Fur-mediated transcriptional and post-transcriptional regulation of FeSOD expression in *Escherichia coli*

Sarah Dubrac and Danièle Touati

INTRODUCTION

Superoxide dismutases (SODs) are ubiquitous enzymes found in nearly all organisms and they play a major role in the multidefence system against oxidative stress (Touati, 1997). They catalyse the dismutation of the superoxide radical (O$_2^-$) to hydrogen peroxide (H$_2$O$_2$) and oxygen (O$_2$). H$_2$O$_2$ is in turn eliminated by catalases and peroxidases (Fridovich, 1997). Two cytoplasmic SODs have been identified in *Escherichia coli*, a manganese SOD (MnSOD) and an iron SOD (FeSOD), encoded by sodA and sodB respectively.

Oxidative stress is potentiated by iron because iron participates, with H$_2$O$_2$, in the Fenton reaction, leading to the formation of the very reactive hydroxyl radical, which may react with any cellular macromolecule (Halliwell & Gutteridge, 1984; Keyer & Imlay, 1996). Iron acquisition and metabolism are therefore strictly regulated and there is increasing evidence for coordination between the regulation of iron homeostasis and defence against oxidative stress (reviewed by Touati, 2000). In particular, synthesis of both the cytoplasmic SODs of *E. coli* (MnSOD and FeSOD) is controlled by the regulator of iron homeostasis, Fur (ferric uptake regulation protein) (Niederhoffer et al., 1990; Tardat & Touati, 1991). In turn the expression of fur is regulated by two global regulator systems of oxidative stress, OxyR and SoxRS (Zheng et al., 1999). Fur, in association with Fe(II), represses the transcription of genes involved in iron uptake (Bagg & Neilands, 1987). More generally, it represses the transcription of genes involved in iron metabolism, general metabolism, stress responses, pathogenicity and various other processes (reviewed by Escolar et al., 1999). The molecular mechanism of the transcriptional repression by Fur is well known. The promoters of Fur-repressed genes contain one or two target DNA sequences called iron boxes, displaying various degrees of similarity to the consensus 5'-'GATAATGATAATCATTATC-3'
sequence (de Lorenzo et al., 1987). This sequence was initially characterized as a 19 bp palindromic sequence (Calderwood & Mekalanos, 1988) and has more recently been described as a combination of three repeats of 5′-NAT(A/T)AT-3′ (Escolar et al., 1998). In conditions of iron repression, Fur associates with Fe(II) and binds as a homodimer to the iron box, rendering the promoter inaccessible to RNA polymerase, thereby inhibiting the initiation of transcription (Escolar et al., 1997). All the genes involved in iron homeostasis are repressed at the transcriptional level by Fur, resulting in a correlation between iron requirements and iron uptake. The sodA promoter contains two iron boxes and sodA transcription has been shown to be repressed by Fur in an iron-dependent manner (Tardat & Touati, 1991, 1993).

A few cases of Fur-mediated activation have been reported. Fur activates the expression of ftn and bfr (iron storage), acnA and yuhA (Krebs cycle) and sodB (FeSOD) (Andrews et al., 1989; Gruer & Guest, 1994; Niederhofer et al., 1990). Positive effects of Fur have also been detected by proteome analysis after acid stress (Hall & Foster, 1996). However, nothing is known about the molecular mechanism underlying these activations. In a previous study on the regulation of sodB expression as a whole, we showed that Fur induced an increase in sodB expression by a factor of seven (Dubrac & Touati, 2000). As demonstrated for Fur-mediated repression, this activation was iron-dependent. The cis-acting elements involved in Fur-mediated activation were located in the 5′ untranslated region (UTR) of the sodB transcript, encompassing a palindromic sequence followed by an AT-rich stretch. The activation appeared to be due, at least partly, to Fur-mediated sodB transcript stabilization. H-NS, a histone-like protein, also regulated sodB expression in a Fur-dependent manner, repressing sodB expression in the absence of Fur.

We investigated further the mechanism of Fur-mediated activation of sodB expression by analysing at the molecular level the transcriptional and post-transcriptional effects of Fur on sodB expression. Fur acted both at transcriptional and post-transcriptional level. These effects appeared to be indirect since purified Fur could not enhance sodB transcription nor bind the sodB transcript in vitro. The previously demonstrated effect of Fur on sodB mRNA stability was found to depend on an endonucleolytic cleavage and to involve RNaseIII and RNaseE. Fur-dependent effect of H-NS interfered with transcription rate and was not additional to the post-transcriptional effect of Fur.

METHODS

Bacterial strains, phages and plasmids. All strains are E. coli K-12 derivatives and are listed in Table 1. Basic genetic manipulations were carried out by standard procedures (Miller, 1992). Δfur::kan, Δfur::cat, bns-1001::Tn5seq1 and Δarc::kan mutations were introduced by P1 transduction, selecting for antibiotic resistance 100% associated with the mutation as described previously (Compan & Touati, 1993). The Δarr::kan mutant was further verified as accumulating 30S RNA precursors (Babitzke et al., 1993). The Φ(sodB-lacZ)₃ and Φ(sodB-lacZ)₂ fusions were described in a previous publication (Dubrac & Touati, 2000).

Specific strain construction. The rne131 allele was introduced by cotransduction with zce726::Tn10 and rne131 mutants were selected from Tet⁺ transductants on the basis of their lack of growth on minimal medium containing 0.4% glycerol as sole carbon source at 44°C (M. Dreyfus, personal communication). The rne1 (ams1) allele was introduced by cotransduction with zce229::Tn10 and the rne1 mutants were selected from Tet⁺ transductants by their inability to grow at 42°C. The ΔtrpC::kan bns-1001::Tn5seq1 rne131 mutant was constructed as follows. The trpB::Tn10 mutation was introduced into a bns-1001::Tn5seq1 mutant by P1 transduction. A P1 lysate on the resulting strain was used to cotransduce the bns mutation and trpB::Tn10 into QC2922 (Δrne::kan). Transductants were selected for tetracycline resistance and screened for bns mutation on the basis of their ability to grow on minimal medium containing 0.5% salicylic acid (Ohta et al., 1999). Tetracycline resistance was eliminated by transduction for trpB¹, selecting for tetracycline prototrophy and bns-1001::Tn5seq1 transductants were selected as described above. The rne131 allele was then introduced by cotransduction with zce::Tn10 and rne131 Tet⁺ transductants were selected as described above.

Media, growth conditions and β-galactosidase assays. Cells were grown in LB medium at 37°C with shaking at 200 r.p.m. Antibiotics were added as required: ampicillin (500 µg ml⁻¹), kanamycin (40 µg ml⁻¹), chloramphenicol (20 µg ml⁻¹) or tetracycline (10 µg ml⁻¹). β-Galactosidase assays were performed as described previously (Compan & Touati, 1993).

In vitro transcription assays. DNA fragments containing the sodBᵢ₄ promoter sequence (597 bp, from -102 to +495), hisL promoter (388 bp, from -285 to +103) and icuA promoter (623 bp, from -133 to +490) were amplified by PCR using oligonucleotides sodB5 (5′-CGGTGTTAGCACCACCAAAGAATTGCCG-3′) and 4218 (5′-GGTCAGGAGTACCCGCGG-3′), biaL5 (5′-CTGACGCCGCTTCCGTATGC-3′) and biaL3 (5′-CCAGCACACATCGCGCTAC-3′), icu2-A (5′-GGCAGCCTATAATACACGCAC-3′) and icu3-A (5′-GTCGGAAAATGTGGCTTC-3′), respectively, from pHS1-4, chromosomal DNA and pDT10, respectively. The amplified products were purified and quantified with 33258 Hoechst reagent as described by Cesaroni et al. (1979). Transcription reactions were performed as described by Escolar et al. (1997), except that the sodB template was used at a concentration of 0.8 nM and 0.4 U RNAP (purchased from Boehringer) was added. Samples were mixed directly with an equal volume of 7 M urea containing tracking dyes and were analysed by 4% polyacrylamide/8% urea gel. The predicted size of the run-off transcription reaction. The lack of any effect of Fur on expression of a hisL-lacZ fusion was demonstrated before beginning these experiments.

DNA gel mobility shift assays. For competition assays, the sodB₁₄ promoter fragment was prepared from pDT10 by digestion with BamHI and HindIII, resulting in a 170 bp fragment containing two iron boxes. This fragment was eluted from the gel, dephosphorylated and end-labelled with ³²P-ATP using kinase (Boehringer Mannheim). Binding assays were performed in a buffer consisting of 10 mM (BisTris)/
Table 1. Bacterial strains and plasmids

<table>
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<th>Strain or plasmid</th>
<th>Genotype</th>
<th>Source or reference</th>
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<tr>
<td>pHT10</td>
<td>pUC19 derivative carrying the iuc region</td>
<td>D. Touati</td>
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**ATCGGCGCTACGCCG-3' was used as probe to the 5S rRNA to normalize the quantity of RNA in each lane.**

**RNA gel mobility shift assays. A radiolabelled DNA fragment was generated by in vitro transcription. To obtain a large amount of transcript, the DNA matrix was fused to the T7 promoter. A 172 bp DNA fragment was generated by PCR using the following oligonucleotides: 5'-TAATACGACTCACTATAGGGGATC-3'.**

**For FeSOD, we used a fragment, from +306 to +495, amplified by PCR, which was used as probe. The oligonucleotide 5'-ACTACCG-**

borate (pH 7), 40 mM KCl, 1 mM MgCl₂, 100 µM MnCl₂, 2 mM DTT, 5% glycerol, 5 µg sonicated salmon-sperm DNA ml⁻¹, 100 µg bovine serum albumin ml⁻¹ (de Lorenzo et al., 1988), 10 nM purified Fur, 1×5×10⁻⁵ pmol end-labelled iuc promoter and the indicated amounts of unlabelled DNA. DNA competitor fragments were added to the binding mixture in a final assay volume of 10 µl. After incubation at 37 °C for 20 min, samples were run on 4% polyacrylamide gels polymerized in 40 mM (BisTris)/borate (pH 7)/100 mM MnCl₂. Blanks with bromophenol blue and xylene cyanol were run in the same gel to visualize the position of the front. Gels were run in the same buffer [40 mM (BisTris)/borate (pH 7)/100 mM MnCl₂] at 200 V.

**RNA isolation. RNA was isolated at various times after the addition of rifampicin (150 µg ml⁻¹) as described by Babst et al. (1996).**

**Northern blotting analysis. RNA (10–20 µg) was subjected to electrophoresis in a 1% agarose gel. Northern blotting and hybridization (at 42 °C in 50% formamide, v/v) were performed essentially as described by Sambrook et al. (1989). An internal sodB fragment, from +306 to +495, amplified by PCR, was used as probe. The oligonucleotide 5'-ACTACCG-**

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containing 10 mM (BisTris)/borate (pH 7), 40 mM KCl, 1 mM MgCl₂, 100 mM MnCl₂ or Fe(NH₄)₂(SO₄)₂, 2 mM DTT and 5% glycerol, according to the conditions for Fur binding established by de Lorenzo et al. (1988). After incubation at 37 °C for 20 min, the samples were analysed as described for DNA gel mobility shift assays (see above).

**Primer extension reactions.** Reverse transcription was carried out at 42 °C with AMV reverse transcriptase, according to the protocol described by Uzan et al. (1988), using a 25-mer oligonucleotide (5'-CGGGATCCGTAGTACGTAGTATCGATGGG-3', 5' end-labelled, complementary to nucleotides +122 to +146 of the sodB gene. DNA sequencing was performed with the same primer by the dideoxy chain termination method with the Sequenase kit version 2.0 (USB) and [α-³²P]dATP (ICN).

**[³H]Uridine labelling of RNA.** Cells were grown to an OD₆₀₀ of about 1 in M9 medium containing 0.2% Casamino acids at 37 °C. [³H]Uridine labelling of RNA, chase and precipitations on filters were performed as described by Mudd et al. (1990). The filters were then dried and the radioactivity retained determined by scintillation counting.

## RESULTS

In a previous report, we showed that the expression of a sodB-lacZ transcriptional fusion was activated in a Fur-dependent manner. This activation was correlated with the Fur-mediated stabilization of the sodB transcript (Dubrac & Touati, 2000). Overall, Fur-mediated activation of sodB expression was significantly stronger (activation factor of seven) than Fur-mediated mRNA stabilization (factor of three to four). Thus RNA stabilization was unlikely to account entirely for the difference in sodB expression between fur and fur⁺ strains. We therefore questioned whether Fur could also interfere with sodB transcription.

**Fur effect on sodB transcription**

In a first assay we investigated whether Fur bound to the sodB promoter region. The capacity of the sodB promoter to compete in vitro with the iuc promoter was tested in gel mobility shift assays. The metal-coordinated Fur bound to the radiolabelled iuc promoter, as shown by de Lorenzo et al. (1988). The shifted Fur–iuc complex disappeared with a 10-times molar excess of unlabelled iuc promoter as competitor, whereas a 500-times molar excess of sodB promoter was required to obtain the same effect (Fig. 1). However, a 500-times molar excess of a non-specific DNA fragment did not act as a competitor. Considering the affinity of the iuc promoter for Fur, i.e. about 5 nM (Wee et al., 1988), we can deduce that the sodB promoter has an affinity of about 250 nM for Fur. Thus, in vitro, Fur bound to the sodB promoter specifically, but with very low affinity.

We investigated the relevance in vivo of this low-affinity binding in vitro using the FURTA test (Stojilkovic et al., 1994). The sodB promoter carried on a high-copy-number plasmid (pUC19 derivative) did not titrate Fur, as shown by the lack of derepression of a fbuF-lacZ fusion (data not shown). This failure of psodB to titrate Fur called into question the biological significance of the observed in vitro binding. However, because of the large number (5000 to 10000) of Fur molecules in the cell (for review see Hantke, 2001) the sensitivity of the FURTA test is not necessarily accurate for a weak promoter. Indeed despite significant Fur titration by the iuc promoter, we observed no significant titration of Fur by the sodA promoter (data not shown), although Fur binding has been clearly demonstrated in vitro (Tardat & Touati, 1993).

It is interesting to note the presence on the target sequence of sodB (Dubrac & Touati, 2000) of an array, ATAT, preceded by a similar degenerate array. Repeats of such arrays have been shown to be involved in interaction with Fur; however, two repeats were not sufficient for stable binding (Escolar et al., 1998).

We further analysed the effect of Fur on the in vitro transcription of sodB. No increase in transcription rate was observed in the presence of Fur (Fig. 2) whereas, under the same assay conditions, Fur fully repressed iuc transcription (data not shown). To normalize the assay, we used as an internal control of in vitro transcription a DNA fragment constitutively expressing a transcript similar in size to that studied; hisL and thrS were used alternately (Fig. 2). Unexpectedly, the relative efficiencies of transcription from psodB and phisL were very different. The efficiency of in vivo transcription from phisL was similar to that from psodB, as shown by expression of a hisL-lacZ transcriptional fusion (data not shown). However, in vitro, a 10-times molar excess of hisL over sodB DNA was required to detect a hisL transcript with about the same intensity (Fig. 2). Increasing the concentration of RNA polymerase by a factor of four had no effect (data not shown). However,
if the hisL DNA was used alone in the assay, transcription efficiency reached the expected value (data not shown). A similar result was obtained with thrS (data not shown). This discrepancy between the relative efficiency of transcription in vivo and in vitro indicates that RNA polymerase has a very strong affinity for the sodB promoter in vitro. This is consistent with there being a factor inhibiting psodB transcription present in vivo but absent in the in vitro assay.

From those results, we concluded that Fur might not directly regulate sodB at the transcriptional level.

**Post-transcriptional Fur-mediated regulation of sodB**

The effect of Fur on sodB mRNA decay may be a general RNA stabilization effect mediated by an indirect effect of Fur on some RNases. We therefore tested whether Fur stabilized the majority of E. coli mRNAs. The half-life of bulk mRNA was about 3 min in the parental fur+ strain and 2.7 min in the fur strain (data not shown). These values are similar to those previously reported for a wild-type strain (2–3 min) (Mudd et al., 1990). Thus, Fur has no effect on the chemical half-life of total mRNA and the effect on the sodB transcript is specific.

**Effect of mutations in major endoribonucleases on the expression of a sodB-lacZ fusion.** The cis elements involved in Fur-mediated protection of mRNA transcript were located in the 5’ UTR of the transcript, excluding a possible initial role of 3’-dependent exonucleases, the only exonucleases known to be present in E. coli. A single mutation in the rnc gene, which encodes RNaseIII, an endoribonuclease that cleaves specific double-stranded RNA structures, had no effect on expression of the fusion or on Fur-dependent activation (Fig. 3a). The rne131 mutation in the gene of RNaseE, a single-strand-specific endoribonuclease, did not significantly affect sodB expression either (Fig. 3b). The rne131 allele encodes a truncated RNaseE lacking the C-terminal part of the protein, preventing the association of RNaseE with the degradosome, but without modifying its rRNA processing activity (Lopez et al., 1999). Similarly, the rne1 mutation, which renders RNaseE catalytic activity heat-sensitive, had no effect (data not shown).

Although neither the rne131 nor the rnc::kan mutations affect expression of the sodB-lacZ fusion, expression was significantly higher (three times) in a fur strain in which both RNase genes were mutated. A slightly higher level of expression was also observed in the fur+ strain (1-2 times) (see Fig. 3c). This suggests that

**Fig. 3.** Effects of various combinations of mutations on expression of a sodB-lacZ transcriptional fusion. Strains were grown in LB medium and assayed for β-galactosidase activity, expressed in units ml⁻¹, as described in Methods. The values shown are means of three experiments and individual values did not differ by more than 15% from the means. □, QC2597 (wild-type); ○, QC2598 (fur mutant); (a) ■, QC2922 (rnc mutant); ●, QC2923 (fur rnc mutant). (b) ■, QC2896 (rne131 mutant); ●, QC2897 (fur rne131 mutant). (c) ■, QC2909 (rne rne131 mutant); ●, QC2911 (fur rnc rne131 mutant).
Different results were obtained with the nne131 strain to prevent this cleavage. Thus both the activity of RNaseIII and the integrity of the carboxy-terminal part of RNaseE must be disrupted for the higher level of sodB transcript inter-fering with sodB expression. However, as stated above, RNaseIII and RNaseE are specific for different sequences or structures and this synergic effect of the double mutation is difficult to explain by classical models.

**Location of an endonucleolytic cleavage involved in the Fur-mediated stabilization of the sodB transcript.** To identify the possible site of endonucleolytic cleavage responsible for the higher level of sodB mRNA decay in the fur strain, we examined the 5’ part of the sodB transcript by primer extension analysis in strains with mutations in genes encoding proteins with various endoribonucleolytic activities. A sodB transcript species shorter than the full-length transcript was identified in the wild-type strain. Cleavage occurred in the palindromic sequence located just downstream from the transcription start site (see Fig. 4). The band corresponding to the cleaved transcript was detected if primer extension was performed on RNA from a wild-type strain or from single nnc and nne131 mutants (Fig. 4a and data not shown), but not if RNA from the nnc nne131 strain was used (Fig. 4a). Cleavage was also detected in a fur strain but the processed transcript was more labile, suggesting greater sensitivity to further degradation (data not shown). Thus both the activity of RNaseIII and the integrity of the carboxy-terminal part of RNaseE must be disrupted to prevent this cleavage.

Different results were obtained with the nne1 mutation of the nme gene which renders the enzyme heat-sensitive. Cleavage was observed in an nnc nne1 strain at non-permissive temperature (Fig. 4b). The expression of the sodB-lacZ fusion in a fur nnc nne1 strain was the same at 30, 42 (Fig. 5b) and 37 °C (not shown), similar to expression in fur (Fig. 5a). This is consistent with cleavage being important for the RNA processing enhanced in a fur background. Noteworthy is the fact that the effect of activation by Fur of sodB expression in the wild-type background was temperature-dependent, being reduced at 30 °C (see Fig. 5a). This is presumably due to a temperature-dependent structural change.

The result obtained with RNA from the nnc nne131 strain was connected with the Fur effect on a sodB-lacZ fusion, Φ(sodB-lacZ), in which the promoter region is partially deleted, the deletion including the cleavage site identified above. The Φ(sodB-lacZ) fusion was more strongly expressed than a fusion involving the sodB wild-type promoter region, Φ(sodB-lacZ). The increase in expression was weak in fur + and significantly higher in fur mutant strains (Dubrac & Touati, 2000). The introduction of nnc nne131 mutations did not affect the expression of the Φ(sodB-lacZ) fusion in either fur + or fur strains (Fig. 5c). Thus deletion of the cleavage site appears to mimic effect of the nnc nne131 mutations.

These results together strongly suggest that cleavage occurs within the palindromic sequence located at the 5’ end of the sodB transcript and that this cleavage is...
correlated with the Fur-dependent activation of sodB expression.

As Fur mediates stabilization of the sodB transcript, we investigated whether Fur protected RNA from degradation by direct binding. sodB RNA was heated and then cooled slowly to allow multiple possible folding. Purified Fur (at concentrations up to 300 nM) did not bind any of the refolded forms of RNA (Fig. 6). The binding assays were performed with manganese as the Fur cofactor because this metal is more stable than iron in its reduced form in aerobiosis. An experiment performed with Fe(II) [Fe(SO₄)]₆(NH₄)₂ as the Fur cofactor gave identical results (not shown).

**H-NS-mediated repression of the sodB expression**

H-NS reduced the level of expression of a sodB-lacZ transcriptional fusion if the fur locus was inactivated (Dubrac & Touati, 2000). To determine the level at which H-NS-mediated regulation occurred, we investigated whether H-NS modulated sodB mRNA degradation. As expected, given that the expression of sodB is H-NS-independent in the fur⁺ strain, similar amounts of sodB transcript were detected in the wild-type and the hns mutant. In an hns fur double mutant, the half-life of the sodB transcript was similar to that in a single fur mutant (data not shown). This result strongly suggests that the observed effect of the hns mutation on sodB-lacZ expression in a fur background was transcriptional.

H-NS is known to bind specifically to curved DNA and thereby modulates transcription (Yamada et al., 1991). A computer analysis was performed to identify possible sections of curved DNA in the sodB promoter region. The bending model was created with the 3D-WEDGE program (G. Micheli, Centro Acidi Nucleici CNR, Rome, Italy). A 1000 bp DNA fragment (−500 to +500 from the translation start site) was tested. The cis elements of the sodB promoter region involved in H-NS-mediated regulation are located in the 60 bp region between the transcription start site and the translation start site (Dubrac & Touati, 2000). Computer analysis showed no potential curvature in the region of the sodB

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**Fig. 5.** Expression of sodB-lacZ transcriptional fusions with and without 5' UTR cleavage of the sodB transcript. Strains were grown in LB medium and assayed for β-galactosidase activity (expressed in units ml⁻¹) as described in Methods. The values shown are means of three experiments and individual values did not differ by more than 15% from the means. (a, b) Effect of rne1 rnc mutations on the expression of sodB-lacZ transcriptional fusion at various temperatures. (a) QC2597 (wild-type) at 30 °C; QC2598 (fur mutant) at 30 °C; QC2597 at 42 °C; QC2598 at 42 °C. (b) QC2975 (rne1 rnc mutant) at 30 °C; QC6014 (rne1 fur mutant) at 30 °C; QC2975 at 42 °C; QC6014 at 42 °C. (c) Measurement of β-galactosidase activity of strains carrying the Φ(sodB-lacZ)₃ fusion. The palindromic region shown in Fig. 4 is truncated in Φ(sodB-lacZ)₃. The sequence 5'-'CAATAAGGCTATTGT-3' is replaced by 5'-'ATCCT-3'. This modification results in the absence of the cleavage site. QC2700 (wild-type); QC2704 (fur); QC2909 (rne131 rnc); QC2911 (fur rne131 rnc).

**Fig. 6.** Effect of Fur on gel mobility shift of the sodB transcript. The sodB transcript was prepared by in vitro transcription using [³H]uridine. It was then heated at 85 °C for 10 min and slowly cooled. About 10000 c.p.m. of radiolabelled mRNA was mixed with purified Fur. Lanes: 1, no Fur; 2, 100 nM Fur; 3, 300 nM Fur.
promoter involved in H-NS repression (data not shown). To confirm this prediction experimentally, we analysed the migration of a PCR-amplified fragment of the sodB promoter containing the region of interest with respect to H-NS repression. Migration in agarose at 4 and 60 °C increased and reduced, respectively, the effect of DNA bending on electrophoretic mobility (Dieckmann & Wang, 1983). We observed no differential migration at the two different temperatures (data not shown). In the light of previous studies showing a very high specificity of H-NS binding for curved DNA, these results strongly suggest that the observed repression of sodB-lacZ expression by H-NS did not result from binding of H-NS to the sodB promoter.

As H-NS repressed expression by a factor of about two, the lower level of expression in the fur strain may have resulted from cumulative effects: loss of RNA stabilization and transcriptional repression via H-NS. Consistent with this, it was predicted that expression of the sodB-lacZ fusion in a strain carrying the fur rne131 rnc hns mutations would reach levels about twice those in the fur rne131 rnc strain, and similar to those in the fur+ strain. No difference was found between the two strains, ruling out a cumulative Fur-dependent effect of RNases and H-NS mutations (data not shown).

This supported the view that the Fur H-NS-mediated effect is indirect and that Fur acts indirectly both at post-transcriptional and transcriptional levels.

DISCUSSION

Fur, which is primarily involved in the regulation of iron metabolism, is now known to regulate a large number of genes. The molecular mechanism of regulation has been studied only for Fur acting as a transcriptional repressor. There are a few reports of positive effects of Fur on gene expression, but the underlying mechanisms were never investigated. It is therefore unknown whether the activation of expression was due to a direct effect of Fur or mediated by an intermediate regulator under Fur control. Our previous study on positive Fur-mediated regulation of the sodB gene encoding FeSOD suggested that regulation occurred at both the post-transcriptional and transcriptional levels. The data presented here show only a weak binding of Fur to the sodB promoter region and no binding to the sodB transcript. Although these data do not authorize us to exclude the possibility that Fur activation of sodB expression is mediated via a direct binding of Fur on the sodB promoter, we favour the hypothesis of indirect regulation. (i) The target region of the Fur effect is located downstream from the transcription start site of sodB, a position more frequently subjected to negative than to positive transcriptional control. (ii) Although DNA-binding assays indicated some specific binding of Fur to the promoter region of sodB, binding efficiency was very low and possibly not significant in vitro. (iii) The inability of purified Fur to activate in vitro transcription and the much higher level of in vitro transcription from psodB than from phisL, despite similar levels of in vivo expression, provide further evidence of an indirect effect and suggest that an inhibitory factor may be missing in the in vitro experiment. (iv) No binding of Fur to RNA was detected, ruling out the hypothesis that Fur acts as an RNA-binding protein. This led us to speculate that an intermediate regulator under Fur control might exist. As the basic level of sodB expression in the fur mutant is high enough, classical methods of selecting mutants for this putative regulator were not possible. An approach involving transcriptome analysis is currently being used.

One clear consequence of Fur regulation was stabilization of the sodB transcript. The higher level of sodB expression in a fur strain in which both major RNase genes were mutated, together with the lack of endonucleolytic cleavage of the sodB transcript in an rne131 rnc strain, suggested that both RNaseE and RNaseIII were involved in sodB mRNA decay. However, the process of RNA degradation is still unclear. In particular, it is not easy to account for the apparent redundancy between the actions of RNaseIII and RNaseE on the sodB transcript. Cleavage within the palindromic sequence at the 5' end of the transcript occurs if one of these RNases is present. There is no known example of cleavage at the same site by these two RNases and it has been clearly demonstrated that whereas RNaseIII is double-strand-specific, RNaseE can cleave in single-stranded AU-rich RNA in a 5'-dependent manner (Mackie, 1998; Nicholson, 1999). This suggests that the sodB transcript is degraded in several steps, including, at the late stage, events dependent on the actions of RNaseIII and RNaseE. Cleavage within the palindromic sequence may be a crucial initial event. RNaseE is an endonucleolytic enzyme involved in mRNA decay and rRNA maturation. It is the central element of the degradosome, a multi-enzyme complex involved in the decay of most mRNA species. The effect of RNaseE was found only in experiments carried out with the rne131 allele, which was originally characterized in the laboratory of M. Dreyfus (Lopez et al., 1999). This mutant produces an RNaseE truncated at the carboxy-terminal end, resulting in inhibition of the formation of the degradosome without the abolition of catalytic activity. Thus, the rne131 mutation permits the further processing of rRNA and strains carrying this allele are viable, but impaired in mRNA decay. In contrast, the rne1 mutation does not seem to inhibit degradosome formation, but at non-permissive temperature, the catalytic activity of RNaseE is abolished (Coburn et al., 1999). The endonucleolytic cleavage of sodB mRNA was observed and no increase of sodB-lacZ expression was found in an rnc rne1 double mutant at non-permissive temperature. This suggests that, rather than the catalytic activity of RNaseE, the interaction between other major components of the degradosome is important.

Fur-dependent RNA stabilization did not fully account for the activation of sodB expression, suggesting that an additional effect, at the transcriptional level, may be mediated by Fur. A Fur-dependent transcriptional effect mediated by H-NS was effectively observed. (i) The
effect of H-NS is not post-transcriptional because bns mutation in the fur strain did not increase sodB mRNA decay, whereas sodB-lacZ expression was increased (two to three times). (ii) The effect of H-NS is probably indirect because examination of the sodB promoter region revealed no putative curved DNA region, specific for recognition by H-NS. (iii) There was no cumulative effect of H-NS and RNA stabilization by inactivation of the major RNases (RNaseIII and RNaseE). This suggests a common Fur-regulated pathway leading to transcriptional and post-transcriptional effects. The numerous bands observed upon the gradual cooling of the psodB RNA region suggested that this RNA may take up numerous conformations. It is possible that all Fur-dependent effects, transcriptional and post-transcriptional, may be mediated by a single intermediate regulator. We speculate that the expression of the gene encoding the intermediate regulatory protein ‘X’ is probably negatively regulated by Fur and positively by H-NS. Only if Fur repression was relieved, could H-NS activate transcription. H-NS is known to be a transcriptional repressor, but two-dimensional protein gel analysis has shown that the expression of many genes is activated by H-NS, although the underlying mechanism is unclear. It has also been demonstrated that H-NS-mediated repression can be eliminated by inducing target-gene expression (reviewed by Atlung & Ingmer, 1997). These findings are consistent with our hypothesis: an ‘X’ target gene may be repressed by Fur and activated by H-NS if Fur repression is relieved.

Few proteins have been shown to bind both DNA and RNA (Shamoo et al., 1993). Such binding may account for the transcriptional and post-transcriptional effects of the putative ‘X’ protein. We favour a model in which the ‘X’ protein binds to DNA in the region between the transcription and translation start sites, slowing down the progression of RNA polymerase, generating two effects: reduction of the efficiency of sodB transcription and formation of RNA structures more accessible to endonucleolytic cleavage. Experiments are under way to determine whether such a model of coupling between DNA transcription and RNA stabilization, consistent with several of the observations reported in this work, operates in reality.

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