FNR-dependent repression of ndh gene expression requires two upstream FNR-binding sites

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The ndh gene of Escherichia coli encodes a non-proton-translocating NADH dehydrogenase (NdhII) that is anaerobically repressed by the global transcription regulator, FNR. FNR binds at two sites (centred at −505 and −945) in the ndh promoter but the mechanism of FNR-mediated repression appears not to be due to promoter occlusion. This mechanism has been investigated using an aerobically active derivative of FNR, FNR* (FNR-D154A), with ndh promoters containing altered FNR-binding sites. FNR* repressed ndh gene expression both aerobically and anaerobically in vivo. Gel retardation analysis and DNase I footprinting with purified FNR* protein confirmed that FNR interacts at two sites in the ndh promoter, and that FNR and RNA polymerase (RNAP) can bind simultaneously. Studies with three altered ndh promoters, each containing an impaired or improved FNR-site, indicated that both FNR-sites are needed for efficient repression in vivo. The α-subunit of RNAP interacted with two regions (centred at −105 and −46), each overlapping one of the FNR-sites in the ndh promoter. Footprints of the FNR*-RNAP-ndh ternary complex indicated that FNR*-binding at −505 prevents the α-subunit of RNAP from docking with the DNA just upstream of the −35 element. Binding of a second FNR* molecule at the −105 site likewise prevents binding of the α-subunit at its alternative site, thus providing a plausible mechanism for FNR-mediated repression based on displacement of the α-subunit of RNAP.

Keywords: NADH dehydrogenase II, FNR, RNA polymerase, transcription regulation, Escherichia coli

INTRODUCTION

The facultative anaerobe Escherichia coli possesses two NADH dehydrogenases, NdhI and NdhII, which serve as primary dehydrogenases in the aerobic respiratory chain (Calhoun & Gennis, 1993; Calhoun et al., 1993). NdhI, encoded by the nuo operon, is a multi-subunit, membrane-bound, proton-translocating enzyme similar to mitochondrial Complex I (Weidner et al., 1992). NdhII, encoded by the ndh gene, is a monomeric, membrane-associating, non-proton-translocating flavoprotein (Young et al., 1981; Matsushita et al., 1987; Hayashi et al., 1989). The nuo operon and the ndh gene are both repressed by anaerobiosis, the former via the ArcB-A two-component sensor-regulator system (Bongaerts et al., 1995) and the latter via FNR (Spiro et al., 1989).

FNR is a global transcription regulator which activates or represses the expression of target genes in response to anaerobiosis. Its response is mediated by the anaerobic acquisition of a [4Fe-4S] centre leading to dimerization, enhanced binding to the FNR operator sequence (TTGAT----ATCAA) and consequent regulation of transcription (Khoroshilova et al., 1993; Green et al., 1996a, b; Lazazzera et al., 1996). It has also been suggested that FNR normally exists as a monomer in vivo and that it only dimerizes on its target DNA (Melville & Gunsalus, 1996). FNR activates transcription by binding to a site centred at about −41 in target promoters where it makes direct activating contacts with RNA polymerase (RNAP) (Bell & Busby, 1994). The mechanism of FNR-mediated repression is less well

Abbreviations: CRP, CAMP receptor protein; FNR, regulator of fumarate and nitrate reduction; GST, glutathione S-transferase; NdhI (NdhII), NADH dehydrogenase I (II); Pndh, ndh gene promoter region; RNAP, RNA polymerase; αCRM, C-terminal domain of the α-subunit of RNA polymerase.
defined. Repression of ndh gene expression involves FNR-binding at two sites: a high-affinity site (FNR I; TTGAT-----ATCAA) which is centred at -50.5 and contains a perfect match to the FNR-site consensus, and a low-affinity site (FNR II; TTGAT-----ACCCCG) which is centred further upstream at -94.5 and matches the consensus in only one half-site (Fig. 1; Sharrocks et al., 1991; Green & Guest, 1994). The importance of FNR II was inferred from footprints of the ndh promoter region of pGS418, including nucleotides from the vector (italicized), and showing: the FNR-sites, FNR I (-50.5) and FNR II (-94.5); the -35 and -10 hexamers; transcription and translation start points (open arrows above sequence); PCR primers; and relevant restriction sites. The lower case g at the junction between insert and vector was found to be deleted. (b) Comparison of the nucleotide sequences of wild-type (+), impaired (-) and improved (*) FNR-sites showing: the FNR-sites, FNR I (-50.5) and FNR II (-94.5); the -35 and -10 hexamers; and relevant restriction sites. The lower case letters. (i) FNR I; (ii) FNR II; (iii) FNR II; (iv) FNR II.

Fig. 1. Nucleotide sequence and mutagenesis of the ndh promoter of E. coli. (a) Nucleotide sequence of the ndh promoter region of pGS418, including nucleotides from the vector (italicized), and showing: the FNR-sites, FNR I (-50.5) and FNR II (-94.5); the -35 and -10 hexamers; transcription and translation start points (open arrows above sequence); PCR primers; and relevant restriction sites. The lower case g at the junction between insert and vector was found to be deleted. (b) Comparison of the nucleotide sequences of wild-type (+), impaired (-) and improved (*) FNR-sites showing: the FNR-sites, FNR I (-50.5) and FNR II (-94.5); the -35 and -10 hexamers; and relevant restriction sites. The lower case letters. (i) FNR I; (ii) FNR II; (iii) FNR II; (iv) FNR II.

METHODS

Bacterial strains, plasmids and phages. The strains of E. coli, plasmids and phages are listed in Table 1. Strain DH5a was the host for propagating plasmids, JRG3462 was the source of the active FNR variant, RK5279 was the β-galactosidase null strain used for assaying ndh-lacZ fusion activities, and BW313 was used for producing uracil-containing DNA. The fnr* gene was transferred from pFRDA154 (kindly provided by S. J. W. Busby, University of Birmingham, UK) to pUC19 and pBR328 as a 1-64 HindIII-fragment, and the fnr* mutation was transferred to the coding region of the expression plasmid pGS771 (Fig. 2a) by PCR amplification of an fnr* fragment and reconstruction of the intact and resequenced fnr* gene in a 156 bp NcoI-HindIII fragment that was subsequently cloned in pGEX-KG. The primary source of ndh promoter DNA was pGS418 (Green & Guest, 1994). A derivative of pALTER-EX1, pGS82, containing the 430 bp EcoRI-BamHI ndh promoter (Pndh) fragment from pGS418, was used to create three altered ndh promoters (Fig. 1b), Pndh + (FNR-I-impaired), Pndh − (FNR-II-impaired) and Pndh* + (FNR-II-improved), first as pGS82 derivatives (pGS829-pGS831, respectively) and then as pUC18 derivatives (pGS832-pGS834) by inserting the EcoRI-BamHI promoter fragments, in order to create plasmids that are directly analogous to pGS418

expression and that repression is not simply due to promoter occlusion but to an FNR-mediated displacement of the α-subunit of RNAP.

In this work the mechanism of ndh repression was investigated by a combination of in vivo and in vitro approaches using an aerobically active FNR variant, FNR* (Kiley & Reznikoff, 1991; Lazazzera et al., 1993). FNR* greatly facilitated the use of most in vitro techniques by eliminating the need for anaerobic activation of the regulator. The results showed that both FNR-sites are required for full repression of ndh gene expression and that repression is not simply due to promoter occlusion but to an FNR-mediated displacement of the α-subunit of RNAP.

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### Table 1. Strains of *E. coli* and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or relevant features</th>
<th>Source or reference</th>
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<td><strong>E. coli</strong></td>
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<td>DH5a</td>
<td>ΔlacU169 (g80lacZΔM15) supE44 hsdR17 recA endA1 gyrA96 thi-1 recA1</td>
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<td>CAG627</td>
<td>lacZΔ76 trpE phoA4 supC4 ma lon rpsL</td>
<td>Green et al. (1996b)</td>
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<td>JRG3462</td>
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<td>RK3279</td>
<td>ΔlacU169 frr-250 ara gyrA non rpsL</td>
<td>V. Stewart, Cornell University, USA</td>
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<td>JRG1991</td>
<td>inDH–lacZ (ΔG211) lysogen of RK3279; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Spiro et al. (1989)</td>
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<td>BW313</td>
<td>Hfr lysA thi relA dut ung</td>
<td>Kunkel et al. (1987)</td>
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<td><strong>Plasmids</strong></td>
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<td>pGS24</td>
<td>pBR322 derivative with frr gene in 1,64 kb HindIII–BamHI fragment; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Shaw &amp; Guest (1982)</td>
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<td>pFRNA1DA54</td>
<td>pGS24 with frr&lt;sup&gt;+&lt;/sup&gt; gene in 1,64 kb HindIII–BamHI fragment, no upstream/vector EcoRI sites; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pUC19 with frr from pGS24; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>pBR328 with frr from pGS24; Cm&lt;sup&gt;R&lt;/sup&gt; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Sharrocks et al. (1990)</td>
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<td>pGS1009</td>
<td>pBR328 with frr&lt;sup&gt;+&lt;/sup&gt; from pFRNA1DA54; Cm&lt;sup&gt;R&lt;/sup&gt; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>Pndh&lt;sup&gt;+&lt;/sup&gt; – derivative of pUC18; Ap&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>lacZ operon fusion vector</td>
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<tr>
<td>pGS997</td>
<td>Pndh&lt;sup&gt;+&lt;/sup&gt; + derivative of pGS994; Te&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
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(Table 1). The substitutions were confirmed by nucleotide sequencing at the intermediate stage. A corresponding set of *Pndh–lacZ* operon fusion plasmids (pGS994-pGS997) was constructed in a low-copy vector (pRW50, kindly provided by S. J. W. Busby) for investigating their regulation by FNR and FNR<sup>R</sup>. Two synthetic primers, S352 (5′-TAGCTAGAATTCTGTGGGTCGGATAAGGCC-3′) and S353 (5′-AGTGGTCAACGTGACCCCC-3′), were used to PCR-amplify four 367 bp *ndb* promoter fragments flanked by EcoRI and BamHI sites (Fig. 1a), prior to subcloning in pRW50 and sequence confirmation (Table 1). An alternative approach, involving the construction of monolysogenic strains containing different *ndh–lacZ* translational fusions (ΔG211) by subcloning the altered EcoRI–BamHI fragments to the corresponding fusion vector and transfer to *JRZS* (Spiro et al., 1989), was attempted but failed. This ultimately proved to be due to a single G deletion and consequent reading-frame shift that had occurred at a *Pndh–Smal* junction during the construction of the parental plasmid, pGS418 (Fig. 1a). This probably explains why the *ndb–lacZ* translational fusions of two plasmids (pGS602 and pGS603) derived from pGS418 had very low activities (Green & Guest, 1994).

#### Microbiological methods.

Cultures were grown routinely at 37 °C with vigorous shaking in L-broth with ampicillin (100 μg ml<sup>−1</sup>), tetracycline (35 μg ml<sup>−1</sup>) or chloramphenicol (10 μg ml<sup>−1</sup>), as required. β-Galactosidase activities expressed from *ndb–lacZ* protein and operon fusions were assayed according to Miller (1972) using cultures grown in L-broth plus glucose (0-4%), either aerobically or anaerobically (in screw-cap bottles filled to the neck). The quoted specific activities (in Miller units) are averages from duplicate samples of at least three independent cultures, variations being no more than 15% from the mean.

#### Protein amplification, purification and properties.

The GST–FNR<sup>R</sup> fusion protein was amplified to 13% of soluble protein (equivalent to 5·5 mg FNR<sup>R</sup> per litre of culture) by inducing aerobic cultures of JRG3462 with IPTG (30 μg ml<sup>−1</sup>). It was purified aerobically from French press extracts (Fig. 2b) by chromatography on GSH-agarose (Sigma) and heparin agarose (Green et al., 1996b). The FNR<sup>R</sup> (FNR-D154A) protein was released by cleavage with thrombin (Sigma) and separated from GST by repeating the GSH-agarose step to give a material containing up to 0·75 atoms Fe and 0·02 atoms acid-labile S per monomer indicating that its [4Fe–4S] clusters had been degraded. The purified FNR<sup>R</sup> protein, stored at 4 °C, was partially dimeric (Lazazzera et al., 1993), and had a fourfold higher affinity for target DNA in footprinting reactions than comparable samples of wild-type FNR.
PCR amplifications were carried out under standard conditions with *Pfu* polymerase (Stratagene) or *Taq* polymerase (Promega). The primers used to amplify the *fnr* fragment from pFNRDA154 were S308, 5'-CTGATGCTGATCGCTCCGGGAAAGCCG-3' (fnr coordinates 511–537), and S167, 5'-ACACAATGTCACCGCATTTG-3' (fnr coordinates 1387–1407) (mismatched bases shown in superscript letters). The former incorporates unique BamHI and NcoI restriction sites to facilitate subcloning during the construction of pGS771.

The Altered Sites *in vitro* Mutagenesis System (Promega) was used with uracil-containing template DNA from pGS828, a derivative of pALTER-EXI, to direct specific substitutions in the FNR-sites of *Pndh* (Fig. 1b). Three phosphorylated primers were used simultaneously to make the desired substitutions and at the same time eliminate or repair the respective *tet* or *bla* genes of the vector.

For gel retardation and DNase I footprinting analyses, wild-type and altered or truncated *ndh* promoters contained in the 458 bp EcoRI–HindIII (–259 to +162) or 285 bp HpaII–HindIII (–90 to +162) fragments were obtained from pGS418 and its derivatives (pGS832–pGS834), were end-labelled with [γ-32P]ATP [3000 Ci mmol⁻¹ (111 TBq mmol⁻¹); Amersham] on the lower (HindIII site), upper (EcoRI site), or both strands according to Sambrook et al. (1989). The procedures for gel retardation and DNase I footprinting have been described previously (Green & Guest, 1994; Maxam and Gilbert G tracks provided corresponding calibrations in the latter. Gels were dried and autoradiographed at –70 °C. RNAP saturated with 76s was obtained from Pharmacia.

**RESULTS**

**Binding of FNR* to the ndh promoter**

The FNR* protein containing a D154A substitution in the putative dimer interface was used to investigate the mechanism of FNR-mediated repression of *ndh* gene expression. It was chosen because the substitution enhances dimer stability, thus providing a form of FNR that by-passes the need for allosteric activation via the highly unstable [4Fe–4S] clusters, and is sufficiently active to allow gel retardation analysis to be done under aerobic conditions (Kiley & Reznikoff, 1991; Lazazzera et al., 1993). The FNR* protein was obtained by enzymic cleavage of an amplified GST–FNR* fusion protein (Fig. 2; see Methods). Its DNA-binding affinity in footprinting reactions was approximately fourfold higher than comparable samples of wild-type FNR. Gel retardation analysis with FNR* supported previous conclusions from DNase I footprinting with FNR that there are two FNR-binding sites in the *ndh* promoter region (Fig. 3a). Both sites were fully occupied when the FNR* concentration exceeded 500 nM and the concentration of FNR* giving 50% retardation of *ndh* DNA (*K*ₐ) was 250 nM. Removal of the upstream site, FNR II, led to the formation of a single FNR*:ndh complex and a lowering of the *K*ₐ to 140 nM (Fig. 3b). This confirms that FNR has a higher affinity for the conserved consensus in FNR I than the divergent site in FNR II. In similar experiments with added RNAP, the formation of a highly retarded complex confirmed that RNAP and FNR* (like FNR) can simultaneously interact with the *ndh* promoter (Fig. 3c). Further confirmation that the regulator and polymerase can bind simultaneously was
Fig. 3. Formation of an RNAP:FNR*:Pndh ternary complex in vitro. Gel retardation analysis of FNR* interactions with wild-type (a) and truncated (b) ndh promoters. Fragments labelled at both ends (0.2 nM, final concentration) were incubated for 5 min at 37 °C with increasing concentrations of FNR* and fractionated immediately in 5% polyacrylamide gel. Lanes: 1, no FNR*; 2–9, 25, 50, 100, 125, 188, 250, 500 and 1000 nM FNR* dimer, respectively. (c) Effects of adding RNAP under the same conditions as in (a). Lanes: 1, no addition; 2, 500 nM FNR*; 3, 200 nM RNAP; 4, 500 nM FNR* and 10 nM RNAP; 5, 500 nM FNR* and 20 nM RNAP; 6, 500 nM FNR* and 40 nM RNAP; 7, 500 nM FNR* and 80 nM RNAP; 8, 500 nM FNR* and 100 nM RNAP; 9, 500 nM FNR* and 150 nM RNAP; 10, 500 nM FNR* and 200 nM RNAP. (d) DNase I footprints of FNR* and RNAP at the ndh promoter. The ndh promoter fragment (1.5 nM) labelled on the upper strand was incubated with no protein (lane 2) or with 1000 nM FNR* (lane 3); 500 nM RNAP (lane 4); 1000 nM FNR* and 500 nM RNAP (lanes 5 and 6), followed in each case by digestion with 1 unit of DNase I. Lane 1: Maxam-Gilbert G track of the same DNA fragment (2.7 nM). The regions protected by FNR* (filled boxes) and RNAP (open box) are indicated.

obtained from DNase I footprints of the ternary complex (Fig. 3d). On its own, FNR* protected two regions of the ndh promoter (−61 to −40 and −105 to −84) corresponding to the FNR-I- and FNR-II-sites identified previously (Sharrocks et al., 1991; Green et al., 1993), and generated a hypersensitive site at an intermediate position, −73 (Fig. 3d). Likewise, RNAP alone protected from −60 to +20 and generated a hypersensitive site at −49. However, when RNAP was added to the 2FNR*:ndh complex, two protected regions were detected: one extended from −61 to +20, combining that of RNAP (−38 to +20) with FNR* at FNR I (−61 to −38); and the other at −105 to −84 corresponded to FNR* bound at FNR II (Fig. 3, lanes 5 and 6). The loss of the RNAP-induced hypersensitive site at −49 and the reappearance of the hypersensitive site at −73 upon adding FNR* indicates that FNR* changes the upstream region of the RNAP footprint and induces distortion in the DNA between the two FNR-binding sites. Not only do these patterns show that FNR* and RNAP can simultaneously occupy the ndh promoter, they also indicate that the upstream contacts between RNAP and Pndh in the −60 to −38 region are prevented when FNR* is bound at FNR I. It is relevant that another
FNR* protein is reported to bind as a monomer at a site in the hemA promoter TTGAT----cgCAt; Melville & Gunsalus, 1996) that is similar to FNR I1. However, even though FNR I has little more than one half-site, the protection patterns indicate that two FNR* subunits are bound. Close scrutiny of the hemA footprint suggests that the same is true for its poor FNR-site.

**FNR and the α-subunit of RNAP compete for overlapping binding sites in the ndh promoter**

Because it appeared that FNR-mediated repression of Pndh (Fig. 4a, lanes 3 and 4), but it was readily recruited to the ndh promoter when FNR* occupied FNR I, the high-affinity site at -50.5 (Fig. 4a, lanes 5 and 6). However, the α-subunit was no longer recruited when both FNR-sites were occupied (Fig. 4a, lanes 11 and 12). This suggests that repression by FNR may require the disruption of essential contacts between the α-subunit and Pndh.

DNase I footprints of the α-subunit: Pndh complex revealed that α occupies two sites in the ndh promoter: a site (-55 to -37) which overlaps FNR I; and a weakly protected site (-113 to -98) which overlaps FNR II (Fig. 4b). The protected regions were confirmed

![Fig. 4. Interactions of the RNAP α-subunit and FNR* with the ndh promoter.](image)

(a) Gel retardation assays with end-labelled Pndh, FNR* and α-subunit. Lanes (μM FNR* dimer or α-subunit): 1, 7, no protein; 2, FNR* (2); 3, 9, α (4); 4, 10, α (10); 5, FNR* (2) and α (4); 6, FNR* (2) and α (10); 8, FNR* (4); 11, FNR* (4) and α (4); 12, FNR* (4) and α (10). The positions of free DNA and retarded complexes are indicated. (b) DNase I footprinting of Pndh: α-subunit binary complexes. DNase I digestion patterns for Pndh in the absence (-) and presence (+) of α-subunit (83 μM dimer) are aligned with a G-track calibration (M) obtained by DMS-piperidine hydrolysis of the same labelled fragment. (c) Densitometric difference profile for the α-binding regions, track (+) minus (-) after normalizing in the unaffected upstream region. Values below the line denote protection whereas those above the line correspond to hypersensitive sites (arrowed).
by densitometric difference analyses; the binding-site profiles are similar and each contains a hypersensitive site, −46 and −102 (Fig. 4c). It is envisaged that the α-subunit is recruited to the weaker upstream location when FNR* is bound to the high-affinity FNR-I-site centred at −50.5, and that α-binding to its weaker upstream site is likewise prevented when FNR* is bound at the low-affinity FNR-II-site centred at −94.5 (Fig. 1a). By precluding interaction between the α-subunit and ndh promoter DNA, FNR* might thus allow the unrestrained α-subunit to inhibit transcription activation.

**Aerobic repression of Pndh by FNR**

The fnr* mutants were detected by virtue of their ability to activate, albeit to a limited extent, the aerobic expression of a narG–lacZ reporter fusion in vivo (Kiley & Reznikoff, 1991). It was therefore of interest to test whether FNR* could likewise repress ndh expression under aerobic conditions. Studies with a λndh–lacZ monolysogen of RK5279 (Δlac fnr) transformed with analogous fnr, fnr* or control plasmids, showed that FNR* partially represses ndh expression under aerobic conditions (Fig. 5a). The degree of aerobic repression was significantly greater with FNR* than FNR, and ndh expression was likewise more severely repressed by FNR* than FNR under anaerobic conditions (Fig. 5a). The anaerobic derepression of ndh expression observed in the absence of FNR or FNR* (Fig. 5a) is mediated by the amino acid response regulator (Arr) which activates ndh expression under these conditions (Spiro et al., 1989; Green & Guest, 1994).

**Effects of altering the FNR-sites in the ndh promoter**

In previous studies on the interaction between FNR and Pndh it was shown that the upstream FNR-II-site is needed for efficient FNR-mediated repression; the role of the higher affinity FNR-I-site was not defined (Green & Guest, 1994). Here, site-directed mutagenesis was used to generate three ndh promoter variants: Pndh +−, in which the relatively poor upstream FNR-II-site was retained but FNR I was impaired by replacing the discriminatory T in each TTGAT half-site; Pndh −+, in which the discriminatory T in the consensus half-site of FNR II was substituted and the only conserved base in the other half-site (CCCGG) was altered, but FNR I was retained; and Pndh*+, in which FNR II was improved by replacing the poor half-site by the consensus motif, and FNR I was unchanged (Fig. 1b).

Impairing the downstream FNR-I-site inhibited FNR* binding but a weak FNR*:Pndh +− retarded complex was formed due to the continued interaction of FNR* with FNR II (Fig. 6a ii). Protection at FNR II is evidenced by the hypersensitive site at −88 in the footprint (Fig. 6b ii), consistent with the inherently low affinity of FNR II for FNR*. Although a FNR*:RNAP:Pndh +− ternary complex was formed, it contrasted with the wild-type complex insofar as the RNAP component of the footprint was not affected by FNR*, as indicated by the retention of the hypersensitive site at −49 (Fig. 6b). The corresponding ndh–lacZ fusion showed that Pndh +− is still subject to anaerobic repression by FNR and FNR* in vivo despite the lack of interaction with
Fig. 6. Interactions between FNR* and RNAP at altered ndh promoters. (i) Pndh+++, wild-type; (ii) Pndh+-; (iii) Pndh--; and (iv) Pndh++. (a) Gel retardation assays of FNR*:Pndh complexes. End-labelled wild-type and variant Pndh fragments (0.5 nM) were incubated with FNR*. Lanes: 1, no FNR*; 2-8, 50, 100, 188, 375, 625, 1000 and 1250 nM FNR* dimer, respectively. (b) DNase I footprints of FNR*:Pndh and RNAP:FNR*:Pndh complexes at wild-type and variant ndh promoters. Equal amounts of Pndh DNA (3 nM) labelled on the lower strand were incubated with FNR* (0.5 μM dimer) and RNAP (0.5 μM protein). Lanes: 1, calibrating G track; 2, no addition; 3, FNR*; 4, RNAP; 5, FNR* and RNAP. Protection by RNAP (open box) and by FNR* at different FNR-sites [unaltered (filled), impaired (open, dashed line) and improved (open, bold line)] are indicated.
FNR I, although the degree of repression by FNR (or FNR*) was reduced from 7- (or 20-) fold for Pndh + + to 2-9- (or 22-) fold for Pndh + −, relative to the corresponding aerobic activities measured in the absence of FNR (Fig. 5b).

Impairing the poor upstream FNR-II-site produced a single FNR*:Pndh + + retarded complex presumably with FNR* bound at the unaltered FNR-I-site (Fig. 6a iii), and a ternary complex with added RNAP (Fig. 6b ii). The pattern of protection again indicates that FNR* prevents contact between RNAP and Pndh + + at the upstream extremity of the RNAP component of the footprint, as was observed with Pndh + + (Fig. 6b). The presence of the weakened FNR-II-site in Pndh + + did not abolish anaerobic repression in vivo (Fig. 5b) although the presence of the hypersensitive site at −88 suggests that FNR* may still interact to some extent at FNR II. Consistent with the impaired binding to Pndh + +, the degree of repression by FNR (or FNR*) in vivo was lowered to 44- (or 20-) fold at Pndh + + compared with 7- (or 20-) at Pndh + +. The degree of repression was also greater than the factor of 2-9 (or 22) observed at Pndh + − where FNR II is the only active FNR-binding site. The 20-fold repression observed with FNR* probably corresponds to the maximum observable degree of repression.

Improving FNR II whilst retaining FNR I enhanced the binding affinity of FNR* for Pndh + +, as witnessed by the formation of retarded complexes at lower FNR* concentrations compared to Pndh + + (Fig. 6a), and by the enhanced protection of FNR II in DNase I footprints (Fig. 6b). Interestingly, the footprint of the improved FNR II resembled that of FNR I in lacking the hypersensitive base at −88 normally present in the unaltered FNR II footprint. This suggests that the DNA in this region adopts different conformations in the two FNR*:Pndh complexes. Improving the upstream site was also accompanied by an increased degree of anaerobic repression in vivo, 103- (or 21-) fold by FNR (or FNR*) with Pndh + + compared to 7- (or 20-) fold with the unaltered promoter, relative to the corresponding aerobic activities measured in the absence of FNR (Fig. 5b).

**DISCUSSION**

The molecular mechanisms of transcription activation by members of the FNR-cAMP receptor protein (CRP) family of transcription regulators have been studied extensively and shown to depend on the formation of specific contacts between the regulator and RNAP which promote transcription initiation (Bell & Busby, 1994; Wing et al., 1995; Guest et al., 1996; Busby & Kolb, 1996). Far less is known about the mechanisms of FNR-mediated transcription repression. Three genes whose anaerobic repression by FNR has been studied at the in vitro level are: fnr and narX (Takahashi et al., 1994) and ndh (Sharrocks et al., 1991; Green & Guest, 1994). The promoter regions of each of these genes contain more than one FNR-site, the most upstream of which seems to play a crucial role in FNR-mediated repression. Thus it would appear that FNR-mediated repression may not simply involve competition between FNR and RNAP for overlapping binding sites (Green & Guest, 1994; Guest et al., 1996). The current studies were simplified by using FNR* (FNR-D154A) protein, which unlike the wild-type protein, retains some activity under aerobic conditions, both in vivo and in vitro (Kiley & Reznikoff, 1991; Lazazzera et al., 1993). This FNR variant, already known to activate narG expression aerobically (Kiley & Reznikoff, 1991), has now been shown to repress ndh expression under aerobic conditions. Studies with ndb–lacZ fusions further showed that impairment of either of the upstream FNR-sites in the ndh promoter (FNR II and FNR I) reduces the level of anaerobic repression. The results extend previous studies in which the importance of the far upstream site (FNR II) had been inferred from the complete loss of repression observed in vivo with a truncated promoter lacking FNR II, and from the sixfold higher FNR concentration needed to repress transcription in vitro from the truncated promoter relative to the intact promoter (Green & Guest, 1994). The DNase I footprints with FNR* likewise confirmed that the bound regulator alters but does not prevent RNAP polymerase from interacting with the ndh promoter. The C-terminal domain of the α-subunit of RNAP (αcrTD) is not required for RNAP assembly or catalytic activity, but it can increase promoter strength by binding upstream of the −35 element and it can also interact with transcription activators. The current work provides direct evidence for α-subunit binding at two upstream sites in the ndh promoter.

The view that the operator sites of global regulators occupy a wider variety of locations in negatively regulated promoters than in positively regulated promoters (Collado-Vides et al., 1991) is upheld for FNR where binding sites are centred at −50.5 and −94.5 in ndh; −0.5 and −103 in fnr; −35.5 in sodA; and −106.5, −75.5 and +107.5 in narX (Guest et al., 1996). Such diversity probably allows each promoter to be regulated to different extents, and this is supported by studies on the effects of repositioning the lac operator (Lanzar & Bujard, 1988). The greatest degree of repression was observed when the operator was located between the −35 and −10 elements, such that repressor binding occludes both promoter elements. An intermediate degree of repression occurred when the operator overlapped both the −10 element and the transcription start point. Here the bound repressor occludes the initiation region but RNAP can still form potentially productive complexes by interacting with the −35 element. The weakest degree of repression was observed when the operator was placed immediately upstream of the −35 element. This is the least effective arrangement for preventing RNAP binding because it leaves exposed the critical −10 and initiation sites (Collado-Vides et al., 1991). The ndh promoter most closely resembles the latter situation, particularly with respect to the weak repression observed when FNR I (−50.5) is the only
functional FNR-site. The presence of tandem FNR-sites in the ndh promoter also resembles the arrangement of CRP-sites in CytR-regulated promoters. Here, repression occurs when CytR binds to two bound CRP dimers and stabilizes the CRP-CytR-CRP-DNA complex solely by protein–protein interactions (Busby & Kolb, 1996). Such a mechanism is unlikely to operate at the ndh promoter because FNR represses on its own.

A plausible model for FNR-mediated repression, accounting for the observed coexistence of FNR and RNAP at the repressed promoter, the presence of two α-binding sites and the partial repression of ndh expression from Pndh+ − and Pndh − +, is shown in Fig. 7. During aerobic growth the inactive form of FNR has a high-affinity FNR-I-site and induces DNA-bending. This may in turn displace αCTD to its weaker binding site (−113 to −98) in the FNR II region (Fig. 3d), thus forming a complex that could still initiate a limited amount of transcription (Fig. 7b). Then, under strictly anaerobic conditions, the amounts of active FNR should be sufficient to occupy both FNR-sites, thereby completely blocking access to the α-binding sites without preventing other interactions between RNAP and the ndh promoter (Fig. 7c). The unbound or ‘orphaned’ αCTD may then in turn inhibit transcription initiation by destabilizing RNAP promoter interactions (Zhou et al., 1994). The coexistence of both repressor and RNAP in a repression complex is not without precedent because RNAP can form a ternary complex with DNA-bound LacI (Straney & Crothers, 1987).

An alternative model, which takes account of the residual binding of FNR* at the impaired FNR II site in Pndh − + (more apparent in footprinting than gel retardation; Fig. 6iii), likewise confers a key role in repression on the weaker regulator interaction at the upstream FNR-II-site. This would involve modifying the above model to indicate that transcription is unaffected when FNR is bound solely at FNR I (Fig. 7b). Although consistent with the abolition of repression observed in vivo when FNR II is deleted, it does not account for the intermediate level of repression observed in vitro when FNR II is removed by truncating the promoter fragment so as to exclude the possibility that an alternative α-binding site is provided by the upstream DNA (Green & Guest, 1994). According to the alternative model, it might be predicted that RNAP would be occluded from the promoter when FNR is bound at both sites. However, the current gel retardation and footprinting studies confirm that two regulator molecules can be bound without displacing RNAP from this promoter.

The preferred model shown in Fig. 7 establishes a direct link between the mechanisms of transcription activation and repression. A key component of transcription activation from class II CRP-dependent promoters is the formation of ‘anti-inhibition’ contacts between CRP and αCTD in order to relieve the inhibitory effects of an otherwise unbound αCTD (Zhou et al., 1994). The evidence for ‘anti-inhibition’ includes the suppression of some crp ‘positive control’ mutations that accompanies the removal of αCTD (West et al., 1993) and the observation that deleting αCTD has no adverse effects on transcription activation at CRP-dependent class II promoters (Igarashi et al., 1991; Kolb et al., 1993). In the case of FNR-mediated ndh repression it would appear that FNR prevents αCTD from docking with the ndh promoter. Presumably, due to specific architectural features of this promoter, particularly the locations of the FNR-sites, bound FNR cannot make the necessary ‘anti-inhibition’ contacts with the displaced or ‘orphan’
\( \xi_{\text{CTD}} \) and transcription is repressed. So, whereas transcription activation requires suppression of the inhibitory effects of unbound \( \xi_{\text{CTD}} \) by contact with the regulator, repression can involve the generation of inhibitory \( \xi_{\text{CTD}} \) by regulator-mediated displacement from the promoter.

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