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A Positive Control Mutant of the Transcription Activator Protein FIS

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The FIS protein is a transcription activator of rRNA and other genes in *Escherichia coli*. We have identified mutants of the FIS protein resulting in reduced *rrnB* P1 transcription activation that nevertheless retain the ability to bind DNA in vivo. The mutations map to amino acid 74, the N-terminal amino acid of the protein's helix-turn-helix DNA binding motif, and to amino acids 71 and 72 in the adjoining surface-exposed loop. In vitro analyses of one of the activation-defective mutants (with a G-to-S mutation at position 72) indicates that it binds to and bends *rrnB* P1 FIS site I DNA the same as wild-type FIS. These data suggest that amino acids in this region of FIS are required for transcription activation by contacting RNA polymerase directly, independent of any other role(s) this region may play in DNA binding or protein-induced bending.

FIS is a 98-amino-acid DNA-binding protein that activates transcription from *Escherichia coli* rRNA promoters (30), several tRNA promoters (23, 24), and the *proP* promoter (35). Originally, FIS was identified as a recombinational enhancer of the *Salmonella* Hin, phage Mu Gin, and phage P1 Cin site-specific inversion reactions (12, 15, 17) and of phage λ excision (32). FIS also plays a role in replication at *oriC* (6, 8), and it serves as a transcriptional repressor (34).

The crystal structure of FIS has been determined (20, 37), and the structure indicates that the C-terminal region of the protein contains a helix-turn-helix DNA binding motif. FIS binds as a dimer (14, 18), and it bends the DNA in the protein-DNA complex 40 to 90°, depending on the particular binding site (7, 27, 31). Genetic studies indicate that FIS-dependent stimulation of the Hin- and Gin-mediated DNA inversion reactions requires both the C-terminal region of the protein (ca. amino acid residues 70 to 93) and the N-terminal section of FIS (19, 26). However, enhancement of λ excision by FIS does not require the N-terminal domain (26), suggesting that the mechanisms by which FIS stimulates λ excision and inversion differ.

The seven rRNA P1 promoters are among the strongest promoters known in *E. coli*. FIS contributes to *rrn* P1 promoter strength by binding to three sites (centered at positions −71, −102, and −143 with respect to the transcription start site [+1] in *rrnB* P1) and activating transcription approximately 10-fold in vivo (30). Binding of FIS to the promoter-proximal FIS site (site I) is responsible for most of this activation (2, 30). In addition, the region between the FIS sites and the −35 hexamer constitutes a third promoter recognition element (besides the −10 and −35 hexamers) that interacts with the α subunit of RNA polymerase (RNAP) and stimulates transcription another 30-fold (28, 29). *rrn* P1 promoters are also regulated in response to nutritional conditions by at least two distinct systems, growth rate-dependent control and stringent control (see references 4 and 10 for reviews). Whereas *rrnB* P1 sequences upstream of −41 are required for maximal promoter activity, *rrnB* P1 promoter sequences downstream of −41 are sufficient for regulation by stringent control (16) and growth rate-dependent control (1a).

We previously examined an extensive collection of *fis* mutants defective in site-specific recombination (26) for their effects on rRNA transcription (9). We identified a class of mutant proteins unable to stimulate *rrnB* P1 transcription yet able to bind and bend DNA in vitro. This and other information (2, 22) indicated that an additional FIS function, presumably interaction with RNAP, is likely to be required for transcription activation. However, this class of activation-defective mutations consisted of deletions and multiple substitutions that may have altered the overall conformation of the protein. Therefore, we concluded that these mutations were not likely to define the region in FIS specifically involved in interactions with RNAP (9).

We report here the identification of potential positive control mutants of *fis* (*fis*^{pc}). These mutants contain single substitutions at three amino acid positions, namely, positions 71 (R to S), 72 (two independent changes of G to C and G to S), and 74 (Q to R). Characterization of the mutant protein with a G-to-S mutation at position 72 (G72S) in vitro confirmed that this mutant protein is defective in transcription activation, yet its DNA binding and bending characteristics are unaffected. This suggests that the loop between α-helices B and C of FIS (37) may define a region responsible for interactions with RNAP.

MATERIALS AND METHODS

Bacterial strains and plasmids. RLG1445 (9) is a *fis* mutant strain (MG1655 Δ*lacX74 fis*::*kan* F′ *proAB lacI*^{q}Z$_{u118}$*fzz*::Tn*5-320*) that carries an *rrnB* P1 promoter-*lacZ* fusion on a λ prophage. The *rrnB* P1 promoter in the fusion lacks FIS binding sites, since it contains sequences from only −48 to +1 with respect to the transcription start site (Fig. 1A) (30). RLG1722 is RLG1445 containing plasmid pKG13 (described below). RLG1739 is the same host strain as RLG1445 but carries the *rrnB-lac* hybrid promoter–*lacZ* fusion on the λ prophage (Fig. 1B) (*rrnB* P1 sequences from −88 to −37 and *lac* P1 sequences from −36 to +2, constructed by modification of pRLG1819 [8a, 28]). There is no *lac* operator in this construct, but the *lac* promoter is activated by FIS (1).

pKG18 is a FIS expression plasmid in which the *fis* gene was derived from pRJ807 (26) and was modified to contain a *Hin*dIII site overlapping the stop codon. The resulting 305-bp *Eco*RI-*Hin*dIII fragment containing the *fis* gene was inserted into the *Eco*RI and *Hin*dIII sites of pKK223-3 (Pharmacia), placing the gene under the control of the *tac* promoter (26). Expression of this inducible *fis* gene was kept partially repressed at all times by the host *lacI*^{q}, since full induction was lethal. The wild-type *fis* gene contains two *Bst*EII sites. Site-directed mutagenesis (21) was used to introduce a conservative change to inactivate the more N-terminal *Bst*EII site (GGTAACC→GGTAACT) without

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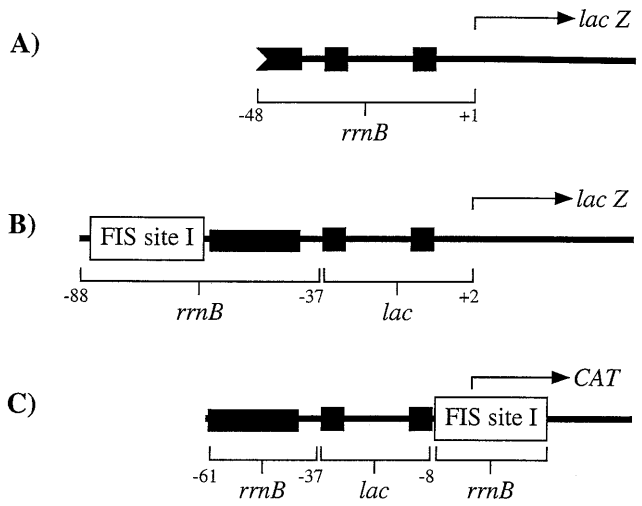

FIG. 1. Structures of reporter fusions used to measure transcription activation and DNA binding by FIS in vivo. Closed squares represent the −10 and −35 hexamers. Closed rectangles represent the *rrnB* P1 UP element. Open rectangles represent FIS site I. (A) *rrnB* P1 promoter-*lacZ* fusion lacking FIS sites in RLG1445 used in feedback derepression screen for FIS activity. The promoter contains DNA sequences from −48 to +1 of *rrnB* P1. The closed half rectangle represents the portion of the UP element present in this promoter. (B) *rrnB-lac* hybrid promoter–*lacZ* fusion in RLG1739 used to measure direct FIS-dependent activation. The promoter contains sequences from −88 to −37 of *rrnB* P1 (including FIS site I and the UP element) and sequences from −36 to +2 of the *lac* core promoter. (C) *rrnB-lacP*-O^{FIS} promoter–*CAT* fusion in plasmid pKG13 used to measure FIS-dependent repression. The promoter contains the *lac* −10 and −35 hexamers, *rrnB* P1 UP element sequences (from −61 to −37) just upstream of the *lac* −35 hexamer, and *rrnB* P1 FIS site I sequences (from −84 to −58) just downstream of the *lac* −10 hexamer (see Materials and Methods).

changing the amino acid sequence (amino acid residue 23) or affecting FIS function.

pKG13 was used for analysis of FIS binding in vivo. It contains the *rrnB-lacP*-O^{FIS} hybrid promoter (Fig. 1C), a *lac* promoter that is repressed by FIS. In this promoter, *rrnB* P1 FIS site I is in the position normally occupied by the *lac* operator. The DNA sequence of the *Bam*HI-*Hin*dIII fragment containing this promoter is 5′-GGATCCTCAGAAAATTATTTTAAATTTCCTCTTTACACT TTATGCTTCCGGCTCGTATGTT*TGATTTGGTTGAATGTTGCGCGGTCA G*AAGCTT-3′ (*Bam*HI and *Hin*dIII sites and *lac* −10 and −35 hexamers are underlined, and *rrnB* sequences containing FIS site I are in italics). Sequences from −37 through −8 are from the *lac* promoter; sequences immediately downstream of the *lac* −10 hexamer contain FIS site I (positions −84 to −58 of *rrnB* P1) and overlap the transcription start site. Sequences from −37 to −61 of *rrnB* P1 (the RNAPα subunit binding site, or UP element [29]) were included immediately upstream of the *lac* −35 hexamer to increase the transcription level from the promoter (28). The *Bam*HI-*Hin*dIII fragment containing the *rrnB-lacP*-O^{FIS} hybrid promoter was inserted in pKK232-8 (Pharmacia), creating an operon fusion to the gene coding for chloramphenicol acetyl transferase (*CAT*). An *Xmn*I fragment containing the hybrid promoter-*CAT* fusion was inserted into pACYC184 digested with *Bsa*AI. This resulted in pKG13, a plasmid containing the hybrid *rrnB-lacP*-O^{FIS} promoter–*CAT* fusion that could be replicated compatibly with the ColE1-derived FIS expression plasmid pKG18 described above.

Mutagenesis. The *fis* gene (from pKG18) was randomly mutagenized by PCR with *Taq* DNA polymerase (39). The primers used were 5′-GTGTGGAATTG TGAGCGGATAAC-3′ (P-R1) and 5′-CTGAAAATCTTCTCTCATCCGCC-3′ (P-Hd). The PCR products were digested with *Eco*RI and *Hin*dIII to generate the *fis* gene segment, gel purified, and ligated into pKK223-3.

Isolation of *fis*^{pc} mutants. To identify FIS mutants unable to activate *rrnB* P1 transcription but capable of DNA binding, we used two sequential genetic screens, screen 1 for reduced transcription activation and screen 2 for normal DNA binding.

An *rrnB* P1 promoter-*lacZ* fusion lacking FIS sites (Fig. 1A) was used as a reporter for the state of feedback derepression of *rrn* P1 promoters (9, 30) and thus as an indicator of the presence of functional FIS protein (see Results). Strains expressing wild-type FIS from a chromosomal or plasmid-encoded *fis* gene produce white colonies on MacConkey-lactose indicator plates. However, strains expressing activation-defective FIS derepress *rrn* P1 core promoters in a *fis*::*kan* host to keep total rRNA expression constant. The increased transcription from the *rrnB* P1 core promoter-*lacZ* fusion produces red colonies on MacConkey-lactose indicator plates. These mutants were then screened for normal FIS-DNA binding by analyzing repression of the *rrnB-lacP*-O^{FIS} promoter–*CAT* fusion described above.

To identify activation-defective mutants, RLG1445 carrying the FIS binding assay plasmid pKG13 was transformed with pKG18 derivatives containing mutagenized *fis* genes and plated on MacConkey-lactose plates. After 40 to 48 h, single red or pink colonies suggesting altered FIS-dependent activation were picked (screen 1). To eliminate mutants unable to bind DNA (screen 2), these colonies were resuspended in 1 ml of LB medium (10 g of tryptone, 5 g of yeast extract, 10 g of NaCl per liter) and diluted 1:1,000, and 15 μl of the dilution was plated on each of two LB agar plates, one containing 100 μg of ampicillin per ml and 100 μg of chloramphenicol per ml and one containing 100 μg of ampicillin per ml and 600 μg of chloramphenicol per ml. Percent survival (number of colonies on a plate containing 600 μg of chloramphenicol per ml per number of colonies on a plate containing 100 μg of chloramphenicol per ml) was used as a measure of FIS binding.

DNA sequence analysis of FIS^{pc} mutants. Plasmids carrying potential mutant *fis* genes of interest were digested with *Eco*RI and *Bsa*BI, ligated into similarly digested pKG18, and transformed into the reporter strains RLG1722 and RLG1739, cells were grown on indicator plates, and β-galactosidase assays were performed to confirm that the colony phenotype resulted from mutations in the plasmid-encoded *fis* gene. The entire mutant *fis* genes were sequenced by use of a double-stranded sequencing protocol and Sequenase (United States Biochemicals).

β-Galactosidase determination. β-Galactosidase activities from the *rrnB-lac* hybrid promoter in RLG1739 (see above) were determined in the presence or absence of wild-type and mutant *fis* genes as described previously (9). Cells were grown logarithmically for at least three generations at 37°C to an optical density at 600 nm of 0.3 to 0.5. The data reported are the average of at least two independent experiments, and the standard errors were less than 20%.

Purification of wild-type and mutant FIS proteins. The purification of FIS proteins was based on that described by Osuna et al. (26). Cells carrying plasmids expressing either wild-type or mutant FIS proteins were grown in LB with ampicillin (100 μg/ml) at 37°C to an optical density at 600 nm of 0.6 to 0.7, induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 1 h, and harvested. A 1.75- to 1.9-g sample of cells (wet weight) was resuspended in 11.5 ml of lysis buffer [50 mM Tris-Cl (pH 8.0), 10% sucrose, 300 mM $(NH_4)_2SO_4$, 0.1 mM phenylmethylsulfonyl fluoride, 2 mM dithiothreitol, 15 mM EDTA, 5 mg of spermidine per ml, 200 μg of lysozyme per ml] and lysed on ice for 1 h. NaCl was added to a final concentration of 1.0 M, and cell debris was removed by centrifugation at 30,000 × *g* for 30 min. The supernatant was dialyzed overnight against 0.3 M HSB buffer (300 mM NaCl, 20 mM Tris-Cl [pH 7.5], 0.1 mM EDTA, 10% glycerol) and loaded onto a 2-ml S-Sepharose column equilibrated with 0.3 M HSB buffer. The column was eluted with a 30-ml 0.3 to 1.0 M NaCl linear gradient. Fractions were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and fractions containing purified FIS were collected and dialyzed overnight into 500 mM NaCl–20 mM Tris-Cl (pH 7.5)–0.1 mM EDTA–50% glycerol for storage at −20°C. The Bio-Rad protein assay was used to determine protein concentration, with previously purified wild-type FIS as a standard.

In vitro transcription. FIS proteins were diluted in buffer containing 20 mM Tris-Cl (pH 7.5), 500 mM NaCl, 50% glycerol, 0.1 mM EDTA, 100 μg of bovine serum albumin (BSA), and multiple-round in vitro transcription assays were performed on a supercoiled template (pRLG589; *rrnB* P1 sequences from −88 to +50) as described previously (9, 30). Transcripts were quantitated after electrophoresis by phosphorimaging (Molecular Dynamics).

DNA binding in vitro. Band shift assays were used to determine the ability of mutant and wild-type FIS proteins to bind DNA in vitro. *rrnB* P1 DNA fragments were purified from pSL9 (*rrnB* P1 sequences from −88 to +50) and pSL11 (−61 to +50), digested with *Bam*HI, and ^{32}P-end-labeled with Sequenase (9). Fragments were gel purified and complexed with various concentrations of wild-type or mutant FIS proteins at 23°C for 10 min in a mixture containing 10 mM Tris-Cl (pH 8), 10 mM $MgCl_2$, 1 mM dithiothreitol, 100 mM NaCl, and 5 μg of sonicated calf thymus DNA per ml. Reaction mixtures were electrophoresed for approximately 3 h on an 8% polyacrylamide gel that had been prerun for at least 1 h in 0.5× Tris-borate-EDTA buffer.

DNase I footprinting. DNA templates containing *rrnB* P1 sequences from −88 to +50 were obtained by digesting pSL9 (9) with *Xho*I (position −168) and *Nhe*I (position +75). The DNA templates were ^{32}P-end-labeled on the bottom strand at position −168. DNA fragments were incubated with 20 to 40 nM wild-type or mutant FIS for 10 min at 22°C in a 25-μl reaction volume containing 120 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris-Cl (pH 7.9), 1 mM dithiothreitol, and 100 μg of BSA. DNase I treatment, processing, and electrophoreses were performed as described previously (30).

DNA bending assays. The extent of protein-induced bending was determined by a circular permutation assay (9, 33). pSL9 was digested with *Bam*HI and *Bgl*II, giving fragments of equal length that placed the FIS site near the end or the center of the fragment, respectively. Fragments were incubated with wild-type or mutant FIS proteins. The extent of bending was determined by comparing the mobility of the complex with the FIS site at the end of the fragment with that at the center of the fragment.

TABLE 1. Properties of *fis* mutants in vivo

fis allele	No. of isolates	Codon change	% Activation[a]	% Repression[b]
Wild type			100	100
None			0	0
R71S	1	GGT→TGT	2	95
G72S	1	GGT→AGT	5	89
G72D	1	GGT→GAT	<1	93
Q74R	6	CAG→CGG	7–10	94

[a] Percent activation was determined from measurements of β-galactosidase activities of a *fis*::*kan* host containing a chromosomal *rrnB-lac* promoter–*lacZ* fusion (Fig. 1B) and the indicated *fis* alleles on plasmids. Percentages were assigned by interpolation between the activation observed in strains containing a wild-type *fis* gene (100%) and no *fis* gene (0%; pKK223-3 vector control).

[b] Percent repression was determined from measurement of the relative plating efficiencies of strains containing the *rrnB-lac*-O^{FIS} promoter–*CAT* fusion (Fig. 1C) in the presence of plasmids containing the indicated *fis* alleles. One hundred percent repression (wild-type *fis* gene) equals 0 to 10% survival on a 600-μg/ml concentration of chloramphenicol. Zero percent repression (no *fis* gene) equals 60 to 70% survival on a 600-μg/ml concentration of chloramphenicol.

RESULTS

Identification of *fis*^{pc} mutants. We used PCR to mutagenize a *fis* gene, fused it to the *tac* promoter on a plasmid, and introduced it into a *fis*::*kan* host. *fis*^{pc} mutants were identified by using two sequential genetic screens. First, mutants defective in transcription activation of *rrn* P1 promoters were identified with a screen based on feedback derepression of *rrn* operons (9) (see Materials and Methods). In strains lacking active FIS, *rrn* P1 core promoter activity increases (derepresses) to compensate for the decrease in transcription activation of the seven rRNA operons (30). This derepression is a consequence of a feedback mechanism that regulates the rate of rRNA and tRNA synthesis in the cell in response to the levels of translationally competent ribosomes (3, 11, 13, 36).

Several classes of loss-of-function mutations in *fis* (e.g., nonsense mutations, mutations leading to unstable proteins, or DNA binding mutations, in addition to the *fis*^{pc} mutations) would be expected to lead to an activation-defective phenotype. Thus, we developed an in vivo screen that allowed us to distinguish those activation-defective *fis* mutants that make stable proteins retaining DNA-binding function. In this screen, FIS capable of DNA binding in vivo works as a repressor of transcription of *CAT* from the *rrnB-lacP*-O^{FIS} promoter (Fig. 1C and Materials and Methods), reducing resistance to high concentrations of chloramphenicol (1 to 10% survival).

We performed five independent mutagenesis reactions and screened a total of 8,000 colonies. Three hundred thirteen colonies exhibited derepressed core *rrnB* P1 transcription (resulting from defective activation) and were subsequently screened in vivo for DNA binding. Nine of the 313 mutants retained DNA binding function in the in vivo repression assay. Plasmids carrying the mutant *fis* genes were isolated and reintroduced into the same reporter strains to verify that the activation and binding phenotypes were attributable to alterations in the plasmid-encoded *fis* gene, and the DNA sequence of the entire *fis* gene was determined for each of the nine mutants.

The nine mutants consist of four different alleles. Each mutation is a single base substitution, and all cluster within a single region of the protein, at amino acids 71 to 74 (Table 1). The Q74R substitution was isolated six times from three independent mutagenesis reactions. Two substitutions for the glycine at position 72 were found, G72S and G72D, and the remaining mutant contained a R71S mutation. The locations of these residues in the primary and tertiary structure of FIS are illustrated in Fig. 2.

Transcription activation by *fis*^{pc} mutants in vivo. As a second measure of FIS-dependent activation of *rrnB* P1, we tested the effects of the mutants on expression of the FIS-activated *rrnB-lac* hybrid promoter–*lacZ* fusion (Fig. 1B). In this assay, loss of FIS-dependent activation reduces β-galactosidase activity, since the *rrnB-lac* hybrid promoter is not subject to the compensatory regulation of the *rrnB* P1 core promoter observed in strains lacking *fis*. Each of the four mutant *fis* alleles resulted in less than 10% FIS-dependent activation of the *rrnB-lac* promoter (and almost complete repression of the *rrnB-lacP*-O^{FIS} promoter) compared with that of wild-type *fis* (Table 1).

Characterization of *fis*^{pc} mutants in vitro. To evaluate the properties of the mutant proteins in vitro, we chose to purify the R71S and G72S mutant proteins, since the substitutions in these proteins are adjacent to, rather than within, the helix-turn-helix motif and since they contain relatively small side chain substitutions. We reasoned that these mutations were less likely to affect DNA bending and more likely to derive from loss of an essential interaction than from interference with a nearby interaction. R71S, G72S, and wild-type FIS proteins were analyzed for their DNA binding, DNA bending, and transcription activation characteristics in vitro, by use of band shift, DNase I footprinting, circular permutation, and in vitro transcription assays.

We found that purified R71S protein binds DNA specifically yet is defective in transcription activation in vitro, confirming the results obtained in vivo. However, R71S protein appears to have unusual oligomerization properties on DNA (data not shown), limiting interpretation of its effect on transcription. Therefore, this mutant was not studied further.

The DNA binding and bending properties of G72S protein are shown in Fig. 3 to 5. The band shift experiment shown in

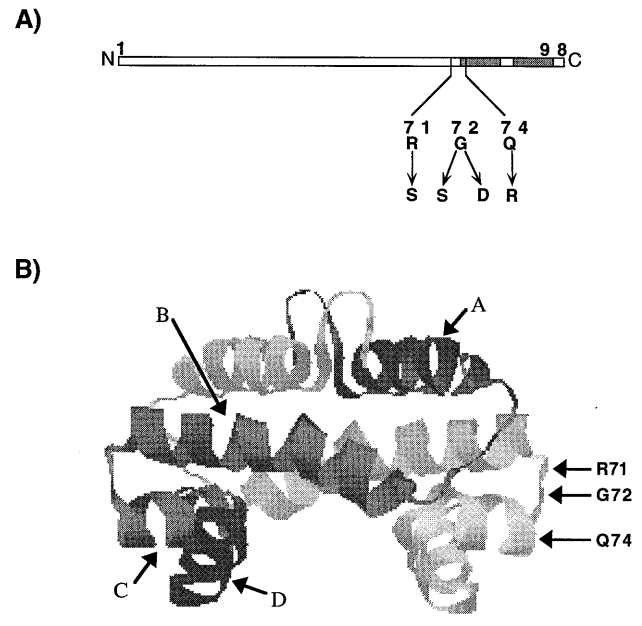

FIG. 2. Positions of *fis*-positive control mutants. (A) Shaded areas indicate the positions of α-helices C and D in the helix-turn-helix DNA binding motif of the 98-amino-acid FIS protein. (B) Ribbon diagram of the crystal structure of the FIS dimer (37). The two monomers are distinguished by different shading. Helices A, B, C, and D are indicated on one monomer, and R-71, G-72, and Q-74 are indicated on the other monomer.

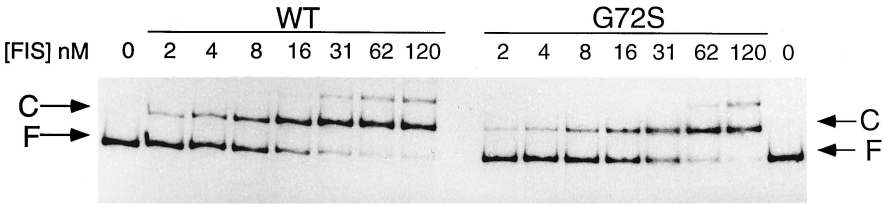

FIG. 3. Comparison of DNA binding properties of wild-type FIS and the G72S mutant protein by the band shift assay. The indicated concentrations of purified wild-type (WT) or G72S mutant (G72S) FIS protein were added to DNA fragments containing *rrnB* P1 sequences from −88 to +50 and electrophoresed as described in Materials and Methods. F, free DNA; C, FIS-DNA complexes (see text).

Fig. 3 indicates that a DNA fragment containing FIS site I forms a complex with G72S FIS that migrates to the same position as the complex containing wild-type FIS protein. (A DNA fragment lacking the FIS binding site did not bind either protein; data not shown.) A second, more-slowly-migrating complex, most likely resulting from binding of more than one FIS dimer, appears at very high concentrations of mutant or wild-type FIS, as reported previously (9, 30), but it is not clear whether this complex has any physiological significance.

The band shift titration results were used to estimate the concentrations of the wild-type and mutant FIS protein preparations active in DNA binding. The DNA binding activities of the wild-type and mutant protein preparations were not grossly different (within about twofold of each other). DNase I footprints were then made under conditions sufficient for full FIS site I occupancy (Fig. 4). The wild-type and G72S proteins resulted in the same patterns of protections and enhancements, suggesting that they bind to FIS site I identically.

FIS bends the DNA sites to which it binds (7). Since we found previously that a mutant FIS protein that bent DNA abnormally was defective in transcription activation (9), we assessed bending by the G72S mutant FIS protein by a circular permutation assay (9). We examined complexes of mutant or wild-type FIS bound to DNA fragments containing the *rrnB* FIS site I located near the center or the end of the fragment (Fig. 5). The electrophoretic mobility of the wild-type FIS-DNA complex with FIS site I at the center of the fragment is reduced relative to that of the complex with the FIS site near the end of the fragment, indicating FIS-induced DNA curvature. The same result is observed for G72S FIS. Thus, within the limits of this assay, the degree of bending exhibited by the mutant protein is identical to that exhibited by wild-type FIS (about 75°) (2a).

The results shown above suggest that the almost complete loss of transcription activation by G72S FIS observed in vivo is not attributable to defective DNA binding or bending. To confirm that the activation defect observed in vivo was a direct effect of the mutant FIS, in vitro transcription reactions were performed in the presence or absence of wild-type or mutant FIS protein at a concentration sufficient for full FIS site occupancy (Fig. 6). Wild-type FIS stimulated *rrnB* P1 transcription 4.6-fold under these conditions, while G72S FIS activated transcription only 1.6-fold (i.e., 17% as well as wild-type FIS; see Fig. 6 legend for calculation). Thus, G72S FIS has all the characteristics of a positive control mutation in that it binds and bends DNA normally yet is defective in transcription activation.

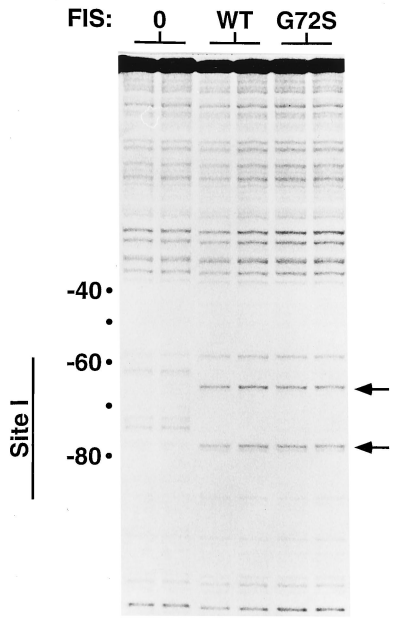

FIG. 4. DNase I footprints of wild-type and G72S proteins on *rrnB* P1. A DNA fragment containing *rrnB* P1 sequences from −88 to +50 labeled on the bottom strand was incubated without FIS (0), with wild-type FIS (WT), or with G72S FIS (G72S) and digested with DNase I as described in Materials and Methods. The limits of the protected region are indicated by the line labeled Site 1, and characteristic enhancements are indicated with arrows.

DISCUSSION

We identified mutations in *fis* that define a region (amino acids 71 to 74) of the protein specifically required for stimu-

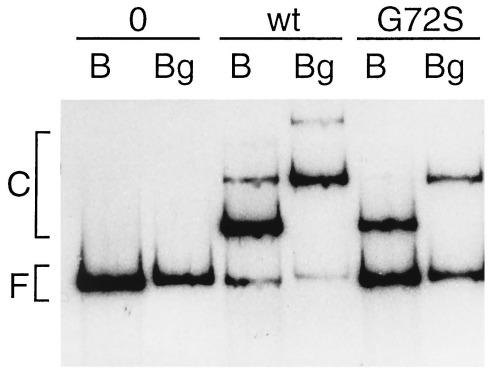

FIG. 5. DNA bending by wild-type and G72S mutant FIS proteins. Circular permutation assays were performed in the absence (0) or presence of wild-type (wt) or G72S mutant (G72S) FIS (32 nM) as described in Materials and Methods. Bg, *Bgl*II DNA fragment containing FIS site I near the center of the fragment; B, *Bam*HI DNA fragment containing FIS site I near the end of the fragment; F, free DNA; C, FIS-DNA complexes.

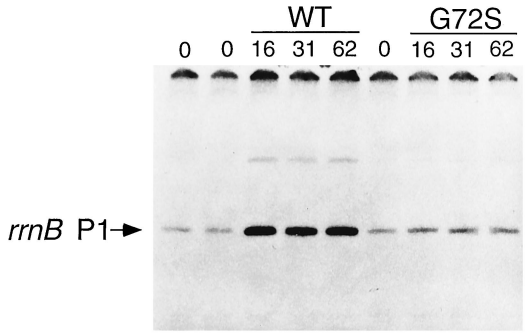

FIG. 6. Effect of wild-type FIS and G72S mutant FIS on activation of *rrnB* P1 transcription in vitro. Supercoiled DNA templates containing an *rrnB* P1 promoter (from −88 to +50) were transcribed in the absence (0) or presence of the indicated nanomolar concentrations of wild-type (WT) or mutant (G72S) FIS. The transcript from *rrnB* P1 is indicated. Percent activation by the mutant FIS protein was calculated as 17% by interpolation: the mutant protein resulted in 1.6-fold activation versus 4.6-fold for wild-type FIS (0.6/3.6 = 17% activation).

lation of *rrnB* P1 transcription. In the FIS crystal structure (20, 37), amino acids 71 to 73 are in a surface-exposed loop between α-helices B and C (the B-C loop), and amino acid 74 is the first amino acid of the helix-turn-helix DNA binding motif (Fig. 2B).

Our results confirm that a function in addition to DNA binding and bending is required for stimulation of transcription. This model is consistent with previous evidence suggesting that FIS and RNAP interact directly: (i) FIS and RNAP are located on the same face of the DNA helix (2); (ii) FIS-dependent activation is face-of-the-helix dependent (22, 38); (iii) RNAP and FIS bind cooperatively to the *rrnB* P1 promoter (2).

Our results suggest a model in which the B-C loop of FIS directly contacts RNAP. Since G72S FIS binds and bends DNA normally but fails to activate transcription, it can be considered a positive control mutant. However, it is not clear at this time whether G-72 contacts RNAP directly. It is also possible that the G72S substitution restricts rotation around the peptide backbone, changing the local structure of the surface-exposed loop such that a side chain of a nearby residue can no longer contact RNAP. Alternatively, any amino acid substitution for G-72 would introduce a longer side chain that could conceivably clash with the target on RNAP, thereby interfering with interactions between nearby amino acids and RNAP.

We found previously that two other mutations in the B-C loop, R71C and N73C, resulted in altered DNA bending as well as decreased transcription activation (8b, 9), suggesting that different amino acids in the surface-exposed loop can affect DNA bending and/or transcription activation. Alanine scanning mutagenesis and other studies are under way to define more precisely the amino acids in the FIS B-C loop region that are essential for interaction with RNAP.

The position of the activation loop in FIS immediately adjacent to its DNA-binding surface resembles the situation found with at least two other transcription factors whose structural determinants are known in detail: CAP (catabolite activator protein) and λ *c*I (λ repressor). CAP positive control mutations also map to a surface-exposed loop (amino acids 156 to 162) immediately preceding its DNA binding motif (5, 40). Extensive in vivo and in vitro analyses of amino acid substitutions indicated that this region of CAP is essential for activation of the *lac* promoter but not for DNA binding or bending (25, 40). Alanine-scanning mutagenesis of these residues indicated that only a substitution at amino acid 158 resulted in a significant loss of CAP-dependent activation. Therefore, it was proposed that T-158 of CAP directly contacts RNAP and activates transcription (25). G-72 of FIS might be analogous to G-162 of CAP, where substitutions are thought to reduce activation indirectly by influencing neighboring amino acids; cysteine, aspartic acid, or serine substitutions at this position in CAP result in loss of activation (5, 40), but an alanine substitution at this position is still 60% active (25). Positive control mutations in λ *c*I that fail to activate the λ P_{RM} promoter are also located next to the DNA-binding surface of that protein (12a).

Among the *fis* mutations defective in site-specific recombination, we had previously identified some that could bind and bend DNA in vitro but failed to activate transcription. These mutations leading to activation defects at *rrnB* P1 consisted of extended deletions or multiple substitutions, predominantly between residues 24 and 34 (9). FIS has a compact structure in which the N-terminal sections of the protein are packed close to the DNA binding motif. We concluded that FIS binding and bending are not sufficient for transcription activation, but since the mutations could potentially have affected overall protein conformation, the positions of these mutations did not necessarily define an activation surface. In light of the present results, i.e., the identification of a *fis*^{pc} mutation adjacent to the DNA-binding surface, we suggest that the more-N-terminal mutations identified previously impair transcription activation indirectly. Furthermore, since we did not identify activation-defective mutants in the N-terminal portion of the protein in the present screen, we conclude that FIS interacts differently with the transcription apparatus than with the inversion apparatus.

The results presented here do not address which subunit in RNAP is contacted by FIS. Our previous studies of FIS-dependent activation of an RNAP holoenzyme containing a truncated α subunit suggested that FIS might interact with a region of RNAP other than the C-terminal domain of α (αCTD) (29). However, recent studies (2a) indicate that the activation observed in the absence of αCTD is much reduced compared with that observed with wild-type α, and this residual activation works by an alternative mechanism to that observed in the presence of wild-type RNAP. Furthermore, our hydroxyl-radical footprinting studies showed that the region of DNA protected by FIS is adjacent to the region of DNA protected by αCTD (2). Thus, the location of the *fis*^{pc} mutations adjacent to a DNA surface that binds αCTD provides further evidence that FIS contacts αCTD.

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