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FEBS Letters 340 (1994) 59–64

FEBS 13696

FEBS LETTERS

Repression of in vitro transcription of the *Escherichia coli fnr* and *narX* genes by FNR protein

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Received 4 October 1993; revised version received 23 December 1993

Abstract

In facultative anaerobes, the anaerobic expression of respiratory genes is regulated by a transcriptional activator, FNR. Transcription in vitro of the *E. coli fnr* gene was repressed by its product, FNR. The transcription of the *E. coli narX* gene encoding the nitrate sensor protein was likewise repressed. DNA truncation experiments for *fnr* and *narX* genes indicated that multiple anaero-boxes in each promoter region are essential for repression by the FNR protein, but they also suggest that factor-independent upstream activation signals are operating with these promoters.

Key words: Transcriptional regulation; FNR; *fnr*; *narX*; Upstream activation signal; *Escherichia coli*

1. Introduction

The expression of respiratory genes in *E. coli* is controlled by two trandscriptional regulatory systems: one is the ArcB/A system for aerobic genes [1]; and the other is the FNR system for anaerobic genes [2]. Twelve FNR-regulated genes from *E. coli* and eight genes from other facultative anaerobes have so far been identified [2], all of these carrying the consensus sequence for the FNR-binding site ('anaero-box' [3]) in each regulatory region. FNR was isolated from *E. coli* in a monomeric form with molecular masses of 30 kDa as estimated by SDS-PAGE [4–6] and 28-kDa by gel filtration and sucrose gradient sedimentation [6]. Sequence analysis of the *fnr* gene indicates that FNR is structurally homologous to cAMP receptor protein (CRP), the transcriptional regulator of catabolite-sensitive genes [2,7,8]. Specific binding of purified FNR to the anaero-box was not detected in gel retardation assays [6], although Guest and colleagues recently succeeded in detecting the DNA-binding activity by DNase I footprinting. Moreover, they also observed both the in vitro transcriptional activation of *FFmelR* (semi-synthetic promoter gene carrying the anaero-box) and the repression of *ndh* (the NADH dehydrogenase gene) by FNR [9], indicating that FNR, like CRP, has a dual function.

To understand the molecular mechanisms of the FNR function, we examined the effect of FNR on in vitro transcription from various *E. coli* promoters with or without the anaero-box. In the *fnr* promoter region, two anaero-boxes have been assigned to regions centered at −105 and +1, whereas three anaero-boxes are present in the overlapping promoter region between the convergently transcribed *narX* (the nitrate sensor protein gene) and *narK* (the nitrate transport protein gene) [3,10]. In the present paper, we report that in vitro transcription initiated by both the *fnr* and *narX* promoters is repressed by FNR. From DNA truncation experiments, an upstream sequence including these anaero-boxes was found to be essential for transcription of both the *fnr* and the *narX* genes.

2. Experimental

2.1. E. coli *FNR and RNA polymerase*

A 1.2-kb *Eco*RI-*Bam*HI fragment carrying the *fnr* gene was isolated from pFNR2, which contains a 1.6-kb *Hin*dIII-*Bam*HI fragment of lambda 8C12 [11] in pGEM3Zf(+), and then inserted into an expression vector pT7-6 between the *Eco*RI and *Bam*HI sites to construct pFNR14 (Fig. 1A). *E. coli* BL21(DE3)pLysS strain [12] was transformed with the pFNR14 and cultured at 37°C in LB medium containing 25 μg/ml of ampicillin and 10.2 μg/ml of chloramphenicol. Expression of FNR was induced by the addition of 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at a culture density of A_{600nm} = 0.4, as measured with a Hitachi 100-10 spectrophotometer. FNR was purified from the cell lysate essentially according to the method of Trageser et al. [5]. During the purification of FNR, two cross-reactive components, a major and

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SSDI 0014-5793(94)00080-F

most probably intact form (M_r = 30 kDa) and a truncated form (M_r = 29 kDa), were identified as reported previously [5]. They were eluted at pH 6.5 and 6.0, respectively, by chromatofocusing in PBE94-Polybuffer 74 (Pharmacia). As the final step of purification, chromatography on a CM-Toyopearl 650M TSK-GEL column (Tosoh) was employed. The 30-kDa intact component eluted at 180 mM NaCl was more than 95% pure with only a slight contamination by the 29-kDa truncated component, as judged by SDS-PAGE. The N-terminal amino acid sequence of the 30-kDa component, determined with a peptide sequencer (Applied Biosystems model 477A), agreed with that predicted from the DNA sequence [7].

The holoenzyme of RNA polymerase was prepared from *E. coli* W3350 as described previously [13].

2.2. *Fragments of* fnr *and* narX/K

The truncated promoter fragments used in this study are shown in Fig. 1, together with locations of the anaero-boxes on the *fnr* and *narX/K* fragments and of the consensus NarL binding sites ('nitrate-boxes' [3]) on the *narX/K* fragment. Sequences assigned as the anaero-boxes on these genes are summarized in Table 1.

A 275-bp fragment containing the region between −167 and +108 of the *fnr* gene was prepared by the PCR method using pFNR2 as the template (Fig. 1A), and FNR-01 and FNR-10 as primers. Two other *fnr* fragments of 231 and 200 bp in length, both lacking the 5′-terminal region of the 275-bp fragment, were also prepared by PCR, using FNR-02/FNR-10, and FNR-03/FNR-10 as synthetic primers.

The overlapping promoter region (~300 bp) between *narX* and *narK* carries three anaero-boxes and three putative nitrate-boxes (Fig. 1B). A 421-bp *narX/K* fragment, containing the overlapping promoter region and parts of the structural genes for both *narX* and *narK* [3,10], was prepared by PCR using pNR24 [10] as the template, and NarK-01/NarK-10 as primers. Two other *narX/K* deletion fragments of 356 and 200 bp in length, both lacking the 3′-terminal region of the 421-bp fragment, were also prepared by PCR, using NarK-01/NarK-14 (for 356 bp) and NarK-01/NarK11 (for 200 bp) as primers.

The nucleotide sequences of eight oligonucleotides synthesized as the above mentioned primers were as follows; FNR-01, 5′-CATTAAACAATTTGTCGCCAG-3′; FNR-02, 5′-TTACCCTTAACAACTTAAGGG-3′; FNR-03, 5′-GATAGACATATATTTACATCT-3′; FNR-10, 5′-CTGGCTGATGCTGCAATCCTG-3′; NarK-01, 5′-TGATTAACCAGGGTGAGCGGA-3′; NarK-10, 5′-GACAGCTCCAGTAGCCCTTTC-3′; NarK-14, 5′-TGTCTGCCACCTTAGTGTCTG-3′; NarK-11, 5′- CTTACCGATGTAAAGCGACTA-3′.

The transcription initiation site (cap) of the *narX* gene was determined by the primer extension method as described previously [17]. A sample of the transcript used as a template was produced from a DNA fragment containing a *narX* cap site by in vitro transcription assay (see below), and a 5′ region of template DNA (21 b single strand DNA, 5′-GGCTTTAAATGAGCAATAACC-3′) was used as the primer.

2.3. *In vitro transcription*

Mixed transcription was carried out under the standard reaction conditions with purified RNA polymerase holoenzyme ($E\sigma^{70}$) [13,19] using template mixtures containing one of the FNR-regulated promoters, *fnr* or *narX*, in addition to *lac*UV5 as an internal control. Dithiothreitol (5.0 mM) was added to all reaction mixtures. RNA products were purified by ethanol precipitation and analyzed by electrophoresis on 7% polyacrylamide gels containing 8 M urea. Gels were exposed to X-ray film, and the ^{32}P distribution was monitored with a Fujix BAS2000 BioImaging Analyzer. The size of run-off transcripts from the *fnr* or *narX* promoters was estimated to be 108 b and 200 b, respectively, whereas that from the *lac*UV5 promoter was 63 b [13].

3. Results

3.1. *Autorepression of* fnr *transcription by FNR*

In vitro transcription of *fnr* was conducted with a 275-bp truncated DNA fragment carrying the *fnr* promoter (Fig. 1A) as a template. From the DNA sequence of this template, we expected an *fnr* transcript of 108 b in length. Throughout mixed transcription experiments, a dominant transcript of approximately 108 b was identified as well as the 63 b *lac*UV5 RNA (Fig. 2A).

By adding increasing amounts of the purified FNR,

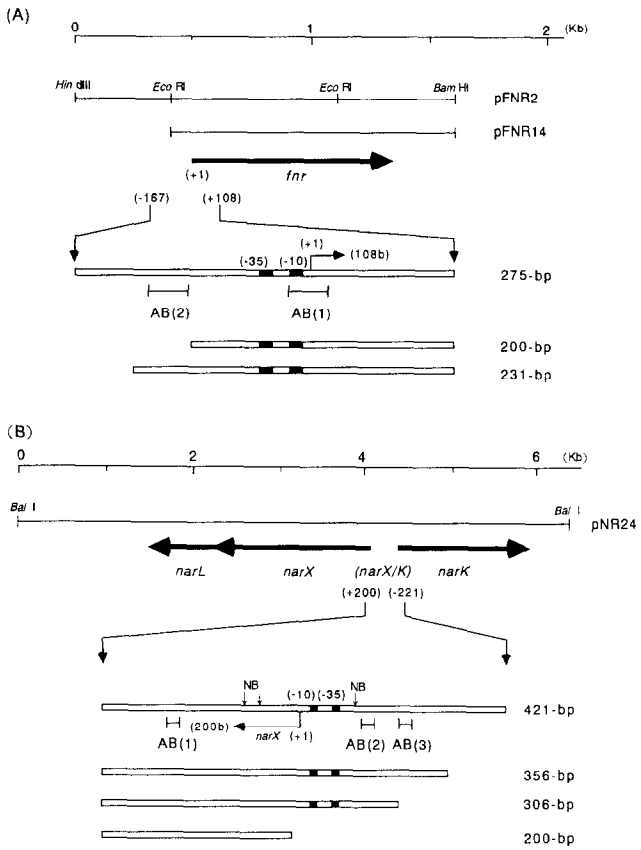

Fig. 1. Truncated promoter fragments of the *fnr* and *narX/K* genes from *E. coli*. The nucleotide sequences of anaero-boxes of each promoter are summarized in Table 1. Locations of the anaero-box (AB) and the major promoter are shown for the *fnr* promoter in (A), and for the *narX* promoter in (B). Nitrate-boxes (NB) in (B) indicate the putative sequence (TACTCCTTA) and the consensus ones for *narK* and *narGHJI* described previously [3,18].

Table 1
Nucleotide sequences assigned as the anaero-boxes (FNR-binding sites) in the regulatory regions of the *fnr* and *narX/K* genes in *E. coli*.

Gene	Anaero-box		
fnr	(1) [−6]	TTGAC----ATCAA	[+8]
	(2) [−109]	TTAAG----TTCAA	[−95]
narX/K	(1) [+101]	ATGTA----CACAT	[+114]
	(2) [−82]	TTGAT----ATCAT	[−69]
	(3) [−100]	TTGAT----ATCAA	[−113]
Consensus		TTGAT----ATCAA	

These anaero-boxes were identified by comparison with the reported consensus sequences [3,4,14,15,16]. The numbers in parenthesis correspond to those shown in Fig. 1. The position of anaero-boxes was determined from the transcriptional startpoints (+1) of each promoter of *fnr* [4] and *narX* (this work).

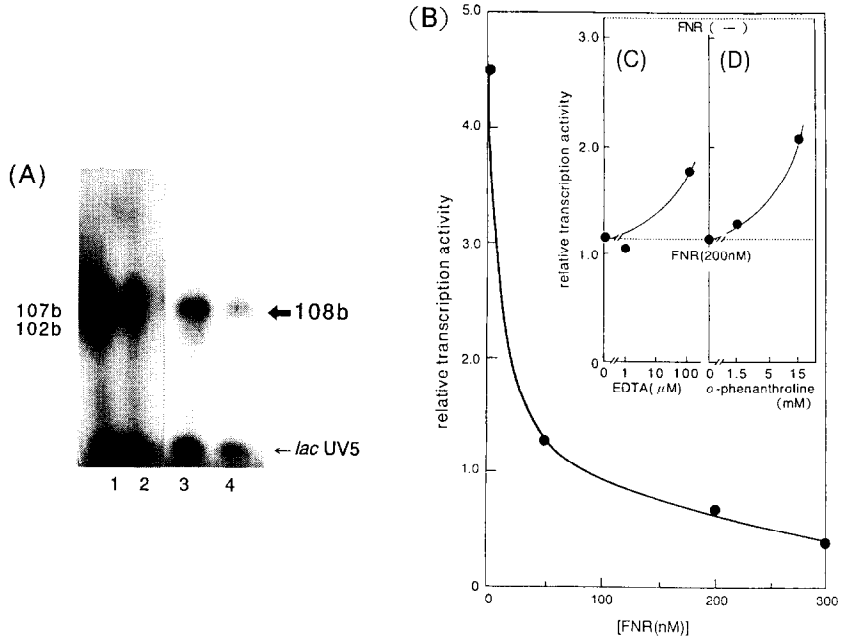

Fig. 2. Autorepression effect of FNR on in vitro transcription initiated by the *fnr* promoter. (A) In vitro transcription of *fnr* fragments of 275-bp (lanes 1–4 with 0, 50, 200 and 300 nM FNR, respectively) was carried out in a mixed transcription system. RNA polymerase holoenzyme, 1 pmol; *fnr* 275-bp fragments, 0.88 pmol; and *lac*UV5, 0.072 pmol in 50 μl reaction mixture. The transcript size was confirmed by two size markers of 107 and 102 b (of which positions are as indicated), provided by transcription of digested pBluescript II KS(STRATAGENE) by restriction enzymes *Apa*I and *Xho*I, respectively. (B) From the autoradiographic intensity of each band in (A) as measured with a Fujix BAS2000 BioImaging analyzer, the relative levels of 108 b RNA, taken as the relative transcription activity, was estimated through normalization based on the level of *lac*UV5 transcript as previously described [19] (C and D). The relative transcription activity without added FNR (upper dotted line indicated by FNR(−)) was repressed by 200 nM FNR to the level indicated by lower dotted line. The releasing effect on the repression by EDTA and *o*-phenanthroline is shown in (C) and (D), respectively.

the synthesis of 108 b RNA was specifically repressed (lanes 1–4 in Fig. 2A), with 300 nM FNR exhibiting approximately 90% repression (Fig. 2B). This observation indicates that the synthesis of the *fnr* transcript is autogenously repressed by its own gene product FNR. The autorepression by FNR was weakened by the addition of EDTA or σ-phenanthroline (see Fig. 2C or 2D), presumably due to conversion of the FNR from active to inactive conformation by chelation of the FNR-associated (Fe(II)) ion [2].

In order to identify the transcriptional regulatory signal(s) on this *fnr* fragment, we prepared a set of upstream deletion fragments and assayed transcript formation in the presence and absence of FNR. The 231- and 200-bp truncated fragment yielded the 108 b transcript (lanes 3, 5 in Fig. 3A) as observed with the 275-bp template (Fig. 2A and lane 1 in Fig. 3A), although the amount was much reduced. The synthesis of 108 b from the 275- and 231-bp templates, possessing two FNR-binding sites (anaero-boxes (1) and (2), in Fig. 1A and Fig. 3B), were both significantly repressed by the addition of FNR (lanes 1–4 in Fig. 3A). In contrast, the 200-bp template carrying only one FNR-binding site (anaero-box (1), in Fig. 1A), the synthesis of the 108 b transcript was not affected by added FNR (lane 5–6 in Fig. 3A and B). The fast-migrating smaller transcript, produced from the 275-bp template as a minor component if detected (lane 1 in Fig. 3A), was also repressed by FNR (lane 2 in Fig. 3A), raising the possibility that the *fnr* gene carries two FNR repressible promoters of different strengths.

3.2. *Repression of* narX *transcription by FNR*

Analysis of the effect of FNR on transcriptional activation and repression was extended to the regulatory region for two other anaerobic operons, *narX* and *narK*. For this purpose, we prepared a DNA fragment of 421-bp *narX/K* (see Fig. 1B).

No *narK* transcript was identified when the 421-bp truncated fragment carrying the *narK* promoter was used. In contrast, we could detect a *narX* transcript of 200 b in length by using the 421-bp truncated template (Fig. 4A). This was confirmed also by the primer extension method (data not shown), indicating that the transcription start site is located 159 bp upstream of the initiation codon for NarX, in a region which contains no effective open reading frame. The 200 b RNA was synthesized from either the original 421-bp template or the 356-bp fragment lacking the upstream region of the *narX* promoter but still carrying all three anaero-boxes (see Fig. 1B). Furthermore, upon the addition of FNR, transcription of the *narX* promoter was significantly repressed (Fig. 4A and B), 250 nM FNR exhibiting approximately 90% repression. Further deletion yielded inactive templates of 200 (Fig. 4C and D) and 306-bp (not

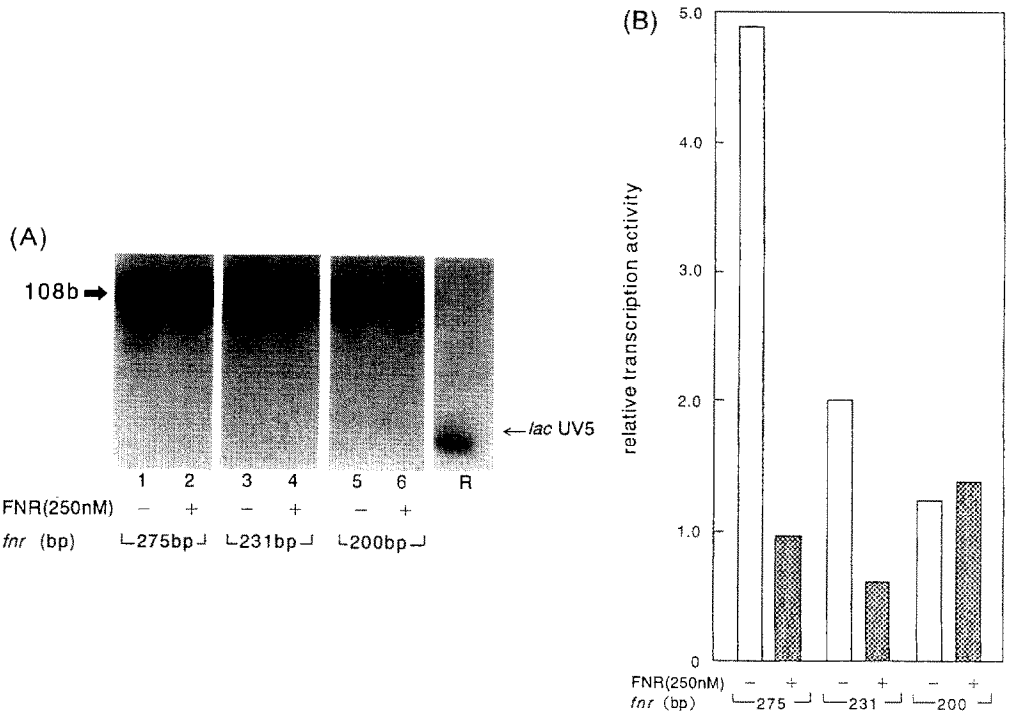

Fig. 3. Effect of the *fnr* promoter length on autorepression by FNR. (A) In vitro transcription assay was performed with 1 pmol of RNA polymerase and ~0.1 pmol for each of the promoter fragments. FNR concentration was 200 nM. Lane R is a 63 b transcript from the *lac*UV5 promoter (0.072 pmol). (B) The relative transcription activity was estimated from the autoradiographic intensity of each band in (A).

shown), each possessing one (box 1) and two (box 1 and box 2) out of three anaero-boxes, respectively (see Fig. 1B). Therefore, the upstream region between −105 and −155 seems to be required for transcription from the *narX* promoter.

4. Discussion

Complex regulatory systems operate in *E. coli* for the expression of anaerobic respiratory genes [1]. All these genes are activated by FNR bound to the anaero-box(es) located near the promoters [2]. In addition to its transcriptional activator function, FNR has been shown to repress the *fnr* and *ndh* genes in vivo [2], and Guest and colleagues [9] have reported repression of in vitro transcription of *ndh* in the presence of FNR. In this study, we have demonstrated autorepression of *fnr* and repression of *narX* by purified FNR.

An in vivo study using a *fnr-lacZ* translational fusion suggested that *fnr* is expressed under both aerobic and anaerobic growth conditions but that its expression is repressed by glucose or hyper-expression of FNR particularly during anaerobic growth [20]. Our template truncation experiments revealed that the involvement of two anaero-boxes in the *fnr* and of three anaero-boxes in the *narX* promoter region is essential for the repression by the FNR protein. A recent review by Stewart [21] stated that two anaero-boxes (corresponding to boxes (2) and (3) of *narX* in Table 1) in the overlapping *narX/K* promoter are required for the transcriptional activation of *narK*. If these palindromic boxes in the overlapping promoter are functioning in transcription in the *narX* direction, the activation effect of FNR should be also observed for transcription of *narX*. To explain the repressive effect on *narX* transcription by FNR as disclosed in the present study, another consensus sequence between +101 and +114 (box (1) of *narX* in Table 1) is proposed to be essential as an additional anaero-box in the overlapping *narX/K* promoter. Based upon this proposition, the dual function of FNR in the overlapping promoter could be interpreted to exhibit repression toward *narX* but activation toward *narK* depending on the arrangement of these three anaero-boxes.

In addition to the specific repression by the FNR protein, the significant decrease in transcription of the *fnr* and *narX* genes was also noticed even in the absence of FNR by truncation of respective upstream sequences. In this respect, we suggest that an upstream sequence is required as a *cis*-element for transcription of both the *fnr* and *narX* genes. The putative 'upstream activation' (or 'extended promoter') signal is located between −92 and −123 for *fnr* and between −105 and −155 for *narX* as is the case for the *E. coli rrnB* P1 promoter (the region between −50 and −154 [22]. Noteworthy is the fact that these regions of *fnr* and *narX* include one of the anaero-boxes (box (2) in Fig. 1A and box (3) in Fig. 1B, respectively). Therefore, one possibility is that the anaero-box

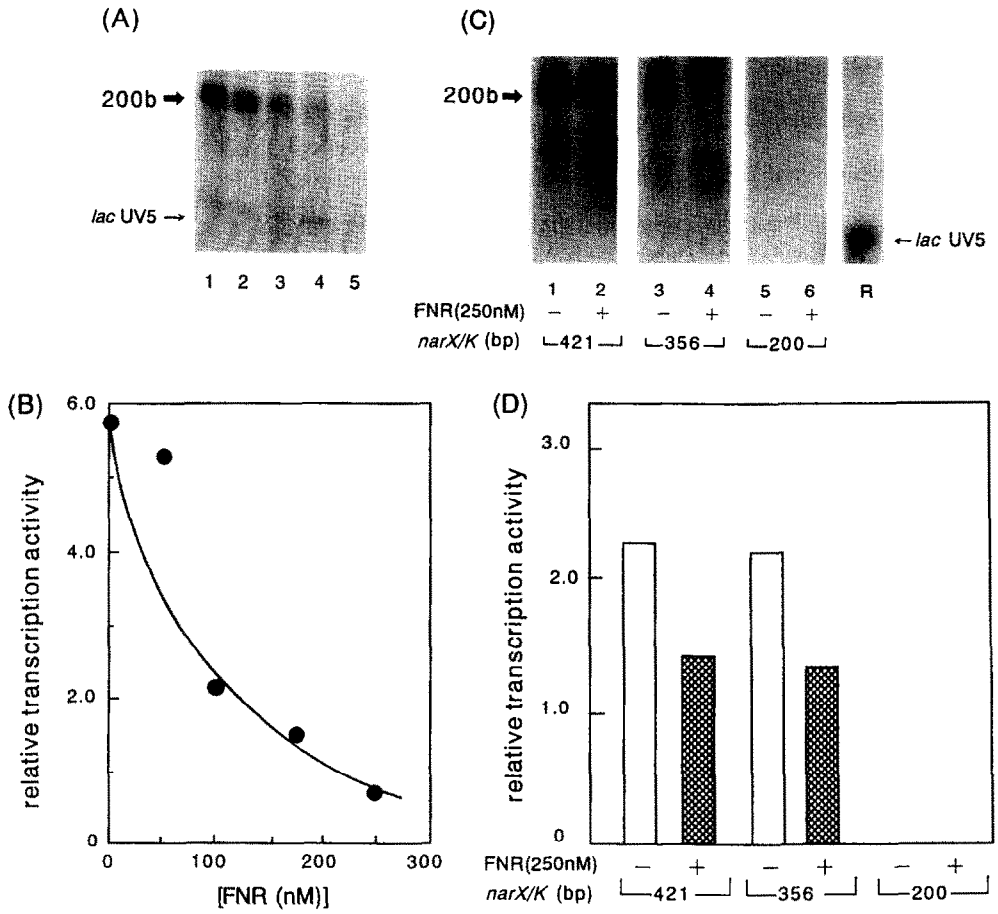

Fig. 4. Effects of FNR on transcription initiated by the *narX* promoter. (A) In vitro transcription of *narX* fragment of 421-bp (lanes 1–5 with 0, 50, 100, 175, and 250 nM FNR, respectively) yielding 200 b transcript. The conditions were the same as described in Fig. 2A except for the use of ~0.1 pmol of *narX/K* fragment. (B) From the autoradiographic intensity of each band in (A), the plot of relative transcription activity was obtained. (C) In vitro transcription assay was carried out with ~0.1 pmol of the various truncated *narX/K* fragments. The concentration of FNR, when added for autorepression, was 250 nM. Lane R is the 63 b transcript from the *lac*UV5 promoter. Other conditions employed were same as described in Fig. 2A. (D) The relative transcription activity was estimated from the autoradiographic intensity of each band in [C].

itself serves as an upstream activation signal of *fnr* or *narX*, for the transcription of 108 b RNA (Fig. 3B) or 200 b RNA (Fig. 4D), respectively, although the consensus sequence for such upstream activation signals has not yet been identified [23].

FNR possesses a cysteine cluster to which iron (Fe(II)) binds only in the active form [2,6]. In this study, we found that the addition of EDTA and *s*-phenanthroline inhibited the repression activity of FNR (see Fig. 2C or D). Despite the sequence homology in the DNA binding domain between FNR and CRP, the two regulatory proteins respond to different signals. In the case of CRP-activated transcription, CRP bound upstream of the promoter interacts with the contact site I on the RNA polymerase *α*-subunit [13], whereas CRP bound within the promoter region interacts with the contact site II of the σ-subunit [24,25]. To reveal the molecular mechanisms of transcription activation and repression by FNR, it is also a prerequisite to determine the contact site(s) for FNR on RNA polymerase.

Acknowledgements: We thank Drs. Y. Kohara (National Institute of Genetics, Japan), S. Tabor (Harvard Medical School, USA) and H. Weiner (Purdue University, USA) for kindly donating lambda 8C12, pT7-6 and BL21(DE3)pLysS strains, respectively. This work was supported by Grants-in-Aid for Scientific Research on Priority Areas (no. 02263209, 03259210, 04254212 to K.T.; no 03354035, 04256105, 04454614 to A.I.) from the Japanese Ministry of Education, Science and Culture, and by a fund (to S.T.) from CIBA-GEIGY Foundation for the Promotion of Science (1991).

References

[1] Iuchi, S. and Lin, E.C.C. (1991) Cell 66, 5–7.
[2] Spiro, S. and Guest, J.R. (1990) FEMS Microbiol. Rev. 75, 399–428.
[3] Noji, S., Nohno, T., Saito, T. and Taniguchi, S. (1989) FEBS Lett. 252, 139–143.
[4] Unden, G. and Guest, J.R. (1985) Eur. J. Biochem. 146, 193–199.
[5] Trageser, M., Spiro, S., Duchene, A., Kojro, E., Fahrenholz, F., Guest, J.R. and Unden, G. (1990) Mol. Microbiol. 4, 21–27.
[6] Green, J., Trageser, M., Six, S., Unden, G. and Guest, J.R. (1991) Proc. R. Soc. Lond. B 244, 137–144.

[7] Shaw, D.J. and Guest, J.R. (1982) Nucleic Acids Res. 10, 6119–6130.
[8] Shaw, D.J., Rice, D.M. and Guest, J.R. (1983) J. Mol. Biol. 166, 241–247.
[9] Sharrocks, A.D., Green, J. and Guest, J.R. (1991) Proc. R. Soc. Lond. B 245, 219–226.
[10] Nohno, T., Noji, S., Taniguchi, S. and Saito, T. (1989) Nucleic Acids Res. 17, 2947–2957.
[11] Kohara, Y., Akiyama, K. and Isono, K. (1987) Cell 50, 495–508.
[12] Studier, F.W., Rosenberg, A.H. Dunn, J.J. and Dubendorff, J.W. (1990) Methods Enzymol. 185, 60–89.
[13] Igarashi, K. and Ishihama, A. (1991) Cell 65, 1015–1022.
[14] Dong, X.R., Li, S.F. and DeMoss, J.A. (1992) J. Biol. Chem. 267, 14122–14128.
[15] Li, S.F. and DeMoss, J.A. (1988) J. Biol. Chem. 263, 13700–13705.
[16] Walker, M.S. and DeMoss, J.A. (1992) J. Bacteriol. 174, 1119–1123.
[17] Domdey, H., Apostol, B., Lin, R.-J., Newman, A., Brody, E. and Abelson, J. (1984) Cell. 39, 611–621.
[18] Tyson, K.L., Bell, A.I., Cole, J.A. and Busby, S.J.W. (1993) Mol. Microbiol. 7, 151–157.
[19] Kajitani, M. and Ishihama, A. (1983) Nucleic Acids Res. 11, 671–686.
[20] Spiro, S. and Guest, J.R. (1987) J. Gen. Microbiol. 133, 3279–3288.
[21] Stewart, V. (1993) Mol. Microbiol. 9, 425–434.
[22] Leirmo, S. and Gourse, R.L. (1991) J. Mol. Biol. 220, 555–568.
[23] Ross, W., Gosink, K.K., Salomon, J., Igarashi, K., Zou, C., Ishihama, A., Severinov, K. and Gourse, R.L. (1993) Science 262, 1407–1413.
[24] Igarashi, K., Hanamura, A., Makino, K., Aiba, H., Aiba, H., Muzuno, T., Nakata, A. and Ishihama, A. (1991) Proc. Natl. Acad. Sci. USA 88, 8958–8962.
[25] Kumar, A., Grimes B., Fujita, N., Makino, K., Malloch, R.A., Hayward, R.S. and Ishihama, A. (1994) J. Mol. Biol. 235, in press.