DNA target sequence and FNR-dependent gene expression

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Abstract FNR proteins are global transcription regulators that respond to fluctuations in environmental oxygen. They recognise a DNA target consisting of an inverted repeat, TTGA-$TN_1N_2N_3N_4$ATCAA (where N_{1-4} represents a non-conserved tetrad, NCT). Analysis of 68 known and predicted FNR sites from the *Escherichia coli* K12 genome revealed a bias toward A or T at positions N_2 and N_3 of the NCT. The effect of the NCT sequence on FNR-dependent transcription in vivo was assessed using a series of class II and class I model promoters with different NCT sequences. Changing the NCT sequence did not affect basal activity but altered anaerobic induction by as much as an order of magnitude. Thus, the NCT sequence is a fundamental component in setting the dynamic range of the FNR switch.

Key words: cAMP receptor protein-FNR family; Oxygen sensing; Transcriptional regulation

1. Introduction

The FNR protein of *Escherichia coli* regulates the global response to the transition between aerobic and anaerobic growth [1,2]. FNR is a member of the cAMP receptor protein (CRP) family of transcription factors, and as such is composed of two basic domains, an N-terminal sensory domain and a C-terminal DNA-binding domain [1]. The sensory domain contains four cysteine residues that act as ligands for an oxygen-labile [4Fe–4S] cluster [1–18]. Acquisition of a [4Fe–4S] cluster initiates the formation of FNR homodimers that bind DNA site-specifically and regulate transcription from target promoters [12,14]. Under aerobic conditions the [4Fe–4S] clusters are disassembled, the FNR dimers dissociate to form non-DNA-binding monomers [12,14,16,18]. The C-terminal domain of FNR contains a helix-turn-helix motif that is responsible for DNA recognition. FNR-regulated promoters possess DNA sequences closely related to the FNR-binding site consensus (TTGATN$_1$N$_2$N$_3$N$_4$ATCAA where N_{1-4} represents any base) [19]. Molecular genetic evidence with reference to the structure of the CRP:DNA complex indicates that protein:DNA interactions are established between FNR residues 209 (Glu), 213 (Arg) and 212 (Ser) and the G, A and first T of each FNR half-site (TTGAT) [20,21]. Thus, the $N_1N_2N_3N_4$ sequence (hereafter referred to as the non-conserved tetrad, NCT) between the FNR half-sites does not interact directly with the FNR protein. Here we report an analysis of a set of FNR sites within the *E. coli* K12 MG1655 genome that reveals that G-C base pairs at positions N_2 and N_3 are less common than A-T. Moreover, in vivo transcriptional analyses using model FNR-activated promoters indicated that the activity of FNR-dependent promoters varied by as much as order of magnitude depending on the sequence of the NCT. Thus, the NCT sequence is shown to be a fundamental component in setting the dynamic range of the FNR switch, despite not being in direct contact with the FNR protein.

2. Materials and methods

2.1. Database interrogation

The *E. coli* MG1655 genome database Colibri (http://genolist.pasteur.fr/Colibri/) was searched for potential FNR-binding motifs within 300 bp upstream of a start codon using the pattern (T, A, C)TGA-nnnnnnTCA(A, T, G). This set was extended by the addition of a further 14 sites that did not fit the search criteria but have been shown experimentally to be present in FNR-regulated promoters.

2.2. Bacterial strains and plasmids

The isogenic *E. coli* strains MC1000 (Δ*lac*) and JRG1728 (Δ*lac* Δ*fnr*) [22] were used as hosts for *lacZ* reporter plasmids based on the low copy number vector pRW50 (tet^R) [23]. Two series of plasmids were constructed by overlap polymerase chain reaction with appropriate oligonucleotide primers using either the model class II FNR-dependent promoter *FF*-41.5 or the model class I FNR-dependent promoter *FF*-71.5 as the template [24]. Each series consisted of 16 different promoters representing all possible combinations of A or G nucleotides in the NCT. The pBR322 derivative pGS196 (amp^R) was used to introduce *fnr* in multicopy [22].

2.3. Growth conditions, β-galactosidase assay and gel retardation assay

β-Galactosidase activity was measured according to Miller [25]. Cultures were inoculated (1:200) from overnight cultures and grown until an OD_{600} of 0.4–0.6 was reached before β-galactosidase activities were estimated. All cultures were grown in Lennox broth [26] containing glucose (0.2%, w/v) and tetracycline (35 μg/ml) at 37°C. Aerobic cultures were grown in vigorously (250 rpm) shaken conical flasks (250 ml) containing 5 ml medium. Anaerobic cultures were grown as 5 ml cultures in anaerobic jars. Anaerobic gel retardation assays were as previously described [14] except that the DNA used was the class II *Eco*RI-*Hin*dIII fragments containing the NCT sequences GAAG and AGGA. Reconstituted FNR protein (0–750 nM) was incubated with the DNA for 10 min before separation of complexes on TBE-buffered 6% polyacrylamide gels.

3. Results and discussion

3.1. Genomic database analysis of potential FNR-dependent promoters

The availability of complete genome sequences provides a

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valuable tool for the study of transcriptional regulation [27–29]. In this instance the *E. coli* K12 genome database was searched for potential FNR-binding sites using the pattern search tools provided for use with the Colibri database (http://genolist.pasteur.fr/Colibri/). The FNR-binding site consensus has been defined as an inverted repeat (TTGAT-$N_1N_2N_3N_4$ATCAA) in which the regions recognised by FNR are separated by four non-conserved base pairs (N_{1-4}), the NCT. All FNR-binding sites within 300 bp of a start codon were included. Database interrogation was further complicated by the similarity of the FNR-binding site (TTGATNNNNATCAA) to that of the CRP-binding site (GTGANNNNNNTCAC). Specificity of each regulator for its site is determined by the bases flanking the common core (T-A for FNR; G-C for CRP), thus, to select against CRP-binding sites a pattern which excluded the CRP-determining bases was used: (T, A, C)TGATNNNNATCA(A, T, G). A consequence of this strategy is that whilst false positives are reduced, it underestimates the number of functional FNR sites. The search yielded 54 potential FNR-binding sites located within 300 bp of the first codon of a gene. This set of potential FNR-regulated genes included 13 previously characterised FNR sites (located within the following promoter regions: *pdhR*, *focA*, *ndh*, *hlyE*, *narK*, *narX*, *narG*, *yfiD*, *tdcD* and *nirB*). Of 41 potential FNR sites all but seven were associated with a predictable transcript start in the RegulonDB database (http://cifn.unam.mx/computational.genomics/regulondb/). Thus, FNR was predicted to repress transcription of seven genes (*phrB*, *hycI*, *ygiE*, *ygiD*, *yhbX*, *gltP* and *yjhB*). Other sites were identified in conventional class II positions (*yhdJ* and *moaA*) and class I positions (*dcuC*, *ybhK*, *yciO*, *ydgG*, *yfbV*, *ygaC*, *fliZ*, *yfgF* and *yhjA*). The remaining sites were located between −124 and −243 in the corresponding promoter regions. As expected some known FNR-responsive genes were not represented in the data set. Therefore, the sequences of a further 14 FNR sites from promoters known to be FNR-responsive in vivo (*dmsA*, *fnr*, *hmp*, *dcuA*, *ansB*, *acnA*, *adhE*, *arcA*, *cydAB*, *nrfA*, *yfiD*, *yieL* and *fdnG*) were added.

The sequences (68 in all) containing the putative FNR-binding sites were aligned and the frequency of each base pair at each position was determined. This revealed previously unrecognised patterns in the distribution of bases within the NCT (Fig. 1). At the two central positions (N_2 and N_3) A-T base pairs are preferred (74% and 72% for positions 2 and 3, respectively), whereas at position 4 (N_4) A-T is present in only

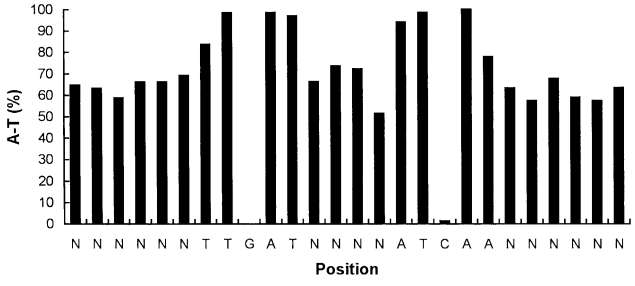

Fig. 1. Distribution of A+T bases at each position within known and predicted FNR-binding sites located <300 bp upstream of a start codon in the *E. coli* K12 (MG1655) genome. Each bar represents the frequency (%) of A+T bases at each position in a data set of 68 FNR sites. The sequence of the FNR consensus site is aligned below the graph.

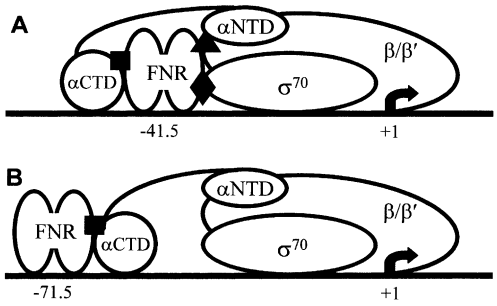

Fig. 2. The organisation of simple class II and class I FNR-dependent promoters. A: At the model class II promoter used here FNR binds at a site centred at −41.5 and three contacts are made with RNA polymerase. The activating region 1 surface of the upstream subunit of the FNR dimer contacts the C-terminal domain of the α-subunit of RNA polymerase (αCTD) (■); activating region 2 of the downstream subunit of FNR contacts the N-terminal domain of the α-subunit of RNA polymerase (αNTD) (▲); and activating region 3 of the downstream subunit of FNR contacts σ^{70} (♦). B: At the model class I promoter used here FNR binds to a site centred at −71.5. Now only activating region 1 of the downstream subunit of the FNR dimer contacts αCTD (■).

52% of FNR sites. Thus, although there is apparently no interaction between FNR and the NCT sequence, these observations suggested that the NCT plays a role in FNR-mediated transcriptional regulation.

3.2. *In vivo analysis of NCT variant promoters*

Most FNR-activated promoters in *E. coli* possess an FNR-binding site located at or around −41.5 bp relative to the transcript start (Fig. 2A) [30]. These promoters are designated class II FNR-dependent promoters and FNR activates transcription by making multiple contacts with RNA polymerase [31–36]. To investigate the biological significance of the apparent selection against G-C base pairs at positions 2 and 3 of the NCT, a series of 16 *lacZ* reporters based upon the model class II FNR-activated promoter *FF*-41.5 [24] were constructed. The series included each of the 16 possible NCT combinations that can be generated using different combinations of A and G bases in each position (Table 1). The β-galactosidase activities of aerobic and anaerobic cultures of MC1000 (Δ*lac*) carrying the indicated reporter fusions were measured, as well as the corresponding activities associated with JRG1728 (Δ*lac* Δ*fnr*) strains to control for any differences in basal promoter activity. Whilst the NCT sequence had little effect on promoter activity in the absence of FNR, it had a marked effect upon the anaerobic activity of the FNR-dependent promoters in the presence of FNR, with activities ranging from 768 to 6461 Miller units (Table 1). The five promoters with highest anaerobic activities all had an A-T pair at position N_3, with the top four having AA at positions N_2 and N_3 (Table 1). In contrast the five promoters with the lowest anaerobic activities all had G at position N_3, with the worst having GG at positions N_2 and N_3 (Table 1). The degree of anaerobic induction was calculated by dividing the anaerobic activity in the presence of FNR by the anaerobic activity in the absence of FNR for each promoter. This also showed that an A-T base pair was located at N_3 in the six most highly induced promoters, which included all the promoters with AA at positions 2 and 3, with highest induction being observed with the sequence GAAG (Table 1). These six promoters were followed in the anaerobic induction ranking

by four promoters that possessed either G-C or A-T at N_3, but when G was present at N_3, positions N_1 and N_2 were both A. The least inducible promoters all had a G-C base pair at position N_3, with the lowest anaerobic induction being for AGGA (Table 1). This suggests that for class II FNR-dependent promoters if position 3 of the NCT is occupied by a G-C base pair anaerobic induction of transcription is low unless both positions 1 and 2 are A-T base pairs.

At class II promoters the FNR site overlaps the −35 element allowing multiple contacts to be established between FNR and polymerase (Fig. 2A). However, FNR can also activate transcription from class I promoters in which the FNR-binding site is located at or around −61.5, −71.5, −82.5, or −92.5 relative to the transcription start [24]. At these promoters the FNR site is remote from the basic promoter elements and only a single contact is made with RNA polymerase (Fig. 2B) [37,38]. Therefore, it is possible that the effects of the NCT sequence could be different at class I promoters. To test this a series of reporters based on the model class I promoter *FF*-71.5 were created [24]. Transcription from each of the 16 promoters was estimated by measuring β-galactosidase activities of aerobic and anaerobic cultures (Table 2). Once again the base composition of the NCT did not significantly affect basal promoter activity in the absence of FNR but had a marked effect upon the anaerobic activity of the promoters in the presence of FNR, with activities ranging from 108 to 1731 Miller units (Table 2). Of the eight most anaerobically active promoters seven had G at position N_4, whereas seven of the eight least active promoters had A at N_4 (Table 2). Interestingly, the analysis of FNR sites in the *E. coli* K12 genome revealed that A-T and G-C were almost equally represented at N_4 (Fig. 1). In contrast to the class II promoters, the anaerobic induction ratio did not reveal any strongly sustained preferences in the NCT sequence. The highly induced class I promoters tended to have a bias towards A-T at position N_2, rather than at N_3 as observed for the class II series. However, in accord with the class II data the least inducible class I promoter had the NCT sequence AGGA. Moreover, as seen with the class II series the most highly inducible promoters displayed a preference for AA at positions 2 and 3 of the NCT (Table 2). Thus, although there are differences in the expression patterns between the class I and class II series, positions 2 and 3 of the NCT have a profound influence on the effectiveness of FNR at both sets of promoters with G_2G_3 dampening anaerobic induction and A_2A_3 enhancing anaerobic induction.

The work presented here provides clear evidence that the four base pairs separating the core motifs that comprise an FNR-binding site influence FNR activity at both class I and class II promoters. This NCT thus constitutes a fundamental component in setting the dynamic range of the FNR switch. Similar observations have been reported for interaction between the 434 repressor and its target DNA, in which the sequence of non-contacted bases at the centre of the 14 bp site affects the affinity of the protein for the DNA [39]. It has been suggested that the ability of the non-contacted bases to be overtwisted in the protein:DNA complex is a key factor in establishing the affinity of the protein for a particular target [40]. Recently, the presence of an N2 amino group on the purine bases at the centre of the binding site has been shown to destabilise the 434:DNA complex, perhaps by sterically or electrostatically inhibiting overwinding of the DNA [41]. To investigate whether the sequence of the NCT affects the affinity of FNR for target DNA an in vivo approach was adopted. It was reasoned that if the NCT sequence affects FNR binding, then overproducing FNR should overcome this and the ratio of the activities of the best and worst promoters should approach 1.0. Thus, strains carrying promoters with the highest and lowest activities (Tables 1 and 2) were transformed with the *fnr* expression plasmid pGS196 [22] and β-galactosidase activities of anaerobic cultures were measured. These experiments showed that overproduction of FNR reduced the difference in transcription at the strongest and weakest promoters of both classes (Table 3). The ratio of the activities of the best and worst class I promoters was reduced from 16.0 to 2.4 upon introduction of multicopy *fnr*. Similarly, the ratio

Table 1
Effect of NCT sequence on in vivo transcription activation at class II FNR-dependent promoters

Sequence of NCT	Aerobic		Anaerobic		Anaerobic induction
	FNR^-	FNR^+	FNR^-	FNR^+	
GAAG	4.7 (0)	403(18)	2.0 (0.5)	6461(120)	3313
AAAG	2.9 (0.4)	355(8)	2.2 (0.7)	4915(81)	2234
GAAA	3.4 (0)	327(9)	1.8 (0.3)	4745(51)	2636
AAAA	3.2 (0.2)	275(14)	2.2 (0.2)	4536(63)	2110
AGAA	3.0 (0)	238(12)	1.3 (0.4)	4190(24)	3223
AAGA	3.1 (0.4)	385(6)	2.2 (0.3)	4154(27)	1888
GGGG	9.4 (0.8)	71(13)	4.9 (0.8)	4071(77)	839
GGAG	4.7 (0.1)	87(7)	1.9 (0.2)	3973(113)	2148
AGAG	7.1 (1.3)	143(9)	4.3 (0.2)	3934(46)	926
AAGG	9.9 (0.2)	355(18)	3.8 (0.5)	3567(41)	951
GGAA	3.6 (0.5)	178(13)	2.3 (0)	2843(91)	1236
AGGG	8.7 (0.4)	137(12)	5.2 (0)	2698(87)	519
GAGG	8.9 (0.1)	87(6)	4.5 (0.2)	2576(56)	579
GAGA	4.0 (1.8)	103(12)	2.8 (0.3)	1718(64)	452
GGGA	4.5 (0.1)	95(8)	2.7 (0.5)	1599(25)	603
AGGA	4.7 (0.1)	292(12)	2.1 (0)	768(41)	366

β-Galactosidase activity was measured from at least three independent cultures of strains (MC1000 Δ*lac* and JRG1728 Δ*lac* Δ*fnr*) carrying an FNR-dependent class II promoter fused to *lacZ*. The DNA sequence of the NCT that separates the FNR site core motifs for each promoter tested is indicated. Cultures were grown to OD_{600} of 0.4–0.6 at 37°C either aerobically or anaerobically as indicated. The degree of anaerobic induction was calculated by dividing the anaerobic activity (Miller units) in the presence of FNR by the anaerobic activity (Miller units) in the absence of FNR for each promoter. Figures in parentheses are the standard deviations from the mean value.

Table 2
Effect of NCT sequence on in vivo transcription activation at class I FNR-dependent promoters

Sequence of NCT	Aerobic		Anaerobic		Anaerobic induction
	FNR$^-$	FNR$^+$	FNR$^-$	FNR$^+$	
AAAG	11.5(0.7)	47(4)	8.1(0.3)	1731(43)	214
GAGG	27.0(14.5)	216(79)	17.0(1.1)	1656(22)	98
GGGA	30.6(12.4)	273(92)	13.0(0.4)	1431(28)	111
GAAG	14.8(0.6)	145(29)	10.3(1.0)	1394(84)	135
AAGG	34.4(0.1)	78(37)	14.8(0.4)	1254(146)	85
GGAG	34.1(16.3)	157(26)	26.6(0.9)	1199(23)	45
AGGG	36.0(0)	91(6)	16.2(3.7)	987(24)	61
GGGG	18.6(1.5)	60(4)	10.9(0)	825(20)	76
GAGA	17.9(0.1)	28(1)	12.2(0.4)	613(16)	50
AAAA	7.9(0.1)	27(5)	5.6(0.4)	554(20)	99
AGAA	9.7(0.7)	36(16)	8.1(0.5)	467(1)	58
GAAA	11.0(0)	30(4)	8.1(0.1)	430(37)	53
AAGA	9.6(0.4)	45(5)	5.7(0.1)	393(7)	70
AGAG	10.9(0.8)	39(9)	8.2(0)	354(36)	43
AGGA	20.2(5.2)	10(1)	11.1(0.1)	116(3)	10
GGAA	7.7(0.1)	19(1)	5.9(0.1)	108(1)	18

β-Galactosidase activity was measured from at least three independent cultures of strains (MC1000 Δ*lac* and JRG1728 Δ*lac* Δ*fnr*) carrying an FNR-dependent class I promoter fused to *lacZ*. The DNA sequence of the NCT that separates the FNR site core motifs for each promoter tested is indicated. Cultures were grown to OD_{600} of 0.4–0.6 at 37°C either aerobically or anaerobically as indicated. The degree of anaerobic induction was calculated by dividing the anaerobic activity (Miller units) in the presence of FNR by the anaerobic activity (Miller units) in the absence of FNR for each promoter. Figures in parentheses are the standard deviations from the mean value.

of the activities of the best and worst class II promoters was reduced from 8.4 to 2.3 upon introduction of multicopy *fnr*. Therefore, it was concluded that the sequence of the NCT probably affects the affinity of FNR for its target DNA. Attempts to estimate the difference in FNR affinity for the best and worst FNR-dependent class II promoters in vitro using gel retardation assays [14] revealed that the differences were too small to quantify in these experiments (not shown). Thus, it would appear that small changes in the affinity of FNR binding have profound effects on transcriptional activity in vivo.

The observations described above imply that NCT sequences are not random but have evolved to fine-tune particular promoters to allow optimum expression of the corresponding genes and proteins. For highly induced genes an A-T-rich NCT is indicated, whereas for poorly induced genes a G-C-rich NCT is indicated. The central two bases (N_2 and N_3) appear to be particularly important in this regard. However, if the sole effect of the NCT sequence is on DNA-binding affinity, and aerobic cultures contain a little active FNR, then the rank order of promoter activities should be the same under aerobic and anaerobic conditions. This, with two exceptions (AAAG and AAGG), was largely the case for the class I series (Table 2), but was not sustained for the class II series (Table 1), suggesting that other factors contribute particularly at the class II architecture (Fig. 2A). Because the DNA in the FNR:DNA complex has been shown to be bent by up to 92° from linearity and this bending is thought to be important for FNR-dependent transcription activation [42], the effects of the various base changes on the consensus bendability of the DNA were investigated using the Plot.It software package [43] (http://www3.icgeb.trieste.it/~dna/index.html). No sustained correlation was observed between the activities of the class II promoters and the predicted bendability of the DNA. However, a correlation was noted between the activities of the class I promoters and the predicted bendability of the DNA (not shown). In the latter case it appeared that the greater the predicted bendability of the DNA the greater the FNR-mediated transcription. This may suggest that DNA bendability modulates the affinity of FNR binding at class I promoters.

Analysis of FNR sites in the *E. coli* K12 genome sequence suggests that most FNR-dependent promoters are tuned for high anaerobic induction. Variation in the relative effectiveness of particular NCT sequences was observed when class I and class II promoters were compared. This may be a reflec-

Table 3
Effect of *fnr* overproduction on in vivo transcription activation at class I and class II FNR-dependent promoters

Sequence of NCT		Promoter activity plus multicopy *fnr*	Activity ratio class I (AAAG : GGAA)		Activity ratio class II (GAAG : AGGA)	
Class I	Class II		Multicopy	Single copy	Multicopy	Single copy
AAAG		2008(107)	2.4	16.0		
GGAA		823(50)				
	GAAG	4938(332)			2.3	8.4
	AGGA	2123(198)				

β-Galactosidase activity was measured from at least three independent cultures of JRG1728 Δ*lac* Δ*fnr* containing *fnr* on a multicopy plasmid (pGS196) and the indicated FNR-dependent promoter *lacZ* fusions representing the class I and class II promoters with the highest and lowest anaerobic activities. Cultures were grown to OD_{600} of 0.4–0.6 at 37°C under anaerobic conditions and promoter activity was estimated by measuring β-galactosidase activities (Miller units). Figures in parentheses are the standard deviations from the mean value. Activity ratios were calculated by dividing the activity of the best promoter by the activity of the worst for each class. The single copy activity ratios are calculated from the data in Tables 1 and 2.

tion of the different FNR:RNA polymerase contacts that are established in order to activate transcription (Fig. 2). The class II architecture and the formation of multiple FNR:RNA polymerase contacts might impose different constraints on the NCT sequence than the class I architecture in which only a single contact is made through a module (the C-terminal domain of the RNA polymerase subunit) that is attached to a highly flexible linker [44].

Only four FNR-dependent promoters with consensus core motifs (TTGATNNNNATCAA), like those used in this study, have been characterised and they have, like the overwhelming majority of FNR-dependent promoters, class II architectures. Published data from the corresponding *lacZ* fusions indicate that the *narK*, *nirB* and *yfiD* promoters are highly induced under anaerobic conditions (48–64-fold) and these all have TA at positions N_2 and N_3 of the NCT [45–47]. The *narG* promoter is less induced (six-fold) and this promoter has GT at positions N_2 and N_3 of the NCT [48]. Although this sample is small, and, in contrast to the model promoters used here, other transcription factors will influence activity, the pattern of expression does support the conclusion that G-C base pairs at N_2 and/or N_3 reduce the effectiveness of FNR.

In summary, we have uncovered a previously unsuspected layer of control over FNR-dependent transcription activation that can be used to tune gene expression without compromising core protein:DNA interactions. It is anticipated that similar strategies could be applied to any transcription factor that recognises a target with a spaced inverted repeat and thus could be of general significance in poising a wide range of transcriptional switches.

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