4028–4034 *Nucleic Acids Research, 1997, Vol. 25, No. 20* © *1997 Oxford University Press*

Transcription activation at Class I FNR-dependent promoters: identification of the activating surface of FNR and the corresponding contact site in the C-terminal domain of the RNA polymerase α subunit

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Received July 7, 1997; Revised and Accepted August 26, 1997

ABSTRACT

A library of random mutations in the *Escherichia coli fnr* gene has been screened to identify positive control mutants of FNR that are defective in transcription activation at Class I promoters. Single amino acid substitutions at D43, R72, S73, T118, M120, F181, F186, S187 and F191 identify a surface of FNR that is essential for activation which, presumably, makes contact with the C-terminal domain of the RNA polymerase α subunit. This surface is larger than the corresponding activating surface of the related transcription activator, CRP. To identify the contact surface in the C-terminal domain of the RNA polymerase α subunit, a library of mutations in the *rpoA* gene was screened for α mutants that interfered with transcription activation at Class I FNR-dependent promoters. Activation was reduced by deletions of the α C-terminal domain, by substitutions known to affect DNA binding by α, by substitutions at E261 and by substitutions at L300, E302, D305, A308, G315 and R317 that appear to identify contact surfaces of α that are likely to make contact with FNR at Class I promoters. Again, this surface differs from the surface used by CRP at Class I CRP-dependent promoters.

INTRODUCTION

The *Escherichia coli* FNR protein is a global activator of transcription initiation which regulates transcription from a large number of promoters in response to oxygen starvation. FNR is related to another global regulator, the cyclic AMP receptor protein (CRP). FNR and CRP are believed to have similar structures and to have evolved from a common origin (reviewed in 1,2). Binding sites for both FNR and CRP span 22 bp, accommodating dimers of each activator. A striking feature of both FNR- and CRP-dependent promoters is that the location of the DNA site for the activator can vary from one promoter to another. Studies with semi-synthetic promoters in which a consensus DNA site for either FNR or CRP was positioned at different distances upstream of the same promoter elements showed that FNR or CRP dimers could activate transcription when they were centred near positions 41, 61, 71, 81 or 91 bp upstream of the transcript start point (3–5).

At promoters where the DNA site for FNR or CRP is centred around 41 bp upstream of the transcription start (known as Class II promoters), FNR and CRP function by making multiple interactions with different parts of the RNA polymerase holoenzyme (RNAP) (reviewed in 6; see also 5). In contrast, at promoters where the DNA site for FNR or CRP is centred further upstream (known as Class I promoters), transcription activation is dependent solely on interactions with the C-terminal domain of the RNAP α subunit (αCTD) (reviewed in 7,8; see also 5). At Class I CRP-dependent promoters a surface-exposed loop on the downstream subunit of the CRP dimer (amino acids 156–164, known as activating region 1) interacts with a contact site in αCTD, resulting in recruitment of αCTD to promoter DNA and an increase in RNAP binding (reviewed in 7,8). Thus, transcription activation at Class I CRP-dependent promoters is suppressed by single amino acid substitutions in activating region 1 of CRP (9,10) and by deletions and single amino acid substitutions in αCTD (11–14).

In contrast, far less is known about interactions between FNR and αCTD at Class I FNR-dependent promoters. A single positive control FNR mutant defective at Class I promoters has been identified (15). This mutant carries the single amino acid substitution SF73 and, based on its properties, Wing *et al.* (5) concluded that FNR must contain an activating surface that was equivalent to activating region 1 of CRP. Using the technique of 'oriented heterodimers', this activating region was shown to be functional in the downstream subunit of the FNR dimer at Class I FNR-dependent promoters (5). In the first part of this work we generated a random library of mutations in the *fnr* gene and screened for FNR mutants that were defective in activation at Class I FNR-dependent promoters. This allowed us to identify the activating surface of FNR (including S73) that is functional at these promoters. In the second part we generated a random library of mutations in the segment of the *rpoA* gene encoding αCTD and screened for mutants that interfere with activation at Class I FNR-dependent promoters. The resulting mutants fall into a number of classes that confirm the role of αCTD in transcription activation and suggest a likely contact site for the activating region of FNR.

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Table 1. Promoters and plasmids used in this work

Promoters (all cloned on fragments with *Eco*RI site upstream and *Hin*dIII site downstream of the transcription start)	
FF(–71.5)	Semi-synthetic FNR-dependent promoter with FNR binding site centred at position –71.5 upstream of the *melR* transcription start (5)
YF(–71.5)	Derivative of *FF(–71.5)* with upstream half of FNR binding site, 5′-AAATTT**G**ATGT-3′ (designated F) changed to 5′-AAATTT**A**ATGT-3′ (designated Y) (5)
FY(–71.5)	Derivative of *FF(–71.5)* with downstream F sequence replaced with Y sequence (5)
FF (–61.5)	Semi-synthetic FNR-dependent promoter with FNR binding site centred at position –61.5 upstream of the *melR* transcription start (5)
p*ndh*	*E.coli ndh* promoter, which is repressed by FNR (15,17)
Plasmids	
pRW50	Broad host range *lac* expression vector for cloning of different promoters on *Eco*RI–*Hin*dIII fragments: encodes resistance to 35 µg/ml tetracycline (18)
pAA121	General cloning vector for *Eco*RI–*Hin*dIII fragments derived from pBR322: encodes resistance to 80 µg/ml ampicillin (19)
pFNR	Plasmid carrying *fnr* gene (and mutant derivatives) cloned in pBR322: encodes resistance to 80 µg/ml ampicillin (15)
pHW1	Plasmid carrying *fnr* gene (and mutant derivatives) cloned in pLG339: encodes resistance to 25 µg/ml kanamycin (5,15)
pLAW2	Plasmid carrying *rpo*A gene (and mutant derivatives) cloned in pBR322: encodes resistance to 80 µg/ml ampicillin (12)

MATERIALS AND METHODS

The Δ*lac* strains *E.coli* M182 *fnr*$^+$ and JRG1728 Δ*fnr* were used throughout this work, as before (5,15). The plasmids and promoters used in this work are listed in Table 1. All the promoters used were cloned on *Eco*RI–*Hin*dIII fragments and were shuttled between pAA121 (for manipulation) and pRW50 (for *lac* fusions and assays). By convention, promoter sequences are numbered with the transcript start as +1, with upstream and downstream sequences denoted by – and + prefixes respectively. Standard recombinant DNA, site-directed mutagenesis and sequencing technologies were used as in our previous work (5,15).

A library of random mutations throughout the *fnr* gene cloned in pFNR was created using error-prone PCR, exploiting the exact protocol described by Bell and Busby (15). From this library, positive control FNR mutants that were defective in FNR-dependent activation at the *FF(–71.5)* promoter were selected, using the protocol used by Bell and Busby (15) to obtain similar mutants at *FF(–41.5)*. Briefly, M182 Δ*lac fnr*$^+$ cells carrying pRW50 containing the *FF(–71.5)* promoter, encoding a *FF(–71.5)*::*lac* fusion, were transformed using electroporation with the pFNR mutant library. Transformants were plated on MacConkey lactose plates containing ampicillin and tetracycline, Lac$^-$ candidates were picked and purified and the pFNR derivative was extracted. We checked the ability of each putative mutant FNR to repress the *ndh* promoter and assayed activation at both *FF(–61.5)* and *FF(–71.5)*. To do this, the mutant pFNR derivative was transformed into JRG1728 Δ*lac* Δ*fnr* cells carrying pRW50 into which the *ndh* promoter or *FF(–61.5)* or *FF(–71.5)* had been cloned. β-Galactosidase activities of transformants grown anaerobically in L-broth supplemented with 0.4% glucose, ampicillin and tetracycline were measured (full details are given in 15). In this study we retained only those FNR mutants that, like wild-type FNR, fully repressed expression from the *ndh* promoter. The sequence of these mutants was deduced (Table 2) and the defect in transcription activation at *FF(–61.5)* and *FF(–71.5)* was quantified (Table 3). Derivatives of pFNR encoding FNR with single alanine substitutions at particular positions were made by PCR (16). In all cases the base sequence of the entire *fnr* gene was confirmed (secondary substitutions are noted in Table 2). Oriented heterodimer experiments (Table 5) were performed exactly as described by Bell and Busby (15) and Wing *et al*. (5).

Mutagenesis of *rpoA* encoded by pLAW2 was performed using protocols derived from Zou *et al*. (12). This plasmid carries a unique *Hin*dIII site adjacent to codon 231 of *rpoA* and a unique *Bam*HI downstream of the *rpoA* stop codon. Using primers flanking the *Hin*dIII and *Bam*HI sites the segment of *rpoA* corresponding to the C-terminal domain (from codon 231) was synthesized using error-prone PCR as above. After restriction with *Hin*dIII and *Bam*HI, the product was cloned into pLAW2 to generate a library of random mutations in the segment of pLAW2 encoding αCTD. M182 Δ*lac fnr*$^+$ cells carrying pRW50 containing the *FF(–71.5)* promoter, encoding a *FF(–71.5)*::*lac* fusion, were transformed using electroporation with the pLAW2 mutant library. Transformants were plated on MacConkey lactose plates containing ampicillin and tetracycline and Lac$^-$ candidates were picked and purified. The pLAW2 derivatives were extracted, mutant *rpoA* sequences were determined and the defects in transcription activation at *FF(–71.5)* were quantified (Table 6).

Table 2. Sequences of 22 *fnr*-positive control (p.c.) mutants

Amino acid substitution	Codon substitution	No. of independent isolates[a]	Repression at p*ndh*[b] (%)
DG43	GAT→GGT	1	100
RH72	CGC→CAC	1	88
SF73	TCC→TTC	1	97
TA118	ACC→GCC	1	98
TP118	ACC→CCC	1[c]	100
MI120	ATG→ATA	2	93
MR120	ATG→AGG	1[d]	100
MT120	ATG→ACG	2	100
MV120	ATG→GTG	3[e]	94
FL181	TTT→CTT	2	101
FS186	TTC→TCC	2	105
SP187	TCC→CCC	2[f]	95
FL191	TTC→CTC	3	99

pFNR derivatives encoding FNR carrying the listed substitutions were isolated after mutagenesis of pFNR as described in the text. In each case the entire *fnr* base sequence was determined. JRG1728 cells carrying pRW50 containing the *ndh* promoter were transformed with different pFNR derivatives and β-galactosidase expression in transformants was measured and compared with a control pFNR plasmid from which the *fnr* gene had been removed.

[a]Independent isolates are defined as substitutions that occurred in different PCR reactions.

[b]Repression was measured *in vivo* using a p*ndh*::*lac* fusion. Values of repression are expressed as percentages of repression achieved with wild-type FNR.

[c]This isolate contained a second amino acid substitution MV223 (ATG→GUG). Data for repression is for FNR carrying the single substitution mutant TP118, constructed by subcloning.

[d]This isolate contained a second amino acid substitution YC230 (TAC→TGC). Data for repression is for FNR carrying the single substitution MR120, constructed by subcloning.

[e]One isolate contained two further amino acid substitution: FL191 (TTC→CTC), KR220 (AAA→AGA).

[f]One isolate contained a second amino acid substitution: FY112 (TTC→TAC).

Table 3. Transcription activation by FNR derivatives carrying different p.c. substitutions

Amino acid substitution	Activation at *FF(–71.5)* (%)	Activation at *FF(–61.5)* (%)
Wild-type FNR	100	100
DG43	42	32
RH72	20	13
SF73	5	6
TA118	15	19
TP118	18	16
MI120	8	9
MR120	3	1
MT120	25	21
MV120	23	15
FL181	52	51
FS186	48	28
SP187	4	9
FL191	31	29
No FNR	0	0

Activation was measured in JRG1728 (Δ*lac* Δ*fnr*) cells carrying *FF(–71.5)* or *FF(–61.5)* fused to *lac* in pRW50. Values are expressed as percentages of transcription activation by wild-type FNR. 100% activation = 2870 nmol/min/mg cell dry wt at *FF(–71.5)* and 2960 nmol/min/mg at *FF(–61.5)*; 0% activation = 390 nmol/min/mg at *FF(–71.5)* and 280 nmol/min/mg at *FF(–61.5)*. Data in the table are averages of three independent assays; in each case the standard deviation from the mean was <10%.

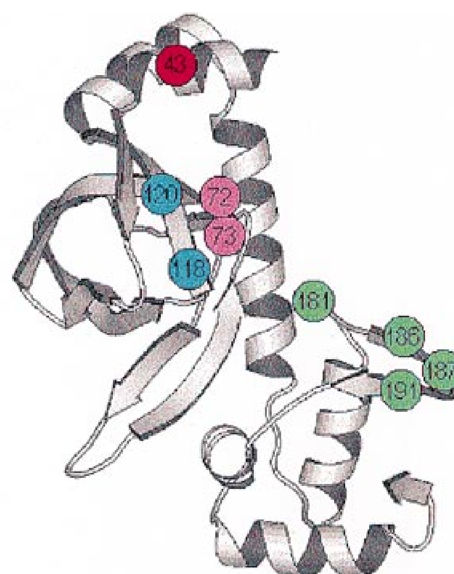

Figure 1. Three-dimensional model of an FNR monomer, without its extended N- and C-terminal domains, based on the known CRP structure taken from the Brookhaven protein database file 1CGP.PDB (a full discussion of the relation between the structures of CRP and FNR is to be found in ref. 2). The model shows the location of amino acid residues substituted in the FNR-positive control mutants described here. Residue D43 (red) is located on α-helix A, residues R72 and S73 (purple) are located on the loop between β-sheets 3 and 4, residues T118 and M120 (blue) are located on β-sheet 8, residue F181 (green) is located on the loop between α-helix D and β-sheet 9 and residues F186, S187 and F191 (green) are located on β-sheet 9.

RESULTS

Isolation of positive control mutants of FNR

The first aim of this work was to identify single amino acid substitutions in FNR that result in a defect in transcription activation at Class I FNR-dependent promoters, but do not affect DNA binding or triggering by anaerobiosis. Following previous work with CRP (9,10), we reasoned that the location of such substitutions would identify the surface of FNR that interacted with αCTD during transcription activation. To identify such 'positive control' mutants we adapted the strategy fully described by Bell and Busby (15). The starting point was the semi-synthetic *FF(–71.5)* promoter, which is completely dependent on FNR (5). This promoter, which contains a consensus DNA site for FNR centred between base pairs 71 and 72 upstream of the *melR* transcription start point (i.e. position –71.5), was cloned into plasmid pRW50 to give a *FF(–71.5)*::*lac* fusion. M182 (Δ*lac fnr*$^+$) cells carrying the resulting recombinant score as Lac$^+$ on indicator plates because chromosomally encoded FNR activates the *FF(–71.5)* promoter. To identify positive control FNR mutants we exploited plasmid pFNR, which carries the cloned *fnr* gene and is compatible with pRW50 derivatives. After mutagenesis of the cloned *fnr* gene by error-prone PCR, pFNR was transformed into M182 cells carrying the *FF(–71.5)*::*lac* fusion. Positive control mutants result in a Lac$^-$ phenotype, since they are unable to interact correctly with RNAP, and yet fold correctly and bind to DNA sites for FNR. Note that mutants that are unable to fold correctly will be unable to suppress activation of *FF(–71.5)* by the chromosomal *fnr* gene and will not be picked by this screen. After performing seven independent error-prone PCR mutagenesis reactions and screening >60 000 colonies, we selected 48 Lac$^-$ colonies apparently containing a *trans*-dominant pFNR derivative that interfered with activation of *FF(–71.5)* by wild-type FNR.

Since FNR mutants defective in DNA binding or triggering by anaerobiosis would also have scored as Lac$^-$ in the first screen, we included a second screening step. This second screening, which was included to eliminate these types of mutants, exploited an FNR-repressible promoter, p*ndh*. Thus, pFNR plasmid DNA isolated from each of the 48 Lac$^-$ colonies was transformed into strain JRG1728 (Δ*lac* Δ*fnr*) cells carrying a p*ndh*::*lac* fusion in plasmid pRW50 and the fusion was used to assess the ability of each mutant to be triggered by anaerobiosis and to bind to DNA targets *in vivo*. We found that 20 of the 48 pFNR derivatives were defective in anaerobically induced repression of the p*ndh*::*lac* fusion and were discarded. We concluded that the remaining 28 pFNR derivatives must encode positive control mutants of FNR: they are significantly defective in transcription activation at *FF(–71.5)*, but are not defective in transcription repression at p*ndh* (see below). The base sequence of each of these derivatives was determined and the amino acid sequence was deduced. This revealed that we had isolated 22 independent mutants (six of the mutants were obtained more than once from the same PCR reaction and were discounted). These 22 mutants were due to amino acid substitutions of nine different residues: D43, R72, S73, T118, M120, F181, F186, S187 and F191. The different substitutions are listed in Table 2: in some cases substitutions were isolated in combination with changes at other positions, but these other changes were found to have little or no effect (see footnote to Table 2). Note that these substitutions include SF73, which we had previously found to interfere with transcription activation at a Class I FNR-dependent promoter (5). Figure 1 presents a model of the

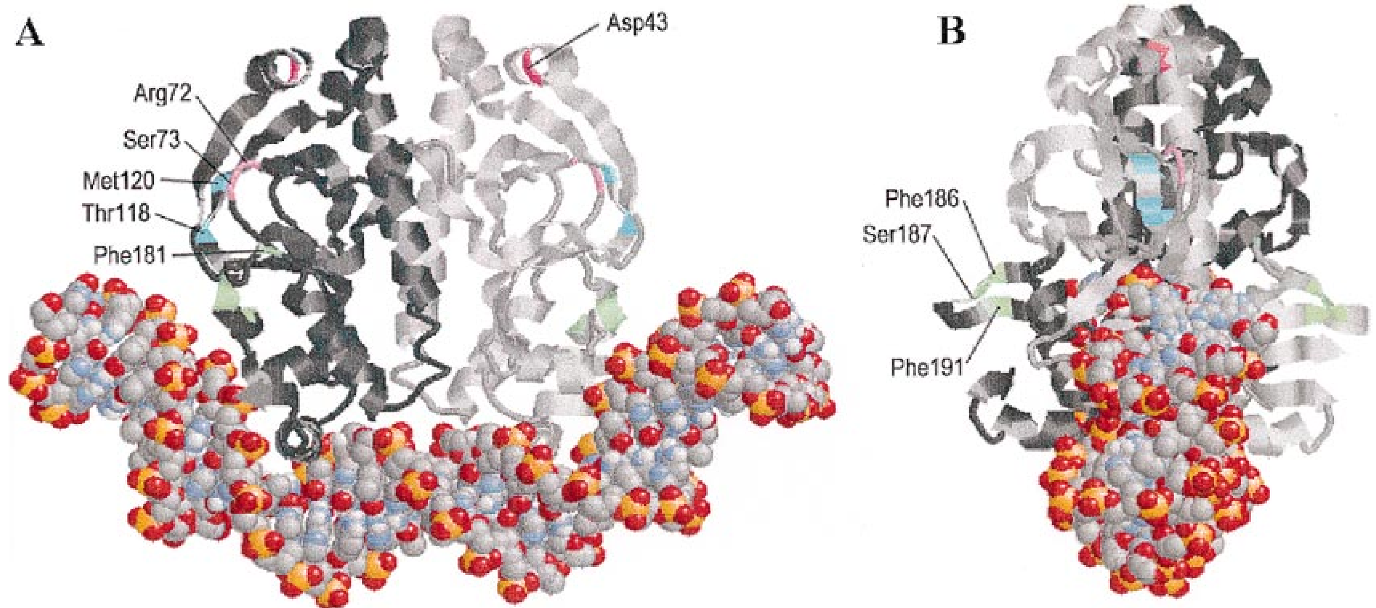

Figure 2. Models of an FNR dimer bound to a target site in DNA viewed side-on and end-on. The models, based on the CRP–DNA structure presented by Schultz *et al.* (26), show the location of substitutions that interfere with activation at Class I FNR-dependent promoters, with colour coding as in Figure 1. Note that the patch identified by green colouring corresponds to activating region 1 of CRP (see Discussion). The patch identified by pink and blue colouring has no functional equivalent in CRP, but, whilst clearly distinct, is on the same face of FNR. Note that this patch is exactly in the direction of the path of the DNA, whilst the 'green' patch (equivalent to activating region 1 of CRP) is off the path of the DNA.

predicted structure of FNR, based on the crystal structure of CRP, showing that the nine substituted residues are all found on the same face of FNR. We conclude that this face must contain the amino acid sidechains that are important for transcription activation by FNR at the *FF(–71.5)* promoter. Figure 2 shows models of FNR bound to a DNA target site, again highlighting the locations of the different positive control substitutions that interfere with FNR-dependent activation of *FF(–71.5)*.

Characterization of positive control mutants of FNR

The effects of the newly isolated substitutions in FNR on transcription activation at Class I promoters with the DNA site for FNR centred at positions –61.5 or –71.5 was determined. To do this, the different pFNR derivatives were introduced into strain JRG1728 (Δ*lac* Δ*fnr*) carrying the test promoters *FF(–71.5)* and *FF(–61.5)* fused to the *lac* operon in plasmid pRW50 and β-galactosidase expression of cells grown anaerobically was measured. The data in Table 3 show that each of the substitutions causes similar substantial reductions in expression from both promoters, confirming the phenotypes that had been observed during the screen. We conclude that the same surface of FNR is likely to be involved during transcription activation at both promoters. To investigate the role of different amino acid sidechains in this surface, a number of different residues were replaced with alanine. R72, S73, G74, T118, M120 and S187 were selected, as these are located in three separate surface-exposed loops where the positive control substitutions had been isolated (Fig. 1). The results in Table 4 show that alanine substitution of S73 and G74 had no effect on transcription activation at *FF(–71.5)*, whilst substitutions at R72, M120 and S187 have only marginal effects (<50%). In contrast, substitution of alanine at T118 greatly reduces activation, suggesting that the sidechain of T118 provides a crucial contact with RNAP. The lack of effect of the SA73 substitution suggests that the consequences of the SF73 substitution, previously reported by us, must be due to indirect effects: for example, the substitution of serine at position 73 by phenylalanine may generate a clash that hinders FNR–RNAP contacts. In control experiments we confirmed that the alanine substitutions had the same effect on activation at the *FF(–61.5)* promoter and that DNA binding was unaffected (as judged by repression of the *ndh* promoter; data not shown).

Table 4. Transcription activation by FNR carrying alanine substitutions

FNR derivative	Activation at *FF(–71.5)* (%)
FNR	100
RA72	78
SA73	109
GA74	102
TA118	15
MA120	78
SA187	58

Activation was measured in JRG1728 (Δ*lac* Δ*fnr*) cells carrying *FF(–71.5)* fused to *lac* in pRW50. Values are expressed as a percentage of activation by wild-type FNR at *FF(–71.5)*. The average activation values for wild-type FNR in nmol/min/mg cell dry wt was 2400. Each FNR derivative carrying an alanine substitution was shown to bind DNA normally, as measured by the ability to repress transcription activation from the FNR-repressible promoter p*ndh* and all alanine scanning mutants were aerobically inactive, as measured in cells grown aerobically (data not shown).

Table 5. Transcription activation by oriented heterodimers at *FF(–71.5)* derivatives

FNR derivatives		Promoter activities		FY/YF
		FY(–71.5)	*YF(–71.5)*	
Control experiment	FNR/EV209	830	900	0.9
Heterodimer experiments	SF73/EV209	2100	470	4.5
	MI120/EV209	2100	440	4.8
	SP187/EV209	1500	350	4.3

Activities were measured in JRG1728 (Δ*lac* Δ*fnr*) cells carrying *FY(–71.5)* or *YF(–71.5)* fused to *lac* in pRW50. Cells contained a pHW1 derivative encoding FNR carrying the EV209 substitution that permits FNR binding to the Y half-site. Cells also contained pFNR derivatives encoding either wild-type FNR or FNR carrying the SF73, MI120 or SP187 substitutions. Cells were grown anaerobically in L-broth supplemented with glucose, tetracycline, kanamycin and ampicillin. Promoter activities are expressed as β-galactosidase activities (nmol/min/mg cell dry wt) and ratios were calculated from three independent sets of data.

Table 6. Sequences of 27 RNA polymerase α mutants that interfere with the *FF(–71.5)* promoter

Amino acid substitution	Codon substitution	No. of isolates	Activity of *FF(–71.5)*[a] (%)
EG261	GAA→GGA	5[b]	70 ± 6
EK261	GAA→AAA	1	61 ± 18
RC265	CGC→TGC	2[c]	44 ± 11
NS268	AAC→AGC	2	80 ± 11
LF300	CTT→TTT	3	65 ± 13
LH300	CTT→CAT	1	80 ± 17
EK302	GAG→AAG	4[d]	61 ± 10
DG305	GAC→GGC	1	60 ± 1
AD308	GCT→GAT	2	57 ± 5
GA315	GGC→GCC	1	77 ± 12
RP317	CGC→CCC	1	66 ± 4
α-257+4	1 bp deletion in codon 258	1[e]	40 ± 2
α-259+7	2 bp deletion in codon 260	1[e]	69 ± 9
α-260	Stop at codon 261	1	33 ± 7
α-262	Stop at codon 263	1	22 ± 9

pLAW2 derivatives encoding α carrying the listed substitutions were isolated after mutagenesis of pLAW2 as described in the text. In each case the entire base sequence of the mutagenized segment of *rpoA* was determined.

[a]Activity was measured *in vivo* using M182 Δ*lac fnr*$^+$ cells carrying a *FF(–71.5)*::*lac* fusion cloned in pRW50. These cells were transformed with pLAW2 derivatives encoding different α mutants and β-galactosidase activities were determined. Activity values (± 1 SD) are expressed as percentages of activity with pLAW2 encoding wild-type α. Note that the observed reduction in expression must be an underestimate of the effect of each mutant since the experiment is performed with chromosomally encoded wild-type α subunits present.

[b]One isolate contained a second amino acid substitution GS279 (GGT→AGT). This second substitution has little or no effect on activation of *FF(–71.5)*.

[c]One isolate contained a second amino acid substitution QL283 (CAG→CTG). This second substitution has little or no effect on activation of *FF(–71.5)*.

[d]One isolate contained a second amino acid substitution EG245 (GAG→GGG). This second substitution has little or no effect on activation of *FF(–71.5)*.

[e]α-257+4, a 1 bp deletion in codon 258, caused a frameshift and changed codon 262 to the stop codon TGA. The amino acid sequence is identical to wild-type α up to amino acid 257, with the addition of four different amino acids (X_{257}GDLN). α-259+7, a 2 bp deletion in codon 260, caused a frameshift and changed codon 267 to the stop codon TAA. The amino acid sequence is identical to wild-type α up to amino acid 259, with the addition of seven different amino acids (Y_{259}GIDCPLC).

Transcription activation by oriented heterodimers

Our results suggest that three separate surface-exposed loops in the FNR structure are involved in transcription activation at Class I FNR-dependent promoters. Since FNR is functional as a dimer, it is possible that each of these regions is functional in both subunits or in either the upstream or the downstream subunit. To investigate this we used the method of 'oriented heterodimers', previously adapted for FNR by Bell and Busby (15). This method relies on the alteration of either the upstream or downstream half-site of the 22 bp FNR binding sequence at a target promoter from 5′-AAATTT<u>G</u>ATGT-3′ (designated F) to 5′-AAATTT-<u>A</u>ATGT-3′ (designated Y). This creates the hybrid binding sites FY or YF, with the altered half-site located either downstream or upstream respectively, and these hybrid sites were incorporated into *FF(–71.5)* to give *FY(–71.5)* and *YF(–71.5)*. Bell and Busby (15) showed that wild-type FNR is unable to recognize the altered half-site Y, whereas FNR carrying the substitution EV209 in the DNA binding helix is able to bind to Y. Thus, heterodimers between wild-type FNR and FNR EV209, which form in cells after introduction of two compatible plasmids encoding different *fnr* genes, bind to target promoters containing the hybrid binding sites with the FNR subunit carrying wild-type binding specificity binding to the F half-site and the FNR E209V subunit with altered DNA binding specificity occupying the Y half-site. In these experiments JRG1728 cells were transformed with plasmid pHW1, a pLG339 derivative encoding FNR EV209. These cells were further transformed with pFNR derivatives encoding FNR with wild-type binding specificity and the positive control substitution SF73, MI120 or SP187 (representative of substitutions in the three surface-exposed loops of FNR that we had identified). The data in Table 5 show that the three positive control substitutions in FNR all interfere with transcription activation at the hybrid promoter *YF(–71.5)* but do not interfere with activation at *FY(–71.5)*. Since the FNR subunit carrying the substitutions is targeted to the F half-site, we conclude that the activating region defined by the substitutions at S73, M120 and S187 are all functional in the downstream subunit at *FF(–71.5)*.

Isolation of mutants in αCTD that interfere with a Class I FNR-dependent promoter

The second aim of this work was to identify single amino acid substitutions in the RNA polymerase α subunit that resulted in a defect in transcription activation at Class I FNR-dependent promoters. Following previous work with CRP (12–14), we reasoned that the location of such substitutions would identify a surface in αCTD that interacted with FNR during transcription activation. To find such mutants we adapted the strategy described by Zou *et al.* (12), using M182 (Δ*lac fnr*$^+$) cells carrying the semi-synthetic *FF(–71.5)* promoter cloned into plasmid pRW50 to give a *FF(–71.5)*::*lac* fusion. These cells score as Lac$^+$ on indicator plates because chromosomally encoded FNR activates the *FF(–71.5)* promoter. To identify substitutions in αCTD that interfere with this activation we exploited plasmid pLAW2, which carries the cloned *rpoA* gene and is compatible with pRW50 derivatives. After mutagenesis of the segment of *rpoA* encoding αCTD by error-prone PCR, pLAW2 was transformed into M182 cells carrying the *FF(–71.5)*::*lac* fusion. After performing three independent error-prone PCR mutagenesis reactions and screening >70 000 colonies, we selected 27 Lac$^-$

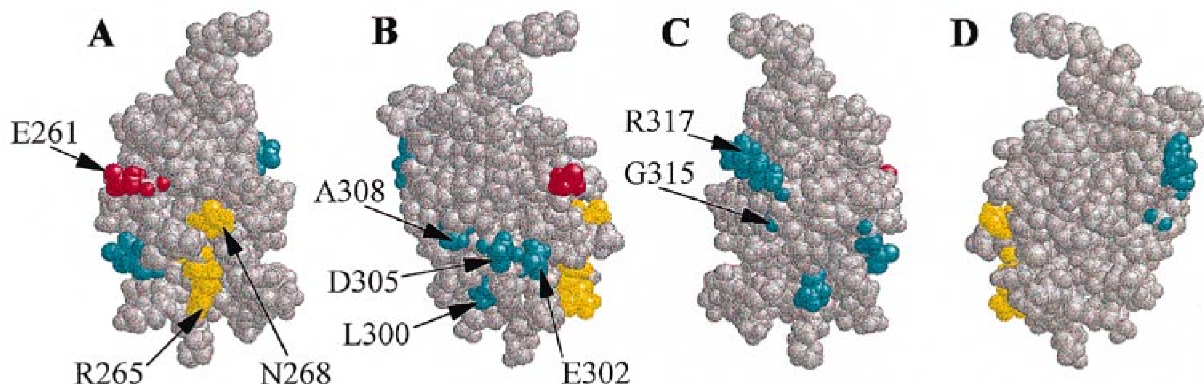

Figure 3. A space-filling model of the predicted structure of αCTD, created from the Brookhaven protein database file 1COO.PDB, showing the location of the residues discussed here. Views (**A**)–(**D**) are related by a rotation of 90° around their *y*-axis. E261 is coloured red, R265 and N268 are coloured yellow and L300, E302, D305, A308, G315 and R317 are coloured blue (see Discussion for details).

colonies apparently containing a pLAW2 derivative encoding α that interfered with activation of *FF(–71.5)* by wild-type FNR. Each mutant pLAW2 derivative was isolated and the base sequence of the mutant *rpoA* gene was determined. Table 6 lists the changes found in each of the derivatives. Four of the mutant plasmids encode truncated αCTD, whilst the others carry single amino acid substitutions, the location of which identify side-chains likely to be involved in transcription activation at *FF(–71.5)*. Figure 3 is a model of the structure of αCTD, showing the location of these sidechains.

DISCUSSION

Many bacterial transcription activators function by making a direct contact with the C-terminal domain of the RNAP α subunit. These activators bind upstream of the RNAP binding elements at target promoters and the role of the activator–αCTD interaction is to recruit RNAP to the promoter. In some cases the flexibility of the linker that anchors αCTD to the rest of the RNAP allows flexibility in the location of the activator binding site on the promoter DNA (reviewed in 8,20). Activators make contact with αCTD via surface-exposed patches (activating regions) that can be defined by the location of positive control substitutions. Similarly, the activation target on αCTD can be investigated by the location of substitutions that interfere with activation. However, to date there is no case where the details of activator–αCTD interactions are clearly understood.

Our results identify the activating region of FNR that interacts with αCTD. Interestingly, according to our model for the structure of FNR the activating surface contains three distinct adjacent surface-exposed loops (residues 71–75, 116–121 and 181–191; Fig. 1). These loops are located on a face of FNR that is distinct from the face that makes contact with the RNAP σ subunit at Class II FNR-dependent promoters (see 5,15). Alanine scanning identifies T118 as providing the crucial sidechain for interaction with αCTD. Presumably the major contact is provided by the 116–121 loop and less crucial contacts are provided by the 71–75 and 181–191 loops (it is likely that D43 is not involved and that the effect of the DG43 substitution is indirect). Our results show that the same activating region is functional at promoters where the FNR site is located at positions –61.5 and –71.5 and is functional solely in the downstream subunit of the FNR dimer. In this respect, FNR and CRP are similar. Interestingly, however, the location and nature of the activating region that interacts with αCTD clearly differs between the two activators. In CRP the activating region that interacts with αCTD is confined to a single β turn from residues 156 to 164 (9,10), corresponding to just one of the surface-exposed loops we have identified in FNR (the 181–191 loop; Fig. 2). However, our results clearly show that the major activating determinant in FNR is located in the adjacent 116–121 loop. Thus, whilst the structures of CRP and FNR may have been conserved, the precise nature of the activating region that interacts with αCTD differs. A possible explanation for this arises from the suggestion that the primary role of activator–αCTD interactions is 'merely' to recruit RNAP to the promoter (21). We suppose that these interactions can be generated in many different ways and, thus, different activators 'solve' their problems in different ways.

Our observation that deletions of αCTD interfere with expression from the *FF(–71.5)* promoter (Table 6) confirms that αCTD is essential for FNR-dependent transcription activation (note that these experiments were performed with plasmid-encoded α introduced in '*trans*' to wild-type α). We have also identified several single amino acid substitutions in αCTD that decrease expression at Class I FNR-dependent promoters (Fig. 3). The interpretation of these results is facilitated by the recent determination of a structure for αCTD (22,23) and by studies of the properties of several mutant α derivatives at UP element-dependent and activator-dependent promoters (see for example 12–14). Expression from *FF(–71.5)* is reduced by substitutions at E261, R265, N268, L300, E302, D305, A308, G315 and R317. Tang *et al.* (13) concluded that E261 was the crucial residue for CRP-dependent transcription activation at the *E.coli lac* promoter, whilst Murakami *et al.* (14) claimed that the same surface of αCTD was involved in both DNA binding and in interaction with CRP at the *lac* promoter. Our screen identified substitutions both at E261 and in the DNA binding surface (R265 and N268) and, whilst it is possible that one or more of these residues does provide a direct contact with both CRP and FNR, it is also possible that the effects of these substitutions are indirect.

Jafri *et al.* (24) reported that the EK261 substitution can interfere with activator-independent transcription initiation and we cannot, at present, exclude the possibility that DNA binding by αCTD is involved in the mechanism by which FNR activates transcription. The remaining substitutions (at L300, E302, D305, A308, G315 and R317) do not define a discrete 'patch' but, rather, appear to cluster in two sub-regions (Fig. 3). It is likely that substitutions within this group identify the contact site for the activating region of FNR and, interestingly, in a previous study of the *Salmonella typhimurium* FNR equivalent, OxrA, Lombardo *et al.* (25) found that substitutions at positions 311 and 317 of αCTD interfered with OxrA-dependent activation of the *S.typhimurium pepT* promoter. Note, however, that it is unlikely that the L300, E302, D305, A308, G315 and R317 sidechains are all involved in contacts with FNR: some of the consequences of substituting these sidechains may be indirect and this will need to be resolved by alanine scanning and *in vitro* studies.

ACKNOWLEDGEMENTS

This work was funded by a BBSRC project grant and a BBSRC CASE studentship to S.M.W. We thank Jeff Cole, Richard Ebright, Jeff Green, John Guest and Patricia Kiley for many helpful suggestions and Christine Webster for expert technical assistance.

REFERENCES

1 Kolb,A., Busby,S., Buc,H., Garges,S. and Adhya,S. (1993) *Annu. Rev. Biochem*., **62**, 749–795.
2 Spiro,S. and Guest,J. (1990) *FEMS Microbiol. Rev*., **75**, 399–428.
3 Ushida,C. and Aiba,H. (1990) *Nucleic Acids Res*., **18**, 6325–6330.
4 Gaston,K., Bell,A., Kolb,A., Buc,H. and Busby,S. (1990) *Cell*, **62**, 733–743.
5 Wing,H., Williams,S. and Busby,S. (1995) *J. Bacteriol*., **177**, 6704–6710.
6 Busby,S. and Ebright,R. (1997) *Mol. Microbiol*., **23**, 853–859.
7 Ebright,R. (1993) *Mol. Microbiol*., **8**, 797–802.
8 Busby,S. and Ebright,R. (1994) *Cell*, **79**, 743–746.
9 Niu,W., Zhou,Y., Dong,Q., Ebright,Y. and Ebright,R. (1994) *J. Mol. Biol*., **243**, 595–602.
10 Zhou,Y., Merkel,T. and Ebright,R. (1994) *J. Mol Biol*., **243**, 603–610.
11 Igarashi,K. and Ishihama,A. (1991) *Cell*, **32**, 319–325.
12 Zou,C., Fujita,N., Igarashi,K. and Ishihama,A. (1992) *Mol. Microbiol*., **6**, 2599–2605.
13 Tang,H., Severinov,K., Goldfarb,A., Fenyo,D., Chait,B. and Ebright,R. (1994) *Genes Dev*., **8**, 3058–3067.
14 Murakami,K., Fujita,N. and Ishihama,A. (1996) *EMBO J*., **15**, 4358–4367.
15 Bell,A. and Busby,S. (1994) *Mol. Microbiol*., **11**, 383–390.
16 Wing,H. (1997) PhD thesis, The University of Birmingham, UK.
17 Spiro,S., Roberts,R. and Guest,J. (1989) *Mol. Microbiol*., **3**, 601–608.
18 Lodge,J., Fear,J., Busby,S., Gunasekaran,P. and Kamini,N.-R. (1992) *FEMS Microbiol. Lett*., **95**, 271–276.
19 Kelsall,A., Evans,C. and Busby,S. (1985) *FEBS Lett*., **180**, 155–159.
20 Ebright,R. and Busby,S. (1995) *Curr. Opin. Genet. Dev*., **5**, 197–200.
21 Dove,S., Joung,J. and Hochschild,A. (1997) *Nature*, **386**, 627–630.
22 Jeon,Y., Negishi,T., Shirakawa,M., Yamazaki,T., Fujita,N., Ishihama,A. and Kyogoku,Y. (1995) *Science*, **270**, 1495–1497.
23 Gaal,T., Ross,W., Blatter,E., Tang,H., Jia,X., Krishnan,V., Assa-Munt,N., Ebright,R. and Gourse,R. (1996) *Genes Dev*., **10**, 16–26.
24 Jafri,S., Urbanowski,M. and Stauffer,G. (1996) *J. Bacteriol*., **178**, 6810–6816.
25 Lombardo,M.-J., Bagga,D. and Miller,C. (1991) *J. Bacteriol*., **173**, 7511–7518.
26 Schultz,S., Shields,G. and Steitz,T. (1991) *Science*, **253**, 1001–1007.