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Cloning and Promoter Identification of the Iron-Regulated *cir* Gene of *Escherichia coli*

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The *cir* gene, which encodes the colicin I receptor protein and is regulated by both cellular iron content and growth temperature, was cloned into a multicopy-number plasmid. Physical mapping and complementation analysis established the position of *cir* between *mgl* and *nfo* on the *Escherichia coli* chromosome. A gene encoding a 32,000-dalton polypeptide was located downstream of and adjacent to *cir*, but did not appear to be part of the same transcriptional unit. A 525-base-pair fragment from the 5′ end of the 1.8-kilobase-pair receptor-coding region directed iron-regulated transcription and translation of a hybrid *cir-lacZ* gene. Two overlapping promoters were identified by determination of the transcriptional start sites and by sequence analysis. A small open reading frame (120 nucleotides) of unknown significance preceded the receptor-coding sequence. Examination of the amino acid sequence of the receptor purified from the outer membrane revealed that the gene product was processed by removal of a signal peptide and that the mature form had an amino acid sequence near its amino terminus which closely resembled that of several other TonB-dependent proteins.

Several of the high-affinity iron chelators (siderophores) used by enteric bacteria to provide intracellular iron have specific outer membrane receptor proteins in common with a number of colicins and phages (30, 40). For example, the 81,000-dalton *fepA* gene product of *Escherichia coli* is required for adsorption of colicin B and binding of the iron-complexed form of enterochelin, the native siderophore of *E. coli* (30, 40). Production of FepA and the biosynthesis of enterochelin are regulated coordinately with the synthesis of the *cir* gene product, the 74,000-dalton outer membrane receptor for colicins Ia and Ib (8, 28).

An inverse relationship exists between transcription of *cir* and the ability of a cell to obtain iron from its growth medium (53). Although the colicin I receptor may be involved in iron acquisition, no specific role has been assigned to this protein in either the transport or assimilation of this essential nutrient. Mutants lacking the receptor have a doubling time indistinguishable from that of the wild type in both iron-replete and iron-deficient media (unpublished data). *E. coli* has the ability to utilize several siderophores produced by other bacteria and fungi found in its natural environment (39, 40), and thus it seems possible that the *cir* gene product may serve as the receptor for an unknown siderophore available to *E. coli* in its native setting.

Translocation of both vitamin B_{12} and iron-siderophore complexes from the outer membrane into the cell is dependent on TonB function, which has been proposed to mediate an energy-dependent coupling or modification of the components of the systems (9, 27, 30, 42). Besides being defective in the uptake of these beneficial substances, *tonB* mutants are insensitive to some phages and to the group B colicins, including colicins Ia and Ib (30). Mutational analysis suggests that the amino-terminal regions of the receptors interact with TonB (25), and sequence analysis of several TonB-dependent transport proteins has revealed significant homology in this region (13, 33).

The molecular basis for the regulation of *cir* and other genes subject to control by iron is just beginning to be understood. The product of the *fur* gene (ferric uptake regulation) is a 16,800-dalton polypeptide necessary for repression of the iron uptake systems of *E. coli* and *Salmonella typhimurium* (46). Recent work has indicated that a dimeric form of this protein is able to form a complex with free ferrous ion and bind to the promoter region of the aerobactin operon in vitro (2a). Furthermore, DNase I protection experiments performed in the same system with purified repressor showed metal-dependent binding in the region containing the −35 and −10 sequences of the major promoter (17). Thus, iron and Fur appear to be corepressors of transcription in the aerobactin operon, inhibiting RNA polymerase binding as long as the supply of iron in the cells remains high. Whereas *fur* has been mapped at 16 min on the *E. coli* genetic map (2) and affects many genes regulated by iron, another mutation known as *cirR* has been described that is closely linked to *cir* (45 min) and results specifically in increased levels of the colicin I receptor under repressing (high-iron) conditions (55). The mutation was *cis* dominant and was not manifested when iron was limiting.

To learn more about the nature of such mutations, the processes that regulate expression of iron-controlled genes, and the functional significance of the *cir* gene product, we have cloned the colicin I receptor gene. Through physical analysis, the distances separating *cir* from neighboring genes of known function were defined. The location, organization, and regulation of the cloned gene were determined. Finally, the *cir* promoter region was sequenced and compared with those which mediate synthesis of other outer membrane proteins in response to iron limitation.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. Bacterial strains are listed in Table 1. Cosmid vector pHC79 determines resistance to ampicillin and tetracycline (26). Cloning vector pBR329 contains resistance determinants for ampicillin, chloramphenicol, and tetracycline (14). Plasmid pJB4J1 is required for thermostability of the operon fusion in strain JK791 (3). Bacteriophage Mu dII1734, which is carried on tetracycline resistance replicon pPO1734, is a small (9.7-kilobase [kb]) transposition-defective derivative of Mu which specifies kanamycin resistance and is capable of

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TABLE 1. Bacterial strains

Strain[a]	Characteristics	Source or reference
GM33	*dam*	J. Cronan
EM23	MC4100 Δ(*lac*) *X74 galU galK* Δ(*araCOIBA-leu*)*7697*	S. Maloy
ML17	*recA supE44 supF* (λ *b2 c*I857 *S*am7)	J. Cronan
MC4100	*araD139* Δ*lacU169 thi rpsL*	T. Silhavy
MH513	MC4100 Φ(*ompF-lac*)	50
LA5539	*lacY galE arg fpk zee-700*::Tn*10*	W. Boos (7)
LA5606	*lacY galE arg ptsF mgl-500*::Tn*10*	W. Boos (7)
LA5641	MC4100 Δ(*glpT-glpA*) *glpR gyrA mgl-500*::Tn*10*	W. Boos (7)
JK1	W3110, *rpsL*	This laboratory
JK354	AN193, *entA fhuA tsx supE44 proC leu trp thi lacY rpsL galK ara mtl xyl azi*	This laboratory
JK589	*E. coli* C600 containing F::Tn*5*	This laboratory
JK693	LA5641 cured of Tn*10*, *cir*	This laboratory
JK706	JK693 *recA* (λ c1857)	This laboratory
JK791	MC4100 Φ(*cir-lac*) *recA*, contains pJB4J1	55
JK820	JK693 *recA*	This laboratory
JK824	*S. typhimurium* LT2 *cir metA trpC rpsL ilv hsdL6 hsdSA29 galE*	This laboratory
JK838	MC4100 *recA cir*	This laboratory
JM103	*endA* Δ(*lac-pro*) *hsdR4 sbcB15 supE rpsL thi*, contains F′ *traD36 proAB*$^+$ *lacI*^{q} Δ*lacZM15*	This laboratory
POII1734	*araD139 ara*::(Mu cts)*3* Δ(*lac*)*X74 galU galK rpsL*, contains pPO1734	12

[a] All strains except JK824 (*S. typhimurium*) are *E. coli* K-12.

forming *lac* transcriptional and translational fusions when integrated in genes in the proper orientation and coding phase (12). Plasmid pMC1871 determines ampicillin resistance and carries the *lacZ* gene lacking a promoter, ribosome-binding site, and initiation codon (Pharmacia, Inc.).

Media and growth conditions. LB broth, M63, and M9 minimal medium were prepared as described by Miller (37) and supplemented where indicated. When low-iron conditions were desired, ethylene diamine-di(hydroxyphenylacetic acid) (EDDA; Sigma Chemical Co.) was used to rapidly chelate free iron. In some experiments, Chelex-100 resin (Bio-Rad Laboratories, Inc.) was used to remove iron from the medium before inoculation (38). When necessary, antibiotics were added to produce the following concentrations: ampicillin, 100 to 500 μg/ml; tetracycline, 20 μg/ml; chloramphenicol, 35 μg/ml; kanamycin, 50 μg/ml; and streptomycin, 100 μg/ml. Compound 2*R*-glyceryl-β-D-galactopyranoside was kindly supplied by Winfried Boos. All cultures were grown at 37°C and aerated by shaking unless otherwise noted.

Recombinant DNA techniques. The procedures compiled by Maniatis et al. (34) were generally used with minor modifications. Isolation of plasmid and phage DNA was carried out by the alkaline lysis methods of Birnboim and Doly (5) as modified (34).

Sensitivity to colicins. Sensitivity to colicins Ia and Ib was determined by cross-streaking overnight cultures of test strains against crude preparations of the colicins on LB agar plates containing appropriate antibiotics. Colicin V sensitivity was determined by the soft agar overlay method (21).

Transposition mutagenesis of plasmids with γδ and bacteriophage Mu dII1734. Guyer (22) has shown that derivatives of pBR322 mobilized by the F factor invariably acquire an insertion of the transposable element γδ (Tn*1000*). Donor strain JK589(pURB4100), containing the F factor, and the streptomycin-resistant recipient strain JK820 were each grown to mid-log phase in LB. Equal volumes from each culture were then combined, incubated for 2 h at 37°C without shaking, and plated on LB agar containing chloramphenicol, tetracycline, and streptomycin. Plasmid DNA was isolated from exconjugates and used to transform strain JK820 before analysis.

A procedure for introducing insertions of the small bacteriophage Mu dII1734 specifically into plasmids was adapted from the methods of Castilho et al. (12). A transducing lysate was prepared from strain POII1734(pURB4000) by temperature induction as described previously (50). Dilutions of the lysate were combined with an equal volume (200 μl) of a mid-log-phase culture of JK693 grown in LB containing 2.5 mM $CaCl_2$. After incubation at room temperature for 25 min without shaking, 5 mM $MgCl_2$, 5 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], and 1.5 ml of LB were added. Cells were incubated for 1 h at 30°C, centrifuged, and suspended in 400 μl of LB. Portions were plated on LB agar containing chloramphenicol and kanamycin and incubated at 37°C overnight. Plasmid DNA was isolated from Lac$^+$ transductants (determined by streaking on MacConkey agar) and used to transform strain JK838, with selection for chloramphenicol and kanamycin resistance. The transformants, which were all Lac$^+$, were analyzed to determine colicin Ia sensitivity, location of the Mu d insertion, and regulation of the gene fusion by iron.

Analysis of plasmid-encoded polypeptides. Proteins synthesized by plasmids were determined by the maxicell system (44) with some modifications (50). Strain JK838 was used as the host strain. Cells were grown in M9 containing 1.0% casein hydrolysate, thiamine (0.1 μg/ml), 0.4% glucose, and 20 μM $FeCl_3$. Incorporation of [^{35}S]methionine was in the sulfate-free medium described by Worcel and Burgi (52).

Assay of β-galactosidase. Measurement of β-galactosidase activity was carried out as described by Miller (37).

Localization of the *cir* promoter fragment. The position of the cloned 525-base-pair (bp) *Hae*III fragment mediating iron-regulated transcription in pMC1871 was determined relative to the *cir* coding region by a procedure based on partial restriction of end-labeled DNA fragments (51). The 2.4-kb *Dra*I fragment from pURB4100 was isolated, treated with alkaline phosphatase, and end-labeled with [γ-^{32}P]dATP (5,000 Ci/mmol; New England Nuclear Corp.). Subsequent digestion with *Hin*dIII produced 1.7- and 0.7-kb fragments, which were separated on a 1% agarose gel and purified. Partial restriction of each fragment was done with *Hae*III. The samples were run on an 8% polyacrylamide gel, and the products were identified by autoradiography. Analysis of the overlapping, end-labeled fragments allowed ordering of the *Hae*III restriction sites within the *Dra*I fragment.

S1 nuclease mapping. To identify the coding strand, single-stranded DNA probes were prepared from M13mp8 derivatives containing the 525-bp *Hae*III fragment cloned in both orientations. Only one of the probes hybridized to cellular RNA in a dot-blot analysis, defining the coding strand. Identification of the start sites of specific RNA transcripts was done by the S1 nuclease mapping technique described by Burke (11). Single-stranded probes were synthesized from M13mp8 templates containing the noncoding strand of either the 525-bp *Hae*III fragment or the 1.3-kb *Pst*I fragment containing the promoter region. Total RNA was isolated from strain JK791(pURB4100) as follows. Cells were grown in two flasks containing LB broth to mid-log phase, at

which time 300 μM EDDA was added to one flask. After 60 min, total RNA was isolated (56). For each S1 digestion reaction mixture, 50 μg of RNA and 100 pg of single-stranded probe were used. DNA fragments were electrophoresed on a 7% polyacrylamide gel containing 8 M urea.

DNA sequence analysis. DNA sequences were determined by the dideoxy chain termination method (45). Fragments were cloned into bacteriophage M13 derivatives, and the resulting templates were used for sequence analysis (35). A computer program from DNASTAR, Inc., was used in the examination of data.

Protein sequence analysis. A culture of strain JK354 (pURB4100) growing exponentially in M63 containing 0.4% glycerol, 0.15% casein hydrolysate, tryptophan (50 μg/ml), 10 mM citrate, and 10 μM $FeCl_3$ was induced for Cir synthesis by incubation with 150 μM EDDA for 25 min. Outer membranes were immediately isolated (23), and the sample was electrophoresed on a preparative 10% polyacrylamide gel with a 6% stacking gel. The gel was soaked in 4 M sodium acetate, and the proteins were visualized by light shadowing. The Cir protein was identified by comparison with the adjacent protein profile of a *cir* derivative which exhibited no band in the 74,000-dalton range. The 74,000-dalton band was excised, and the protein was electroeluted. After extensive dialysis in 0.1% sodium dodecyl sulfate, approximately 200 pmol of protein was sequenced by Edman degradation at the University of Illinois Biotechnology Center.

RESULTS

Identification of a cosmid encoding the *E. coli* colicin I receptor protein. A library constructed by cloning into the *Bam*HI site of cosmid vector pHC79 was available to us for screening (A. Klages, M. Lakshman, and J. Cronan, unpublished). The source of the cloned DNA was a partial *Mbo*I digest of the chromosome from colicin I-sensitive *E. coli* GM33. Transducing lysates were prepared from individual clones of the library by temperature induction of the resident lambda prophage of the host strain ML17 (19). A suitable recipient strain for transductions was constructed by curing of the transposon Tn*10* located in *mgl* in strain LA5641 by the method of Bochner et al. (6) and screening the cured derivatives for colicin I resistance. Since *cir* and *mgl* lie in proximity on the *E. coli* genetic map (7), the resulting *cir* mutation in strain JK706 was probably the result of a deletion or inversion of DNA in this region and therefore nonrevertible. The recipient strain was grown overnight in LB containing 0.4% maltose, centrifuged, and suspended in 0.5 volume of 10 mM $MgSO_4$. Transductions were carried out by incubating dilutions of each lysate with recipient cells at room temperature for 20 min, followed by dilution into LB, growth for 1 h at 30°C, and plating with ampicillin selection. Transductants obtained with each lysate were then screened for colicin Ia sensitivity.

After 135 clones had been analyzed, one cosmid, designated pCC99, was found which was capable of conferring colicin sensitivity on strain JK706. Restriction endonuclease analysis revealed an inserted DNA fragment of roughly 30 kb located in the *Bam*HI site of pHC79. Cells cured of the cosmid by growth in nonselective medium regained colicin resistance.

pCC99 derivatives and structural mapping of other genes in the *cir* region. Cosmid pCC99 was digested with restriction endonuclease *Pst*I and ligated with plasmid pBR329 opened at the single *Pst*I site in the β-lactamase coding sequence. The ligation mixture was then used to transform strain JK706, selecting for chloramphenicol resistance. Cultures of transformants were cross-streaked against colicin Ia. Plasmid DNA isolated from colicin-sensitive transformants contained a 5.7-kb insert consisting of three *Pst*I fragments of unequal size. A physical map of this plasmid, pURB4000, is shown in Fig. 1. Since physical analysis of pCC99 showed the three *Pst*I fragments to be contiguous (data not shown), plasmid pURB4000 was probably formed by ligation of a partial *Pst*I digestion product with the vector.

Plasmid pURB4000 conferred sensitivity to colicin Ia

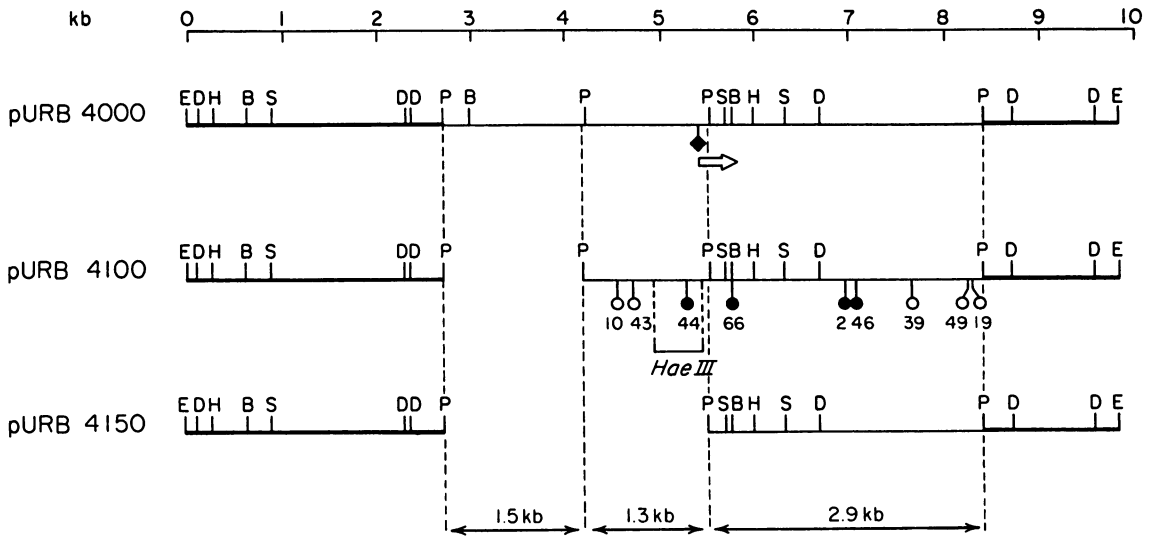

FIG. 1. Physical maps of plasmids containing DNA from the *cir* region. Heavy lines represent pBR329 DNA. Cloned chromosomal DNA is represented by thin lines. The locations of γδ insertions in pURB4100 are indicated: ●, mutations which inactivated production of colicin receptor; ○, mutations that did not inactivate colicin receptor production. The position of the Mu dII1734 insertion in pURB4000 described in the text is also shown (◆), and the direction of transcription of the hybrid gene formed is indicated by the arrow. The 525-bp *Hae*III fragment containing the *cir* regulatory region is indicated below the restriction map of pURB4100. Restriction endonuclease sites are abbreviated as follows: B, *Bam*HI; D, *Dra*I; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I. Numbers indicate specific mutations.

when transformed into six independent *cir* mutants of *E. coli*. Furthermore, a colicin Ia-resistant strain of *S. typhimurium*, JK824, was also rendered colicin sensitive after transformation with the plasmid. This implies that the proteins involved in colicin I binding in *E. coli* and *S. typhimurium* may be functionally equivalent.

In addition to conferring sensitivity to colicin Ia on these strains, the plasmids in the transformed cells of three *E. coli* strains tested made the strains susceptible to killing by colicin Ib and colicin V, which also require the *cir* gene product for their activity (16).

Since *cir* had been mapped previously by P1 transduction between *mgl* and *fpk* on the chromosome (7), we examined the cloned inserts for the presence of these genes. Strains LA5539 (*fpk*) and LA5606 (*mgl lacY galE*) were both transformed with pCC99 and pURB4000. Complementation of the *fpk* and *mgl* mutations was tested by measuring growth in the presence of 1 mM fructose and 50 μM 2*R*-glyceryl-β-D-galactopyranoside, respectively, as previously described (7). Complementation of the *mgl* mutation but not *fpk* was observed in pCC99 transformants, whereas pURB4000 complemented neither mutation.

The *mgl* genes have been cloned by Harayama et al. (24), and a restriction map of the region was determined. Comparison of the physical maps of their recombinant plasmid, pUH21, and pCC99, each of which contains *mgl*, revealed that portions of the DNA contained in these two plasmids were probably identical (Fig. 2). In addition, the *nfo* gene has recently been located on a Clarke-Carbon plasmid (15) and is believed to map near *cir* (B. Weiss, personal communication). The physical map of this plasmid, pLC38-27, is also shown in Fig. 2 and clearly overlaps with that of the cosmid pCC99. To confirm that pLC38-27 actually contained *cir*, this plasmid was transformed into strain JK838 and was found to confer sensitivity to colicin Ia. The combination of restriction and complementation data allowed us to map *cir* between the *nfo* and *mgl* genes, as shown in the composite physical map in Fig. 2.

Plasmids lacking specific *Pst*I restriction fragments of pURB4000 were constructed by subcloning. pURB4100 was created by isolation of a partial digestion product comprising the 1.3- and 2.9-kb *Pst*I restriction fragments of pURB4000 from an agarose gel, followed by ligation with *Pst*I-digested pBR329. This plasmid therefore lacked the 1.5-kb *Pst*I fragment of the original insert (Fig. 1). Plasmid pURB4150 was created by ligation of the gel-purified 2.9-kb *Pst*I fragment with pBR329 in the same manner (Fig. 1). The inserts of both plasmids were in the same orientation as that of pURB4000. Mutations in *cir* were complemented in strains containing pURB4100 but not in strains with pURB4150. These data indicated that DNA from both the 1.3- and 2.9- but not the 1.5-kb *Pst*I fragment is required for expression of the cloned gene.

Localization of the *cir* coding region. To further define the location of the *cir* gene, insertions of the mobile genetic element Tn*1000* (γδ) were isolated in pURB4100 (Fig. 1). By introducing pURB4100 into the F^+ strain JK589 and mating with JK820 (see Materials and Methods), we were able to isolate a series of plasmids containing insertions at various positions in the cloned DNA. Purified derivatives were transformed into JK820 and tested for the ability to confer colicin Ia sensitivity. From the correlation between the sites of these insertions and the ability to encode functional receptor protein, we estimate that the *cir* gene constituted at least 1.8 kb of the insert contained in pURB4100.

Determination of plasmid-synthesized proteins and the direction of transcription of *cir*. The polypeptides encoded by several recombinant plasmids were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of ^{35}S-labeled proteins in maxicells (Fig. 3). Strain JK838, transformed with the test plasmids, was grown in minimal medium containing excess iron before labeling because the receptor-coding plasmids inhibited growth of the host strain in low-iron medium (see below). The cloned DNA contained in pURB4000 (lane C) and pURB4100 (lane D) encoded two major polypeptides: a 74,000-dalton protein corresponding in size to the colicin I receptor, and a 32,000-dalton protein of unknown function.

To determine the direction of transcription of *cir* on the cloned DNA, insertion derivatives of pURB4000 were cre-

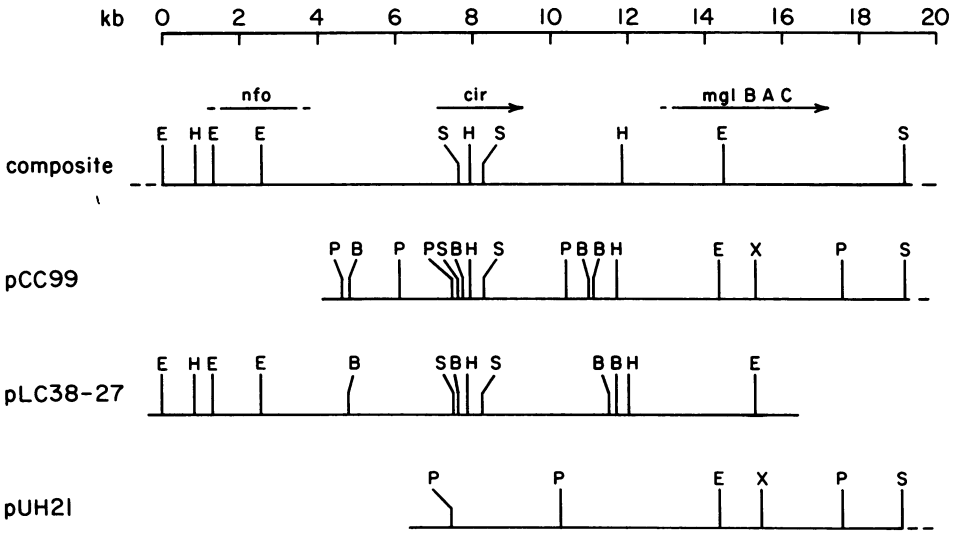

FIG. 2. Comparison of cosmid pCC99 with previously isolated recombinant plasmids. Only the relevant portions of the restriction maps are shown. Plasmids pLC38-27 and pUH21 have been determined to contain the *nfo* and *mgl* genes, respectively (15, 24). A composite map showing the relative locations of the three genes in this region is drawn at top. Only *Eco*RI, *Hin*dIII, and *Sal*I sites are shown in the composite map since these were the only ones determined for all three inserts. Abbreviations are as in Fig. 1, with the addition of X, *Xho*I.

ated by using the gene fusion-forming phage mini-Mu dII1734 (12). Following mutagenesis of the plasmid (see Materials and Methods), transductants which formed dark red colonies on MacConkey agar plates were tested for sensitivity to colicin Ia. One colicin-resistant isolate was chosen for further study. Strain JK838(pURB4000::Mu dII1734-18) was analyzed by the maxicell procedure and found to be lacking the 74,000-dalton receptor protein (Fig. 3, lane F). However, the plasmid did encode a 123,000-dalton product which was probably a hybrid polypeptide consisting of portions of both the *cir* and *lacZ* gene products. A 29,000-dalton protein was also observed which was of the size expected for the kanamycin phosphotransferase protein encoded by the mini-Mu d phage. Synthesis of β-galactosidase was regulated by iron, as discussed below. The position and orientation of the inserted phage DNA, determined by restriction endonuclease analysis, indicated that transcription proceeded to the right in the cloned insert, as depicted in Fig. 1. This is in agreement with the data of Middendorf et al. (36), who concluded that *mgl* and *cir* are transcribed in the same direction.

Plasmid pURB4150, which contained only the 2.9-kb *Pst*I restriction fragment and consequently lacked the amino-terminal region of *cir*, did not synthesize the colicin receptor but did produce the smaller protein (lane E). Plasmids pURB4100::γδ19 and pURB4100::γδ39 contained insertions of γδ located downstream from the receptor coding region (Fig. 1). The γδ39 mutation inactivated production of the 32,000-dalton protein (lane H), whereas the γδ19 mutation, which was located much further downstream from *cir*, did not affect its synthesis (lane G). These data indicate that the entire gene for the 32,000-dalton protein is contained on the 2.9-kb *Pst*I fragment cloned in pURB4150. Insertions of γδ that inactivated *cir* and therefore prevented production of the 74,000-dalton gene product (lane I, pURB4100::γδ44, and lane J, pURB4100::γδ46) did not affect production of the 32,000-dalton protein. Thus, the gene encoding this protein

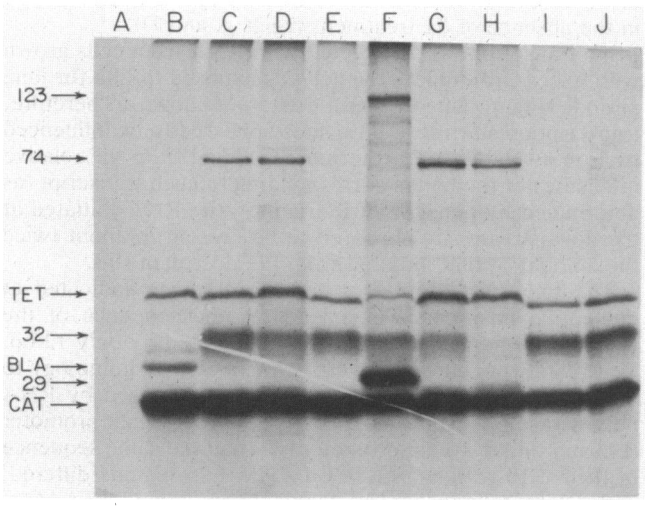

FIG. 3. Polypeptides encoded by plasmids in UV-irradiated, ^{35}S-labeled cells of strain JK838. Samples were analyzed by electrophoresis on a 12% polyacrylamide running gel with a 6% stacking gel. Molecular masses are indicated (in kilodaltons). Lanes: (A) no plasmid; (B) pBR329; (C) pURB4000; (D) pURB4100; (E) pURB4150; (F) pURB4000::Mu dII1734-18; (G) pURB4100::γδ19; (H) pURB4100::γδ39; (I) pURB4100::γδ44; (J) pURB4100::γδ46. Abbreviations: BLA, β-lactamase; CAT, chloramphenicol acetyltransferase; TET, tetracycline resistance protein.

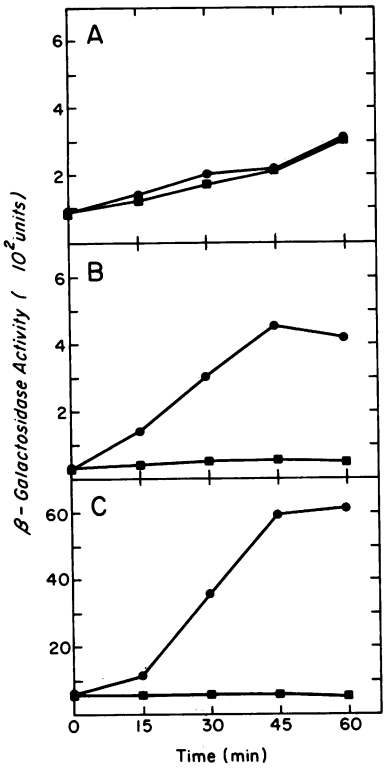

FIG. 4. Regulation of β-galactosidase synthesis in strains containing plasmid and chromosomal gene fusions. Cultures of exponentially growing cells in LB broth were divided into two flasks. To one flask, 300 μM EDDA was added, and incubation of both flasks was continued. Portions were removed at 15-min intervals and stored on ice until assayed for enzyme activity (37). Strains used were (A) JK406, (B) JK791(pURB4000), and (C) JK838(pURB 4000::Mu dII1734). Symbols: ●, with EDDA; ■, no EDDA.

does not appear to be part of an operon with *cir*. No polypeptides were observed to be encoded by the region upstream of *cir*.

Regulation of the cloned gene. In M63 minimal medium containing 0.4% glycerol, tetracycline (20 μg/ml), and 50 μM $FeCl_3$, strain JK791(pURB4000) grew at 37°C with a doubling time nearly identical to that of the same strain containing pBR329. However, JK791(pURB4000) did not grow in the same medium treated with Chelex-100 to chelate iron. JK791(pBR329) grew normally under these conditions. A mutant capable of growth under conditions of iron limitation was isolated from a culture of JK791(pURB4000) which had grown in the iron-deficient medium after prolonged incubation. Plasmid isolation and restriction endonuclease analysis revealed a variant plasmid, pURB4001, containing a 1.5-kb insertion of unknown origin in the 1.3-kb *Pst*I fragment. Several other derivatives of pURB4000 which did not encode receptor production were isolated, and these, too, did not inhibit growth of the host strain in low-iron medium (S. Steenbergen, unpublished results). We therefore suspect that overproduction of the *cir* gene product by the multicopy plasmid under inducing conditions is deleterious.

Regulation of the cloned gene by iron was examined by using the *cir-lac* protein fusion contained on plasmid pURB4000::Mu dII1734-18. β-Galactosidase activity was measured at regular intervals following the rapid chelation of iron from LB broth cultures by EDDA at 37°C (Fig. 4). The enzyme levels observed in strain JK838(pURB4000::Mu

TABLE 2. Regulation of β-galactosidase activity by the *cir* promoter region

Strain	Iron concn in medium	β-Galactosidase activity (U)[a]
JK1 (control)	High	60
	Low	83
JK791(pMC1871) (control)	High	10
	Low	207
EM23(pH3)	High	85
	Low	802

[a] Growth of strains and measurement of enzyme activity were carried out as described in the legend to Fig. 4, with LB broth treated for 60 min with 300 μM EDDA to create low-iron conditions during exponential growth.

dII1734-18) were approximately 10-fold higher than those observed in JK791(pURB4000), which contained a chromosomal operon fusion in *cir*. The difference most likely reflects the multicopy nature of the plasmid, although this could also be due to differences in protein stability or translation initiation between the protein and the operon fusion. Both chromosomal and plasmid-located fusions exhibited maximal expression by 45 min after induction. Activity was increased 10- to 12-fold by addition of EDDA, while activity in noninduced cultures remained constant. EDDA had no effect on enzyme levels in control strain JK406, which contained a chromosomal *ompF-lac* operon fusion. A threefold increase in overall activity was apparent in cultures of JK406; however, this increase was independent of iron availability and probably due to other factors which are known to influence expression of porin proteins (32).

Similar results showing regulation of synthesis of the cloned receptor in response to iron availability were obtained in experiments measuring specific binding of ^{125}I-labeled colicin Ia to cells (data not shown).

We examined the possibility that the presence of multiple copies of the *cir* coding region might affect expression of a chromosomal *cir-lac* operon fusion by dilution of cytoplasmic regulatory factors. Since control of chromosomal gene expression in cells containing pURB4000 or pURB4001 (insertion in *cir*) was not considerably different than that observed when vector alone was present (data not shown), there appears to be no significant titration of any *trans*-acting elements by the DNA sequences contained in the plasmids.

Cloning of the promoter region. The promoter region of *cir* was identified by a procedure in which the large *Dra*I fragment containing all but the carboxyl terminus of the *cir* structural gene was purified from pURB4100 and digested separately with *Hae*III, *Alu*I, and *Rsa*I. The products were ligated into the *Sma*I-generated blunt cloning site of the promoter detection vector pMC1871. Tetracycline-resistant transformants of strain EM23 were cross-streaked on MacConkey plates against a paper strip saturated with EDDA. Only isolates containing plasmids with a 525-bp insert from the *Hae*III digestion exhibited red color near the EDDA, whereas those containing the vector with the same insert in the opposite orientation, or other inserts, or lacking any insert were white throughout the streak. The fragment was mapped, as described in Materials and Methods, to the region which the γδ data and direction of transcription suggest contains the amino-terminal portion of the *cir* coding sequence (Fig. 1).

Regulation of enzyme production in this pMC1871 derivative (pH3) was analyzed for comparison with the induction patterns observed with fusions to the complete *cir* gene. Strain EM23 containing pH3 exhibited a 10-fold increase in β-galactosidase levels following exposure of the culture to EDDA for 60 min, as did the control strain JK791 (pMC1871), which contained the chromosomal *cir-lac* fusion (Table 2). These results parallel those seen in Fig. 4, in which the regulation of the entire cloned gene was compared with that of the chromosomal copy. No activity was detected in EM23(pMC1871) without an insert or with the *Hae*III fragment in the opposite orientation. Since the regulatory properties of the small fragment so closely resembled those of the complete gene, it appeared to contain all or most of the sequence information pertinent to control of transcription by iron.

Sequence determination and analysis of transcriptional start sites. The sequence of the 640-bp *Hae*III-*Pst*I segment of DNA responsible for iron-regulated transcription of *cir* was determined by the dideoxynucleotide chain terminator method (Fig. 5). Then, to determine how many transcripts were initiated in the promoter region and where their start sites lay on the DNA sequence, S1 nuclease protection assays were carried out (Fig. 6). Radioactive probes were prepared from the noncoding strands of the 525-bp *Hae*III fragment and the 1.3-kb *Pst*I fragment (see Materials and Methods). The probes were hybridized with total RNA isolated from log-phase cells of strain JK791(pURB4100) grown in the presence or absence of EDDA.

With the *Hae*III probe and RNA isolated from iron-depleted cultures, two major protected fragments were observed with lengths of 223 and 236 bp (Fig. 6, lane B). To ensure that these transcripts were initiated in the promoter fragment and elongated in the direction of the *cir* coding region, the 1.3-kb *Pst*I fragment containing the *Hae*III fragment was also used as a probe. Since the *Pst*I restriction site was 116 bp downstream of the *Hae*III site, any *cir*-specific transcripts were expected to protect a DNA fragment 116 bases larger than the protected *Hae*III fragment. As shown in Fig. 6 (lane E), both protected fragments were increased appropriately in size when the *Pst*I probe instead of the *Hae*III probe was used. Neither probe was degraded in the absence of S1 treatment (lanes A and D).

No transcripts were detected with RNA from cells grown with excess iron unless the gel was exposed to film for long periods (Fig. 6, lanes C and F; data not shown). Therefore, transcription starting at both sites appeared to be influenced by iron availability. Furthermore, judging from the relative intensities of the bands corresponding to each transcript (as determined by densitometric tracing), the RNA initiated at the downstream site appeared to be present in about twice the amount of that initiated from the upstream site.

The two start points for transcription are indicated at nucleotide positions 290 and 303 in the sequence of the promoter region (Fig. 5) and were separated by only 12 bp. Upstream from each site were sequences with homology to the canonical sequences known to be recognized by RNA polymerase molecules (43). Both the downstream promoter (P1) and the upstream promoter (P2) had the same sequence in their −10 regions, TATCGT. The −35 regions differed, with P1 containing TTGATA and P2 containing TTAACA. Both promoters exhibited the most highly favored 17-bp spacing between their respective −10 and −35 regions.

Two open reading frames were identified in the sequence: a short open reading frame beginning at position 348 and extending to position 470, and a longer one extending from position 463 through the end of the determined sequence. The small open reading frame, if translated, would encode a polypeptide consisting of 40 amino acids. A potential ribo-

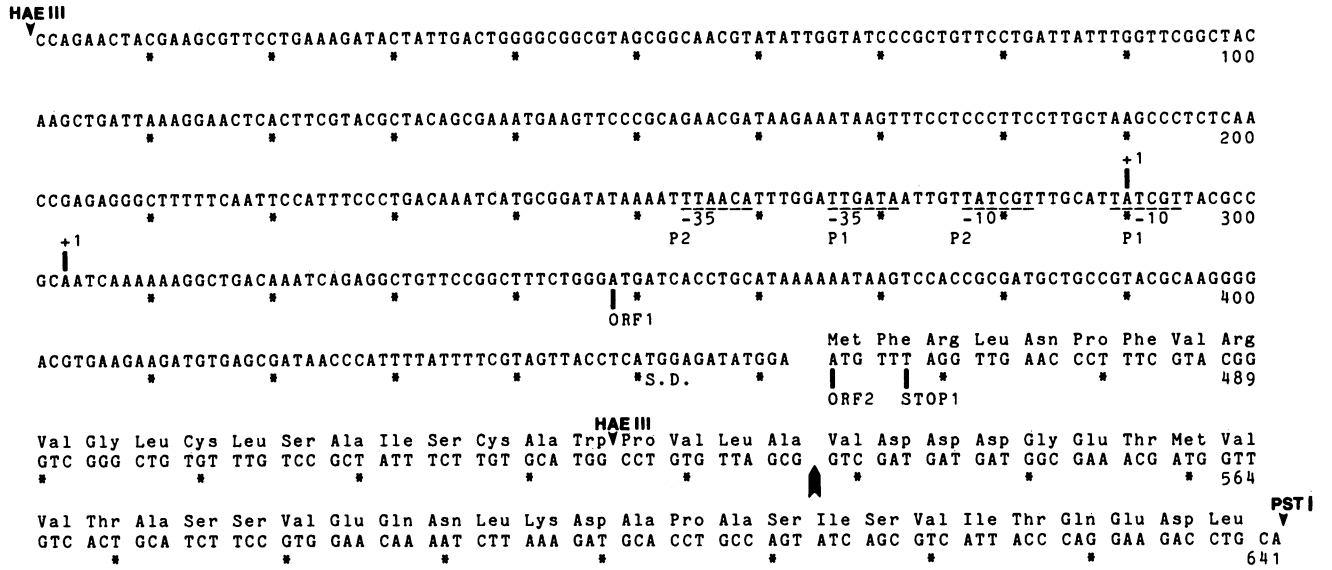

FIG. 5. Nucleotide sequence of the regulatory region of *cir*. The sequence shown begins at the *Hae*III site upstream of the promoters and extends to the *Pst*I site contained within the large open reading frame (ORF). The major transcription start sites and their corresponding −10 and −35 regions are indicated, as are the two open reading frames and the prospective ribosome-binding site (S.D.) for the larger one. The arrow shows the putative site of cleavage for processing of Cir to the mature form.

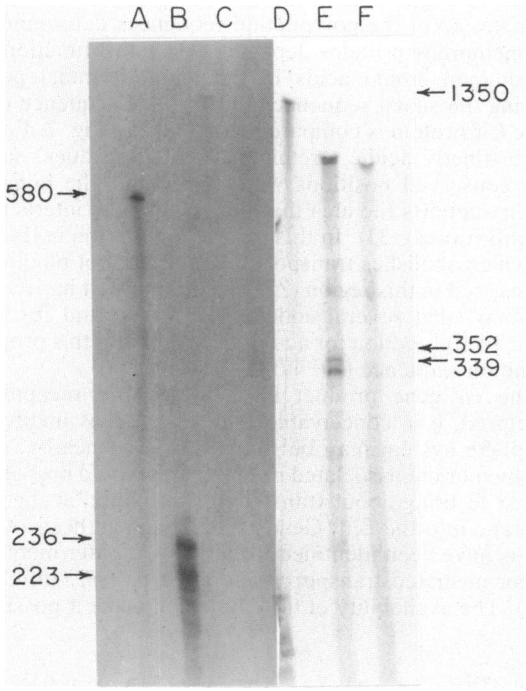

FIG. 6. S1 mapping of transcripts initiated in the promoter region. S1 analysis was carried out as described in the text with total cellular RNA and single-stranded, ^{32}P-labeled DNA consisting of 55 bases from M13mp8 plus the 525-bp *Hae*III or the 1.3-kb *Pst*I fragment. The sizes of the protected fragments (in bases) were determined by running sequencing ladders. Lanes: (A) *Hae*III probe and no S1 nuclease; (B) *Hae*III probe and RNA from iron-limited cells; (C) *Hae*III probe and RNA from iron-replete cells; (D) *Pst*I probe and no S1 nuclease; (E) *Pst*I probe and RNA from iron-limited cells; (F) *Pst*I probe and RNA from iron-replete cells.

some-binding site (48) was found about 18 bp upstream of the proposed start codon; however, this sequence may be too far away from the start codon to function efficiently in translation initiation.

The long open reading frame was also preceded by a sequence with homology to the 3′ end of 16S rRNA and was considered likely to encode the first portion of the colicin receptor protein. To confirm this, a small amount of the Cir protein was purified for analysis from a strain containing pURB4100. Twenty amino acids from the amino-terminal end of the mature protein were determined, and the order of residues matched that predicted from the DNA sequence, beginning at the valine residue at codon 26 in the long open reading frame (Fig. 5). The residues encoded prior to this codon appeared to make up a signal sequence typical of exported proteins (49). That the *cir* gene product was a processed protein was also indicated by experiments directly comparing its molecular weight when synthesized in maxicells (74,000) with that measured in an in vitro transcription-translation system in which processing does not occur (approximately 78,000) (data not shown).

DISCUSSION

The mapping data available for the region around 46 min are among the most confusing for the *E. coli* chromosome. Contradictory results obtained by different researchers have led to the suggestion that some of the strains used may contain genetic rearrangements of DNA in this region (1). The isolation of *cir* on a 30-kb fragment afforded us the opportunity to examine directly the relationship of some genes in this region by physical analysis. Comparison of the restriction maps of pCC99 containing *cir*, pUH21 containing *mgl* (24), and pLC38-27 containing *nfo* (15) revealed extensive overlapping regions. Also, pCC99 contained *mgl*, and pLC38-27 contained *cir* as well as *nfo*. Therefore, the order of genes in this region is *mgl cir nfo*. From 3 to 4 kb separate both *nfo* and *mgl* from the *cir* structural gene. A gene encoding an unidentified 32,000-dalton protein was located

just downstream of *cir*, between *cir* and the *mgl* operon. Since deletion of the *cir* promoter and amino terminus did not affect synthesis of this protein, we may surmise that the unidentified gene is not part of a *cir* operon.

Regulation of receptor synthesis by iron was retained in cloned sequences of plasmid pURB4100, and the time course of derepression was nearly identical to that observed with the single chromosomal copy. Attempts to demonstrate a titration effect on chromosomal *cir* expression by using multiple plasmid copies of the regulated sequence were unsuccessful. Although the copy numbers of these plasmids were not determined, the results suggest that any repressor or activator complex required to regulate *cir* would have to be present in high enough levels to control expression of many more genes than would normally be present in the cell. This could be achieved by high constitutive synthesis of the *trans*-acting proteins or by autoregulation, so that as the free regulator protein concentration drops, synthesis increases. A sequence has been observed in the upstream region of the *fur* gene with homology to the site bound by Fur protein in the aerobactin operon (17). It would be interesting to determine whether differences exist in the relative affinities of a regulatory protein, such as Fur, for the various iron-controlled genes or in the stoichiometry of binding of such proteins.

A 525-bp *Hae*III restriction fragment cloned into a promoterless *lac* expression vector contained all the appropriate signals for production of a hybrid protein with β-galactosidase activity. In addition, this fragment contained the sequence information necessary for derepression of transcription during iron limitation. Two major start sites for transcription, separated by only 12 nucleotides, were identified by in vivo S1 mapping. Both promoters had an identical sequence in their −10 regions which differed by 2 bp from the canonical sequence (TATAAT) thought to be important for interaction with σ^{70} holoenzyme RNA polymerase in *E. coli* (43). The two dissimilar −35 regions differed at only one position from the consensus sequence (TTGACA). Unlike the situation reported for the aerobactin operon, in which only one of the two promoters identified appears to be important in vivo (4, 17), both *cir* promoters may be utilized with similar efficiency. The activity of each promoter is influenced by iron availability. Since the two promoters are in such close proximity and actually overlap, it would not be possible for RNA polymerase to be bound at each site simultaneously. Although the two promoters appear to be functionally similar with regard to regulation by iron, there may be certain conditions in which the relative frequency of transcription initiation at the two start sites differs. For example, since *cir* expression is known to be decreased at the transcriptional level by elevating growth temperatures (54), it is possible that RNA polymerase preferentially binds at the weaker promoter during growth at higher temperatures. It is interesting that the ratio of the amounts of the two transcripts, as estimated by S1 analysis, was on the order of 2:1 or 3:1, which is identical to the decrease in transcriptional activity of *cir* during growth at 42°C. It is also conceivable that the presence of two RNA polymerase-binding sites serves as an "antenna" to localize the molecule in greater concentration in the region, thereby amplifying expression (43).

Several iron-regulated genes from *E. coli* have been cloned and sequenced (4, 13, 31, 33), but in only one other case has the transcriptional start site been determined to identify the promoter region (4). Comparison of the sequence of the *cir Hae*III fragment with the published sequence of the aerobactin operon regulatory region revealed striking homology between the major promoter (P1) of aerobactin and the region containing the downstream promoter (P1) of *cir*. Both promoters contained the same contiguous 9 bp in the −35 region (ATTGATAAT). Many areas of lesser homology also existed in this region. Most notably, the *cir* sequence TGGATTGATAATTGTTATC, which contains the −35 region of P1 and much of the −10 region of P2, was nearly 70% homologous to a consensus Fur recognition site recently proposed (17), GATAATGA TAATCATTATC. Strong similarity in the 5′ noncoding sequences of other iron-regulated genes, such as *fepA* (33) and *fhuA* (13), to *cir* was also noted, particularly in the region including and downstream of the −35 region of P1.

The sequence TGACTGGGG beginning at position 34 resembles a 9-bp sequence which has been observed at various distances upstream of several genes encoding TonB-dependent receptors (33). However, the significance of this sequence in regulation, if any, is unknown.

To date, all of the polypeptide sequences determined for outer membrane proteins dependent on TonB function contain common amino acids in the amino-terminal portion following the signal sequence (13, 33). The sequence of the mature Cir protein is compared with these in Fig. 7. Following a distinctly acidic stretch of 6 to 12 residues, several highly conserved positions were observed. The homology with Cir supports the idea that this region may interact with the TonB protein (33). In this regard, a mutation in the *btuB* gene which abolishes transport function but not binding has been mapped in this region (25). Furthermore, it has recently been found that several colicins (B, M, Ia, and Ib) which require TonB function for activity also contain this proposed recognition sequence (29, 47).

If the *cir* gene product is a siderophore receptor, as conjectured, it is conceivable that other genes involved in this uptake system may be found clustered nearby. Additional membrane-associated polypeptides would probably be required to bring about transfer of iron bound at the outer membrane into the cell. Genes specifying synthesis of such proteins have been identified for all known outer membrane receptor-mediated transport systems in *E. coli* (10, 18, 20, 41, 57). The availability of this clone will make it possible to

FepA	Gln	Glu	Pro	Thr	Asp	Thr	Pro	Val	Ser	His	Asp	Asp	Thr	Ile	Val	Val	Thr	Ala	Ala	Glu	Gln	Asn	
BtuB							Gln	Asp	Thr	Ser	Pro	Asp	Thr	Leu	Val	Val	Thr	Ala	Asn	Arg	Phe	Glu	
FhuA						Ala	Val	Glu	Pro	Asp	Glu	Asp	Thr	Ile	Thr	Val	Thr	Ala	Ala	Pro	Ala	Pro	
IutA							Gln	Gln	Thr	Asp	Asp	Glu	Thr	Phe	Val	Val	Ser	Ala	Asn	Arg	Ser	Asn	
Cir							Val	Asp	Asp	Asp	Gly	Glu	Thr	Met	Val	Val	Thr	Ala	Ser	Ser	Val	Glu	

FIG. 7. Comparison of Cir with outer membrane receptor proteins dependent on TonB for transport function (13, 25, 31, 33). Only the amino-terminal portions of the mature proteins are shown, with those residues that are identical in all five sequences indicated by underlining.

determine whether synthesis of the 32,000-dalton protein is iron regulated. The 2.4-kb region upstream of *cir* in pURB4000 thus far appears to be silent and encoded no polypeptides detectable in maxicells. It is possible that structural genes exist in this region but are not expressed on the cloned insert.

One distinctive feature of *cir* which has not been observed for the other iron-regulated genes of *E. coli* is that a small open reading frame lies between the promoter region and the large structural gene downstream. It will be interesting to determine whether this sequence is translated and whether it affects expression of *cir*. Furthermore, unlike the aerobactin operon, which appears to make use of only one major promoter in vivo, *cir* possesses two overlapping promoters. Comparison with the promoter organization of related operons awaits determination of their start sites. Genetic and biochemical analysis involving the *cir* regulatory region should delineate those features important for control by iron and other environmental factors.

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