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Expression, isolation and properties of Fur (ferric uptake regulation) protein of *Escherichia coli* K 12*

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Summary. The cloned *fur* (ferric uptake regulation) gene of *Escherichia coli* K12 was ligated to an expression vector which was inducible with nalidixic acid. The Fur protein was isolated in a single step by immobilized metal-ion-affinity chromatography over zinc iminodiacetate agarose. The amino acid composition of the isolated protein agreed with that predicted from the gene sequence and indicated post-transcriptional removal of the N-terminal methionine residue. All four cysteines were shown to be present as thiols. Proteolysis with trypsin and chymotrypsin yielded large fragments identifiable on polyacrylamide gel electrophoresis. Various divalent metal ions were found by a nitrocellulose filter binding assay to effect non-specific interaction of the Fur dimer with DNA with a dissociation constant of 7×10^{-12} M. A much smaller value, 2.5×10^{-17} M, was measured by gel mobility retardation assay for binding of Fur to a DNA fragment containing the operator sequences of the aerobactin promoter.

Key words: Fur repressor — Iron regulation

Abbreviations: *Bistris,* 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)-propane-1,3-diol; *DMSO,* dimethylsulfoxide; *EDTA,* ethylenediaminetetraacetate; *Hepes,* 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; *kb,* kilobase; *kDa,* kilodalton; *LB,* Luria broth; *PMSF,* phenylmethylsulfonyl fluoride; *SDS-PAGE,* sodium dodecyl sulfate/polyacrylamide gel electrophoresis; *Tris,* 2-amino-2-hydroxymethylpropane-1,3-diol; *TEMED,* tetramethylethylenediamine

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Introduction

In the past few years considerable information has developed regarding the distribution, structure and membrane physiology of a group of compounds, generically termed siderophores, which are required for high-affinity iron assimilation in aerobic and facultative anaerobic microorganisms (Neilands 1981, 1982). The level of understanding is greatest in the case of *Escherichia coli,* which forms at least two siderophores, enterobactin and aerobactin, and which has the capacity to transport several other siderophores of bacterial and fungal origin. The aerobactin system of *E. coli* is of special interest since the presence of this iron-assimilation pathway is believed to be a factor conferring virulence on a normally innocuous organism (Warner et al. 1981).

The cloning of the entire regulatory, biosynthetic and transport functions of the aerobactin operon borne on plasmid ColV-K30 of clinical isolates of *E. coli* afforded an opportunity for investigation of the mechanism of control of siderophore systems (Bindereif and Neilands 1983), which have been known for more than 30 years to be tightly regulated by the level of iron present in the growth medium (Garibaldi and Neilands 1956). A major advance was registered by Ernst et al. (1978), who detected a mutation in *Salmonella typimurium* labelled *fur* (ferric uptake regulation), which resulted in constitutive expression of all known siderophore-related functions in the bacterium. Hantke (1981) isolated a corresponding mutant in *E. coli* and tentatively mapped the lesion near *lac.*

Having in hand the restriction map (Bindereif and Neilands 1983) of the aerobactin operon of pColV-K30 enabled the construction of a *lacZ* fusion in this operon and the isolation of a constitu-

tive mutant, which was found to map at 15.7 min on the chromosome (Bagg and Neilands 1985). This proved not to be an additional *fur*-type mutation, however, since Hantke (1984) revised the *fur* locus to about 15.5 min, cloned the gene and determined its sequence (Schaffer et al. 1985). Additional mutants, constitutive for iron transport (*fur* phenotype), have been isolated in this and other laboratories and all, thus far, map to the same locus.

In order to investigate the mode of action of the Fur protein its gene was re-cloned in this laboratory (Bagg and Neilands 1987a). In the present paper we describe transfer of the *fur* gene to an inducible expression vector and isolation of the Fur protein by single-column affinity chromatography on zinc iminodiacetate agarose (Porath et al. 1975). The availability of the Fur protein has led to the conclusion that it behaves as a classical repressor, requiring only ferrous iron as co-repressor (Bagg and Neilands 1987b) to bind the operator of the aerobactin promoter (de Lorenzo et al. 1987). We also report here some of the significant properties of the isolated protein, including its affinity for a site, or sites, contained in a 156-bp fragment derived from the promoter-operator region of the aerobactin operon.

Materials and methods

Materials. All chemicals used were of reagent grade. Hepes, nalidixic acid, streptomycin sulfate, kanamycin sulfate, PMSF and pepstatin A were from Sigma Chemical Co. (St. Louis, Mo.). Iminodiacetate agarose (6% crosslinkage) was a generous gift of Dr. J. Porath. A similar gel (4% crosslinkage) offered by Pierce Chemical Co. (Rockford, Ill.), appeared to be as effective. Restriction enzymes were from Bethesda Research Laboratories. Trypsin and chymotrypsin were purchased from Sigma Chemical Co. (St. Louis, Mo.). RNA polymerase was a gift of Karen Arndt of the Chamberlin laboratory of this department and was prepared by the procedure of Gonzales et al. (1977).

Bacterial strains. The bacterial cultures and plasmids were from laboratory stocks. Media, such as LB, were prepared as described by Miller (1972).

Assays. Protein was measured by the Bradford (1976) procedure using a dye concentrate obtained from Bio-Rad and bovine serum albumin (Sigma) as standard. Fractions from the metal-affinity column were surveyed for Fur protein by SDS-PAGE, using 15% gels and Coomassie blue stain. Thiol groups were measured by the method of Ellman (1959). Amino acid analysis was performed in the Protein Structure Research Laboratory (University of California, Davis). Proteolysis was carried out by the method of Blackburn (1970).

The Fur-operator interaction was investigated by using a modification of the nitrocellulose filter binding assay described by Miller (1972) and by a gel mobility retardation assay.

The buffer for filter binding experiments was 10 mM KCl, 10 mM Tris/Cl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM dithiothreitol and 5% DMSO. The purified Fur protein was incubated at 37° C for 30 min with a 5′-labelled 156-bp *Sau*3A fragment of plasmid pVC3, which includes the operator region of the aerobactin operon (de Lorenzo et al. 1987) in binding buffer containing 100 μM activating metal ion, 20 μg/ml bovine serum albumin and 5 μl/ml salmon sperm DNA. A 1-ml aliquot was then filtered during a 45 to 60-s interval with Membranfilter (Schleicher and Schüll, BA 85/5) and washed once with the same volume of binding buffer containing 100 μM activating metal ion solution. The filter was dried and dissolved in 8 ml Scint-A (Packard) scintillation cocktail prior to measurement of radioactivity.

The altered gel migration of the *Sau*3A fragment due to binding to the Fur protein or RNA polymerase was assayed according to the procedure of Dr. V. de Lorenzo of this laboratory. Briefly, the 15-μl assay mixture consisted of 10 μl binding buffer, 1 μl of a solution of the operator fragment to give a final concentration of 1.0 pM, and various amounts of Fur or RNA polymerase. The binding buffer contained 10 mM Bistris/boric acid (pH 7.5), 5 μg/ml salmon sperm DNA, 5% glycerol, 100 μg/ml bovine serum albumin, 1 mM $MgCl_2$ and 40 mM KCl. To provide a relatively redox-stable divalent heavy metal capable of performing as an effective substitute for Fe(II) as activating ion (Bagg and Neilands 1987b), $MnCl_2$ was included in the binding buffer at a level of 100 μM. After a 15-min incubation at 37° C, the assay mixture was loaded on a polyacrylamide gel containing 5.25% acrylamide:bisacrylamide (30:0.8), 40 mM Bistris/boric acid (pH 7.5), 100 μM $MnCl_2$, 0.03% ammonium persulfate, and 30 μl TEMED. The electrode buffer consisted of 40 mM Bistris/boric acid and 100 μM $MnCl_2$. The gel was run for 45 min at 100 V, dried and analyzed by radioautography.

Results

Construction of expression vector pMON2064

The *fur* gene of *E. coli* K12 was cloned into plasmid pF3 (Bagg and Neilands 1987a) by an alternative procedure to that used by Hantke (1984). In order to produce large quantities of Fur protein, an expression vector for the *fur* gene was constructed. The *fur* gene was first mutagenized to introduce cloning sites which would enable its transfer to a suitable vector. An *AccI* fragment containing the *fur* gene was cloned from pF3 into the single-stranded phage vector, M13mp8, from which deoxyuridine-containing, single-stranded DNA template was prepared. Two oligonucleotide primers were simultaneously used to prime synthesis of the complementary strand of this phage DNA. The oligonucleotides specified alterations of the *fur* gene sequence such that an *NcoI* restriction site would occur at the initiator methionine codon and, by reference to the sequence reported by Schaffer et al. (1985), a *Hind*III restriction site at the 3′ end of the gene. Transfection and propagation of the phage DNA thus ob-

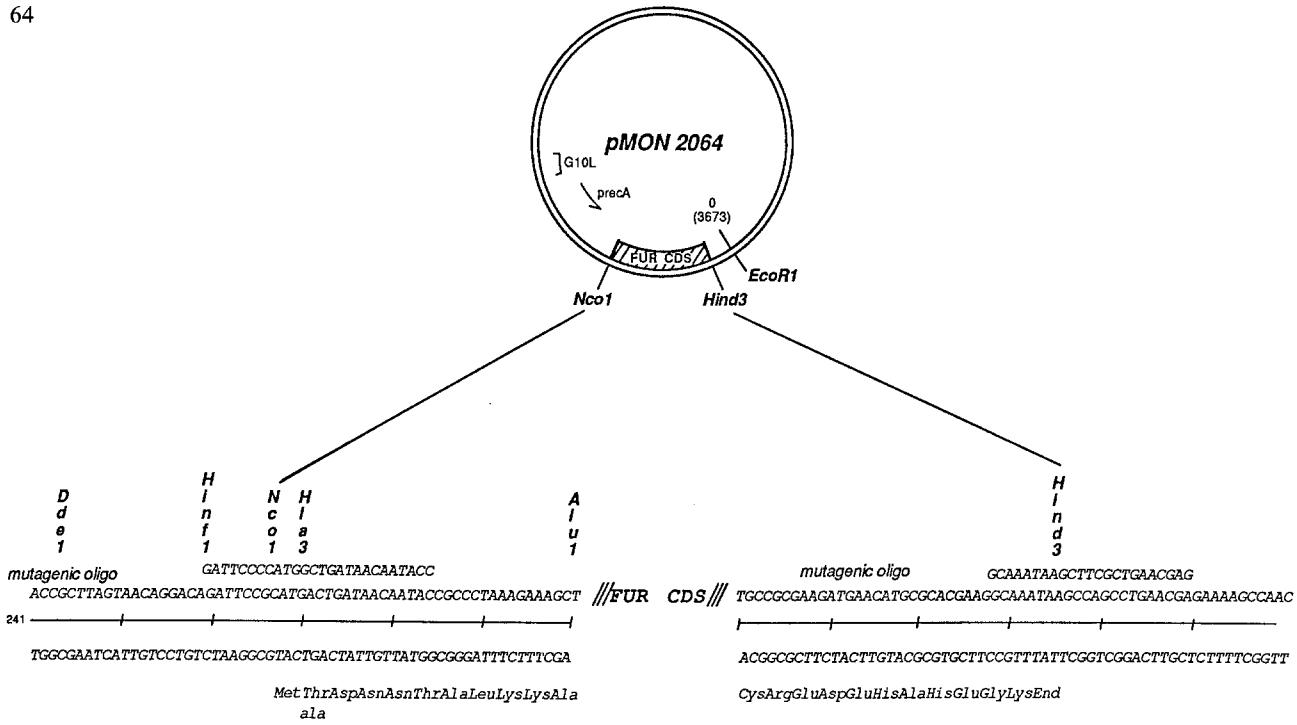

Fig. 1. Plasmid MON2064, a derivative of pBR327, used for expression of the *fur* gene. See text for method of construction. *CDS*, coding sequence; *precA*, promoter/operator of *recA*; *G10L*, translation initiation sequence of coliphage T7 gene 10

tained in *E. coli* JM101, which rapidly degrades the uracil-containing template strand, yielded progeny phage having the expected restriction pattern. The translation product of the mutagenized *fur* gene differs from the wild gene in carrying an alanine rather than a threonine residue at position 2 of the translation product. This change was a consequence of the introduction of the *Nco*I site.

The altered *fur* gene was transferred to a plasmid vector in order to place the promoter-operator sequence of the *E. coli recA* gene and the translation initiation sequence of coliphage T7 gene 10 at the 5′ end of the *fur* gene (Fig. 1). The replication and ampicillin-resistance functions of this plasmid are derived from pBR327. The expression vector so obtained, pMON2064, was transformed into *E. coli* JRB45 *(lac, ara, lon, strA, supF)* and used for production of the Fur protein.

Isolation of the Fur protein

E. coli JRB45 pMON2064 was inoculated into 16 ml LB containing 200 μg/ml ampicillin. The culture was poured into 0.5 l of the same medium and grown overnight. Exactly 50 ml of this culture was inoculated into each of 10 Fernbach flasks containing 1 l LB medium. After about 3 h of growth on a gyrorotary shaker at 37° C, at which time the $A_{600\,nm}$ had reached a value of 1.0–1.2, 5 ml 0.1 M NaOH containing 50 mg nalidixic acid was added to each flask and the shaking continued for an additional 4 h. The culture was centrifuged and the pellet washed with 0.1 M Tris pH 7.6 to yield 36 g wet cells, which were stored in the deep freeze.

To initiate a preparation of Fur, a 12-g portion of cell paste was thawed in 25 ml 50 mM Tris/Cl pH 8.0 containing 20 mM EDTA. After addition of 3 ml glyerol, 0.5 ml acetone containing 6 mg PMSF and 50 μl of an aqueous solution containing 0.1 mg pepstatin A, the cell suspension was stored for 30 min at room temperature. The suspension was then sonicated and the cell debris was removed by centrifugation. Streptomycin sulfate was added to the supernatant to give a concentration of 1% and the nucleic acids removed by centrifugation at 20000 *g* for 20 min. The clarified supernatant was brought to 80% saturation in ammonium sulfate and, after standing overnight at 5° C, the precipitate was collected and dissolved in the least volume of 20 mM Tris/Cl, pH 8.0.

The preparation, which contained about 200 $A_{275\,nm}$ units, was then applied to a column (1.5 × 14.5 cm) of iminodiacetate agarose which had been equilibrated with excess 20 mM $ZnSO_4$ and washed with 60 ml 0.5 M K_2SO_4 in 20 mM Tris/Cl pH 8.0. The ultraviolet absorbance of the effluent was monitored with a photocell attached

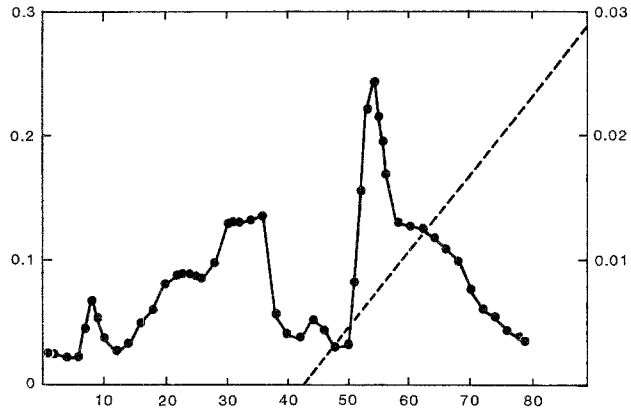

Fig. 2. Ultraviolet absorption profile of column fractions. Ultraviolet absorption of fractions obtained by glycine wash and histidine elution steps from a zinc iminodiacetate agarose column. Fractions 0–41 were collected by elution with 25 mM glycine in 20mM Tris/Cl pH 8.0. Elution with a 0–50 mM histidine gradient was initiated at fraction 42. Analysis of fractions 30 and 36 by SDS-PAGE showed the RecA protein to be the main component, together with small amounts of Fur. Fractions from the histidine elution proved to be pure, or virtually pure, 17-kDa Fur protein. A_{280} (●—●), Histidine concentration, M (- - - -)

to a strip chart recorder. The column was washed in succession with 150 ml 0.5 M ammonium sulfate in 20 mM Tris pH 8.0 and a similar volume of 0.1 M Bistris pH 6.5, using a flow rate of 15 ml/h. A final wash at 5 ml/h was performed with 25 mM glycine in 20 mM Tris/Cl pH 8.0 and the Fur protein eluted from the column with a linear gradient of 0–50 mM DL-histidine in the same buffer. Figure 2 shows the $A_{280\,nm}$ profile of fractions obtained from the glycine wash and histidine elution steps.

The yield of Fur protein, using the dye-binding assay and bovine serum albumin standard, was approximately 130 mg. The pooled fractions from the histidine gradient elution were brought to 80% saturation in ammonium sulfate and the precipitate dissolved in 20 mM Hepes (pH 7.6) containing 10 mM EDTA. The solution of the Fur protein was passed through a small Sephadex G-25 column equilibrated with metal-free Hepes buffer. The protein, which in more concentrated solution exhibited a slight amber color, was then concentrated with Centriprep 10 (Amicon Co, Danvers, Mass.).

Properties of the Fur protein

Purity. Figure 3 shows the SDS-PAGE profiles of a cell supernatant and the isolated Fur protein. The gel was scanned. Fur protein was found to be 35% in the cell supernatant and after purification over 95%. The small amounts of higher-molecular-mass protein which sometimes contaminated the product could be removed by a column of diethylaminoethyl-cellulose (Whatman, DE52) equilibrated with 0.1 M Bistris (pH 6.5). Fur protein eluted in a single band with 0.15 M NaCl in the same buffer.

Ultraviolet absorbance. A solution of Fur in 20 mM Tris/Cl, pH 8.0, which assayed at 1.0 mg/ml by the Bradford reagent, had an $A_{275\,nm}$ of 0.4. This low value is expected since the gene sequence (Schaffer et al. 1985) indicates the protein is devoid of tryptophan.

pI. The protein was subject to isoelectric focusing by the method given in the catalog of the Hoeffer Scientific Instrument Co. A value of 5.85 was found. This procedure further confirmed the purity of the material shown in Fig. 3 (data not shown).

Solubility. The solubility of the Fur protein was determined to be about 10 mg/ml in water or 10 mM Hepes (pH 7.6). In 100mM Hepes, pH 7.6, the solubility increased to 160 mg/ml. These data are for either 4°C or room temperature.

Thiol content. The thiol content was determined to be 3.4 mol/mol Fur, suggesting that all four cysteines predicted by the gene sequence are present in the reduced state.

Amino acid content. The amino acid analyses were compatible with the composition inferred from

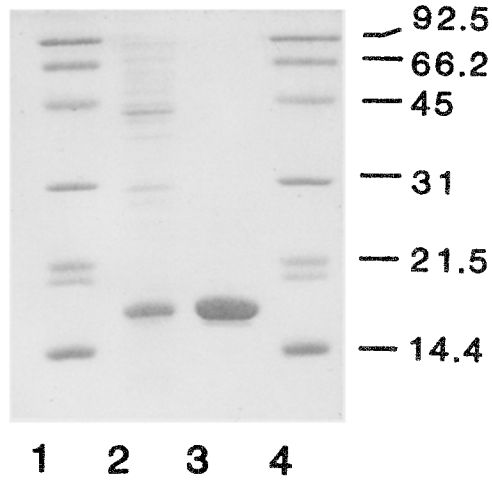

Fig. 3. SDS-PAGE of Fur preparations. *Lanes 1 and 4:* molecular mass standards (values in kDa on right). *Lane 2:* protein profile of cell supernatant of *E. coli* JRB45 (pMON2064), following induction. *Lane 3:* isolated Fur protein. Analyses were by Coomassie blue stain. Total protein added to each lane was 5 μg

the gene sequence, with the exception of alanine and threonine. These were present at 11.1 and 5.7 instead of 10 and 7 residues per molecule, respectively, which was a result of the method of cloning. The analyses indicated the presence of a single residue of methionine, per molecule.

Proteolytic digestion. Digestion of Fur to completion with trypsin afforded on SDS-PAGE analysis a single detectable band with a molecular mass of 7.5 kDA while similar treatment with chymotrypsin yielded two bands with value of 8 and 6 kDa (data not shown). In each case the ratio of protein/enzyme used was 100:1. The proteolytic fragments have not yet been examined for repressor activity.

Protein-DNA interactions. An attempt was made to demonstrate specific association of Fur with operator DNA by the filter-binding assay. For this purpose two *Sau*3A fragments of pVC3 were used, one of which was a 156-bp operator-containing sequence and the other a 145-bp control sequence. Since pVC3 contains a single copy of the aerobactin promoter, it seemed adequate to compare the two similar-sized DNA fragments. However, in the presence of Mn^{2+} as activating metal ion, Fur bound to both the operator and control fragments with a Hill coefficient of 2 and a dissociation constant of approximately 7×10^{-12} M (data not shown). This suggests that Fur has some capacity for non-specific binding, as a dimer, to DNA.

The Fur protein has been shown to be a repressor (Bagg and Neilands 1987a, b). To determine whether Fur acts at the level of initiation of transcription, direct competition between RNA polymerase and Fur was monitored by acrylamide gel electrophoresis. Figure 4 shows the retardation of gel migration due to protein binding to the radioactively end-labelled *Sau*3A fragment of pVC3. The two proteins, when individually complexed with the operator fragment, moved to different positions on the gel thus enabling identification of the complexes. At lower concentrations, both proteins altered the migration of the operator fragment to afford a single band in the gel. Above 100 nM Fur protein and 30 nM RNA polymerase, the operator fragment was seen to move to yet other positions, indicating that the protein might extend its binding domain beyond the primary operator sequences of the fragment. This is in agreement with the data of de Lorenzo et al. (1987), who defined the operator sequence by footprinting analysis. The half-saturation concentration of the Fur protein was estimated as less than 5 nM, suggesting the dissociation constant of the Fur-operator complex to be smaller than 2.5×10^{-17} M. In this gel mobility retardation assay, the Fur protein obviously exhibits much higher affinity for the operator fragment when compared to the filter-binding assay (see above). The half-saturation concentration of RNA polymerase was found to be 30 nM. This indicates that the binding affinity of Fur to the fragment is higher than that of RNA polymerase.

After equilibration of Fur and operator fragment, various amounts of RNA polymerase were added to test for replacement. The Fur protein starts to be replaced at 20 nM RNA polymerase (lanes in left half of Fig. 5). It is rather surprising to note that in the presence of 50 nM Fur, RNA polymerase shows half-saturation at 30 nM,

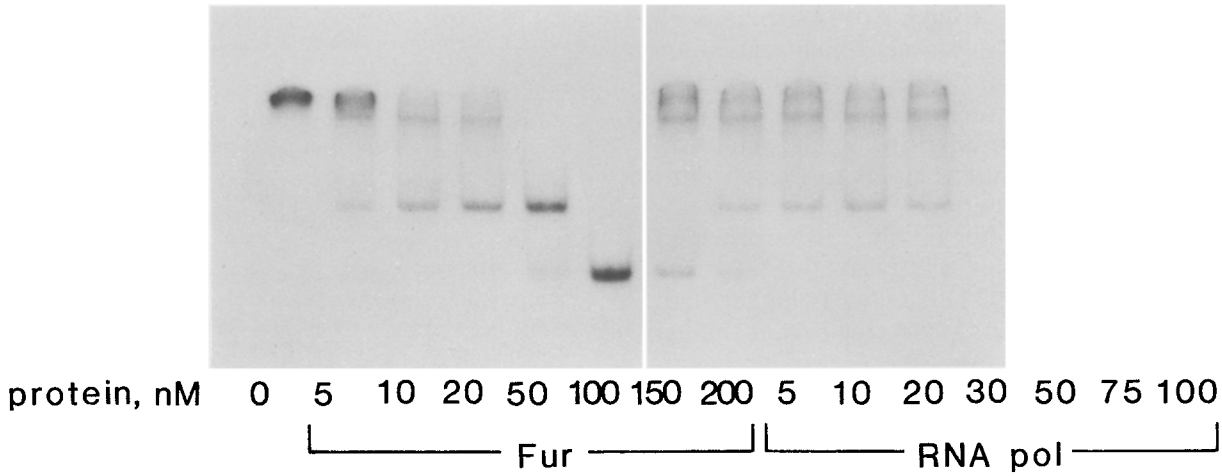

Fig. 4. Gel mobility retardation assay. An approximately 1 pM solution of the 156-bp *Sau*3A fragment of pVC3 end-labelled with [^{32}P]dATP was incubated with the indicated amounts of Fur or RNA polymerase in the presence of 100 μM $MnCl_2$ (see Materials and methods for details). Each protein altered the mobility of the probe DNA to yield a distinct band in the 5.25% polyacrylamide gel

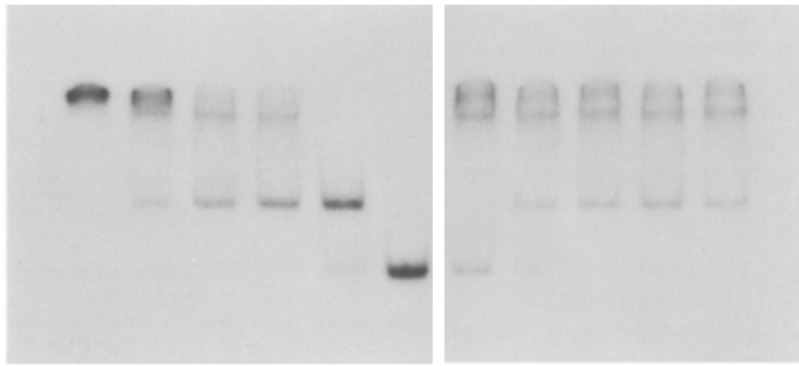

Fig. 5. Interaction of Fur and RNA polymerase with promoter/operator DNA fragment. Assay conditions were the same as in Fig. 4 except that the indicated amounts of RNA polymerase were added to a mixture of operator fragment and 50 nM Fur protein preincubated for 15 min *(left half)*. The *right half* of the figure shows the result of adding the indicated amounts of Fur protein to the mixture preincubated for 15 min with 30 nM RNA polymerase

which is the same as that observed with RNA polymerase alone. Also, if the operator fragments were preincubated with RNA polymerase, Fur protein was not able to replace efficiently the polymerase (lanes in right half of Fig. 5). Apparently, the binding of RNA polymerase to the operator fragment is not affected by the Fur protein. However, under these experimental conditions, Fur affinity for the fragment is decreased in the presence of RNA polymerase. Elimination of $MnCl_2$ and addition of 1 mM EDTA to the assay mixture and to the electrophoresis system resulted in inability of binding of Fur, but not RNA polymerase, to the *Sau*3A promoter fragment (data not shown).

Discussion

In principle, several types of mutations leading to the *fur* phenotype are possible. The one isolated from *E. coli* in this and other laboratories (Hantke 1981) reflects the absence of a 17-kDa protein which functions as a repressor (Bagg and Neilands 1987b). Operator mutants have not yet been reported, possibly because the repressor and RNA polymerase binding sites may be coincident or, alternatively, functional operator sites may be scattered over the DNA upstream of the regulated genes. Unfortunately, the original *fur* mutation in *S. typhimurium* (Ernst et al. 1978) was not mapped and has not yet been cloned.

It is apparent that a vector bearing the *recA* promoter is an effective vehicle for expression of the *fur* gene. Provided that the culture is not grown past an $A_{600\,nm}$ of 1.2, addition of the inducer arrests growth and orders the cells to synthesize the RecA and Fur proteins. The molecular mass differences and other properties of these proteins renders their separation a relatively straightforward procedure.

Fractionation with ammonium sulfate proved ineffective for purification of Fur. Precipitation of the protein began at 10% salt and was not completed at salt concentrations exceeding 60%. As may be seen from the solubility data, the protein exhibits a marked tendency for salting-in.

Fur is a metal-binding protein rather than a metalloprotein and hence it cannot be entirely specific for Fe(II) (Bagg and Neilands 1987b). This, plus the fact that Fur contains 12 histidine residues per molecule, suggested use of the metal-chelating supports developed by Porath et al. (1975). Zinc was chosen as the metal ion coordinated to the column since, unlike Fe(II), it is redox-stable. Other divalent heavy metal ions may be as effective as zinc.

The preparative method herein described has provided specimens of pure protein which have enabled in vitro studies proving the repressor role of Fur (Bagg and Neilands 1987b) and have led to a definition of an 'iron box' consensus sequence, 5′-GATAATGATAATCATTATC, in operators

sensing the Fur-Fe(II) complex (de Lorenzo et al. 1987). The important questions which remain are the affinities of various divalent heavy metal ions for Fur, their stoichiometries and sites of binding to the protein and, finally, the mode of attachment of the ferrous-Fur complex to the operator of iron-regulated genes.

A nitrocellulose filter binding assay provided a dissociation constant of 7×10^{-12} M for the Fur-operator complex. This value is higher than the number 2.5×10^{-17} M obtained from the gel retardation assay by over five orders of magnitude. Binding of Fur may distort the protein in a manner wich diminishes its attraction for the operator. Fur affinity for the nitrocellulose paper may be relatively weak, or perhaps only an operator-multiprotein complex, or aggregate, can bind to the filter. In any case, the nitrocellulose filter-binding assay seems to be a very insensitive method for the measurement of this physical constant. Following completion of this work, a study by Klig et al. (1987) on *trp* repressor-operator interaction appeared in which it was reported that specific binding occurs only at a high salt concentration.

Due to its superior sensitivity, the gel retardation assay system might be adequate for determination of the physical parameters of Fur-operator binding (Hendrickson and Schleif 1984). Interaction of Fur and RNA polymerase with the promoter region of the aerobactin operon confirms the repressor hypothesis as the function for the protein (Bagg and Neilands 1987b). In regard to the intimate mechanism of regulation, the recent work of Straney and Crothers (1987) is of special interest. These workers found that in the *lac* system, the regulation of which shares many features with the aerobactin system, repressor and polymerase can form a ternary complex with the promoter/operator in which contacts to the DNA are different from those made by the proteins alone.

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