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Deletion Analysis of the *fis* Promoter Region in *Escherichia coli*: Antagonistic Effects of Integration Host Factor and Fis

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Fis is a small DNA-binding and -bending protein in *Escherichia coli* that is involved in several different biological processes, including stimulation of specialized DNA recombination events and regulation of gene expression. *fis* protein and mRNA levels rapidly increase during early logarithmic growth phase in response to a nutritional upshift but become virtually undetectable during late logarithmic and stationary phases. We present evidence that the growth phase-dependent *fis* expression pattern is not determined by changes in mRNA stability, arguing in favor of regulation at the level of transcription. DNA deletion analysis of the *fis* promoter (*fis* P) region indicated that DNA sequences from −166 to −81, −36 to −26, and +107 to +366 relative to the transcription start site are required for maximum expression. A DNA sequence resembling the integration host factor (IHF) binding site centered approximately at −114 showed DNase I cleavage protection by IHF. In *ihf* cells, maximum cellular levels of *fis* mRNA were decreased more than 3-fold and transcription from *fis* P on a plasmid was decreased about 3.8-fold compared to those in cells expressing wild-type IHF. In addition, a mutation in the *ihf* binding site resulted in a 76 and 61% reduction in transcription from *fis* P on a plasmid in the presence or absence of Fis, respectively. Insertions of 5 or 10 bp between this *ihf* site and *fis* P suggest that IHF functions in a position-dependent manner. We conclude that IHF plays a role in stimulating transcription from *fis* P by interacting with a site centered approximately at −114 relative to the start of transcription. We also showed that although the *fis* P region contains six Fis binding sites, Fis site II (centered at −42) played a predominant role in autoregulation, Fis sites I and III (centered at +26 and −83, respectively) seemingly played smaller roles, and no role in negative autoregulation could be attributed to Fis sites IV, V, and VI (located upstream of site III). The *fis* P region from −36 to +7, which is not directly regulated by either IHF or Fis, retained the characteristic *fis* regulation pattern in response to a nutritional upshift.

Fis is an 11.2-kDa DNA-binding and -bending protein that was first identified for its ability to stimulate DNA inversion reactions mediated by the Hin, Gin, and Cin family of recombinases (24, 29, 31). Hence, this protein was termed factor for inversion stimulation (Fis). Subsequently, Fis was shown to be involved in other cellular processes, including bacteriophage λ DNA excision and integration (2, 3, 50), regulation of initiation of DNA replication at *oriC* (12, 19, 54), and regulation of gene expression (13). Fis can stimulate transcription of rRNA and tRNA operons (23, 36, 47) and several structural genes (1, 20, 56) and can also negatively regulate the transcription of various genes, including its own (3, 20, 39, 55). Roles played by Fis appear to be especially significant under conditions of rapid cell growth and high temperature, since *fis* cells grow slower than wild-type cells in rich medium but not minimal medium (37, 38) and since extensive filamentation is observed with *fis* cells grown at 44°C (12).

fis is transcribed from a single promoter located 1,025 bp upstream from its start codon (4, 39). An approximately 1,400-base mRNA transcribed from this promoter encodes both the 294-bp *fis* gene and a 963-bp open reading frame (*orf-1*) located upstream of *fis*, indicating that *fis* and *orf-1* are organized as an operon. Although the function of *orf-1* is not known, its presence in both *Escherichia coli* and *Salmonella typhimurium* (41) and its strong similarity to *nifR3* (function also unknown) from *Rhodobacter capsulatus* and *Azospirillum brasilense* (14, 33) suggest that it encodes an important protein.

Fis levels in *E. coli* and *S. typhimurium* are extremely low or undetectable during stationary phase, but when cells are batch cultured in rich medium, its levels increase from less than 250 to over 25,000 dimers per cell by 75 to 90 min (4, 38, 41, 50). Thereafter, Fis protein levels decrease to less than 1% of its peak as cells enter stationary phase. A similar regulation pattern observed with *fis* mRNA suggested that much of the regulation occurs at the mRNA level (4, 39, 41). The magnitude of *fis* expression is related to the nutritional quality of the medium, so that higher Fis protein and mRNA levels are measured in nutritionally richer medium (4, 38). This expression pattern may be important in determining the roles of Fis in its various cellular functions.

Negative autoregulation accounts for about a sixfold decrease in *fis* mRNA levels and about a two- to fourfold decrease in β-galactosidase activity measured from *lacZ* fusions to the *fis* promoter (*fis* P) region (4, 39, 41). Six Fis-binding sites have been identified in the *E. coli fis* P region, two of which (Fis sites I and II) overlap the region protected by RNA polymerase (4). Prebound Fis prevented subsequent binding of RNA polymerase, suggesting that Fis might act by hindering RNA polymerase binding to *fis* P. Fis sites I and II have been shown to participate in autoregulation (39), whereas the roles of the additional four Fis sites in this region have not previously been investigated. Although *fis* autoregulation controls the magnitude of *fis* mRNA levels, the overall growth phase-dependent *fis* expression pattern can still be observed in the absence of Fis (4, 41). Hence, growth phase-dependent *fis* expression is not controlled by *fis* autoregulation.

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fis is also negatively regulated by the stringent response. Cells subjected to amino acid starvation exhibit a rapid decrease in *fis* mRNA levels, an effect that can be reversed by the addition of chloramphenicol (39). Stringent control and growth phase-dependent *fis* regulation seemingly require distinct molecular mechanisms, since the latter is largely unaffected in a *relA spoT* double mutant (4). Since neither autoregulation nor stringent control by itself appears to determine the overall *fis* expression pattern observed in response to a nutritional upshift, we anticipate that additional regulatory processes exist that regulate the expression of this gene.

In this work, we investigated the possibility that other processes affect the regulation of *fis* expression. We present evidence that *fis* mRNA decay rates do not show significant variation during different stages of the *fis* mRNA expression, suggesting that transcriptional control is the primary determinant of the *fis* expression pattern. A set of DNA deletions within the *fis* P region was used to identify potential regulatory sequences. We found that the region from −166 to −81 relative to the transcriptional start site, which contains a match to the consensus sequence for the integration host factor (IHF), was required for maximum transcription from *fis* P. We showed that IHF bound to a site centered approximately at −114, causing an increase in *fis* P transcription in a position-dependent fashion. The roles played by the six Fis sites in autoregulation were also examined. We found that Fis site II, centered at approximately −42, played a predominant role in autoregulation while Fis sites I and III appeared to play minor roles in this process. Measurements of relative *fis* mRNA levels generated from minimal *fis* promoter sequences (−36 to +7) indicated that this region was sufficient for achieving the characteristic *fis* regulation pattern and supported previous observations (39).

MATERIALS AND METHODS

Chemicals, enzymes, and growth media. All chemicals were from Sigma Chemical Co., Fisher Scientific Co., VWR Scientific, Life Technologies Inc. (Gibco BRL), or Promega Corp. Bacterial culture media were from Difco Laboratories. Enzymes were purchased from New England Biolabs Inc. or Promega Corp. unless otherwise indicated. Purified IHF was a generous gift from Howard Nash, National Institutes of Health, Bethesda, Md. The radioisotopes [γ-^{32}P] ATP and [α-^{32}P]dATP were from Amersham Life Science Inc. Oligonucleotides were synthesized in a Perkin-Elmer automated DNA synthesizer operated within the Department of Biological Sciences, University at Albany, Albany, N.Y.

Bacterial cultures were grown at 37°C in Luria-Bertani (LB) medium (48). To select for appropriate drug resistance, 100 μg of ampicillin per ml, 50 μg of kanamycin per ml, or 12 μg of chloramphenicol per ml was added to the growth medium.

Bacterial strains and plasmids. RZ211 [F$^-$ Δ(*lac pro*) *thi ara str recA56 srl*] (30) and RJ1561 (RZ211 *fis*::*767*) (28) were used for most of the β-galactosidase assays and primer extension assays. Strains MC4100 [F$^-$ λ$^-$ *araD139* Δ(*argF-lac*) *U169 rpsL1 relA1 deoC1 ptsF25 rboR flb5301*] and HP4110 (MC4100 *ihfA*::Tn*10*) were obtained from Prasanta Datta (University of Michigan) and were also used for β-galactosidase assays. Strains MC1000 [*araD139* Δ(*ara-leu*)*7697* Δ*lacX74 galU galK strA*] (7) and MC1000 *ihfB*::Cam (obtained from B. Ely) were used for primer extension analysis.

Plasmid pRJ807 carries an *Eco*RI-*Hin*dIII fragment containing *E. coli fis* under *tac* promoter control as described previously (40). pDR114 was made by cloning an *Alu*I-*Alu*I DNA fragment, containing the *fis* P region from −250 to +55, into the blunted *Xba*I site of pDR112. pDR112 is pUC18 with a *Bam*HI-*Xba*I-*Bgl*II restriction site sequence between the *Sma*I and *Pst*I sites. pRJ805 (obtained from R. C. Johnson) carries a *Kpn*I-*Kpn*I DNA fragment, containing the region from −756 to +866, within the *Kpn*I site of pUC18 such that *fis* P directs transcription toward the *Hin*dIII site.

pRJ800 is a pBR322-derived plasmid containing the (*trp-lac*)*W200* fusion preceded by the pUC18 polylinker region (4). All DNA deletions across the *fis* promoter region were placed in the polylinker region of pRJ800 such that expression of the (*trp-lac*)*W200* fusion was under *fis* P control. pRJ1028 (obtained from R. C. Johnson) carries the *Hin*dIII-*Nco*I fragment from pRJ805 (containing the region from −373 to +80) within the *Hin*cII site of pRJ800.

The deleted *fis* P regions and their respective plasmids are represented in Fig. 2A. pTP137 was made by cloning the *Kpn*I-*Nco*I (blunt-ended) DNA fragment from pRJ805 into the *Kpn*I and *Sma*I sites of pRJ800. pTP123 contains the *Hin*dIII (blunt-ended)-*Sal*I DNA fragment from pRJ805 cloned within the *Sma*I and *Sal*I sites. For pTP124, the *Hin*dIII (blunt-ended)-*Eco*RV DNA fragment from pRJ805 was cloned within the *Sma*I site. For pTP122, the *Dra*I-*Hin*dIII DNA fragment from pRJ1028 was cloned into the *Sma*I and *Hin*dIII sites.

Plasmids pRJ1069, pRJ1060, pRJ1071, pRJ1064, and pRJ1075 (containing various DNA deletions upstream of *fis* P) were made by applying a unidirectional exonuclease III digestion procedure (26) to the *Xba*I- and *Sac*I-cleaved pRJ1028. Precise deletion boundaries were determined by DNA sequencing.

Other DNA sequences containing various portions of the *fis* P region were generated by PCR with *Taq* polymerase from Boehringer Mannheim Corp. as specified by the manufacturer. Various oligonucleotides used in these reactions were designed such that a *Kpn*I or *Xba*I restriction site would be generated upstream or downstream, respectively, of the desired *fis* P region with either pRJ805 or pRJ1028 as the template. The resulting products were cloned within the *Kpn*I and *Xba*I sites of pRJ800 to generate pTP118, pTP119, pTP120, pTP121, pTP126, pTP127, pTP138, and pTP184. For pTP132, the amplified region from −139 to +107 was cleaved with *Dra*I (at −81) and *Xba*I, and cloned within the *Sma*I and *Xba*I sites of pRJ800. For pTP116, two complementary oligonucleotides extending from positions −35 to +7 were synthesized, annealed, and ligated into the *Sma*I site of pRJ800 such that the sequence from −36 to +7 was generated. All *fisp* regions synthesized by PCR were verified by DNA sequencing.

Point mutations in the *ihf* site were also generated by PCR. To construct pTP130, the sequence from −139 to +107 was amplified from pRJ805 with the upstream primer 5′-dGGGGTACCAGTCACTATTTTC**A**CATATGAA**T**ATTT TTATGAGTA (point mutations in bold; *Kpn*I site underlined) and the downstream primer 5′-dGCTCTAGAAGGTCTGTCTGTAATGCC (*Xba*I site underlined) and cloned within the *Kpn*I and *Xba*I sites of pRJ800. pTP185 was constructed in a two-step process by a megaprimer procedure (5). First, the region from −139 to +107 was amplified from pRJ805 with the same downstream primer used to construct pTP130 and a similar upstream primer that lacked the *Kpn*I site. The resulting fragment was used as a megaprimer for a second PCR together with a primer that annealed to the upstream polylinker region in pRJ1028. The resulting mutated *fis* P region from −373 to +107 was cloned within the *Kpn*I and *Xba*I sites of pRJ800.

pKW125 is similar to pRJ1028 except that it contains a 5-bp insertion between nucleotide positions −60 and −61 such that a *Bam*HI site is created. The *fis* P regions from −373 to −61 and from −61 to +80 were amplified independently, creating *Bam*HI sites at position −61 that were used to ligate these two fragments. The desired fragment was purified by acrylamide gel electrophoresis and cloned within the *Eco*RI and *Hin*dIII sites of pRJ800. pKW232 contains a 10-bp insertion between nucleotide positions −60 and −61 such that both an *Xho*I site and a *Bam*HI site are created. The region from −373 to −60 in pKW125 was amplified with a primer that created a *Xho*I site followed by a *Bam*HI site next to position −61. The PCR product was cleaved with *Bam*HI and used as a megaprimer for amplification of the region from −373 to +80 in pKW125. The resulting fragment was cloned within the *Xba*I and *Sph*I sites in pRJ800.

DNA sequencing reactions. DNA sequencing was performed on alkali-denatured double-stranded plasmid DNA with Sequenase version 2.0 (U.S. Biochemicals) as specified by the supplier.

β-Galactosidase assays. β-Galactosidase assays were performed as described previously (35). Overnight bacterial cultures were diluted 75-fold in LB medium and grown at 37°C for 90 min with constant shaking. Values represent an average from at least three independent assays.

RNA analysis. Total RNA was prepared essentially as described previously (8). For measurements of *fis* mRNA half-lives, saturated RZ211 cultures were diluted 25-fold in LB medium and grown at 37°C with shaking. After 15, 40, 70, and 100 min of growth, 130 ml of cell culture was removed and treated with 400 μg of rifampin at 37°C. After 0, 2, 4, 8, 10, and 12 min of rifampin treatment, 20 ml of cell culture was collected and used for preparation of total cellular RNA. The RNA concentration was measured spectrophotometrically by using the equivalence of 1 optical density absorbance unit at 260 nm (OD_{260}) to 40 μg/ml RNA.

Northern blot hybridizations were performed at 42°C with a formamide hybridization solution (48). A 550-bp *Eco*RI-*Bgl*II DNA fragment from plasmid pRJ807 containing *fis* was used as a ^{32}P-labeled probe. *fis* mRNA signals were detected by autoradiography and quantified by densitometry.

Primer extensions were performed essentially as described previously (25). Total RNA was isolated from cultures of RJ1561 carrying different *fisp*-containing plasmids. These cultures were diluted 50-fold in LB medium and grown at 37°C for 60 min, such that mRNAs generated from *fis* P were near peak levels. DNA primer (2 pmol) with the sequence 5′-dGCTGATATTGTCCGATG (annealing to *fis* mRNA from +40 to +56) and 10 μg of total RNA were used in these reactions. Primer-extended products were separated electrophoretically on 8% polyacrylamide–8 M urea gels, autoradiographed, and quantified by densitometry or by direct counts-per-minute measurements with a Betascope 603 blot analyzer (Betagen). Based on the fraction of primer-extended product to free primer, we estimate an over 200-fold excess of primer to *fis* P-dependent mRNA in these reactions. Background *fis* mRNA signals originating from chromosomal *fis* P were measured from primer-extended products obtained with 10 μg of RNA from RJ1561 containing pRJ800. Values for *fis* mRNA signals were corrected for the background signal, which never exceeded 20% of the total signal measured. For analysis of plasmid pTP116 (−36 to +7), a primer with the sequence

5′-dCTGACAAAAGCTTGCATGCCTG was used, which hybridizes to the top DNA (sense) strand of the pRJ800 polylinker-*trp* junction downstream of the *fis* P region. As expected, no signals were detected from chromosomally derived *fis* mRNA when this primer was used.

Gel mobility shift assays. The DNA-binding conditions used in gel mobility shift assays with IHF were as described previously with some modifications (32). IHF was diluted to various concentrations in high-salt buffer (50 mM Tris-HCl [pH 7.4], 800 mM KCl, 10% glycerol, 2 mg of bovine serum albumin [BSA] per ml). From each IHF dilution, 1 μl was mixed with 3,000 cpm of the ^{32}P-end-labeled *Bam*HI-*Bgl*II DNA fragment from pDR114, 2 μl of 10× binding buffer (500 mM Tris-HCl [pH 8.0], 25 mM EDTA), and 3.8 μl of 50% glycerol in a final volume of 20 μl. Following incubation for 10 min at 25°C, 5 μl of gel-loading buffer (50 mM Tris-HCl [pH 8.0], 2.5 mM EDTA, 40 mM KCl, 0.1 mg of BSA per ml, 10% glycerol, 0.02% bromophenol blue) was added and the IHF-DNA complexes were electrophoretically separated from free DNA in 8% polyacrylamide gels (60:1 acrylamide/bisacrylamide ratio) at 20 mA with 1× TBE (89 mM Tris base, 89 mM boric acid, 2.5 mM EDTA) as the running buffer. The gels were fixed in 10% acetic acid, dried under vacuum, and autoradiographed.

DNase I footprinting. DNase I protection analysis was performed essentially as described previously (6). The *Bam*HI-BglII DNA fragment from pDR114 containing the *fis* P region from −250 to +55 labeled with ^{32}P at either one of its 3′ ends was used. For the binding reactions, different amounts of purified IHF were combined with 20,000 cpm of labeled DNA fragment, 50 mM Tris-HCl (pH 8.0), 40 mM KCl, 2.5 mM EDTA, 0.1 mg of BSA per ml, and 10% glycerol in a final volume of 45 μl. Incubation was carried out for 5 min at 25°C. The DNase I cleavage products were separated in 8% polyacrylamide–8 M urea gels.

RESULTS

***fis* mRNA half-life.** The transient *fis* mRNA expression pattern that occurs in response to a nutritional upshift could be due to changes in transcriptional activity from *fisp*, changes in *fis* mRNA decay rates, or both. If *fis* mRNA stability played an important role in generating its characteristic expression pattern, longer *fis* mRNA half-lives would be expected during the time when cells accumulate *fis* mRNA than during the time when *fis* mRNA levels decrease (Fig. 1A). From the slopes of the *fis* mRNA decay, mRNA half-lives of 1.8, 2.1, 2.0, and 2.1 min were obtained from cell cultures grown for 15, 40, 70, and 100 min, respectively (Fig. 1B to E). These values fall within 18 s or less of each other and thus exhibit little variation. Moreover, these small variations do not obey a pattern that allows a correlation between *fis* mRNA half-lives and the relative intracellular levels, since the shortest half-life (1.8 min) was obtained from cells that are rapidly accumulating *fis* mRNA. The results suggest that the *fis* mRNA expression pattern is not regulated at the level of *fis* mRNA decay and should therefore be regulated primarily at the level of transcription.

Deletion analysis of the *fis* P region in the absence of Fis. To identify DNA sequences that might be involved in regulating *fis* expression, we generated a set of deletions affecting DNA sequences upstream and downstream of *fis* P (Fig. 2A). The resulting sequences were placed in plasmid pRJ800 such that expression of a (*trp-lac*)*W200* fusion on this plasmid was dependent on transcription from *fis* P. β-Galactosidase activities in both RZ211 and RJ1561 (RZ211 *fis*::*767*) cells carrying the different plasmids were measured. Because deletion of the region from +81 to +107 relative to the transcriptional start site was found to have negligible effects on *fis* P expression in either RZ211 or RJ1561 cells (Fig. 3C), the effects of DNA deletions upstream of *fis* P were compared in DNA constructs having downstream boundaries at either +81 or +107 (Fig. 3A).

In the absence of Fis (RJ1561 cells), a stepwise deletion of the DNA region from −756 to −167 resulted in small reductions in β-galactosidase activity (around 0 to 10% of maximum), suggesting that these upstream sequences have minimum effects on *fis* P expression. However, when the DNA region upstream of −81 was removed, transcription decreased to about 40% of maximum levels, suggesting that the region from −166 to −82 is required for full expression.

Further DNA deletion to −65 resulted in a moderate increase in β-galactosidase activity, whereas deletion to −50 resulted in a moderate decrease. A deletion to −36 resulted in almost no change in activity relative to the deletion to −50. Thus, in the absence of additional upstream DNA sequences, the region from −50 to −37 does not appear to affect *fis* P expression. When the region from −36 to −27 was further deleted, transcription was abolished. This deleted region (CC TTTCATCT) contains a poor match to the consensus −35 promoter sequence for σ70 RNA polymerase. Either the sequence TTCATC (matches to consensus underlined [Fig. 2B]), with a 16-bp spacing between the −10 and −35 regions, or TTTCAT, which further deviates from consensus but exhibits the preferred 17-bp spacing, might serve as the promoter −35 region. In either case, this result indicates that the region from −36 to −27 is essential for transcription and is likely to function as a −35 promoter region.

To verify that differences in β-galactosidase activities measured reflected differences in transcription initiating from *fis* P in the various deletion constructs, we examined the relative *fis* mRNA levels in RJ1561 carrying pRJ800-based plasmids containing various deleted *fis* P regions. After 60 min of growth in LB medium at 37°C, total cellular RNA was isolated and mRNA levels originating at *fis* P were measured by primer extension analysis (Fig. 3B). The results indicate that relative *fis* mRNA levels produced from different DNA deletion constructs in RJ1561 obeyed a pattern similar to that resulting from measurements of β-galactosidase activity.

Effect of Fis sites on *fis* P expression. Because of *fis* autoregulation, β-galactosidase activities generated from *fis* P are higher in RJ1561 compared to RZ211 cells (4, 39, 41). A measure of *fis* autoregulation can be obtained from β-galactosidase activity ratios of RJ1561 to RZ211 cells for each deletion construct. For unknown reasons, the pRJ800 vector control reproducibly gave twofold-higher β-galactosidase activities in RJ1561 cells than in RZ211 cells. This effect could not be attributed to plasmid copy number differences, since these were found to be similar (about 8% variation) for different plasmid constructs in the two strains. Hence, *fis* autoregulation values were corrected for this background effect. Deletion of a Fis site important for autoregulation should result in a decrease in autoregulation efficiency.

Deletion of Fis site VI in pRJ1071 had almost no effect on autoregulation (Fig. 3A; Table 1). Removal of the region from −166 to −139 in pTP126, which removes most of Fis site V, caused a small decrease in *fis* P transcription in RJ1561. A greater decrease was observed in RZ211 than in RJ1561. This effect can be expressed as an increase in negative autoregulation from 3.3- to 4.4-fold (Table 1). Thus, Fis bound to site V might somehow help alleviate some of the negative regulation caused by Fis bound to other sites. When Fis site IV was also disrupted in pTP127, *fis* autoregulation efficiency further increased to 5.0-fold. However, this additional effect cannot necessarily be attributed to Fis site IV, since an *ihf* site has also been deleted in this construct (see below).

Further deletion of Fis site III in pTP121 resulted in a small decrease in autoregulation, from 5.0- to 4.6-fold (Table 1). In pTP120, where the first four nucleotides of Fis site II were replaced by vector DNA sequence, an additional decrease to 4.2-fold was observed. However, when most of Fis site II was disrupted in pTP119, leaving Fis site I and the putative −35 and −10 promoter sequences intact, repression was only 1.7-fold. Thus, Fis site II plays a predominant role in this process.

When all the Fis sites, including Fis site I, were deleted in

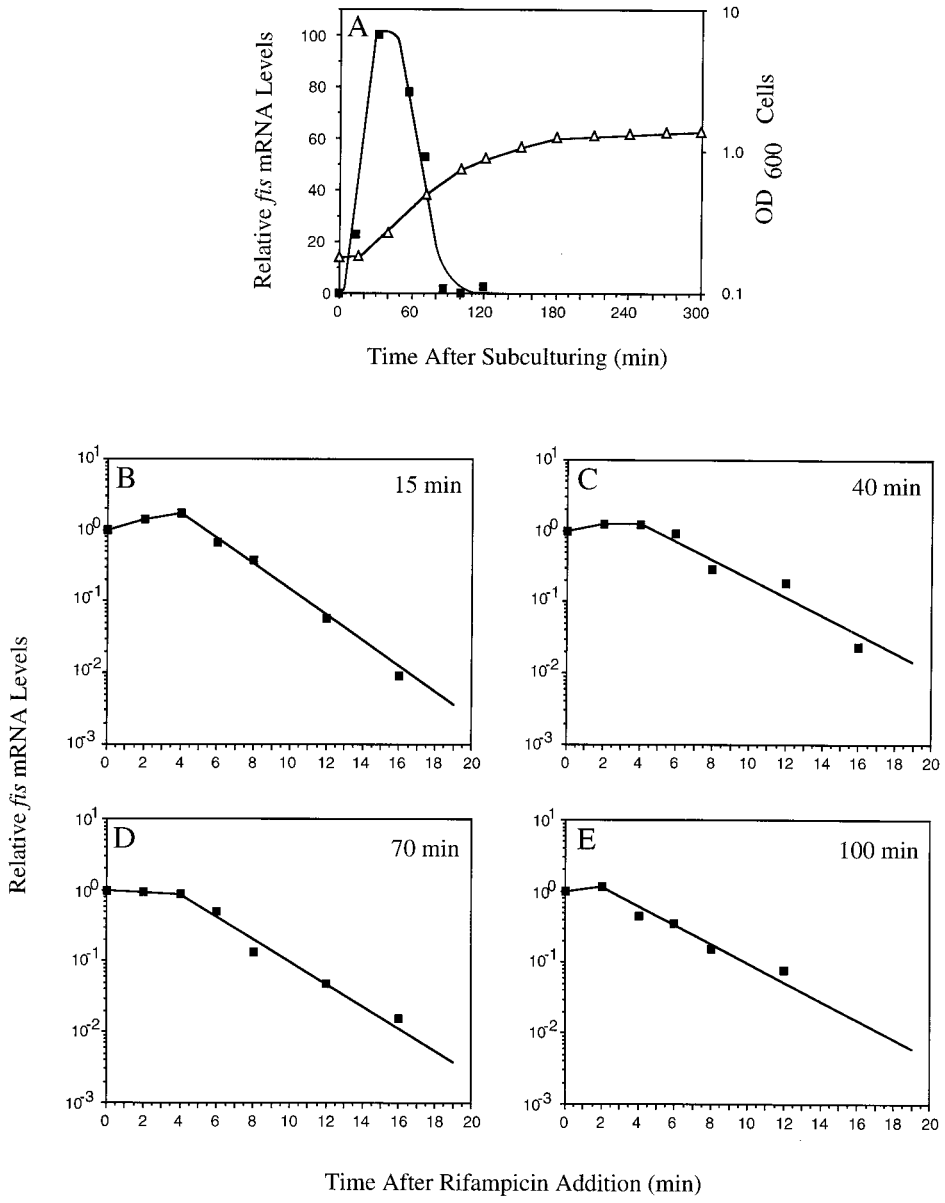
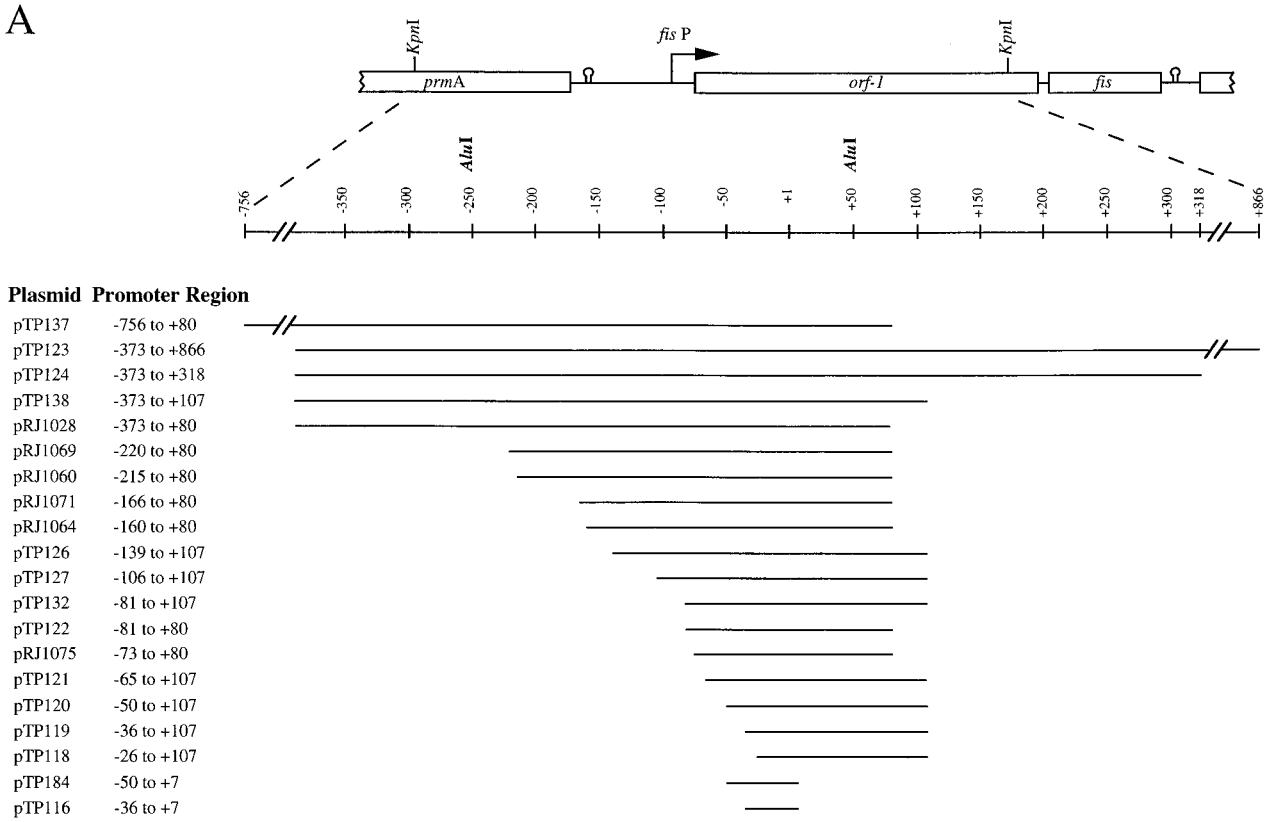


FIG. 1. Determination of *fis* mRNA half lives. (A) *fis* mRNA expression pattern. RZ211 cells were grown overnight, diluted 25-fold in LB medium, and grown at 37°C. At various times after subculturing, cells were harvested for preparation of total cellular RNA. Northern blots were performed with 10 μg of total RNA from each sample and with a *fis*-containing 550-bp DNA fragment as the ^{32}P-labeled probe. Symbols: ■, Relative *fis* mRNA levels determined by densitometric measurements of the *fis* mRNA signal on an autoradiograph (a 100% value was assigned to the maximum band intensity measured and all other values are shown relative to this one); △, cell density as measured by light absorbance at 600 nm. (B to E) *fis* mRNA decay rate after 15 min (B), 40 min (C), 70 min (D), and 100 min (E) of subculturing RZ211 cells in LB medium. At these times, 130 ml of cell culture was removed and treated with 40 μg of rifampin per ml at 37°C with shaking. At various times after the addition of rifampin, the cells were harvested for preparation of total cellular RNA. Northern blot analyses were performed, and relative *fis* mRNA levels (■) were quantified as in panel A. A 100% value was assigned to the *fis* mRNA levels measured at the time of rifampin addition (0 min), and all other values are shown relative to this one.

pTP116, autoregulation decreased to background levels, suggesting that Fis bound to site I alone accounts for a 1.7-fold repression. However, when only Fis site II was present in pTP184, *fis* P repression was 4.1-fold, a value similar to that when both Fis sites I and II were present in pTP120 (4.2-fold). Thus, while Fis sites I and III can participate in *fisp* repression, Fis site II can account for almost all the autoregulation detected in our assays.

Effect of downstream DNA sequences on *fis* P expression. The possibility existed that other DNA sequences downstream of *fis* P, besides Fis site I, somehow affected the expression of this promoter. To investigate this, RZ211 and RJ1561 cells containing various plasmids with DNA sequences starting at −373 and ending at +866 (pTP123), +318 (pTP124), +107 (pTP138), or +80 (pRJ1028) were used to measure β-galactosidase activities. A deletion of the downstream DNA regions from +866 to +318 and from +107 to +80 showed negligible effects on transcription in either strain (Fig. 3C). However, deletion of the DNA region from +318 to +107 resulted in about a 40% decrease in β-galactosidase activity measured in RZ211 and a 25% decrease in RJ1561 cells. Results from primer extensions of mRNA originating from *fis* P in these



B

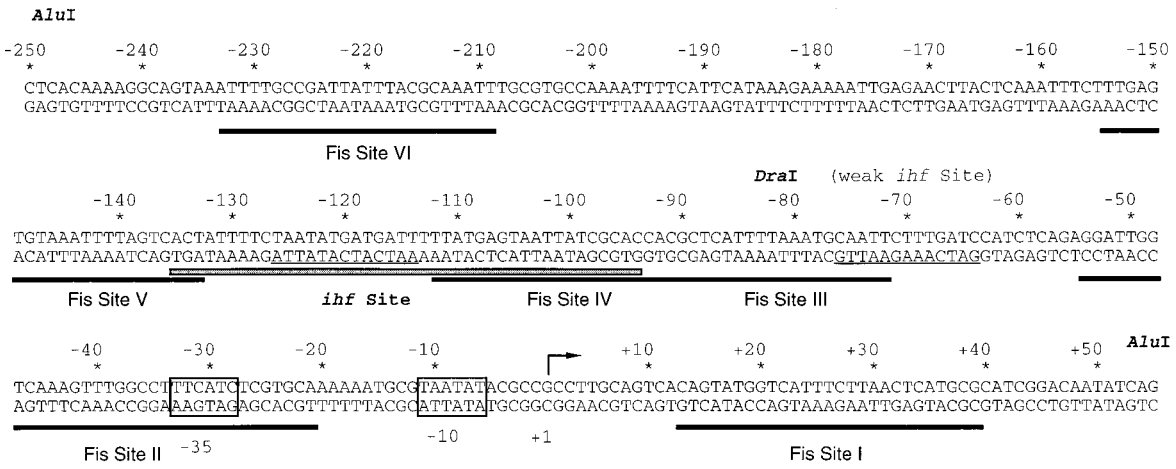


FIG. 2. DNA deletions affecting the *fis* P region. (A) Diagrammatic representation of DNA deletion constructs containing various lengths of DNA sequences upstream or downstream of *fis* P. The top portion represents the region on the *E. coli* chromosome containing the *fis* operon, consisting of *fis*, an upstream open reading frame (*orf-1*), which are indicated by open rectangles, and *fis* P, which is indicated by an arrow. *prmA*, the gene encoding L11 methyltransferase, is located upstream of the *fis* operon (51). Inverted repeats that are presumed to function as transcription termination signals are indicated with ♀. The linear scale underneath represents the *Kpn*I-*Kpn*I DNA fragment extending from −756 to +866 relative to the transcriptional start site (+1). The double slanted lines (//) represent sections of DNA not drawn to scale. The positions of two *Alu*I restriction sites in this region are indicated. Solid lines in the lower section represent DNA regions that remained intact in the various deletion constructs. The extents of these DNA regions and names of pRJ800-based plasmids that contain them are indicated on the left. (B) DNA sequence of the *fis* promoter region within the *Alu*I-*Alu*I DNA fragment. The nucleotide sequence is numbered relative to the transcriptional start site (+1), which is indicated by an arrow. *Alu*I and *Dra*I restriction sites are labeled above the sequence. Open boxes denote sequences presumed to represent the −10 and −35 promoter regions for *fis* P. DNA regions protected from DNase I cleavage by Fis (■) or IHF (▨) are indicated below the sequence. Fis binding sites I through VI are centered at approximately nucleotide positions +26, −42, −83, −101, −143, and −221, respectively. Nucleotide sequences resembling the *ihf* consensus sequence are underlined for both the *ihf* site at −114 and a weaker site at −64. The sequence of the entire 2,647-bp *fis* operon including the sequence presented here is deposited with GenBank under the accession number M95784.

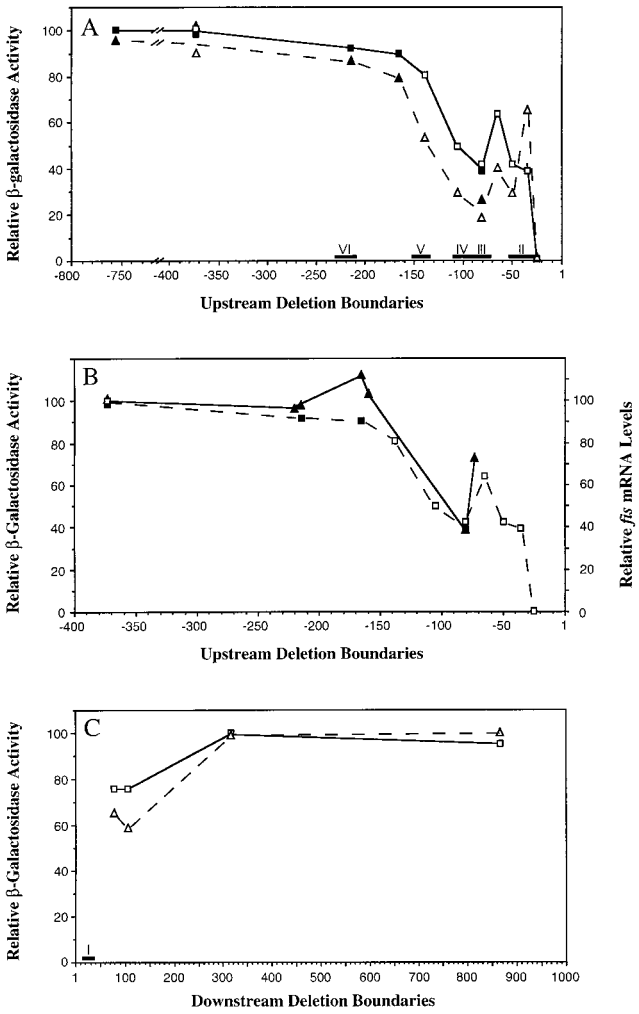


FIG. 3. Effects of DNA deletions on *fis* P transcription in vivo. (A) Effects of upstream DNA sequences on *fis* P transcription. Relative β-galactosidase activities are from RZ211 (▲, △) or RJ1561 cells (■, □) transformed with pRJ800-based plasmids carrying various lengths of DNA sequences upstream of *fis* P. The abscissa denotes nucleotide positions of upstream deletion boundaries in each of the *fis* promoter regions analyzed. Since the region from +80 to +107 shows little or no effect on transcription (C), DNA deletion constructs that contained fixed downstream boundaries at either +80 (▲ and ■) or +107 (△ and □) were used. We assigned 100% values to β-galactosidase activity levels generated by pTP137 (carrying sequences from −786 to +80) in RZ211 cells (121 Miller units) or RJ1561 cells (725 Miller units). All other values are shown as a proportion of these two values in the respective strains. ▬, approximate positions of Fis binding sites II through VI. (B) Relative mRNA levels from *fis* P in pRJ800-based plasmids. Overnight cultures of RJ1561 carrying plasmids with deletion constructs were diluted 50-fold in LB medium and grown at 37°C. After 60 min, the cells were harvested and used for preparation of total RNA. Primer extensions were performed with 10 μg of total RNA to detect mRNA initiating at *fis* P, which was quantified as described in Materials and Methods. A value of 100% was assigned to *fis* P mRNA levels produced from pRJ1028 (−373 to +80), and all other values of *fis* mRNA are given relative to this one. Symbols: ▲, relative *fis* mRNA levels; ■, □, relative β-galactosidase activity from RJ1561 containing various deletion constructs shown for comparison. β-Galactosidase activity from RJ1561 carrying pRJ1028 (722 Miller units) was used as the 100% value. (C) Effects of downstream DNA deletions on *fis* P transcription. β-Galactosidase activities generated from pRJ800-based plasmids carrying DNA sequences extending from −373 to various nucleotide positions downstream of *fis* P (+80, +107, +318, +866) were measured in RZ211 cells (△) or RJ1561 cells (□). The abscissa denotes nucleotide positions of deletion boundaries downstream of the *fis* P transcription start site. Values obtained for pTP123 (−373 to +866) in RZ211 (192 Miller units) and in RJ1561 (889 Miller units) are shown as 100%, and all other values are relative to these. The approximate position of Fis site I (▬) is indicated.

TABLE 1. Effect of Fis sites on autoregulation

Plasmid	*fis* P region	β-Galactosidase activity for[a]:		RJ1561/RZ211 ratio[b]	Fold repression[c]	Fis site(s) present
		RZ211	RJ1561			
pTP138	−373 to +107	114 ± 1	722 ± 12	6.3	3.2	I–VI
pRJ1071	−166 to +80	99 ± 3	655 ± 14	6.6	3.3	I–V
pTP126	−139 to +107	67 ± 3	584 ± 3	8.7	4.4	I–IV
pTP127	−106 to +107	36 ± 2	363 ± 8	10.1	5.0	I–III
pTP121	−65 to +107	51 ± 2	465 ± 30	9.1	4.6	I–II
pTP120	−50 to +107	36 ± 6	305 ± 6	8.5	4.2	I–II[d]
pTP119	−36 to +107	82 ± 6	282 ± 3	3.4	1.7	I
pTP184	−50 to +7	45 ± 1	369 ± 6	8.2	4.1	II[d]
pTP116	−36 to +7	188 ± 7	409 ± 14	2.2	1.1	None
pTP118	−26 to +107	2 ± 0.1	4 ± 0.7	2.0	1.0	I
pRJ800	Vector only	3 ± 0.2	6 ± 0.5	2.0	1.0	None

[a] β-Galactosidase activities are given in Miller units (35). Values represent means and standard deviations of at least three independent assays.
[b] Values were obtained by dividing the β-galactosidase activities obtained from RJ1561 cells by those obtained from RZ211 cells.
[c] Fold repression was calculated by dividing the RJ1561/RZ211 ratios of β-galactosidase activities obtained for each plasmid by the same ratio obtained for the pRJ800 vector control.
[d] Fis site II in these plasmids lacks the upstream-most 4 bp from its DNase I-protected region.

plasmids exhibited a similar pattern (not shown), indicating that this reduction in activity can be attributed to a reduction in mRNA levels specifically initiated from *fis* P. Hence, the region from +107 to +318 is required for maximum expression of *fis* P.

Effect of IHF on *fis* P expression. Upon closer examination of the DNA sequence from −166 to −81 shown here to be required for maximum *fis* P activity, we noticed a close match to the *ihf* consensus sequence, WATCAANNNNTTR (10, 15, 21, 22), located from −114 to −126 (Fig. 2B, bottom strand). To determine if IHF played a role in the regulation of *fis* expression, we compared mRNA levels derived from chromosomal *fis* in MC1000 and MC1000 *ihfB* cells at various times

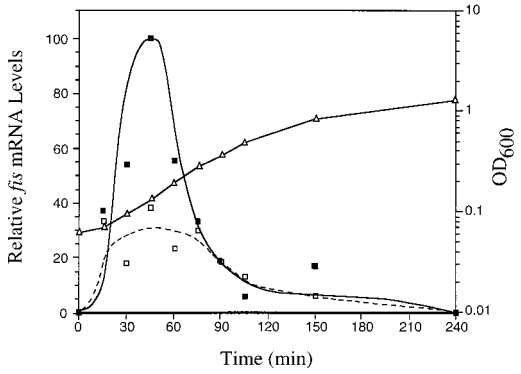


FIG. 4. Effect of IHF on *fis* mRNA levels. Overnight cultures of MC1000 or MC1000 *ihfB* were diluted 25-fold in LB and grown at 37°C with shaking. Samples were withdrawn at various times thereafter and harvested for total RNA preparation. Primer extension reactions were performed with RNA obtained from an equivalent amount of cells (0.5 optical density at 600 nm [OD_{600}] unit) and a primer that anneals to the top strand from +40 to +56 relative to the *fis* P start of transcription. Primer-extended products were separated on a 8% polyacrylamide–8 M urea gel, autoradiographed, and quantified by laser densitometry. Relative *fis* mRNA levels are indicated for MC1000 cells (■) and MC1000 *ihfB* cells (□). A value of 100% was assigned to the maximum *fis* mRNA levels in the entire data set, and all other values are shown as a percentage of this one. △, growth of MC1000 as measured by OD_{600}. The growth pattern of MC1000 *ihfB* was similar to that of MC1000.

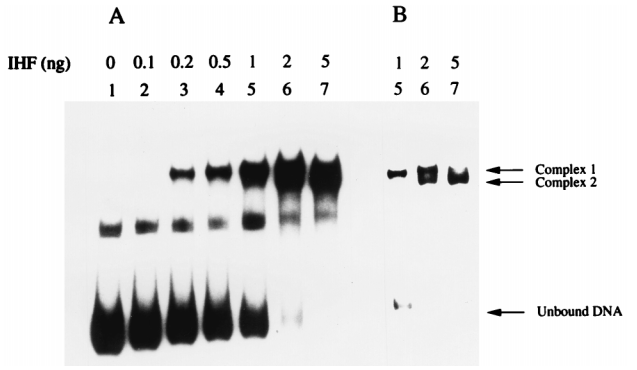


FIG. 5. IHF binding to the *fis* promoter region. (A) A gel mobility shift assay was performed with a ^{32}P-labeled DNA fragment containing the *fis* P region from −250 to +55 shown in Fig. 2B and the indicated amounts of purified IHF. Samples were separated on an 8% polyacrylamide gel and subjected to autoradiography. Indicated above each lane are the lane numbers and the amounts of IHF used in each binding reaction. (B) A shorter exposure of lanes 5 to 7 in panel A. Gel positions of IHF-bound complexes 1 and 2, as well as the unbound DNA fragment, are indicated by arrows on the right. An additional DNA contaminant band is present in all lanes.

after subculturing stationary-phase cells in LB medium at 37°C (Fig. 4). These results show that maximum *fis* mRNA levels in MC1000 *ihfB* cells reach only about 30% of that in MC1000 cells, indicating that IHF is required for maximum *fis* expression in vivo. The effect of IHF was further verified by comparing β-galactosidase activities generated from pRJ1028 in MC4100 and HP4110 (MC4100 *ihfA*::Tn*10*) cells (see Table 3). These results show that in vivo transcription from *fis* P on a plasmid is about 3.8-fold higher in the presence of IHF than in its absence.

To determine if IHF could specifically interact with the *fis* promoter region, we performed gel mobility shift assays with purified IHF protein and a linear DNA fragment containing the region from −250 to +55 (Fig. 5). These results show that IHF interacts with this DNA fragment, with as little as 0.2 ng of IHF. Based on measurements of relative amounts of bound and unbound DNA complexes, we estimate that IHF binds this region with an apparent K_d of about 2.2 nM. With a 10-fold-larger amount of IHF (2 ng), a second IHF-DNA complex, with a slightly higher electrophoretic mobility than the complex observed at lower IHF concentrations, can be detected (Fig. 5B).

Results from DNase I footprinting show that IHF protects the region from −135 to −94 on the bottom strand and from −126 to −101 on the top strand when as much as 5 or 10 ng of IHF is used (Fig. 6). The *ihf* consensus match (−126 to −114) is asymmetrically positioned relative to the protected region, as has been shown for other *ihf*-binding sites (10, 21, 22). An AT-rich region, also known to be asymmetrically positioned on the other half of IHF-protected regions, is located in the region from −105 to −99. In the presence of 60 ng of IHF, very weak DNase I protection could be observed in the region from −88 to −37 on the top DNA strand and from −79 to −49 on the bottom strand. This region contains a sequence from −76 to −64 that also resembles the *ihf* consensus sequence (21), although not as closely as the one from −126 to −114. Based on the DNase I protected regions and the distribution of the *ihf* consensus sequence in a crystal structure of an IHF-DNA complex (45), we estimated the centers of the higher- and lower-affinity *ihf*-binding sites to reside at −114 and at −64, respectively.

To determine if the effect of IHF on *fis* expression could be

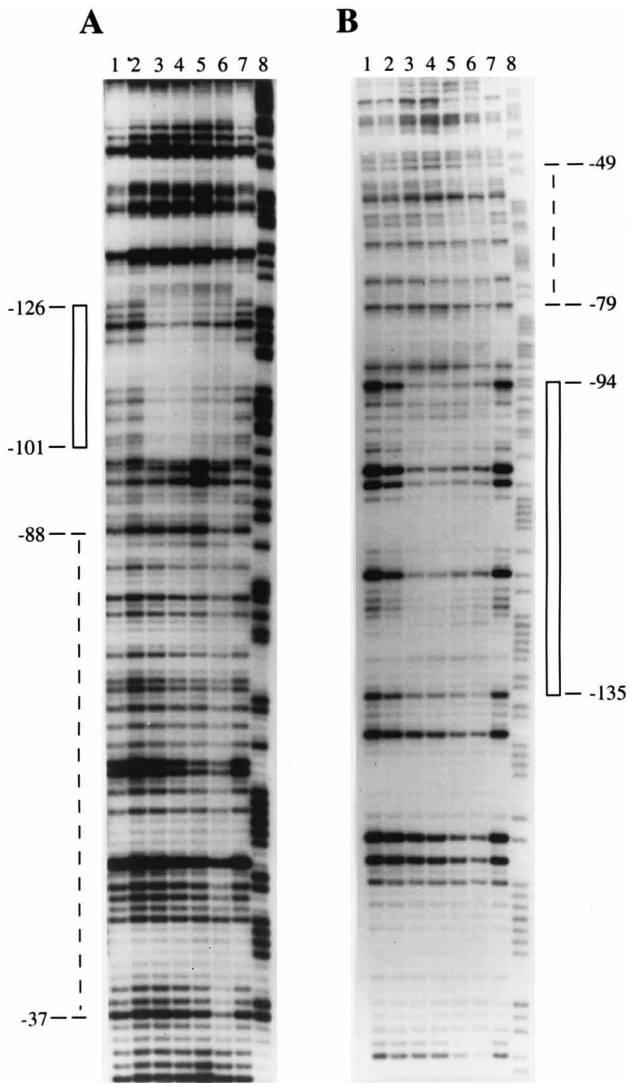


FIG. 6. DNase I protection of the *fis* P region by IHF. The top DNA strand (A) and the bottom DNA strand (B) as represented in Fig. 2B are shown. The ^{32}P-labeled *Alu*I-*Alu*I DNA fragment was incubated with 0 ng (lanes 1 and 7), 1 ng (lanes 2), 5 ng (lanes 3), 10 ng (lanes 4), 20 ng (lanes 5), and 60 ng (lanes 6) of IHF prior to DNase I treatment. In lanes 8, Maxam-Gilbert cleavage reaction mixtures for G and A nucleotides (34) were electrophoresed in parallel and used as size markers. The region protected by IHF is indicated on the side by an open bar, while a region weakly protected by IHF at higher concentrations is indicated by dashed lines.

mediated by IHF binding to the higher-affinity site, we generated four point mutations at positions known to be highly conserved among *ihf*-binding sites (21), leaving the remaining DNA sequences in the region from −373 to +107 unaltered in pTP185 (Table 2). The four point mutations were: −126T to A, −125A to C, −118T to A, and −117G to T. We were unable to detect IHF binding to this mutated *fis* P region in gel mobility shift assays, even when the IHF concentration was increased 64-fold over that required to detect binding with the wild-type DNA template (data not shown). Thus, formation of either IHF-DNA complex normally observed in these assays requires an intact *ihf* site at −114.

The effect of the mutations in the *ihf* site on *fis* P expression was examined by comparing β-galactosidase activities between pTP185 (mutated *ihf* site) and pTP138 (wild-type *ihf* site).

TABLE 2. Effect of *ihf* site mutations on *fis* P activity

Strain	Plasmid	*fis* P region	β-Galactosidase activity[a]	% Activity[b]	*ihf* site[c]
RJ1561	pTP138	−373 to +107	722 ± 12	100	wt
	pTP185	−373 to +107	279 ± 7	39	mut
	pTP126	−139 to +107	584 ± 3	81	wt
	pTP130	−139 to +107	301 ± 7	42	mut
RZ211	pTP138	−373 to +107	114 ± 1	100	wt
	pTP185	−373 to +107	27 ± 1	24	mut
	pTP126	−139 to +107	67 ± 3	59	wt
	pTP130	−139 to +107	27 ± 1	24	mut

[a] β-Galactosidase activities are given in Miller units. Values represent the mean and standard deviation of at least three independent assays.
[b] Relative β-galactosidase activities within each strain are given relative to those of pTP138, which was assigned a value of 100.
[c] wt, the *ihf* site at −114 remained intact in these plasmids; mut, the *ihf* site at −114 contains four point mutations which abolish IHF binding.

These mutations result in 76 and 61% reduction in transcription in RZ211 and RJ1561 cells, respectively (Table 2), strongly suggesting that the positive effect exerted by IHF on *fis* P transcription is mediated, at least in part, by IHF binding to the site identified at −114.

Because IHF is known to bend DNA by as much as 140° (49), it can sometimes function as an architectural element to bring specific DNA sequences upstream of the *ihf* site in close proximity to RNA polymerase at the target promoter (22). However, sequences upstream of −166 can be replaced with plasmid DNA without substantial effects on *fis* P expression (Fig. 3A). When the region upstream of −139 was replaced with vector DNA in pTP126, transcription was about 81% of maximum levels in RJ1561 and 59% in RZ211 cells (Table 2). This DNA construct replaces most sequences upstream of the *ihf* site centered at −114 with plasmid DNA. If the region from −139 to +107 contains a mutated *ihf* site (pTP130), *fis* P activity further decreases to about 42% and 24% maximum activity in RJ1561 and RZ211 cells, respectively. This suggests that significant IHF stimulation can be achieved without specific DNA sequences upstream of −139.

To determine if IHF can also regulate *fis* P independently of the *ihf* site at −114, β-galactosidase activities from the *fis* P region lacking a functional *ihf* site at −114 were measured in MC4100 and HP4110 cells (Table 3). In MC4100, pTP185 produced about 20% β-galactosidase activity (93 U) compared to the wild-type promoter in pRJ1028 (456 U). This is consistent with results obtained for RZ211. Deletion of the *ihf* site at −114 (in pTP127) yielded about 22% β-galactosidase activity (103 U) compared to pRJ1028. Similar results were obtained when both *ihf* sites were deleted in pTP120. In addition, point mutations that either improved or weakened the match to the *ihf* consensus for the site at −64 (−64G to A, −67C to T, −68A to G, and −69A to G) while leaving the *ihf* site at −114 intact had no significant effect on transcription (not shown). Thus, the putative *ihf* site at −64 does not appear to affect *fis* P expression.

TABLE 3. Effect of IHF on β-galactosidase activity from *fis* P

Plasmid[a]	*fis* P region	β-Galactose activity for[b]:		Fold activation[c]
		MC4100	HP4110	
pRJ1028	−373 to +80	456 ± 35	119 ± 9	3.8
pTP185	−373 to +107	93 ± 8	111 ± 10	0.8
pTP127	−106 to +107	103 ± 19	126 ± 18	0.8
pTP120	−50 to +107	103 ± 9	143 ± 12	0.7
pRJ800	Vector only	17 ± 2	17 ± 12	1.0

[a] pTP185 contains the mutated *ihf* site at −114; pTP127 lacks the *ihf* site at −114; pTP120 lacks both the −114 and −64 *ihf* sites.
[b] β-Galactosidase activities are given in Miller units. Values represent the mean and standard deviation of at least three independent assays.
[c] Fold activation by IHF was obtained for each plasmid by dividing the β-galactosidase activity in MC4100 cells by that in HP4110 cells.

In HP4110, β-galactosidase levels showed little variation irrespective of the presence of *ihf* sites, indicating that the deletions or point mutations did not significantly affect transcription in the absence of IHF. These values were also comparable to those obtained in MC4100 carrying pTP185, pTP127, or pTP120. The slightly higher values obtained for these three plasmids in HP4110 than in MC4100 (16 to 30% higher) could be attributed to a comparable increase in plasmid copy number (about 20%) in the former. Based on these observations, IHF does not affect *fis* P indirectly or by interacting with sites other than that at −114.

To examine the position-dependency of the *ihf* site centered at −114, we inserted 5 or 10 bp between positions −61 and −60 and determined their effects on *fis* P transcription (Table 4). The 5-bp insertion, which positions the *ihf* site on the opposite side of the DNA helix relative to its original position, decreases transcription to 26 and 49% of maximum levels in RZ211 and RJ1561 cells, respectively. A 10-bp insertion in the same position, which approximately restores the helical phasing of the *ihf* site and increases its distance to the promoter, partially increases transcription to 39 and 74% of maximum levels in RZ211 and RJ1561 cells, respectively. Effects comparable to those in RZ211 were observed in MC4100 cells. However, neither the 5-bp nor the 10-bp insertion affected *fis* P expression in HP4110 cells, suggesting that the effects observed in MC4100 cannot be attributed to upstream sequences functioning independently of IHF. Thus, the ability of IHF to stimulate transcription is dependent on its position relative to *fis* P.

***fis* expression pattern in the absence of *ihf* and Fis sites.** It was previously observed that although Fis exerts a negative effect on the magnitude of *fis* mRNA levels, its overall expression pattern was similar to that observed in the absence of Fis (4, 41). Here we observed that although IHF has a positive effect on *fis* expression, *fis* mRNA levels are still subject to growth phase-dependent expression in *ihfB* cells (Fig. 4). The *fis* P region from −36 to +7 (pTP116), which lacks all known Fis- and *ihf*-binding sites, responded to conditions of nutritional upshift by generating a greater than 35-fold increase in mRNA levels that peak after about 45 min of growth (Fig. 7). Thereafter, *fis* mRNA levels decreased to very low levels by 150 min and were undetectable by 300 min, when the cells had entered the stationary phase. Hence, this limited promoter sequence exhibits growth phase-dependent regulation, demonstrating that while Fis and IHF exert antagonistic effects on the *fis* promoter, they are not necessary for growth phase-dependent regulation of *fis* expression.

DISCUSSION

Regulation of *fis* P by IHF. We present evidence that IHF interacts with a site centered approximately at −114 relative to *fis* P to stimulate transcription from this promoter by about three- to fourfold in vivo. DNA deletion analysis first pointed to the region from −166 to −82 as a requirement for maximum *fis* P expression (Fig. 3). This region contains a good match to the *ihf* consensus sequence (10, 15, 21), and indeed, DNase I protection experiments revealed an *ihf*-binding site extending

TABLE 4. Effect of *ihf* site position on β-galactosidase activity from *fis* P

Plasmid[a]	Insertion (bp)[a]	RZ211		RJ1561		MC4100 β-Gal[b]	HP4110 β-Gal[b]	Fold IHF activation[d]
		β-Gal[b]	% Activity[c]	β-Gal[b]	% Activity[c]			
pRJ1028	0	126 ± 4	100	722 ± 45	100	456 ± 35	119 ± 9	3.8
pKW125	5	33 ± 1	26	352 ± 18	49	107 ± 8	110 ± 15	1.0
pKW232	10	49 ± 2	39	532 ± 22	74	189 ± 20	107 ± 30	1.8

[a] *fis* P regions in these plasmids contained the wild-type sequence from −373 to +80. The 5-bp insertion in pKW125 and the 10-bp insertion in pKW232 are between positions −60 and −61.
[b] β-Galactosidase activities (β-Gal) are given in Miller units. Values represent the mean and standard deviation of at least three independent assays.
[c] Percent β-galactosidase activities are given relative to that of pRJ1028 within each strain, which was assigned a value of 100%.
[d] Fold IHF activation was obtained for each plasmid by dividing the β-galactosidase activity in MC4100 cells by that in HP4110 cells.

from −135 to −94 (Fig. 6). A mutation in this site causes a severe reduction in IHF binding and results in a three- to fourfold reduction of *fis* P expression (Tables 2 and 3). Finally, peak *fis* mRNA levels originating from the chromosomal *fis* P in MC1000 *ihfB* cells are about 30% that of MC1000 cells (Fig. 4) and β-galactosidase levels generated from *fis* P on a plasmid are about 3.8-fold higher in MC4100 cells than in HP4110 (MC4100 *ihfA*) cells (Table 3).

Our DNase I protection assay showed, at best, weak protection in the region from −79 to −49, with about a 12-fold-higher IHF concentration than that required for binding at −114. Gel mobility shift assays revealed two closely migrating IHF-DNA complexes formed at different IHF concentrations. Since four point mutations designed to destroy the *ihf* site at −114 prevented detection of either IHF-DNA complex, an intact *ihf* site at −114 is somehow required for both complexes. These two IHF-DNA complexes may represent two different conformational states of IHF bound at −114. Alternatively, the IHF-DNA complex formed at higher IHF concentrations may result from weak interactions between IHF and the site centered at −64. However, we did not detect an effect on *fis* P expression by this weaker *ihf* site.

Significant IHF stimulation of *fis* P occurs in *fis* cells without the need for specific DNA sequences upstream of −135. This is in contrast to what has been suggested for several σ54 promoters in various bacterial species, where IHF-induced DNA bending facilitates interactions between an activator protein (NIFA or NRI) and the σ54 RNA polymerase (9, 22, 27). If IHF plays an architectural role in bringing upstream DNA sequences in contact with RNA polymerase bound at *fis* P, such interactions must be largely nonspecific.

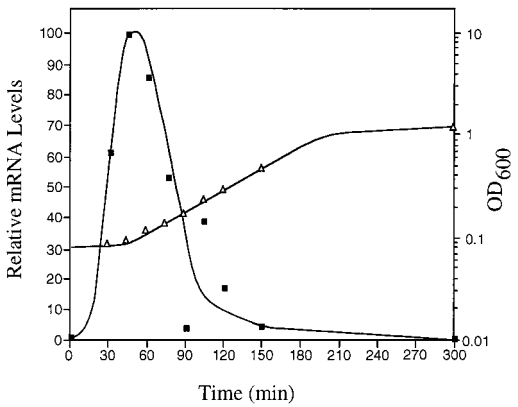


FIG. 7. *fis* mRNA expression pattern by the *fis* P region from −36 to +7. Overnight-grown RJ1561 (*fis*) cells containing pTP119 were diluted 50-fold in prewarmed LB medium and grown at 37°C with shaking. Samples were withdrawn at various times thereafter and harvested for total RNA preparation. Primer extension reactions were performed as in Fig. 4 with a primer that anneals to plasmid DNA sequences extending from +47 to +30 relative to the transcriptional start site. A value of 100% was assigned to the maximum *fis* mRNA level from the data (45 min), and all other values are shown as a percentage of this one. ■, relative *fis* mRNA levels; △, cell growth as determined by optical density at 600 nm (OD_{600}).

In the case of the nonoverlapping promoters of the *ilvGMEDA* operon, IHF represses transcription of the upstream $ilvp_G1$ promoter and stimulates transcription of the downstream $ilvp_G2$ promoter (42). IHF-mediated activation of $ilvp_G2$ is not strictly dependent on the helical phasing or distance of the IHF-binding site relative to this promoter, suggesting that interactions between IHF and RNA polymerase or between upstream DNA sequences and RNA polymerase are not required (43). Instead, it appears that an IHF-induced DNA bend alters the structure of the $ilvp_G2$ −10 region so as to favor formation of an open complex (44). However, this does not appear to be the mechanism by which IHF stimulates *fis* P. A 5-bp insertion between *fis* P and the *ihf* site caused about a fourfold reduction in transcription in the presence of Fis and about a twofold reduction in its absence. These effects resembled those of a mutated *ihf*-binding site at −114. A 10-bp insertion between the *ihf* site and *fis* P resulted in a partial restoration of transcription in the presence or absence of Fis. Thus, activation by IHF appears to be position dependent, since it is sensitive to distance and possibly helical phasing relative to *fis* P.

Stimulation of *fis* P by IHF may resemble the situation with the bacteriophage Mu Pe and bacteriophage λp_L1 promoters. In these promoters, IHF stimulation is face-of-the-helix dependent (16, 52) and requires the C-terminal domain of the α-subunit (α-CTD) of RNA polymerase (17, 52, 53). In the case of Mu Pe, an UP-element-like region was found to be positioned from −39 to −51 relative to the transcription start site and appeared to be required for IHF stimulation (53). Hence, an IHF-induced DNA bend may stimulate transcription in these promoters by facilitating interactions between IHF and α-CTD, by enhancing interactions between α-CTD and an UP element-like sequence, or both. No DNA sequence similarity exists between the *fis* P region from −60 to −40 and the UP element found in several other promoters (46). However, as has been suggested (18), an IHF-induced DNA conformational change might facilitate interactions between α-CTD and an UP element-like sequence located further upstream from the promoter. Alternatively, IHF may directly contact RNA polymerase to stimulate transcription.

Intracellular IHF levels are about 5- to 10-fold higher in stationary phase than in early logarithmic phase (11). Although in vivo IHF levels have been estimated to be in the range of 6 to 30 μM (11), its free and effective intracellular concentrations have been measured to be in the range of 15 to 35 nM (57). If our in vitro estimates of K_d for IHF binding to the *fis*

P region (~2.2 nM) could be taken as an approximation of its K_d in vivo, IHF would be capable of interacting with this region during the logarithmic and stationary phases. If this were the case, IHF would not be expected to play a physiological role as a transcriptional regulator of *fis* P, unless other proteins (such as Fis) regulate the binding activity of IHF.

Role of Fis sites in regulation of *fis* P. The presence of multiple copies of the *fis* P region on plasmids can potentially reduce the effective cellular concentrations of important *trans*-acting factors and thereby weaken their effects on regulation. Nevertheless, our deletions of the *fis* P region on a plasmid allowed an assessment of the roles played by some of the Fis-binding sites previously identified in this region. Deletion of Fis site VI showed virtually no effect on the regulation of *fis* P. However, disruption of Fis site V resulted in a moderate increase in *fis* autoregulation from 3.3- to 4.4-fold. Additional disruption of Fis site IV resulted in a further increase in *fis* autoregulation efficiency to 5.0-fold. However, since the same deletion also removes the *ihf* site at −114, we cannot attribute this effect exclusively to Fis site IV. Indeed, point mutations within the *ihf*-binding site alone also lead to an increase in autoregulation from 3.2- to 5.2-fold (Table 2). Presumably, Fis exerts a more efficient negative control in the absence of IHF-dependent stimulation. An interesting possibility is that Fis bound to site V collaborates with IHF bound at −114 to increase transcription.

Disruption of Fis site III caused a small loss in autoregulation from 5.0- to 4.6-fold. However, disruption of Fis site II resulted in the greatest loss in autoregulation, with a drop from 4.6- to 1.7-fold. When Fis site I was also deleted and no Fis sites were present, autoregulation was reduced to background levels, suggesting that Fis bound to site I is capable of a modest 1.7-fold negative regulation. These results are consistent with previous work showing that Fis sites I and II can regulate transcription from the *fis* promoter (39). However, here we also show that Fis site II, by itself, is capable of achieving the same levels of autoregulation as when both Fis sites I and II are present. Thus, while Fis sites I and III may assist in autoregulation, Fis bound to site II can account for most of the autoregulation observed.

Two other observations are consistent with Fis bound to site II acting as the predominant autoregulator. First, we showed that although the sequences predicted to serve as a −35 promoter region for *fis* P represent poor matches to the consensus sequence, their deletion completely abolishes transcription from this promoter. Thus, RNA polymerase interactions with this region are essential for basal transcriptional activity in vivo. Since the DNase I-protected region for Fis site II extends from −54 to −21 (4), its occupancy by Fis can be expected to hinder interactions between RNA polymerase and the −35 promoter sequence. Second, it was previously observed that Fis site II showed a higher affinity for Fis binding than did Fis sites I or III (4). Thus, a more stable interaction between Fis and site II may further enhance its regulatory effectiveness.

Effect of downstream sequences on *fis* P expression. DNA sequences downstream of *fis* P were also noted to affect transcription independently of *fis* autoregulation. Deletion of the region from +107 to +318 showed a reproducible decrease in transcription, suggesting that this region is required for maximum expression of this promoter. Since this effect was confirmed by primer extension analysis of *fis* mRNA levels, it cannot be attributed to other promoters potentially present in this downstream region but, instead, is correlated with a decrease in mRNA levels specifically initiating from *fis* P. Whether this region contains sequences contributing to greater stability of *fis* mRNA remains an open question.

Deletion of downstream sequences from +8 to +107 caused a moderate increase in transcription in the absence of *fis* autoregulation. This effect can be attributed to the region from +8 to +80, since deletion of the region from +81 to +107 showed little or no effect on *fis* P expression. The proximity of this region to the *fis* promoter may allow it to serve as a potential binding site for a negative regulator other than Fis.

Regulation of *fis* expression pattern. Rapid changes in *fis* mRNA levels occur in response to a nutritional upshift. This pattern cannot be explained by corresponding changes in *fis* mRNA decay rates. *fis* mRNA half-lives during the early to mid-logarithmic growth period, when the most dramatic changes in *fis* mRNA levels occur, ranged from only 1.8 to 2.1 min. Even the small differences in our measurements of these half-lives did not obey a pattern that could be correlated with that of *fis* mRNA levels. Therefore, the unusual *fis* expression pattern must be attributed to an efficient control of transcription.

The growth phase-dependent *fis* expression pattern can be observed in the absence of Fis (4) or IHF (Fig. 4). Moreover, consistent with previous observations (39), we showed that the DNA sequence from −36 to +7, which lacks binding sites for Fis and IHF, as well as other sequences that can affect levels of *fis* P transcription, is capable of exhibiting its characteristic expression pattern (Fig. 7). This promoter sequence on a plasmid generates a greater-than-35-fold increase in mRNA levels when stationary phase cells are subcultured in LB. After its peak at about 45 min, the levels rapidly decrease to less than 5% of maximum by 150 min and become undetectable by 300 min. Although this minimal promoter sequence is capable of exhibiting stringent control (39), regulation by stringent control is unlikely to be observed in the highly rich medium LB. Indeed, growth phase-dependent *fis* expression is still observed in *spoT relA* strains grown in LB medium (4). Single substitutions generated within this limited promoter region are currently being investigated to further our understanding of this regulatory mechanism in vivo.

The magnitudes of *fis* mRNA and protein levels have been shown to increase with the nutritional quality of the growth medium (4, 38). Antagonistic roles played by IHF and Fis may be important in establishing a suitable balance in *fis* mRNA levels under different growth conditions. In addition, DNA regions that moderately affect *fis* P transcription independently of Fis or IHF might contribute to this effect. This work has uncovered DNA sequences within the *fis* P region that influence the expression of this promoter. Further efforts will focus on these sequences to characterize more precisely their roles in *fis* regulation.

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