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Promoter Mapping and Transcriptional Regulation of the Iron Assimilation System of Plasmid ColV-K30 in *Escherichia coli* K-12

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The promoter of the high-affinity iron assimilation system coded in an approximately 8-kilobase-pair segment of the large *Escherichia coli* plasmid ColV-K30 was localized to a 0.7-kilobase *Hin*dIII-*Sal*I fragment by in vitro runoff transcription. By an S1 nuclease protection assay, with in vitro-transcribed RNA and total in vivo-synthesized RNA, the major start site for transcription was mapped within this fragment and found to be identical in vitro and in vivo. A minor initiation site was located about 50 base pairs upstream from the major site. DNA sequencing of the *Hin*dIII-*Sal*I fragment revealed the presence of two promoter-like structures within an extremely AT-rich region with transcriptional initiation sites at 30 and about 80 base pairs upstream from the initiation codon for the first structural gene. Numerous potential secondary structures were found in the DNA sequence around the major promoter. The major transcriptional start site was determined precisely by sequencing the 5′ end of in vitro-transcribed RNA. The effect of iron on both the level of specific RNA, as determined by a quantitative S1 nuclease mapping assay, and on β-galactosidase activity in a *iucA′-′lacZ* protein fusion, showed that the aerobactin operon is regulated at the transcriptional level. The iron-regulatory sequences are contained within a 152-base-pair *Sau*3A fragment of the promoter region.

Escherichia coli, although normally an innocuous inhabitant of the large intestine in humans, is also the most common gram-negative bacterium isolated from the bloodstream. Since the concentration of free ferric iron in body tissues and fluids is estimated to be not more than 10^{-18} M, considerable research has centered on the mechanism whereby these bacteria obtain the iron needed for growth. Most aerobic and facultative anaerobic bacteria form high-affinity, virtually Fe(III)-specific ligands generically termed siderophores (27). *E. coli* and many other gram-negative bacteria synthesize enterobactin, a catechol-type siderophore. Clinical isolates of *E. coli* frequently form, in addition, a hydroxamate-type siderophore, aerobactin (29, 32, 38). In many of these strains the genetic determinants for aerobactin biosynthesis and transport reside on extrachromosomal elements such as the large ColV-type plasmids.

It has been known for many years that both catechol- and hydroxamate-type siderophore ligands are induced by culture of the microorganisms at low levels of iron (11). However, the molecular mechanism of regulation of siderophore systems by iron has remained obscure. We have selected the aerobactin gene complex of the ColV plasmid as a relatively simple siderophore biosynthetic and transport system amenable to investigation with the contemporary tools of molecular biology. Recently we reported (3) the cloning of this system on a 16.3-kilobase (kb) *Hin*dIII fragment containing the genes for both aerobactin biosynthesis and the 74,000-dalton outer membrane receptor for its ferric complex. The system is still iron regulated in the cloned sequences (3).

In this paper we describe the transcriptional organization and iron regulation of the gene complex, mapping of the iron-regulated promoter, and the DNA sequence analysis of the promoter region. The mapping, expression, and function of the five aerobactin biosynthesis-transport gene products detected (4) in ^{35}S-labeled minicells will be described in a subsequent publication.

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MATERIALS AND METHODS

Materials and bacterial strains. Restriction enzymes, S1 nuclease, and polynucleotide kinase were obtained from Bethesda Research Laboratories and used according to the supplier's suggested conditions. RNases T1, U2, and PhyM were from P. L. Biochemicals, and DNA polymerase I large fragment (Klenow fragment) was from Boehringer Mannheim Biochemicals. [α-^{32}P]dCTP (3,000 Ci/mmol) and [γ-^{32}P]ATP (3,000 Ci/mmol) were purchased from Amersham Corp.

E. coli K-12 strain 294 *endA hsdR thi pro* was obtained from M. J. Chamberlin; *E. coli* BN3040Nal^r F$^-$ *proC leuB trpE thi entA cir* (15) carrying the ColV-K30 plasmid was obtained by conjugation of the BN3040Nal^r strain with *E. coli* LG1315 (40). *E. coli* SE5000 F$^-$ *araD139* Δ (*argF-lac*) *U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR recA56* (37) was used as the host for pABN40.

DNA templates and in vitro transcription. Plasmid pABN5 has been described previously (3), and pABN15 is an *Ava*I deletion mutant of pABN5 (4). In vitro transcription reactions were carried out at 37°C for 8 min under the conditions of Chamberlin et al. (8). Purified *E. coli* RNA polymerase was a very generous gift from M. J. Chamberlin and was used in a fivefold molar excess over the DNA template. Rifampin (10 μg/ml) was added after 1 min to inactivate free RNA polymerase. After the reaction the products were phenol extracted, ethanol precipitated, and fractionated by gel electrophoresis on a 2% vertical agarose gel containing 0.1% sodium dodecyl sulfate in a Tris-borate-EDTA buffer (21).

S1 nuclease protection assays. S1 mapping was performed by the procedure of Weaver and Weissmann (39). The DNA probe, a single-stranded, 0.7-kb *Hin*dIII-*Sal*I fragment 5′ end-labeled at the *Sal*I site, was prepared by 5′ end labeling of *Sal*I-digested pABN15 in the forward reaction (21), followed by digestion with *Hin*dIII and purification of the

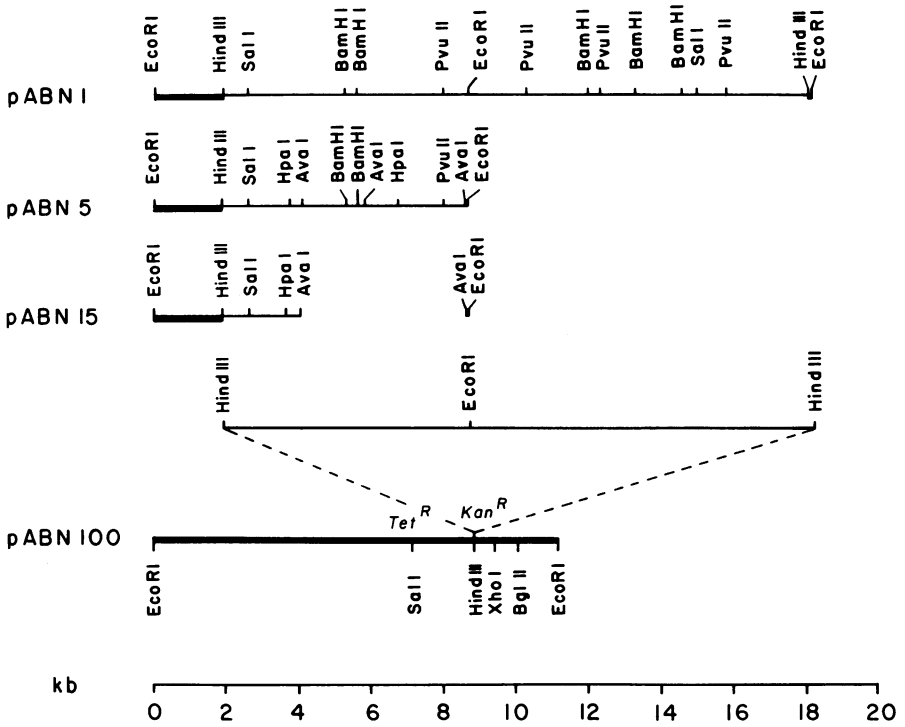

FIG. 1. Restriction maps of recombinant plasmids (3). Plasmid maps are shown linearized at the *Eco*RI site of the vector DNA, which is represented by heavy lines (pPlac [3] for pABN1, the 1.9-kb *Hin*dIII-*Eco*RI fragment of pPlac for pABN5, and pRK2501 [18] for pABN100). The inserts of all plasmids are lined up relative to the 16.3-kb *Hin*dIII-fragment of pABN1. In plasmid pABN5, additional restriction sites were mapped.

single-stranded 0.7-kb fragment on a 5% polyacrylamide strand-separating gel (21). For in vitro S1 mapping, plasmid pABN5, double digested with *Hin*dIII and *Hpa*I, was transcribed in vitro without rifampin by using the conditions of Chamberlin et al. (8). For in vivo S1 mapping, total RNA was isolated by the procedure of Aiba et al. (1). Exactly 50 μg of total RNA and approximately 100 ng of DNA probe were used per reaction; for the S1 nuclease digestion, 50 U was used per reaction.

Construction of a β-galactosidase protein fusion. For the construction of plasmid pABN40, the 654-base-pair (bp) *Hin*dIII-*Sal*I fragment was gel purified and partially digested with *Sau*3A. The single-stranded ends were filled in by treatment with the large (Klenow) fragment of DNA polymerase I, and the blunt-ended fragments were ligated into plasmid pBML1034 (37) linearized by *Sma*I. *E. coli* SE5000 was transformed, and ampicillin-resistant *lac*$^+$ transformants were identified as blue colonies on L-broth plates containing 30 μg of ampicillin per ml and 40 μl of a 2% 5-bromo-4-chloro-3-indolyl-D-galactopyranoside solution. Plasmid DNA was purified from several of these transformants by the Holmes and Quigley procedure (16). The insert size was determined by double digestion with *Eco*RI and *Bam*HI, both of which flank the cloning site. A recombinant plasmid containing a ca. 160-bp *Eco*RI-*Bam*HI insert was designated pABN40 and selected for sequencing (22) of the linker-insert junction.

Assay of β-galactosidase. Strains were grown in nutrient broth (34). At an optical density at 650 nm between 0.1 and 0.2, cultures were divided into two samples. α,α′-Bipyridyl (200 μM) was added for induction, and 20 μM $FeSO_4$ was added for repression. Before and after induction-repression, samples were taken at various time points, cells were lysed (33), and β-galactosidase activity was assayed (24).

DNA and RNA sequencing. The chemical modification method of Maxam and Gilbert (22) and the terminator method of Sanger et al. (35) were applied to determine the DNA sequence of the 0.7-kb *Hin*dIII-*Sal*I fragment. Nucleotides 1 through 260 and 645 through 375 were determined by the chemical modification method, whereas nucleotides 1 through 420, 410 through 575, 415 through 260, and 645 through 571 from M13 recombinant bacteriophages (23) were determined by the chain terminator method. DNA sequence analysis programs from the Biomathematics Computation Laboratory (Department of Biochemistry and Biophysics, University of California, San Francisco) were applied to the DNA sequence data. Only 412 nucleotides (*Hin*dIII site to start codon of the first structural gene) are reported.

For RNA sequencing *Sal*I-digested plasmid pABN15 (10 μg) was transcribed in vitro (45 min, 37°C). After the removal of free ribonucleotides by gel filtration, the mixture of template DNA and runoff RNA products was treated with calf intesting alkaline phosphatase and 5′ end-labeled with polynucleotide kinase and [γ-^{32}P]ATP (750 μCi). The major runoff RNA transcript of 260 nucleotides was then purified on a 5% polyacrylamide–8.3 M urea gel. It was submitted to partial alkaline hydrolysis and partial RNase digestions (T1, U2, PhyM) as described by Donis-Keller et al. (9).

RESULTS

In vitro transcription. To characterize the transcriptional organization of the cloned aerobactin gene complex, in vitro transcription was performed with purified *E. coli* RNA polymerase with two subclones of the gene complex as templates. Plasmid pABN5 carries the complete aerobactin biosynthesis gene complex and a fragment of the gene for the 74,000-dalton outer membrane receptor protein (Fig. 1), whereas plasmid pABN15 is a subclone of pABN5 and

contains the 2.1-kb *Hin*dIII-*Ava*I fragment of pABN5. These plasmids were doubly digested with *Hin*dIII and a second restriction enzyme to give restriction fragments extending from the *Hin*dIII site to various points in the gene complex. The complete digests were used as templates in an in vitro transcription system, and the [α-^{32}P]CTP-labeled runoff transcripts were analyzed by gel electrophoresis.

A single major runoff transcript was formed with all of the various templates used (Fig. 2). Three minor RNA transcripts of 1.7, 1.2, and about 0.6 kb were commonly found in all reactions and most likely originated from the 1.9-kb vector DNA.

The formation of a single major transcript suggests the existence of one strong major promoter and a single operon structure at least 6.8 kb in length. From the sizes of the runoff transcripts, which ranged from larger than 6 kb to less than 640 nucleotides, we concluded that the major promoter is located in the far left side on our map of plasmid pABN5 (Fig. 1), within the 0.7-kb *Hin*dIII-*Sal*I fragment, transcribing from left to right. Using the purified *Hin*dIII-*Sal*I fragment as the template, the major runoff transcript was about 260 nucleotides in length; a minor transcript of approximately 320 nucleotides could be seen only after overexposure (data not shown). This placed the major promoter about 260 bp upstream from the *Sal*I site.

Mapping of the transcriptional start sites by S1 nuclease assay and RNA sequencing. To determine the precise transcription start sites in vitro and in vivo, S1 nuclease protection assays were performed. The DNA probe used in all these experiments was the sense strand of the 645-bp *Hin*dIII-*Sal*I fragment labeled at the 5′ *Sal*I end. RNA was either in vitro transcribed from plasmid pABN5 doubly digested with *Hin*dIII and *Hpa*I, which gives an approximately 1.3-kb runoff transcript, or in vivo isolated as total RNA from *E. coli* BN3040Nal^r carrying the iron assimilation system on the ColV-K30 plasmid and grown under low-iron conditions.

After S1 digestion, the major protected DNA fragment is about 260 nucleotides long and the same in vitro and in vivo (Fig. 3, lane B; Fig. 4). In vitro, however, there is a minor protected DNA fragment corresponding to a minor RNA initiation site about 50 bp upstream from the major in vitro-in vivo initiation site. No protection could be seen without

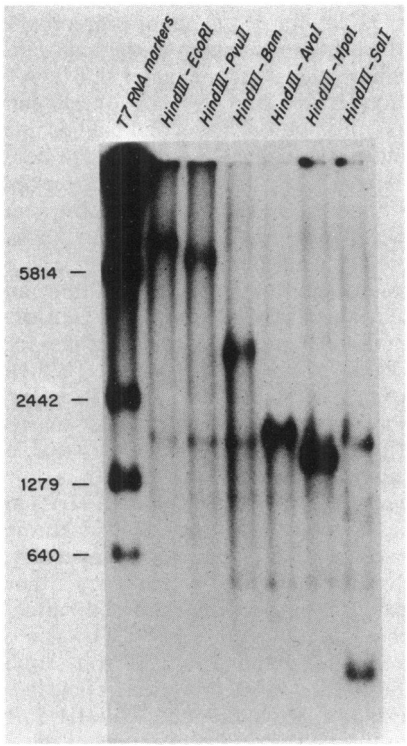

FIG. 2. In vitro transcription of the aerobactin gene complex. Subclone pABN5 was doubly digested with *Hin*dIII and *Eco*RI, *Pvu*II, or *Bam*HI; subclone pABN15 was doubly digested with *Hin*dIII and *Ava*I, *Hpa*I, or *Sal*I. The double digests were transcribed in vitro as described in the text. The runoff transcripts were fractionated on a 0.1% sodium dodecyl sulfate–2% agarose gel. As size markers RNAs transcribed by T7 RNA polymerase from T7 DNA were used (12). The positions of these T7 transcripts are given in bases.

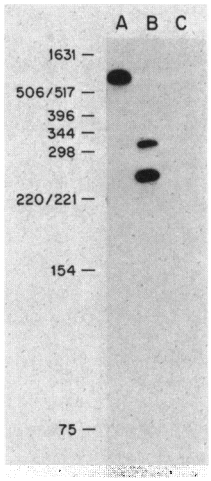

FIG. 3. S1 mapping of in vitro-transcribed RNA. S1 mapping was carried out as described in the text with the single-stranded 0.7-kb *Hin*dIII-*Sal*I fragment 5′ end labeled at the *Sal*I site as the DNA probe (A) and the RNA transcribed in vitro from *Hin*dIII-*Hpa*I-digested pABN15 DNA. DNA fragments (B) were analyzed by electrophoresis on a 8.3 M urea–5% polyacrylamide gel with end-labeled *Hin*fI fragments of pBR322 as size markers. Lane C is a control without RNA.

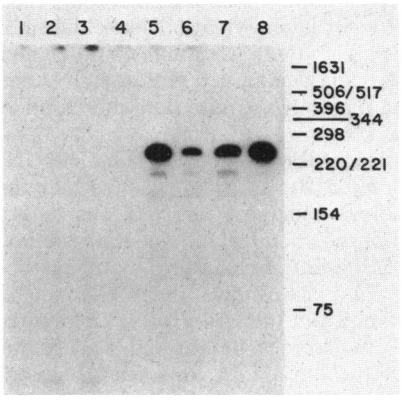

FIG. 4. S1 mapping of in vivo-transcribed RNA. S1 mapping was carried out as described in the text, the single-stranded 0.7-kb *Hin*dIII-*Sal*I fragment 5′ end-labeled at the *Sal*I site as the DNA probe and total RNA isolated from *E. coli* strains grown under the following conditions: *E. coli* BN3040Nal^r grown iron limited (lane 1) and iron starved (lane 2); *E. coli* BN3040Nal^r(pColV-K30) grown iron replete (lane 3), iron limited (lane 4), and iron starved (lane 5); *E. coli* 294(pABN100) grown iron replete (lane 6), iron limited (lane 7), and iron starved (lane 8).

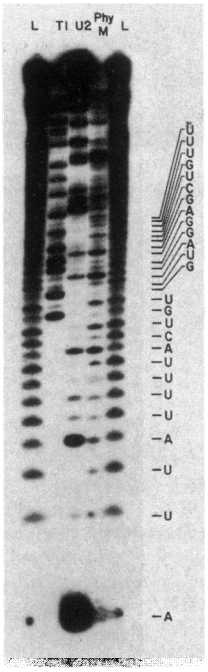

FIG. 5. 5′-Terminal sequence analysis of the major in vitro-transcribed RNA. Synthesis, end labeling, isolation, and sequencing of the major in vitro-transcribed RNAs were carried out as described in the text. Partial alkaline hydrolysis (L) was done in 50 mM NaOH–1 mM EDTA at 100°C for 30 s. For partial RNase digestions, 0.1 U of RNase T1 per 5 μg of RNA (T1), 0.1 U of RNase U2 per 5 μg of RNA (U2), and 1U of RNase PhyM per 5 μg of RNA (PhyM) were used.

added RNA (Fig. 3, lane C) or by adding total RNA from an *E. coli* strain without the ColV iron assimilation system grown under iron-limited and iron-starved conditions (Fig. 4, lanes 1 and 2).

This result agrees well with the sizes of the in vitro runoff transcripts and confirms the position of the promoter sites identified by DNA sequence analysis (see below).

For mapping the major initiation site to a precise nucleotide the major in vitro-transcribed RNA was 5′ end-labeled and gel purified, and its 5′-terminal base sequence was determined by partial enzymatic RNase digestions. At least 30 nucleotides from the 5′ terminus could be identified (Fig. 5). The RNA sequence agreed with the DNA sequence and identified the major RNA initiation site as an A residue at position 379.

DNA sequence of the promoter region. The DNA sequence of the 0.7-kb *Hin*dIII-*Sal*I fragment, in which the major and minor promoters had been located, was determined by a combination of the chemical modification method of Maxam and Gilbert (22) and the chain terminator method of Sanger et al. (35). Figure 6 shows the DNA sequence in the antisense strand from the *Hin*dIII site to the presumed start codon ATG (nucleotides 409 through 411) of the first structural gene of the operon. A long open reading frame was identified (unpublished data). The coding sequence is preceded, with a spacing of 7 bp, by a strong ribosome-binding site, AGGAGcTG (nucleotides 394 through 401), with complementarity to a sequence near the 3′ end of 16S rRNA (indicated by uppercase letters), as originally proposed by Shine and Dalgarno (36).

The most striking feature of this DNA sequence is an unusually AT-rich region (80% AT) compared with the average AT content of the *E. coli* chromosome of 49% (30) extending over almost 120 bp (nucleotides 270 through 387) and flanked by G+C-rich sequences. Both major and minor promoters, as identified by in vitro transcription and S1 mapping, were identified in the DNA sequence by homology with the *E. coli* consensus sequence of Hawley and McClure (14). As to the major promoter, P1, both the −10 region, cATAAT (nucleotides 369 through 374), and the −35 region, TTGAtA (nucleotides 346 through 351), deviate only in one nucleotide each from the consensus sequence (deviations indicated by lowercase letters). The −10 and −35 regions of the major promoter are separated by 17 nucleotides which is considered the optimal spacing for a strong promoter (14). The minor promoter, P2, shows close homology to the consensus sequence in its −10 region, TAaAAT (nucleotides 313 through 318), but deviates more in its −35 region, aTGtat (nucleotides 291 through 296). The two regions are separated by 16 nucleotides. Determination of the 5′-terminal sequence of the major in vitro-transcribed RNA (discussed below) further confirmed the site of the main promoter, P1, by placing the major RNA initiation site at nucleotide 379.

The second striking feature of this sequence is the potential to form multiple secondary structures in DNA or RNA (Fig. 7). These dyad symmetries are all clustered in the AT-rich region, in particular around the major promoter P1. Since many of these symmetries are overlapping, it is apparent that alternative secondary structures can be formed.

Furthermore, two palindromic sequences and one extensive inverted repeat symmetry were found in the AT-rich region (Fig. 7). Two of the symmetry centers are very close to the −35 or −10 region of the minor promoter, P2, and one is in the middle of the −35 region of the major promoter P1. The first palindrome is the hexamer ATTAAT centering around nucleotides 298 and 299 and overlapping the −35 region of the P2 promoter. The second palindromic sequence centers around nucleotides 320 and 321 and consists of the dodecamer AAAATTAATTTT, which overlaps the −10 region of the minor promoter P2 and has the same hexamer sequence in the symmetry center as the first palindrome and imperfect symmetry further extending on either side thus spanning a total region of 38 bp. In addition, an extensive inverted repeat symmetry spanning a region of 71 bp and centered around nucleotide 350 was identified in the middle of the −35 region of the major promoter. When the DNA sequences around the promoters P1 and P2 were compared, it became clear that extensive homology existed between two ca. 50-bp-long contiguous sequences (nucleotides 285 through 329 and 332 through 385) (Fig. 7).

Regulation of promoter P1-specific RNA levels by iron. The S1 mapping assay was used quantitatively to measure specific RNA levels in vivo. Total RNA was isolated from *E. coli* BN3040Nal^r(pColV-K30), which carries the aerobactin gene complex in a single copy per cell, after growth in nutrient broth medium with 50 μM $FeSO_4$ (iron replete), no $FeSO_4$ (iron limited), and 200 μM α,α′-dipyridyl (iron starved). Total RNA was used in an S1 nuclease assay with the single-stranded 645-nucleotide *Hin*dIII-*Sal*I probe 5′ end-labeled at the *Sal*I site. The products of the S1 nuclease digestion were analyzed on denaturing gels. Under iron-replete growth conditions, no specific RNA initiated at the major promoter could be detected (Fig. 4, lane 3) and under iron-limiting conditions a very low level of specific RNA existed (Fig. 4, lane 4), and under iron starvation the level of transcripts drastically increased by more than 10-fold (Fig. 4, lane 5). This result proves directly that the levels of RNA initiated at the major promoter are regulated by iron.

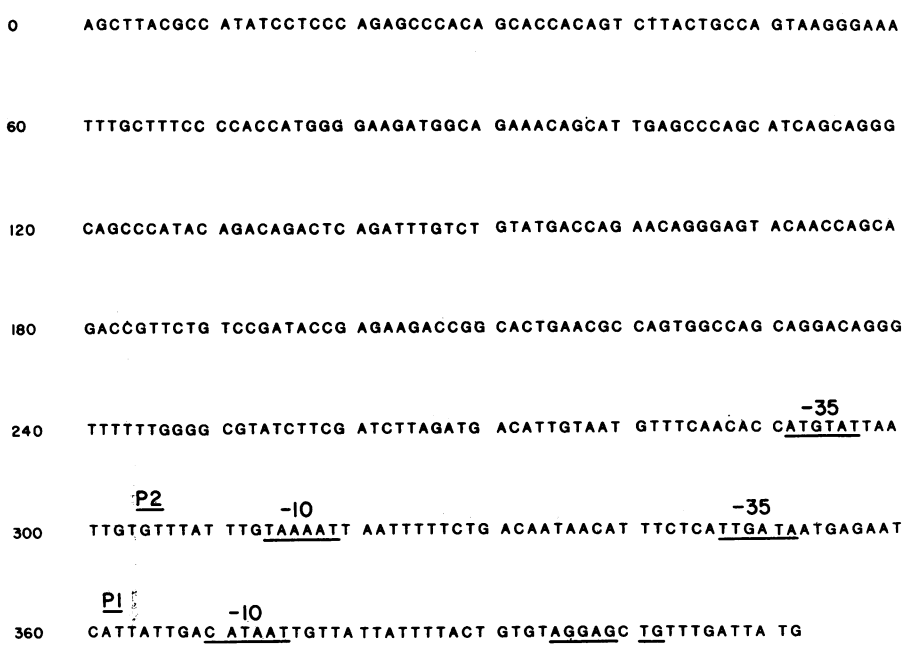

FIG. 6. Nucleotide sequence of the promoter and 5' flanking regions of the aerobactin operon. The DNA sequence was determined as described in the text and is numbered from the *Hin*dIII site, which defines the vector-insert junction at the left end of the aerobactin operon to the presumed start codon of the first structural gene of the operon. Major and minor promoters, P1 and P2, respectively, and the Shine-Dalgarno sequence are indicated by underlining.

During the initial cloning of this system on multiple-copy-number plasmids, we observed that iron regulation of expression was retained only to a limited extent (3). The attribution of this to an increased gene dosage effect was tested by recloning the 16.3-kb *Hin*dIII fragment, which covers the complete system, into a low-copy-number vector plasmid, pRK2501 (18). Plasmid pRK2501 is a derivative of the RK2 plasmid and is maintained at a copy number of eight per *E. coli* chromosome. The orientation of the insert in the resulting recombinant plasmid, pABN100 (27.4 kb), was determined by restriction mapping (Fig. 1). Iron regulation of gene expression in *E. coli* 294(pABN100) and in strains carrying the single-copy ColV-K30 plasmid was measured by the accumulation of aerobactin in the culture supernatant and found to be very similar (data not shown).

To test whether regulation was maintained in the low-copy-number derivative pABN100, quantitative S1 nuclease mapping assays were performed with total RNA from *E. coli* 294(pABN100). Figure 4 (lanes 6 through 8) shows that there was a low basal level of specific RNA even under iron-replete growth conditions, but that the RNA level was induced 4-fold under iron-limited conditions and 11-fold under iron-starved conditions. The basal RNA level might be due to the slightly elevated copy number of pABN100, as compared to the single-copy ColV-K30 plasmid. Nevertheless these experiments demonstrate that the regulatory sequences responsible for induction and repression by iron of specific RNA levels were contained in the cloned DNA.

Iron-regulated expression of a β-galactosidase fusion protein. To study the regulation of the aerobactin operon, a defined protein fusion was constructed in vitro that brought the *lacZ* gene under the control of the iron-regulatory region. To eliminate any potential contribution of aerobactin sequences to the stability of the hybrid mRNA, the fusion was constructed to include as few aerobactin sequences as possible. The result was plasmid pABN40 (Fig. 8). It contains, in a 152-bp *Sau*3A fragment (nucleotides 260 through 411, Fig. 6), the aerobactin promoter region and the Shine-

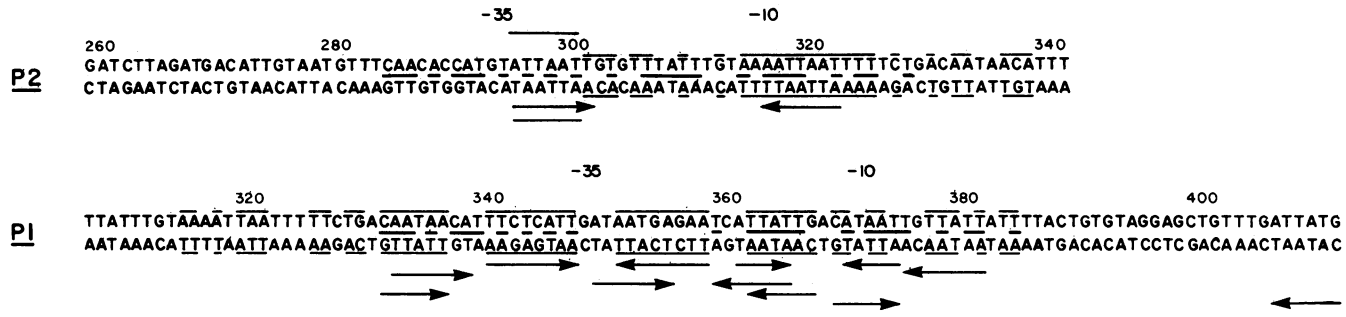

FIG. 7. Possible secondary structures and sequence homologies in the promoter region. The DNA sequences around the major and minor promoter, P1 and P2, respectively, are shown. The −10 and −35 regions are indicated above the sequence. The inverted repeat and palindromic symmetries are shown by arrow pairs under the sequence and by over- and underlining. Symmetry centers are at nucleotides 298 and 299, 320 and 321, and 350. Lines between the DNA strands mark sequences homologous in the two promoter regions.

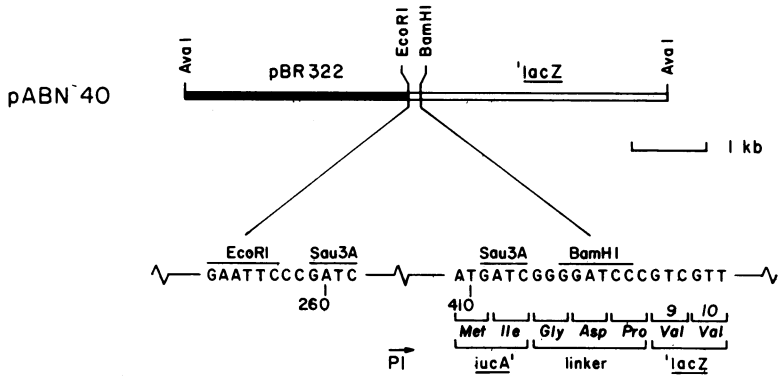

FIG. 8. Structure of plasmid pABN40. A partial restriction map of plasmid pABN40 is linearized at the pBR322 *Aval* site. The vector plasmid is pMBL1034 (37), which consists of pBR322 sequences (■) and a promoterless *lacZ* gene (□). Into the *Sma*I site of the *Eco*RI-*Sma*I-*Bam*HI linker sequence the 152-bp *Sau*3A fragment containing the aerobactin promoter region was inserted by blunt-end ligation. The DNA sequences of the two vector-insert junctions and the predicted amino acid sequence of the fusion protein are outlined at the bottom.

Dalgarno sequence extending up to the codon following the presumed start codon of structural gene (*iucA*) of the operon. A 9-bp linker sequence connects in frame to the *lacZ* structural gene. The structure of the recombinant plasmid was confirmed by determining the size of the *Eco*RI-*Bam*HI insert and by sequencing from the *Bam*HI site through the *Sau*3A site at least 40 bases into the insert.

To determine whether iron-regulatory sequences are contained within this 152-bp fragment, the time course of β-galactosidase activity in nutrient broth cultures of *E. coli* SE5000(pABN40) was followed before and after induction and repression (Fig. 9). Repression was relatively rapid; after 45 min β-galactosidase activity had reached a basal level of ca. 40 U per unit of absorbancy to 600 nm. Induction was slower and reached the fully induced level of ca. 1,200 U per unit of absorbancy at 600 nm only after 150 min. The iron concentration in nutrient broth of ca. 3 μM (K. Nakamura, unpublished observations) may be sufficient to allow some accumulation of intracellular iron, which would explain the slow induction kinetics. In a control experiment with *E. coli* SE5000 carrying the vector plasmid pMBL1034, which consists of pBR322 sequences and a promoterless *lacZ* gene, no β-galactosidase activity could be detected under the same conditions.

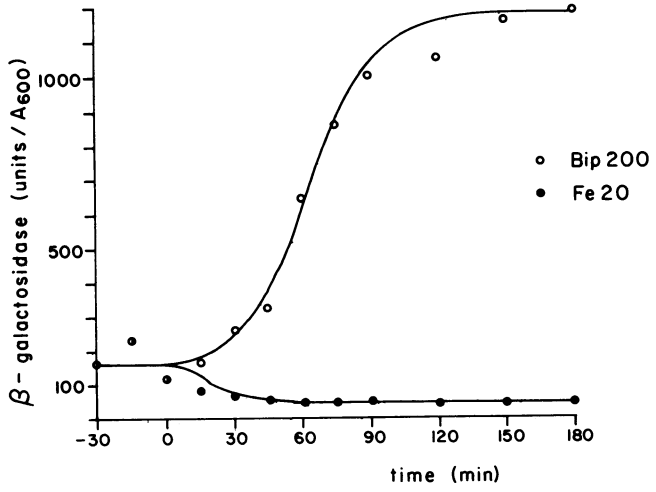

FIG. 9. Iron-regulated expression of the β-galactosidase fusion protein. Strain SE5000(pABN40) was grown in nutrient broth, divided into two samples at time zero, and induced by the addition of the Fe(II) chelator α,α'-bipyridyl (Bip200) and repressed by the addition of 20 μM $FeSO_4$ (Fe20) as described in the text. The time course of β-galactosidase specific activity in units of enzyme activity per unit of absorbancy at 600 nm (A_{600}) of cell growth before and after induction and repression at time zero is shown.

From these data we conclude that both promoter and regulatory sequences are located within the 152-bp *Sau*3A fragment. Since the fused mRNA should contain only about 30 nucleotides upstream of the start codon, the observed control is most likely exerted primarily at the transcriptional level.

DISCUSSION

A prime reason to study the aerobactin-mediated iron assimilation system of *E. coli* is that it could exhibit the same iron-regulatory pattern as do other more complicated systems in microorganisms. We cloned the entire aerobactin gene complex from the ColV-K30 plasmid on a ca. 8-kb DNA fragment, the cloned sequence being still iron regulated. Considerable progress has been made in the past few years in the genetic analysis of this particular system. The discoverers of the ColV plasmid-coded iron uptake system (41) had identified the two components, siderophore biosynthesis and transport. In addition to the plasmid-coded specific genes, several more generalized functions coded by chromosomal genes (*fhuCDB*, *tonB*, and *exbB*) were required for the uptake of ferric aerobactin (5–7). Using *lac* operon fusions constructed with phage Mu d *lac*, Braun and Burckhardt (5) found that the aerobactin system of the ColV plasmid is under the control of the chromosomal *fur* gene; a mutation in the *fur* gene caused constitutive expression of all genes involved in ferric iron uptake studied so far (10, 13). These include *fhuA* coding for the ferrichrome receptor, *fepA* specifying the enterobactin receptor and part of the enterobactin system, and *cir* for which no function in iron transport has yet been found (28). However, there are differences in the level of expression and in the induction kinetics of these iron-regulated proteins (19). In addition, a unique membrane protein of molecular weight 90,000 had been detected by its inverse regulation, namely, repression by low-iron growth (19). To corroborate any model for the mechanism of iron regulation we need to accumulate information about transcriptional organization, promoter-operator structures, and sequences from several iron-regulated systems. This information will also be a prerequisite for the

study of the interaction of regulatory proteins and regulatory DNA sequences and the reconstitution in vitro of an iron-regulated transcription system. We have initiated this biochemical approach by analyzing the promoter-operator region of the cloned aerobactin system.

By in vitro transcription, S1 nuclease mapping, and sequence analysis of DNA and RNA we have mapped promoters active in vitro and in vivo. A major promoter, P1, and a minor upstream promoter, P2, both operational in vitro, were shown to be tandemly arranged and separated by about 50 bp. The major transcriptional start site was mapped at nucleotide 379 downstream from the *Hin*dIII site at the left end of the gene complex. Transcription is directed from left to right as shown. The minor in vitro promoter, P2, showed no in vivo activity under different iron growth conditions and is most likely not relevant for regulation. Defined 5'-promoter deletions would be necessary to unequivocally prove this point. We conclude from in vitro transcription that at least the left *Hin*dIII-*Eco*RI fragment, 6.8 kb in length and containing the biosynthetic genes, is transcribed as a single unit. This conclusion was supported by determination of the DNA sequence between the first two structural genes of the operon, where a strong Shine-Dalgarno sequence, but no new promoter sequence, was found (data not shown). Both deletion of the promoter-containing 0.7-kb *Hin*dIII-*Sal*I fragment from the recombinant plasmid pABN1 (data not shown) and Tn*5* insertions in several sites in the biosynthetic genes (S. Cho, unpublished observations) reduced to a very low level expression of the 74,000-dalton polypeptide, the gene for which maps farthest downstream in the complex.

This leads to the conclusion that the entire gene complex, some 8 kb in length and consisting of aerobactin biosynthesis and transport genes, is organized in a single-operon structure.

Cloning of the aerobactin operon has allowed for the first time direct comparison of promoter-specific RNA levels under different iron growth conditions. The levels of RNA initiated at the major promoter are strictly regulated by the iron status of the cell. Most likely this reflects an iron-dependent initiation of transcription at the major promoter P1. Iron regulation was also studied by measuring the regulation of β-galactosidase levels in an in vitro-constructed protein fusion of the putative aerobactin promoter-operator region and the presumed first two *iucA* codons with a promoterless *lacZ* gene. Iron regulation of expression of the fusion protein over a 30-fold range strongly suggests a transcriptional regulatory mechanism. However, we cannot rule out the possibility that at least part of the iron regulation effect is contributed by iron-dependent mRNA stability. The absence of an iron-regulated promoter within the 6.4-kb *Bam*HI fragment covering the genes for the 53,000- and the 74,000-dalton proteins was reported by Krone et al. (20). An *iucC'-'lacO lacZ* operon fusion constructed in vitro (2), which had intact the 5'-flanking region up to the *Hin*dIII site, gave induction ratios similar to those described for plasmid pABN40, but resulted in 30- to 50-fold higher absolute β-galactosidase levels. The reason for this is at present unclear. The construction of pABN40-analogous fusions extending to various points in the 5'-flanking region may clarify the different expression levels.

In summary, these results allowed us to conclude that at least the highly inducible expression of the complete system under iron starvation is due to high levels of mRNA from the major promoter P1. The residual, low-level expression of at least the last gene of the operon (*iutA*), coding for the ferric aerobactin receptor protein in the absence of the iron promoter P1, may be explained by a weak constitutive downstream promoter. This would be physiologically significant, because it would allow *E. coli* to take up exogenous siderophore without expressing the biosynthetic genes.

We also present in this paper the first DNA sequence of an iron-regulated promoter from any organism, and palindromic sequences were found in the promoter region. The significance of these symmetry structures is not known at this time, but we suspect that they may be important as single or multiple recognition sites for iron-regulatory factors such as a repressor protein. Complete repression depended on a low copy number of the gene complex, suggesting that a regulatory protein may be present in the cell in limiting concentrations. Our present hypothesis is that a common repressor protein, with iron acting as corepressor, plays a key role in the mechanism of iron-regulated gene expression (19, 26). Genetic evidence for a common regulatory mechanism in several iron-regulated genes (5, 13) supports this hypothesis.

It seems likely that there exist families of iron-regulated genes in microorganisms including other functions besides iron transport. The classical example would be the iron-controlled diphteria toxin production of *Corynebacterium diphteriae* (31). In fact, a model for toxin gene regulation by iron similar to ours has been proposed (25). Other bacterial toxins also appear to be under iron regulation (17).

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