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Operator Sequences of the Aerobactin Operon of Plasmid ColV-K30 Binding the Ferric Uptake Regulation (*fur*) Repressor

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The promoter region of the pColV-K30-encoded operon specifying biosynthesis and transport of the siderophore aerobactin was subjected to deletion analysis to determine the smallest DNA sequence affording iron regulation of a *iucA'-'lacZ* gene fusion. A 78-base-pair (bp) region containing the main (P1) promoter retained the character of inducibility under iron starvation. A 250-bp fragment carrying this sequence was examined for protection against DNase I by the Fur protein, the product of a gene (*fur*) required for negative control of several iron-regulated functions. The DNase I footprints, in the presence of various divalent heavy-metal ions added as corepressors, revealed two contiguous binding sites with different lengths and affinities for Fur. Increased concentrations of the protein appeared to elicit formation of repressor oligomers which bind to the upstream and downstream regions of the P1 promoter in a metal-dependent fashion, but with a presently undefined stoichiometry. The primary site for Fur binding spans 31 bp and contains two overlapping symmetry dyads which share the sequence 5'-TCATT-3'. It also contains extensive homology with a 19-bp consensus sequence for iron-regulated genes as deduced from comparison with the *fhuA* and *fepA* putative promoter sequences.

Escherichia coli displays a characteristic response to iron starvation which is shared by many aerobic and facultative anaerobic microorganisms (3, 18). Under such conditions ([Fe] ≤ 1 to 5 μM), the transcription of a collection of genes or groups of genes scattered through the chromosome is coordinately activated in a manner resembling an iron-controlled regulon. The function of several of these genes is well characterized, like those involved in the production and transport of the siderophore enterobactin (*ent*, *fep*, and *fes* genes [4, 8]) or the transport of some exogenously provided ferrisiderophores (*fhuABCDE* genes [3, 11]). In other cases the function of the genes, such as those for the colicin Ia receptor (*cir* [22]) or the Fiu protein (11), although strongly induced by iron starvation, is presently unknown. Iron control is in all instances mediated by negative regulation via the product of the *fur* gene (1, 10), the absence of which triggers the expression of the genes to constitutive levels. This negative regulation may be the only one which controls the expression of a particular gene or may be superimposed on other control circuits (10). The *fur* gene also regulates expression of some genes that can be obtained by the *E. coli* cells from an exogenous donor. This is the case for the determinants of the aerobactin-mediated iron transport system, typically carried in pColV plasmids (1).

The *fur* gene and its product, the Fur protein (size, 17 kilodaltons), have been the subject of recent studies. After the report of its cloning (12) and nucleotide sequence (20), the protein was purified to homogeneity (S. Wee et al., manuscript in preparation), and its activity as a DNA-binding repressor was assessed in vitro (A. Bagg and J. B. Neilands, Biochemistry, in press). The capacity of Fur, acting in concert with Fe^{2+} or certain other divalent heavy-metal ions as corepressors, to bind to the promoter region of the iron-regulated aerobactin operon was indicated by a variety of techniques (Bagg and Neilands, in press). However, the precise operator sequence remained unknown.

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The promoter of the aerobactin operon is a convenient model for studies of DNA-Fur interaction in an iron-regulated system. A previous report from our laboratory (2) identified a 156-base-pair (bp) *Sau*3A1 restriction nuclease fragment located just upstream of the first structural gene (*iucA*) of the operon as containing all the signals required to afford iron regulation of an *iucA'-'lacZ* fusion. Furthermore, quantitative S1 mapping showed the iron control to take place at the transcriptional level. Two promoterlike sequences termed P1 and P2 were observed in the nucleotide sequence of this *Sau*3A1 fragment. Only the main promoter (P1) displayed activity in vivo. Research from this and other laboratories has disclosed the complete genetic arrangement of this virulence-related iron transport system (6, 7, 9).

The present study was undertaken to characterize the operator sequence within the aerobactin promoter. For this purpose, we used plasmids with deletions through the upstream zone of the aerobactin operon in the gene fusion vector pMLB1034 (21), resulting in a collection of iron-regulated *iucA'-'lacZ* fusions. Analysis of the deletions and footprinting experiments with the purified *fur* product allowed eventual identification of the Fur-binding sequence.

MATERIALS AND METHODS

Strains, plasmids, and enzymatic assays. *E. coli* 413 (*lacZ recA*) from the laboratory collection was used as the recipient for all recombinant plasmids. Unless indicated, restriction endonucleases and other enzymes were purchased from Pharmacia Fine Chemicals (Piscataway, N.J.) and were used according to the directions of the supplier. Plasmids pABN40 (2) and pMLB1034 (21) have been previously described. All DNA manipulations were performed by standard techniques (16). Plasmid pVC4 (Fig. 1) was constructed by cloning in the *Sma*I site of pMLB1034 a 410-bp *Hin*dIII-*Sau*3A1 fragment of pABN15 (6) made blunt ended with the Klenow fragment of DNA polymerase (16) (New England BioLabs, Inc., Beverly, Mass.) in the presence of the four deoxynucleotide triphosphates. The *Sma*I site of vector pMLB1034 was lost during cloning. pCON1 was obtained by

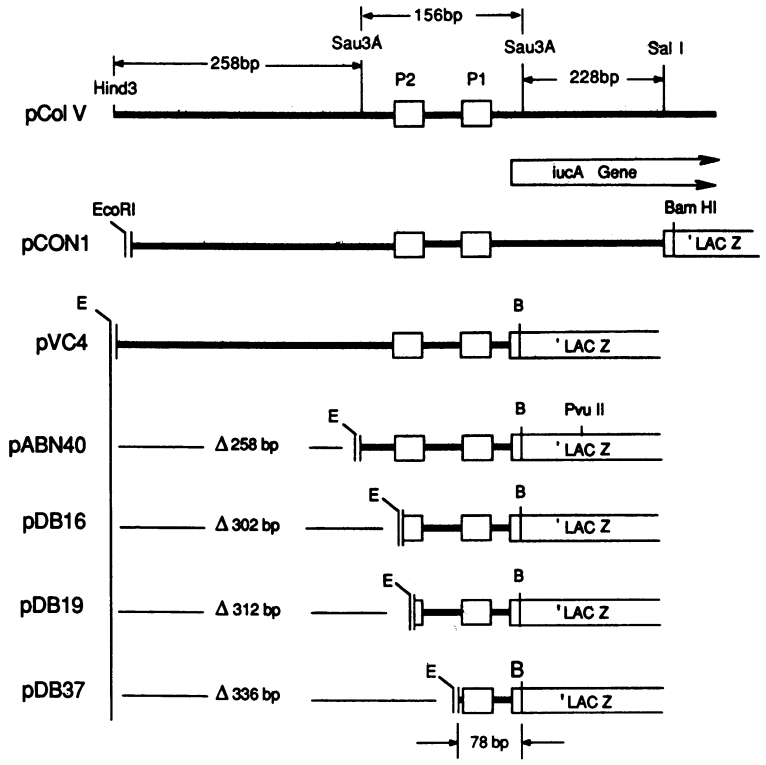

FIG. 1. Organization of gene fusion plasmids carrying the aerobactin promoter region. The relevant parts of the constructions are shown lined up with certain restriction sites of the aerobactin region of the original pColV-K30 plasmid and the position of the *iucA* gene sequence. Inserts in the gene fusion vector pMLB1034 are drawn as solid lines. *Eco*RI (E) and *Bam*HI (B) sites from the vector in all cases flank the insertions, which were cloned at its *Sma*I site via blunt-end ligations. The *Sma*I site in the vector was therefore lost, its previous position being indicated by a vertical line to the right of the *Eco*RI sites (see text for details). Three *Bal* 31 deletions (pDB16, pDB19, and pDB37) are shown, the rest behaving like those in terms of iron regulation (Table 1). pABN40 is a deletion obtained without *Bal* 31 treatment (2). The vector-insert boundaries of pVC4, pABN40, pDB16, pDB19, and pDB37 are known from their sequence, and those of pCON1 are approximate. The complete nucleotide sequence of the region has been published (2).

treating for a short time the 638-bp *Hin*dIII-*Sal*I fragment of pABN15 with a low concentration of *Bal* 31 nuclease. This resulted in a loss of about 10 bp from both ends. The resulting fragments were inserted at the *Sma*I site of pMLB1034. One transformant yielding a blue colony on LB-Xgal (5-bromo-4-chloro-3-indolyl-β-galactoside) medium (21) was selected for further analysis to confirm the structure of pCON1 (Fig. 1) in which the *Sma*I site was also lost. β-Galactosidase was measured in Miller units (17).

***Bal* 31 deletions.** Plasmid pABN40 (2) was digested with *Eco*RI restriction endonuclease and treated subsequently with a low concentration of *Bal* 31 nuclease (16). Samples were taken at different times, pooled, phenol extracted, and treated with *Bam*HI. This latter digestion was run in a preparative 10% polyacrylamide–Tris borate–EDTA (TBE) gel (16) from which the DNA fragments in the range 140 to 50 bp were fractionated, eluted, and recloned in *Sma*I-*Bam*HI-digested vector pMLB1034 (21). Owing to ligation of blunt ends at the *Bal* 31-digested extremes, *Sma*I sites were lost in all cases. The ligation mixtures corresponding to the different sizes of deleted fragments were individually used to transform *E. coli* 413. A total of 30 ampicillin-resistant colonies giving blue color in LB-Xgal plates (21) were picked for further analysis. Once the approximate sizes of the *Eco*RI-*Bam*HI inserts of the recombinant plasmids had been determined, seven were recloned in M13mp9 and sequenced by the dideoxy method to determine precisely the extent of the deletions. Sequencing followed closely a protocol provided by the Amersham pamphlet titled *M13 Cloning and Sequencing Handbook* (Amersham Corp., Arlington Heights, Ill.).

Footprint experiments. The *fur* gene of *E. coli* K-12 was cloned by a procedure (A. Bagg, Ph.D. thesis, University of California, Berkeley, 1986) that is an alternative to that used by Hantke (12). The cloned gene was then ligated downstream of the *recA* promoter in pMON2064 to enable induction of its expression with nalidixic acid. The Fur protein was adsorbed to zinc iminodiacetate agarose and eluted with histidine. Full details of the expression, isolation, and properties of the Fur protein will be reported elsewhere (S. Wee et al., in preparation). The protection against DNase I nicking exerted by the Fur protein on a restriction fragment carrying the P1 promoter sequence was examined by the procedure of Johnson et al. (14) with minor modifications. Plasmid pABN40 was digested with *Eco*RI and 3′-labeled with [α-^{32}P]dATP plus dTTP in the presence of the Klenow fragment of DNA polymerase (New England BioLabs). A second digestion with *Pvu*II was made to segregate the label. A 250-bp segment containing the promoter region of the aerobactin operon was purified from this second digestion. The end labeled fragment (Fig. 2) was diluted to a concentration of 0.1 to 0.5 nM in 100 μl of a buffer consisting of 10 mM bis(2-hydroxyethyl)imino-tris (hydroxymethyl)methane (BisTris) (pH 7.0), 2 mM $MgCl_2$, 1 mM $CaCl_2$, 0.1 mM EDTA, 0.1 M KCl, 100 μg of bovine serum albumin per ml and 2.5 μg of sonicated salmon sperm DNA per ml. Different

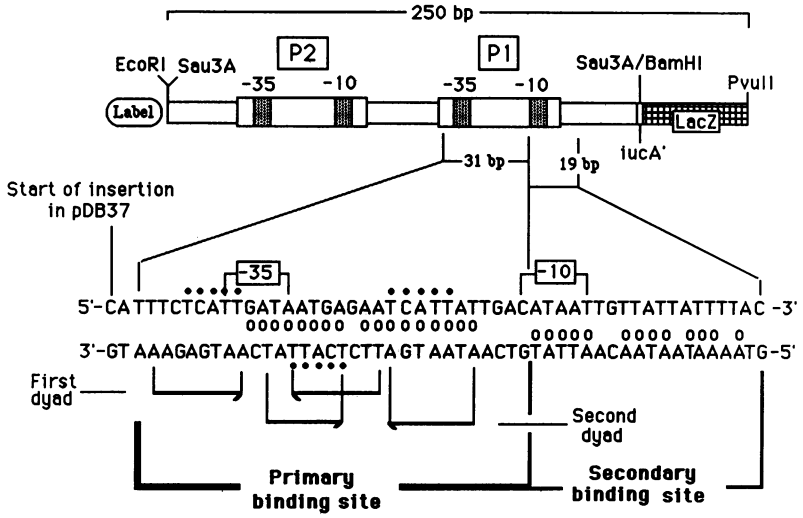

FIG. 2. Sequences protected by the Fur protein in the promoter region of the aerobactin operon. The top of the figure shows the organization of the 250-bp fragment from pABN40 used for the footprinting experiments, some relevant restriction sites, and the positions of the P1 and P2 promoters. The complete nucleotide sequence of this fragment can be found in reference 2. The fragment was 3' labeled at its *Eco*RI end. The bottom part of the figure displays the sequences of the primary (31 bp) and secondary binding sites of the Fur protein around the P1 promoter. The −35 and −10 regions are indicated, as well as the two overlapping symmetry dyads (arrows), the shared 5'-TCATT-3' sequences (●), and the start of the smallest iron-controlled promoter deletion. Note that owing to the end-labeling procedure, the actual footprinted sequences correspond to the lower strand of DNA. Circles between the strands (○) indicate the sequence matchings found in both binding sites with the proposed consensus 5'-GATAATGATAATCATTATC-3' (see Fig. 6 and text for explanation). In the secondary binding site, the best fit is found in the bottom strand (13 bp).

amounts of the purified Fur protein were then added. Other components added are specified for each particular experiment. The tubes with the different mixtures were preincubated for 2 min at 37°C, after which 2.5 ng of DNase I (Worthington Diagnostics, Freehold, N.J.) was added and the incubation continued for 3.5 min. DNA was precipitated by adding, per tube, 40 μg of carrier tRNA, 25 μl of 7.5 M ammonium acetate, and 2.5 volumes of ethanol. The pellets were directly redissolved in 80% formamide sequencing sample buffer and loaded in either long (37-cm) or short (23-cm) 7% polyacrylamide–7 M urea–TBE thin sequencing gels (16). After electrophoresis and drying, the autoradiographs of such gels revealed the protected regions, the positions of which were determined by running sequencing ladders as size markers.

RESULTS

Deletion analysis of aerobactin promoter region. Figure 1 shows a number of plasmids carrying rightward deletions in the promoter region of the aerobactin operon of pColV-K30 fused in frame to the *lacZ* gene sequence of pMLB1034 (21). The deleted sequences span from the *Hin*dIII site located 375 bp upstream of the transcription start site of *iucA*, the first structural gene of the operon, to 9 bp upstream of the −35 region of the main P1 promoter. As expected, further rightward deletions resulted in the loss of any transcriptional activity from the insert. Table 1 shows the values of low-iron-induced β-galactosidase in *E. coli* 413 cells harboring the different plasmids. No significant difference was found among strains carrying deletions with the same *iucA'-'lacZ* gene fusion (pVC4, pDB16, pDB19, pDB37). In contrast, the downstream fusion corresponding to pCON1 afforded a substantial increase of the induced level, while the repressed figures were similar to those of the other fusions. In any case, all of them showed a rather similar pattern of about 20- to 50-fold induction under low-iron conditions. Owing to the multicopy nature of the fusions, differences in repressed conditions were not considered substantial in regard to regulation. This suggested that sequences conferring the inducibility were maintained in the smallest deletion (pDB37), consisting of a 78-bp insert from pColV-K30 containing the P1 promoter, the Shine-Dalgarno sequence, and 6 bp of the *iucA* gene. These data exclude a significant role of the sequences located upstream of the P1 promoter in the iron regulation of the system.

Fur footprint of promoter region. Owing to the superior redox stability of Mn^{2+} in an aerobic environment and its similar coordination character to Fe^{2+}, the binding ability of the Fur protein to an end-labeled DNA fragment carrying the

TABLE 1. Induction of β-galactosidase in iron-regulated gene fusions

Strain (plasmid)[a]	β-Galactosidase[b]		
	Basal level[c]	+100 μM $FeCl_3$[d]	+200 μM 2,2' bipyridyl[d]
413(pCON1)	45	22	3,100
413(pVC4)	50	20	954
413(pABN40)	47	22	872
413(pDB16)	96	74	924
413(pDB19)	56	24	965
413(pDB37)	12	8	745

[a] *E. coli* 413 was individually transformed with the plasmids carrying the collection of gene fusions shown in Fig. 1 and listed below.

[b] Miller units (17).

[c] Cultures of the different transformants were grown in LB plus amplicillin (200 μg/ml) to OD_{600} = 0.2, and the β-galactosidase value at that time was considered the basal level.

[d] β-Galactosidase levels 4 h after adding to the exponentially growing (OD_{600} = 0.2) cultures either 100 μM $FeCl_3$ or 200 μM of the iron chelator 2,2' bipyridyl. Figures given are the mean value between two independent experiments.

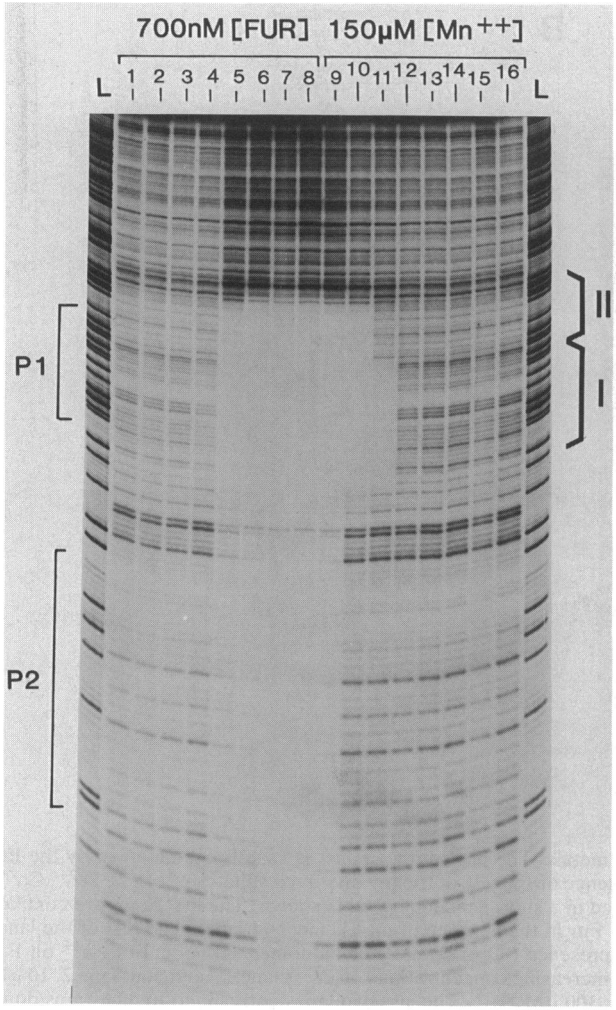

FIG. 3. DNase I footprints of the Fur protein of the promoter region of the aerobactin operon. The organization of the 3'-end-labeled fragment used for the footprinting experiments is shown in Fig. 2. This fragment (about 0.1 nM) was treated with DNase I in the presence of various concentrations of Fur protein monomer and Mn^{2+} as described in the text. The figure shown is the autoradiography of a long sequencing gel loaded with the products of the corresponding digestions. The lanes labeled L are sequencing ladders (T tracks from a known sequence) used as size markers to localize precisely the footprinted regions. Left lanes (1 to 8), Digestions in 700 nM Fur monomer with increasing concentrations of Mn^{2+}: 1, 400 μM 2,2'-bipyrydyl, no Mn^{2+}; 2, no Mn^{2+}; 3, 25 μM Mn^{2+}; 4, 50 μM Mn^{2+}; 5, 75 μM Mn^{2+}; 6, 100 μM Mn^{2+}; 7, 125 μM Mn^{2+}; 8, 150 μM Mn^{2+}. Right lanes (9 to 16), Digestions in 150 μM Mn^{2+} with decreasing concentrations of Fur; 9, 200 nM Fur; 10, 50 nM Fur; 11, 20 nM Fur; 12, 15 nM Fur; 13, 10 nM Fur; 14, 5 nM Fur; 15, 2 nM Fur; 16, 1 nM Fur. Locations of primary and secondary protected sequences are indicated at the right of the figure (I and II, respectively), while the regions corresponding to P1 and P2 promoters are shown to the left.

sequences of both P1 and P2 promoters was first studied in the presence of Mn^{2+}. Figure 3 shows the regions protected from DNase I nicking under different Mn^{2+} and Fur monomer concentrations. In the absence of divalent metal ion no protection was observed, even with rather high concentrations of Fur (700 nM). However, beyond a certain threshold of Mn^{2+} (50 to 75 μM in our assay mixture), increasing concentrations of Fur protein started to protect additional zones of the fragment. When the divalent metal was in excess, the first protected area detected was at about 20 nM Fur and covered 31 bp (Fig. 3). We will refer hereafter to this region as the primary binding site, which spans from 7 bp upstream of the −35 region of the P1 promoter down to the first base pair of its −10 sequence (Fig. 2). Lower concentrations of Fur resulted in a fading of the protected area, but not in a reduction to a smaller sequence. On the other hand, higher concentrations of Fur initiated protection of a contiguous downstream site of 19 bp, the secondary binding site (Fig. 2 and 3). Still further increases lead to the protection of sequences located upstream of the primary binding site, reaching the P2 promoter sequence and beyond. This protection, although directional, did not display defined boundaries. To study this extended protection at high Fur concentrations, we performed the experiment shown in Fig. 4. Here, the protection with or without divalent metal was examined at Fur monomer concentrations above 0.7 μM. The results shown in Fig. 4 further confirm that Fur is

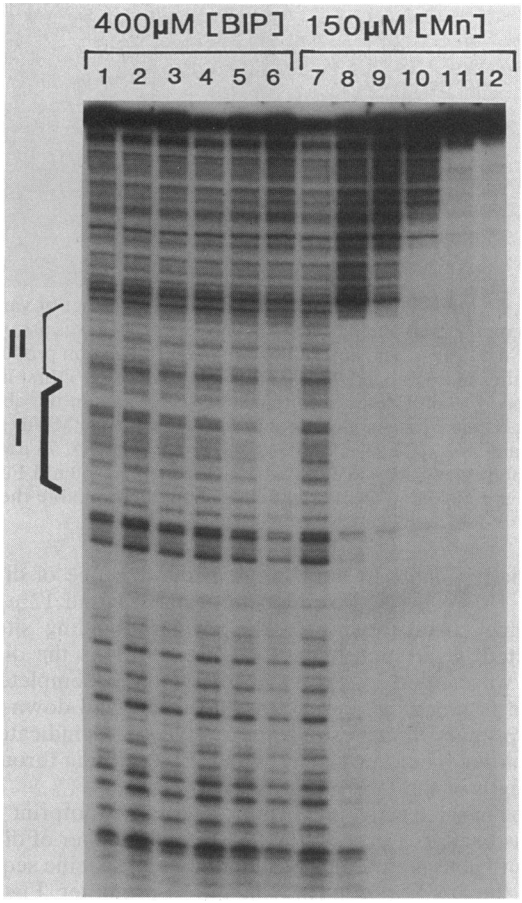

FIG. 4. DNA binding at high repressor concentrations. DNase I nicking assays on the end-labeled DNA fragment shown in Fig. 2 were performed as indicated in the text. The digestion products were analyzed on a short sequencing gel, the autoradiograph of which is shown. Left lanes (1 to 6) correspond to digestions made in the presence of 400 μM 2,2'-bipyridyl (BIP), which removes metal contaminants. Right lanes (7 to 12) are the same digestions in 150 μM Mn^{2+}. Fur concentrations: 1 and 7, controls without Fur; 2 and 8, 0.7 μM Fur; 3 and 9, 1.4 μM Fur; 4 and 10, 3 μM Fur; 5 and 11, 6 μM Fur; 6 and 12, 12 μM Fur. The locations of the primary and secondary binding sites (see text) are indicated at the left of the gel (I and II, respectively).

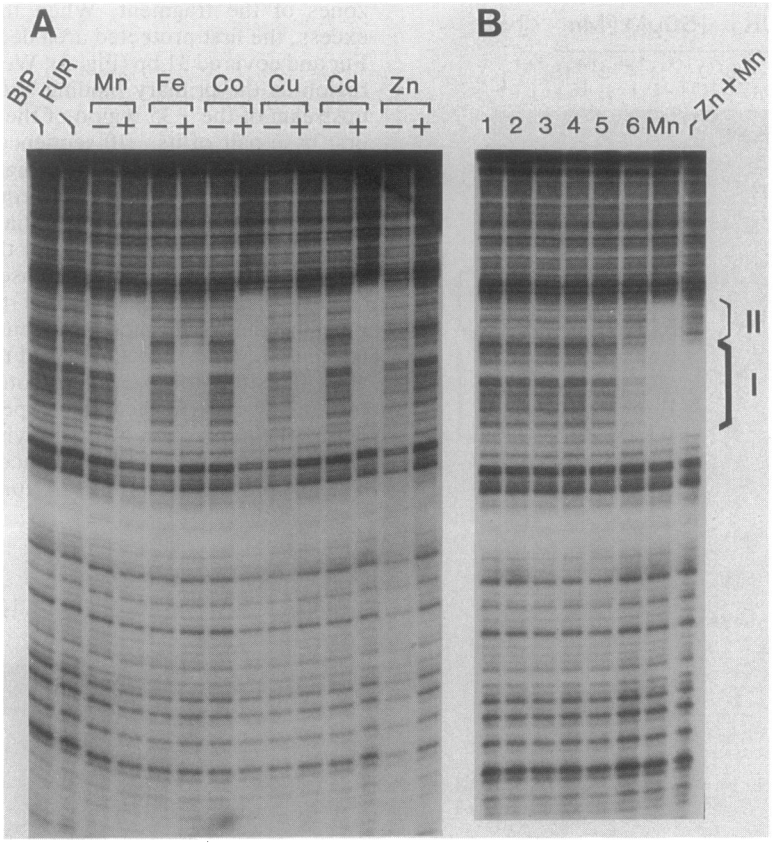

FIG. 5. Metal specificity of Fur binding. (A) Effect of various divalent metals. The protection against DNase I nicking given by the Fur protein on the end-labeled fragment shown in Fig. 2 was assayed in the presence of 150 μM of freshly dissolved sulfates of Mn^{2+}, Fe^{2+}, Co^{2+}, Cu^{2+}, Cd^{2+}, Zn^{2+}. The autoradiograph of the digestion products fractionated in a short sequencing gel is shown. The first lanes are controls with either no metal and 400 μM 2,2'-bipyrydyl (BIP) (first lane) or 60 nM Fur in the absence of metals (second lane). The rest of the lanes correspond to digestions with indicated metal ions in the absence (−) or presence (+) of 60 nM Fur monomer. (B) Effect of Zn^{2+} on Fur binding. DNase I digestions were performed with 60 nM Fur monomer and increasing concentrations of Zn^{2+}: lane 1, no metal; lane 2, 10 μM Zn^{2+}; lane 3, 25 μM Zn^{2+}; lane 4, 50 μM Zn^{2+}; lane 5, 75 μM Zn^{2+}; lane 6, 100 μM Zn^{2+}. The last two lanes correspond to digestions done in the presence of 60 nM Fur and 150 μM Mn^{2+} or 60 nM Fur and 150 μM Mn^{2+} plus 100 μM Zn^{2+}. Primary and secondary binding sites (I and II) are indicated at the right edge of the figure. Note the weak protection at 75 to 100 μM Zn^{2+} and inhibition of the Mn^{2+} effect.

essentially unable to bind DNA in the absence of divalent metal. However, at Fur concentrations beyond 12 μM the sequence corresponding to the primary binding site was protected. If at such high Fur concentrations the divalent metal was added in excess (150 μM), the complete end-labeled fragment sequence both upstream and downstream of the primary binding site was protected. This indicates that a metal-dependent polymerization of the protein throughout the length of the DNA took place.

Metal-specific activation of Fur. The same footprint protocol was used to ascertain the ability of a number of divalent metals to activate the Fur protein to bind a specific sequence within the DNA stretch carrying the P1 promoter. Figure 5A shows the results of such assays. Co^{2+}, Cd^{2+}, Cu^{2+}, and a freshly prepared solution of Fe^{2+} in addition to Mn^{2+} were able to exert such activation when added in excess to the footprinting reaction. If binding to DNA sequences with decreasing affinity is taken as an approximate estimation of Fur activity, then the apparent activation ability of the divalent metals is $Co^{2+} \geq Mn^{2+} \geq Cd^{2+} \geq Cu^{2+}$. The activity of Fe^{2+} could not be properly estimated under our conditions owing to its rapid oxidation to Fe^{3+}. The effect of Zn^{2+} was studied separately (Fig. 5B). At 150 μM this ion did not produce any detectable effect. However, a lower concentration of the ion (70 to 100 μM) afforded a partial activation of the protein (Fig. 5B). In addition, Zn^{2+} decreased the activation by Mn^{2+} (Fig. 5B).

DISCUSSION

In this paper we describe a double approach to locate an operator site for the Fur protein within the aerobactin promoter: (i) definition of the smallest region capable of affording iron regulation of an *iucA'-'lacZ* gene fusion (Table 1) and (ii) detection of the actual contact of the operator sequences with the Fur protein in the presence of a number of divalent heavy-metal cations.

The analysis of a collection of gene fusions linked to *Bal* 31 deletions through the promoter region limited the putative regulatory sequences to a 78-bp insert of pColV-K30 in pDB37. In this smallest deletion, the entire P2 promoter has been removed as well as three symmetry dyads noted as present in the aerobactin promoter (2). Another fusion, pCON1, carrying sequences both upstream and downstream of the P1 promoter displayed a higher induced level while keeping the general regulation profile. This could be a consequence of enhanced stability of the fusion protein or more efficient initiation of translation rather than the pres-

ence of regulatory sequences within the *iucA* gene. In any case, the 76-bp insert of pDB37 contains enough specific DNA sequence to afford iron control of the corresponding gene fusion. This is consistent with the previously reported lack of transcripts from the P2 promoter observed in vivo at either high- or low-iron conditions (2).

A recent in vitro study from this laboratory (Bagg and Neilands, in press), in which the effect of isolated Fur protein was examined by transcription-translation assays of a *lacZ* operon fusion, protection of a *Hin*fI site in the promoter, and alteration of electrophoretic mobility of promoter fragments on gels, provided evidence for the repressor function for the product of the *fur* gene. In the present work, the actual sequences bound to the Fur protein were determined by footprinting with DNase I (14). In most cases, Mn^{2+} was used instead of Fe^{2+} as an activator of Fur owing to the superior stability of Mn^{2+} in an aerobic environment at biological pH.

Figure 2 summarizes the footprint experiments. In the presence of a number of divalent heavy metals (Mn^{2+}, Fe^{2+}, Co^{2+}, Cu^{2+}, Cd^{2+}, and partially with Zn^{2+}), Fur binds primarily to a DNA sequence of 31 bp within the promoter region. The absence of divalent metals results in a dramatic decrease in the DNA-binding ability of the repressor (Fig. 4). Although all the metals assayed activated the repressor, the observed effects were not equivalent (Fig. 5). For instance, the effect of Zn^{2+}, in comparison to its close relative Cd^{2+}, suggests the possibility of additional metal-binding events besides those which lead to Fur activation. Zn^{2+} may provoke a Fur conformation unsuitable for DNA binding. The inhibition of Mn^{2+} activation by a lower concentration of Zn^{2+} (Fig. 5B) indicates that this ion may bind apart from the activation site, with a deleterious effect. Other divalent metals such as Mg^{2+} and Ca^{2+}, which are present in the footprint buffer, did not have any effect on Fur activity.

Comparison of Figures 3 and 4 facilitates an estimate of the range of metal-dependent activation of Fur. Twelve micromolar Fur (monomer) in the absence of metals afforded approximately the same footprint as 20 nM repressor in 150 μM Mn^{2+}. The increase of activity in the presence of divalent metal is therefore on the order of 10^3. It appears that the level of expression of iron-controlled genes will be a function of the concentration of both the Fur protein and its cognate metal ion, the latter believed to be the ferrous form of iron (Bagg and Neilands, in press).

The DNA sequence to which Fur primarily binds was protected as a single unit since lowering the Fur concentration through a critical level (20 to 10 nM repressor monomer; Fig. 3 and data not shown) resulted in a uniform fading of the complete region rather than the appearance of a shorter protected zone. Furthermore, the same 31-bp sequence was protected at high Fur concentrations in the absence of metals (Fig. 4). The upstream boundary of the protected region is just 2 bp downstream from the start of the insert sequence in the smallest plasmid deletion (pDB37) which showed iron control of the corresponding gene fusion.

Sequences other than the primary binding site were protected with a stringent metal dependency as the Fur concentration was increased. A well-defined secondary binding site of 19 bp was identified at 50 nM Fur (Fig. 3). Further increases in Fur concentration resulted in the protection of, first, the upstream region of the primary binding site, and eventually, of the complete DNA sequence of the end-labeled fragment (Fig. 3 and 4). While these sequence-unspecific protections might have little physiological significance, they suggest the ability of the Fur protein to form

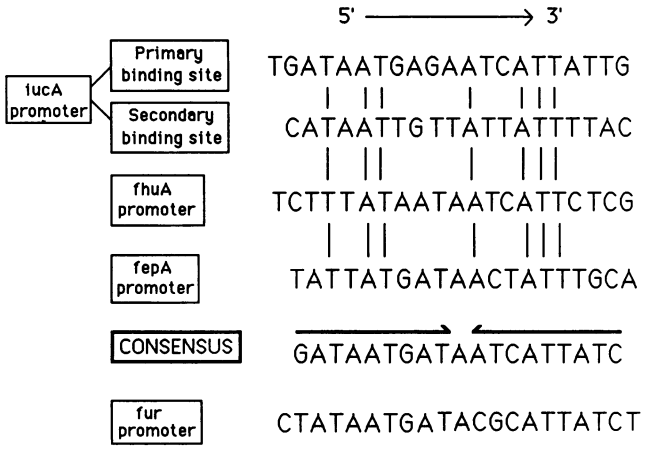

FIG. 6. Comparison of sequences from promoter regions of iron-regulated genes. Nucleotide sequences from the actual binding sites for the Fur protein in the aerobactin promoter are shown lined up with sequences found at the upstream regions of iron-regulated genes *fhuA* (5) and *fepA* (15). A sequence from the *fur* promoter region (20) has also been included for comparison (bottom). A tentative consensus sequence for Fur recognition is shown (see text for details). The most conserved nucleotides are indicated with vertical lines. This consensus fits almost exactly the second dyad in the aerobactin operator and, with some mismatches, the secondary binding site as well (Fig. 2). This sequence with dyad symmetry could bind one Fur dimer. Sequences progressively different from the consensus would be expected to bind further repressor units with decreasing affinity.

metal-dependent ordered polymers. Random binding to DNA is ruled out by the fact that a preference in the direction of the protected zones was observed. Linear polymerization requires the monomer unit to have two binding domains to attach contiguous units.

Unusual features of the primary binding site are its length and its symmetry. Classical DNA-binding regulatory proteins such as the lambda repressor, the Cro protein, or CAP bind about one turn of DNA helix (10 bp) per repressor unit so that a repressor dimer occupies two consecutive turns, typically as the result of binding a palindromic sequence (19). This leads to the protection of a region in the range of 20 bp (19). In other instances, as for the protein AraC (13), the protected regions bracket a sequence of about 40 bp. However, the whole zone protected by AraC displays an overall dyad symmetry distributed through three major grooves of operator DNA (13). In our case, however, the Fur-footprinted sequence showed an apparently complex symmetry in which two overlapping dyads share a sequence, 5'-TCATT-3' (Fig. 2), which is repeated three times through the binding site with a uniform spacing of 3 bp. Taking into account that the nicking by DNase I reveals quite precisely the sequence actually bound to the repressor, it is possible that both dyads play a role in recognition. However, without more information about the repressor molecule itself, models to account for the observed protection must remain speculative. The amino acid sequence of the Fur protein (20) does not closely resemble that of other DNA-binding proteins.

A plausible model is suggested by the results of the in vitro transcription-translation system (Bagg and Neilands, in press) as well as by comparisons with published sequences of the upstream regions of other iron-regulated genes, such as *fhuA* (5) and *fepA* (15). Sequences in the putative pro-

moter regions of the latter two genes, when compared with those in the primary and secondary binding sites of the aerobactin promoter, show a certain degree of homology: an apparent 19-bp dyad consensus sequence 5′-GATAATG ATAATCATTATC-3′ could be proposed from those comparisons (Fig. 6). Interestingly, the putative promoter region of the *fur* gene itself (20) also has a stretch with significant homology with the consensus sequence mentioned above (Fig. 6).

Other models are also possible since sequence comparisons should be taken with caution. Unfortunately, all the previous work (5, 15, 20) on iron-related genes of *E. coli* does not include experimental characterization of the promoter regions. Such data are only available for the aerobactin operon (2). Additional sequences of iron-controlled promoters are clearly to be desired.

ACKNOWLEDGMENTS

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