
Mutational analysis of the nucleotide sequence at the FNR-dependent *nirB* promoter in *Escherichia coli*

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ABSTRACT

During anaerobic growth of *E.coli*, the FNR protein activates transcription initiation at the *nirB* promoter. After chemical synthesis using deliberately contaminated nucleotides, we isolated a series of recombinant plasmids with single point mutations or one base pair deletions in the *nirB* promoter. The effects of these alterations on the anaerobic induction of promoter activity were measured. Mutations that abolish anaerobic induction identify the -10 hexamer sequence whilst changes that allow reduced induction suggest positions involved in FNR binding. Comparison of the nucleotide sequence of the *nirB* promoter with other promoters that are regulated by FNR show clear homologies, suggesting consensus sequences for FNR binding sites, and confirming that some of the point mutations described here do indeed act by weakening FNR binding.

INTRODUCTION

Escherichia coli contains a number of genes that are only expressed during anaerobic growth. Many of these genes are regulated by the product of the *fnr* gene (FNR), which, during anaerobic growth, activates transcription initiation at a number of promoters (1-3). One such gene, *nirB*, encodes the NADH - dependent nitrite reductase apoenzyme (EC 1.6.6.4) (4): DNA carrying the *nirB* gene has been cloned in this laboratory (5) and the nucleotide sequence has been determined (J.A.C. and S.B., in preparation). We recently located the transcription startpoint just upstream of the *nirB* structural gene (6) and used a crude deletion analysis to determine the extent of the promoter sequences (7). Transcription from this promoter is induced during anaerobic growth and this induction requires FNR. From the deletion analysis we showed that the nucleotide sequences from position -87 upstream of the transcription startpoint to +36 downstream are sufficient for FNR-dependent transcription of the *nirB* promoter (7). Further we showed that transfer of the *nirB* sequence from -30 to -54 to the same positions in the *galPl* promoter conferred FNR-dependence on transcription initiation at *galPl* suggesting that the FNR-binding site was located in this zone. Interestingly Spiro and Guest

(15) identified nucleotide sequences in this zone that are common to other FNR-dependent promoters.

One of our aims is to understand the structure of FNR-dependent promoters in order to investigate the mechanism of action of FNR. In particular we should like to know which bases are important for FNR binding and transcription initiation by RNA polymerase. However, although the nucleotide sequences at several FNR-dependent promoters have been determined (Fig 3), neither mutational analyses nor successful *in vitro* experiments have been reported. In this study we have used recombinant DNA techniques to introduce mutations into a DNA fragment carrying the nirB promoter sequence from -87 to +36 with a view to identifying some of the important bases. The effects of mutations on nirB promoter activity are described. Some of the mutations are located in sequences that are common to other FNR-dependent promoters.

MATERIALS AND METHODS

Using the method described by Oliphant et al (8), random point mutations were introduced into a DNA fragment carrying the nirB promoter sequence: our protocol is illustrated schematically in Fig 1. Two synthetic oligonucleotides were synthesised and purified: one carried the sequence 5' CGCGAATTC 3' followed by the nirB promoter sequence from -87 to -24 whilst the other was 5' CACAAGCTTC 3' followed by the nirB sequence from +36 to -33. The oligonucleotides were synthesised by Dr. J. Fox of the University of Birmingham Macromolecular Analysis Service with a Biotech Instruments BT 8500 synthesiser using cyanoethyl phosphoamidite derivatives of the four bases supplied by American Bionetics Inc., Emeryville, California. To introduce mutations throughout the sequence each of the four base derivatives was contaminated with 0.3% of the other three base derivatives. The two synthetic oligonucleotides were hybridised and extended using Klenow enzyme and four deoxynucleotide triphosphates as shown in Fig 1: the result of this was a double stranded fragment of DNA carrying nirB sequence from -87 to +36 flanked by an EcoRI - containing linker upstream of -87 and a HindIII - containing linker downstream of +36. After cleavage of this DNA with EcoRI and HindIII the fragment was cloned into the replicative form of M13mp11 for nucleotide sequence determination using the Amersham M13 cloning and sequencing kit.

To determine the effects of mutations on nirB promoter activity, EcoRI - HindIII fragments carrying mutant promoter sequences were transferred to the

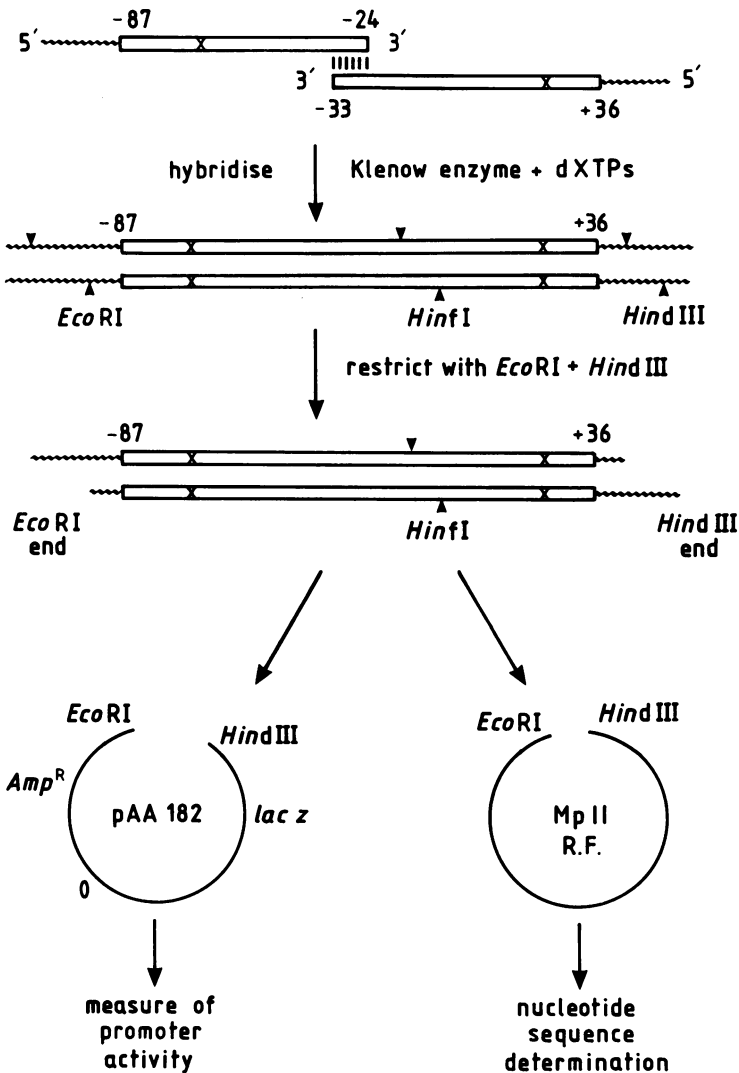


Figure 1 Mutagenesis of the *nirB* promoter sequence. The top line illustrates two synthetic oligonucleotides each carrying a linker sequence (wavy line) upstream of *nirB* promoter sequence (open box). The two oligonucleotides were hybridised at their 3' ends and extended using Klenow enzyme to give a duplex carrying *nirB* sequence from -87 to +36, with an *EcoRI* linker upstream and a *HindIII* linker downstream (line 2). The location of an internal *HinfI* site is shown. After restriction of the duplex with *EcoRI* and *HindIII* (line 3) the *nirB* promoter fragment was cloned into M13mpl1 for nucleotide sequence determination (bottom line). Fragments were also cloned into pAA182 for the measurement of promoter expression.

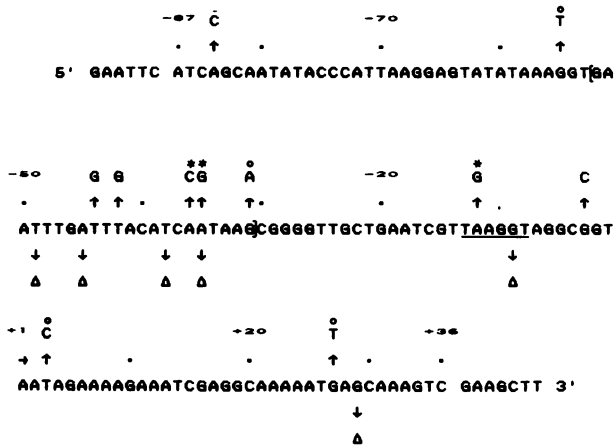


Figure 2 Nucleotide sequence of the *nirB* promoter region. The top strand of the promoter sequence from -87 to +36 is shown together with the *EcoRI* and *HindIII* linker sequences upstream and downstream, respectively. The sequence is numbered with the transcription start as +1 marked with an arrow indicating the direction of transcription. The -10 hexamer sequence is underlined and the nucleotide sequences proposed to be involved in FNR binding are bracketed. The position and nature of point mutations discussed here are described by arrows above the sequence and the positions of 1 base pair deletions are indicated by arrows below the sequence. Asterisks identify point mutations that reduce the anaerobic induction ratio to below 6 and circles indicate changes that have no significant effect on the ratio.

lacZ expression vector, pAA182 using the Δ *lac* host M182 or an *fnr* derivative (6,7). Promoter activity was estimated by measuring levels of β -galactosidase expression in cells grown aerobically or anaerobically exactly as previously described (4, 6, 7).

Standard methods for handling recombinant DNA were used throughout this work as described by Maniatis et al. (9).

RESULTS

Characterisation of point mutations in the *nirB* promoter

Using the phosphoamidite method of chemical synthesis and the strategy outlined in Fig 1, a DNA fragment was made carrying *nirB* promoter sequence from -87 to +36 with an *EcoRI* site upstream of -87 and a *HindIII* site downstream of +36: the nucleotide sequence is shown in Fig 2. As the oligonucleotides were synthesised using deliberately contaminated precursors we expected mutations to occur throughout the *nirB* promoter sequence. The synthetic 130 bp *EcoRI* - *HindIII* fragment was therefore purified and inserted into M13mp11: 38 mp11 derivatives were purified and the nucleotide sequence

TABLE I β -galactosidase expression from pAA182 carrying the nirB promoter with different point mutations

Mutation or Deletion in <u>nirB</u> promoter sequence	β -galactosidase expression (%) during		Ratio of anaerobic/aerobic activity
	aerobic growth	anaerobic growth	
Wild type	6	100	16.6
A - C at -84	6	99	16.5
G - T at -55	6	106	17.6
T - G at -44	5	44	8.8
T - G at -42	6	55	9.2
A - C at -36	9	14	1.6
A - G at -35	9	48	5.3
G - A at -31	7	105	15.0
A - G at -12	4	4	1.0
G - C at -3	7	75	10.7
T - C at +3	5	70	14.0
G - T at +27	2	31	15.5
Δ at -49	2.5	14	5.6
Δ at -45	5	6	1.2
Δ at -38	4	5	1.2
Δ at -35	3.5	4	1.1
Δ at -9	4	5	1.0
Δ at +29	5	49	16.3
no promoter	1	1	1.0

M182 cells containing pAA182 carrying the nirB promoter sequence with different mutations were grown to A_{600} of 0.3-0.4 in L Broth supplemented with 0.1% glucose and 80ug/ml Ampicillin. Aerobic growth was ensured by vigorous shaking and anaerobic growth was due to the use of unshaken narrow tubes. Cultures were lysed and β -galactosidase levels were determined as described by Miller (21). Activities are tabulated as a percentage of the expression found with the wild type promoter grown anaerobically (corresponding to 9000 Miller units). Each figure is the average of at least three independent determinations: the measurements were reproducible to within 10% error.

of the promoter insert in each case was determined. Of the 38 cloned fragments, 8 contained the wild type sequence (as in Fig 2), 8 contained single point mutations and 6 contained one base pair deletions, whilst the remaining 16 fragments carried more than one mutation. The 16 cases with multiple mutations included 7 fragments with two mutations, 1 fragment with three mutations, 4 fragments with four mutations, 3 fragments with 5 mutations and in 1 case there was a major rearrangement in the nirB sequence. Three of the fragments with multiple changes contained single point mutations which could be isolated using the HinfI site located from -20 to -16 (Figs 1 & 2). The remainder were discarded. This mutagenesis thus yielded 11 different point mutations and 6 different one base pair deletions (Fig 2).

Effects of mutations on nirB promoter activity

To measure the effects of the point mutations on nirB promoter activity, EcoRI - HindIII fragments carrying the different promoter sequences were cloned into the lacZ expression vector pAA182 (Fig 1) such that β -galactosidase expression was dependent on transcription initiation at the nirB promoter. The Δ lac host, M182, was transformed with the recombinant plasmids and β -galactosidase levels were measured in cells grown either aerobically or anaerobically. The results of these measurements are summarised in Table I. The Table also gives the anaerobic induction factor for each mutant promoter i.e. the ratio of expression during growth in anaerobic conditions to expression in aerobic conditions.

With the wild type nirB promoter sequence, expression is induced 16-fold on transfer to anaerobic growth conditions. Within the limit of experimental error this induction is unaffected by the point mutations at -84, -55, -31, +3, +27 or the deletion at +29. With the point mutation at +27 and the deletion at +29, although the induction factor is unaltered, expression is reduced 2 to 3-fold during both aerobic and anaerobic growth. As the anaerobic induction of the nirB promoter is unaltered by these mutations and as they are located at the nirB translation startpoint, it is most likely that the observed effects are due to decreases in the rate of translation rather than in transcription initiation: in similar studies with the E. coli gal operon promoters, it was found that changes in translation rates resulted in decreased β -galactosidase expression by pAA182 derivatives (10).

The point mutations at -44, -42 and -3 resulted in small decreases in anaerobic induction, the changes at -36 and -35 caused more severe decreases,

whilst the mutation at -12 caused decreases during both anaerobic and aerobic growth and totally suppressed anaerobic induction. Of the deletions, those at -45, -38, -35 and -9 caused almost total loss of promoter activity and anaerobic induction, whilst a small induction ratio was found with the deletion at -49. The measurements were repeated with the recombinant plasmids in an *fnr* derivative of the Δ *lac* host, M182: in each case the anaerobic induction was totally suppressed (data not shown).

DISCUSSION

Although many alternatives are available for *in vitro* mutagenesis (11), the use of random-sequence oligodeoxynucleotides is the most direct method for creating mutations throughout a short nucleotide sequence. However this study was hampered by three problems, namely, the synthesis of fragments with no mutation, the synthesis of fragments with multiple mutations and the creation of 1 bp deletions in the sequence. Whilst the first two problems are unavoidable the problem of deletions was unexpected and was, presumably, due to inefficient capping during the synthesis.

The nucleotide sequences essential for activity of most positively activated promoters in *E. coli* are located either in the -10 sequence or in the binding site for the activator (12). In the case of the *nirB* promoter we had previously tentatively identified the -10 sequence hexamer as 5' TAAGGT 3' (6) on the basis of homologies with other -10 sequences (13). However the effect of the mutation at -12 provides experimental evidence that this is the case: the mutation alters the A at the second position in the hexamer, a base that is highly conserved between different *E. coli* promoters, and thus by implication is important for promoter function. The fact that this mutation reduces transcription to background levels argues strongly that the -10 hexamer is indeed 5' TAAGGT 3': this is further confirmed by the effects of the deletion at -9. This result implies that σ^{70} is involved in the expression of *nirB* rather than an alternative such as σ^{54} : RNA polymerase containing σ^{54} does not recognise this type of -10 hexamer (14). Interestingly the region between the -10 hexamer and the transcription startpoint contains the unusual nucleotide sequence 5' GGCGG 3': the small but significant effects of the mutation at -3 implies that this sequence may play some role.

We have previously shown that nucleotide sequences between -30 and -54 are sufficient to confer FNR-dependence on transcription initiation (7): the binding site for FNR is thus likely to be contained in this sequence and the

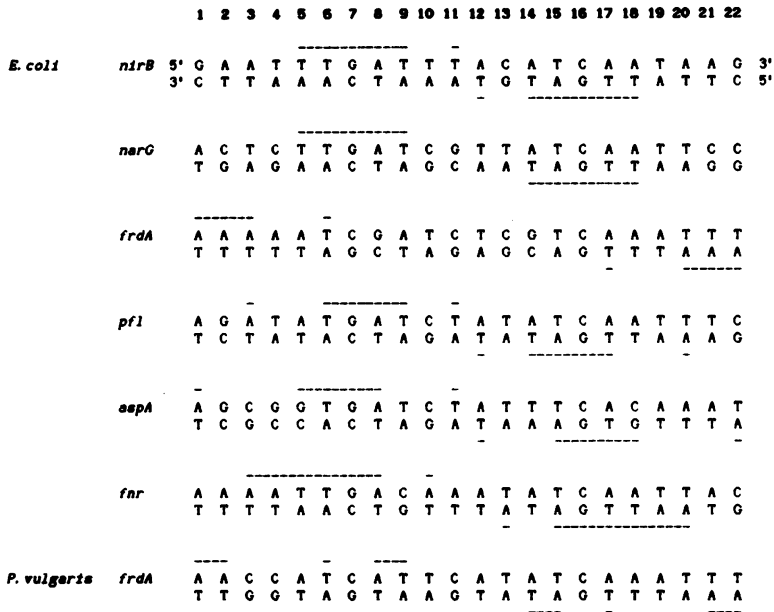


Figure 3 Comparison of nucleotide sequences at different FNR - dependent promoters. The figure shows comparisons of 22bp tracts of sequence from *nirB* (positions -52 to -31 from Fig 3); *narG* (positions -59 to -38: from ref 22); *frdA* (positions -57 to -36: from ref 23 and 24); *pfl* (G. Sawers, personal communication); *aspA* (coordinates 369 to 390; from references 15 and 25); *fnr* (coordinates 483 to 504; from reference 26) and the *frdA* gene from *P.vulgaris* (coordinates 179 to 200; from reference 27). Two-fold symmetry in these sequences centred between positions 11 and 12 is shown by dotted lines above or below the symmetric bases.

mutations described here may pinpoint key bases involved in binding. The point mutation at -36 reduces FNR-dependent stimulation of *nirB* promoter activity by 10-fold whilst mutations at -35, -42 and -44 cause only a 2-3 fold reduction. Deletions throughout this sequence at -35, -38, -45 and -49 also greatly reduce promoter activity. To assess the significance of these results we searched the published nucleotide sequences around other FNR-regulated promoters for homologies with this zone in the *nirB* sequence. We found that the 22 base pair sequence from -52 to -31 at the *nirB* promoter is significantly homologous with sequences at the promoters of the *E. coli* *narG* (nitrate reductase), *frd* (fumarate reductase), *pfl* (pyruvate formate lyase), *aspA* (aspartase) and *fnr* (FNR protein) genes, and the *P.vulgaris* *frdA* gene. Fig 3 shows these sequences aligned to display the homologies: this alignment, which is identical to that suggested by Spiro and Guest (15) after

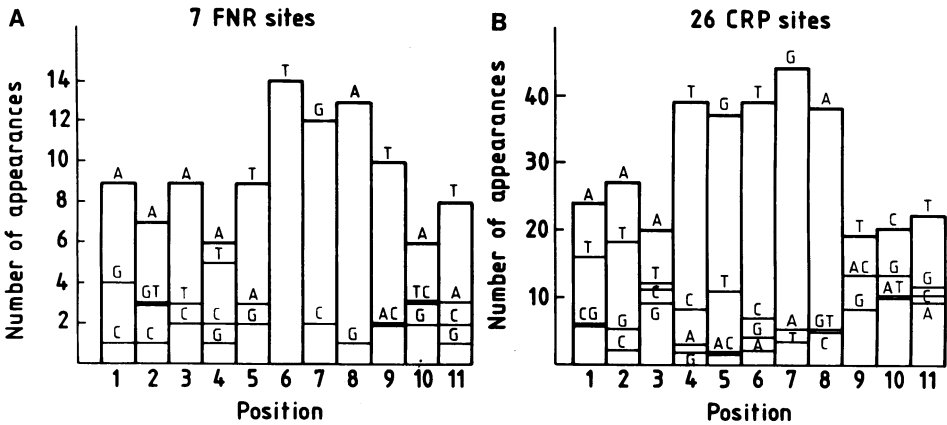


Figure 4 Frequency of appearance of bases at FNR and CRP binding sites. Part A is a histogram depicting the number of times (ordinate) the four bases, A, G, C and T appear at each of the 11 positions in the 14 proposed half sites for FNR binding listed in Fig 3. We argue that each half site is a binding sequence for a monomer of FNR. Part B is a similar analysis of the nucleotide sequences of 26 known CRP binding sites taken from references 17, 19 and 20. The ordinate represents the number of times each base appears at the 11 base pair binding sequence for the CRP monomer.

a similar search, shows that all of the sequences contain an imperfect inverted repeat centered between positions 11 and 12 of the 22 base pair sequence. The appearance of twofold symmetry suggests that FNR might bind as a dimer to these sequences with each subunit contacting bases in just one half of the inverted repeat. Interestingly the FNR protein is highly homologous to the CRP protein (16), another transcription activator, which also binds as a dimer to 22 base pair sequences carrying inverted repeats, with each subunit of the dimer contacting one half of the inverted repeat (17, 18). Because of this we have considered each 22 bp sequence as two 11 bp half-sites and compared the sequence of the half-sites by aligning the sequences running 5' - 3' from positions 1 to 11 in Fig 3 with the sequences running 5' - 3' from positions 22 to 12. This operation was performed on the 7 FNR binding sites listed in Fig 3 and the frequency of appearance of each base is illustrated as a bar chart in Fig 4A. For comparison, Fig 4B is a similar chart showing the frequency of appearance of the four bases throughout the 11 bp half-sites in 26 known CRP binding sites (17, 19, 20). This analysis suggests that the preferred half site binding sequence for FNR is 5' AAAaTTGATaT 3' where bases present in 50% or more of the cases are in capitals. This sequence is clearly very similar to the preferred half-site sequence for CRP binding which is 5' aAaTGIGATct 3' (Fig 4B). This strongly

suggests that the homologous sequences identified in Fig 3 are indeed FNR binding sites, and that FNR binds as a dimer to an inverted repeat sequence. It is striking that potential -10 hexamer sequences are located downstream of these sequences at the narG and frd promoters at the same distance as the -10 hexamer 5'TAAGGT3' in the nirB sequence.

The point mutations at -44, -42 -36 and -35 in the nirB promoter (Fig 2) all fall at positions where the actual sequence corresponds to the "consensus" for FNR binding (positions 9, 11, 17 and 18, respectively, in Fig 3). Although all the changes reduce anaerobic induction of the nirB promoter (Table I) the mutations at -35, -42 and -44 have only 2 to 3-fold effects suggesting that symmetry at these positions is not absolutely necessary for some FNR binding or activity. In contrast, anaerobic induction is reduced 10-fold by the mutation at -36: interestingly the A:T base pair at this position (together with a symmetric T:A) is found in each of the 7 putative FNR-binding sequences that we examined (compare positions 6 and 17 in Fig 3), suggesting that these bases may provide a crucial contact for FNR binding. Clearly our results with the mutations and deletions are consistent with the model for FNR binding suggested by sequence comparisons. However, definitive proof must await successful in vitro experiments.

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