Volume 329, number 1,2, 55–58 FEBS 12859 August 1993

A role for iron in transcriptional activation by FNR

Jeffrey Green and John R. Guest

The Krebs Institute for Biomolecular Research, Department of Molecular Biology & Biotechnology, University of Sheffield, Western Bank, Sheffield S10 2UH, UK

Received 15 June 1993

FNR is a transcriptional regulator which controls the expression of target genes in response to anoxia in *Escherichia coli*. The mechanism by which FNR senses and responds to anaerobiosis is unknown but indirect evidence suggests that an iron cofactor is involved. Using $KMnO_4$ as a probe for DNA melting at active promoters, footprinting studies have now shown that the ferrous iron chelator, ferrozine, inhibits open complex formation in vivo, and that FNR with a high iron-content is essential for open complex formation in vitro. Since open complex formation is an essential pre-requisite for transcription, it is concluded that transcriptional activation by FNR is mediated by a ferrous iron cofactor.

FNR; Transcriptional regulation; Iron; Footprinting; Redox-sensing; *Escherichia coli*

1. INTRODUCTION

The FNR protein of *Escherichia coli* belongs to a family of transcriptional regulators which are structurally related to the cAMP receptor protein (CRP) or catabolite activator protein (CAP). FNR regulates gene expression in response to oxygen starvation whereas CRP activates catabolite-sensitive genes in response to cAMP (glucose starvation). It is predicted that FNR contains all of the secondary structural elements in CRP but FNR differs in being monomeric rather than dimeric, in possessing a cysteine-rich N-terminal extension, and in its ability to bind up to one atom of iron per monomer [1–3].

The mechanism by which FNR senses and responds to anoxia is unknown but several lines of evidence suggest that the cysteine-rich N-terminal region forms part of an iron-binding redox-sensing domain, and that transcriptional activation by FNR is controlled by a redox-responsive iron cofactor. First, the activation and repression of FNR-dependent promoters is prevented deleting residues 3–30 or by single substitutions of four of the five cysteine residues (C20, C23, C29 and C122, but not C16) [4–6]. Second, chelating agents mimic oxygen in preventing the anaerobic activation or repression of FNR regulated genes [7] and by increasing the rate of cysteine-residue carboxymethylation in the FNR of permeabilized cells [8]. Third, purified FNR protein contains two forms differing in the presence of a disulphide bond linking C122 to one of the N-terminal cysteine residues, and the proportion of the oxidised form increases in aerobic bacteria [9]. It has also been shown that the in vivo activation of FNR is reversible, depending on the availability of iron and the oxygen status [10], and that FNR responds to redox rather than to oxygen per se [11].

In previous studies [12] it was shown a high iron content is not essential for site-specific binding of FNR to target DNA although it improves the binding affinity about 1.7- to 2-fold with different promoters [12]. Here, studies with the FNR-activated *FF-melR* promoter show that iron is essential for the formation of open complex in vitro, and that a ferrous iron chelating agent, ferrozine, inhibits open complex formation in vivo.

Correspondence address: J.R. Guest, Department of Molecular Biology and Biotechnology, University of Sheffield, Western Bank, Sheffield S10 2UH, UK.

Abbreviations: CRP, cAMP receptor protein.

2. EXPERIMENTAL

2.1. *Bacterial strains, plasmids and other materials*

Two strains of *E. coli K12*: JM101 (*fnr*$^+$) and an *fnr*-deletion strain JRG1728 (*fnr*$^-$), each containing a plasmid (pGS422) in which the promoter region of an FNR-dependent *melR-lacZ* fusion, *FF-melR* (kindly provided by Dr S. Busby), is located in a 343 bp *Eco*RI–*Hin*dIII fragment [12]. Standard procedures were used for the isolation and manipulation of DNA. RNA polymerase saturated with σ^{70} was obtained from Pharmacia, other enzymes from NBL, and [α-^{35}S]dATP from Amersham International.

2.2. *Footprinting studies*

In vivo $KMnO_4$ footprinting was performed according to Heltzel et al. [13]. The *fnr*$^+$ and *fnr*$^-$ strains were grown aerobically and anaerobically to mid-exponential phase in L-broth supplemented with glucose (0.5%) and ampicillin (100 μg/ml) before transfer to minimal medium A containing ampicillin (50 μg/ml) [14]. Cultures were treated with rifampicin (200 μg/ml, for 5 min) and probed with $KMnO_4$ (6.25 mM for 4 min). Plasmid DNA was then isolated, digested with *Eco*RI, and end-labelled with [α-^{35}S]dATP using DNA polymerase I (Klenow

fragment) [15]. The labelled *FF-melR* promoter fragment was released by *Hin*dIII digestion and then processed, cleaved and analysed on 8% denaturing polyacrylamide-urea sequencing gels as described previously [13].

In vitro $KMnO_4$ footprinting [16] was performed with FNR protein purified in the presence of added iron (high-iron FNR, 0.8 mol iron/monomer) or in the absence of iron (low-iron FNR, 0.14 mol iron/monomer) as described previously [3]. Combinations of RNA polymerase (10 pmol) and FNR (20 pmol) were incubated with the 343 bp, end-labelled *FF-melR* promoter fragment of pGS422 in buffer containing Tris-HCl pH 8.0 (20 mM), $MgCl_2$ (10 mM), EDTA (0.1 mM), KCl (120 mM), and glycerol (5% by vol), and then probed for 2 min with $KMnO_4$ (8.8 mM). The reactions were stopped by adding β-mercaptoethanol (1 M) and the DNA was processed and analysed as described previously [16].

3. RESULTS AND DISCUSSION

Melting of the DNA helix associated with the activation of transcription at a promoter can be detected by reacting the transcriptional complexes with $KMnO_4$ both in vivo and in vitro. $KMnO_4$ reacts preferentially with unpaired thymine and cytosine bases in the RNA polymerase–promoter complex, and rifampicin improves the signal intensities observed in vivo by stalling these complexes.

3.1. *The effect of ferrozine on open complex formation at the FF-melR promoter in vivo*

The effect of the ferrous iron chelating agent ferrozine on FNR-dependent open complex formation at the *FF-melR* promoter was investigated using in vivo $KMnO_4$ footprinting. The *FF-melR* promoter is a semi-synthetic promoter generated by replacing the natural CRP binding site in the promoter region of a *melR-lacZ* gene fusion with an FNR consensus sequence. As a result, the fusion becomes an anaerobically activated member of the FNR modulon [17]. Open complex formation at the *FF-melR* promoter in anaerobic cultures of the *fnr*$^+$ strain was apparent from the enhanced reactivity of the A-T base pairs at positions −11, −9 and −8 relative to the transcription start site in in vivo footprints (Fig. 1, lane 1). No such reactivity was detected with cultures grown in the presence of ferrozine (Fig. 1, lane 2) or with aerobic cultures (not shown). Under these conditions the in vivo footprints resembled those for anaerobic cultures of the *fnr*$^-$ strain (Fig. 1, lane 3).

The results show that open complex is formed only under anaerobic conditions when FNR is present and they confirm previous observations that chelating agents inhibit the FNR-dependent expression of appropriate *lacZ* fusions [7]. The inhibitory effects of chelators on *lacZ* expression could be prevented by the prior addition of metal ions, ferrous iron being the most effective. However, although the latter studies suggested that iron may function in a putative redox-sensing and response pathway, the complexities of the in vivo situation meant that a precise role in transcription regulation could not be inferred. It can now be concluded that

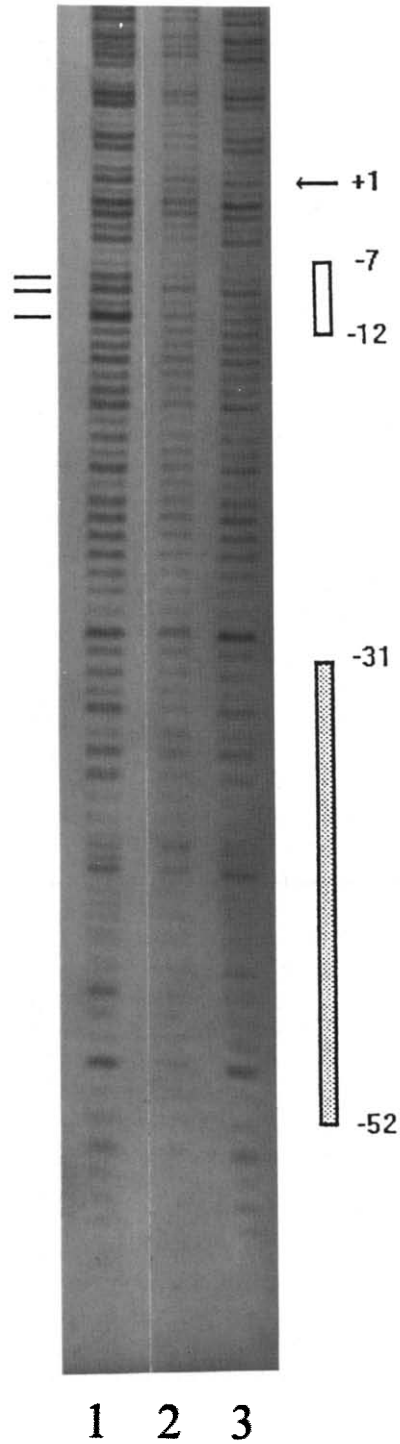

Fig. 1. In vivo $KMnO_4$ footprints from anaerobically grown JM101 (*fnr*$^+$) and JRG1728 (*fnr*$^-$) transformed with pGS422. Lane 1, *fnr*$^+$; lane 2, *fnr*$^+$ grown in the presence of ferrozine (0.4 mM); lane 3, *fnr*$^-$. Nucleotide numbering is from the transcriptional start point (+1), and the −10 region (open box), FNR binding site (shaded box), and FNR-dependent hyper-reactive bases (horizontal bars), are indicated.

ferrozine has a direct effect on open complex formation. The fact that the inhibitor ferrozine, is a ferrous iron chelator, further implies that ferrous iron has an important role in trancriptional activation by FNR.

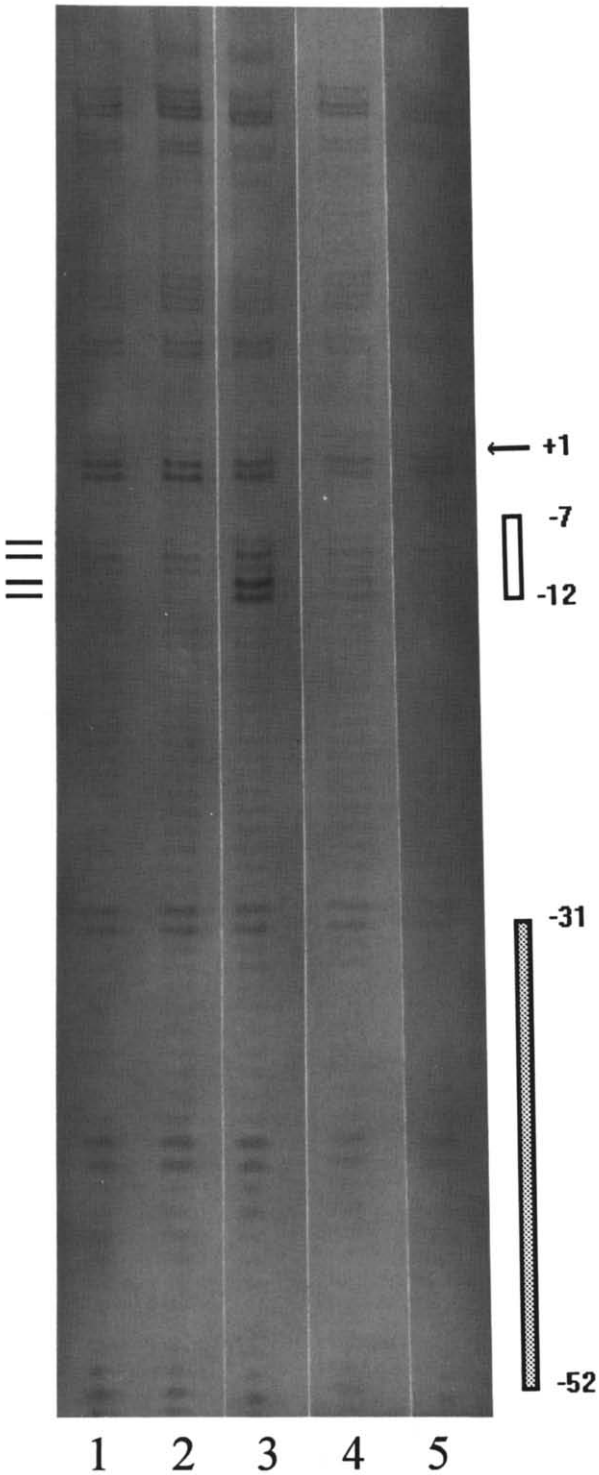

Fig. 2. In vitro $KMnO_4$ footprints of the *FF-melR* promoter. An end-labelled 343 base pair *Eco*RI–*Hin*dIII fragment containing the *FF-melR* promoter was incubated without further addition (lane 1) or with high-iron FNR (lane 2), high-iron FNR and RNA polymerase (lane 3), low-iron FNR and RNA polymerase (lane 4), and RNA polymerase alone (lane 5). The nucleotide positions are numbered from the transcriptional start point (+1), and the –10 region (open box), FNR-binding site (shaded box), and FNR-dependent hyper-reactive bases (horizontal bars), are indicated.

3.2. *The effect of the iron content of FNR on open complex formation in vitro*

In order to better define the role of iron in FNR-dependent gene activation, in vitro $KMnO_4$ footprints were performed using FNR protein containing a high and low iron content, 0.8 mol/FNR monomer and 0,14 mol/FNR monomer, respectively. Enhanced reactivity at positions −12, −11, −9 and −8, was observed with high-iron FNR (Fig. 2, lane 3), but it was barely detectable with low-iron FNR (Fig. 2, lane 4) and absent with no FNR (Fig. 2, lane 1). Thus it is clear that the presence of bound iron is essential for DNA-melting and open complex formation at the FNR-activated *FF-melR* promoter.

It was previously shown that FNR with a high iron content is capable of activating in vitro transcription from the *FF-melR* promoter [12], although a high iron content is not required for site-specific DNA-binding [3,12]. The present studies now show iron is essential for open complex formation by the FNR-RNA polymerase complex. It seems likely that iron is involved in the mechanism used by FNR for sensing and responding to redox state, and it would seem that even after isolation under aerobic conditions, preparations of high-iron FNR contain sufficient of the active form to promote open complex formation and generate specific transcripts. The proposed switch between active and inactive FNR has not been reproduced in vitro. So far, attempts to reactivate low-iron FNR by reincorporating iron under a variety of conditions have failed. Nevertheless, most of the evidence, including that reported here, points to FNR having a redox-sensing iron cofactor which can activate transcription when ferrous iron is present.

Acknowledgements: This work was supported by the Science and Engineering Research Council.

REFERENCES

[1] Spiro, S. and Guest, J.R. (1990) FEMS Microbiol. Rev. 75, 399–428.
[2] Unden, G. and Trageser, M. (1991) Antonie van Leeuwenhoek 59, 65–76.
[3] Green, J., Trageser, M., Six, S., Unden, G. and Guest, J.R. (1991) Proc. Roy. Soc. B 244, 137–144.
[4] Spiro, S. and Guest, J.R. (1988) Mol. Microbiol. 2, 701–707.
[5] Sharrocks, A.D., Green, J. and Guest, J.R. (1990) FEBS Lett. 270, 119–122.
[6] Melville, S.B. and Gunsalus, R.B. (1990) J. Biol. Chem. 265, 18733–18736.
[7] Spiro, S., Roberts, R.E. and Guest, J.R. (1989) Mol. Microbiol. 3, 601–608.
[8] Trageser, M. and Unden, G. (1989) Mol. Microbiol. 3, 593–599.
[9] Green, J., Sharrocks, A.D., Green, B., Geisow, M. and Guest, J.R. (1993) Mol. Microbiol. 8, 61–68.
[10] Engel, P., Trageser, M. and Unden, G. (1991) Arch. Microbiol. 156, 463–470.
[11] Unden, G., Trageser, M. and Duchene, A. (1990) Mol. Microbiol. 4, 315–319.

[12] Sharrocks, A.D., Green, J. and Guest, J.R. (1991) Proc. Roy. Soc. B 245, 219–226.
[13] Heltzel, A., Lee, I.W., Totis, P.A. and Summers A.O. (1990) Biochemistry 29, 9572–9584.
[14] Miller, J.H. (1972) Experiments in Molecular Genetics, Cold Spring Harbour Laboratory, Cold Spring Harbour, NY.
[15] Davies, R.W. (1988) in; Gel Electrophoresis of Nucleic Acids (Rickwood, D. and Hames, B.D., Eds.) pp. 164–165, IRL Press, Oxford.
[16] Sasse-Dwight, S. and Gralla, J.D. (1989) J. Biol. Chem. 264, 8074–8081.
[17] Bell, A.I., Gaston, K.L. and Busby, S.J.W. (1989) Nucleic Acids Res. 17, 3865–3874.