Evidence of an Unusually Long Operator for the Fur Repressor in the Aerobactin Promoter of Escherichia coli*

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Lucia Escolar‡, José Pérez-Martín, and Víctor de Lorenzo§

From the Department of Microbial Biotechnology, Centro Nacional de Biotecnología CSIC, Campus de Cantoblanco, 28049 Madrid, Spain

Production of the siderophore aerobactin in Escherichia coli is transcriptionally metalloregulated through the iron-dependent binding of the Fur (ferric uptake regulator) to a large region (>100 base pairs) within the cognate promoter in the pColV-K30 plasmid. We show in this article that such an unusually long operator results from the specific addition of degenerate repeats 5′-NAT(A/T)AT-3′ and not from a fortuitous occupation of the DNA adjacent to the primary binding sites by an excess of the repressor. Furthermore, the protection pattern revealed by DNase I and hydroxyl radical footprinting reflected a side-by-side oligomerization of the protein along an extended DNA stretch. This type of DNA-protein interactions is more like those observed in some eukaryotic factors and nucleoid-associated proteins than typical of specific prokaryotic regulators.

Prokaryotic transcriptional regulators bind DNA to repress or activate expression of specific genes or groups of genes (1). Although the sequences recognized can be extremely diverse, most regulatory proteins naturally bind discrete target sites within the bacterial genome. However, some regulators (typically the nucleoid-associated proteins; Ref. 2) are also known to bind somewhat degenerated sequences or structural motifs, thus spreading DNA-protein interactions along extended nucleotide sequences. This feature is shared with a variety of eukaryotic regulators, typically those containing zinc fingers such as the transcription factor TFIIIA (3). In this respect, the Fur protein of Escherichia coli displays both properties found in specific transcriptional factors and those in more global regulators. Fur is the product of the fur (ferric uptake regulation) gene (4–7), which controls transcription of iron-dependent sequences (the so called polymerization region; Fig. 1) is bound by the repressor to the consensus, the massive protection of the further upstream adjacent sites named I and II can be justified by their similarity to a palindromic 5′-GATAATGATAATCATTATC-3′, 19-bp1 consensus box (26, 31, 32). More recently, we have reinterpreted such a consensus as the combination of three repeats of the simpler motif 5′-NAT(A/T)AT-3′ (33), in which the thymines would be the bases determining the type of contact of the Fur protein with such a minimal unit of interaction. The corollary of this interpretation is that extended sites for Fur binding could be naturally or artificially assembled by simply adding multiple adjacent 5′-NAT(A/T)AT-3′ hexamers to a minimum of three repeats. This is a very attractive possibility, because it would permit the generation of repertoires of binding sites of varying extensions and affinities, which would allow Fur to act in some promoters as a very specific regulator and in others as a more general co-regulator (12). Although this notion has been substantiated using synthetic DNA sequences consisting of synthesized 5′-NAT(A/T)AT-3′ hexamers (33), it is unclear whether long sequence-dependent Fur operators are operative in natural iron-regulated promoters. The promoter of the operon responsible for the biosynthesis of the aerobactin siderophore (referred hereafter as Paer) is particularly interesting in this respect (26, 32, 33, 34). Unlike other promoters controlled by Fur in which the operator involves a clear-cut target sequence (13, 24, 27–29), Paer is bound by the repressor to three distinct extents depending on the concentration of the protein (Fig. 1 and see below). Although Fur binding to the adjacent sites named I and II can be justified by their similarity to the consensus, the massive protection of the further upstream sequences (the so called polymerization region; Fig. 1) is intriguing, because it does not contain clear Fur consensus boxes. Such an extensive occupation of the promoter by the repressor spreading over 100 bp has been revealed not only by DNase I and hydroxyl radical footprinting (32, see below) but also visualized directly through electron microscopy (35). Other iron-regulated promoters appear to undergo such an ample occupation as well (25, 36), so it might be a genuine phenomenon and not an unspecific protection caused by an excess of the protein.

In this work, we show that the as yet unaccounted binding of the Fur protein to the 5′ upstream region of the aerobactin promoter is due to the functionality of a long operator composed of nine adjacent 5′-NAT(A/T)AT-3′ hexamers. This operator,

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‡ Recipient of a Fellowship of Fundación Ramón Areces. Present address: Inst. fur Genetik. Biozentrum, Weinbergweg, 22 06120 Halle (Saale) Germany.
§ To whom correspondence should be addressed: Dept. of Microbial Biotechnology, Centro Nacional de Biotecnología-CSIC, Campus de Cantoblanco, 28049 Madrid, Spain. Tel.: 34-91-585-4536; Fax: 34-91-585-4506; E-mail: vdlorenzo@cnb.uam.es.

1 The abbreviation used is: bp, base pair(s).
which is entirely sequence-dependent, becomes effective only following the occupation of the other two sites. These results support the notion that the binding of Fur to DNA is mediated by the recognition of hexamer repeats and that an increasing number of adjacent repeats allow a co-operative binding of the repressor mediated by lateral protein-protein interactions. Furthermore, we argue that this type of interaction, which has features reminiscent of some transcription factors (37), endows the protein with the ability to behave both as a very specific repressor and as a more general regulator.

**EXPERIMENTAL PROCEDURES**

**General Procedures**—The Fur protein used in all the assays was purified to homogeneity following the metallo-affinity purification protocol of Wee et al. (17). According to Ref. 8, such purification protocol yields a Fur protein containing 1 atom of zinc/repressor monomer, whose DNA binding ability is responsive to Mn²⁺ in our assays system (see below). Protein Fur concentrations indicated through this work refer to the protein monomer. DNA techniques were run according to published protocols (38).

**DNA Templates for Footprinting Assays**—The organization of the DNA fragments used in footprinting assays is shown in Fig. 2. The fragment wt1 is a 368-bp EcoRI-PvuII segment from plasmid pUC-LE15 that contains the region spanning positions -128 to +32 of the aerobactin promoter region (using as a reference the transcription start site of the main promoter P1) as an EcoRI-BamHI plus a vector-born unrelated BamHI-PvuII extension of 208 bp. The strategy for creating the promoter termed Δ50 is sketched in Fig. 2A as well. Primers were devised for amplification of the sequence -50 to +32 (thus excluding the P2 promoter) and the upstream extension region. This fragment was recloned in pUC19 using the EcoRI and BamHI sites present in the amplified fragments (BamHI already present and EcoRI entered with the rightward primer). To get a template of identical size to wt1 for the footprint assay, a primer was engineered that contained a terminal NcoI site located at exactly the same distance as the EcoRI site of the wt1 promoter (Fig. 2B). Such a segment was then entered at the single HindIII site of the previous pUC19 derivative. This new plasmid contained an insert in the vector that spans the new promoter construct Δ50. The fragment generated after restriction with Ncol-DalII allowed a base-wise comparison of its footprint with the wild type fragment because the end-sites, EcoRI or Ncol, were located exactly at the same point.

For the second series of templates shown in Fig. 2B, modified variants of the aerobactin promoter with increasing distance between the Fur boxes (BamHI fragment) are indicated below, as well as the location of the radioactive label (asterisk) in the DNA fragments assayed. B, modified variants of the aerobactin promoter with increasing distance between the Fur boxes I and II. The boundary between the two boxes was entered with a novel ClaI site, which was further employed for addition of 2, 10, or 14 bp (new bases in bold type) as explained under “Experimental Procedures.” The mutated segments were cloned back to pUC19 and used as the source of the end-labeled EcoRIPvuII restriction fragments employed in the footprintings.

**Footprinting with DNase I and Hydroxyl Radicals**—DNA-protein interactions were probed with DNase I as described in Refs. 32 and 33. Samples were preincubated for 5 min at 37 °C with the amounts of the Fur protein indicated in each case. Each tube was then added with 2.5 

![Fig. 1. Organization of the aerobactin promoter region.](image1)

**Fig. 1.** Organization of the aerobactin promoter region. The overall arrangement of functional elements within the DNA segment placed at 5′ in respect to the aerobactin gene cluster is shown. The promoter region includes two -10/−35 hexamers that define promoters P1 (proximal) and P2 (distal). The primary target DNA sequences for the Fur protein (sites I and II) and the upstream extension are pointed as defined by DNase I footprinting (Ref. 32 and Fig. 2), with an indication of the two segments with a maximal coincidence with the 19-bp consensus Fur binding sequences (5′-GATAATGATAATACATTAT-3′, Fur boxes). The transcription start sites of each of the promoters is indicated as well.

![Fig. 2. Pwo promoter variants used as templates for DNA footprinting analysis.](image2)

**Fig. 2.** Pwo promoter variants used as templates for DNA footprinting analysis. A, the fragment wt1 is a 368-bp EcoRI-PvuII segment from plasmid pUC-LE15 spanning positions -128 to +32 of the aerobactin promoter region as an EcoRI-BamHI plus a vector-born unrelated BamHI-PvuII extension. The promoter variant termed Δ50 was created by amplifying the sequence -50 to +32 as an EcoRI and BamHI fragment, combining it with an Ncol-EcoRI extension of identical size of that of the wt1 segment and cloning the whole in pUC19 (see text for explanation). The Ncol-PvuII segment present in the resulting plasmid has its Ncol end located at exactly the same distance from the Fur boxes as the EcoRI site of the wt1 fragment, thus allowing a faithful comparison of its footprint with the wild type fragment. The relative position of each fragment in respect to the functional motifs of the aerobactin promoter (led by the iucA gene) are indicated below, as well as the location of the radioactive label (asterisk) in the DNA fragments assayed. B, modified variants of the aerobactin promoter with increasing distance between the Fur boxes I and II. The boundary between the two boxes was entered with a novel ClaI site, which was further employed for addition of 2, 10, or 14 bp (new bases in bold type) as explained under “Experimental Procedures.” The mutated segments were cloned back to pUC19 and used as the source of the end-labeled EcoRIPvuII restriction fragments employed in the footprintings.

![Footprinting with DNase I and Hydroxyl Radicals](image3)
The Fur Operator in the Aerobactin Promoter

![Figure 3](image_url)

**RESULTS AND DISCUSSION**

**Visualization of a Continuous Pattern of Fur-DNA Interactions through the Aerobactin Promoter Region**—To match faithfully the extensions of each binding site for the Fur protein along the *Paer* promoter with the specific bases involved in protein-DNA contacts, we conducted the experiment shown in Fig. 3. In it, we compared directly the protections caused by growing repressor concentrations under the aerobic conditions of the experiment (11, 12, 14). To address this issue, we engineered a ClaI site next to the Fur box of site I (primary binding site of the protein). This site was employed to insert extra bases that changed the relative orientation of the downstream site II from 2, 10, or 14 bp (Fig. 2B). The expected result of such insertions was to either offset moderately the two target sequences (+2) or to separate them but keep the phase of the DNA helix (+10) or to entirely offset and separate sites I and II (+14). The resulting promoter variants were then footprinted with DNase I in the presence of growing concentrations of Fur-Mn^{2+}, with the results shown in Fig. 4.

The 2-bp insertion (Fig. 4) between the sites entered a change in the extension and strength of the occupation of site II. The first 31 bp (site I) were protected to the same extension and the same protein concentration as the wild type promoter. However, occupation of site II (which was displaced further downstream: upwards in Fig. 4) required a significantly higher Fur-Mn^{2+} level. Interestingly, the protection of the 5' extension region occurred at the very same protein concentration as the wild type promoter, thus suggesting that such an extension is entirely independent of the presence of protein bound to site II. Although these results indicated that occupation of site II is co-operative with that of site I, they do not rule by themselves that both sites are indeed independent. This issue, however, was unequivocally ascertained by the results of the -10 and +14 promoters. Regardless of the maintenance of the DNA helix phase (+10) or its full disruption (+14), the increased distance between sites I and II resulted in the inability of the downstream site to bind any protein. In both cases, the protection of site I was in all comparable with the wild type *Paer*...
promoter. These data favor the notion that site II is an extension of site I rather than a separate target sequence. As in the case of the template, the separation of the sites by longer insertions did not affect at all the upstream 5′ extensions, which were detected to the same extent and apparent intensity as in the wild type promoter.

Extensive Binding of Fur to the DNA Adjacent to the Primary Binding Site in the Aerobactin Promoter Is Sequence-specific—The results above gave a preliminary hint on whether the lateral enlargements of the protection caused by Fur on most iron-regulated promoters of E. coli (24, 28, 29, 14) is sequence-specific or whether they just reflect an artifactual occupation caused by a high protein concentration in vitro. This is a reasonable doubt, because such prolongation not always matches the 19-bp consensus Fur box (24, 28, 29, 14). The data of Fig. 4 show that not any sequence adjacent to site I within the aerobactin promoter is suitable to become protected by a high concentration of the repressor. Furthermore, extensions require a certain frame and distance in respect to the primary site. However, the secondary site does include a sequence stretch similar to the 19-bp consensus, thereby suggesting that frame, distance, and specific sequence are all necessary for the enlargement toward the site II.

This is, however, less clear at the third protected region, where the extension phenomenon is far more dramatic that in site II (Fig. 3). In this case, the upstream protection observed spans 60 additional base pairs. Although such a protection has a clear directionality and defined boundaries, the sequence involved does not show any significant homology with the reference 19-bp Fur consensus box (26). It is thus conceivable that such a massive protection is not specific and therefore irrelevant to understand the metalloregulation of the promoter. To ascertain this question, we simply prepared a new DNA template (Fig. 2A) in which we faithfully replaced the upstream DNA by an unrelated sequence. The substitution was such that a DNA fragment of a size identical to that bearing the wild type Paer promoter could be examined in parallel in DNase I footprinting assays. The results shown in Fig. 5 indicated that the unrelated sequence failed to bear any visible extension of the footprint, even at the higher protein concentrations. Furthermore, the 5′ boundary of the protection was located exactly at the point where the heterologous sequence started (marked with an arrow in Fig. 5). We thus conclude that the binding of Fur to the third region is indeed sequence-dependent. We argue below that this cannot be explained with the generally accepted 19-bp consensus model, but it is perfectly compatible with the notion that a shorter 5′-NAT(A/T)AT-3′ motif is the basic unit of Fur binding.

Reinterpretation of the Fur Operator within the Aerobactin Promoter—The data presented in this work support the hypothesis (33) that Fur binding sites do not follow the standard palindromic organization of target sequences for regulators in prokaryotic promoters (1). Instead, Fur operators of different extensions can be formed by addition, in any orientation, of a minimum of three NAT(A/T)AT hexamers.
timers (33), this report shows that this is the case also for a natural promoter such as that of the aerobactin operon, whose extended binding sites for the repressor cannot be easily explained with the generally accepted 19-bp consensus model. Fig. 6 shows a reinterpretation of the pattern of Fur-DNA interactions in the promoter as the result of a single, enlarged operator that is formed by additions of up to 18 boxes whose frame give a maximum match to the reference AT(A/T)AT pentamer. Such boxes are separated in all cases by one intervening extra base. The one exception is at the boundary between the site I and the protected upstream region, which lacks such an additional base, a fact that is faithfully reflected in the hydroxyl radical footprint of the region (Fig. 3). It seems that either the deletion of 1 base or the addition of two bases between boxes (as in the artificial promoter +2, see Fig. 4) flaws the co-operative occupation of adjacent hexamers but does not inhibit it. In fact, it is revealing that such a naturally existing deletion between site I and the upstream extension is required to frame maximally the further upstream sequence to the reference NAT(A/T)AT motif. But how does this hypothesis equate the actual data?

The sequence that is protected by the lowest concentrations of Fur-Mn$^{2+}$ includes 31 bp and, according to the hydroxyl radical footprinting of Fig. 3, consists of a whole of five adjacent hexamers, three of them with a nearly perfect match to NAT(A/T)AT. The side repeats contain less conserved T residues, and thus their occupation requires a higher repressor concentration, which establishes the pause in the protection that is clearly revealed by DNase I footprint (Fig. 3) and which defines site II. Such second site would include three additional repeats. This extension certainly requires protein-protein interactions with the repressor already bound to site I, to compensate the divergence in the sequence. In fact, some hexamers have only a limited match with the consensus. Thus, the downstream sequence may not bind by itself to the Fur-Mn$^{2+}$ complex, but it does in the context of the whole promoter. Finally, the long upstream extension can also be sorted out as an array of adjacent Fur-binding hexamers frameshifted by one base in respect to the sequence of boxes included in sites I and II. Although such a shift may explain the lower affinity, the hydroxyl radical data of Fig. 3 shows that the shift resettles the pattern of protein-DNA interactions to the maximum match with the NAT(A/T)AT array. It thus appears that the suboptimal alignment with the primary sites and the considerable sequence divergence of the upstream region are balanced by a higher number of boxes that, as a result, produce an unusually long operator.

**Conclusion**—Although not to the same dramatic extent as the aerobactin system, many if not all iron-regulated promoters...
of *E. coli* (14, 22, 24, 28, 29, 36) contain Fur target sequences that spread beyond the core iron box. No natural Fur binding sites have been found to give less than a 31-bp footprint with DNase I, although the minimal operator is only 19 bp. It thus looks likely that such adjacent sequences are not casual but are indeed arrayed in a configuration of various 6-bp repeats with a potential to interact specifically with the Fur protein as a whole. Extended sites might tolerate a degree of divergence in the sequences involved, which could be compensated by the higher overall affinity. These additional contacts might strengthen the overall binding of the DNA segment to the regulator and do explain why the protection is not limited to the consensus Fur box. The 6-bp box criteria accounts for the variability and extension of the sequences protected by Fur in most iron-regulated promoters and is also compatible with the relatively high amount of Fur molecules (approximately 5000) found inside the cell (16, 41). The published DNase I footprinting assays on several promoters (14, 24, 28, 29) can be consistently reinterpreted as arrays of hexameric sequences akin to those of the aerobactin promoter, in which the key T residues are conserved to various degrees. This mode of Fur-DNA interaction, in which new Fur molecules must necessarily bind adjacent hexamers through side-by-side oligomerization, explains the gradual physiological response observed in Fe$^{2+}$-responsive systems, because it would make possible an entire range of repression levels of iron-controlled promoters (12). The affinity for specific promoters would vary depending on the number of repeats present on each operator and the conservation of their sequences, thus generating a hierarchy of transcriptional responses depending on small changes in the iron status of the cell. Such an ability of Fur to control promoters through extensive DNA-protein interactions makes this protein to be mechanistically closer to general regulators than to specific transcriptional factors. In fact, because Fur is a Zn-containing protein (8), it is curious that the type of DNA-protein interactions reported here have certain reminiscence to the occupation of adjacent DNA sites by individual zinc fingers within eukaryotic transcription factors such as transcription factor TFIIIA (3, 37).

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