Contributions of [4Fe-4S]-FNR and Integration Host Factor to *fnr* Transcriptional Regulation[▽]

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Maintaining appropriate levels of the global regulator FNR is critical to its function as an O_2 sensor. In this study, we examined the mechanisms that control transcription of *fnr* to increase our understanding of how FNR protein levels are regulated. Under anaerobic conditions, one mechanism that controls *fnr* expression is negative autoregulation by the active [4Fe-4S] form of FNR. Through DNase I footprinting and in vitro transcription experiments, we observed that direct binding of [4Fe-4S]-FNR to the predicted downstream FNR binding site is sufficient for repression of the *fnr* promoter in vitro. In addition, the downstream FNR binding site was required for repression of transcription from *fnr'-lacZ* fusions in vivo. No repression of *fnr* was observed in vivo or in vitro with the apoprotein form of FNR, indicating that repression requires the dimeric, Fe-S cluster-containing protein. Furthermore, our in vitro and in vivo data suggest that [4Fe-4S]-FNR does not bind to the predicted upstream FNR binding site within the *fnr* promoter. Rather, we provide evidence that integration host factor binds to this upstream region and increases in vivo expression of P*fnr* under both aerobic and anaerobic conditions.

The ability of *Escherichia coli* to efficiently sense and respond to O_2 is primarily controlled by the global regulatory protein FNR (22, 42, 58). FNR is selectively active as a transcription factor under anaerobic growth conditions, where it has been shown to control the transcription of hundreds of genes, many of which are necessary for adaptation to O_2-limiting growth conditions (10, 16, 25, 46). The large number of genes whose expression is regulated by changes in O_2 and the dramatic reprogramming of metabolic pathways have made the study of FNR and its regulon ideal for a system level approach. The primary mechanism of regulation is the direct inactivation of FNR via the O_2-dependent destruction of its [4Fe-4S] cluster, which is required for its activity (20, 28, 33). Recent studies indicate that this inactivation mechanism is optimized for normal cellular levels of FNR protein (2,600 to 4,100 molecules per cell) (56) since excess FNR protein (even twofold) escapes O_2 inactivation (4, 37, 38, 53). Defining the mechanisms that control FNR protein levels is important in understanding the global response to O_2.

Both transcriptional and posttranscriptional control of FNR protein levels has been observed, providing a foundation for understanding how FNR levels are regulated. Under aerobic growth conditions, proteolysis decreases FNR protein levels (12, 38), while under anaerobic conditions, FNR represses its own transcription (24, 41, 44, 47, 54). While the mechanism that regulates FNR proteolysis has been elucidated (12, 38), a detailed analysis of *fnr* transcription has not been carried out. In vivo studies using either *fnr::lacZ* transcriptional or translational fusions demonstrated that the *fnr* promoter was repressed approximately two- to threefold in an FNR-dependent

manner under anaerobic conditions (24, 41, 44, 47, 54). Surprisingly, repression of *fnr::lacZ* was shown to be further enhanced when *fnr* was expressed from a multicopy plasmid (44, 54), suggesting that repression is limited by FNR levels under anaerobic conditions. Two sequence elements that showed similarity to the FNR consensus binding site (TTGAT-N_4-ATCAA) (22) were identified within the *fnr* promoter region (Fig. 1). The predicted upstream FNR binding site (<u>TTA</u>A<u>G</u>-N_4-<u>TTCAA</u>) is centered at bp −103.5 relative to the transcription start site, whereas the predicted downstream FNR binding site (<u>TTGAC</u>-N_4-<u>ATCAA</u>; underlined nucleotide match the consensus) is centered at bp −0.5 and overlaps the transcription start site (22). While binding of reconstituted [4Fe-4S]-FNR to the predicted downstream site has been reported in vitro (21), the contribution of this site or the predicted upstream site to P*fnr* repression was not examined.

In this study, we determined the roles of the two predicted FNR binding sites in the regulation of *fnr* transcription using both in vitro and in vivo approaches. DNase I footprinting and in vitro transcription experiments were used to determine whether direct binding of [4Fe-4S]-FNR to either the upstream or the downstream site was sufficient for P*fnr* repression. β-Galactosidase activities from wild-type or mutant *fnr::lacZ* fusions in which base substitutions were made within the upstream or downstream binding sites were monitored in both anaerobically and aerobically grown strains. In addition, the involvement of other transcription factors, in addition to FNR, in the regulation of *fnr* transcription was investigated.

MATERIALS AND METHODS

FNR protein purification. Isolation of [4Fe-4S]-FNR was carried out as described previously (38, 55), using a Pharmacia fast-performance liquid chromatography system equipped with a BioRex-70 cation-exchange column (Bio-Rad Laboratories) in a Coy anaerobic chamber with an atmosphere of 80% N_2, 10% CO_2, and 10% H_2. To further enrich for the dimeric, cluster-containing form of FNR, the [4Fe-4S]-FNR preparation was subject to size exclusion chromatogra-

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5′-AAACAATTTGTGCCAGCTTGTTCACACTTTTA

TGTAAAGTTACCCTTAACAACTTAAGGGTTTTCAA
                                upstream site
  -117

ATAGATAGACATATATTTCACATCTAATATCGGAAT

IHF   -89

TCTCTGCTGTTAAGGTTTGCTTAGACTTACTTGCT
                                    -35

CCCTAAAAAGATGTTAAAATTGACAAATATCAATT
extended -10   -10   downstream site
              -14

ACGGCTTGAGCAGACCTATGATCCCGGAAAA-3′
                    fnr
  +11   start
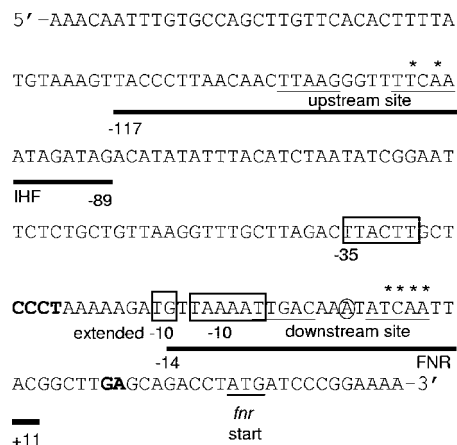
FIG. 1. *fnr* promoter region. Shown are nucleotides −163 to +40 relative to the transcription start site (circled). The predicted upstream and downstream FNR binding sites are underlined, and the asterisks indicate the bases in both of the sites that were mutated in this study. The *fnr* start codon is also underlined, and the −35, −10, and extended −10 promoter elements are boxed. The bold horizontal lines mark the areas of protection by FNR or IHF from DNase I cleavage as determined in this study. Positions of enhanced DNase I cleavage are in bold.

phy under anaerobic conditions as previously described (40) and the pooled dimeric FNR protein fraction was analyzed for protein, iron, and sulfide content (2, 26, 27). The isolated [4Fe-4S]-FNR was ~100% occupied with [4Fe-4S] clusters, calculated on the basis of the sulfide content (27). Apo-FNR was purified as described previously (38), using a Pharmacia fast-performance liquid chromatography system equipped with a 5-ml Hi-Trap heparin column (Amersham), followed by concentration with a 1-ml Hi-Trap heparin column.

DNase I footprinting. DNA fragments containing the *fnr* promoter region were isolated from plasmid pPK7665 (bp −155 to +25 relative to the transcription start site) or pPK8221 (bp −214 to +25) (Table 1) with either HindIII and BamHI or EcoRI and BamHI. A Klenow fragment (New England Biolabs) was used to 3′ radiolabel the HindIII or EcoRI end of the DNA fragment with [α-^{32}P]dATP (~3,000 Ci mmol^{-1}, i.e., ~110 TBq mmol^{-1}) (GE Healthcare). Labeled DNA fragments were isolated from a nondenaturing 5% acrylamide gel and were subsequently purified with elutip-d columns (Schleicher and Schuell). DNase I footprinting was carried out in a Coy anaerobic chamber in a total volume of 20 μl by incubating 6 nM DNA and either isolated [4Fe-4S]-FNR (100 to 400 nM), apo-FNR (200 to 400 nM), integration host factor (IHF) (250 to 750 nM), or cyclic AMP receptor protein (CRP) (0.5 to 4 μM) proteins for 30 min at 37°C in 40 mM Tris (pH 7.9), 70 mM KCl, 100 μg ml^{-1} bovine serum albumin, and 1 mM dithiothreitol. Cyclic AMP (cAMP) was added to a final concentration of 0.2 mM where indicated. DNase I (2 μg ml^{-1}) and MgCl$_2$ (10 mM) were added, and after 30 s, the reaction was terminated by the addition of 300 mM sodium acetate and 20 mM EDTA. The reaction mixtures were then ethanol precipitated, resuspended in loading dye (8 M urea, 0.5× TBE [Tris-borate-EDTA], 0.05% bromophenol blue, 0.05% xylene cyanol), heated for 30 s at 90°C, and loaded onto a 7 M urea–8% polyacrylamide gel in 0.5× TBE buffer. A+G sequencing ladders were generated as previously described (35). The reaction products were visualized by phosphorimaging and ImageQuant software.

In vitro transcription assays. The *fnr* promoter regions (bp −155 to +25 or −214 to +25 relative to the transcription start site) were PCR amplified using pRZ7411 (Table 1) (32) as a template and primers containing XhoI and BamHI sites, digested with XhoI and BamHI, and cloned into pPK7179, a pUC19-*spf′* derivative containing an XhoI site (25). The resulting plasmids (pPK7665 or pPK8221) were purified with a QIAfilter Maxi kit (QIAGEN). Assays were carried out in a Coy anaerobic chamber using isolated [4Fe-4S]-FNR, IHF, and CRP proteins. Supercoiled plasmid DNA (2 nM) (pPK7179 or pPK8221) was incubated with the indicated protein(s), 5 μCi (185,000 Bq) of [α-^{32}P]UTP (~3,000 Ci/mmol, i.e., ~110 TBq mmol^{-1}), unlabeled UTP (50 μM), and 500 μM final concentrations each of ATP, CTP, and GTP (GE Healthcare) for 30 min at 37°C in 40 mM Tris (pH 7.9), 70 mM KCl, 100 μg ml^{-1} bovine serum albumin, 1 mM dithiothreitol, and 10 mM MgCl$_2$. cAMP (0.2 mM) was present where indicated. Eσ^{70} RNA polymerase (50 nM) (Epicentre) was added, and

each reaction (in a 20-μl total volume) was terminated after 5 min by adding 10 μl of 95% (vol/vol) formamide, 20 mM EDTA, 0.05% (wt/vol) bromophenol blue, and 0.05% (wt/vol) xylene cyanol FF (USB Corporation). After the mixture was heated to 90°C for 30 seconds, 5 μl was loaded onto an 8% polyacrylamide–7 M urea gel (0.5× TBE). Upon exposure to a PhosphorImager screen, transcripts were quantified using Molecular Dynamics ImageQuant software and *fnr* transcription was normalized to the amount of RNA-1 transcript (13). Each assay was repeated at least three times.

Construction of strains and plasmids. The construction of *fnr* promoter-*lacZ* fusions involved two steps. First, pPK7035 (Table 1) (25) plasmid derivatives containing base substitutions within the downstream FNR binding site in the *fnr* promoter region were made via site-directed mutagenesis of pRZ7411 to create pPK6979 (Table 1). DNA fragments containing the wild-type or downstream mutant *fnr* promoter region (bp −155 to +25 or −214 to +25 relative to the transcription start site) were PCR amplified using pRZ7411 or pPK6979 as a template and primers containing XhoI and BamHI sites, digested with XhoI and BamHI, and cloned into pPK7035 to create pPK6978 (bp −115 to +25 of P*fnr*), pPK6981 (bp −155 to +25 of downstream mutant P*fnr*), or pPK8278 (bp −214 to +25 of P*fnr*) (Table 1). Base substitutions within the upstream FNR binding site were made via site-directed mutagenesis of pPK6978 and pPK6981 to create pPK6980 (bp −155 to +25 of upstream mutant P*fnr*) and pPK7000 (bp −155 to +25 of downstream and upstream mutant P*fnr*), respectively. Base substitutions within the predicted CRP binding site were made via site-directed mutagenesis of pPK8278 to create pPK8429 (bp −214 to +25 of P*fnr* with a mutation in the CRP binding site).

The second step involved PCR amplification of the *lacI*-Kn promoter-*lacZ* fragment from the pPK7035 plasmid derivatives and recombination into the chromosome as previously described (14). Kn promoter-*lacZ* fusions were introduced into MG1655 and its FNR$^-$ derivative, PK4811, via P1 transduction and selection for kanamycin resistance. Transduction with P1 was also used to introduce *himA::tet*, *crp::cat*, and *arcA::cat* from strains DPB102, DM0068, and PK7510 (Table 1), respectively, into strains containing wild-type or mutant *fnr* promoter-*lacZ* fusions.

β-Galactosidase assays. β-Galactosidase activity was measured in strains containing wild-type or mutant *fnr* promoter-*lacZ* fusions as described previously (39). Cells were grown aerobically or anaerobically to an optical density at 600 nm of ~0.2 in either M9 minimal medium with 0.2% (wt/vol) glucose (or 0.2% [wt/vol] fructose where indicated), 10 μM ferric ammonium citrate, and 0.2 μM ammonium molybdate or LB as previously described (55). Casamino Acids or chloramphenicol were added to the medium where indicated. To terminate cell growth and any further protein synthesis, either chloramphenicol (final concentration, 20 μg ml^{-1}) or tetracycline (final concentration, 10 μg ml^{-1}) was added and cells were placed on ice until assayed for β-galactosidase activity (39). β-Galactosidase assays were repeated at least three times. β-Galactosidase activity was normalized to account for the difference in cell numbers per ml of culture for aerobically and anaerobically grown cells as determined via viable plating assays (56). At an optical density at 600 nm of 0.4, aerobic and anaerobic cultures contained (2.6 ± 0.2) × 10^8 and (4 ± 0.3) × 10^8 cells ml^{-1}, respectively. Therefore, β-galactosidase activity was normalized by multiplying the aerobic values by a factor of 1.5.

RESULTS

In vivo negative autoregulation requires the presence of [4Fe-4S]-FNR. To determine if negative autoregulation requires the [4Fe-4S] form of FNR, expression of P*fnr′-lacZ* (bp −155 to +25 of P*fnr* relative to the transcription start site) was monitored in anaerobically grown strains expressing either wild-type FNR or FNR mutants (FNR-CA23 and FNR-CA122) that were previously shown to not contain [4Fe-4S] clusters (29, 32, 34, 37, 50, 53). Consistent with previous studies (24, 41, 44, 47, 54), expression from P*fnr* was repressed approximately twofold when the chromosomal copy of *fnr* was present (Fig. 2). In addition, P*fnr* expression was further decreased approximately fourfold in the presence of plasmid-derived wild-type FNR. In contrast, repression of P*fnr* was approximately five- to sixfold less efficient in strains expressing the FNR-CA23 and FNR-CA122 mutants than P*fnr* repression by plasmid-derived wild-type FNR. These data support the

TABLE 1. *E. coli* strains and plasmids used in this work

| Construct	Relevant genotype	Reference or source
Strains		
PK22	BL21 (DE153) Δ*crp*-bs990 *rpsL* Δ*fnr*ΩSp^r/Sm^r *zcj-3061*::Tn*10*	32
BW25993	*lacI*^{q} Δ*lacZ*$_{WJ16}$ *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	11
DBP102	*himA452*::mini-*tet* Δ(*lac pro*) *rpsL*	3
DM0068	*crp*::*cat*	30
BW25113	*lacI*^{q} *hsdR514* Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	11
PK7510	BW25113 *arcA*::*cat*	This laboratory
MG1655	F$^-$ λ$^-$ *rph*-1	This laboratory
PK4811	MG1655 but Δ*fnr*ΩSp^r Sm^r	This laboratory
PK6988	MG1655 (−155 to +25) P*fnr'*-*lacZ*	This study
PK6997	PK4811 (−155 to +25) P*fnr'*-*lacZ*	This study
PK6990	Same as PK6988 but bp +4 to +7 of P*fnr* changed to 5'-CTGG-3'	This study
PK6999	Same as PK6997 but bp +4 to +7 of P*fnr* changed to 5'-CTGG-3'	This study
PK7608	Same as PK6988 but *arcA*::*cat*	This study
PK7609	Same as PK6997 but *arcA*::*cat*	This study
PK7610	Same as PK6988 but *himA*::*tet*	This study
PK7611	Same as PK6997 but *himA*::*tet*	This study
PK8208	Same as PK6988 but *crp*::*cat*	This study
PK8209	Same as PK6997 but *crp*::*cat*	This study
PK6989	Same as PK6988 but bp −100 to −98 of P*fnr* changed to 5'-CCG-3'	This study
PK6998	Same as PK6997 but bp −100 to −98 of P*fnr* changed to 5'-CCG-3'	This study
PK7602	Same PK6989 but bp +4 to +7 of P*fnr* changed to 5'-CTGG-3'	This study
PK7603	Same as PK6998 but bp +4 to +7 of P*fnr* changed to 5'-CTGG-3'	This study
PK7616	Same as PK6989 but *himA*::*tet*	This study
PK7617	Same as PK6998 but *himA*::*tet*	This study
PK8293	MG1655 (bp −214 to +25) P*fnr'*-*lacZ*	This study
PK8294	PK4811 (−214 to +25) P*fnr'*-*lacZ*	This study
PK8295	Same as PK8293 but *crp*::*cat*	This study
PK8296	Same as PK8294 but *crp*::*cat*	This study
PK8434	Same as PK8293 but bp −142 to −138 of P*fnr* changed to 5'-CTGAG-3'	This study
PK8435	Same as PK8294 but bp −142 to −138 of P*fnr* changed to 5'-CTGAG-3'	This study
PK7667	PK6997 but with pRZ7411	This study
PK7668	PK6997 but with pACYC184	This study
PK7669	PK6997 but with pPK852	This study
PK7670	PK6997 but with pPK436	This study
PK7671	PK6997 but with pPK853	This study
PK7672	PK6997 but with pPK438	This study
PK7673	PK6997 but with pPK6928	This study
PK7674	PK6997 but with pPK6929	This study
RZ7416	λ (bp −481 to +55) P*fnr'*-*lacZ*	This laboratory
RZ7426	Same as RZ7416 but Δ*fnr*ΩSp^r Sm^r	This laboratory
PK8242	Same as RZ7416 but *arcA*::*cat*	This study
PK8243	Same as RZ7426 but *arcA*::*cat*	This study
Plasmids		
pPK823	Ap^r; bp +1 to +1115 of *fnr* in NdeI and BamHI sites of pET-11a	32
pPK7179	Ap^r; pUC19-*spf'* with XhoI site replacing SalI site	25
pPK7665	bp −155 to +25 of P*fnr* in XhoI and BamHI sites of pPK7179	This study
pPK8221	bp −214 to +25 of P*fnr* in XhoI and BamHI sites of pPK7179	This study
pACYC184	Cm^r	8
pRZ7411	Cm^r; HindIII-BamHI of *fnr*; bp −521 to +1115 of *fnr* in pACYC184	32
pPK6979	Same as pRZ7411 but bp +4 to +7 of P*fnr* changed to 5'-CTGG-3'	This study
pPK7035	Kn^r gene from pHP45Ω and BamHI-NdeI fragment from pRS1553 into pBR322	25
pPK6978	bp −155 to +25 of P*fnr* in XhoI and BamHI sites of pPK7035	This study
pPK6980	Same as pPK6978 but bp −100 to −98 of P*fnr* changed to 5'-CCG-3'	This study
pPK6981	Same as pPK6978 but bp +4 to +7 of P*fnr* changed to 5'-CTGG-3'	This study
pPK7000	Same as pPK6981 but bp −100 to −98 of P*fnr* changed to 5'-CCG-3'	This study
pPK8429	Same as pPK8278 but bp −142 to −138 of P*fnr* changed to 5'-CTGAG-3'	This study
pKD46	Phage λ *gam-bet-exo* genes under P*araB* control	B. Wanner
pPK852	Same as pRZ7411 but *fnr*-CS20	This laboratory
pPK436	Same as pRZ7411 but *fnr*-CS23	32
pPK853	Same as pRZ7411 but *fnr*-CS29	This laboratory
pPK438	Same as pRZ7411 but *fnr*-CS122	32
pPK6928	Same as pRZ7411 but *fnr*-CA23	38
pPK6929	Same as pRZ7411 but *fnr*-CA122	38
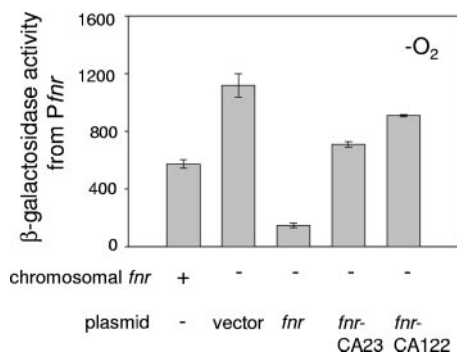
FIG. 2. Effect of plasmid-derived FNR protein levels on *fnr′-lacZ* expression. Δ*fnr* strains expressing wild-type FNR or FNR mutants from pACYC184 were grown in M9 minimal glucose medium containing a final concentration of 20 μg ml⁻¹ of chloramphenicol under anaerobic growth conditions. β-Galactosidase activity from *fnr ′-lacZ* (bp −155 to +25) is also shown for the wild-type strain, containing *fnr* present in a single copy. FNR-CA23 and FNR-CA122 are mutant proteins that do not ligate [4Fe-4S] clusters (29, 32, 34, 37, 50, 53). Error bars represent the standard errors for three independent experiments.
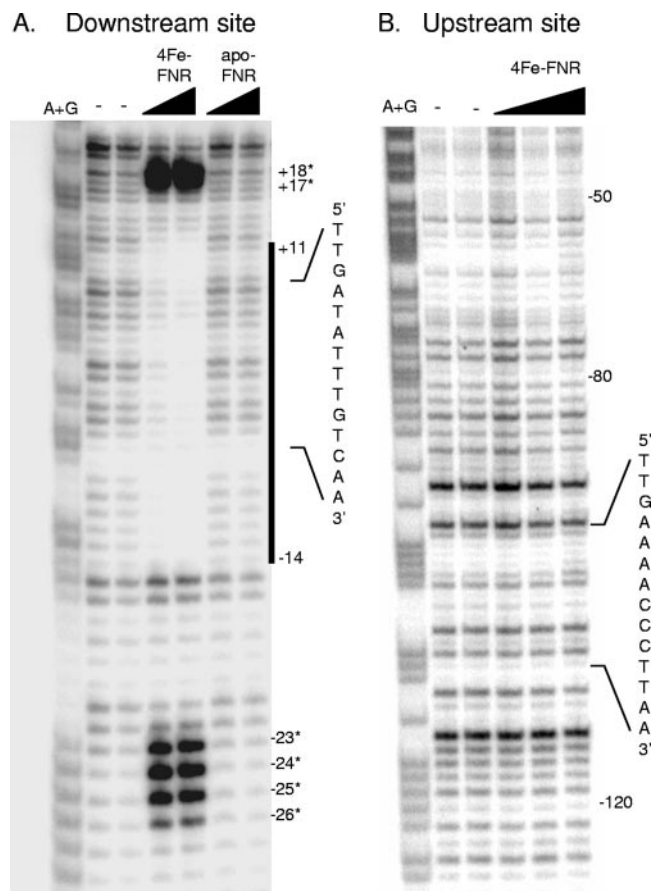

FIG. 3. Footprinting of FNR at the predicted downstream (A) and upstream (B) FNR binding sites within the *fnr* promoter region. The DNA sequences for the predicted FNR binding sites are indicated at the right of each panel. Numbers indicate the distances relative to the transcription start site. Samples were electrophoresed with Maxam-Gilbert (A+G) ladders made using the same DNA. (A) DNase I cleavage of the EcoRI-BamHI DNA fragment from pPK7665 (Table 1), in which the 3′ EcoRI end was radiolabeled. FNR protein concentrations are given from left to right in terms of nM total protein: [4Fe-4S]-FNR (4Fe-FNR), 200 and 400; apo-FNR, 200 and 400. The area of protection from DNase I by [4Fe-4S]-FNR is indicated with a vertical line. Asterisks indicate the positions of enhanced DNase I cleavage. (B) DNase I cleavage of the HindIII-BamHI DNA fragment from pPK7665, in which the 3′ HindIII end was radiolabeled. [4Fe-4S]-FNR (4Fe-FNR) concentrations are given from left to right in terms of nM total protein: 100, 200, and 400.

notion that negative autoregulation in vivo requires the presence of active [4Fe-4S]-FNR.

FNR directly binds to the predicted downstream site but not the upstream site in vitro. To address whether FNR directly binds to both of the predicted FNR binding sites within the *fnr* promoter region, in vitro DNase I footprinting experiments with [4Fe-4S]-FNR were performed. At the proposed downstream FNR binding site, a clear pattern of protection was observed from bp −14 to +11 relative to the *fnr* transcription start site by [4Fe-4S]-FNR (200 to 400 nM) (Fig. 3A). In addition, strong enhancements of DNase I cleavage were present at positions −26 through −23 and at positions +17 and +18. The presence of the [4Fe-4S] cluster was required for FNR to bind to the downstream site, since no enhancements or regions of protection were detected when equivalent amounts of apo-FNR were used in the assay (Fig. 3A). In contrast, no enhancements or regions of protection were detected for the predicted upstream FNR binding site by either [4Fe-4S]-FNR (Fig. 3B) or apo-FNR (data not shown). These data indicate that [4Fe-4S]-FNR binds only to the downstream site under the in vitro solution conditions used in this study.

To test whether binding of this downstream site is sufficient for repression of P*fnr*, in vitro transcription assays were carried out with purified RNA polymerase and a plasmid template containing bp −155 to +25 of the *fnr* promoter region. In the absence of FNR protein, distinct transcripts that initiated from the *fnr* promoter (expected sizes of 135 and 134 nucleotides) and the control RNA-1 promoter (13) were detected (Fig. 4). In the presence of increasing amounts of [4Fe-4S]-FNR protein (0 to 500 nM), the amount of *fnr* transcript decreased in a concentration-dependent manner, whereas no effect on the amount of RNA-1 transcript was observed. Since the in vitro transcription assays were carried out under reaction conditions similar to those for the DNase I footprinting experiments, these data suggest that binding of [4Fe-4S]-FNR to the downstream site is sufficient to repress P*fnr* transcription in vitro.

Repression of P*fnr* transcription in vivo also requires the downstream FNR binding site but not the upstream binding site. To determine if either of these sequence elements plays a role in regulating transcription of P*fnr* in vivo, expression from wild-type or mutant *fnr* promoters (bp −155 to +25 relative to the transcription start site) fused to *lacZ* was measured (Fig. 5). Analysis of mutations within the upstream (−110TTAAG-N₄-TCCGA−97) or downstream (−7TTGAC-N₄-ACTGG+7) (base substitutions are underlined) site showed that only the downstream site was required for repression of P*fnr*, in agreement with the in vitro results. Surprisingly, the mutation in the upstream site caused an approximately twofold decrease in expression from the mutant *fnr* promoter in both FNR⁺ and FNR⁻ strains. Furthermore, the mutations in the
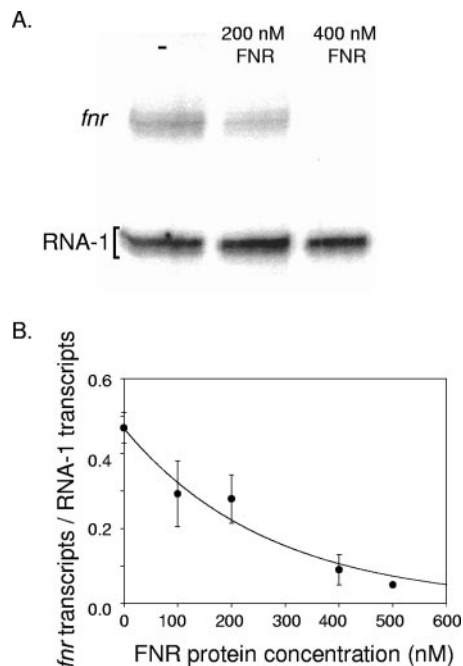
FIG. 4. Effect of [4Fe-4S]-FNR on P*fnr* transcription in vitro. (A) Assay mixtures contained 2 nM plasmid DNA containing bp −155 to +25 of the *fnr* promoter region relative to the transcription start site, 50 nM Eσ[70] RNA polymerase, and, where indicated, 200 nM or 400 nM [4Fe-4S]-FNR protein. (B) Quantified data showing the amounts of in vitro transcription from P*fnr* in the presence of increasing concentrations of [4Fe-4S]-FNR. P*fnr* transcription was normalized by dividing the amount of the *fnr* transcript by the amount of the RNA-1 control transcript. Error bars represent the standard errors for three independent experiments.

FIG. 5. In vivo expression from wild-type or mutant *fnr'-lacZ* (−155 to +25) promoter fusions from anaerobically (A) or aerobically (B) grown cells measured using β-galactosidase assays. FNR[+] and FNR[−] strains are represented by the white and gray bars, respectively. "−" indicates that base substitutions (Fig. 1) were made within the predicted upstream FNR binding site, the predicted downstream FNR binding site, or both sites. "+" indicates that the wild-type sequence is present. β-Galactosidase activity from the *fnr'-lacZ* promoter fusions was normalized by correcting for the difference in cell numbers ml^{−1} of culture for aerobically and anaerobically grown cells as explained in Materials and Methods. Error bars represent the standard errors for three independent experiments.

downstream and upstream sites appear to act independently, since substitutions in both of the sites resulted in both a loss of negative autoregulation and an overall approximately twofold decrease in *fnr* expression. Collectively, these results indicate that only the downstream FNR binding site is required for negative autoregulation, while the upstream site may play a role in transcription activation of P*fnr* by an FNR-independent mechanism.

Activation of P*fnr* occurs under aerobic and anaerobic growth conditions. The effect of the mutation of the upstream DNA element indicated that an unknown factor may function to weakly activate *fnr* expression under anaerobic conditions. To determine whether disruption of the upstream element also affected P*fnr* expression under aerobic growth conditions, β-galactosidase activity from the wild-type and mutant *fnr* promoters was measured in aerobically grown cells (Fig. 5B). As expected (24, 41, 44, 47, 54), no repression of P*fnr* by FNR was observed under aerobic growth conditions. However, mutation of the upstream element caused the same approximately twofold decrease in expression in aerobically grown cells as that observed under anaerobic growth conditions. The possibility that mutation of this upstream sequence decreased the function of a second *fnr* promoter was eliminated since in vivo mapping of transcription start sites within the wild-type *fnr* promoter region indicates that there is a single transcription start site similar to that observed in vitro (data not shown).
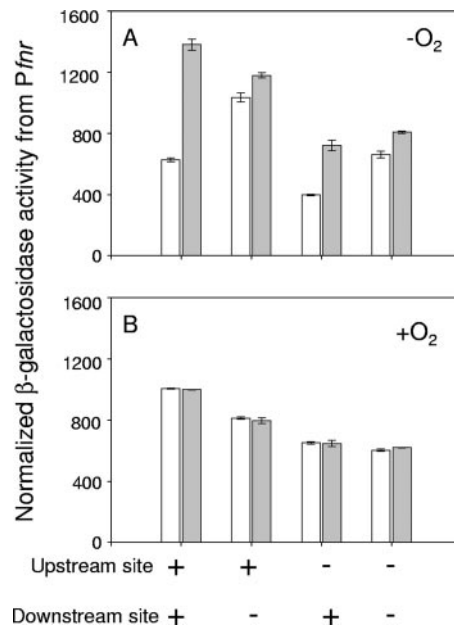
Rather, these data are consistent with the notion that another transcription factor may be recruited by the upstream site to activate P*fnr* expression under both aerobic and anaerobic growth conditions.

IHF increases transcription from the *fnr* promoter in vivo. To examine whether other transcription factors regulate P*fnr*, we tested whether the DNA-bending protein IHF had an effect on *fnr* transcription, since it has been shown to be involved in the regulation of several FNR-dependent promoters, such as *narG*, *nir*, *nrfA*, *dmsA*, *ubiC*, *ndh*, *pfl*, *sodA*, and *narK* (5–7, 9, 19, 31, 36, 43, 48, 51, 52). In addition, the DNA sequences that IHF binds to (consensus site YAANNNNTTGAW, where W is A or T, Y is T or C, and N is any nucleotide) (15, 23, 60) show some similarity to the sequence within this upstream region of the *fnr* promoter (−110TTAAGGGTTTTCAA−97). An approximately twofold decrease in P*fnr* expression was observed in both aerobic and anaerobic cells lacking IHF (Fig. 6). Furthermore, the IHF-dependent increase in *fnr* expression was not observed in the construct containing the mutant upstream site (Fig. 6), suggesting that IHF binds to the upstream element. In support of this notion, the results of DNase I footprinting experiments revealed that the region of DNA from bp −117 to −89 relative to the *fnr* transcription start site was protected in the presence of 0.5 μM IHF (Fig. 7) and includes the sequence of DNA that was originally predicted to be the upstream FNR binding site centered at bp −103.5. These data
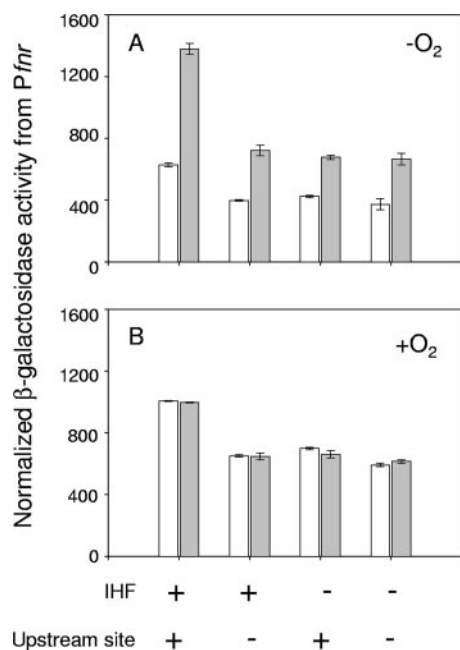
FIG. 6. In vivo expression from wild-type or upstream mutant *fnr'-lacZ* promoter fusions in anaerobically (A) or aerobically (B) grown cells lacking IHF. FNR$^+$ and FNR$^-$ strains are represented by the white and gray bars, respectively. "−" indicates that the strains are IHF$^-$, whereas "+" indicates that the strains are IHF$^+$. For the predicted upstream FNR binding site, "−" indicates that base substitutions (Fig. 1) were made within the upstream sequence, whereas "+" indicates that the wild-type sequence is present. β-Galactosidase activity from the *fnr'-lacZ* promoter fusions was normalized by correcting for the difference in cell numbers ml^{-1} of culture for aerobically and anaerobically grown cells as explained in Materials and Methods. Error bars represent the standard errors for three independent experiments.
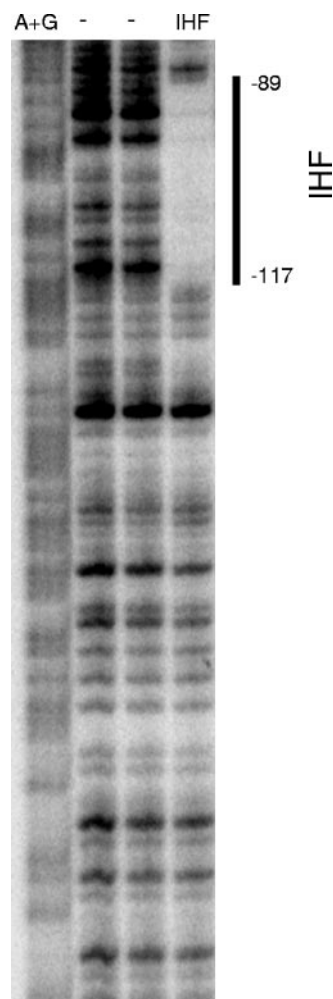

FIG. 7. Footprinting of the *fnr* promoter region by IHF. Samples contained the HindIII-BamHI DNA fragment from pPK8221 (Table 1), in which the 3′ HindIII end was radiolabeled. The area of protection from DNase I by 0.5 μM IHF is indicated with a vertical line, and numbers indicate the distances relative to the transcription start site. Samples were electrophoresed with Maxam-Gilbert ladders (A+G) made using the same DNA.

suggest that binding of IHF to the *fnr* promoter enhances transcription.

In vitro transcription experiments revealed that IHF did not alter transcription from the *fnr* promoter (data not shown), raising the possibility that IHF works in conjunction with another transcription factor to regulate transcription of P*fnr* as has been previously observed for several FNR-dependent promoters (5–7, 9, 19, 31, 36, 43, 48, 51, 52). A recent study, which evaluated the distribution of CRP binding sites along the *E. coli* chromosome, identified a potential CRP binding site within the *fnr* promoter region centered at bp −145.5 relative to the transcription start site (18). Although DNase I footprinting revealed a weak binding site in this position, no increase in P*fnr* transcription was observed in vitro in the presence of purified CRP-cAMP (0.5 to 1 μM), either in the presence or in the absence of IHF (0.5 μM) (data not shown). Furthermore, no effect of CRP was found in vivo by either mutation of the CRP binding site or use of strains that lacked CRP (data not shown), indicating that under the conditions tested, CRP does not regulate the FNR promoter.

We also tested whether the anaerobic regulator ArcA plays a role in regulating *fnr* transcription since recent reverse transcription-PCR studies have shown that levels of *fnr* transcript are slightly higher in cells lacking ArcA than in wild-type cells (49). However, we found that expression from P*fnr* (bp −155

to +25 or −418 to +55) in ArcA$^-$ cells was similar to that in wild-type cells under both aerobic and anaerobic growth conditions (data not shown). Thus, it is unclear how IHF increases the expression of the *fnr* promoter.

DISCUSSION

In this study, we have defined the role of the two predicted FNR binding sites in *fnr* transcription. In addition to defining one sequence element required for negative autoregulation by FNR, we found a second element for IHF binding, which, through an unknown mechanism, enhances P*fnr* expression. Thus, these studies have expanded our knowledge of how FNR is regulated at the transcriptional level and have provided new insights into how FNR protein levels are achieved for the global response to O$_2$.

Regulation of *fnr* repression. While our data indicate that [4Fe-4S]-FNR represses its own synthesis by binding to the

predicted downstream FNR binding site within the *fnr* promoter region, the mechanism by which [4Fe-4S]-FNR prevents RNA polymerase from transcribing the *fnr* gene is not known. Given that the downstream FNR binding site is centered at bp -0.5 relative to the transcription start site, these results may suggest that [4Fe-4S]-FNR represses P*fnr* by blocking RNA polymerase from binding to P*fnr* through steric hindrance. However, further investigations are needed to examine this hypothesis. Although the downstream site contains 9 out of 10 bp which match the consensus FNR binding site, previous findings (44, 54) and data presented in this study suggest that the *fnr* promoter is not fully saturated by endogenous levels of [4Fe-4S]-FNR. For example, repression of P*fnr* was increased approximately fourfold in vivo when FNR was expressed from a multicopy plasmid and was larger (approximately fivefold) in vitro than in vivo (approximately twofold) when FNR was expressed from the chromosome. Whether this is due to competition between FNR and RNA polymerase for binding to P*fnr* and/or reflects additional sequence requirements for FNR binding in vivo is not known. Furthermore, it appears that FNR binding sites cannot be predicted based on bioinformatic data alone. For example, FNR did not bind to the predicted upstream site, which contains 7 out of 10 bp that match the consensus site. In contrast, FNR has been shown to directly bind to two sites within the FNR-repressed *ndh* promoter (19), one of which contains only 6 out of 10 bp that match the consensus site. Perhaps the efficiency of FNR binding is also influenced by differences in the architecture of FNR-dependent promoters.

Regulation of *fnr* activation. Although our studies indicate that the predicted upstream site is not an FNR binding site, we found that this sequence binds IHF and increases expression of *fnr* under both aerobic and anaerobic conditions. This finding is also in agreement with a previous study which indicated that DNA sequences upstream of bp position -41 relative to the transcription start site are important for maximal *fnr* expression (47). While IHF alone had no effect on P*fnr* transcription in vitro, it is possible that conditions of the assay may have bypassed a role for IHF or that another transcription factor, along with IHF, is required to activate the *fnr* promoter. Alternatively, IHF may function by preventing another transcription factor from repressing P*fnr*. Indeed, this appears to be the case for the *nir* promoter, in which binding of IHF to the IHF II site decreases the repression of *nir* mediated by IHF and Fis binding at other sites (7). Our studies suggest that neither CRP nor ArcA is this transcription factor even though a previous study indicated that expression of *fnr* is slightly higher (approximately twofold) in ArcA$^-$ cells under microaerobic growth conditions (49). However, a recent study, which mapped the distribution of Fis binding sites across the *E. coli* genome, identified a potential Fis binding site within the *fnr* promoter region (17). Thus, further investigation is necessary to determine the role of Fis and other transcription factors in regulating *fnr* transcription.

Relevance of negative autoregulation in O$_2$ sensing. Negative autoregulation is not an uncommon regulatory mechanism found in *E. coli*. In fact, it has been reported that over 40% of known *E. coli* transcriptional factors are subject to negative autoregulation (45). Mathematical modeling and studies with synthetic gene circuits have indicated that negative autoregulation decreases the response times of transcription networks because the steady-state concentration of the transcription factor is achieved faster (1, 45, 57, 59). Since *E. coli* lives in environments with regular changes in O$_2$ tension, rapidly achieving a new steady-state level of active FNR may provide an advantage during adaptation to various growth conditions by quickly allowing a new transcription rate for the FNR regulon.

In addition to providing a means for rapidly reaching steady-state levels of [4Fe-4S]-FNR, the amount of [4Fe-4S]-FNR protein produced by the negative autoregulation mechanism also seems optimal for the efficient inactivation of FNR by O$_2$. Even though under standard aerobic growth conditions the O$_2$ concentration in the medium can never exceed $\sim$220 μM at 37°C, it is in excess relative to the cellular concentration of FNR ($\sim$7 μM) (56). Previous kinetic studies suggest that the rate of FNR inactivation is moderately fast at 220 μM O$_2$ (half-life of $\sim$30 seconds at 25°C) (56). Despite this, it has been shown that even small increases in FNR protein result in increased FNR activity under aerobic growth conditions, indicating that excess FNR is not efficiently inactivated, presumably as a result of insufficient time to inactivate the additional protein (4, 37, 38, 53). Thus, we hypothesize that under anaerobic conditions, negative autoregulation also prevents [4Fe-4S]-FNR from exceeding a critical level beyond which it can be efficiently inactivated. Taken together, negative autoregulation appears to provide an optimal balance of FNR protein levels, directing a new rate of synthesis of the FNR regulon under anaerobic conditions and allowing the efficient inactivation of [4Fe-4S]-FNR upon exposure to O$_2$. Future studies involving construction of mathematical models will be necessary to test these predictions.

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