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 rrrB 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 rrrB 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 RNA promoters (30), several rRNA promoters (23, 24), and the prop P 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 rmB P1 promoter strength by binding to three sites (centered at positions −71, −102, and −143 with respect to the transcription start site [+1] in rmB 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). rmB 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 10 and 11 for reviews). Whereas rmB P1 sequences upstream of −41 are required for maximal promoter activity, rmB P1 promoter sequences downstream of −41 are sufficient for regulation by stringent control (16) and growth rate-dependent control (14).

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 rmB 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 (fisD*). 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 lac::X74 fis::kan F’ proAB lacI-lacZ::Zap::lacZ-520) that carries an rmB P1 promoter-lacZ fusion on a λ prophage. The rmB 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 rmB-lac hybrid promoter-lacZ fusion on the λ prophage (Fig. 1B). RMB P1 sequences from −88 to −37 and lacP1 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 pJR807 (26) and was modified to contain a HindIII site overlapping the stop codon. The resulting 305-bp EcoRI-HindIII fragment containing the fis gene was inserted into the EcoRI and HindIII 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 lacP-lacI, since full induction was lethal. The wild-type fis gene contains two BarEII sites. Site-directed mutagenesis (21) was used to introduce a conservative change to inactive the more N-terminal BarEII site (GGTAACC--GGTAACT) without
FIS-DNA binding by analyzing repression of the rmB-lacP-OFS promoter-CAT fusion described above.

To identify activation-defective mutants, RLG1445 carrying the FIS binding assay plasmid pKGl8 was transformed with pKGl8 derivatives containing mutating lacZ fusions and plated on 10 mM IPTG to induce expression for 3 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 mutants. Plasmids carrying potential mutant fis genes of interest were digested with EcoRI and BstBI, ligated into similarly digested pKGl8, 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 rmB-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. (30). Genes 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. Cell density was monitored by measurement of the increase in absorbance 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-HCl [pH 8.0], 10% sucrose, 300 mM KCl, 0.1 mM phenylmethylsulfonyl fluoride, 2 mM diithiothreitol, 15 mM EDTA, 5 μg of spermidine per ml, 200 μg of lysosome 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 dailedyzed 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, 10% bovine serum albumin (BSA), and multiple-round in vitro transcription assays were performed on a supercoiled template (pRLG589). Plasmid DNA sequences from −88 to +50 were obtained as previously described (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. rmB-1 lac DNA fragments were purified from pRLG589 (rmB-1 lac) sequences from −88 to +50 and pSL13 (−61 to +50), digested with BamHI, and 32P-end-labeled with Sequenase (9). Fragments were gel purified and complexed with various concentrations of wild-type or mutant FIS proteins at 25°C for 10 min in a mixture containing 10 mM Tris-Cl (pH 8.0), 10 mM MgCl2, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, and 5% (vol/vol) 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.

DNA footprinting. DNA templates containing rmB-1 lac sequences from −88 to +50 were obtained by digesting pSL9 (lacZ (position −168) and Xhol (position +75). The DNA templates were 32P-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 MgCl2, 1 mM dithiothreitol (pH 7.9), 1 mM dithiothreitol, and 100 μg of BSA. DNase I treatment, processing, and electrophoreses were performed as described previously (30).

DNA binding assays. The extent of protein-induced bending was determined by a cross-blotting assay for low-activity mutations (33). PL9 was digested with BamHI and BstBI, 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.

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 rmB P1 UP element. Open rectangles represent FIS site I. (A) rmB 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 rmB P1. The closed half rectangle represents the portion of the UP element present in this promoter. (B) rmB-lac hybrid promoter-lacZ fusion used in RLG1739 to measure direct FIS-dependent activation. The promoter contains sequences from −88 to −37 of rmB P1 (including FIS site I and the UP element) and sequences from −36 to −2 of the lac core promoter. (C) rmB-lacP-OFS promoter-CAT fusion in plasmid pKGl3 used to measure FIS-dependent repression. The promoter contains the lac −10 and −35 hexamers, rmB P1 UP element sequences (from −61 to −37) just upstream of the lac −35 hexamer, and rmB P1 FIS site 1 sequences (from −94 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. pKGl3 was used for analysis of FIS binding in vivo. It contains the rmB-lacP-OFS hybrid promoter (Fig. 1C), a lac promoter that is repressed by FIS. In this promoter, rmB P1 FIS site 1 is in the position normally occupied by the lac operator. The DNA sequence of the BamHI-HindIII fragment containing this promoter is 5′-GAATTTGCGAGTCTGCAGAATAATTAAAATTCGTTCCTTASTACTTTATGTCGGCAGCCGCGTTGTAAGCTT-3′(BamHI and HindIII sites and −10 and −35 hexamers are underlined, and rmB sequences containing FIS site 1 are in italics). Sequences from −37 through −8 and −36 through −2 (from the promoter) are transcribed immediately downstream of the lac −10 hexamer contain FIS site 1 positions (−84 to −58 of rmB P1) and overlap the transcription start site. Sequences from −37 to −61 of rmB P1 (the RNAP 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 BamHI-HindIII fragment containing the rmB-lacP-OFS hybrid promoter was inserted in pKK232-8 (Pharmacia), creating an operon fusion to the gene coding for chloramphenicol acetyl transferase (CAT). An XmnI fragment containing the hybrid promoter-CAT fusion was inserted into pACYC184 digested with BstBI. This resulted in pKGl3, a plasmid containing the hybrid rmB-lacP-OFS promoter-CAT fusion that could be replicated compatibly with the ColE1-derived expression plasmid pKGl8 described above.

Mutagenesis. The fis gene (from pKGl8) was randomly mutagenized by PCR with Tau DNA polymerase (39). The primers used were 5′-GGTGGTAGAATTGGAGCCGATA-3′ (P-R1) and 5′-CTGCAAATCTCTCTCACTCCGC3′ (P-Hd). The PCR products were digested with EcoRI and HindIII to generate the fis gene segment, gel purified, and ligated into pKK223-3.

Isolation of fis mutants. To identify FIS mutants unable to activate rmB 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 rmB P1 promoter-lacZ fusion lacking FIS sites (Fig. 1A) was used as a reporter for the state of feedback derepression of rm B promoters (9, 30) and thus as an indicator of the presence of functional FIS protein (see Results). Staining with the 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 rmB P1 core promoters in a fis:kan host to keep total RNA expression constant. The increased transcription from the rmB P1 core promoter-lacZ fusion produces red colonies on MacConkey-lactose indicator plates. These mutants were then screened for normal
RESULTS

Identification of fis" 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" 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" 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"P promotor (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" 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"P promotor) compared with that of wild-type fis (Table 1).

Characterization of fis" 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 binding 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

### Table 1. Properties of fis mutants in vivo

| fis allele | No. of isolates | Codon change | % Activation | % Repression |
|-----------|----------------|--------------|--------------|--------------|
| Wild type | 100            | 100          |              |              |
| None      | 0              | 0            |              |              |
| R71S      | 1              | GGT→TGT      | 2            | 95           |
| G72S      | 1              | GGT→AGT      | <1           | 93           |
| G72D      | 1              | GGT→GAT      |              |              |
| 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 fis:kan 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 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).

![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.](http://jb.asm.org/Downloaded from http://jb.asm.org/)
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 rRNA P1 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 rRNA 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.

**DISCUSSION**

We identified mutations in fis that define a region (amino acids 71 to 74) of the protein specifically required for stimu-
1.6-fold activation versus 4.6-fold for wild-type FIS (0.6/3.6 protein was calculated as 17% by interpolation; the mutant protein resulted in indicated nanomolar concentrations of wild-type (WT) or mutant (G72S) FIS. 

Moter (from 2 transcription in vitro. Supercoiled DNA templates containing an mBB 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 mBB 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).

FIG. 6. Effect of wild-type FIS and G72S mutant FIS on activation of mBB P1 transcription in vitro. Supercoiled DNA templates containing an mBB 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 mBB 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 mBB 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 mBB 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 CI (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 CI that fail to activate the λ PRRM 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 mBB 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 fisR2 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 fisR2 mutations adjacent to a DNA surface that binds αCTD provides further evidence that FIS contacts αCTD.

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