAFDHLDDMHQ
AmpR    135  DPAAEGLDYTIRYGGGAWHDTDA    QYLCSALMSPLCSPTLASQIQTP ADILKFPLLRSYRRDE WA
MetR    133  ALQQGELDLVMTSDILPRSELHYSPMFDFEVRLVLAPDHPLASKTQI TP EDLASETLLIYPVQRSRLD
NahR    141  ALQNGTVDLAVGLLPNLQTGFFQRRLLQNHYVCLCRKDHPVTREPLTLERFCSYGHVRVIAAGTGHGEVD

IciA    203  AFLQQNFDLPPGSVPCHIVNSSEAFVQLARQGTTCCMIPHLQIEKELASGELIDLTPGLFQRRM LYW H
AmpR    199  LWMQAAGEAPPSPTHNVMVFDSSVTMLEAAQGGMGVAIAPVRMFTHLLSSERI VQPFLTQIDLGSYWIT
MetR    201  VWRH  FLQPAGISPLLKSVDNTLLLIQMVAARMGIAALPHWVVESVERQGLV VTKTLGD    GLW S
NahR    211  TYMT RVGIRRDIRLEVPHFAAVGHILQRTDLLATVPIRLADCCVEPFGLSALPHPVVLPEIAINMFWHA

IciA    271  RFA  PESRMMRKVTDALLDYGHKVLRQD  297
AmpR    268  RLQSRPETPAMREFSRWLTG    VLHK  291
MetR    263  RL    YAAVRDATS         VRR   276
NahR    280  KYHKDLANIWLRQLMFDLFTD         300
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FIG. 4. Amino acid homology between IciA, AmpR, MetR, and NahR. Shaded letters indicate identical amino acid residues or conservative valine-leucine-isoleucine substitutions. Helix-turn-helix DNA-binding motif is boxed. Organisms are *E. coli* (Ec), *C. freundii* (Cf), *S. typhimurium* (St), and *P. putida* (Pp).

the purified IciA protein enabled the cloning and sequencing of the *iciA* gene reported in this study.

The DNA sequence upstream of the *iciA* reading frame did not reveal canonical −35 or −10 promoter sequences. Yet, elevated expression of the gene is achieved from a cloned restriction fragment that contains 650 bp upstream of the translational start of *iciA* (Table 1). Deletion of upstream DNA within 345 bp from the translational start did produce a 70% drop of expression, as judged by gel-shift assays of crude extracts (data not shown). Thus, sites present several hundred base pairs upstream of the open reading frame may be needed for expression of the *iciA* gene. Downstream of the open reading frame, 13 bp from the stop codon, an inverted repeat is present that might be involved in transcriptional termination. These data suggest that the *iciA* gene is organized as a monocistronic operon, the transcription and regulation of which requires further study.

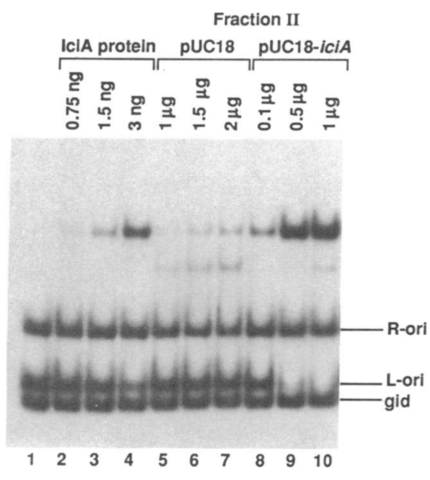

FIG. 5. Gel-shift assays of IciA protein activity in extracts of wild-type and overproducing cells. The 638-bp *Cla* I restriction fragment (Fig. 1) was cleaved with *Sau*96I and end-labeled with ^{32}P as described (17). Gel-shift assays detected binding of the IciA protein to the L-ori DNA fragment. The radioactively labeled fragments were cut out from the gel and quantitated in a liquid scintillation counter. One unit of IciA protein activity shifts one-fourth of the input L-ori fragment (1.5 fmol). Lanes: 1, no protein; 2–4, purified IciA protein (0.75 ng, 1.5 ng, and 3 ng, respectively); 5–7, crude extracts from cells harboring pUC18 (1 µg, 1.5 µg, and 2 µg, respectively); 8–10, crude extracts from cells harboring pUC18-iciA (1.8-kb *Sal* I; 0.1 µg, 0.5 µg, and 1 µg, respectively).

The amino acid sequence of IciA has homology to a family of bacterial regulatory proteins, grouped as the LysR family (23). Characteristics common to most of these proteins are (*i*) mass of 30–36 kDa, (*ii*) function as transcriptional regulators, (*iii*) negative regulation of their own expression, and (*iv*) presence of a helix-turn-helix DNA-binding motif. A role of IciA in regulating its own (or other genes) expression is not known and will be investigated in future studies. In the MetR protein of *E. coli* that also exhibits significant homology to

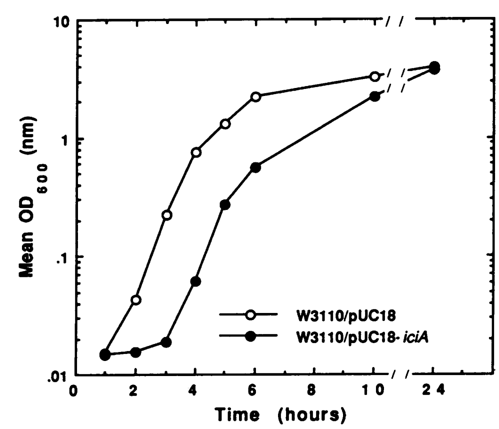

FIG. 6. Growth in rich medium of *E. coli* harboring pUC18 with or without the *iciA* gene. Because not all 38 experiments (see *Results*) contained the same time points, the data for this figure are presented only for experiments in which regular hourly values were collected. These values include all experiments whether or not a lag occurred.

Table 1. Overproduction of IciA in cells bearing the high-copy-number plasmids with the *iciA* gene

	Total protein, mg	Total activity, units $\times 10^{-3}$	Specific activity,* units $\times 10^{-3}$/mg
W3110/pUC18	24.2	8.2	0.34
W3110/pUC18-iciA	25.0	272	10.9

*IciA activity was determined in fraction II, the crude ammonium sulfate fraction.

the IciA protein (data not shown), four leucine residues between amino acids 19 and 40 have been proposed as responsible for leucine zipper dimerization (29). Both the IciA protein (17) and the MetR protein of *E. coli* (29) behave as dimers in solution. However, participation of the leucines of the MetR protein in homodimerization via leucine-zipper motifs conflicts with a potential helix-turn-helix DNA-binding domain spanning the same N-terminally located amino acids.

The role of the IciA protein *in vivo* is unknown. Overexpression of *iciA* on high-copy-number plasmids does cause a pronounced lag in the outgrowth of *E. coli* cells. This transient growth inhibition has yet to be connected to any replication inhibition. We also observed that an *iciA* null mutant of *E. coli* is viable and has the same growth rate as wild type (unpublished results). Investigations on the correct timing of replication initiation in this mutant should elucidate its role in chromosomal replication.

Cloning and sequencing of the *iciA* gene provide the basis not only for physiological studies but have also allowed the overexpression and purification of the protein in large quantity (unpublished results) for definition of its biochemical properties.

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