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The *Escherichia coli* K-12 NarL and NarP Proteins Insulate the *nrf* Promoter from the Effects of Integration Host Factor▽

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The *Escherichia coli* K-12 *nrf* operon promoter can be activated fully by the FNR protein (regulator of fumarate and nitrate reduction) binding to a site centered at position −41.5. FNR-dependent transcription is suppressed by integration host factor (IHF) binding at position −54, and this suppression is counteracted by binding of the NarL or NarP response regulator at position −74.5. The *E. coli acs* gene is transcribed from a divergent promoter upstream from the *nrf* operon promoter. Transcription from the major *acs*P2 promoter is dependent on the cyclic AMP receptor protein and is modulated by IHF and Fis binding at multiple sites. We show that IHF binding to one of these sites, located at position −127 with respect to the *nrf* promoter, has a positive effect on *nrf* promoter activity. This activation is dependent on the face of the DNA helix, independent of IHF binding at other locations, and found only when NarL/NarP are not bound at position −74.5. Binding of NarL/NarP appears to insulate the *nrf* promoter from the effects of IHF. The *acs-nrf* regulatory region is conserved in other pathogenic *E. coli* strains and related enteric bacteria but differs in *Salmonella enterica* serovar Typhimurium.

The *Escherichia coli* K-12 *nrf* operon encodes a periplasmic nitrite reductase, which is responsible for the reduction of nitrite to ammonium ions (8). Transcription of this operon is driven from a single promoter (*pnrf*), and expression is induced in the absence of oxygen by the FNR protein (regulator of fumarate and nitrate reduction), a global transcription activator that controls the expression of many genes required for anaerobic respiration (2). FNR binding to a single site, centered at position −41.5, is sufficient for maximum induction of *pnrf* (3, 25). However, FNR-dependent activation is suppressed by the binding of integration host factor (IHF), a nucleoid-associated protein, to a site located at position −54 (IHF I). This suppression is reversed by the binding of NarL or NarP at position −74.5. NarL and NarP are homologous response regulators that are controlled by the NarX and NarP sensor kinases in response to the presence of nitrite or nitrate (reviewed in references 9 and 24). NarL/NarP binding at position −74.5 results in displacement of IHF from the IHF I site and, hence, in nitrite/nitrate-dependent activation of *pnrf* (6).

Directly upstream of the *nrf* operon is the divergently transcribed *acs* gene, which encodes acetyl coenzyme A synthetase (16). Expression of *acs* is principally dependent on the *acs*P2 promoter, which controls a divergent transcript that initiates at a location 280 bp upstream of the *nrf* transcription start point (1) (Fig. 1). Transcription from *acs*P2 is totally dependent on activation by the cyclic AMP receptor protein (CRP), which binds at two sites (CRP I and CRP II) (Fig. 1). CRP-dependent activation of *acs*P2 is modulated by binding of the nucleoid-associated proteins IHF and Fis to tandem sites, namely, IHF II and III and Fis II and III (4) (Fig. 1). Most of our previous studies of *pnrf* activity focused on DNA sequences up to position −87. The crowded nature of the *acs-nrf* intergenic region prompted us to investigate whether *acs*P2 and its many regulators have any effect on expression from *pnrf*. Here we show that *pnrf* activity is stimulated by IHF binding to the IHF III site, but only in the absence of NarL/NarP. We report that the *acs-nrf* regulatory region is conserved in other related pathogenic strains but differs in *Salmonella enterica* serovar Typhimurium.

MATERIALS AND METHODS

Bacterial strains, plasmids, and DNA fragments. The bacterial strains, plasmids, and promoter fragments used in this work are listed in Table 1, and the oligonucleotides used are listed in Table 2. Standard methods for cloning and manipulating DNA fragments were used. By convention, locations at the *nrf* promoter are labeled with the transcript start point designated +1 and with upstream and downstream locations prefixed with "−" and "+," respectively. All promoter fragments carry an upstream EcoRI linker and a downstream HindIII linker. Single base substitutions in *pnrf* are denoted pNX, where "N" is the position of the substitution relative to the transcript start and "X" is the substituted base in the nontemplate strand of the promoter. For routine DNA manipulations and as a source of DNA fragments for gel retardation and footprinting analysis, fragments were cloned into plasmid pAA121 or pSR. To measure promoter activities, fragments were cloned into the *lac* expression vector pRW50. Derivatives of pAA121 and pSR were maintained in host cells using medium supplemented with 100 μg ml^{-1} ampicillin, while pRW50 derivatives were maintained with 15 μg ml^{-1} tetracycline.

Construction of *pnrf433* promoter fragment. The *pnrf433* promoter fragment, which encodes *pnrf* sequences from positions −302 to +131, was constructed using PCR. Primers acsP2 and nrfDown were used to amplify the *nrf* promoter region from JCB387. The resulting product was restricted with EcoRI and HindIII and cloned into the pRW50 vector.

Construction of *pnrf53* derivatives carrying mutations in the upstream promoter region. The p175G (Fis II) and p153G (CRP II) substitutions were introduced into the *pnrf53* fragment by mega-primer PCR. The *nrf* promoter DNA was amplified using primer D4600 and either primer Fis II or CRP II, with pAA121/*pnrf53* as the template. The purified PCR products were then used in a second round of PCR with the primer D5431 and pAA121/*pnrf53*. Products were restricted with EcoRI and HindIII and cloned into pAA121 that had been cut

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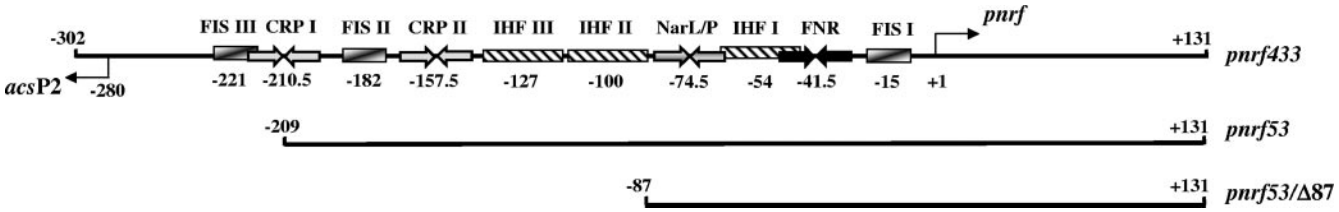


FIG. 1. Organization of the *nrf-acs* intergenic region. The figure shows a schematic representation of the *pnrf433*, *pnrf53*, and *pnrf53*/Δ87 promoter fragments and the important elements involved in regulation of the *nrf* and *acs*P2 promoters. The upstream boundary for each promoter fragment is given, and the locations of transcription start sites for *pnrf* and *acs*P2 are indicated by arrows. FNR, CRP, and NarL/NarP binding sites are represented by inverted arrows, while IHF and Fis binding sites are depicted by boxes. The central base pair of each DNA binding site is indicated, and all numbering is in relation to the transcription start of the *nrf* promoter (+1).

with EcoRI and HindIII. The p104Gp103C (IHF II) and p124Gp123C (IHF III) substitutions were introduced into *pnrf53* by conventional PCR. The p104Gp103C mutation was introduced into *pnrf* using primers D4600 and IHF II, with pAA121/*pnrf53* as the template. The product was cut with HindIII and SphI and cloned into HindIII-SphI-restricted pAA121/*pnrf53*. For the p124Gp123C mutation, *pnrf* DNA was amplified using primers D5431 and IHF III, with pAA121/*pnrf53* as the template. The PCR product was restricted with EcoRI and SphI and cloned into pAA121/*pnrf53* that was also cut with EcoRI and SphI. The p124Gp123G substitution was also combined with the *pnrf53*/p104p103C, *pnrf53*/p54Gp51G, *pnrf53*/+5, and *pnrf53*/+10 promoter fragments. In all cases, an NsiI-HindIII-restricted promoter fragment was subcloned into pRW50/*pnrf53*/p124Gp123C that had been cut with NsiI and HindIII.

Construction of *pnrf-nir* fusion promoters. The *pnrf-nir* fusion promoters were constructed using mega-primer PCR. The downstream region of *pnir* (positions −60 to +36) was amplified using primers Nrf-Nir and D4600, with pAA121/*pnir7150* as the template. The purified PCR product was then used in a second round of PCR with the primer D5431, with pAA121/*pnrf53* as the template. The product was cloned into pRW50 by using EcoRI and HindIII. The p124Gp123C (IHF III) substitution was introduced into the *pnrf-nir* fusion by using pAA121/*pnrf53*/p124Gp123C as the template in the final PCR.

Construction of *nrf* promoter fragments from other enteric bacteria. The DNA sequences of the *nrf* promoters from other enteric bacteria were compiled from *coli*BASE (http://colibase.bham.ac.uk), an online database for *E. coli*, *Shigella*, and *Salmonella* comparative genomics (7). The *nrf* promoter DNA from *S. enterica* serovar Typhimurium was amplified by a PCR using the primers nrfUP STM and nrfDown STM. Deletion of sequences upstream of position −87 from the *S. enterica* serovar Typhimurium *nrf* promoter was achieved using primers nrfSTM 87E and nrfDown STM. PCR products were restricted with EcoRI and BamHI and cloned into pRW50.

Proteins. Purified IHF protein was prepared by the method of Nash et al. (20), and purification of the NarL-maltose binding protein fusion (MBP-NarL) was carried out as detailed by Li et al. (17). The mature native NarL protein was used after the MBP moiety had been cleaved from MBP-NarL, using the protease factor Xa (New England Biolabs) (17).

TABLE 1. Bacterial strains, plasmids, and promoter fragments used in this work

Strain, plasmid, or promoter	Relevant characteristics	Reference or source
Strains		
E. coli K-12 strains		
JCB387	Δ*nir* Δ*lac*	21
JCB3884	JCB387 *narL narP253*::Tn*10*d(Cm)	25
JRG1728	Δ*fnr* Δ*lac*	26
Other strains		
S. enterica serovar Typhimurium LT2	Wild type	I. Henderson
Plasmids		
pAA121	Cloning vector for EcoRI-HindIII fragments derived from pBR322	14
pSR	pBR322 derivative containing a λ *oop* transcription terminator	15
pRW50	Broad-host-range *lacZ* fusion vector for cloning promoters on EcoRI-HindIII fragments; contains the RK2 origin of replication	18
Promoters		
pnrf433	*E. coli nrf* promoter fragment carrying nucleotide sequences from positions −302 to +131	This work
*pnrf*53	*E. coli nrf* promoter fragment carrying nucleotide sequences from positions −209 to +131	25
*pnrf*53/Δ87	*E. coli nrf* promoter fragment carrying nucleotide sequences from positions −87 to +131	10
*pnrf*53/+5	*E. coli nrf53* promoter fragment carrying 5-bp insertion at position −56	3
*pnrf*53/+10	*E. coli nrf53* promoter fragment carrying 10-bp insertion at position −56	3
*pnrf*53/p54Gp51G	*E. coli nrf53* promoter fragment carrying T-to-G and C-to-G substitutions at positions −54 and −51	3
pnrf53/STM	*S. enterica* serovar Typhimurium *nrf* promoter fragment carrying nucleotide sequences from positions −246 to +133	This work
pnrf53/Δ87 STM	*S. enterica* serovar Typhimurium *nrf* promoter fragment carrying nucleotide sequences from positions −87 to +133	This work
pnir7150	*E. coli nir* promoter fragment carrying nucleotide sequences from positions −150 to +36	25

TABLE 2. Oligonucleotide primers used for this study

Name	Sequence (5′–3′)
acsP2	GGGGAATTCCATGCTTTTGTTCTCCTTG TAGG
nrfDown	CCCGGATCCCTGAAGATACGGCGTGCG
D4600	GTAGTCGGTGTGTTCAC
Fis II	GCAAATAAACGGGAGGGTAATTTTTG AAGG
CRP II	GAAGGTCAGGAACAAAAGTTGATTA ATTC
D5431	ACCTGACGTCTAAGAAACC
IHF II	CCCGAATTCGCATGCTGTGCAAAAAGA GGAAG
IHF III	CCCCGCATGCATAACTGCAGCTTCCTC AAAG
Nrf-Nir	CTAAAGTGGTATTTTACATGCATGTGAA TTTGATTTAC
nrfUP STM	CCCGAATTCCCGGGGATCACGCAAAAA GGGAACTGTGC
nrfDown STM	CCCGGATCCCTGAAGAAACGGCGTGCG
nrfSTM 87E	CCCGAATTCGTTACTAACTCTAAAGTGG

Gel retardation assays. Gel retardation assays using purified IHF and Fis were carried out as detailed by Browning et al. (3). Purified *nrf53* promoter fragments were end labeled with [γ-^{32}P]ATP, and approximately 0.5 ng of each fragment was incubated with various amounts of each protein. The reaction buffer contained 10 mM potassium phosphate (pH 7.5), 100 mM potassium glutamate, 1 mM EDTA, 50 μM dithiothreitol, 5% glycerol, and 25 μg ml^{-1} herring sperm DNA. The final reaction volume was 10 μl. After incubation at 37°C for 20 min, samples were run in 0.25× Tris-borate-EDTA in a 6% polyacrylamide gel (12 V cm^{-1}) containing 2% glycerol and analyzed using a Bio-Rad FX molecular imager and Quantity One software (Bio-Rad).

DNase I footprinting experiments. Footprinting experiments were performed on ^{32}P-end-labeled *pnrf53* fragments, using the protocols of Savery et al. (23). Each reaction mix (20 μl) contained a final concentration of 1.35 nM template DNA. The buffer composition was 20 mM HEPES (pH 8.0), 50 mM potassium glutamate, 5 mM $MgCl_2$, 1 mM dithiothreitol, 500 μg ml^{-1} bovine serum albumin, and 25 μg ml^{-1} herring sperm DNA. When NarL was used in DNase I footprinting experiments, the NarL protein was preincubated with 50 mM acetyl phosphate at 37°C for 45 min (10). Samples were analyzed by denaturing gel electrophoresis. Gels were calibrated with Maxam-Gilbert G+A sequencing reactions of the labeled fragment and quantified using a Bio-Rad FX molecular imager and Quantity One software (Bio-Rad).

Assays of *nrf* promoter activity. To assay expression from *pnrf* derivatives cloned into the *lac* expression vector pRW50, different host strains were transformed, and β-galactosidase activity was measured as described by Jayaraman et al. (12), using the Miller protocol (19). Cells were grown in minimal medium (minimal salts with 0.4% glycerol, 10% Lennox broth, and 40 mM fumarate) (22). Where indicated, sodium nitrite was also added to cultures to a final concentration of 2.5 mM. For aerobic growth, cells were shaken vigorously, while for anaerobic growth, they were held static in growth tubes (150 mm long and 15 mm in diameter). Aerobic cultures were grown to an optical density at 650 nm of 0.2 to 0.3, anaerobic cultures were grown to an optical density at 650 nm of 0.4 to 0.6, and cells were assayed exactly as described previously (3). β-Galactosidase activities are reported in nmol of *o*-nitrophenyl-β-D-galactopyranoside (ONPG) hydrolyzed under our assay conditions min^{-1} mg^{-1} dry cell mass, and each activity reported is the average of three independent determinations.

RESULTS

Sequences upstream of position −87 stimulate anaerobic expression from the *nrf* promoter. Previously, we showed that the *pnrf53*/Δ87 promoter fragment, which carries *nrf* sequences from positions −87 to +131, possesses all of the elements necessary for the regulation of the *nrf* promoter (3) (Fig. 1). Since it was unclear how *acs*P2 and far-upstream sequences might influence *pnrf* activity, we constructed promoter fragments that carry *nrf* upstream sequences to positions −302 (*pnrf433*) and −209 (*pnrf53*) (Fig. 1). Fragments were subcloned into the *lacZ* expression vector pRW50 to generate *pnrf*::*lacZ* transcriptional fusions, and β-galactosidase activities in the Δ*lac narL narP* strain JCB3884 were determined. The results in Fig. 2 show that the anaerobic expression levels from the *pnrf433* and *pnrf53* fragments were identical. Thus, sequences upstream of position −209, which include the *acs*P2 promoter and the Fis III and CRP I binding sites, do not influence expression from *pnrf*. In contrast, anaerobic expression from the *pnrf53*/Δ87 fragment was decreased twofold, indicating that a *cis*-acting element located between positions −209 and −87 stimulated FNR-dependent transcription.

To locate the element responsible, point mutations were introduced into the *pnrf53* fragment (positions −209 to +131), which disrupted the DNA binding sites Fis II (p175G), CRP II (p153G), IHF III (p124Gp123C), and IHF II (p104p103C). Gel retardation assays confirmed that the substitutions interfered with the binding of the relevant factors (Fig. 3) (1). Mutated *pnrf53* fragments were subcloned into pRW50, and β-galactosidase expression was examined in JCB3884. The results in Fig. 2 indicate that only the disruption of IHF III decreased anaerobic expression similarly to that observed for the *pnrf53*/Δ87 fragment. Thus, we concluded that IHF III is necessary for the stimulation of FNR-dependent transcription. Note that the promoter activities of all derivatives were completely dependent on FNR, as expression was undetectable in the *fnr* null strain JRG1728 (data not shown).

Since the *nrf-acs* intergenic region contains three IHF sites, we investigated whether IHF bound to the IHF III site functioned independently of IHF bound to other sites within *pnrf*. The p124Gp123C substitution (IHF III) was therefore combined with mutations that disrupted IHF binding to either IHF

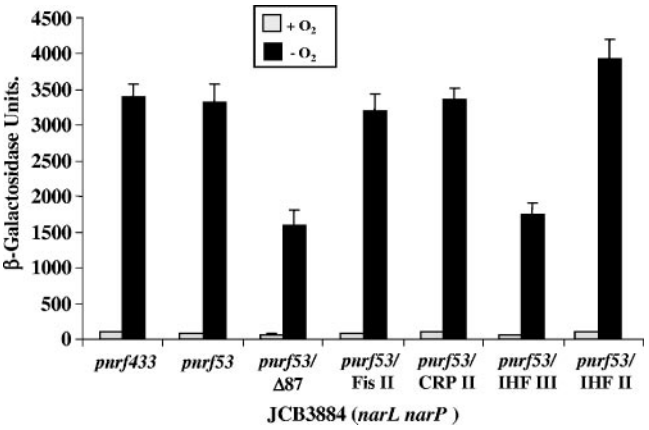


FIG. 2. IHF III activates FNR-dependent transcription at *pnrf*. The figure illustrates measured β-galactosidase activities of JCB3884 (*narL narP*) cells carrying pRW50 and containing different *nrf* promoter fragments. The *pnrf433*, *pnrf53*, and *pnrf53*/Δ87 fragments contain upstream *pnrf* DNA from positions −302, −209, and −87, respectively, and all fragments end at position +131 (see Fig. 1). Substitutions were introduced into the *pnrf53* fragment to disrupt the DNA binding sites Fis II (p175G), CRP II (p153G), IHF III (p124Gp123C), and IHF II (p104p103C). Cells were grown aerobically or anaerobically in minimal salts medium, and β-galactosidase activities are expressed as nmol of ONPG hydrolyzed min^{-1} mg^{-1} dry cell mass. Each activity is the average of three independent determinations.

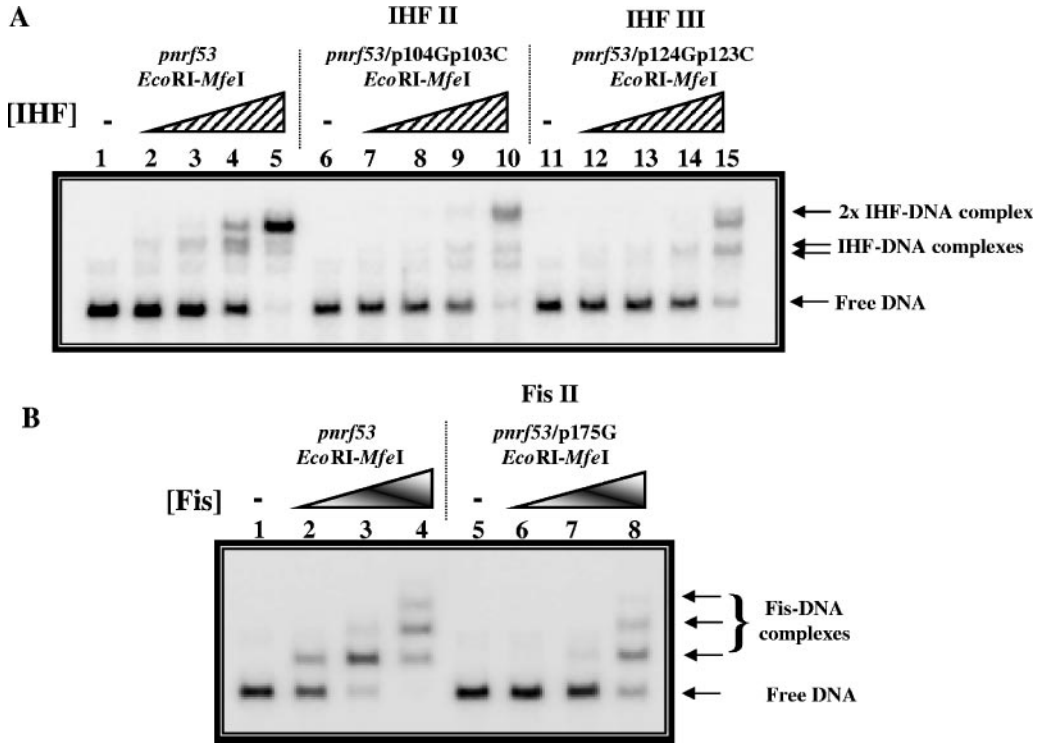


FIG. 3. Gel retardation assays with the *nrf* promoter. The figure shows gel retardation assays with *pnrf53* promoter fragments incubated with purified IHF (A) and Fis (B) proteins. (A) ^{32}P-end-labeled *pnrf53* EcoRI-MfeI fragments were incubated with increasing concentrations of purified IHF protein, as follows: lanes 1 to 5, wild-type *pnrf53*; lanes 6 to 10, *pnrf53*/p104Gp103C (IHF II); and lanes 11 to 15, *pnrf53*/p124Gp123C (IHF III). The concentrations of IHF protein in the reactions were as follows: lanes 1, 6, and 11, no protein; lanes 2, 7, and 12, 25 nM; lanes 3, 8, and 13, 50 nM; lanes 4, 9, and 14, 0.1 μM; and lanes 5, 10, and 15, 0.2 μM. (B) ^{32}P-end-labeled *pnrf53* EcoRI-MfeI fragments were incubated with increasing concentrations of purified Fis protein, as follows: lanes 1 to 4, wild-type *pnrf53*; and lanes 5 to 8, *pnrf53*/p175G (Fis II). The concentrations of Fis protein in the reactions were as follows: lanes 1 and 5, no protein; lanes 2 and 6, 0.11 μM; lanes 3 and 7, 0.22 μM; and lanes 4 and 8, 0.44 μM. Note that *pnrf53* EcoRI-MfeI fragments carry *pnrf* sequences from positions −209 to −50 and, therefore, only carry the IHF II, IHF III, and Fis II binding sites.

I (p54Gp51G) (see reference 3) or IHF II (p104Gp103G). Promoter fragments were subcloned into pRW50, and expression was examined in JCB3884. The data presented in Table 3 show that the disruption of IHF III results in similar decreases in anaerobic expression for *pnrf53* derivatives carrying substitutions in either IHF I or IHF II. Therefore, we concluded that IHF bound to the IHF III site acts independently of IHF bound to other sites.

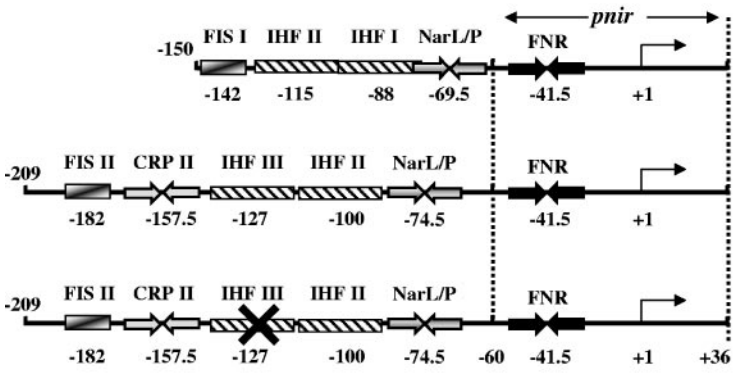
To examine whether the effect of IHF III was dependent on the face of the helix, the p124Gp123C substitution was intro-

TABLE 3. β-Galactosidase activities of JCB3884 cells carrying different *nrf* promoter fragments[a]

Promoter	Presence of site			β-Galactosidase activity		% Decrease in β-galactosidase activity[b]
	IHF I	IHF II	IHF III	$+O_2$	$-O_2$	
pnrf53	+	+	+	80	3,600	
pnrf53/p124Gp123C	+	+	−	50	2,100	42
pnrf53/p54Gp51G	−	+	+	180	7,300	
pnrf53/p124Gp123C p54Gp51G	−	+	−	140	5,000	32
pnrf53/p104Gp103G	+	−	+	70	3,500	
pnrf53/p124Gp123C p104Gp103G	+	−	−	80	2,400	31
pnrf53/+5	−	+	+	210	7,700	
pnrf53/+5 p124Gp123C	−	+	−	190	8,200	
pnrf53/+10	−	+	+	90	6,100	
pnrf53/+10 p124Gp123C	−	+	−	120	3,900	36

[a] The p54Gp51G, p104Gp103C, and p124Gp123C substitutions disrupt the IHF I, IHF II, and IHF III sites, respectively, as indicated. In the *pnrf53*/+5 and *pnrf53*/+10 promoter fragments, the IHF I site has been disrupted by the insertion of 5 or 10 bp of DNA at position −56. Measured β-galactosidase activities of JCB3884 cells carrying pRW50 containing the different *nrf* promoter fragments are shown. Cells were grown aerobically and anaerobically in minimal salts medium. β-Galactosidase activities are expressed as nmol of ONPG hydrolyzed min^{-1} mg^{-1} dry cell mass. Each activity is the average of three independent determinations that varied <10%.

[b] Decrease in anaerobic expression due to the p124Gp123C substitution in IHF III.



Promoter	β-Galactosidase activity in JCB3884 (*narL narP*) +O_2	-O_2
pnir7150	70	1500
pnrf-nir	220	4900
pnrf-nir/ IHF III	150	2200

FIG. 4. Upstream sequences from *pnrf* can regulate the *nir* promoter. The figure shows a schematic representation of the *pnir7150* promoter fragment and two *pnrf-nir* fusion promoters. The *pnir7150* fragment contains *nir* sequences from positions −150 to +36. The *pnrf-nir* fusion promoters carry the *nir* core promoter sequences from positions −60 to +36 and *nrf* upstream sequences from positions −209 to −60. FNR, CRP, and NarL/NarP binding sites are represented by inverted arrows, while IHF and Fis binding sites are depicted by boxes. The position of the transcription start site for *pnir* is shown by an arrow, and the location of the center of each DNA binding site is indicated (5). The *pnrf-nir*/IHF III promoter fragment contains the p124Gp123C substitutions which disrupt the IHF III site, shown with an "X." The figure also lists measured β-galactosidase activities of JCB3884 (*narL narP*) cells carrying each promoter subcloned into pRW50. Cells were grown aerobically and anaerobically in minimal salts medium plus 0.4% glucose. β-Galactosidase activities are expressed as nmol of ONPG hydrolyzed min^{-1} mg^{-1} dry cell mass. Each activity is the average of three independent determinations that varied <10%.

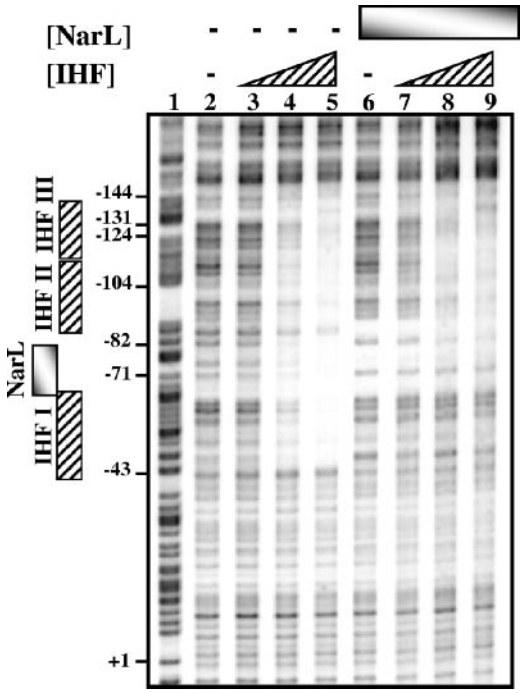


FIG. 5. NarL prevents IHF binding to the IHF I site. The figure shows the results of in vitro DNase I footprint experiments with purified IHF and NarL. An end-labeled *pnrf53* EcoRI-HindIII fragment was incubated with purified IHF and NarL proteins and subjected to DNase I footprint analysis. The concentration of IHF was as follows: lanes 2 and 6, no protein; lanes 3 and 7, 0.23 μM; lanes 4 and 8, 0.46 μM; and lanes 5 and 9, 0.93 μM. The concentration of NarL was as follows: lanes 2 to 5, no protein; and lanes 6 to 9, 0.8 μM. Gels were calibrated using Maxam-Gilbert G+A sequencing reactions (lane 1), and relevant positions are indicated. The locations of the NarL/NarP and IHF binding sites are indicated by vertical boxes.

duced into the *pnrf53*/+5 and *pnrf53*/+10 promoter fragments, which carry 5- and 10-bp DNA insertions, respectively, at position −56. Note that the insertion of DNA at position −56 completely disrupts IHF binding to IHF I (3). The results in

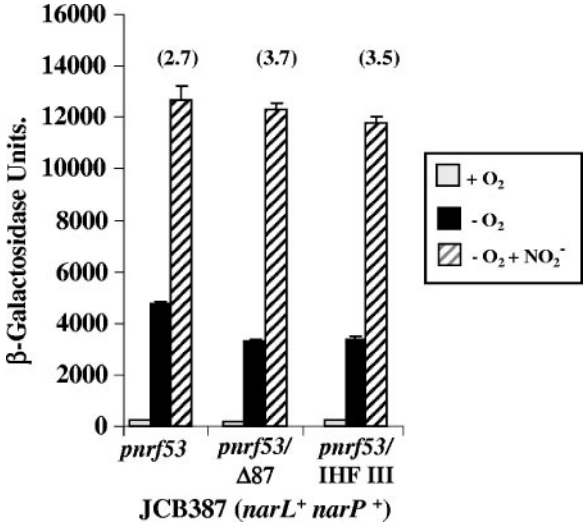


FIG. 6. NarL insulates *pnrf* from the effect of IHF III. The figure shows measured β-galactosidase activities of JCB387 cells carrying pRW50 containing different *nrf* promoter fragments. The *pnrf53* and *pnrf53*/Δ87 fragments contain upstream *pnrf* DNA from positions −209 and −87, respectively, and all fragments end at position +131 (see Fig. 1). The p124Gp123C substitutions disrupt the IHF III site. Cells were grown aerobically and anaerobically in minimal salts medium, and nitrite was added to a final concentration of 2.5 mM where indicated. β-Galactosidase activities are expressed as nmol of ONPG hydrolyzed min^{-1} mg^{-1} dry cell mass. Each activity is the average of three independent determinations. The numbers in parentheses indicate *x*-fold increases observed due to nitrite.

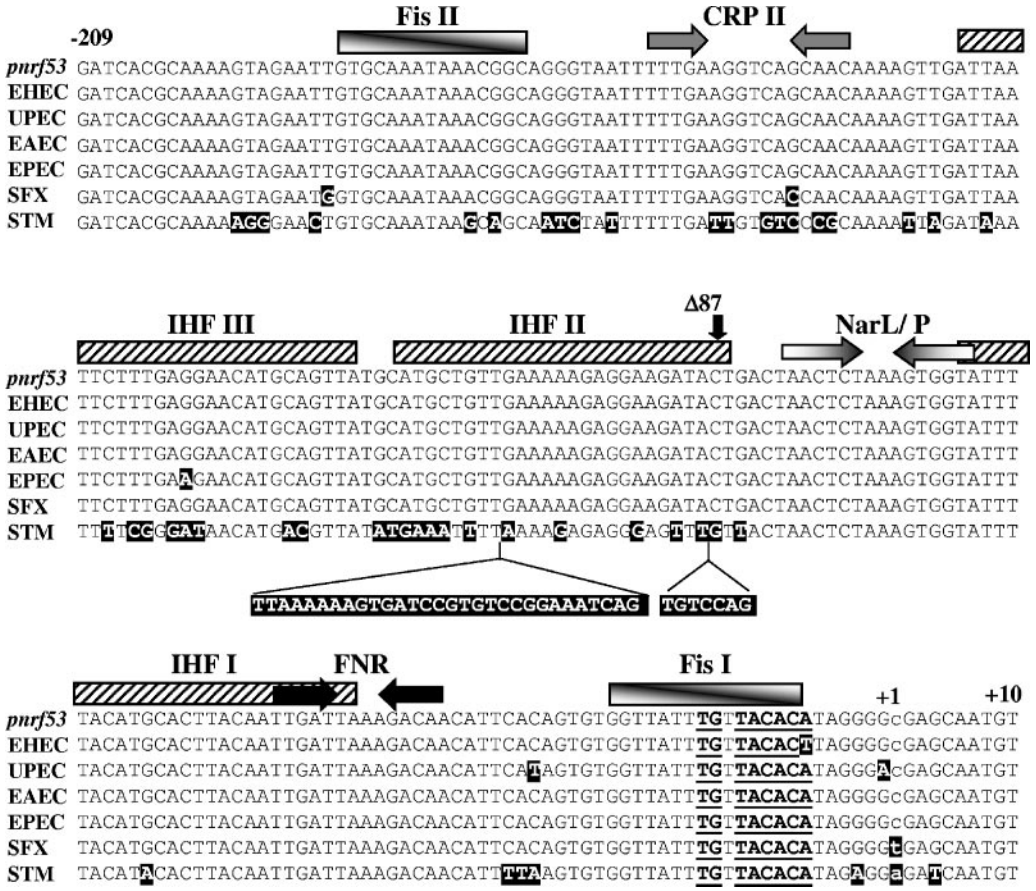


FIG. 7. Alignment of *nrf* promoter sequences from different enteric bacteria. The figure shows the sequence of the *E. coli* K-12 *pnrf53* fragment from positions −209 to +10 (NC00913), aligned with the *nrf* promoter regions from enterohemorrhagic *E. coli* (EHEC) (NC002695), uropathogenic *E. coli* (UPEC) (NC004431), enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), *Shigella flexneri* (SFX) (NC004741), and *S. enterica* serovar Typhimurium (STM) (NC003197). The location of the transcription start site for *pnrf53* is indicated in lowercase. The locations of FNR, CRP, and NarL/NarP binding sites are represented by inverted arrows, while IHF and Fis binding sites are depicted by boxes. The insertion of sequences within the upstream promoter region of the *S. enterica* serovar Typhimurium promoter is indicated. Differences between the *pnrf53* fragment and other promoters are highlighted by black boxes. The position at which upstream sequences were deleted from the *S. enterica* serovar Typhimurium promoter to generate the *pnrf53*/Δ87 STM promoter fragment is indicated by Δ87.

Table 3 show that the mutation of IHF III in the *pnrf53*/+10 promoter fragment resulted in a decrease in anaerobic expression, while no effect was observed for the *pnrf53*/+5 promoter fragment. Thus, IHF III must be correctly positioned for IHF-mediated stimulation to occur.

Like that from the *nrf* promoter, expression from the *E. coli* *nir* promoter (*pnir*) is induced under anaerobic conditions by the binding of FNR to a site centered at position −41.5 (13) (Fig. 4). To investigate whether the IHF III site from *pnrf* could regulate the *nir* promoter, the *nrf* upstream sequences (positions −209 to −60) were fused to the *nir* core promoter sequences (positions −60 to +36) to generate a *pnrf-nir* fusion promoter. β-Galactosidase activities of the wild-type *pnir7150* promoter (sequences from −150 to +36), the *pnrf-nir* fusion, and a *pnrf-nir* fusion in which IHF III was disrupted (i.e., the p124Gp123C substitution), each of which was subcloned into pRW50, were then determined in JCB3884. The data in Fig. 4 demonstrate that all three promoters were still induced by anaerobiosis and that the IHF III site activated anaerobic expression in the *pnrf-nir* fusion. Thus, the IHF III site from *pnrf* can be transplanted to another FNR-dependent promoter with similar architecture and still stimulate transcription initiation. Note that mutation of the *nir* −10 hexamer from TAAGGT to TGAGGT in both *pnir* and the *pnrf-nir* fusion decreased anaerobic expression to basal levels, indicating that expression was completely dependent on *nir* core promoter sequences (data not shown).

NarL/NarP can compensate for the loss of IHF III. Previously, we demonstrated that NarL activates FNR-dependent transcription at *pnrf* by displacing IHF bound to the IHF I site (3, 6). To investigate whether IHF bound to the IHF II and IHF III sites influences this competition, we examined the binding of purified IHF and NarL to the *pnrf53* fragment by using DNase I footprinting. The results in Fig. 5 show that IHF alone protected an extended region of ~100 bp, encompassing IHF I, IHF II, and IHF III (lane 5), while NarL alone protected the NarL/NarP binding site centered at position −74.5 (lane 6). When both proteins were present (lanes 7 to 9), protection of the IHF II, IHF III, and NarL/NarP binding sites was observed, but the IHF I site was not protected. Thus, IHF

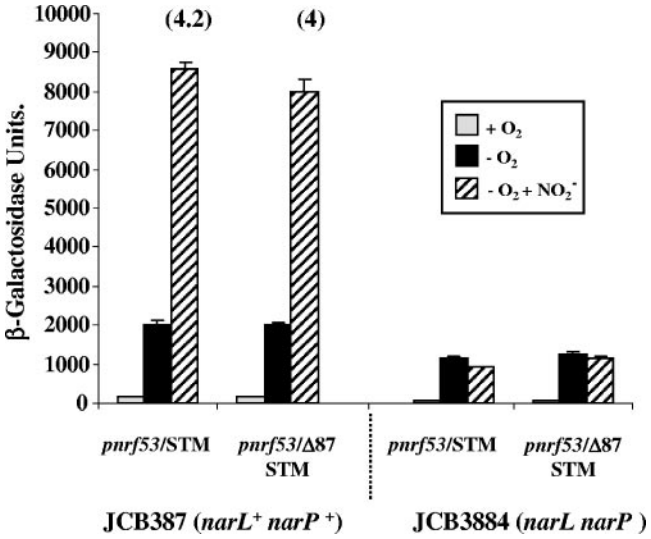


FIG. 8. Expression of *nrf* promoter fragments from *S. enterica* serovar Typhimurium. The figure shows measured β-galactosidase activities of *S. enterica* serovar Typhimurium *nrf* promoter fragments (STM) subcloned into pRW50 in JCB387 and JCB3884 (*narL narP*) cells. In the *pnrf*/Δ87 STM fragment, sequences upstream of position −87 have been deleted (see Fig. 7). Cells were grown aerobically and anaerobically in minimal salts medium, and nitrite was added to a final concentration of 2.5 mM where indicated. β-Galactosidase activities are expressed as nmol of ONPG hydrolyzed min^{-1} mg^{-1} dry cell mass, and each activity is the average of three independent determinations. The numbers in parentheses indicate *x*-fold increases observed due to nitrite in JCB387.

bound to the IHF II and IHF III sites does not interfere with the ability of NarL to bind DNA and displace IHF from IHF I.

To test how IHF III influences nitrite induction at *pnrf*, the promoter activities of *pnrf53*, *pnrf53*/Δ87, and a *pnrf53* fragment carrying the p124Gp123C substitution were measured in the $narL^+$ $narP^+$ strain JCB387. Fragments were subcloned into pRW50, and β-galactosidase activities were determined under aerobic, anaerobic, and anaerobic nitrite-rich conditions. The results in Fig. 6 show that in the presence of nitrite, all three promoters displayed the same level of nitrite induction. Thus, IHF binding at site III does not contribute significantly to promoter activity when nitrite is present. This indicates that NarL/NarP can compensate for the loss of IHF III and that the effect of IHF III is only observed in the absence of NarL/NarP binding.

Upstream sequences do not stimulate the *S. enterica* serovar Typhimurium *nrf* promoter. Figure 7 shows an alignment of the *E. coli* K-12 *pnrf53* fragment (positions −209 to +10) with the *nrf* promoter regions from other pathogenic *E. coli* strains and enteric bacteria. For most of the *nrf* promoters, upstream sequences are conserved, but the *S. enterica* serovar Typhimurium promoter possesses large insertions within IHF II. To examine whether upstream sequences at the *S. enterica* serovar Typhimurium promoter activate in vivo, we subcloned into pRW50 the *S. enterica* serovar Typhimurium promoter and a derivative in which sequences upstream of position −87 were deleted. β-Galactosidase activities were then measured in both JCB387 and JCB3884 (*narL narP*). The results in Fig. 8 indicate that deletion of upstream sequences had no significant effect on *pnrf* expression in either strain. We concluded that upstream sequences do not contribute to FNR-dependent transcription at the *S. enterica* serovar Typhimurium promoter. Note that expression from all promoters was dependent on FNR, as promoter activity was negligible in the *fnr* null strain JRG1728 (data not shown).

DISCUSSION

Our experiments show that FNR-dependent transcription at the *nrf* promoter is stimulated by the binding of IHF to an upstream site, centered at position −127. The action of IHF from this site is independent of IHF bound to other sites within *pnrf* (i.e., IHF I and IHF II) and dependent on the face of the helix. The stimulatory effect of IHF III can be transplanted to another FNR-dependent promoter with similar promoter architecture. At present, the mechanism by which IHF bound to the IHF III site stimulates transcription initiation is unclear. Recall that at the phage λ P_L promoter, IHF bends upstream DNA and allows the C-terminal domain of the α subunit of RNA polymerase to contact a distal UP element (11). Thus, IHF bound to IHF III may also enable promoter-bound RNA polymerase to make similar contacts. The organization of the *nrf* upstream region is clearly conserved in other pathogenic *E. coli* strains and related pathogenic bacteria, with the exception of *S. enterica* serovar Typhimurium, where the insertion of DNA within IHF II appears to disrupt the ability of IHF to activate transcription from IHF III.

The role of IHF at the *nrf* promoter is to set its activity under conditions where nitrite/nitrate-dependent activation via NarL/NarP is minimal. Under these conditions, IHF has both negative and positive effects on FNR-dependent activation due to its binding at the IHF I and IHF III sites. At present, it is unclear whether changes in conditions can alter the fine balance between repression and activation. The effects of IHF are observed only in the absence of NarL/NarP. Thus, NarL and NarP protect FNR-dependent transcription at *pnrf* from both the negative and positive effects of IHF and appear to function like insulator proteins rather than conventional transcription activators, which stimulate promoter activity by recruitment of RNA polymerase. Interestingly, at the *E. coli nir* promoter, IHF binding to the IHF I site (centered at position −88) represses FNR-dependent activation, while IHF binding to the IHF II site (centered at position −115) activates the promoter (5). Thus, IHF achieves the same pattern of regulation at both the *nir* and *nrf* promoters by binding to different locations.

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