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Fnr-, NarP- and NarL-Dependent Regulation of Transcription Initiation from the *Haemophilus influenzae* Rd *napF* (Periplasmic Nitrate Reductase) Promoter in *Escherichia coli* K-12

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Periplasmic nitrate reductase (*napFDAGHBC* operon product) functions in anaerobic respiration. Transcription initiation from the *Escherichia coli napF* operon control region is activated by the Fnr protein in response to anaerobiosis and by the NarQ-NarP two-component regulatory system in response to nitrate or nitrite. The binding sites for the Fnr and phospho-NarP proteins are centered at positions −64.5 and −44.5, respectively, with respect to the major transcription initiation point. The *E. coli napF* operon is a rare example of a class I Fnr-activated transcriptional control region, in which the Fnr protein binding site is located upstream of position −60. To broaden our understanding of *napF* operon transcriptional control, we studied the *Haemophilus influenzae* Rd *napF* operon control region, expressed as a *napF-lacZ* operon fusion in the surrogate host *E. coli*. Mutational analysis demonstrated that expression required binding sites for the Fnr and phospho-NarP proteins centered at positions −81.5 and −42.5, respectively. Transcription from the *E. coli napF* operon control region is activated by phospho-NarP but antagonized by the orthologous protein, phospho-NarL. By contrast, expression from the *H. influenzae napF-lacZ* operon fusion in *E. coli* was stimulated equally well by nitrate in both *narP* and *narL* null mutants, indicating that phospho-NarL and -NarP are equally effective regulators of this promoter. Overall, the *H. influenzae napF* operon control region provides a relatively simple model for studying synergistic transcription by the Fnr and phospho-NarP proteins acting from class I and class II locations, respectively.

Facultative aerobes such as *Escherichia coli* can respire with a variety of terminal electron acceptors, including oxygen, nitrate, dimethyl sulfoxide (DMSO), and fumarate. Synthesis of the corresponding respiratory enzymes is regulated in response to the preferred acceptors, oxygen and nitrate. During anaerobic growth, the Fnr protein (*f*umarate and *n*itrate *r*eductases) activates transcription initiation at many operons, including the *narGHJI* and *dmsABC* operons encoding the respiratory enzymes cytochrome *b*-linked nitrate reductase and DMSO reductase, respectively (17, 21, 59).

The Fnr protein is homologous to the well-studied Crp protein (cyclic AMP receptor protein; also known as Cap, catabolite gene activator protein). The Crp and Fnr proteins bind as homodimers to DNA sites of dyad symmetry upstream of regulated promoters, from whence they stimulate transcription initiation. The Crp protein is activated upon binding its allosteric effector, cyclic AMP, whereas the Fnr protein is activated upon assembly of its oxygen-labile iron-sulfur cluster (23, 28).

Two types of simple Crp- and Fnr-dependent transcription control regions are defined (6, 8). Class I control regions have a Crp or Fnr binding site located 60 or more nucleotides (nt) upstream of the transcription initiation point (8, 65). From these distal locations, Crp and Fnr make specific contacts with the RNA polymerase α-subunit carboxyl-terminal domain (α-CTD). Class II control regions have a Crp or Fnr binding site located near position −40 with respect to the transcription initiation site, overlapping or replacing the −35 promoter element. From this proximal location, Crp and Fnr make specific contacts with both the α-CTD and the σ^{70} subunit of RNA polymerase. Several examples of class I and class II Crp-dependent promoters are known (8). However, expression from most known Fnr-dependent promoters, including those for the *narG* and *dmsA* operons, is activated through Fnr class II mechanisms (6, 31).

As defined formally, class I and II promoters are regulated by only a single activator protein, whereas promoters controlled by multiple activators are designated class III (8). Nevertheless, we refer here to multiply activated promoters as class I or class II in order to denote the locations of the respective regulatory protein binding sites.

Transcription initiation for a subset of Fnr-activated operons is further regulated by nitrate, which induces synthesis of enzymes for nitrate respiration and represses synthesis of enzymes for respiration of other anaerobic acceptors. Response to nitrate is mediated by the NarX sensor kinase, which controls phosphorylation of the NarL response regulator (57). Phospho-NarL binds to upstream sites in the *narG* operon control region to stimulate transcription activation in synergy with the Fnr protein (10, 61), and it also binds to operator sites in the *dmsA* operon control region to repress transcription (3).

The *E. coli napFDAGHBC* operon encoding cytochrome *c*-linked nitrate reductase (periplasmic nitrate reductase) contains an Fnr site centered at −64.5 nt with respect to the major transcription initiation point and therefore is a rare example of a class I Fnr-activated operon (9, 11). Expression of the $napF_{E.\ coli}$ ($napF_{Ec}$) operon is further induced by nitrate and nitrite, acting through the NarQ-NarP two-component regulatory system,

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TABLE 1. *E. coli* K-12 strains

Strain	Genotype	Reference or source
VJS676	F^- λ^- prototroph Δ(*argF-lacIZYA*)*U169*	56
Derivatives of strain VJS676		
VJS4797	$\lambda\Phi(napF_{Ec}$-*lacZ*) Δ85	11
VJS5101	$\lambda\Phi(napF_{Ec}$-*lacZ*) Δ85 *narL215*::Tn*10*	11
VJS5109	$\lambda\Phi(napF_{Ec}$-*lacZ*) Δ85 *narP253*::Tn*10*d(Cm)	11
VJS5117	$\lambda\Phi(napF_{Ec}$-*lacZ*) Δ85 *narL215*::Tn*10 narP253*::Tn*10*d(Cm)	11
VJS6621	$\lambda\Phi(napF_{Hi}$-*lacZ*) Δ110	This study
VJS6623	$\lambda\Phi(napF_{Hi}$-*lacZ*) Δ110 *narL215*::Tn*10*	This study
VJS6625	$\lambda\Phi(napF_{Hi}$-*lacZ*) Δ110 *narP253*::Tn*10*d(Cm)	This study
VJS6627	$\lambda\Phi(napF_{Hi}$-*lacZ*) Δ110 *narL215*::Tn*10 narP253*::Tn*10*d(Cm)	This study
VJS6906	$\lambda\Phi(napF_{Hi}$-*lacZ*) Δ260	This study
VJS6907	$\lambda\Phi(napF_{Hi}$-*lacZ*) Δ260 (NarP/NarL site mutant)	This study
VJS6908	$\lambda\Phi(napF_{Hi}$-*lacZ*) Δ260 (Fnr site mutant)	This study

a paralog of the NarX-NarL system (53). The phospho-NarP protein binds to a site centered at −44.5 nt with respect to the major transcription initiation point to activate transcription in synergy with the Fnr protein (11, 13). Thus, in this context, the phospho-NarP protein can be considered a class II activator.

The $napF_{Ec}$ operon control region exhibits complexities that may limit its utility as a simple model for studying Fnr class I and phospho-NarP class II transcription activation. The phospho-NarL protein also binds to the site centered at −44.5 but fails to stimulate transcription activation. Thus, it competes for binding with, but antagonizes activation by, the phospho-NarP protein (11, 13). In addition, the major promoter for $napF_{Ec}$ operon transcription overlaps a minor promoter of uncertain physiological significance (9, 13, 54). Finally, expression of the $napF_{Ec}$ operon is also regulated by the molybdate-responsive ModE protein, which binds to a site centered at −134.5 with respect to the major transcription initiation point (41, 54).

We report here our analysis of the *Haemophilus influenzae* Rd *napF* control region, which we transplanted into *E. coli* as a $\Phi(napF_{Hi}$-*lacZ*) monocopy operon fusion (where $napF_{Hi}$ denotes $napF_{H.\ influenzae}$). Results demonstrate that in *E. coli* at least, transcription from the $napF_{Hi}$ promoter (i) is stimulated by the Fnr protein from a site centered at −81.5 nt upstream of the transcription initiation point, (ii) is further stimulated by either the phospho-NarP or phospho-NarL protein from a site centered at −42.5, and (iii) is not responsive to molybdate limitation or ModE protein control. Therefore, this control region provides a relatively simple example of a promoter that is controlled by an Fnr class I transcription activation mechanism, in synergy with phospho-NarP or phospho-NarL bound at a class II location.

(Studies with *H. influenzae* presented here were submitted by Catherine T. Yen in 1998 as part of an undergraduate thesis for the Cornell University Division of Biological Sciences Honors Program.)

MATERIALS AND METHODS

Strains and plasmids. Strains and plasmids are listed in Table 1. Genetic crosses were performed by P1 *kc*-mediated generalized transduction (42). Null alleles of *nar* regulatory genes have been described previously (46). Standard methods were used for restriction endonuclease digestion, ligation, transformation, and PCR amplification of DNA (38).

Culture media and conditions. Defined, complex, and indicator media for genetic manipulations were used as described previously (38). Defined medium to grow *E. coli* cultures for enzyme assays and for RNA extraction was buffered with 3-{*N*-morpholino}propanesulfonic acid (MOPS) as previously described (56). The initial pH of this medium is set at 8.0 to ameliorate nitrite toxicity. Because the pK_a of MOPS is 7.2, the buffering capacity of this medium continually increases as acidic fermentation products accumulate; at harvest, cultures typically had a pH value of about 7.5. Medium for culturing *H. influenzae* was full-strength heart infusion broth supplemented with NAD and hemin (1) and buffered with MOPS (pH 8.0). The respiratory oxidants $NaNO_3$ (40 mM), $NaNO_2$ (5 mM), DMSO (40 mM), and sodium fumarate (40 mM) were added as indicated.

Cultures were grown at 37°C to the mid-exponential phase. Culture densities were monitored with a Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., New York, N.Y.) equipped with a number 66 (red) filter. Anaerobic cultures for enzyme assays and for RNA extraction were grown in screw-cap tubes as described previously (32).

Enzyme assays. β-Galactosidase activities were determined at room temperature (approximately 21°C) by following the hydrolysis of *o*-nitrophenyl-β-D-galactopyranoside in $CHCl_3$–sodium dodecyl sulfate-permeabilized cells. Specific activities are expressed in arbitrary (Miller) units (22).

Nitrate reductase activities were determined at room temperature by following the production of nitrite in intact cells (56). Cells were suspended in 0.32 M potassium phosphate, pH 7.1, and stored on ice. Samples (0.8 ml) were mixed with 0.1 ml of 0.5 mg ml^{-1} benzyl viologen. Reactions were started by adding 0.1 ml of a mixture containing 4 mg ml^{-1} $Na_2S_2O_4$, 4 mg ml^{-1} $NaHCO_3$, and 0.5 M $NaNO_3$. Reactions were terminated by vigorous vortex mixing (to oxidize the viologen), and 1 ml each of sulfanilic acid and *N*-1-napthylethylenediamine solutions was added. Specific activities are expressed in arbitrary units analogous to Miller units (56).

All cultures were assayed in duplicate, and reported values are averaged from at least two independent experiments.

Construction of $napF_{Hi}$ control region alterations. The source DNA for the $napF_{Hi}$ control region was a plasmid pUC8 shotgun subclone, designated GHIEP28, isolated for the *H. influenzae* genome sequencing project (16) and purchased from the American Type Culture Collection (Manassas, Va.). Oligonucleotide-directed site-specific mutagenesis was used to introduce substitutions into the $napF_{Hi}$ operon control region or its flanking sequences. Mutagenesis followed the ampicillin selection protocol (33). PCRs were performed with a high-fidelity thermostable DNA polymerase (Accuzyme; Bioline USA, Reno, Nev.).

Following each round of mutagenesis, the DNA sequence for the entire fragment was determined to eliminate isolates with spurious nucleotide substitutions. The control region cassettes were then recloned into the operon fusion vector pRS415 (51). The resulting $\Phi(napF_{Hi}$-*lacZ*) operon fusions were crossed into bacteriophage λRS45 (51), and monocopy lysogens were identified by a whole-colony PCR test (45).

Transcript analysis. Analysis by rapid amplification of cDNA ends (5′-RACE) (47), also termed anchored PCR, used reagents purchased from Invitrogen Life Technologies (5′-RACE system, version 2.0; Invitrogen Life Technologies, Carlsbad, Calif.) and was performed essentially as described by the manufacturer's instructions. Oligonucleotide primers used were as follows: 5′-GGGTAAC GCCAGGGTTTTCC (gene-specific primer 1 in *lacZ*), 5′-GTTTTCCCAGTCA CGAC (gene-specific primer 2 in *lacZ*; M13 forward primer), 5′-GGCCACGC

GTCGACTAGTACGGGIIGGGIIGGGIIG (abridged anchor primer), 5′-AA GCTTAGTGAATCCGTAATCATGGTCATAG (gene-specific primer 3 in *lacZ*), and 5′-GGCCACGCGTCGACTAGTAC (universal amplification primer).

RESULTS

***H. influenzae* is a facultative aerobe.** *Haemophilus* spp. are classified phylogenetically as members of the gamma subdivision of the proteobacteria and are very close relatives of the enterobacteria (14). *H. influenzae* is indigenous to the mucous membranes of the human upper respiratory tract, and many strains cause infections of the middle ear (otitis media) or respiratory tract (40). Although defined media have been developed (25), even complex media must be supplemented with NAD (factor V) and hemin (factor X).

H. influenzae Rd is known to use both oxygen and nitrate as respiratory oxidants (62). More-extensive analysis of the hemin-independent species *H. parainfluenzae* revealed that oxygen, nitrate, and fumarate serve as electron acceptors. Furthermore, in *H. parainfluenzae*, the amount and types of cytochromes are regulated in response to oxygen and nitrate availability, and nitrate reductase synthesis is induced by nitrate during anaerobic growth (52, 63). Thus, like their enterobacterial relatives, *Haemophilus* spp. regulate the synthesis of respiratory enzymes in response to growth conditions.

The *H. influenzae* Rd strain KW20 genome encodes very few transcriptional regulatory proteins (16). Nevertheless, it does encode the regulators that in *E. coli* are known to control anaerobic respiration: the Fnr and ModE proteins and the NarQ-NarP two-component system, described in the introduction, as well as the ArcB-ArcA two-component system, which controls citrate cycle enzyme synthesis in response to ubiquinone-dependent respiration (18). Together with the physiological studies summarized immediately above, this indicates that regulation of respiratory gene expression in *H. influenzae* Rd is likely very similar to that in *E. coli*.

As deduced from the respective DNA sequences, the *H. influenzae* Fnr protein (257 residues) shares 80% sequence identity over 236 residues with the *E. coli* Fnr protein (254 residues). The Fnr_{Hi} protein (encoded by the *fnr* gene; locus tag HI1425) contains all four Cys residues shown to be essential for Fnr_{Ec} function (20, 28), and the two protein sequences are identical in a 32-residue span that encompasses the helix-turn-helix DNA binding domain (29). The fnr_{Hi} gene complements an *E. coli fnr* null allele (19, 39).

As with *E. coli*, the *H. influenzae narP* (locus tag HI0726) and *narQ* (locus tag HI0267) genes are unlinked (16). The $NarP_{Hi}$ protein (208 residues) shares 59% identity over 204 residues with the $NarP_{Ec}$ protein (215 residues), and the $NarQ_{Hi}$ protein (567 residues) shares 38% identity over 204 residues with the $NarQ_{Ec}$ protein (566 residues). Identities along the multidomain NarQ sequences are localized in discrete patches corresponding to discrete functions (53). The $narP_{Hi}$ gene is essential for nitrate induction of nitrate reductase synthesis (this study; see below), and the $narQ_{Hi}$ gene complements an *E. coli narQ* null allele (C. T. Yen and V. Stewart, unpublished data).

NarP-dependent nitrate respiration by *H. influenzae*. We cultured *H. influenzae* Rd strain KW20 and its *narP* null derivative, strain MGH90 (22), as described in Materials and Methods. Both strains grew well with aeration, exhibiting exponential-phase doubling times of approximately 1 h and achieving relatively high culture densities of about 120 Klett units after 8 h of cultivation (data not shown). Both strains grew more slowly with DMSO or fumarate as the electron acceptor, with exponential-phase doubling times of approximately 3.5 to 4 h and culture densities of only about 50 Klett units. Cultures with no added electron acceptor exhibited very slow growth to a culture density of about 30 Klett units.

The $narP^+$ strain grew relatively well with nitrate as the electron acceptor, exhibiting an exponential-phase doubling time of approximately 1 h and achieving a final culture density of about 60 Klett units. By contrast, the *narP* null strain grew very slowly with nitrate (doubling time of >8 h) and achieved a final culture density of only about 40 Klett units. Thus, the $narP^+$ gene was specifically required only for nitrate respiration.

The *H. influenzae* Rd genome contains the *napFDAGHBC* operon encoding cytochrome *c*-linked nitrate reductase but does not contain a *narGHJI* operon for cytochrome *b*-linked nitrate reductase (44). We measured periplasmic nitrate reductase specific activity as described in Materials and Methods. The $narP^+$ strain synthesized about 25 U of activity after anaerobic growth with fumarate as the electron acceptor and about 120 U after anaerobic growth with nitrate plus fumarate. The *narP* null strain by contrast synthesized only about 5 U irrespective of added nitrate. Enzyme activity was insensitive to azide (55), as expected for cytochrome *c*-linked nitrate reductase (44). Thus, the $narP^+$ gene was required for nitrate induction of periplasmic nitrate reductase synthesis in *H. influenzae*.

***$napF_{Hi}$* transcription initiation point in *E. coli*.** We constructed a $\Phi(napF_{Hi}\text{-}lacZ)$ operon fusion in the moderate-copy-number plasmid pRS415 as described in Materials and Methods. The insert encompasses a sequence from a native EcoRI site at position −260 with respect to the transcription initiation site, within the upstream conserved hypothetical gene designated HI0341, through a BamHI site, introduced via oligonucleotide-directed site-specific mutagenesis, within the $napF_{Hi}$ coding region (Fig. 1). We introduced this plasmid into the wild-type *E. coli* strain VJS676, grew cultures anaerobically in the presence of nitrate, and used the method of rapid amplification of cDNA ends (47) to determine the 5′ end of the $napF_{Hi}$ mRNA as described in Materials and Methods.

Results (not shown) identified the G residue denoted by the asterisk in Fig. 1 as position +1. We conclude that this G is the transcription initiation point in *E. coli*. In contrast to the $napF_{Ec}$ control region, which contains two distinct initiation points (54), we found no evidence for additional transcription initiation points for the $napF_{Hi}$ control region.

Regulated expression of the *$napF_{Hi}$* control region in *E. coli*. We isolated λ specialized transducing bacteriophage for two different $\Phi(napF_{Hi}\text{-}lacZ)$ operon fusion constructs and made monocopy lysogens, as described in Materials and Methods. The first construct, denoted $\Phi(napF_{Hi}\text{-}lacZ)$ Δ260, is described above (Fig. 1). The second construct, denoted $\Phi(napF_{Hi}\text{-}lacZ)$ Δ110, contains a sequence from the same downstream BamHI site to an EcoRI site (66) overlapping position −110 (Fig. 1).

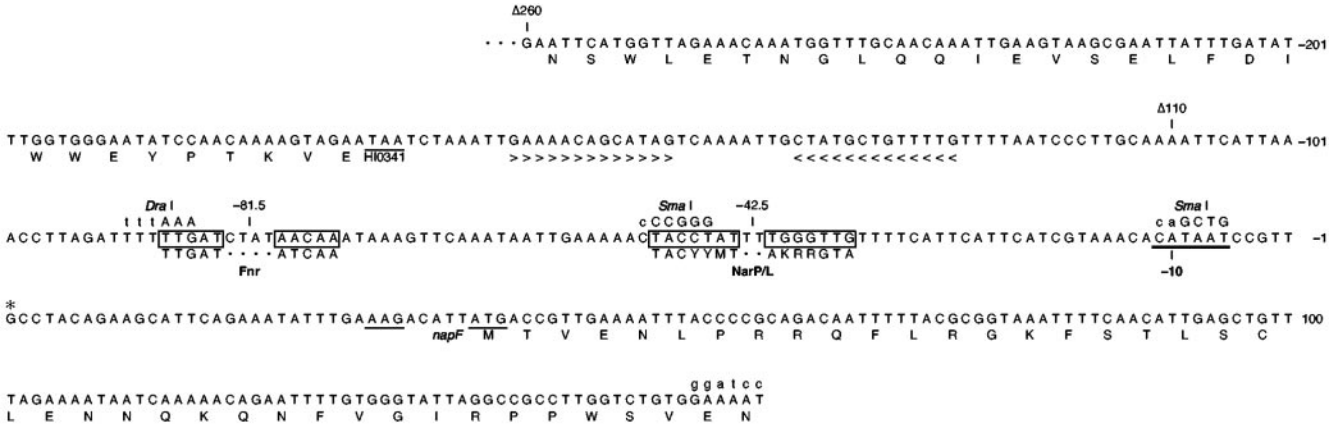

FIG. 1. $napF_{Hi}$ control region sequence. Numbering is with respect to the transcription initiation point, indicated with an asterisk. The −10 promoter element is indicated with a thick underline, and translation initiation (Shine-Dalgarno element and initiation codon) and termination sequences are indicated with thin underlines. Sequences for binding the Fnr and phospho-NarP or -NarL proteins are boxed; consensus sequences are shown below boxes. Restriction endonuclease sites introduced to inactivate *cis*-acting regulatory sequences are indicated, with uppercase lettering denoting nucleotide changes from the wild type. The downstream BamHI restriction site introduced for constructing Φ($napF_{Hi}$-*lacZ*) operon fusions is indicated in lowercase. Nucleotides in the inverted repeat forming the likely intrinsic terminator downstream of the HI0341 coding region are indicated with arrowheads.

Thus, the two constructs differ only in the extent of the upstream sequence present.

We cultured the lysogenic strains to the mid-exponential phase with oxygen, nitrate, nitrite, or no added electron acceptor and measured β-galactosidase activity as described in Materials and Methods. Results are shown in Table 2. Expression levels from both the Φ($napF_{Hi}$-*lacZ*) Δ260 construct and the Φ($napF_{Hi}$-*lacZ*) Δ110 construct were qualitatively similar. Aerated cultures synthesized negligible levels of β-galactosidase, whereas anaerobic cultures synthesized readily measured amounts, demonstrating that Φ($napF_{Hi}$-*lacZ*) expression was induced by anaerobiosis. In anaerobic cultures, added nitrate and nitrite resulted in further increases in β-galactosidase synthesis. Thus, the $napF_{Hi}$ control region in *E. coli* exhibited transcription activation by anaerobiosis and further activation by nitrate or nitrite.

The overall level of β-galactosidase synthesized was greater in strains carrying the Φ($napF_{Hi}$-*lacZ*) Δ110 construct than in those with the Φ($napF_{Hi}$-*lacZ*) Δ260 construct (Table 2). A probable intrinsic transcription terminator (43) lies between the end of the upstream HI0341 gene and the Δ110 deletion endpoint (Fig. 1). It is likely that the lower overall levels of β-galactosidase synthesis in strains carrying the Φ($napF_{Hi}$-*lacZ*) Δ260 construct result from this terminator restricting low-level readthrough transcription from the upstream bacteriophage λ sequence (51).

***cis*-acting sites regulate expression of the $napF_{Hi}$ control region in *E. coli*.** The consensus sequence for Fnr binding contains inverted repeats of the pentamer TTGAT separated by 4 nt (48). The consensus sequence for phospho-NarP and phospho-NarL binding contains inverted repeats of the heptamer TACYYMT (where Y represents C or T and M represents A or C) separated by 2 nt (12). Sequence inspection of the $napF_{Hi}$ control region reveals likely sites for binding the Fnr and phospho-NarP proteins centered at positions −81.5 and −42.5, respectively (Fig. 1). A likely −10 promoter element for σ^{70}-RNA polymerase recognition (consensus TAT AAT) (24) is positioned appropriately with respect to the transcription initiation point.

To evaluate the in vivo roles for these sites, we used oligonucleotide-directed site-specific mutagenesis to introduce multiple nucleotide substitutions. These substitutions (which simultaneously introduced new restriction endonuclease sites) changed 3 nt in the upstream Fnr half-site, 5 nt in the upstream phospho-NarP half-site, and 4 nt in the −10 promoter element (Fig. 1).

Plasmid-borne Φ($napF_{Hi}$-*lacZ*) Δ260 constructs carrying the Fnr and phospho-NarP site substitutions were converted to

TABLE 2. Effects of oxygen, nitrate, and nitrite on expression from Φ($napF_{Hi}$-*lacZ*) constructs

Strain	Fusion[b]	Site[c]	LacZ sp act[a]				Activation by:		
			$+O_2$	$-O_2$	$+NO_3^- - O_2$	$+NO_2^- - O_2$	O_2	NO_3^-	NO_2^-
VJS6621	Φ($napF_{Hi}$-*lacZ*) Δ110		3	150	1,280	420	25	8.5	2.8
VJS6906	Φ($napF_{Hi}$-*lacZ*) Δ260		<1	16	390	50	>16	24	3.1
VJS6907	Φ($napF_{Hi}$-*lacZ*) Δ260	NarP/NarL	<1	10	7	5	>10	1	1
VJS6908	Φ($napF_{Hi}$-*lacZ*) Δ260	Fnr	<1	<1	2	<1			

[a] Strains were cultured to the mid-exponential phase in MOPS medium (defined medium with glucose) with the terminal electron acceptor as indicated.
[b] Location of upstream endpoint in construct.
[c] Mutant regulatory protein binding site in control region (see Fig. 1).

TABLE 3. Effects of *narL* and *narP* null alleles on on expression from Φ(*napF*$_{Hi}$-*lacZ*) and Φ(*napF*$_{Ec}$-*lacZ*) constructs

Strain	Fusion	Genotype		LacZ sp act[a]			Activation by:	
		narL	*narP*	$-NO_3^- - O_2$	$+NO_3^- - O_2$	$+NO_2^- - O_2$	NO_3^-	NO_2^-
VJS4797	Φ(*napF*$_{Ec}$-*lacZ*) Δ85	+	+	160	1,760	3,040	11	19
VJS5101	Φ(*napF*$_{Ec}$-*lacZ*) Δ85	−	+	220	8,940	7,010	40	32
VJS5109	Φ(*napF*$_{Ec}$-*lacZ*) Δ85	+	−	89	140	130	1.6	1.5
VJS5117	Φ(*napF*$_{Ec}$-*lacZ*) Δ85	−	−	91	88	84	1.0	0.9
VJS6621	Φ(*napF*$_{Hi}$-*lacZ*) Δ110	+	+	200	2,080	770	10	3.8
VJS6623	Φ(*napF*$_{Hi}$-*lacZ*) Δ110	−	+	200	1,660	1,180	8.3	5.9
VJS6625	Φ(*napF*$_{Hi}$-*lacZ*) Δ110	+	−	190	1,640	350	8.6	1.8
VJS6627	Φ(*napF*$_{Hi}$-*lacZ*) Δ110	−	−	180	180	150	1.0	0.8

[a] Strains were cultured to the mid-exponential phase in MOPS medium (defined medium with glucose).

specialized transducing phage and used to form monocopy lysogens as described above. We cultured these strains and measured β-galactosidase activity in the same experiments described above for the wild-type versions (Table 2). Expression from the construct carrying the Fnr site alterations remained at the low level characteristic of aerobic cultures irrespective of culture conditions. Expression from the construct carrying the phospho-NarP site alterations exhibited essentially wild-type induction by anaerobiosis, but further induction by nitrate or nitrite was abolished. These results demonstrate that the sequence motifs identified by visual inspection are indeed the authentic binding sites for regulation by the Fnr and phospho-NarP proteins.

The plasmid-borne Φ(*napF*$_{Hi}$-*lacZ*) Δ260 construct carrying the −10 promoter element substitutions failed to direct synthesis of measurable levels of β-galactosidase activity (data not shown), demonstrating that the −10 sequence identified by visual inspection is essential for *napF*$_{Hi}$ expression in *E. coli*. We did not convert this construct into a specialized transducing phage.

NarP and NarL proteins regulate expression of the *napF*$_{Hi}$ control region in *E. coli*. We next determined the effects of null alleles in the *narP* and *narL* genes, encoding the nitrate-responsive regulators, on expression of the *napF*$_{Hi}$ control region in *E. coli*. To provide controls, we included analogous strains carrying the Φ(*napF*$_{Ec}$-*lacZ*) Δ85 fusion, which lacks the upstream ModE binding site (11, 41).

Patterns of expression from the Φ(*napF*$_{Ec}$-*lacZ*) fusion (Table 3) were essentially as described previously (11). In the *narP*$^+$ *narL*$^+$ strain, β-galactosidase synthesis was induced about 10-fold and 20-fold during growth with added nitrate and nitrite, respectively. Induction was increased in the *narL* null strain but essentially eliminated in the *narP* null strain. These patterns of expression have been interpreted as revealing phospho-NarP activation and phospho-NarL-dependent antagonism of expression from the *napF*$_{Ec}$ control region (11).

Patterns of expression from the Φ(*napF*$_{Hi}$-*lacZ*) fusion (Table 3) were strikingly different. In the *narP*$^+$ *narL*$^+$ strain, β-galactosidase synthesis was induced about 10-fold and 4-fold by growth with added nitrate and nitrite, respectively (see also Table 2). Induction by nitrate was unaffected by introduction of either the *narL* null or the *narP* null allele. However, the level of induction by nitrite was increased in the *narL* null strain and decreased in the *narP* null strain. As observed also with the Φ(*napF*$_{Ec}$-*lacZ*) fusion, expression in the *narP narL* double null strain remained at the anaerobic level irrespective of added nitrate or nitrite. These results indicate that phospho-NarP and phospho-NarL are equally effective activators of expression from the *napF*$_{Hi}$ control region in nitrate-grown cultures. The different expression levels in nitrite-grown cultures in the *narL* versus *narP* null strain likely reflect differences in nitrite signaling by the cognate sensors, NarX and NarQ (53).

ModE protein does not regulate expression from the *napF*$_{Hi}$ control region in *E. coli*. Finally, we determined the effect of a null allele in the *modE* gene, encoding the molybdate-responsive regulator (49). Expression from the full-length *napF*$_{Ec}$ control region requires both the *modE*$^+$ gene and the *cis*-acting ModE protein binding site centered 70 nt upstream of the center of the Fnr binding site (41, 54). *H. influenzae* Rd contains the *modE*$^+$ gene as well as several operons whose expression is likely regulated by the ModE protein (58). However, neither computer analysis (58) nor visual inspection (Fig. 1) has identified a candidate ModE protein binding site in the *napF*$_{Hi}$ control region.

We examined Φ(*napF*$_{Hi}$-*lacZ*) expression in *E. coli* in response to molybdate and *modE* genotype as previously described (54). As controls, we included strains carrying the Φ(*napF*$_{Ec}$-*lacZ*) Δ146 and Φ(*napF*$_{Ec}$-*lacZ*) Δ123 fusions, which, respectively, retain and lack the upstream ModE protein binding site (54). Expression from both the Φ(*napF*$_{Hi}$-*lacZ*) Δ260 fusion and the Φ(*napF*$_{Hi}$-*lacZ*) Δ110 fusion was indifferent to molybdate and *modE* genotype (data not shown). We conclude that expression from the *napF*$_{Hi}$ control region is not regulated by the ModE protein in *E. coli* and is likely not regulated by the ModE protein in *H. influenzae*.

DISCUSSION

Surrogate genetics exploits a well-characterized host to study gene function from a related but experimentally less-tractable species (37). The close phylogenetic affiliation between *E. coli* and *H. influenzae* (14) makes the former a suitable surrogate for the latter. Thus, *E. coli* has been used as a host for studying aspects of *H. influenzae* gene function, including regulation by the Fnr and ArcB proteins (19, 30, 39). Results on control of Φ(*napF*$_{Hi}$-*lacZ*) expression in *E. coli* presented here therefore may provide a close approximation for control of *napF*$_{Hi}$ operon expression in *H. influenzae*. Nevertheless, our primary motivation for studying Φ(*napF*$_{Hi}$-*lacZ*)

expression in *E. coli* was to develop a relatively simple native model system for studying Fnr class I and phospho-NarP class II transcription activation mechanisms.

Results with *E. coli* suggest that the phospho-NarP protein directly activates transcription from the Fnr class I *napF* promoter (11, 13) and that the phospho-NarL protein directly activates transcription from the Fnr class II *narG* and *fdnG* operon promoters (34, 35). By contrast, the phospho-NarL and -NarP proteins act indirectly to stimulate transcription initiation from the Fnr class II *nirB* and *nrfA* promoters (7, 67). For discussion, we adopt the hypothesis that phospho-NarP and -NarL directly activate transcription from the $napF_{Hi}$ promoter.

Transcription activation from Nar-regulated promoters is synergistic: the magnitude of expression is much greater when both the Fnr regulator and the NarL or NarP regulator are active than when either is inactive (for examples, see Tables 2 and 3). One model to explain synergistic transcription activation is that the two activators make contact simultaneously with distinct components of RNA polymerase (2, 26). This distinct-contact model can explain results from studies with the Fnr class II *narG* operon promoter, which we summarize here before considering how analogous models might apply to transcription activation at the Fnr class I *napF* operon promoters.

Transcription activation by phospho-NarL in synergy with Fnr (class II). The Fnr protein interacts both with region 4 of the σ^{70} subunit and with the α-CTD subunit to stimulate *narG* operon transcription initiation from a class II site (position −42.5) (4, 31, 32, 36). These deduced interactions are supported by analogy to the well-studied homolog Crp (6). Fully induced *narG* operon transcription additionally requires the phospho-NarL protein, acting from upstream (class I) positions (57). The hypothesis that the Fnr and phospho-NarL proteins make distinct contacts with RNA polymerase is supported by two observations on expression of the *narG* operon control region.

The first observation concerns positive control missense substitutions in the *fnr* and *rpoD* genes that significantly decrease *narG* operon transcription initiation. For most of these mutants, addition of nitrate to generate phospho-NarL protein results in near-wild-type levels of expression (31, 36). This indicates that the second transcription activator (phospho-NarL) can overcome blocks to action by the first (Fnr).

The second observation concerns the *narG* operon promoter −10 region (5′-TACCTT), which is a relatively poor match to the consensus. Alteration to a consensus −10 sequence (5′-TATAAT) bypasses the need for Fnr activation (60), much as the UV5 alteration in the *lacZ* operon promoter bypasses the need for Crp activation (50). Remarkably, transcription initiation from this mutant *narG* promoter is stimulated by the phospho-NarL protein even in an *fnr* null mutant (60). This demonstrates that, given the proper promoter structure, the phospho-NarL protein can stimulate transcription initiation independently of the Fnr protein.

Class I transcription activation by Fnr. Class I control regions have a Crp or Fnr binding site located upstream of the promoter, and studies with model control regions reveal optimal spacing of −61.5, −71.5, −82.5, or −92.5 nt from the transcription initiation point to the center of the dyad (8, 65). To date, the only well-characterized Fnr class I control regions have been synthetic constructs based on the *melR* promoter (32, 64, 65) and the native $napF_{Ec}$ control region (9, 11, 13, 54). Here, we present evidence that the $napF_{Hi}$ control region Fnr protein binding site is centered at position −81.5 (Fig. 1; Table 2) and therefore represents an additional characterized example of a native class I Fnr-activated promoter. Other recently described class I Fnr-activated promoters are those for the *ydjX* gene (27) and also for the *hcp-hcr* operon, transcription of which requires phospho-NarP or -NarL for full expression (15).

Transcription activation by phospho-NarP or -NarL in synergy with Fnr (class I). Large-scale analysis of positive control mutants has not yet been applied to analysis of the *napF* operon control regions. Our working hypothesis is that activation of transcription from these promoters also involves distinct contacts to RNA polymerase. Phospho-NarP or -NarL, bound at a class II position (Fig. 1), is in position to make contact both with region 4 of the σ^{70} subunit and with the α-CTD, whereas the Fnr protein, bound at a class I position, is in position to make contact with the α-CTD (5).

Transcription from both the $napF_{Ec}$ and the $napF_{Hi}$ operon control regions is activated synergistically by the Fnr and phospho-NarP proteins. However, transcription from the native $napF_{Ec}$ control region is antagonized rather than activated by the phospho-NarL protein, leading to the notion that phospho-NarL is not an effective transcription activator from a proximal (class II) binding site (11, 13). Recently, however, we found that transcription initiation from a mutant version of the $napF_{Ec}$ control region, lacking the minor promoter P2, is stimulated by the phospho-NarL protein (54). Here, we present evidence that phospho-NarL and -NarP proteins, bound at a class II position in the wild-type $napF_{Hi}$ control region, stimulated transcription initiation equally well in nitrate-grown cultures (Table 3). Therefore, given the appropriate promoter context, the phospho-NarL protein can stimulate transcription initiation from a class II position. Presumably, the structure of the wild-type $napF_{Ec}$ control region prevents contacts between RNA polymerase and phospho-NarL but not between RNA polymerase and phospho-NarP. This would imply that the two Nar response regulators make different contacts to RNA polymerase.

Similar transcription control regions in proteobacteria. The distances between the centers of the Fnr and phospho-NarP binding sites for the $napF_{Ec}$ and $napF_{Hi}$ control regions are 20 and 39 nt, respectively. This suggests that the DNA helical phase is important for their synergistic transcription activation functions. We therefore used the National Center for Biotechnology Information World Wide Web portals to search for additional examples of potential upstream Fnr and downstream phospho-NarP binding sites with center-to-center spacing in increments of approximately 10 nt (results not shown). Several such cases were apparent in the regions upstream of the *napF* operon in genome sequences from members of the families *Pasteurellaceae* and *Vibrionaceae*, as well as in the regions upstream of the *ccmA* operon, encoding cytochrome *c* maturation functions, from members of the family *Pasteurellaceae*. These and a few additional examples all have center-to-center spacing near 30 or 40 nt. Each of these genomes contains genes for the NarQ-NarP (but not the NarX-NarL) two-component system (53). Therefore, the architecture of the

napF$_{Hi}$ control region may be broadly representative of those subject to synergistic activation by the Fnr and phospho-NarP proteins in a range of species classified in the gamma subdivision of the proteobacteria.

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