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The N-terminal part of the *E.coli* DNA binding protein FIS is essential for stimulating site-specific DNA inversion but is not required for specific DNA binding

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ABSTRACT

FIS protein is involved in several different cellular processes stimulating site-specific recombination in phages Mu and λ as well as transcription of stable RNA operons in *E.coli*. We have performed a mutational analysis of *fis* and provide genetic and biochemical evidence that a truncated version of FIS lacking the N-terminal region is sufficient for specific DNA binding and for stimulating λ excision. These mutants also retain their ability to autoregulate *fis* gene expression. Such mutant proteins, however, cannot stimulate the enhancer dependent DNA inversion reaction.

INTRODUCTION

Prokaryotic site-specific recombination systems provide several examples for the involvement of small auxiliary DNA binding proteins in mediating the interaction of distant sites on DNA (1). One such case is the stimulation of Gin and Hin-mediated DNA inversion by the *E.coli* FIS protein (2, 3, 4). Site-specific inversion of the G segment of phage Mu requires the functional interaction of three DNA sites. In addition to the two recombination sites that are recognized by the phage encoded recombinase Gin (5) a *cis*-acting recombinational enhancer (3, 4) bound by the FIS protein is required for efficient recombination *in vivo* and *in vitro*. The *gin* enhancer carries three FIS binding sites that are bent by binding of FIS (6, 7, 8). The activity of the recombinational enhancer is dependent on the integrity of the FIS binding sites and on the distance between the sites (7, 8, 9, 10). A relative degenerate consensus sequence for FIS binding was deduced from a mutational analysis of the recombinational enhancer of the *cin* system in phage P1 (9).

The *fis* gene has been cloned and insertional inactivation demonstrated that *fis* is not an essential gene (11, 12). The structure of the FIS dimer has been solved by X-ray crystallography and revealed the presence of a helix-turn-helix motif at the C-terminus of FIS (14). The putative recognition helices (13) within the dimer have, however, an unusual relative arrangement (14) suggesting that DNA binding is accompanied with significant structural changes of the DNA or of FIS. Indeed FIS induced DNA bending has been observed for several sites (8, 10, 15, 16). For site-specific DNA inversion a topologically defined synaptic intermediate has to be assembled that is characterized by the introduction of two interdomainial nodes within the supercoiled substrate molecule (6, 17). Models have been proposed in which FIS and the enhancer are part of the synaptic intermediate and act to stabilize its structure by protein-protein interactions between FIS and Gin (2, 7). Support for such a model was obtained by an electron microscopical analysis which detected FIS and the enhancer in a synaptic intermediate after chemical crosslinking (18). An alternative model suggests that FIS acts primarily to introduce or stabilize interdomainial crossings in the supercoiled substrate thereby favoring the formation of the topologically correct synapse (17). Such a mechanism would not necessarily require specific protein-protein interactions between Gin and FIS.

The activity of the FIS protein in *E.coli* is not confined to the stimulation of DNA inversion. A constantly growing number of processes are discovered where FIS is involved: FIS was shown to stimulate excision of phage λ (19, 20), FIS affects Mu development (21) and has recently been shown to be an activator of rRNA and tRNA transcription (22, 23). FIS also acts as a repressor of its own transcription (O. N., unpublished). This raises the question whether FIS operates by the same mechanism in all these systems.

To further understand how FIS mediates the activity of the recombinational enhancer in phage Mu, we have isolated *fis* mutants that are affected in DNA binding and/or stimulation of G inversion. We report here the identification of a functional domain in the FIS protein that is crucial for G inversion but not required for specific DNA binding. Furthermore this domain is dispensable for stimulation of λ excision as well as for autoregulation of *fis* gene expression *in vivo*.

MATERIALS AND METHODS

Bacterial strains, plasmids and phages

Bacterial strains: WK6(λ*c*I+) (24), WK6*mutS*(λcI+) (24, 25), CSH50*fis*::Kan (12) and K12ΔHIΔ*trp fis*::Kan (24). CSH50-*fis*::Kan(λ *c*I857) was constructed by lysogenizing CSH50*fis*::Kan with λ *c*I857. CSH50*fis*::Kan(λFP1) (O. N., unpublished) is a

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derivative of CSH50*fis*::Kan harboring a single copy transcriptional fusion of the *fis* operon to *lac*Z on a resident λ prophage. λFP1 carries sequences from position −750 to +1062 of the *fis* operon fused to *lac*Z. λFP1 contains only the first 46 bp of the *fis* gene and does not express FIS protein. Plasmid pJT110 (26, 15) was a kind gift from A. Landy. pUHE25 (27, 28) was kindly provided by H. Bujard. Plasmids pAK3 (5), pBRminiG(−) (3) and pIR2 (24) were used to monitor DNA inversion.

Proteins

Protein concentrations were determined by the method of Bradford (31). FIS and mutant FIS proteins were purified from strain K12ΔHIΔ*trp fis*::Kan as published (32) with the modifications described by Choe et al. (33). Gin was isolated as described (34) with some modifications.

Manipulations of DNA and construction of plasmids

All DNA manipulations followed established protocols (35). DNA sequencing was performed by the dideoxy method of Sanger et al. (36). Construction of pLMCfis2 and pLMAfis2: The *Pvu*II-*Hin*dIII fragment of pCF221 (12) carrying the *fis* gene was cloned into pT7-5 (37) to generate PCF355. The *Eco*RI-*Bgl*II fragment from PCF355 was then cloned into the respective sites of pMa5-10 (29) and pMc5-10 (29) to generate pLMAfis1 and pLMCfis1. This places *fis* under the control of the λP_L promoter. Subsequently sequences of the translational initiation region were changed by site-directed mutagenesis to AAAGAGGTGACAGATCTATG. This generated plasmids pLMAfis2 and plMCfis2 respectively. The inversion test plasmid pFD4 was derived from pMD3lacZ (30) by cloning a *Sma*I-*Nae*I fragment from pLMA5-8gin (30) carrying the *gin* gene under the control of the λP_L promoter into the *Xba*I site of pMD3lacZ after filling in the ends with Klenow polymerase. pUHEfis2 and pUHEfis2 derivatives were constructed by inserting the *fis* cassette from pLMCfis2 as a *Eco*RI-*Hin*dIII into the respective sites of pUHE25 that carries the synthetic, IPTG-inducible promoter PA1-04/03 (27, 28). The *lacI*^{q} from pKT101-Iq (a kind gift from U. Günthert) was cloned into the *Eco*RI site of pACYC184 to generate pIQ1. PTZME7 was generated by cloning the *Bam*HI fragment of pHB136 (8) comprising the Mu enhancer (position 53−178) (3) in the *Bam*HI site of pTZ18R (Pharmacia).

Mutagenesis of *fis*

fis mutants were generated with pLMCfis2 and pLMAfis2 by the gapped duplex method (25) using either nitrous acid treated single stranded DNA of pLMAfis2 or by using degenerate oligonucleotides covering parts of the *fis* gene.

Chemical mutagenesis with nitrous acid. The first step was to chemically mutagenize single stranded DNA from plMAfis2 (coding strand of *fis*) with nitrous acid as described (24). The mutagenized single stranded DNA was then hybridized to the *Bgl*II-*Hin*dIII fragment of pLMCfis2 DNA to generate a gapped duplex molecule (25). After filling in the gap with Klenow DNA polymerase and T4 DNA ligase the DNA was transformed into the repair deficient strain WK6*mut*S(λcI$^+$). Transformants were selected for chloramphenicol resistance. Since pLMCfis2 (Ap^s/Cm^R) and pLMAfis2 (Ap^R/Cm^s) carry different genetic markers, this step enriches for pLMCfis2 derivatives carrying mutations targeted to the *fis* gene. DNA was prepared from pools of transformants and mutants were identified as described below. With this approach the following mutants were isolated: A34V/T23A, V16L/A77V, K25E, K32E, E59G, T75A, N84S, R85C, T87M. For all mutants the whole *fis* gene was sequenced.

Mutagenesis with degenerate oligonucleotides. A set of 9 overlapping 35-mers covering the *fis* gene were synthesized with each nucleotide precursor contaminated with 3% of the three others (38). These were used to prime DNA synthesis on a gapped duplex prepared from single stranded DNA of pLMAfis2 and the *Eco*RI-*Hin*dIII fragment of pLMCfis2. Gapped duplex DNA was further processed as described above. From a pool of mutants generated this way mutant V16G was obtained. The C-terminal nonsense mutants as well as mutants R85V, L88D, T87A, R89L/N84K, G86D and K91E were obtained from a pool of mutants generated with a set of 10 mutagenic oligonucleotides covering codons 85−94 of FIS. In each oligonucleotide (30-mers) a single codon was mutated by using all 4 nucleotide precursors at three positions. These were used as primers as described above.

Construction of N-terminal deletion derivatives of *fis*

The deletions were generated by cloning synthetic DNA fragments into the *Bgl*II/*Hpa*I sites of pLMCfis2 to generate mutants FISΔ5, FISΔ10, FISΔ12, FISΔ15. In the mutants the first 5 to 15 codons of *fis* were deleted and replaced by ATG. Deletion derivatives FISΔ26 and FISΔ39 were generated by site-directed mutagenesis using oligonucleotides and a gapped duplex DNA generated with the *Eco*RI/*Hin*dIII fragment of pLMCfis2. The N-terminal amino acid sequences generated were: $MALRDS_{30}$ in FISΔ26 and $MAQLNG_{44}$ in FISΔ39 (numbers denote the position of the respective residue in the wt sequence).

In vivo analysis of G inversion

The effect of *fis* mutants on Gin mediated recombination was analysed *in vivo* by transforming strain CSH50*fis*::Kan harboring the inversion test plasmid pFD4 with the mutagenized plasmid pools of pLMCfis2. Transformants were grown on MacConkey lactose plates at 37°C. After 24 h the colonies were screened for their Lac phenotype. Red colonies were scored as *fis*$^+$ (+); intermediate phenotypes were scored as (+/−); mutants giving rise to white colonies after 24 h were scored as defective (−). Each clone isolated was subsequently tested individually.

Preparation of cell extracts for *in vitro* analysis of FIS mutant proteins

Mutant FIS proteins were overexpressed in K12ΔHIΔ*trp fis*::Kan by heat induction. After inducing the cultures at 42°C for 1.5 h, 4 ml of culture were centrifuged and the cell pellet resuspended in 0.4 ml 50 mM Tris-HCl pH 8.0, 300 mM NaCl, 0.5% Brij58, 1.5 mM EDTA, 20 μg/ml PMSF. Extracts were prepared by sonification, diluted 3-fold in 20 mM Tris-HCl pH 7.5, 150 mM NaCl, 50 mM DTT, heated to 100°C for 5 min to inactivate nucleases and centrifuged before use. 1 μl extract contained approx. 5−20 ng FIS.

In vitro recombination assays

FIS activity in extracts was analysed using the test plasmid pAK3 (5). Recombination was performed for 10 to 30 min in 25 μl TEAM buffer (34) containing 1 μg pAK3 DNA, 100 ng Gin and 2−5 μl of FIS extract. After stopping the reaction by heating to 80°C for 10 min or by digestion with proteinase K (300 μg|ml), the DNA was restricted with *Pst*I and analysed on 2% agarose gels.

DNA binding assays

For gel retardation assays 5'-end labeled DNA fragments (approx. 2 ng) were incubated with various concentrations of FIS in 25 μl of buffer containing 25 mM Tris-HCl pH 7.5, 100 mM NaCl, 1 mM EDTA, 400 μg/ml BSA, 2 mM DTT, 16 μg/ml sonified calf thymus DNA. After incubation at 20°C for 5 min 5 mg/ml sucrose and 25 μg/ml Bromphenol blue were added and the samples applied to a 5% polyacrylamide gel running at 100–150 V in TBE buffer (35). Footprinting of FIS on the recombinational enhancer was performed with the *SalI-EcoRI* fragment of pTZME7. The *SalI* site was labeled at the 5'-end with polynucleotide kinase. Binding reactions were done in 170 μl at 20°C in 25 mM Tris-HCl pH 7.5, 75 mM NaCl, 0.1 mM EDTA, 1 μg/ml calf thymus DNA, 30 μg/ml BSA. After incubating with FIS for 5 min 17 μl of a DNaseI solution (6.3 μg/ml in 100 mM $MgCl_2$) were added. DNaseI treatment was terminated after 2 min by addition of 5μg tRNA, 25 μl of 3M sodium acetate, 20 μl of 25 mM EDTA and 230 μl phenol/chloroform. After extraction with phenol/chloroform and ethanol precipitation the DNA was analysed on a 6% sequencing gel.

In vivo analysis of λ excision

For analysing the effect of FIS on phage λ excision, CSH50*fis*::Kan(λ*c*I857) harboring plasmid pIQ1 and the mutant derivatives of pUHEfis2 was used. For induction of *fis* the strains were grown in the presence of 50 μM IPTG. Phage lysates were prepared by heat induction and titrated on CSH50 as indicator following standard techniques (39).

LacZ assays

LacZ activity was determined as described (40) from cultures grown overnight at 37°C. The LacZ units are those of Sadlèr and Novick (40) multiplied by 1000 to make them approximately equivalent to those of Miller (39).

RESULTS

Isolation of *fis* mutants affected in stimulating G inversion

For analysing the stimulatory effect of *fis* on G inversion *in vivo* we developed the test plasmid pFD4. pFD4 encodes the recombinase *gin* and a G segment carrying a promoterless *lacZ* gene. Site-specific inversion of the G segment of pFD4 fuses *lacZ* to a promoter located outside the invertible element leading to a switch from Lac$^-$ to Lac$^+$. This switch is monitored on MacConkey lactose plates. Since FIS is required for efficient recombination, transformation of the *fis*$^-$ strain CSH50*fis*::Kan with pFD4 gives rise to white colonies on MacConkey lactose plates. Subsequent transformation of this strain with the compatible plasmid pLMCfis2 expressing the wild type *fis* gene gives rise to Lac$^+$ colonies after 16–20 h. Using this complementation assay we screened pools of mutagenized pLMCfis2 plasmids and isolated a collection of *fis* mutants (see Materials and Methods). Since we were particularly interested in mutants that express stable FIS protein we examined the expression of FIS in the mutants by probing western blots with FIS antiserum (not shown). Selected mutants were then sequenced and analysed (Table 1; Fig. 1). Mutants T75A and N84S were isolated as clones where recombination could be detected earlier than for the wild type (wt) control. In the mutants K25E and K32E inversion occurred, but was significantly delayed. All other mutants were strongly down for stimulating recombination.

Table 1. Analysis of FIS mutants.

	Stimulation of G inversion in vitro[a]	in vivo[b]	Binding to enhancer[a]	Protein expression[c]
FIS-wt	+	+	+	+
pMc5-10	−	−	−	−
V16G	−	−	+	+
A34V/T23A	−	−	+	+
A34V	−	−	+	n.t.
V16L/A77V	−	−	+/−	+
FISΔ5	+	+	+	+
FISΔ10	+	+	+	+
FISΔ12	−	−	+	+
FISΔ15	−	−	+	+
FISΔ26	−	−	+/−	+
FISΔ39	−	−	−	−
K25E	+/−	+/−	+	+
K32E	+/−	+/−	+	+
E59G	−	−	−	+
T75A	+	+	+	+
N84S	+	+	+	+
R85C	−	−	−	+
R85V	−	(−)	−	+
G86D	−	(−)	−	+
T87A	+/−	(−)	+/−	+
T87M	+/−	−	+/−	+
L88D	−	−	−	+
R89L/N84K	−	−	−	+
K91E	−	(−)	−	+
K94–TAA	−	−	−	+
K93–TAA	−	−	−	+
L88–TAA	−	−	−	−

[a] activity was determined in crude extracts as described in Materials and Methods. −: activity was not detected in extracts. +/−: weak activity was detectable with high concentration of extracts.

[b] determined as described in Materials and Methods.
+: fully active; +/−: reduced activity in vivo; (−): strongly impaired in vivo
−: no activity detectable *in vivo*;

[c] determined by probing western blots with FIS antiserum (not shown) n.t.: not tested.

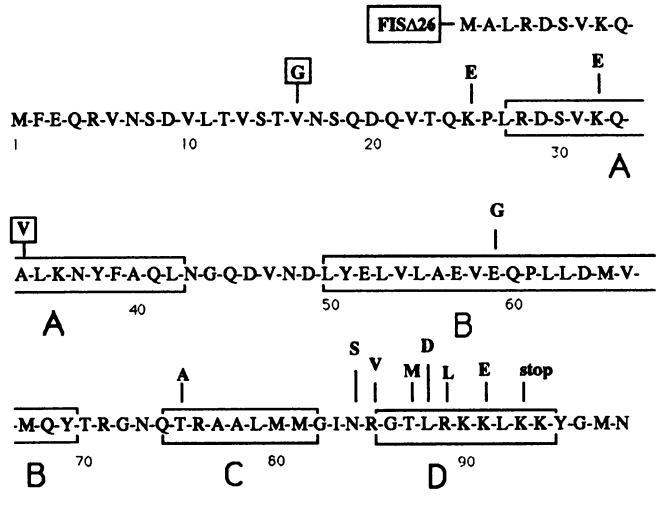

Figure 1. Amino acid sequence of FIS and location of selected mutations. The sequence of FIS is shown in one letter code. The α-helices A to D are boxed. Helix C and D form the helix-turn-helix motif (14). Mutations are shown in bold face and those that disrupt the ability of FIS to stimulate G inversion without abolishing specific DNA binding are boxed.

Mutant FIS proteins were then analysed for the ability to stimulate recombination *in vitro*. For this purpose we prepared crude extracts as outlined in Materials and Methods. For most of the mutants the *in vitro* phenotype using extracts (Table 1) correlated with the *in vivo* findings (Table 1). Small quantitative differences observed are likely to be caused by differences in FIS concentration used *in vitro* and those expressed *in vivo*. The crude cell extracts were subsequently used in gel retardation assays (41, 42) with a Mu enhancer fragment to determine the DNA binding properties of the mutant proteins.

The binding assays showed that *fis* mutants that have lost the ability to stimulate DNA inversion fell into two classes: mutants that were affected in DNA binding and mutants that retained the ability to bind the DNA specifically. These two classes are described below.

FIS mutants affected in DNA binding

The results of binding assays performed with crude extracts are given in Table 1. For most mutants analysed there was a good correlation between DNA binding and the ability to stimulate recombination *in vivo* and *in vitro*. All mutants with a strong defect in DNA binding have amino acid exchanges in the C-terminal part of FIS carrying the helix-turn-helix motif, except for E59G, which is located in the central region of FIS. By analysing FIS mutants with nonsense mutations in the C-terminus we found that a stop codon at amino acid residue 93 results in a mutant FIS protein that is defective for DNA binding, but is stable *in vivo* while a mutant with a stop codon at position 88 gave barely detectable levels of protein as assayed by western blotting and presumably is unstable *in vivo*. (Table 1).

Since crude extracts allow only a qualitative estimate of FIS activity we have purified mutant FIS proteins R85V, T87A, T87M, R89L/N84K, N84S and T75A for a more detailed analysis. As shown in Fig. 2 the purified mutant proteins R85V and R89L/N84K did not produce stable complexes of defined mobility in retention assays and were defective for stimulating G inversion *in vitro*. Purified FIS R85V showed a slight stimulatory effect at high protein concentration. The purified proteins FIS T87A and T87M which weakly stimulate G inversion *in vitro* (Fig.2B) bound the enhancer with an approx. 4 to 8-fold reduced affinity (Fig.2A). In addition, the protein-DNA complexes formed had an increased electrophoretic mobility compared to wild type (Fig. 2A). Altered electrophoretic mobilities of protein DNA complexes were also observed for mutants T75A and N84S which are however fully active in stimulating recombination both *in vivo* and *in vitro* (Fig. 2).

Using two circularly permutated 371 bp DNA fragments from pJT110 which carry the strong FIS binding site in λ attP we analysed whether the effects on complex mobility reflect differences in FIS induced DNA bending (Fig. 3). For FIS T87A and T87M the aberrant complex mobility was dependent on the location of the FIS binding site relative to the ends of the fragment. This may indicate that DNA binding of FIS T87A and FIS T87M induces a different DNA conformation than binding of wt FIS because the electrophoretic mobility of protein-DNA complexes is dependent on both the location and the degree of DNA bending (43, 15). For the other mutant proteins tested only small differences relative to wt FIS were observed.

In summary, the finding that mutations in the C-terminal region of FIS have severe effects on DNA binding demonstrates the importance of the helix-turn-helix motif for DNA binding.

A class of mutant FIS proteins that bind DNA specifically but fail to stimulate G inversion

While the mutations described above affect recombination and binding to a similar degree three mutants were isolated that bind

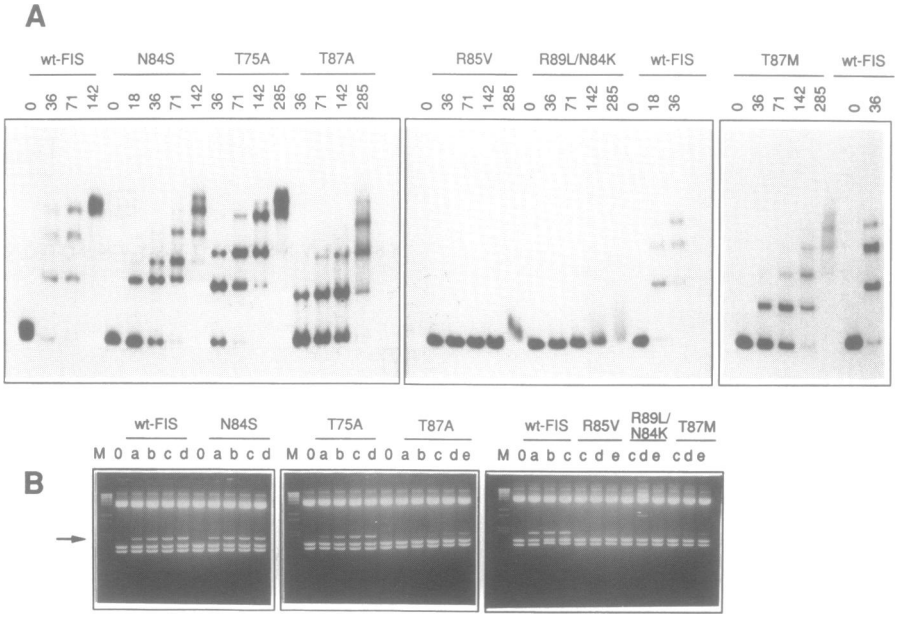

Figure 2. *In vitro* characterization of purified FIS mutant proteins. (A) Analysis of DNA binding properties of FIS mutants. Binding was analysed on 5% polyacrylamide gels with the end labeled *Bam*HI-*Ava*I enhancer fragment of pBRminiG(−). The numbers correspond to the nanomolar protein concentration used (nM). (B) *In vitro* G inversion was carried out with the inversion substrate pAK3 for 10 min. The arrow points to the position of a restriction fragment indicative for the inversion product. The mutant proteins were used in the following concentrations : (0) no FIS; (a) 30 nM; (b) 60 nM; (c) 120 nM; (d) 240 nM; (e) 480 nM. M: size marker (*Bst*EII digested λ DNA). The additional band seen in lane R89L/N84K (d) results from incomplete digestion of the substrate DNA with *Pst*I.

to the recombinational enhancer specifically but are defective for stimulating recombination (V16G, A34V/T23A, V16L/A77V). FIS V16G and the double mutant A34V/T23A bind the enhancer efficiently in crude extracts while the double mutant V16L/A77V showed only weak binding activity in extracts (Table 1; Fig. 4). Mutations T23A and A34V were introduced in the *fis* gene individually by site-directed mutagenesis. The mutation A34V conferred the same phenotype as the double mutant A34V/T23A while the T23A mutation did not interfere with *fis* activity (not shown). The behavior of point mutants V16G and A34V shows that a single mutation in the N-terminal region of FIS can destroy the ability of FIS to stimulate recombination without significantly affecting specific DNA binding activity.

To further elucidate the role of the N-terminus we constructed a series of N-terminal deletion derivatives of FIS (Table 1 and Materials and Methods). Removal of up to 10 amino acids from the N-terminus (FISΔ5 and FISΔ10) did not affect stimulation of G inversion nor DNA binding in extracts by the respective mutant proteins. FIS mutants FISΔ12, FISΔ15 and FISΔ26, however, were defective for stimulating G inversion while DNA binding was normal (Table 1). In crude extracts little binding activity was detected for FISΔ26. Since the purified protein bound with high affinity (see below) the low binding activity in crude extracts most likely reflects poor expression. A deletion derivative lacking the first 39 amino acids (FISΔ39), on the other hand, expressed no detectable protein and presumably is unstable *in vivo*. These analyses demonstrate that the region between amino acid residues 10 to 34 of FIS plays a crucial role in stimulating G inversion.

FIS mutant proteins V16G and FISΔ26 were purified and further analysed *in vitro*. No stimulation of DNA inversion was detectable with the purified mutant proteins even at concentrations 10-fold higher than the amount required to stimulate recombination with wt FIS (Fig.4C).

To analyse DNA binding, the affinity of the purified mutant proteins for the recombinational enhancer was estimated from gel retardation and footprinting assays. As shown in Fig. 4A and 4B the mutant proteins bind to the enhancer with affinities comparable to wild type FIS protein. No significant differences in the mobilities of FIS DNA complexes are detectable between wt FIS and FIS V16G with either the enhancer fragment (Fig. 4A), or the two circularly permutated attP fragments (Fig.3). For the mutant protein FISΔ26 the enhancer complexes have a slightly higher electrophoretic mobility. This is most likely a consequence of the smaller size of this deletion derivative. These results suggest that the N-terminal mutants bend the DNA normally. Furthermore, the mutant FIS proteins induce the same characteristic DNaseI hypersensitive sites in the enhancer as wt FIS (Fig.4B). This provides further evidence for normal DNA binding and bending properties of these mutant proteins (44).

To investigate whether the mutants are affected in the assembly of the synaptic complex we used reaction conditions where a recombination intermediate accumulates in the presence of ethylene glycol.(45). These intermediates have double strand breaks at the recombination sites and contain the recombinase covalently attached to the ends of the fragments (45). Such a

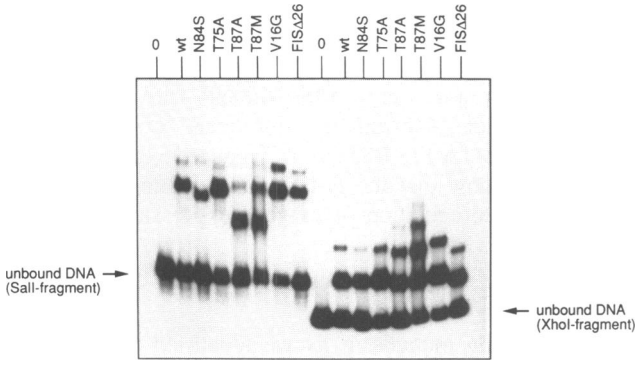

Figure 3. Binding of FIS and purified mutant proteins to attP. FIS was bound to the *SalI* fragment carrying the FIS site in the centre (left) and to the *XhoI* fragment carrying the binding site at the end of the fragment (right). Both DNA fragments were isolated from pJT110 and 5'-end labeled. The fragments were incubated with the FIS proteins indicated. (0) no FIS; 10 ng were added for wt FIS and FIS N84S; 20 ng were added for FIS T75A, FIS V16G and FISΔ26; 40 ng were added for FIS T87M and FIS T87A. Complexes were analysed on a non-denaturing 5% polyacrylamide gel. The different mobility observed for the unbound *SalI* and *XhoI* fragments of identical size is due to the intrinsic curvature of attP (26).

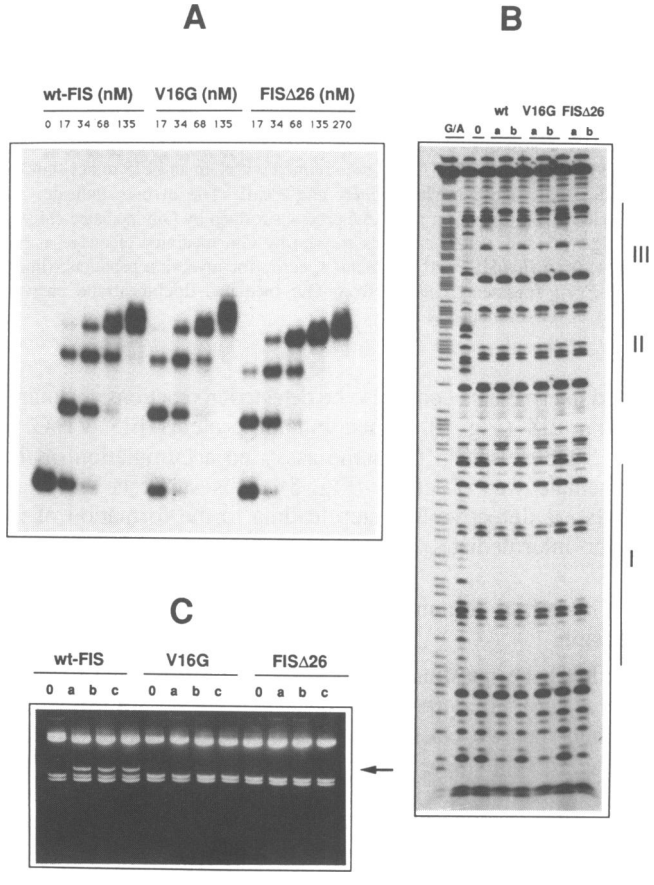

Figure 4. Analysis of N-terminal FIS mutant proteins. (A) Analysis of DNA binding by gel retardation. Binding of FIS to the 5'-end labeled *Bam*HI-*Ava*I enhancer fragment of pBRminiG(−) was performed with the protein concentrations indicated and analysed on a 5% polyacrylamide gel. (B) Footprinting wt and mutant FIS proteins on the recombinational enhancer with DNaseI. (0) no protein added; (a) 16 nM FIS; (b) 160 nM FIS. Lane (G/A) are products of a Maxam and Gilbert G+A sequencing reaction of the same fragment. The FIS sites in the enhancer are marked on the left (I, II, III). (C) Analysis of G inversion. Inversion was carried out with increasing amounts of wild type and mutant FIS proteins V16G and FISΔ26. (0) no added FIS protein; lanes (a) 60 nM; lanes (b) 120 nM; lanes (c) 240 nM FIS.

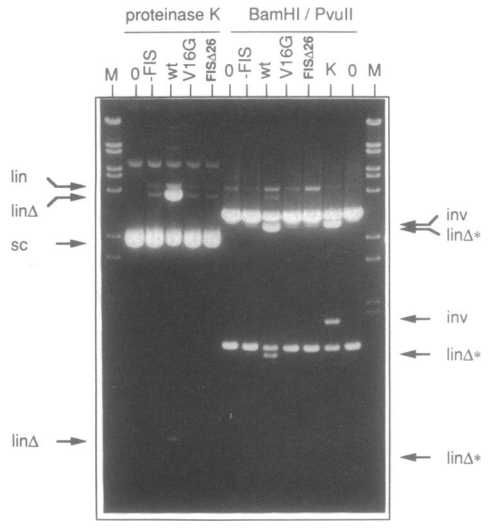

Figure 5. Analysis of the effect of N-terminal FIS mutant proteins on the formation of recombination intermediates. The inversion test plasmid pIR2 (4 μg) was incubated with Gin (70 nM) and FIS (145 nM) in 100 μl cleavage buffer (20 mM triethanolamine pH 7.5; 5 mM EDTA; 100 mM NaCl; 50% (v/v) ethylene glycol) for 30 min at 37°C. The reaction was terminated by proteinase K digestion for 20 min at 37°C (300 μg/ml proteinase K; 0.5% SDS). The DNA was purified by phenol extraction and ethanol precipitation. Half of the sample was digested with a mixture of *Bam*HI and *Pvu*II. Products were analysed on a 1% agarose gel. (M) DNA size marker (*Bst*EII digested λ DNA); (0) no added Gin; (−FIS) no added FIS; (K) pIR2 DNA was recombined with Gin and FIS under standard conditions and digested with *Bam*HI and *Pvu*II. The arrows indicate: (sc) supercoiled pIR2; (lin) linear pIR2, the products formed by Gin mediated cleavage at a single gix site; (linΔ) products formed by Gin mediated cleavage at both gix sites; (inv) *Bam*HI-*Pvu*II fragments specific for inversion products; (linΔ*) *Bam*HI-*Pvu*II fragments derived from Gin mediated double strand cleavage products at one or both gix sites.

cleaved intermediate could also be detected in reactions containing Gin and FIS (Fig. 5). When mutant proteins FIS V16G or FISΔ26 were used in the same assay no accumulation of this intermediate was observed (Fig. 5). This suggests that these mutants are defective in a step leading to the formation of the synaptic intermediate.

The N-terminal domain of FIS is not required for stimulating λ excision

To address the question whether the same functional domains of FIS are needed in the different systems where FIS is involved we tested the two classes of *fis* mutants for their effect on λ excisive recombination. When phage λ is induced in the *fis*⁻ host CSH50*fis*::Kan(λ*c*I857) the yield of infectious phage is about 1×10^8 p.f.u./ml which is about 100 to 200-fold lower than in CSH50(λ*c*I857) or in the presence of a plasmid complementing *fis* (Table 1 and not shown). For complementation studies with *fis* mutants we constructed plasmid pUHEfis2 and the respective *fis* mutant derivatives that express *fis* from an IPTG inducible promoter (see Materials and Methods). In the presence of IPTG pUHEfis2 lead to an about 200-fold increase in phage titer compared to cells harboring the vector plasmid pUHE25 (Table 2). A significant increase in phage titer was also observed without induction with IPTG. This is presumably due to incomplete repression of the promoter which drives *fis* expression. As shown in Table 2 the binding proficient mutants that fail to support G inversion stimulate λ excision to a level comparable to wt FIS.

Table 2: Effect of FIS on λ excision

Resident plasmid in λ lysogen[a]	Phage titer (p.f.u./ml) -IPTG	Phage titer (p.f.u./ml) +IPTG [b]	stimulation of λ excision[c]
pUHE25	1.3×10^8	1.4×10^8	1
pUHEfis2	2.2×10^9	2.5×10^{10}	192
pUHEfisV16G	3×10^9	2×10^{10}	154
pUHEfisΔ26	3.4×10^9	1.8×10^{10}	138
pUHEfisT87M	8.3×10^7	1×10^8	0.8
pUHEfisL88D	9×10^7	1×10^8	0.8
pUHEfisR85V	1×10^8	8×10^8	6
pUHEfisR89L/N84K	1×10^8	1×10^8	0.8

[a] Plasmids were tested in host strain CSH50*fis*::Kan(λ*c*I857) harboring plasmid pIQ1.
[b] *fis* expression was induced by IPTG (50 μM) about 2 h before phage induction.
[c] phage titers from lysogens induced in the presence of IPTG were divided by the titer of the *fis*⁻ control (pUHE25).

Mutants strongly affected in binding are also strongly affected in stimulating λ excision. For mutant R85V a weak stimulation of λ excision was observed. The finding that the N-terminal deletions of FIS are able to support λ excisive recombination suggests that stimulation of λ excision is mediated primarily through the DNA binding activity of FIS.

The N-terminal domain of FIS is dispensable for autoregulating *fis* gene expression

We have recently found that FIS autoregulates its own transcription (O. N., unpublished). To investigate whether the N-terminus of FIS is involved in this process, we tested the autoregulatory activity of *fis* mutants. Activity of the *fis* promoter was studied using a single copy transcriptional fusion of the *fis* operon to *lac*Z (O. N., unpublished; see Materials and Methods). LacZ expression of the fusion was threefold lower in the wild type background than in the *fis*⁻ background (CSH50*fis*::Kan (λFP1)), (not shown). For complementation studies plasmids pUHEfis2 and pIQ1 were introduced in strain CSH50*fis*::Kan (λFP1). When FIS expression from pUHEfis2 was induced with IPTG a 10-fold decrease in LacZ expression was observed compared to the uninduced control (Table3). This assay was then used to test mutant derivatives of pUHEfis2 (Table 3). The binding proficient mutants FIS V16G and FISΔ26 were as active in the repression assay as wt FIS. Mutants with DNA binding defects, however, showed no significant effect on LacZ expression except for FIS R85V which reduced expression 2-fold. These results show that the N-terminal domain of FIS is not required for autoregulation of *fis* gene expression.

DISCUSSION

By characterizing mutants in *fis* we identified two functional domains in the protein. The C-terminal part of FIS forms the DNA binding domain while the N-terminal domain is required for mediating the stimulatory effect on G inversion. We further demonstrate, that the N-terminal part of FIS is dispensable for the ability of FIS to act as a repressor of its own transcription and for stimulating λ-excision.

The structure of the FIS dimer, recently solved by X-ray crystallography (14), suggests explanations for several of the phenotypes we observed in FIS mutants. The C-terminal deletion mutants which lack parts of the helix-turn-helix motif are defective

Table 3: Autoregulation of *fis* gene expression.

Resident plasmid in *LacZ* fusion strain[a]	LacZ-activity[b] without induction	LacZ-activity[b] after induction	Relative LacZ activity[c]
pUHE25	945	815	1,2
pUHEfis2	775	80	9,7
pUHEfisV16G	829	120	6,9
pUHEfisΔ26	750	92	8,2
pUHEfisT87M	893	620	1,4
pUHEfisL88D	1000	670	1,5
pUHEfisR85V	935	460	2,0
pUHEfisR89L/N84K	945	798	1,2

[a] The *fis* operon *LacZ* fusion strain CSH50*fis*::Kan(λFP1) harboring the compatible Lac repressor plasmid pIQ1 was used as recipient.
[b] LacZ activities (units) were determined as described in Materials and Methods, the induced cultures were grown in the presence of 50 μM IPTG.
[c] for each strain the LacZ activity obtained without IPTG induction was divided by the value obtained after induction.

for DNA binding (Fig.1). The mutations R85V and R85C might directly interfere with a protein DNA contact, since the side chain of residue R85 within the recognition helix is both positively charged and solvent exposed. The DNA binding defect of mutant L88D may be caused by a structural distortion of the helix-turn-helix motif because L88 is a highly conserved hydrophobic residue in helix-turn-helix motifs and involved in stabilizing the structure of the bihelical fold (13). Mutation K91E probably also leads to a structural defect. The positively charged residue is facing the core of FIS with the side chain being in close proximity to the negatively charged side chain of residue E59. The substitution of the positively charged lysine residue for the negatively charged glutamate in mutant K91E may interfere with the proper relative arrangement between the helix-turn-helix motif and the protein core. Consequently, a similar structural distortion might be the reason for the binding defect conferred by the mutation E59G.

Mutants T87A and T87M are particularly interesting since their effect on DNA binding is complex. The mutations lead both to an apparent decrease in affinity and to the formation of protein DNA complexes with altered electrophoretic mobility. Assuming that wt FIS and the mutant proteins bind to the same sites this may be due to either a change in the protein induced DNA conformation or to an altered shape or charge of the mutant protein itself. Because the mutations do not change the charge of the protein and because the effect on mobility is dependent on the location of the FIS binding site relative to the ends of the DNA fragment (Fig. 3) we think it likely that the mutations at position 87, besides affecting binding affinity, also change the geometry of the FIS induced DNA conformation. It is therefore difficult to distinguish whether the defects of mutants T87M and T87A result from decreased binding affinity or from altered DNA bending.

Mutants FIS N84S and T75A also show changed binding patterns with the enhancer fragment (Fig. 2). These changes, however, do not lead to a loss of enhancer function. Therefore changes in complex mobilities do not allow direct conclusions about enhancer function. For FIS N84S the first complex comigrates with the wt complex, while complexes II and III differ in their mobility relative to wt. This could indicate, that the FIS sites become occupied in a different order compared to wt FIS and raises the possibility that residue 84 of FIS contributes to binding site selection.

The existence of *fis* mutants that are defective in stimulating G inversion, but are fully competent to stimulate λ excision and to repress *fis* gene expression suggests that FIS mediates these activities via different mechanisms. The central difference between these systems is that for the stimulation of G inversion the enhancer must cooperate with the recombination sites over large distances while in λ excision FIS acts only on proteins bound in its vicinity. The mechanisms underlying the autoregulation of FIS expression and the stimulation of λ excision may be similar because the N-terminal part of FIS is dispensable for both reactions. This suggests that the DNA binding and bending properties of FIS are sufficient for these systems FIS has been shown to bind to attR (15, 19) and to the *fis* promoter region (O. N., unpublished) *in vitro* and it is hence likely that the observed FIS effects are a direct consequence of FIS interacting with these sites. We cannot, however, formally exclude the possibility that the effects observed are indirect e.g. are caused by some unknown FIS effects on cell physiology.

For autoregulation by FIS we suppose that binding of FIS interferes directly with binding of RNA polymerase.

Unfortunately, the N-terminus which we have defined as essential for G inversion is not resolved in the X-ray structure which showed only scattered electron density for the N-terminal 24 amino acids (14). This has been interpreted to be a consequence of local flexibility within the N-terminal domain of FIS. The X-ray data suggest, however, that the unresolved part is located on the side of the FIS dimer opposite the helix-turn-helix motif (14). This makes models very attractive that propose that the N-terminal domain of FIS acts with other components of the synaptic complex. Evidence for the presence of FIS in the synaptic complex has been obtained by electron microscopy (18). The N-terminus of FIS could be interacting directly with Gin or with another site on the DNA molecule.

In the activation deficient mutant V16G, the structural integrity of the N-terminus may be affected while mutation A34V which is located within helix A (see Fig. 1) may either provide a contact point to the synaptic complex or may interfere with the interaction of the flexible activating domain with the core of FIS. The other FIS mutants mapping to this region (K25E, K32E) may have similar effects they confer, however, only a mild phenotype.

If the N-terminus of FIS is involved in a protein-protein interaction with Gin it could be responsible for the assembly of a stable synaptic complex. In this case mutants of FIS lacking the N-terminus would be excluded from such stable complexes while the association of the wt FIS-enhancer complex with the synapse via the N-terminus is strongly favoured. Such cooperative interactions would be consistent with the relative low inhibitory effect the FIS mutants lacking the N-terminus have on G inversion (data not shown). The idea that the N-terminus of FIS acts in a step that follows binding to the enhancer sequence is also suggested by our observation that the N-terminal FIS mutants do not assemble the reaction intermediate in the presence of ethylene glycol.

Since topological experiments with Gin mutants that efficiently recombine without FIS have raised doubts about the importance of specific Gin-FIS contacts for generating the correct synapse topology (46), the possibility that the N-terminus of FIS contacts another DNA site or another FIS-DNA complex within or outside the synaptic complex must also be considered. In any case, our analysis has revealed that FIS fulfills its function in G inversion via two domains, one interacting with the enhancer and the other likely to contact some other component of the synaptic complex.

While this manuscript was written an independently performed mutational analysis of *fis* has been published (47). The authors have used the Hin mediated recombination system which is closely related to the Gin system for the isolation of *fis* mutants. Where their data and the data presented here overlap they are fully consistent.

The finding that the N-terminus of FIS is dispensable for stimulating λ excision and autoregulating *fis* expression raises the intriguing question whether any of the other activities of FIS in *E.coli* require the integrity of the N-terminal domain or whether its function is confined to the DNA inversion reaction.

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