Dual Overlapping Promoters Control napF (Periplasmic Nitrate Reductase) Operon Expression in Escherichia coli K-12

Valley Stewart,1,2* Peggy J. Bledsoe,1 and Stanly B. Williams2†

Section of Microbiology, University of California, Davis, California 95616,1 and Section of Microbiology, Cornell University, Ithaca, New York 148532

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Escherichia coli elaborates a flexible respiratory metabolism, involving differential synthesis of isoenzymes for many oxidation and reduction reactions. Periplasmic nitrate reductase, encoded by the napFDAGHBC operon, functions with concentrations of nitrate that are too low to support respiration by membrane-bound nitrate reductase. The napF operon control region exhibits unusual organization of DNA binding sites for the transcription regulators Fnr and NarP, which activate transcription in response to anaerobiosis and nitrate, respectively. Previous studies have shown that the napF operon control region directs synthesis of two transcripts whose 5′ ends differ by about 3 nucleotides. We constructed mutant control regions in which either of the two promoter −10 regions is inactivated. Results indicate that the downstream promoter (P1) was responsible for Fnr- and NarP-regulated napF operon expression, whereas transcription from the upstream promoter (P2) was activated only weakly by the Fnr protein and was inhibited by phospho-NarP and -NarL proteins. The physiological function of promoter P2 is unknown. These results establish the unconventional napF operon control region architecture, in which the major promoter P1 is activated by the Fnr protein bound to a site centered at −64.5 with respect to the transcription initiation site, working in conjunction with the phospho-NarP protein bound to a site centered at −44.5.

Escherichia coli, a facultative aerobe, synthesizes a diverse repertoire of anaerobic respiratory enzymes in response to electron acceptor availability (12, 34). Enzyme synthesis is controlled at the level of transcription initiation by the transcription activators Fnr, an iron-sulfur protein responsive to anaerobiosis (15, 16), and the NarL and NarP proteins, response regulators whose phosphorylation is controlled by the sensors NarX and NarQ in response to nitrate and nitrite (9, 33). Together, these regulatory proteins act to ensure a hierarchal response to electron acceptors in accordance with their standard redox potentials.

Periplasmic nitrate reductase (NapABC enzyme) is encoded by the napFDAGHBC operon, in which napA encodes the catalytic subunit, napB and napC encode cytochromes c, napD encodes a protein likely required for enzyme assembly, and napF, napG, and napH encode proteins that couple ubiquinol oxidation to nitrate reduction (2, 3, 13, 24). This operon was initially identified by Choe and Reznikoff as an anonymous anaerobically expressed gene at map coordinate 46.5 (aeg-46.5 locus), whose expression is activated by the Fnr protein and inhibited by the NarL protein (6). In E. coli, the NapABC enzyme permits nitrate respiration during growth with low concentrations of nitrate (24, 31, 35).

Transcriptional control regions for several E. coli operons encoding anaerobic respiratory enzymes have been characterized. The napF operon transcriptional control region is exceptional. First, in the other operons, the Fnr protein binding site is centered at or near position −41.5 with respect to the transcription initiation site (15) and the NarL-NarP binding sites are upstream of the Fnr binding site (9, 33). By contrast, the napF operon Fnr site is centered at −64.5 and the NarL-NarP site is located downstream, centered at position −44.5 (Fig. 1) (7, 9, 10). Second, nitrate and nitrite effectively activate transcription in narP+ narL null strains, but not in narL− strains (6, 8, 10, 26). Evidence suggests that the phospho-NarL protein competes with the phospho-NarP protein for binding to the site centered at −44.5 but that only the phospho-NarP protein is able to activate transcription from this location (8, 10). Third, a ModE protein binding site centered at −134.5 confers molybdate-responsive napF operon expression, but deletion of this site renders expression independent of the modE+ gene (21). The mechanism by which the molybdate-responsive ModE protein controls napF operon expression has not been determined. Finally, in continuous culture, expression of the napF operon is maximal during growth with a relatively low concentration of nitrate, 1 mM (35).

Both in vivo and in vitro analyses revealed two initiation sites for napF operon transcription (7, 10). The upstream site (denoted T2 in Fig. 1) was observed in aerated cultures, whereas the downstream site (denoted T1 in Fig. 1) was observed in anaerobic cultures supplemented with nitrate (7). In a defined in vitro transcription system, the T2 transcript is weakly synthesized irrespective of added Fnr protein, but its synthesis is inhibited in reactions containing phospho-NarP or phospho-NarL proteins. By contrast, the T1 transcript is synthesized only in reactions containing both Fnr and phospho-NarP proteins (10). Overlapping potential −10 elements are positioned appropriately with respect to these two transcription initiation sites (denoted P2 and P1 in Fig. 1). Here we report experiments designed to determine the roles of these two promoters.
were then recloned into the vector pRS414. The resulting nate isolates with spurious nucleotide substitutions. The control region cassettes tagenesis, the DNA sequence for the entire fragment was determined to elimi-
mM glucose as the carbon source, and the respiratory oxidants NaNO₃ and
was set at 8.0 to ameliorate nitrite toxicity. Because the pKa
of MOPS is 7.2, the buffering capacity of this medium
empirically to support growth to the mid-exponential phase (about 35 to 40 Klett
C. Culture densities were monitored with a Klett-
°Cultures were grown at 37

Culture media and conditions. Defined, complex, and indicator media for
genetic manipulations were used as described previously (20). Defined medium
grow cultures for enzyme assays was buffered with 3-[N-morpholino]propane-
sulfonic acid (MOPS) as previously described (32). The initial pH of this medium
was set at 8.0 to ameliorate nitrite toxicity. Because the pKa 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 of about 7.5.
Medium for batch cultures grown to the mid-exponential phase contained 80
mM glucose as the carbon source, and the respiratory oxidants NaNO₃ and
NaNO₂ were added to 40 and 5 mM, respectively. Medium for overnight cultures
arrested in the mid-exponential phase (30) contained glucose (6 mM), glucose
plus NaNO₃ (4 and 10 mM, respectively), or glucose plus NaNO₂ (6 and 8 mM,
respectively) as indicated in Tables 2 to 6. These concentrations were determined
and reported values are averages from at least two independent experiments.
Activities are expressed in arbitrary units (22). All cultures were assayed in duplicate,
results are virtually identical to those obtained with primer

Materials and methods

Strains and plasmids. Strains and plasmids are listed in Table 1. Control
region nucleotide sequences are depicted in Fig. 1. Genetic crosses were
performed by P1Cre-mediated generalized transduction (22). Null alleles of nar
regulatory genes (26) and the napF1::Km allele (31) have been described previ-
ously. Standard methods were used for restriction endonuclease digestion, liga-
tion, transformation, and PCR amplification of DNA (20).
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respectively) as indicated in Tables 2 to 6. These concentrations were determined
empirically to support growth to the mid-exponential phase (about 35 to 40 Klett
units).
Cultures were grown at 37°C. Culture densities were monitored with a Klett-
Summern photoelectric colorimeter (Klett Manufacturing Co., New York,
N.Y.) equipped with a number 66 (red) filter. Anaerobic cultures for enzyme
assays were grown in screw-cap tubes as described previously (32).
Enzyme assay. β-Galactosidase activities were determined at room tempera-
ture (approximately 21°C) by monitoring the hydrolysis of o-nitrophenyl-β-
galactoside in CHCl₃-sodium dodecyl sulfate-permeabilized cells. Specific activ-
ities are expressed in arbitrary units (22). All cultures were assayed in duplicate,
and reported values are averages from at least two independent experiments.
Construction of napF operon control region alterations. Oligonucleotide-di-
rected site-specific mutagenesis was used to introduce substitutions into the napF
operon control region. Mutagenesis followed either the ampicillin selection pro-
tocol (17) or the QuickChange protocol (Stratagene Cloning Systems, La Jolla,
Calif.) as described previously (1). PCRs were performed with a high-fidelity thermostable DNA polymerase (Accuzyme; Bioline USA, Reno, Nev.).
Plasmid pVJS1523 contains the napF operon control region on a 465-bp DNA
fragment from an engineered EcoRI site at position −146 to an engineered BamHI site downstream of napF codon 76 (8). Following each round of mu-
tagenesis, the DNA sequence for the entire fragment was determined to elimi-
nate isolates with spurious nucleotide substitutions. The control region cassettes
were then recloned into the vector pRS414. The resulting Φ(napF-lacZ) operon
fusions were crossed into bacteriophage λR345 (29), and monocyte lysogens were
identified by a whole-colony PCR test (25).

Transcript analysis. Primer extension experiments were performed essentially
developed previously (39). The primer 5'-CTTGTGCAGCGAGGACATGGA
TGAG corresponds to napF codons 11 to 17 in the template strand, and there-
fore its proximal (3') end is 108 nucleotides (nt) downstream of the transcription
initiation site T1. The primer was radio labeled at the 5' end and used for
extension reactions on RNA samples isolated from wild-type and mutant strains
cultured to the mid-exponential phase anaerobically in the absence or presence
of nitrate. Extension products were resolved on a thin polyacrylamide-urea slab
gel and visualized by phosphorimager analysis. The same primer was used for
parallel DNA sequencing reactions to provide size markers for the extension
products.
5' rapid amplification of cDNA ends (RACE) analysis (11), also termed
anchored PCR (18), used reagents purchased from Invitrogen Life Technologies
(Carlsbad, Calif.) and was performed essentially as described by the manufac-
turer's instructions. Gene-specific primers were 5'-GGGTAACGCCAGGGTT
TTC for the first round (cDNA synthesis) and 5'-CTTAGTGAATTCCGTAA
TCATGTCATAG for the second round (PCR). Anchor primers were 5'-GG
CCAGGCGTCGACTAGTACGGIGIGGGIGGII (where I stands for
inosine) for dCTP-tailed cDNA products and 5'-GCGCCAGCGGTCGACTAGT
AACCCCACCCCCCCCAC for dGTP-tailed cDNA products.

Results

Transcription initiation sites. Choe and Reznikoff reported
results from primer extension experiments to define the 5' ends of napF operon mRNA species (7). The 5' ends of mRNA
extracted from cultures grown anaerobically with nitrate
mapped to the A residue indicated as T1 in Fig. 1, whereas
those from aerated cultures mapped to the pair of G residues
3 to 4 nt upstream (indicated as T2). To confirm these results,
we employed 5' RACE analysis, as described in Materials and
Methods, on RNA extracted from anaerobic cultures grown
in the absence or presence of nitrate. We sequenced approxi-
mately 12 of the resulting cDNA clones obtained from each of
the two culture conditions over several independent experi-
ments. Results (not shown) revealed 5' mRNA ends from
anaerobic cultures at both G residues (T2) and 5' mRNA ends
from anaerobic cultures with nitrate at the A residue (T1) and
also at the T residue immediately upstream (Fig. 1). These
results are virtually identical to those obtained with primer
extension by Choe and Reznikoff (7).

To simplify discussion and retain consistency with previous
FIG. 1. The napF operon control region. Numbering is with respect to the transcription initiation site T1. Thick upper- and underlines, −10 and
−35 elements. Sequences for binding the NarP and NarL, Fnr, and ModE proteins are boxed; consensus sequences are shown below. Deletion
end points of control region constructs used in this study are indicated. Thin underlines, Shine-Dalgarno regions for the
ecO gene and the divergently transcribed eco gene.

in controlling napF operon transcription. Results indicate that
promoter P1 is largely responsible for Fnr- and NarP-regulated
expression of the napF operon.

E. coli napF operon promoters
Plasmids

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</table>

Plasmids

| pRS415 | Ap⁺; lacZ operon fusion vector | 29 |
| pVJS1523 | Ap⁺; napF control region; EcoRI (–146) to BamHI (+305) in pGEM7Zf(+) | 8 |

In this study, we assign the A residue (T1) +1 for numbering the sequence. For specific reference to the T2 transcript, we designate the G residue at position −3 the transcription initiation site (Fig. 1 and 2).

Two mutationally separable napF operon promoters. As noted previously (7, 10), the two transcription initiation sites are associated with overlapping potential −10 promoter elements (TAATATCTT; Fig. 1). The upstream element, which we denote P2, has two mismatches (underlined) from the −10 element consensus sequence (TAATAT versus TATAAT). The downstream element, which we denote P1, also has two mismatches (TATCTT). A potential −35 element has three mismatches from the consensus sequence (TATGCA versus TTGACA); it is located 16 bp upstream of the P2 −10 element.

**FIG. 2.** Promoter region nucleotide substitutions. The binding sites for the Fnr protein and the NarP and NarL proteins are indicated by boxes. The consensus sequences for the Fnr, −35, and −10 elements are in boldface. Substitutions are indicated above or below the corresponding consensus sequences. Spacers of 5 and 10 bp between the Fnr binding site and the NarP and NarL binding sites are in lowercase letters and underlined.
and 19 bp upstream of the P1 −10 element (7). This −35 element overlaps the distal end of the NarP-NarL binding site centered at position −44.5 (Fig. 1).

We constructed mutant control regions carrying multiple contiguous substitutions designed to ablate these specific promoter elements individually. For simplicity, we omit description of several substitutions that were made and analyzed in exploratory experiments, the results from which are congruent with those presented here. Changes in the −10 region were designed to eliminate either the P2 or the P1 element without affecting the other (Fig. 2). Changes in the −35 region were designed to eliminate this element without affecting the NarP-NarL protein binding site (Fig. 2). Finally, a −10 region change was designed to eliminate both elements P2 and P1, by altering their common 3-bp core sequences.

Each set of nucleotide substitutions was introduced into a λΦ(napF-lacZ) specialized transducing phage and integrated into the E. coli chromosome in monocopy as described in Materials and Methods. Strains were cultured in defined medium under four different growth conditions: aerated, anaerobic with nitrate, and anaerobic with nitrite.

Materials and Methods. Strains were cultured in de

<table>
<thead>
<tr>
<th>Strain</th>
<th>Version of promoter(a)</th>
<th>LacZ sp act(b)</th>
<th>Levels of activation by:</th>
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<td></td>
<td>−35 P2 P1</td>
<td>+O_2 −NO_3(^{-}) −NO_3(^{-}) +NO_3(^{-}) +O_2</td>
<td>−O_2 NO_3(^{-}) NO_3(^{-})</td>
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<tr>
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<td>VJS7431</td>
<td>− + −</td>
<td>30</td>
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\(^{a}\) Strains were cultured overnight in MOPS defined medium with limiting glucose. NO_3\(^{-}\), nitrate or nitrite.

\(^{b}\) Wild-type (+) or mutant (−) for indicated promoter elements (see Fig. 2). The P2 − P1 alteration affects the common 3-bp core sequence.

We used primer extension analysis, as described in Materials and Methods, to examine mRNA 5′ ends. During anaerobic growth in the absence of nitrate, the wild-type and P2 − P1 constructs directed synthesis of modest levels of mRNA with 5′ ends corresponding to transcript T2 (Fig. 3, lanes 1 and 2), whereas during anaerobic growth in the presence of nitrate the wild-type and P2 − P1 constructs directed the synthesis of substantial levels of mRNA with 5′ ends corresponding to transcript T1 (Fig. 3, lanes 5 and 6). We were unable to detect transcription from the P2 − P1 and P2 − P1 constructs during anaerobic growth in the absence and presence of nitrate, respectively (Fig. 3, lanes 3 and 4). Overall these patterns support the results from measuring Φ(napF-lacZ) expression. Further analysis is necessary, however, to confirm that P1 and P2 are essential for nitrate and nitrite induction of napF operon transcription.

The alterations to the common 3-bp core sequences of the P2 and P1 elements (Fig. 2) eliminated Φ(napF-lacZ) expression under all conditions (Table 2). This demonstrates that these two promoters are solely responsible for napF operon transcription. The alterations to the −35 element had relatively modest effects on Φ(napF-lacZ) expression both singly and in combination with alterations to the P2 and P1 elements (Table 2). Considering also the relatively weak match of this element to the −35 consensus and its proximity to the NarP-NarL binding site, we therefore draw no conclusions regarding its role in napF operon expression.

FIG. 3. Primer extension analysis of promoter mutations. RNA was extracted from wild-type (WT), P2 − P1 (P2), and P2 − P1 (P1) strains grown anaerobically in the absence (−) or presence (+) of nitrate as indicated. T1 and T2, transcription initiation sites (Fig. 1); G, A, T, and C, lanes for DNA sequencing reactions. These data are from a single experiment, with samples electrophoresed on the same gel; the gel lane images have been rearranged from the original order.
ther, this demonstrates that these control region substitutions did not result in the formation of alternate promoters.

Effects of fur, narP, and narL null alleles on expression from the two napF operon promoters. The fur null allele sharply reduced $\Phi(napF-lacZ)$ expression from both the wild-type and $P_2^- P_1^+$ control regions but by contrast had only a mild effect on expression from the $P_2^+ P_1^+$ control region (Table 3). These results indicate that the Fnr protein is a strong transcription activator for the $P_1$ promoter but only a modest activator for the $P_2$ promoter.

Previous experiments determined that, during anaerobic growth, $\Phi(napF-lacZ)$ expression is induced about 10-fold by nitrate and about 20-fold by nitrite. In a narL null strain, nitrate induction is induced about 20-fold, whereas in a narP null strain induction by either nitrate or nitrite is virtually nil. This reflects the roles for the NarP and NarL proteins in activating and antagonizing, respectively, transcription initiation (8). These observations are recapitulated in Table 4.

Overall, expression patterns from the $P_2^+ P_1^+$ and the $P_2^- P_1^+$ control regions were very similar. In both narP$^- narL^+$ and narP$^+ narL^-$ null strains, $\Phi(napF-lacZ)$ expression was induced during growth with nitrate (Table 4). This induction was more pronounced in the narL null strains, reflecting the antagonistic role for the phospho-NarL protein in controlling napF operon expression (8). Additionally, the narP narL double-null strains both exhibited about a 10-fold reduction in basal-level $\Phi(napF-lacZ)$ expression. Previously, an analogous observation was made with a narQ narX double-null strain lacking both nitrate sensors (26). This suggests that low levels of phosphorylated NarP and NarL proteins may be present during growth in the absence of an inducer and are able to stimulate significant levels of transcription.

There was one striking exception to the similar expression patterns from these two control regions: the narP null narL$^+$ strain, carrying the $P_2^- P_1^+$ control region, exhibited about a 25-fold induction in response to nitrate (Table 4; strain VJS7454). This was due in significant measure to the nearly 10-fold decrease in basal-level anaerobic expression in the absence of nitrate (28 Miller units in narP null narL$^+$ strains versus 200 Miller units in narP$^+ narL^+$ strains). Thus, the mutant control region differed from the wild type in its response to the NarL protein.

Anaerobic $\Phi(napF-lacZ)$ expression from the $P_2^+ P_1^-$ control region was inhibited two- to threefold by nitrate when either the narP$^+$ or narL$^-$ gene was present, suggesting that

### Table 3. Alterations in the promoter region and an fur null allele influence $\Phi(napF-lacZ)$ expression

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<td>- -</td>
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<td>240</td>
</tr>
<tr>
<td>VJS7440</td>
<td>-</td>
<td>+ -</td>
<td>120</td>
<td>100</td>
</tr>
</tbody>
</table>

$^a$ Strains were cultured overnight in MOPS defined medium with limiting glucose. $\delta$, nitrate or nitrite.

### Table 4. Alterations in the promoter region and narL and narP null alleles influence $\Phi(napF-lacZ)$ expression

<table>
<thead>
<tr>
<th>Strain</th>
<th>Version of narL:</th>
<th>Promoter:</th>
<th>LacZ sp act$^b$ without $\delta$</th>
<th>Level of activation by $\delta$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>narL  narP</td>
<td>P2 P1</td>
<td>$-\delta +\delta$</td>
<td>$+\delta$</td>
</tr>
<tr>
<td>VJS4789</td>
<td>+</td>
<td>+ +</td>
<td>220</td>
<td>2,000</td>
</tr>
<tr>
<td>VJS7442</td>
<td>-</td>
<td>+ +</td>
<td>280</td>
<td>7,430</td>
</tr>
<tr>
<td>VJS7452</td>
<td>+</td>
<td>- +</td>
<td>160</td>
<td>320</td>
</tr>
<tr>
<td>VJS7455</td>
<td>-</td>
<td>- +</td>
<td>20</td>
<td>30</td>
</tr>
<tr>
<td>VJS6990</td>
<td>+</td>
<td>+ -</td>
<td>200</td>
<td>2,970</td>
</tr>
<tr>
<td>VJS7444</td>
<td>-</td>
<td>+ -</td>
<td>260</td>
<td>9,070</td>
</tr>
<tr>
<td>VJS7454</td>
<td>+</td>
<td>- +</td>
<td>28</td>
<td>690</td>
</tr>
<tr>
<td>VJS7457</td>
<td>-</td>
<td>- +</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>VJS6988</td>
<td>+</td>
<td>+ -</td>
<td>240</td>
<td>73</td>
</tr>
<tr>
<td>VJS7443</td>
<td>-</td>
<td>+ -</td>
<td>250</td>
<td>120</td>
</tr>
<tr>
<td>VJS7453</td>
<td>+</td>
<td>- +</td>
<td>270</td>
<td>80</td>
</tr>
<tr>
<td>VJS7456</td>
<td>-</td>
<td>- +</td>
<td>260</td>
<td>410</td>
</tr>
</tbody>
</table>

$^b$ Strains were cultured overnight in MOPS defined medium with limiting glucose. $\delta$, nitrate or nitrite.
the NarL and NarP proteins antagonize expression from the P2 promoter.

Together, these results indicate that transcription initiation from the P1 promoter is strongly regulated by the Fnr, NarP, and NarL proteins and accounts for most of the observed Φ(napF-lacZ) expression in wild-type strains. By contrast, transcription initiation from the P2 promoter seems to be nearly constitutive, exhibiting only weak activation by the Fnr protein and weak inhibition by the NarL and NarP proteins (Tables 3 and 4).

Spacing between the Fnr and NarP protein binding sites. To explore constraints on the spacing between the binding sites for the Fnr and NarP proteins, we constructed two napF operon control region variants with insertions of 5 and 10 nt (Fig. 2). For comparison, we also measured Φ(napF-lacZ) expression from a mutant control region carrying a single-nucleotide substitution (−69G→A) in the Fnr site (8).

Wild-type Φ(napF-lacZ) expression is induced approximately 10-fold by anaerobiosis and an additional 10-fold by nitrate. The previously described −69G→A change mimics an fnr null allele: anaerobic induction and nitrate induction both are sharply decreased (8). The +5 and +10 spacing changes both reduced Φ(napF-lacZ) expression to the same baseline level as the Fnr site substitution (data not shown). We concluded that the Fnr protein binding site cannot readily be moved relative to the NarP protein binding site and so did not pursue more-extensive analysis of this point.

Effects of a modE null allele on expression from the two napF operon promoters. Molybdate (MoO₄²⁻) availability controls anaerobic respiratory gene expression (23) through the molybdate-responsive transcriptional regulator ModE (28). Recently, McNicholas and Gunsalus (21) identified a ModE binding site centered at position −134.5 in the napF operon control region (Fig. 1) and demonstrated that the ModE protein controls Φ(napF-lacZ) expression.

Previous 5’ deletion analysis (8) resulted in two deletions (Δ146 and Δ123) that produce napF operon control regions that differ only in the presence or absence of the ModE protein binding site (Fig. 1). We therefore examined the effect of a modE null allele on Φ(napF-lacZ) expression from these two control region variants. The modABC operon encodes the high-affinity molybdate uptake system, which efficiently scavenges trace molybdate (28). Therefore, these experiments employed modB null strains in order to achieve internal molybdate limitation (23).

In the Δ146 strain, which retains the ModE protein binding site, Φ(napF-lacZ) expression was induced during growth with added molybdate, and this induction required the modE⁺ allele (data not shown). By contrast, in the Δ123 strain, in which the ModE protein binding site is deleted, Φ(napF-lacZ) expression was independent of both molybdate limitation and the modE⁺ allele (data not shown; Table 5). These results are fully congruent with those of McNicholas and Gunsalus (21).

We next examined the effect of a modE null allele on nitrate regulation of Φ(napF-lacZ) expression from P2 − P1⁺ and P2⁺ P1 control region variants in cultures of modB⁺ strains. (These promoter alterations are in the Δ146 version of the napF operon control region, which contains the ModE protein binding site.) Results (Table 5) indicate that the modE null allele similarly affected expression from all three promoter combinations: overall expression was decreased about two- to threefold, but the patterns and magnitude of nitrate regulation were not affected. We conclude that promoters P1 and P2 are subject to equivalent regulation by the ModE protein.

**Effects of narX, narQ, and napA null alleles on napF operon expression.** A previous study of napF operon regulation (26) used a Nap⁻ nap:ßgalKM53 insertion (6). The resulting strain exhibited elevated basal-level napF operon expression, as revealed through β-galactosidase activity, in comparison to the Nap⁺ Φ(napF-lacZ) specialized transductants employed in subsequent work (8). To determine the basis for this difference, we examined the effects on Φ(napF-lacZ) expression of null alleles of the narX and narQ genes encoding the Nar sensors, as well as the napA structural gene. These experiments employed a narL null strain background to eliminate the antagonistic effects of the NarL protein on the induction of napF operon transcription.

A narX null allele had no effect, whereas a narQ null allele resulted in an approximately threefold elevation of basal expression and a twofold reduction in induced expression (Table

<table>
<thead>
<tr>
<th>Strain†</th>
<th>Deletion‡</th>
<th>Version of*:</th>
<th>Promoter:</th>
<th>LacZ sp act* without O₂</th>
<th>Level of activation by NO₃⁻</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>modE</td>
<td></td>
<td>−NO₃⁻</td>
<td>+NO₃⁻</td>
</tr>
<tr>
<td>VJS4789</td>
<td>Δ146</td>
<td>+</td>
<td>+</td>
<td>380</td>
<td>2,440</td>
</tr>
<tr>
<td>VJS8548</td>
<td>Δ146</td>
<td>−</td>
<td>+</td>
<td>140</td>
<td>920</td>
</tr>
<tr>
<td>VJS6990</td>
<td>Δ146</td>
<td>+</td>
<td>−</td>
<td>290</td>
<td>3,890</td>
</tr>
<tr>
<td>VJS8550</td>
<td>Δ146</td>
<td>−</td>
<td>+</td>
<td>130</td>
<td>1,790</td>
</tr>
<tr>
<td>VJS6998</td>
<td>Δ146</td>
<td>+</td>
<td>−</td>
<td>270</td>
<td>54</td>
</tr>
<tr>
<td>VJS8549</td>
<td>Δ146</td>
<td>−</td>
<td>+</td>
<td>120</td>
<td>30</td>
</tr>
<tr>
<td>VJS4796</td>
<td>Δ123</td>
<td>+</td>
<td>+</td>
<td>410</td>
<td>2,590</td>
</tr>
<tr>
<td>VJS8551</td>
<td>Δ123</td>
<td>−</td>
<td>+</td>
<td>460</td>
<td>2,630</td>
</tr>
</tbody>
</table>

† Strains were cultured overnight in MOPS defined medium with limiting glucose.

‡ +, wild type; −, mutant (see Fig. 2).

* All strains carry the modB⁺ allele.

* Control region deletion in Φ(napF-lacZ) construct (see Fig. 1).
6). A napA null allele likewise produced an approximately threefold elevation of basal expression (Table 6). Thus, expression of the napF operon is sensitive to both narQ regulatory gene and nap structural gene integrity.

**DISCUSSION**

The napF operon exhibits a unique pattern of expression and a unique control region architecture in comparison to other characterized _E. coli_ Fnr- and Nar-regulated operons. Studies cited in this paper focus on determining the basis for the two distinct transcription initiation sites. Evidence suggests that overlapping −10 elements direct the synthesis of these transcripts. Interpretation of these results rests on the assumption that substitutions designed to inactivate one promoter have little influence on the activity or regulation of the other. This seems a reasonable supposition; neither of the −10 elements has an upstream TG (extended −10) motif, and therefore the changes designed to inactivate the promoter P2−10 element are predicted not to influence the promoter P1−10 element.

Analysis of synthetic promoters indicates that the Fnr protein, like the paralogous cyclic AMP receptor protein (Crp), can activate transcription when bound to a site centered at position −61.5 (5, 38). The napF operon P2 promoter appears to represent a natural example of this type of Fnr-dependent promoter. However, introduction of an fnr null allele had relatively little effect on Φ(napF-lacZ) expression in the P2− P1− strain (Table 3). Instead, it was the P2− P1+ promoter, for which the Fnr binding site is centered at −64.5, that exhibited a strong requirement for the fnr+ gene for Φ(napF-lacZ) expression. This conclusion is supported by results from in vitro transcription experiments in which the Fnr protein stimulated synthesis of the T1 transcript but not the T2 transcript (10). Interestingly, position −64.5 is not a permissive location for the Fnr protein to activate transcription from the synthetic promoter (38). One obvious difference between the synthetic promoter and the napF operon promoter is that transcription from the latter is also activated by the NarP protein bound to a site centered at position −44.5.

Based on results obtained with the P2− P1+ and P2+ P1− mutants, we conclude that the P1 promoter is the primary target for both Fnr and NarP activation of transcription initiation (Table 4). However, transcription from the P2 promoter was modestly inhibited (about threefold) by the NarP and NarL proteins in response to nitrate. These conclusions are congruent with results from in vitro transcription experiments (10).

**Why two promoters for napF operon expression?** Examples of overlapping promoters abound in the literature. One familiar example is from the _E. coli_ _gld_ operon (36), in which the two promoters are reciprocally regulated by the Crp protein. Overlapping promoters of unknown physiological function are present in the _E. coli lac_ operon control region (27).

In P2− P1+ and P2+ P1− strains cultured without nitrate, Φ(napF-lacZ) basal-level expression was reduced by approximately 10-fold in napP _narL_ double-null mutants (Table 4). This suggests that the low levels of phospho-NarP (and phospho-NarL) are present even with no added inducer. In continuous cultures, Φ(napF-lacZ) expression is maximal during growth with only about 1 mM added nitrate (35). Thus, the napF control region seems to be tuned for efficient response to low nitrate levels, even the very low levels contaminating deionized water and commercial-grade culture medium components.

By contrast, Φ(napF-lacZ) basal-level expression from the P2− P1+ control region remained high even in the napP _narL_ double-null mutant (Table 4). We do not understand the mechanistic basis for this. Nevertheless, the results overall are consistent with the notion that the P2 promoter serves as the default promoter for _napF_ operon expression under growth conditions when the P1 promoter is not activated (i.e., aerobiosis or anaerobiosis with no added nitrate or nitrite). This idea is supported by the different patterns of transcript synthesis in response to inducing versus noninducing conditions (Fig. 3) (7, 10). Furthermore, the P2 promoter might be activated under growth conditions that we have not explored.

**Activation of napF operon transcription by the phospho-NarL protein.** For strains with the wild-type control region, Φ(napF-lacZ) expression is activated in _narP_ _narL_ null strains, but not in the reciprocal _narP_ _narL_+ strains (8). Likewise, the phospho-NarP protein, but not the phospho-NarL protein, in conjunction with the Fnr protein, activates in vitro transcription from the _napF_ P1 promoter (10). These observations led to the notion that the phospho-NarL protein cannot activate transcription when bound close to the promoter (position −44.5) (8). However, Φ(napF-lacZ) expression from the P2− P1+ construct was efficiently induced by nitrate in a _narL_+ _narP_ null strain (Table 4). This induction was due in large measure to the roughly 10-fold decrease in basal expression described above. This indicates that, at least

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**TABLE 6.** Null alleles of the _narX, narQ, and napA_ genes influence Φ(napF-lacZ) expression

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype&lt;sup&gt;a&lt;/sup&gt;</th>
<th>LacZ sp act&lt;sup&gt;b&lt;/sup&gt; without O&lt;sub&gt;2&lt;/sub&gt;</th>
<th>Level of activation by:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>narX</em></td>
<td><em>narQ</em></td>
<td><em>napA</em></td>
</tr>
<tr>
<td>VJS4799</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VJS5723</td>
<td>−</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VJS5743</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>VJS5123</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>VJS5724</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> Strains were cultured to the mid-exponential phase in MOPS medium (defined medium with glucose). NO<sub>3</sub>−, nitrate or nitrite.

<sup>b</sup> All strains carry a narL null allele (see Table 1 for full genotypes).
in the P2− P1+ mutant, phospho-NarL can activate transcription when bound adjacent to the promoter.

Negative regulation of the NarP response regulator by the NarQ sensor. Considerable evidence suggests that the NarX sensor serves as a negative regulator of NarL function but not NarP function (26, 31, 37). An equivalent role for the NarQ sensor as a negative regulator of NarP function was suggested by the patterns of mrfA (respiratory nitrate reductase) operon expression (26), but this conclusion was tempered by parallel studies on napF operon expression in which the operon exhibited high basal-level expression in the Nap− nap:λp lacMu53 insertion employed. Nap+ Δ(napF-lacZ) specialized transductants exhibit relatively low basal-level napF operon expression (8). Indeed, introduction of a napF null allele resulted in elevated basal-level expression (Table 6). This apparent autoregulation of napF operon expression can be explained by low levels of nitrate as a contaminant in the culture medium (19). The likely physiologically functional periplasmic nitrate reductase in E. coli is to scavenge nitrate in low concentration (24, 35), so it seems plausible that low-concentration nitrate might persist in cultures of Nap− strains.

Strikingly, the narQ null narX− strain also exhibited elevated basal-level Ψ(napF-lacZ) expression, whereas the narQ+ strains did not (Table 6). This implies a negative role for the NarQ protein in countering inappropriate, NarX-dependent phosphorylation in the absence of inducer. Presumably, this negative influence reflects phospho-NarP phosphatase activity. Thus, the Nar regulatory system has an element of symmetry, in which only the cognate sensor-regulator pairs (NarQ-NarP and NarX-NarL) exhibit negative regulatory interactions.

ACKNOWLEDGMENTS

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REFERENCES


