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Dramatic Changes in Fis Levels upon Nutrient Upshift in *Escherichia coli*

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Fis is a small basic DNA-binding protein from *Escherichia coli* that was identified because of its role in site-specific DNA recombination reactions. Recent evidence indicates that Fis also participates in essential cell processes such as rRNA and tRNA transcription and chromosomal DNA replication. In this report, we show that Fis levels vary dramatically during the course of cell growth and in response to changing environmental conditions. When stationary-phase cells are subcultured into a rich medium, Fis levels increase from less than 100 to over 50,000 copies per cell prior to the first cell division. As cells enter exponential growth, nascent synthesis is largely shut off, and intracellular Fis levels decrease as a function of cell division. Fis synthesis also transiently increases when exponentially growing cells are shifted to a richer medium. The magnitude of the peak of Fis synthesis appears to reflect the extent of the nutritional upshift. *fis* mRNA levels closely resemble the protein expression pattern, suggesting that regulation occurs largely at the transcriptional level. Two RNA polymerase-binding sites and at least six high-affinity Fis-binding sites are present in the *fis* promoter region. We show that expression of the *fis* operon is negatively regulated by Fis in vivo and that purified Fis can prevent stable complex formation by RNA polymerase at the *fis* promoter in vitro. However, autoregulation only partially accounts for the expression pattern of Fis. We suggest that the fluctuations in Fis levels may serve as an early signal of a nutritional upshift and may be important in the physiological roles Fis plays in the cell.

A group of small DNA-binding proteins referred to as nucleoid associated or histone-like exists in bacteria (8, 41). In addition to functioning directly in specific reactions, some of these proteins have been shown to contribute to the structure of the bacterial nucleoid. Examples of members of this family include HU, IHF, H-NS, and Fis. The basic, 11.2-kDa Fis protein binds DNA as a homodimer and was initially identified by its ability to stimulate Hin-, Gin- and Cin-mediated DNA inversion (15, 23, 24; for a review, see reference 11). Subsequently, Fis has been shown to stimulate both excisive and integrative recombination of bacteriophage λ DNA (1, 2, 45). Recent work has shown that Fis binds the upstream activation region (UAR) of rRNA and tRNA operons and increases their transcription activities (32, 33, 39). In addition, it has been demonstrated that Fis binds the *Escherichia coli* origin of replication (*oriC*) and may play a role in initiation of DNA replication (10, 12).

The regulatory implications as well as the mechanism by which a single protein such as Fis can function in such unrelated systems as site-specific DNA recombination, DNA replication, and transcription are of great interest. Although Fis binds DNA in a site-specific manner, the proposed consensus sequence for Fis-binding sites (G/TNNYRNNA/TNNYRNNC/A) is highly degenerate (11, 19), and it is likely that, in addition to the specificity of the nucleotide sequence, Fis may recognize other structural features of the DNA. When bound to DNA, Fis is able to bend its target by 60 to 90° (12, 13, 44). The DNA-binding and -bending activities of Fis are contained within the carboxy-terminal region (25, 35), which has been shown by X-ray crystallography to contain a helix-turn-helix DNA-binding structure (26, 47). An additional functional region, located closer to the amino terminus, is required for the stimulation of Hin- and Gin-mediated inversion but not for the stimulation of λ excision (25, 35), suggesting that Fis plays different roles in these two mechanistically distinct site-specific recombination reactions. Thus, the precise mechanism by which Fis functions may be largely determined by its immediate nucleoprotein context.

Since Fis is known to bind many different sites with various affinities, the intracellular concentration of Fis could affect how it acts in different biological mechanisms. Thompson et al. (45) initially observed that crude extracts contained varying amounts of Fis-binding activity at different times during the growth of an *E. coli* culture. Growth phase-dependent expression of Fis is a possible means by which Fis can regulate a number of biological processes or be differentially distributed among high- and low-affinity binding sites during the growth cycle. In this work, we characterize the fluctuations in the intracellular Fis levels with respect to cell growth, at both the protein and mRNA levels. The increase in intracellular Fis levels is caused by a rapid but transient increase in *fis* mRNA levels in response to a nutritional upshift. The *fis* promoter region is identified and characterized with respect to the transcription initiation sites, RNA polymerase-binding sites, and Fis-binding sites. Finally, we present evidence that Fis plays a role as an autoregulator and that its binding to the promoter can exclude binding of RNA polymerase. The implications of the unusual expression pattern of Fis for controlling specific aspects of cell growth are discussed. While this work was being prepared for publication, two other groups reported variations in intracellular Fis levels in response to nutrient upshift at both the protein (33) and mRNA (34) levels. Where the data overlap, the results are generally consistent.

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MATERIALS AND METHODS

General methods. Cells were propagated on liquid or solid Luria-Bertani (LB) medium or on supplemented M9 salts, prepared as described previously (29). Fis was purified to homogeneity in this laboratory by S. E. Finkel. RNA polymerase was prepared in the R. Burgess laboratory and was a kind gift from R. Gourse. DNA restriction and modification enzymes used were purchased from Boehringer Mannheim, unless indicated otherwise, and antibiotics were obtained from Sigma.

DNA sequencing was performed on M13 single-stranded DNA templates or on alkaline-denatured plasmid DNA preparations with the Sequenase enzyme (U.S. Biochemical Corp.) as described by the supplier.

For β-galactosidase assays, cells were subcultured 1:50 in LB and grown at 37°C for 45 min. β-galactosidase assays were performed as described previously (29). For the experiment for which the results are shown in Table 2, β-galactosidase activity was measured after cells were similarly subcultured for 1 h in the presence of 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to induce synthesis of plasmid-encoded wild-type or mutant Fis.

Strains and plasmids. *E. coli* K-12 strains MC1000 [*araD139* Δ(*ara-leu*)*7697* Δ*lacX74 galU galK rpsL*, obtained from M. Casadaban] and MG1655 (F^- λ^- prototroph, obtained from R. Gourse) and its derivatives were used for most of the protein and RNA determinations reported in this paper. CAG4000 (from C. Gross) is MG1655 Δ*lacX74*, and RJ1802 is CAG4000 *fis*::*767*. The *fis*::*767* (22) and *fis-985* (this paper) mutations, which replace the central half of the *fis* coding sequence (amino acids 22 to 73) with the *neo* gene from Tn*5* or the *str*/*spc* gene from R100.1, respectively, were introduced into different strains by P1*vir*-mediated transduction. To construct the *fis-985* substitution, the *Hin*dIII cassette encoding the *str*/*spc* gene from pHP45 (37) was ligated between the two *Bst*EII sites in the *fis* gene in pRJ753 after the ends were made blunt with T4 DNA polymerase to give pRJ985 and then transferred into the *E. coli* chromosome as described previously (22). The *lacI*::Tn*10 lacPL8UV5* mutations were transduced into CAG4000 and RJ1802 to give RJ1809 and RJ1810, respectively, from RJ1794 (laboratory collection) by selecting for tetracycline resistance and lac^+. CF1651, containing the *relA*Δ*251*::*kan* null mutation (28, 46), and CF1693, containing null mutations in both *relA* and *spoT* (*relA*Δ*251*::*kan spoT*Δ*120*::*cat* [46]) in MG1655, were originally from M. Cashel. RJ1799 is MG1655 *fis-985*, and RJ1882 is CF1693 *fis-985*.

The *fis* promoter region was cloned by selecting for kanamycin- and ampicillin-resistant plasmids from a *Bam*HI library prepared in pUC9 of DNA from RJ1529 [*fis*::*767* Δ(*lac-pro*) *ara str*]. A subclone, pRJ804, that contains the *Kpn*I fragment (nucleotides 1 to 1622 in Fig. 4C) in pUC18 was used as the starting material for further subcloning and fragment preparation.

The *fis-lacZ* operon fusion plasmids were constructed by using pRZ5202 (31, 38) or pRJ800, which is identical to pRZ5202 except that it contains the polylinker sequence from pUC18. In these vectors, DNA fragments were cloned upstream of the (*trp-lac*)*W200* fusion. The DNA fragments inserted were as follows: pRJ906 contains the sequence between the *Kpn*I (nucleotide 1 in Fig. 4C) and *Nco*I (nucleotide 844) sites, pRJ920 contains the sequence between the *Sau*3A (nucleotide 829) and *Hpa*I (nucleotide 1832) sites, pRJ960 contains the sequence between the *Dra*I (nucleotide 678) and *Eco*RV (nucleotide 1078) sites, and pRJ976 contains the sequence between the *Hin*dIII (nucleotide 386) and *Nco*I (nucleotide 844) sites. In addition, each of these fragments has also been cloned upstream of *lacZ* in pRS415 (43). The relative β-galactosidase values measured using these constructs were similar to those reported in Fig. 6, except that higher numbers of units were obtained. A *fis* promoter fusion to *lacZ* was constructed as a single copy in the chromosome by recombining into λRS45 the fusion in pRJ1046, which contains the *Hin*dIII (nucleotide 386) to *Nco*I (nucleotide 844) fragment cloned into pRS415, as described by Simons et al. (43). The resulting phage, λRJ1083, was lysogenized as a single copy into RJ1733 (F′ *proAB* $lacI^{sq}$ Z_{u118} Δ(*lac-pro*) *fis*::*767 ara str*) to yield RJ1838.

Western blots. Fis antibodies were generated by inoculating a female New Zealand White rabbit with 0.5 mg of Fis in complete Freund's adjuvant. Booster injections of 0.25 mg of Fis in incomplete Freund's adjuvant were made every 6 to 8 weeks. Serum was prepared from blood harvested 10 days after boosting as described by Harlow and Lane (16). Protein samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto 0.1-μm-pore-size nitrocellulose filters (purchased from Schleicher & Schuell) with a Bio-Rad electroblotter. The Western blots (immunoblots) were incubated in a 1:500 or 1:1,000 dilution of rabbit anti-Fis serum and developed by using the Bio-Rad goat anti-rabbit alkaline phosphatase kit.

Densitometry of Western blots and autoradiographs was performed with an LKB model 2202 laser densitometer. Levels of Fis protein were determined by including standards of known Fis concentration mixed with extracts from *fis* mutant cells on the same gel and subjecting them to densitometry. The amount of Fis present in a sample was extrapolated from the values obtained from the standards. The number of molecules per cell was obtained by assuming that a Fis monomer is 11.2 kDa and dividing the number of molecules present in a sample by the number of cells known to have been loaded. Typically, samples for a time course were analyzed on several gels.

Analysis of RNA. Total cellular RNA was prepared as described by Case et al. (5). The total amount of RNA per cell was calculated by dividing the total micrograms of RNA obtained in each sample by the number of cells used as starting material.

Northern (RNA) hybridizations were performed in 50% formamide as described previously (40). The antisense strand of the *fis* gene was used as the ^{32}P-labeled DNA probe and was prepared by primer extending the *fis* gene located on plasmid pRJ807 (35). The amount of total RNA corresponding to the same number of cells was analyzed for each time point.

Primer extension was performed essentially as described by Hartz et al. (17). For analysis of the *fis* promoter activity, a primer with the sequence 5′ dGCTGATATTGTCCGATG which hybridizes to the top strand of the DNA sequence shown in Fig. 4C in the region from nucleotide 815 to 799 was used. For analysis of the *lacPL8UV5* promoter activity, the *lac* universal primer (5′dGTTTTCCCAGTCACGAC) was used. DNA primers were labeled at their 5′ ends with [γ-^{32}P]ATP and T4 polynucleotide kinase, as described previously (40). For each sample, 10 μg of total RNA was analyzed. The relative intensities of the primer extended products were determined by densitometry measurements of autoradiographs, and the values were adjusted to reflect the relative absorbance per 10^9 cells or per unit of optical density at 600 nm of cells as indicated.

DNase I footprinting. DNase I protection analysis was performed essentially as described by Bruist et al. (4). Fis-DNA binding reactions were performed by c ombining 5 to 40 ng of Fis with 50,000 cpm of [^{32}P]-end-labeled DNA (at either their 5′ or 3′ end) (40) in 45 μl of solution containing 20 mM Tris-HCl (pH 7.5) and 80 mM NaCl and incubating the mixture at room temperature for 5 min. RNA polymerase-DNA binding reactions were performed by combining 1 to 20 μg of σ^{70} RNA polymerase holoenzyme with the ^{32}P-labeled DNA fragment (50,000 cpm) in 45 μl of standard transcription buffer (30 mM Tris-HCl [pH 7.9], 100 mM KCl, 10 mM $MgCl_2$, 0.1 mM Na_2-EDTA, 0.1 mM dithiothreitol) in the presence or absence of 100 μM G and C ribonucleotides and followed by a 10-min incubation at 37°C. When Fis and RNA polymerase were added in the same binding reaction, standard transcription buffer was used. Nonspecific or closed RNA polymerase-promoter complexes were dissociated by challenge with 100 μg of heparin per ml for 45 s at room temperature. DNase I incubations were done for 30 s, and samples were prepared for electrophoresis as described previously (4). DNA sequencing reactions specific for G and A nucleotides were performed as described previously (27).

Nucleotide sequence accession number. The sequence of the 2,647-bp *fis* operon described here has been deposited with GenBank under the accession number M95784.

RESULTS

Transient expression of Fis upon initiation of growth. The intracellular levels of Fis protein during the course of the *E. coli* growth cycle were examined. Saturated overnight cultures were diluted such that a sufficient number of cells could be collected immediately after subculturing to measure the amount of Fis per cell by quantitative Western blotting. In the experiment for which the results are illustrated in Fig. 1A, an overnight LB culture of MC1000 was diluted 100-fold into LB medium to a density of 6×10^7 cells per ml and grown at 37°C. Cells that have been in stationary phase for at least 10 h contain less than 100 copies of Fis. Upon subculturing, the number of Fis molecules per cell rapidly increases to a peak of about 60,000 within 90 min. This corresponds approximately to the time of the first cell division as measured by cell concentration (CFU per milliliter) and the initial stages of exponential growth as measured by the increase in the optical density of the culture. After 90 min, the amount of Fis decreases rapidly as the cells continue to grow exponentially. This expression pattern has been observed in the backgrounds of 10 different *E. coli* strains (data not shown) and at different growth temperatures in LB (Table 1). In all cases, the timing of the Fis peak occurred within the first two cell divisions, and the levels were between 50,000 and 100,000 molecules per cell. Fis levels also peak near the time of the first cell division in defined media though the magnitude of the peak is not as high as that observed in LB and appears to reflect the nutritional quality of the culture medium (Table 1). Thus, we conclude that the transition from stationary to exponential growth is accompanied by a large fluctuation in Fis levels. These batch culturing conditions, however, do not allow us to determine the level of Fis under long-term exponential growth.

Two observations indicate that the rapid decline in Fis levels is not caused by selective proteolysis but instead is caused by a decrease in nascent Fis synthesis. First, Fis levels when expressed from the *lac* promoter continue to increase throughout exponential growth (data not shown), suggesting that Fis is stable throughout late logarithmic and

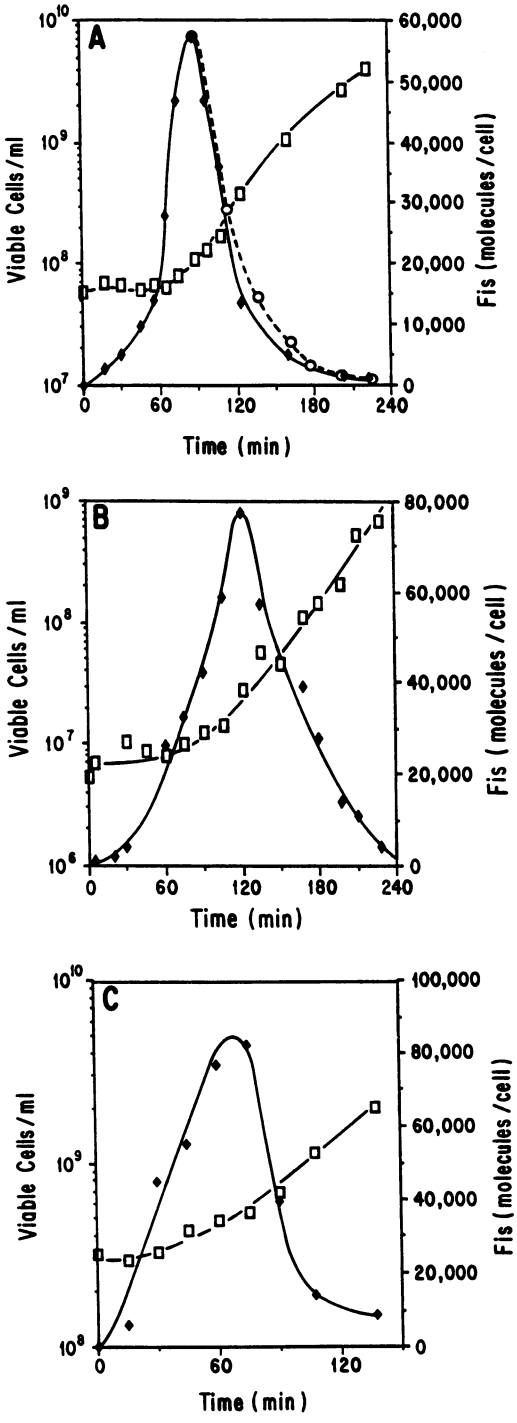

FIG. 1. Expression pattern of Fis protein in batch culture. (A) A stationary overnight culture (MC1000) was diluted 1/100 in prewarmed LB and incubated at 37°C. Samples were removed at intervals and assayed for Fis levels as described in Materials and Methods. ○, amount of Fis per cell that would be predicted if Fis synthesis ceased at 90 min and intracellular Fis levels were then diluted as a function of cell division with a generation time of 25 min. (B) Fis levels were examined in MC1000 cells that had been diluted 1/500 in prewarmed LB and grown at 37°C. (C) The pattern of Fis expression in used LB medium is shown. After MC1000 cells were subcultured and grown in LB for 120 min, they were removed from the LB by centrifugation and filtration. The used LB was then used to subculture a saturated overnight culture at a 1/20 dilution. □, cell growth; ◆, Fis level per cell.

TABLE 1. Fis protein levels under different growth conditions

Medium (temp)[a]	Strain	Initial cell density[b]	Cell density at peak[c]	Fis molecules/cell at peak[d]
LB	MG1655	1.9×10^8	4×10^8	98,000
LB	MC1000	6×10^7	1×10^8	57,000
LB	MC1000	7×10^6	3×10^7	78,000
LB (30°C)	MC1000	8×10^7	1.3×10^8	96,000
LB (42°C)	MC1000	7×10^7	1.2×10^8	88,000
M9 + glc	MG1655	5×10^7	1.8×10^8	7,500
M9 + glc, CAA	MG1655	5×10^7	1.7×10^8	21,000
M9 + glc, CAA, trp	MG1655	5×10^7	1.0×10^8	29,000

[a] Growth was done at 37°C unless otherwise specified. Supplements were 0.2% glucose (glc), 0.2% Casamino Acids (CAA), and 20 μg of L-tryptophan (trp) per ml as noted.
[b] Concentration of cells per milliliter immediately after subculturing.
[c] Concentration of cells per milliliter at the time of maximum Fis levels.
[d] Maximum levels of Fis monomers per cell.

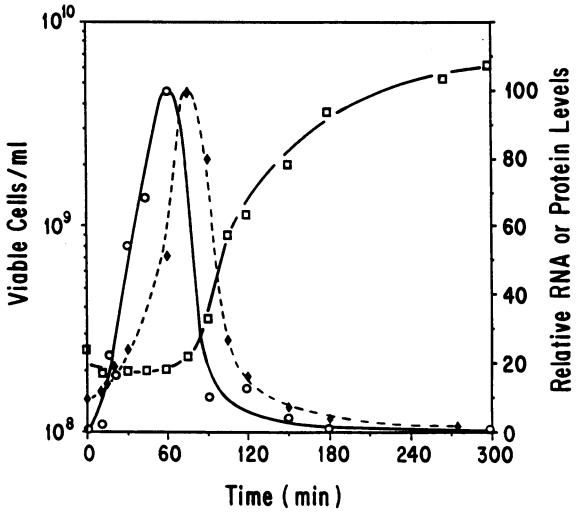

FIG. 2. *fis* RNA and Fis protein expression in subcultured cells. MC1000 cells from a saturated overnight culture were diluted 1/20 in prewarmed LB and grown at 37°C. At intervals, 20-ml samples of culture were removed and assayed for both Fis protein and *fis* RNA, as described in Materials and Methods. □, cell concentration; ◆, relative number of Fis molecules per cell; ○, relative levels of *fis* RNA per cell. The Fis protein levels ranged from less than 100 molecules per cell at 0 min to 98,000 molecules per cell at 75 min (100% value); relative *fis* mRNA levels (measured by densitometry from an autoradiograph of electrophoretically separated products) ranged from background (less than 30 absorbance units/10^9 cells) at 0 min to 28,590 absorbance units/10^9 cells at 60 min (100% value).

stationary phases. Second, the decrease in Fis levels is quite similar to that predicted if Fis synthesis were to be stopped at 90 min (the peak of Fis concentration) and intracellular Fis levels were then diluted by a cell division every 25 min (as illustrated by the dotted line in Fig. 1A). Thus, the normal synthesis of Fis under these culturing conditions appears to be efficiently switched off as cells enter exponential growth, a conclusion that is supported by the RNA analysis described below.

We next asked whether the shutoff of Fis synthesis is influenced by external signals, such as cell density or a component in the medium. The expression of Fis was monitored in cultures where stationary-phase cells were diluted 1/500 or 1/20 in LB and compared with the 1/100 dilution results shown in Fig. 1A. As shown in Fig. 1B and Table 1, the peak of Fis synthesis in a 1/500 dilution occurred at a cell density of 3×10^7 cells per ml, which is significantly lower than 1.0×10^8 when cells were diluted 1/100 (Fig. 1A). When cells were diluted 1/20, the peak of Fis synthesis was approximately 100,000 molecules per cell and occurred at a density of 4.0×10^8 cells per ml (Fig. 2). This demonstrates that the termination of Fis synthesis and the magnitude of the Fis peak are not a strict function of cell density. The precise timing of the peak varies and is generally correlated with the length of the lag phase. It is possible that the medium plays a role in the decrease of Fis expression observed in these batch culturing conditions, either by depletion of an activating signal or by accumulation of an inhibitory signal. This was tested by growing cells in LB for 120 min, at which time Fis levels in the culture are decreasing rapidly (Fig. 1A). Cells were then removed from the medium by centrifugation and filtration, and the same medium was used to subculture stationary-phase cells. Normal expression of Fis is observed with the used medium (Fig. 1C), suggesting that there is no stable component in the medium that provokes the decrease in Fis synthesis. It is possible, however, that the rapid decrease in Fis expression may be signalled by a labile factor or by a change in the relative concentration of a medium component during cell growth.

Regulation of *fis* mRNA levels. In order to determine whether Fis levels could be controlled by changes in transcription, the relative levels of *fis* mRNA throughout the growth cycle were measured by Northern (RNA) blot analysis. Figure 2 shows the result of an experiment in which both *fis* RNA levels and Fis protein levels were assayed from a 1/20 subculture. Like the protein measurements, *fis* mRNA is expressed on the basis of cellular concentration rather than as a fraction of total RNA, since total cellular RNA levels fluctuate approximately 10-fold with cell growth (for example, Fig. 8C shows a 7.5-fold fluctuation). The *fis* message is hardly detectable immediately after subculturing but then rapidly increases approximately 1,000-fold, reaching a peak within 60 min. *fis* message levels then decrease quickly and become undetectable by 150 min after subculturing. Fis protein levels in this experiment reached a peak 75 min after subculturing and then rapidly declined. The similarity between expression patterns of Fis protein and *fis* message in batch culture suggests that Fis expression is regulated largely at the RNA level.

When stationary-phase cells are subcultured, the expression of *fis* mRNA occurs mainly during lag phase and decreases during exponential growth. To determine whether *fis* expression is restricted to the initiation of cell growth, we examined the expression pattern of *fis* when exponentially growing cells are subjected to a sudden increase of nutrients. A saturated overnight culture was diluted 10^7-fold in M9 salts supplemented with 0.2% glucose and allowed to grow at 37°C for approximately 20 generations, well after the predicted peak of Fis expression has occurred and when the cells are in steady-state exponential growth. At this time, a one-fifth volume of a 5× LB concentrate was added, and the cells were assayed for *fis* mRNA expression (Fig. 3). Within 40 min of the nutritional upshift, *fis* mRNA levels increase 11-fold. When *lacZ* mRNA levels (expressed in a *lacI*::Tn*10* *lacPL8UV5* strain) were measured as a control, they were shown to increase only twofold during this time period (data not shown). Soon after the increase in *fis* mRNA levels, the rate of cell division abruptly increases. A similar response to

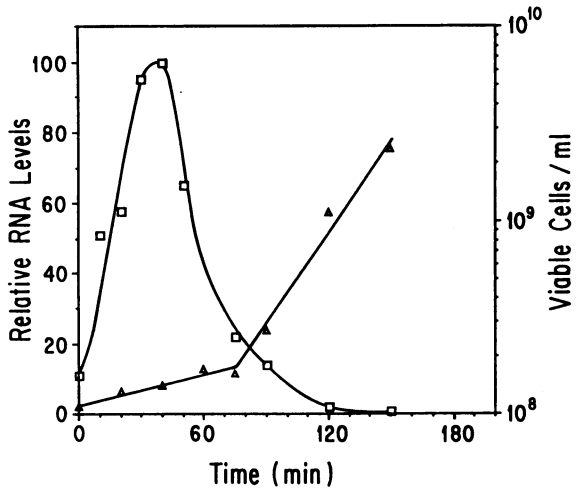

FIG. 3. *fis* response to nutritional upshift in mid-logarithmic growth phase. RJ1809 cells (*fis*$^+$) were subcultured at an initial density of approximately 100 cells per ml in M9 salts supplemented with glucose (0.2%). Cells were grown to a density of 2×10^8 cells per ml (optical density at 600 nm, 0.23), at which time a one-fifth volume of 5× concentrated LB (without sodium chloride) was added to the culture. Beginning at the time of LB addition, 20 ml of cell culture was withdrawn, the titer (CFU) of viable cells was determined, and total cellular RNA was prepared for primer extension analysis, as described in Materials and Methods. △, cell concentration; □, relative *fis* mRNA levels per cell. A value of 100% was assigned to the maximum intensity of *fis* mRNA signal (23,320 absorbance units/10^9 cells) measured by densitometry from an autoradiograph of electrophoretically separated products. The intensity of the *fis* mRNA signal detected immediately following the addition of LB (0 min) was 2,120 absorbance units/10^9 cells; after 150 min, the *fis* mRNA signal was undetectable (less than 30 absorbance units/10^9 cells). While the increase in the rate of cell division upon addition of LB displayed a lag of approximately 75 min, the rate of increase of the optical density of the culture increased almost immediately.

nutritional upshift is observed at the protein level (data not shown). This experiment demonstrates that increased *fis* expression in response to an increase in the nutritional quality of the medium is not restricted to initiation of growth.

Structure of the *fis* operon and identification of the *fis* promoter. DNA sequence analysis of the region surrounding *fis* indicates an extended open reading frame (ORF1) immediately upstream of the *fis* coding sequence (Fig. 4A and C). ORF1 is probably translated because in-frame fusions to the *lacZ* coding sequence generate substantial β-galactosidase activity (data not shown). The function of this reading frame is not known; cells containing an insertion of the *neo* gene from Tn*5* into the *Nru*I site of ORF1 (Fig. 4A and C, nucleotide 1389) are viable and the only observed phenotype of this insertion is the absence of Fis expression. The polar effect of an insertion into ORF1 suggests that *fis* and ORF1 are cotranscribed as a polycistronic message. ORF1 is preceded by a 330-bp region with no open reading frames (in the same orientation) greater than 49 amino acids. Preceding this intercistronic region is an open reading frame of greater than 100 amino acids. Downstream of *fis* is an 85-bp region of DNA with no extensive open reading frames followed by a reading frame of greater than 100 amino acids. Between *fis* and the downstream open reading frame is a possible rho-independent terminator sequence (Fig. 4A and C).

Two RNA species hybridize to a probe consisting of the antisense strand of the *fis* coding sequence (Fig. 5A). The size of the 1,400-base RNA is consistent with a transcript that contains both *fis* and ORF1. The 860-base RNA either is a processed product of the 1,400-base RNA or is generated by a second promoter located within the ORF1 region. The latter explanation is unlikely because *lacZ* fusion data described below show no promoter activity within ORF1. Furthermore, a probe from the 5′ region of ORF1 hybridizes to both the 1,400-base and an ~550-base RNA (data not shown); the latter would be consistent with the 5′ fragment that would result from processing.

Operon fusions to *lacZ* identified the DNA region responsible for *fis* promoter activity. As illustrated in Fig. 6, a *lacZ* fusion to the region between *Hin*dIII and *Nco*I present on pRJ976 promotes high β-galactosidase activity (95 units), while a fusion to the DNA between codon 13 of ORF1 and codon 17 of Fis (pRJ920) provides only 7 units of β-galactosidase activity. Expression of β-galactosidase in cells carrying pRJ960 (34 units) indicates that significant promoter activity is still present when DNA upstream of the *Dra*I site is removed. This evidence suggests that *fis* promoter activity is located between the *Dra*I and *Nco*I sites of the *fis* operon.

The precise transcription initiation site was mapped by extending a primer that hybridizes to the region from nucleotide position 815 to 799 (Fig. 4C). Figure 5B shows that primer extension products identify two transcription initiation sites at the G and C residues located 34 and 32 bp, respectively, upstream of the first AUG in ORF1 (Fig. 4B). A message whose transcription was to initiate at this site and extend approximately 1,400 bases would terminate approximately 80 bases beyond the termination codon of *fis*, within the vicinity of the potential rho-independent terminator sequence (Fig. 4A and C). Upstream of the start of transcription are putative −10 and −35 regions that resemble a σ^{70} promoter.

The DNA upstream of ORF1 was footprinted with σ^{70} RNA polymerase, and two heparin-resistant binding sites were detected (Fig. 7). The footprint extending from −48 to +26 relative to the *fis* mRNA start site was detected only when the initiating ribonucleotides G and C were included in the binding reaction (Fig. 7A, compare lanes 9 and 10). This protected region is in the appropriate position for initiating transcription at the start sites identified by primer extension and, therefore, identifies the DNA sequence encompassing the *fis* promoter. The upstream RNA polymerase-binding site from −66 to −124 relative to the *fis* mRNA start site was readily detectable at two- to fourfold lower RNA polymerase concentrations and in the absence of ribonucleotides (Fig. 7). The relevance of this binding site is not known; attempts to reveal a transcript initiating in either direction from this site by using primer extension have so far been unsuccessful. Therefore, binding of RNA polymerase to the downstream binding site probably accounts for the *fis* transcription we observe.

Negative regulation of *fis* mRNA. To test whether the presence of Fis protein affects the expression of the *fis* gene, RNA was prepared from otherwise isogenic *fis* null mutant and *fis*$^+$ cells at various times during the growth cycle and the relative levels of *fis* mRNA were determined by primer extension by using excess labelled primer. As shown in Fig. 8A and B, intracellular *fis* mRNA levels are extremely low or undetectable in both *fis* mutant and *fis*$^+$ cells immediately after dilution but increase rapidly upon growth of the culture. In *fis*$^+$ cells, levels of *fis* mRNA peak 40 min after subculturing and then rapidly decrease to a point where no *fis* mRNA is detectable 60 to 75 min after subculturing. In *fis*

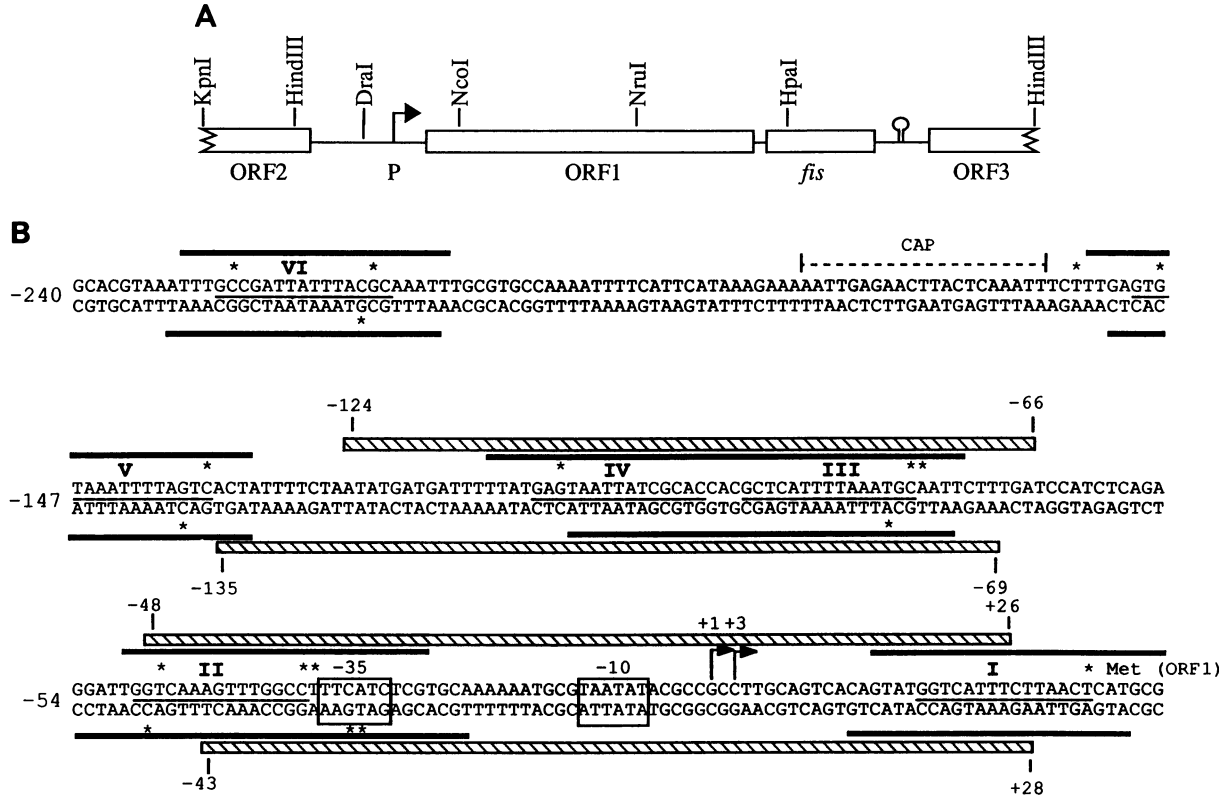

FIG. 4. (A) Schematic diagram of *fis* operon and surrounding DNA. Open reading frames are depicted as open boxes. The relative locations of relevant restriction sites, the transcription start site, and the putative transcriptional terminator are indicated. (B) *fis* promoter region corresponding to nucleotides 520 to 798 in panel C. The nucleotide sequence in this panel is numbered relative to the transcriptional start sites +1 and +3 (shown by arrows). The extents of the regions protected from DNase I cleavage by RNA polymerase (▨) and by Fis (■) are shown for both the top and the bottom strands. Two RNA polymerase- and six Fis-binding sites are shown. The * denotes nucleotides that are hypersensitive to DNase I cleavage in the presence of Fis. The underlined sequences indicate the predicted Fis recognition sequence on the basis of their alignment with the hypersensitive sites (11) and their similarity to the proposed consensus sequence (11, 19). The −10 and −35 regions for the *fis* promoter are boxed. A sequence resembling a CAP-binding site is shown by dotted lines. The significance of this site is not apparent since we have not observed a difference in the growth phase regulation of Fis in *cap* mutant cells grown in rich medium. The initial methionine (Met) codon for the upstream open reading frame (ORF1) is labeled on the sequence. (C) DNA sequence of *fis* operon and surrounding regions. Open reading frames are shown with the three-letter amino acid codes below the corresponding nucleic acid sequence. While no significant similarities have been found between the translation products of ORF1 or ORF2 and proteins in the existing data bases (NBRF-PIR and Swiss-Prot), the predicted ORF3 translation product displays limited identity to a variety of bacterial DNA methylases. Relevant restriction sites are underlined and labeled above the sequence and the +1 transcriptional start site for the *fis* operon is designated by an arrow. A sequence containing an inverted repeat that resembles a transcriptional terminator is also underlined. There are 12 differences between the sequence reported here and the sequence between nucleotides 385 and 1785 reported by Ninnemann et al. (34). They report an additional A between nucleotides 459 and 460, only two G residues where we find three from nucleotides 481 to 483, G C instead of C G at nucleotides 495 and 496, C G instead of a G C at positions 552 and 553, C G instead of G C at positions 1184 and 1185, C G instead of G C at positions 1559 and 1560, and C G instead of G C at positions 1589 and 1590. We have confirmed the accuracy of our sequence.

mutant cells, *fis* transcript levels are approximately sixfold higher at the peak and become undetectable 150 to 180 min after subculturing. This indicates that Fis is negatively regulating its own expression at the RNA level and significantly contributes to the rapid shutoff of Fis expression.

In order to investigate possible mechanisms by which Fis is negatively regulating its own expression, we footprinted Fis to DNA containing the *fis* promoter region (Fig. 7A and B and 4C). Fis binds to six sites between the *Nco*I site and the *Hin*dIII site with affinities that are comparable to those of the binding sites within the Hin recombinational enhancer (apparent K_d, approximately 10^{-9} M [4]). Site II, which extends from −22 to −54, overlaps the −35 region of the promoter sequence and is positioned within the region that is footprinted by RNA polymerase. This appears to be the strongest Fis-binding site because it binds Fis at half the concentration required for the other sites in this region and because it is the only site that remains partially protected after being challenged with 100 μg of heparin per ml (Fig. 7C, lane 10). Site I, which extends from +12 to +39, also overlaps the *fis* promoter RNA polymerase-binding site. Sites III and IV extend from −72 to −112 and are included within the upstream RNA polymerase-binding site. Additional protected regions, which appear at higher Fis concentrations and are probably the result of lower-affinity Fis-binding sites, can also be detected, for example between sites I and II and downstream of site I. The lower-affinity site between sites I and II would extend over the *fis* −10 region. The locations of these Fis-binding sites with respect to the RNA polymerase-binding sites suggest that the mechanism of Fis-mediated repression may involve direct exclusion of RNA polymerase from the promoter. To test this, we

C

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 KpnI
GGTACCCATCCAACCACCTCTCTGTGCCTGCAATGGCTCGACAGCCTCGATTTAACCGGTAAAACAGTCATCGACTTTGGCTGTGGTTCCGGCATTCTGG    100
GlyThrHisProThrThrSerLeuCysLeuGlnTrpLeuAspSerLeuAspLeuThrGlyLysThrValIleAspPheGlyCysGlySerGlyIleLeuAla
                                    EcoRV
CGATCGCGGCGCTGAAACTGGGTGCAGCAAAAGCCATTGGTATTGATATCGATCCGCAGGCGATTCAGGCCAGCCGCGATAACGCCGAACGTAATGGCGT    200
  IleAlaAlaLeuLysLeuGlyAlaAlaLysAlaIleGlyIleAspIleAspProGlnAlaIleGlnAlaSerArgAspAsnAlaGluArgAsnGlyVal

TTCTGACCGTCTGGAACTCTACTTACCGAAAGATCAGCCAGAAGAAATGAAAGCCGACGTGGTGGTCGCTAACATCCTTGCAGGCCCATTACGTGAACTG    300
 SerAspArgLeuGluLeuTyrLeuProLysAspGlnProGluGluMetLysAlaAspValValValAlaAsnIleLeuAlaGlyProLeuArgGluLeu
                                                                                HindIII
GCACCGTTAATCAGCGTCCTGCCGGTTTCAGGCGGTTTGCTGGGCCTTTCCGGTATTCTGGCAAGCCAGGCAGAGAGCGTTTGTGAAGCTTATGCCGATA    400
AlaProLeuIleSerValLeuProValSerGlyGlyLeuLeuGlyLeuSerGlyIleLeuAlaSerGlnAlaGluSerValCysGluAlaTyrAlaAspSer

GCTTCGCACTGGACCCGGTCGTGGAAAAAGAAGAGTGGTGCCGTATTACCGGTCGTAAGAATTAACCTTCGCATCGCCGTAGGGTGACGCGGGGCGAAGT    500
  PheAlaLeuAspProValValGluLysGluGluTrpCysArgIleThrGlyArgLysAsn  ORF2

GCGAGCAAGCTCACAAAAGGCACGTAAATTTGCCGATTATTTACGCAAATTTGCGTGCCAAAATTTTCATTCATAAAGAAAAATTGAGAACTTACTCAAA    600
                                                                                DraI
TTTCTTTGAGTGTAAATTTTAGTCACTATTTTCTAATATGATGATTTTTATGAGTAATTATCGCACCACGCTCATTTTAAATGCAATTCTTTGATCCATC    700
                                                  +1
TCAGAGGATTGGTCAAAGTTTGGCCTTTCATCTCGTGCAAAAAATGCGTAATATACGCCGCCTTGCAGTCACAGTATGGTCATTTCTTAACTCATGCGCA    800
                                                                                ORF1 MetArgIle
                                    NcoI
TCGGACAATATCAGCTCAGAAATCGCCTGATCGCAGCGCCCATGGCTGGCATTACAGACAGACCTTTTCGGACGTTGTGCTACGAGATGGGAGCCGGATT    900
  GlyGlnTyrGlnLeuArgAsnArgLeuIleAlaAlaProMetAlaGlyIleThrAspArgProPheArgThrLeuCysTyrGluMetGlyAlaGlyLeu

GACAGTATCCGAGATGATGTCTTCTAACCCACAGGTTTGGGAAAGCGACAAATCTCGTTTACGGATGGTGCACATTGATGAACCCGGTATTCGCACCGTG   1000
 ThrValSerGluMetMetSerSerAsnProGlnValTrpGluSerAspLysSerArgLeuArgMetValHisIleAspGluProGlyIleArgThrVal
                                                                EcoRV
CAAATTGCTGGTAGCGATCCGAAAGAAATGGCAGATGCAGCACGTATTAACGTGGAAAGCGGTGCCCAGATTATTGATATCAATATGGGTTGCCCGGCTA   1100
GlnIleAlaGlySerAspProLysGluMetAlaAspAlaAlaArgIleAsnValGluSerGlyAlaGlnIleIleAspIleAsnMetGlyCysProAlaLys

AAAAAGTGAATCGCAAGCTCGCAGGTTCAGCCCTCTTGCAGTACCCGGATGTCGTTAAATCGATCCTTACCGAGGTCGTCAATGCAGTGGACGTTCCTGT   1200
  LysValAsnArgLysLeuAlaGlySerAlaLeuLeuGlnTyrProAspValValLysSerIleLeuThrGluValValAsnAlaValAspValProVal

TACCCTGAAGATTCGCACCGGCTGGGCACCGGAACACCGTAACTGCGAAGAGATTGCCCAACTGGCTGAAGACTGTGGCATTCAGGCTCTGACCATTCAT   1300
 ThrLeuLysIleArgThrGlyTrpAlaProGluHisArgAsnCysGluGluIleAlaGlnLeuAlaGluAspCysGlyIleGlnAlaLeuThrIleHis
                                                                        NruI
GGCCGTACACGCGCCTGTTTGTTCAATGGAGAAGCTGAGTACGACAGTATTCGGGCAGTTAAGCAGAAAGTTTCCATTCCGGTTATCGCGAATGGCGACA   1400
GlyArgThrArgAlaCysLeuPheAsnGlyGluAlaGluTyrAspSerIleArgAlaValLysGlnLysValSerIleProValIleAlaAsnGlyAspIle

TTACTGACCCGCTTAAAGCCAGAGCTGTGCTCGACTATACAGGGGCGGATGCCCTGATGATAGGCCGCGCAGCTCAGGGAAGACCCTGGATCTTTCGGGA   1500
  ThrAspProLeuLysAlaArgAlaValLeuAspTyrThrGlyAlaAspAlaLeuMetIleGlyArgAlaAlaGlnGlyArgProTrpIlePheArgGlu

AATCCAGCATTATCTGGACACTGGGGAGTTGCTGCCCCCGCTGCCTTTGGCAGAGGTTAAGCGCTTGCTTTGCGCGCACGTTCGGGAACTGCATGACTTT   1600
 IleGlnHisTyrLeuAspThrGlyGluLeuLeuProProLeuProLeuAlaGluValLysArgLeuLeuCysAlaHisValArgGluLeuHisAspPhe
            KpnI
TATGGTCCGGCAAAAGGGTACCGAATTGCACGTAAACACGTTTCCTGGTATCTCCAGGAACACGCTCCAAATGACCAGTTTCGGCGCACATTCAACGCCA   1700
TyrGlyProAlaLysGlyTyrArgIleAlaArgLysHisValSerTrpTyrLeuGlnGluHisAlaProAsnAspGlnPheArgArgThrPheAsnAlaIle

TTGAGGATGCCAGCGAACAGCTGGAGGCGTTGGAGGCATACTTCGAAAATTTTGCGTAAACAGAAATAAAGAGCTGACAGAACTATGTTCGAACAACGCG   1800
  GluAspAlaSerGluGlnLeuGluAlaLeuGluAlaTyrPheGluAsnPheAla                fis  MetPheGluGlnArgVal
                        HpaI
TAAATTCTGACGTACTGACCGTTTCTACCGTTAACTCTCAGGATCAGGTAACCCAAAAACCCCTGCGTGACTCGGTTAAACAGGCACTGAAGAACTATTT   1900
  AsnSerAspValLeuThrValSerThrValAsnSerGlnAspGlnValThrGlnLysProLeuArgAspSerValLysGlnAlaLeuLysAsnTyrPhe

TGCTCAACTGAATGGTCAGGATGTGAATGACCTCTATGAGCTGGTACTGGCTGAAGTAGAACAGCCCCTGTTGGACATGGTGATGCAATACACCCGTGGT   2000
 AlaGlnLeuAsnGlyGlnAspValAsnAspLeuTyrGluLeuValLeuAlaGluValGluGlnProLeuLeuAspMetValMetGlnTyrThrArgGly

AACCAGACCCGTGCTGCGCTGATGATGGGCATCAACCGTGGTACGCTGCGTAAAAAATTGAAAAAATACGGCATGAACTAATTCAGGTTAGCTAAATGCT   2100
AsnGlnThrArgAlaAlaLeuMetMetGlyIleAsnArgGlyThrLeuArgLysLysLeuLysLysTyrGlyMetAsn

TGATTAAAAAGGCGCTACTCGGCATGGGGAAGCGCCTTTTTTATAGGTGTCACAAAGGGAGTGACCATGAGAACAGGATGTGAACCGACCCGGTTTGGTA   2200
                                                        ORF3 MetArgThrGlyCysGluProThrArgPheGlyAsn

ATGAAGCTAAGACCATTATTCACGGTGATGCGCTTGCCGAACTTAAAAACATCCCCGCCGAAAGTGTCGATCTGATCTTTGCCGACCCACCATATAACAT   2300
  GluAlaLysThrIleIleHisGlyAspAlaLeuAlaGluLeuLysAsnIleProAlaGluSerValAspLeuIlePheAlaAspProProTyrAsnIle

CGGTAAAAATTTTGATGGTCTGATCGAAGCCTGGAAAGAAGATCTGTTTATCGACTGGCTGTTTGAAGTGATTGCAGAGTGCCACCGCGTTCTGAAAAAG   2400
 GlyLysAsnPheAspGlyLeuIleGluAlaTrpLysGluAspLeuPheIleAspTrpLeuPheGluValIleAlaGluCysHisArgValLeuLysLys

CAGGGCAGCATGTACATCATGAACAGTACGGAAAACATGCCCTTTATCGATCTCCAGTGCCGCAAGC                                      2467
GlnGlySerMetTyrIleMetAsnSerThrGluAsnMetProPheIleAspLeuGlnCysArgLys
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FIG. 4—*Continued.*

assayed the ability of RNA polymerase to form a heparin-resistant complex on DNA that was prebound by Fis (Fig. 7C, lanes 9 and 10). The results indicate that prebinding of Fis prevents RNA polymerase from forming a stable complex at both the functional *fis* promoter and the upstream polymerase-binding site.

Genetic and biochemical analyses indicate that Fis contains at least two functional domains: a C-terminal region

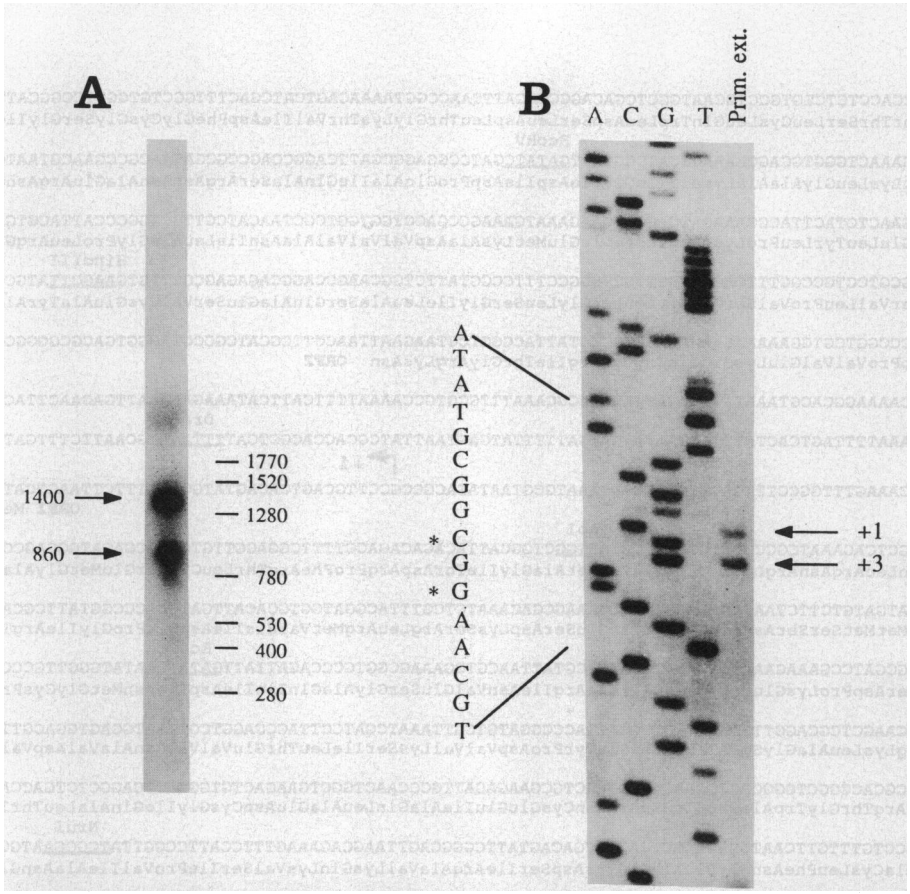

FIG. 5. Determination of size and start site of *fis* mRNA. (A) Northern blot of *fis* mRNA. RJ1809 cells were subcultured in LB, grown for 40 min, and used for preparation of total cellular RNA. Northern blots were done as described in Materials and Methods with the *fis* antisense strand as a ^{32}P-labeled DNA probe. The two *fis* mRNA signals (1,400 and 860 bases) are indicated by arrows. The positions and sizes (in bases) of the RNA size standards are indicated on the right. (B) *fis* mRNA start sites. Total RNA (10 μg) from RJ1809 was subjected to primer extension analysis with a ^{32}P-5′-end-labeled primer that specifically annealed to the top strand (+56 to +40) of the *fis* promoter region shown in Fig. 4B. Primer-extended products were subjected to electrophoresis in an 8% sequencing gel and then autoradiography. Dideoxy DNA sequencing reaction mixtures of the *fis* promoter region using the same labeled primer were electrophoresed in parallel (lanes A, C, G, and T). The lane labeled "prim. ext." contains the primer-extended products of mRNA synthesized from the chromosomal *fis* promoter. The two major products are indicated on the right by arrows, and their positions on the sequence are indicated on the left by asterisks.

that is involved in DNA binding and bending and an N-terminal region that is additionally required to promote recombination by the DNA invertases (25, 35). While mutations within the N-terminal region have not been found to significantly affect Fis-stimulation of bacteriophage λ excisive recombination, some mutations within this region inhibit transcriptional activation of *rrnB* P1 (14). To determine which regions of Fis are required to mediate repression,

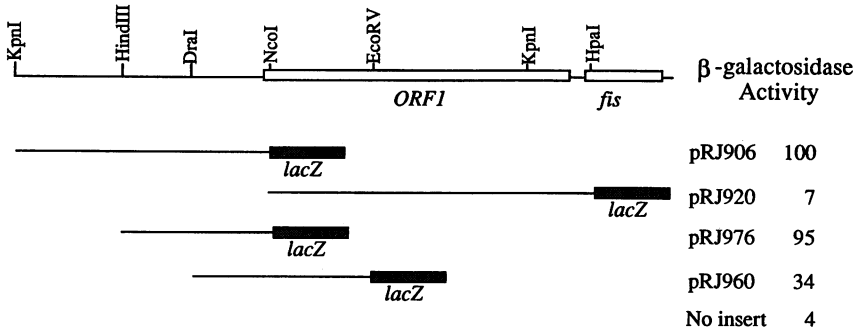

FIG. 6. Schematic diagram of *fis-lacZ* promoter fusions. ORF1 and *fis* coding regions are denoted as open boxes, and relevant restriction sites are illustrated. The section of *fis* operon DNA included in each fusion is shown as a line fused to the *lacZ* sequence shown as a stippled box. The β-galactosidase values (in Miller units), representing the averages of three experiments, are listed on the right.

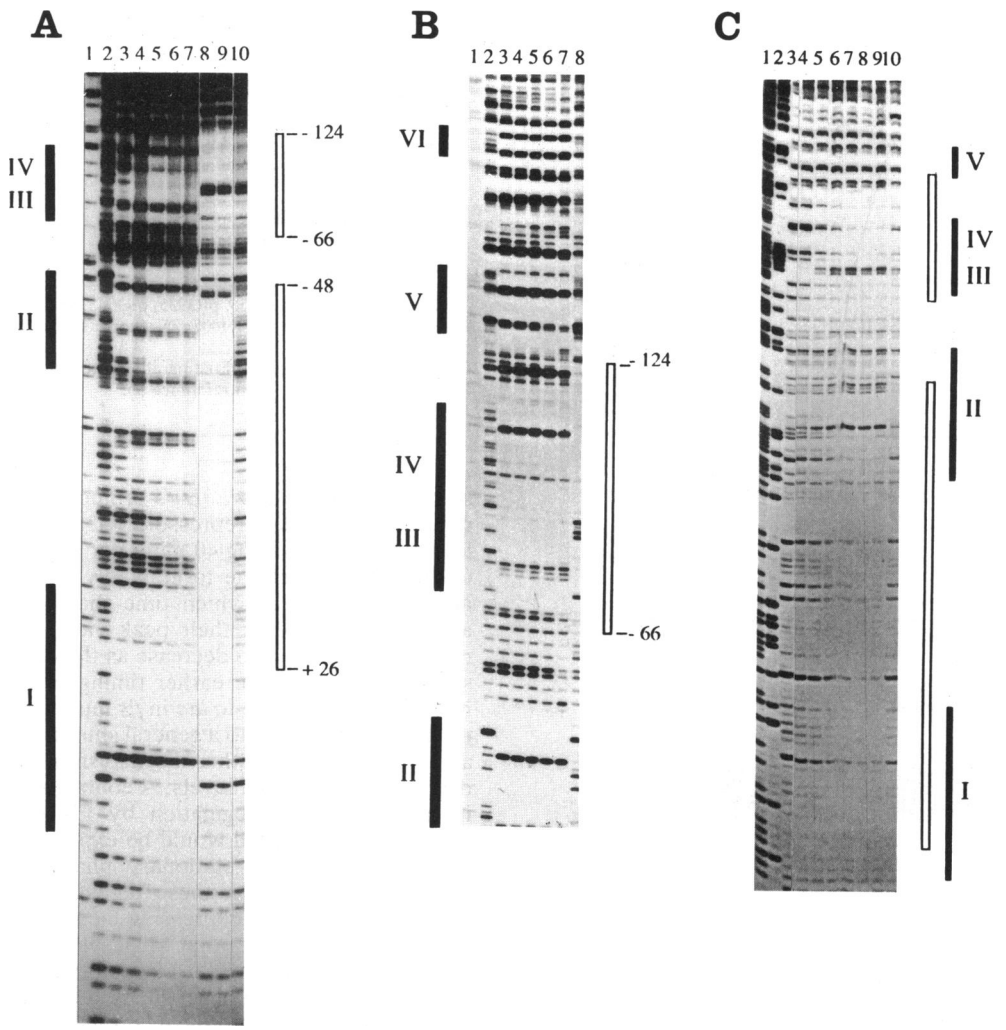

FIG. 7. DNase I footprinting of Fis and RNA polymerase within the *fis* promoter region. Binding conditions and DNase I footprinting were described in Materials and Methods. (A) The *Hin*dIII-*Nco*I DNA fragment containing the *fis* promoter (see Fig. 4A and C) was labeled with ^{32}P at the 3′ end of the *Nco*I site (top strand). Results are shown for G-specific DNA sequencing reactions (lane 1); DNase I cleavage performed in the absence of protein (lane 2), in the presence of 5, 10, 15, 20, and 40 ng of Fis (lanes 3 to 7, respectively), or in the presence of 5 and 10 μg of σ^{70} RNA polymerase holoenzyme and 100 μM GTP and CTP (lanes 8 and 9); and DNase I cleavage in the presence of 10 μg of RNA polymerase without ribonucleotides (lane 10). Protected regions for four Fis sites are shown on the left (filled bars); protected regions for 2 RNA polymerase binding sites are shown on the right (open bars). The borders of the protection by RNA polymerase are denoted as nucleotide positions relative to the *fis* transcriptional start site. (B) DNase I reaction conditions were the same as those described for panel A except that the electrophoresis was run longer. Results are shown for G-specific DNA sequencing reactions (lane 1) and DNase I cleavage reactions in the absence of proteins (lane 2), in the presence of 5, 10, 15, 20, and 40 ng of Fis (lanes 3 to 7), or in the presence of 2.5 μg of RNA polymerase without ribonucleotides (lane 8). Regions protected by Fis and RNA polymerase are shown as described for panel A. Only a portion of Fis site 2 was detected in this autoradiograph. (C) The *Hin*dIII-*Nco*I DNA fragment was labeled at the 5′ end of the *Nco*I site (bottom strand). Results for G-specific (lane 1) and G + A-specific (lane 2) DNA sequencing reactions are shown. DNase I cleavage conditions were as follows: no protein (lane 3); 0.5, 1, 2.5, 5, 7.5, and 10 μg of RNA polymerase in the presence of 100 μM GTP and CTP (lanes 4 to 9, respectively); and DNA bound first with 10 ng of Fis for 5 min at room temperature and then with 10 μg of RNA polymerase and incubated for 10 min at 37°C prior to DNase I cleavage (lane 10). The RNA polymerase protected regions are shown as open bars, and the locations of the high-affinity Fis-binding sites are indicated as closed bars for reference.

plasmids containing mutant *fis* genes were introduced into a *fis*::*767* strain lysogenic for a λ phage containing the *fis* promoter region fused to *lacZ* (RJ1838). The results from β-galactosidase assays show that C-terminal Fis mutants that are unable to bind DNA (Thr75Pro and Arg85Cys) do not efficiently repress the *fis* promoter, while N-terminal mutants that can bind DNA but cannot function in Hin-mediated DNA inversion (Δ17-21, Δ24-29, and Ala34Pro) effectively repress the *fis* promoter (Table 2). Thus, the DNA-binding activity of Fis appears to be sufficient for mediating autoregulation.

As shown in Fig. 8A and B, *fis* mRNA levels become undetectable during the growth of the culture, even in cells deficient in Fis protein synthesis. To determine whether the timing of the decrease in *fis* mRNA levels in *fis* mutant cells is a function of a general decrease in mRNA synthesis, we compared the expression patterns of *fis* and *lac* mRNA in *fis* cells (Fig. 8C). *lac* mRNA was expressed from a chromo-

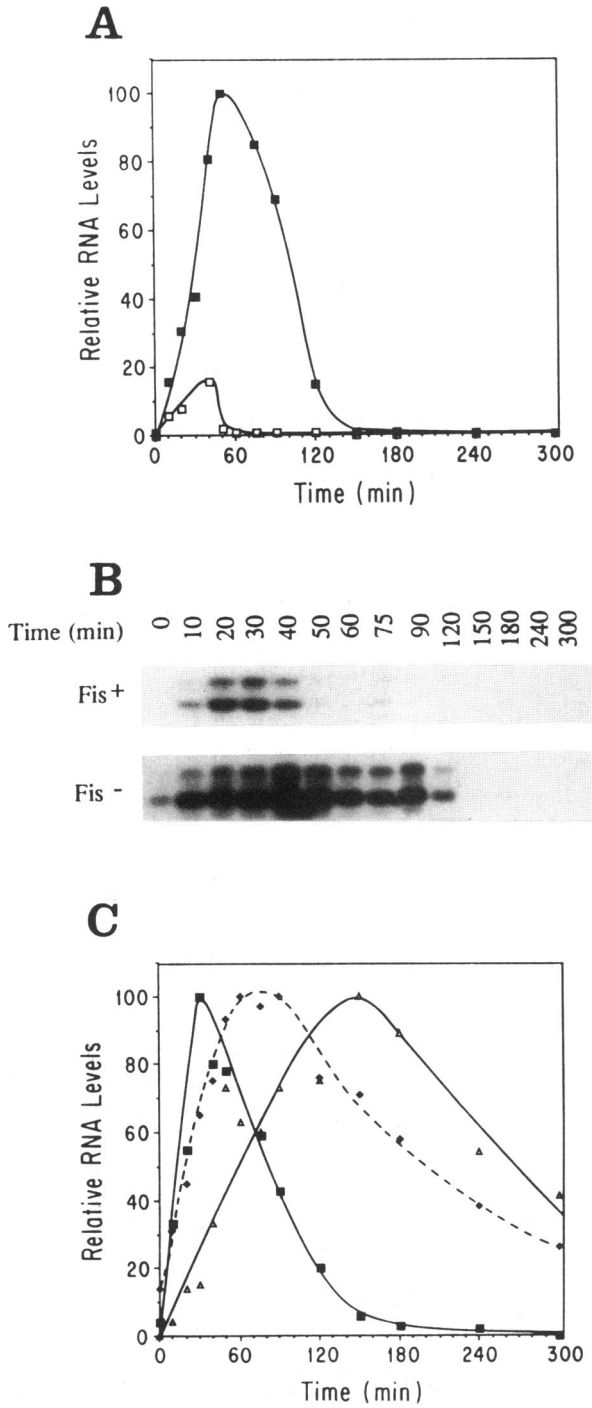

FIG. 8. *fis* autoregulation. (A) Saturated cultures of RJ1809 (*fis*$^+$) cells (□) and RJ1810 (*fis*) cells (■) were diluted 20-fold in LB medium and grown at 37°C. At intervals after subculturing, samples were taken and analyzed for relative *fis* mRNA levels per cell by primer extension. *fis* mRNA from *fis* mutant cells is measurable by this method because the *fis*::*767* mutation contains an insertion only in the *fis* coding sequence, leaving ORF1 and the *fis* promoter intact. Densitometry measurements of the primer-extended products after electrophoresis and autoradiography were adjusted to reflect actual amounts of total cellular RNA per cell obtained at each time point. A 100% value was assigned to the maximum value for *fis* mRNA measured in RJ1810 cells (85,560 absorbance units/10^9 cells). All other values, including those of RJ1809 cells, are shown as a proportion of the 100% value; less than 30 absorbance units/10^9 cells was detected after 240 min in RJ1810 and after 60 min in RJ1809 cells. (B) Autoradiography of *fis* mRNA primer extension products after electrophoresis on an 8% sequencing gel from RJ1809 cells (denoted Fis$^+$) and from RJ1810 cells (denoted Fis$^-$). At each time point, 10 μg of total RNA was used in the primer extension reactions. (C) Comparison of relative mRNA levels of *lac* (△) (derived from a chromosomal copy of *lacPL8UV5* [*lacI*::Tn*10*]) with *fis* (■) in RJ1810 cells. Experimental conditions and determination of the mRNA levels were the same as those described for panel A. Relative total RNA levels per cell (measured in micrograms per 10^9 cells) (◆) are shown. A 100% value was assigned to each maximum level of *lac* mRNA, of *fis* mRNA, or of total RNA per cell obtained.

TABLE 2. Effects of Fis mutations on *fis* expression

Multicopy plasmid[a]	Fis protein	β-Galactosidase units[b]	*fis* repression	DNA-binding activity[c]
pUC18		301	−	−
pRJ807	WT[d]	40	+	+
pRJ955	Δ17-21	47	+	+
pRJ956	Δ18-29	55	+	+
pRJ935	Ala34-Pro	82	+	+
pRJ943	Thr75-Pro	284	−	−
pRJ948	Arg85-Cys	212	−	−

[a] Plasmids pRJ955, pRJ956, pRJ935, pRJ943, and pRJ948 (transformed into RJ1838) are equivalent to pRJ807 (35) except that they contain the specified Fis mutations.
[b] Average of values from three parallel experiments.
[c] Results were taken from reference 35.
[d] WT, wild type.

somal copy of the *lacPL8UV5* promoter in a strain that does not contain the *lac* repressor (RJ1810). While *fis* mRNA levels begin decreasing 40 min after subculturing in LB, *lac* mRNA levels continue to gradually increase until 150 min after subculture, at which time the *fis* mRNA levels are approximately 7% of their peak values. At this point *lac* mRNA levels begin to decrease as the cells are approaching stationary phase. The earlier timing of the decrease of *fis* mRNA levels relative to *lac* in *fis* mutant cells indicates that the former is not due to a general shutoff in mRNA synthesis and suggests that another mechanism is contributing to the decrease of *fis* mRNA levels. A control system that operates in addition to autoregulation by Fis protein to keep *fis* mRNA levels reduced would be expected to be required in late-exponential and stationary phases since Fis protein levels are very low at these times. Such a system could involve an additional repressing factor or an intrinsic property of the *fis* promoter that causes its activity to be reduced early in the growth cycle. Possibilities for this mechanism include stringent response (6), ribosomal feedback control (21), and a sensitivity of the *fis* promoter to changes in DNA supercoiling density.

Ninnemann et al. (34) showed that *fis* is subject to stringent control and proposed that the stringent response is contributing to the repression of *fis* during late exponential and stationary phases. To test this idea, we examined the *fis* expression pattern in *relA* mutant cells. RelA (ppGpp synthetase I) is the major enzyme responsible for the production of ppGpp in response to amino acid starvation. In turn, ppGpp mediates repression of sensitive promoters, such as rRNA or tRNA promoters, in what is termed the stringent response (6). Cells that are deficient in RelA expression are therefore deficient in exhibiting the stringent response. If the stringent response were playing a significant role in the

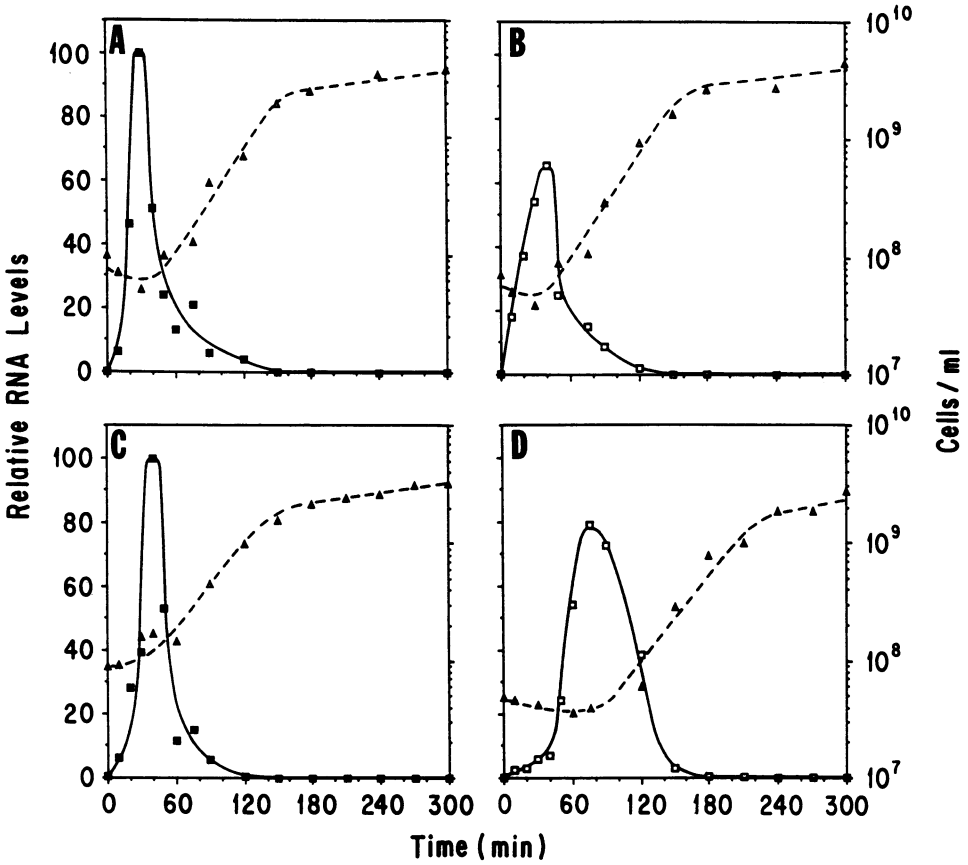

FIG. 9. Effect of ppGpp levels on *fis* mRNA expression pattern. Experimental conditions were the same as described in the legend to Fig. 8. Results are shown for primer extension analysis of *fis* mRNA in wild-type cells RJ1809 (■) (A), *relA* mutant cells CF1651 (□) (B), *fis* mutant cells RJ1799 (■) (C), and *relA spoT fis* mutant cells RJ1882 (□) (D). All values in panels A and B are shown as percentages of the maximum *fis* mRNA level in RJ1809. Similarly, all values in panels C and D are shown as percentages of the maximum *fis* mRNA level in RJ1799. △, cell growth.

repression of *fis*, particularly when Fis levels are low, one would predict that in *relA* mutant cells, *fis* transcript or Fis protein levels would be elevated during late exponential or stationary phase. However, this was not observed when *relA* mutant cells were subcultured in LB and assayed for *fis* expression. As illustrated in Fig. 9A and B, the pattern of expression and relative *fis* mRNA levels are comparable in *relA*$^+$ and *relA* mutant cells throughout the batch culture. Fis protein levels in *relA* mutant cells also show a pattern and level of expression similar to those in *relA*$^+$ cells (results not shown). These experiments show that *fis* is efficiently repressed during late exponential and stationary phases in cells incapable of mounting a stringent response.

A small amount of ppGpp can also be synthesized by ppGpp synthetase II, whose activity can be eliminated by *spoT* null mutations (18, 46). In order to determine whether ppGpp levels contribute at all to *fis* regulation, *fis* mRNA levels were compared for RJ1799 (*fis*) and RJ1882 (*relA spoT fis*). These strains were defective in Fis protein synthesis so that Fis autoregulation would not mask any regulatory effect mediated by ppGpp. Both strains gave a similar peak of *fis* mRNA that is coincident with the onset of cell growth (Fig. 9C and D). The expression of *fis* is delayed approximately 35 min in RJ1882, but this can be attributed to the greater lag time and slightly lower growth rate for RJ1882 than for RJ1799. We conclude that ppGpp is not an important determinant in controlling the levels of Fis during growth in LB, even under conditions when Fis autoregulation is not operating.

DISCUSSION

We have found that the levels of Fis protein vary dramatically during the course of growth of an *E. coli* culture. As noted previously (33, 34, 45), Fis levels are extremely low in stationary-phase cells. However, upon subculturing stationary-phase cells in LB, the level of Fis protein increases over 500-fold during the initial lag phase, reaching a peak of 50,000 to 100,000 copies per cell as cells enter exponential growth. After one or two cell divisions, Fis levels decrease in a manner that is consistent with a near total shutoff of nascent synthesis. Changes in the levels of *fis* mRNA with respect to the growth phase can largely account for the Fis expression pattern. *fis* mRNA levels increase approximately 1,000-fold upon subculturing, peak shortly before the protein levels, and then drop precipitously. The possibility of regulatory contributions by translational control remains to be investigated. To our knowledge, the expression pattern of Fis is unique among *E. coli* proteins.

The dramatic changes in Fis levels could have significant regulatory consequences. If one were to assume two chromosome equivalents and 75,000 Fis molecules (32,500 dimers) at the peak of Fis expression, sufficient Fis would be present to bind every 250 bp in the *E. coli* chromosome.

Therefore, Fis could be transiently binding to many sites of relatively low affinity throughout the chromosome and causing the activation or repression of a variety of operons or processes. In addition to regulatory effects mediated by binding to specific sites of variable affinities, the large changes in Fis levels could have a significant impact on overall chromosome structure.

Regulation of Fis expression. Control of Fis synthesis appears to respond to the nutritional status of the media. The most dramatic changes in Fis levels are seen after stationary-phase cells have been subcultured in LB medium. This can be considered an extreme case of a nutritional upshift. A rapid increase in *fis* expression is also observed when exponentially growing cells in minimal glucose medium are shifted to a richer LB medium. Thus, the burst in Fis expression is not solely a consequence of initiation of growth. However, since a nutritional upshift is always followed by an increase in growth rate, these experiments do not distinguish whether Fis is directly responding to the nutrients in the medium or to the increase in growth rate or both.

It is apparent that under certain conditions the promoter controlling *fis* is quite strong, though a comparison of the *fis* promoter sequence with known promoters predicts that the promoter should be relatively weak (30). The *fis* promoter sequence deviates from the consensus sequences in 5 of 12 nucleotides composing the −35 and −10 regions, which are separated by only 16 bp instead of the preferred 17 bp. In addition, a G+C-rich motif is present around the transcription initiation site. This promoter structure displays some similarities to stable RNA promoters (21), which raises the possibility that regulatory mechanisms involved in the control of stable RNA synthesis may be shared with *fis*. We have also found that RNA polymerase binds to a second region beginning 80 bp upstream of the transcription start site. RNA polymerase interacts at this site on a linear DNA fragment with higher affinity than it does to the *fis* promoter and does not require the presence of nucleotides to form heparin-resistant complexes. The importance of this RNA polymerase-binding site is not understood since growth phase regulation is observed when it is deleted (34, 36).

What is responsible for the transient nature of Fis expression seen following a nutrient upshift? The shutoff of Fis synthesis occurs at low cell densities: 10^8 cells per ml after a 1/100 dilution or 3×10^7 cells per ml after a 1/500 dilution of cells. At these densities in LB, it would seem unlikely that the cells are sensing a nutritional downshift that signals the *fis* promoter to reduce its activity. Indeed, LB medium taken from a mid-log-phase culture is still competent for nutritional upshift. Fis protein itself is negatively regulating its own synthesis (this paper, 34). *fis* mRNA levels reach an approximately sixfold-higher peak concentration in *fis* mutant cells after subculture compared with that in *fis*$^+$ cells and remain elevated for a longer time. In vitro experiments have shown that Fis binds to multiple sites located throughout the *fis* promoter region. The footprints of Fis at two of the high-affinity sites (I and II) overlap the region in which RNA polymerase binds at the *fis* promoter. The binding of Fis was shown to exclude the binding of RNA polymerase. Thus, Fis may be autoregulating its own synthesis by competing with RNA polymerase for binding to the *fis* promoter. The fact that only mutations in Fis that significantly reduce DNA binding are defective in autoregulation is consistent with this model (this paper, 25). While the binding of Fis to sites I and II may be of greatest importance in Fis autoregulation, the presence of six Fis-binding sites in the region raises the possibility that Fis binding may result in a highly wrapped nucleoprotein structure that contributes to repression.

In addition to Fis autoregulation, other regulatory events must also help bring about the decrease in Fis expression because *fis* mRNA levels still decrease in mutant cells deficient in Fis protein synthesis. In the batch culture experiments described in this paper, *fis* mRNA levels decrease well before *lac* mRNA and total RNA levels decrease, even in *fis* mutant cells. Indeed, *lac* mRNA levels continue to rise until cell growth begins to slow as the culture enters stationary phase (Fig. 8C). The fact that *fis* mRNA levels in wild-type cells are undetectable when Fis protein levels are also very low also supports the notion of an additional repressing mechanism acting upon the *fis* promoter. Ninnemann et al. (34) have demonstrated that Fis is subject to stringent control and have proposed that regulation by the stringent control system may be playing a critical role in growth phase regulation of Fis. We have observed, however, that normal growth phase expression of Fis occurs in the absence of both RelA and SpoT, the enzymes responsible for ppGpp synthesis. Moreover, there is no detectable increase in *fis* mRNA levels in late exponential or stationary phase in a *relA* or *relA spoT* mutant, as would be predicted if ppGpp levels were significantly influencing the decrease in *fis* expression during later stages of growth.

Other systems that normally regulate cellular growth rate may be operating to modulate Fis expression, and these systems may contribute to the transient nature of Fis synthesis. For example, Fis expression may be exquisitely sensitive to control by the ribosome feedback system in which excess ribosomes are believed to repress expression from stable RNA promoters (7). Preliminary experiments with cells growing in defined medium with Casamino Acids in a chemostat under limiting glucose indicate that the steady-state levels of Fis do vary as a function of growth rate. Approximately fourfold-higher levels of Fis were measured in cells growing with a generation time of 70 min (~14,000 molecules per cell) compared with the levels in the same culture growing with a 6-h generation time (3). In order to obtain an indication of the steady-state levels of Fis when cells are growing very rapidly in LB, we measured Fis protein levels after cells had been subcultured at a very low density (~50 cells per ml) and harvested after about 6 h or approximately 20 generations. Under these conditions, the measured Fis levels were found to be unexpectedly high (~50,000 molecules per cell) and to remain relatively constant at cell densities at which levels were found to drop in the 1/500-diluted culture. These findings suggest that under prolonged growth, cellular levels of Fis are influenced by the growth rate. The low Fis levels measured in the standard batch cultures during mid-exponential growth phase may indicate that true steady-state conditions are never achieved, even when cells are diluted 500-fold. Further studies under carefully controlled conditions will be required to fully understand the physiological and environmental parameters involved in controlling Fis expression levels during prolonged balanced growth.

Physiological relevance of the Fis expression pattern. It is particularly interesting to note that the expression pattern of Fis is reminiscent of that of eukaryotic primary response or immediate early genes in that Fis is expressed in a rapid and transient manner, the rapid increase in *fis* mRNA levels is independent of de novo protein synthesis, and most significantly, Fis is capable of activating a variety of other systems. For example, Fis has been shown to bind two sites within the *E. coli* origin of replication, *oriC*, and *fis* mutant

cells show some defects in DNA replication (10, 12). Fis has also been shown to function as a transcriptional activator of operons whose gene products are required for translation. These include operons involved in rRNA, tRNA, and translation elongation factors (32, 33, 39). The abrupt changes in Fis levels may be particularly relevant to this later activity as the rapid changes in cellular growth rate in response to changes in environmental conditions are accompanied by rapid changes in intracellular ribosome concentrations. Since the synthesis rate of rRNA is the major determinant that controls ribosome biosynthesis (21), Fis may be involved in facilitating the rapid response of ribosome biosynthesis to changes in the environment. In support of this, many strains of *E. coli* and *Salmonella typhimurium* that are deficient in Fis expression display increased lags upon subculturing, suggesting they have a reduced ability to quickly respond to a nutrient upshift (20). During steady-state growth the various levels of Fis seen with different growth rates may be important in fine tuning the rate of synthesis of ribosomes and other translation components. However, other systems that also control stable RNA synthesis can probably compensate for a loss of Fis to allow relatively good growth in its absence.

The shutoff of Fis synthesis in late-exponential phase appears to be an important step in the programmed series of events that occur as bacterial cells enter stationary phase. These steps are thought to be critical in enabling efficient survival and perhaps the rapid resumption of growth when favorable conditions are encountered (42). When Fis is constitutively expressed so that cells contain high levels of Fis during stationary phase, they have higher death rates than wild-type cells. Cells that do recover show prolonged lags before exponential growth is resumed (9). Thus, high levels of Fis may cause a physiological imbalance during stationary phase that is detrimental both to survival and to the efficient resumption of growth under renewed favorable conditions.

In summary, Fis may be functioning as an important regulator that can communicate the nutritional quality of the environment to various cellular processes. How the quality of the growth medium is so rapidly transduced to the control of Fis synthesis remains to be determined. It is also not understood how changing Fis levels affect the control of DNA replication. Perhaps Fis may also play a role in coupling the cell cycle to growth rate.

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