It has been shown that Fis activates transcription of the ribosomal promoter \textit{rrnB} P1; however, the mechanism by which Fis activates \textit{rrnB} P1 transcription is not fully understood. Paradoxically, although Fis activates transcription of \textit{rrnB} P1 \textit{in vitro}, transcription from the promoter containing Fis sites (as measured from \textit{rrnB} P1-lacZ fusions) is not reduced in a \textit{fis} null mutant strain. In this study, we further investigated the mechanism by which Fis activates transcription of the \textit{rrnB} P1 promoter and the role of Fis in rRNA synthesis and cell growth in \textit{Escherichia coli}. Like all other stringent promoters investigated so far, open complex of \textit{rrnB} P1 has been shown to be intrinsically unstable, making open complex stability a potential regulatory step in transcription of this class of promoters. Our results show that Fis acts at this regulatory step by stabilizing the interaction between RNA polymerase and \textit{rrnB} P1 in the absence of NTPs. Mutational analysis of the Fis protein demonstrates that there is a complete correlation between Fis-mediated transcriptional activation of \textit{rrnB} P1 and Fis-mediated stabilization of preinitiation complexes of the promoter. Thus, our study indicates that Fis-mediated stabilization of RNA polymerase-\textit{rrnB} P1 preinitiation complexes, presumably at the open complex step, contributes prominently to transcriptional activation. Furthermore, our \textit{in vivo} results show that rRNA synthesis from the P1 promoters of several rRNA operons are reduced 2-fold in a \textit{fis} null mutant compared with the wild type strain, indicating that Fis plays an important role in the establishment of robust rRNA synthesis when \textit{E. coli} cells are emerging from a growth-arrested phase to a rapid growth phase. Thus, our results resolve an apparent paradox of the role of Fis \textit{in vitro} and \textit{in vivo} in the field.

In nutrient-rich media, most RNA polymerase (RNAP)\textsuperscript{1} molecules inside rapidly dividing \textit{Escherichia coli} cells are engaged in transcription of a small set of genes in the genome, whose products, such as rRNA and tRNA, are primarily involved in translation (1, 2). However, when cells are shifted from nutrient-rich to nutrient starvation conditions, the cellular transcription machinery is reprogrammed to respond to the environmental cue, a process called the stringent response (for a review, see Ref. 3). One of the most dramatic effects of the stringent response is to “turn off” the expression of those genes that are most active in nutrient-rich conditions; thus, those genes are stringently regulated, and the promoters from these genes are called stringent promoters.

We and others have shown that one unique feature shared by all stringent promoters tested is that the interactions between RNAP and stringent promoters are intrinsically unstable (4, 5). Preinitiation complexes of stringent promoters are unstable because the steps prior to the first phosphodiester bond formation are reversible, with the intermediate closed and open complexes being in rapid equilibrium. The intrinsically unstable preinitiation complexes of stringent promoters probably represent the isomerized open complex that is competent for RNA synthesis. Formation of the first phosphodiester bond results in an initially transcribing complex, which becomes very stable. Contrary to stringent promoters, open preinitiation complexes at nonstringent promoters are generally very stable, and backward reactions to closed complexes are negligible (6). Moreover, we found that several rifampicin-resistant RNAPs, purified from the \textit{rpoB} mutants that exhibited the stringent response phenotype even in rich media (thus, they are called stringent RNAPs), further destabilize preinitiation complexes with the stringent promoters tested when compared with wild type RNAP (5, 7). As expected, all of these \textit{rpoB} mutations confer a slow growth phenotype (~50% of growth rate of wild type cells in rich media), and total RNA synthesis is reduced in these stringent RNAP mutants compared with wild type strain (8, 9). Other RNAP mutants that exhibited partial stringent response phenotypes have also been studied with similar results (10). Taken together, we and others proposed that a potential regulatory step in transcription of stringent promoters is at the interaction between RNAP and the promoter, presumably at the stability of the open complex, and hence factors that act at this regulatory step would modulate transcription of stringent promoters and the stringent (nutrient starvation) response (5, 10).

The \textit{rrnB} P1 promoter is one of the best studied stringent promoters in \textit{E. coli} (for a review, see Ref. 11). Because the sequences of the promoters/regulatory regions are conserved in all of the seven rRNA operons (12), it is likely that the \textit{rrnB} P1 is representative of the P1 promoters of all rRNA operons. There are multiple elements (both \textit{cis} and \textit{trans}) that contribute to the strength and the regulation of \textit{rrnB} P1 (13–19). Among them, Fis functions as a trans-activator of \textit{rrnB} P1 transcription (15).
Fis is a nucleoid-associated and site-specific DNA-binding protein that plays an important role in gene expression and site-specific DNA recombination (20, 21). The expression of fis is subject to growth phase regulation, with its peak expression around the first doubling time after overnight cultures are diluted into a fresh rich medium; thus, the cellular level of Fis is highest at the very early log phase and lowest at the stationary phase (22, 23). Fis exists as a dimer in solution, and its crystal structure is known (22–26). Mutational analyses indicate that the domains in Fis that are important for its different functions are distinct (27, 28). For example, the “activation patch” of Fis, responsible for transcriptional activation of several promoters including rnrB P1, is located in residues Gln68, Arg71, Gly72, and Gln74, within and flanking the β-turn connecting helices B and C adjacent to the C-terminal helix-turn-helix DNA binding motif (29–32), whereas residues near the N terminus (Val16, Asp20, and Val22) are critical for the DNA invasion activity (26–28). Although there are three Fis binding sites in rnrB P1, most activation by Fis is attributable to site 1 of the promoter, where Fis interacts with the C-terminal domain of the α subunit of RNAP (15, 30, 32, 33). However, other rrn promoters, such as rrnA P1, require all three Fis binding sites for effective activation (34, 35).

The mechanism underlying the activation of rnrB P1 by Fis has not been fully elucidated, although much is known about the interactions between RNAP and Fis at the ribosomal promoter (30, 36). Paradoxically, although Fis activates transcription of rnrB P1 in vitro, transcription from the promoter containing Fis sites (as measured from rnrB P1-lacZ fusions) is not reduced in a fis null mutant strain (15, 37). Moreover, the effects of the fis mutations on cell growth are inconsistent and often negligible compared with wild type cells (15, 37, 38). To account for the apparent contradictions between the in vitro and in vivo results by Fis, a feedback mechanism was proposed for the regulation of rnrB P1 (15, 37).

In this study, we investigated the mode of action by Fis on the transcriptional activation of rnrB P1. We demonstrated that Fis stabilizes the interaction between RNAP and the rnrB P1 promoter, probably at the step of open preinitiation complexes in the absence of NTPs, thus leading to enhanced transcription. Furthermore, our results demonstrate that Fis plays an important role in establishing robust rRNA synthesis when cells emerge from an arrested state in stationary phase to a rapidly growing state in early log phase.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—Nucleotides, [γ-32P]UTP, and [γ-32P]ATP were purchased from Amersham Biosciences. Glutamic acid, potassium glutamate, and harnep were from Sigma. Magnesium glutamate was from Fluka. Plasmid pRLG1617 containing the rnrB P1 promoter (−88 to +1) and rnrB P1T12 terminators was kindly provided by Wilma Ross and Richard Gourse (University of Wisconsin, Madison, WI) and described (5). Plasmid pDJ4335, a derivative of pRLG1617, contains a mutant rnrB P1 promoter in which all the G/C in the discriminator sequence upstream of +1 was changed into A/T by PCR-mediated mutagenesis. The DNA sequences of the two primers for the amplification of the rnrB P1 DNA (−88 to +1) and introduction of the discriminator mutations were 5′-CGGATCCCAGCTGATTTGTTGTTGAAT (upper strand) and 5′-CCGATCTTTTAACTTATTAGGATTAT (lower strand). The lower strand primer has mutations in the discriminator region (underlined in the above sequences). PCR was performed using pRLG1617 DNA as template. The PCR product was digested with EcoRI and HindIII and then inserted into the EcoRI-HindIII sites of pRLG1617 to replace the wild type rnrB P1 sequence. The plasmid supercoiled DNA was purified with a Qiagen plasmid purification kit. RNAPs were purified as described (39). The stringent RNAPs (Rpb1114, Rpb3770, and Rpb3449) were described (5). The wild type and mutant Fis proteins used in this study were overproduced and purified as described elsewhere (30, 32).

**Bacterial Strains and Bacteriological Techniques**—Basic bacterial techniques were used as described previously (40). The E. coli strains used were K12 MG1655 (laboratory collection from Carol Gross, University of California, San Francisco) and its derivatives. The fis null mutant strains were constructed by phage P1 transduction with a lysate made from strain RLG1351 (MG1655 Δ inx744/λ rnrB P1 fis::kan) (5), and Kan’ transductants were selected.

**In Vitro Transcription Assays**—Reactions were performed at room temperature (−23 °C) essentially as described previously (5). The transcription reactions with 0.05 mM [γ-32P]ATP, 1.5 mM [γ-32P]UTP, and [γ-32P]CTP were incubated at 37 °C in a 50 mM Tris-HCl buffer (pH 8.0), 10 mM MgCl2, and the indicated concentrations of KCN/NaCl. The latter concentrations of supercoiled plasmid and RNAPs were 4–5 and 2–4 nM, respectively. Each of the Fis proteins (wild type and the mutants) was titrated, and the concentration (usually ~150 nM) that gave maximal activation of the rnrB P1 effect was used in the reactions as indicated. The reaction mixtures were preincubated for 15 min, and reactions were initiated by the addition of NTPs (0.2 mM ATP, CTP, and GTP and 0.02 mM UTP including 5 μCi of [γ-32P]UTP). When indicated, heparin (10 μg/ml for KCN and 100 μg/ml for potassium glutamate) was added with NTPs at the same time. After 10 min, reactions were terminated by the addition of equal volumes of 5% (w/v) formamide, 20 mM EDTA 0.1% bromphenol, and 0.1% xylene cyanol and analyzed on a 6% sequencing gel (National Diagnostics). The signals were visualized and quantified using PhosphorImager equipment (STOR 650; Amersham Biosciences).

**Determination of the Kinetics of Inactivation of Preinitiation Complexes at rnrB P1 by the DNA Competitor Heparin**—Conditions were the same as described above for in vitro transcription with the following modifications. After preincubation of RNAP, Fis protein, and DNA template for 15 min, heparin was added into reactions at time 0. Aliquots were taken and mixed with NTPs at specified time intervals after the addition of inhibitor. The transcription reactions were allowed to continue for another 10 min before being terminated and analyzed as described above. The data were analyzed, and half-lives were calculated. To ensure consistency in the measurement of half-lives of preinitiation complexes in the presence of Fis mutant proteins, we always included wild type Fis in the same set of experiments.

**RNA Extraction**—Overnight cultures were diluted 20-fold in fresh prewarmed LB medium and grown at 37 °C. Total RNA was extracted from 3 ml of cell cultures after dilution at the indicated time intervals by a hot phenol procedure (41). The RNA samples were further purified by use of RNaseasy Mini Kit (Qiagen) and eluted from the RNeasy column in 30 μl of diethylpyrocarbonate-treated water according to the manufacturer’s instructions. The RNA concentration was determined by measuring the optical density at 260 nm.

**Primer Extension Analysis**—Primer NSL186 (5′-GGGTTGCTGATA-ATACGGC-3′) was used in extension reactions to detect the RNA synthesized exclusively from the P1 promoters of four E. coli rRNA operons (rnrB, rnrC, rnrR, and rnrN). Primer NSL939 (5′-ACGAGATACACC-GTAAAATCC-3′) was used to determine the RNA synthesized from the omaA promoters, P1 and P2. The expected extension products were 119 bases for rnrB P1 and 93 and 89 bases for omaA P1 and P2, respectively. About 1 μg of total RNA and 2 pmol of each 32P-labeled primer were used in the extension reactions, using the AMV reverse transcriptase primer extension system (Promega) at 42 °C in accordance with the manufacturer’s protocol. After primer extension, the samples were loaded on 6% sequencing gel and analyzed by the PhosphorImager. The length of the transcripts was determined using corresponding sequencing reactions as references.

**Measurement of the Stability of the Leader rRNAs Initiated at the rnr P1 Promoters in Vivo**—The overnight cultures of both MG1655 and MG1655 fis (DJ2592) were diluted 20-fold in fresh prewarmed LB medium and grown at 37 °C. After 30 min of incubation, rifampicin was added to the cells to a final concentration of 250 μg/ml, and samples were removed for RNA extraction at various intervals (0, 20, 40, 60, 120, and 240 s). For time 0, the RNA was extracted immediately before the addition of rifampicin. The RNAs were extracted, processed, and analyzed by primer extension for the leader rRNAs initiated at the rnr P1 promoter as described above. The amount of RNA samples used for primer extension assay was 1 μg for MG1655 and 2.5 μg for MG1655 fis. The products of primer extension were resolved on 6% sequencing gel and analyzed by phosphorimaging.
Stringent RNAPs That Further Destabilize the Intrinsically Unstable Preinitiation Complexes at rrnB P1 Display Enhanced Activation by Fis—To understand the mechanism by which Fis activates transcription of rrnB P1, we initially analyzed the effect of Fis on the transcription of the promoter with the stringent RNAPs described above (Fig. 1). Although Fis activates transcription of rrnB P1 by wild type RNAP greatly only at high concentrations of KCl or NaCl (150 mM) in vitro (15), the experiments were performed with low salt (30 mM KCl), because the syntheses of rrnB P1 by the stringent RNAPs were extremely sensitive to high salt (5). Previously, we showed that whereas stringent RNAPs (RpoB114, RpoB3449, and RpoB3370) are defective in transcription of rrnB P1, resulting in a 2–3-fold reduction in transcripts compared with the amount obtained in the absence of the DNA competitor (compare lanes 3, 5, and 7 in Fig. 1A with lanes 3, 5, and 7 in Fig. 1C), consistent with the fact that the preinitiation complexes formed with these mutant RNAPs are significantly more unstable than wild type RNAP. Moreover, the Fis-mediated transcriptional activation of rrnB P1 by the stringent RNAPs was significantly increased in the presence of heparin, ranging from 4- to 6-fold. Note that these stringent RNAPs were still defective in transcription of rrnB P1 compared with wild type RNAP in the presence of Fis.

It has been shown that these stringent RNAPs further destabilize the preinitiation complexes at rrnB P1 compared with wild type RNAP (5). The fact that Fis activated transcription from rrnB P1 by the stringent RNAPs to a greater extent than by wild type RNAP under the conditions used indicates that Fis manifests a greater effect on the relatively unstable initiation complexes. Thus, our results suggest that Fis stabilizes metastable preinitiation complexes of the rrnB P1 promoter, leading to increased transcription.

FIG. 1. Effect of Fis on the transcription of rrnB P1 by wild type (WT) and stringent mutant RNAPs in vitro. In vitro transcription assays were performed with 30 mM KCl in the absence (A and B) or the presence of heparin (C and D) as described under “Experimental Procedures.” A and C show autoradiograms of representative in vitro transcription assays. The transcripts from rrnB P1 (—170 nucleotides) and RNAI (—110 nucleotides) are indicated. The amounts of rrnB P1, normalized to RNAI, made by different RNAPs in the absence of heparin (B) or in the presence of heparin (D), were plotted. The results presented are the average of three independent experiments, and the numbers in parenthesis represent the -fold activation by Fis.

A

B

C

D

with lane 1 in Fig. 1A), indicating that the preinitiation complexes were relatively stable at 30 mM KCl. Under this condition, Fis activated the transcription only marginally (1.6-fold). However, heparin exacerbated the defects of the stringent RNAPs in transcription of rrnB P1, resulting in a 2–3-fold reduction in transcripts compared with the amount obtained in the absence of the DNA competitor (compare lanes 3, 5, and 7 in Fig. 1A with lanes 3, 5, and 7 in Fig. 1C), consistent with the fact that the preinitiation complexes formed with these mutant RNAPs are significantly more unstable than wild type RNAP. Moreover, the Fis-mediated transcriptional activation of rrnB P1 by the stringent RNAPs was significantly increased in the presence of heparin, ranging from 4- to 6-fold. Note that these stringent RNAPs were still defective in transcription of rrnB P1 compared with wild type RNAP in the presence of Fis.
little rrnB P1 transcription occurred in the presence of heparin and salt concentrations above 50 mM even with Fis (data not shown). With potassium glutamate, which is the major intracellular salt in E. coli (43), Fis activates transcription of rrnB P1 from 200 to 500 mM either in the absence or presence of heparin (Fig. 2). Thus, we initially measured the half-life of RNAP-rrnB P1 complexes both at 40 mM KCl and 200 mM potassium glutamate.

At 40 mM KCl in the absence of Fis, only a very small amount of rrnB P1 transcripts were synthesized by the wild type RNAP after a 15-s incubation with heparin, indicating that only a few complexes competent for initiation remained (Fig. 3A). Although the fast decay of the preinitiation complexes prevented us from obtaining an accurate value of the half-life, it was estimated to be much less than 15 s. However, in the presence of Fis, the preinitiation complexes became relatively resistant to heparin challenge (Fig. 3A), and the half-life of the RNAP-rrnB P1 complexes increased to about 30 s (Fig. 3B).

Similarly, Fis also increased the half-life of wild type RNAP-rrnB P1 preinitiation complexes when 200 mM potassium glutamate was used (Fig. 3, C and D). With potassium glutamate, the half-life of the RNAP-rrnB P1 complexes in the absence of Fis was about 7 min, whereas in the presence of Fis, the half-life of the RNAP-rrnB P1 complexes increased to about 16 min. With either salt, Fis had no effect on the stability of preinitiation complexes at the RNAI promoter under the conditions used.

We also determined the effect of Fis on the stability of RNAP-rrnB P1 preinitiation complexes with one of the least defective mutant RNAPs: RpoB3449. At 40 mM KCl, even in the presence of Fis, the interaction between RpoB3449 and rrnB P1 was too weak to measure the half-life of the RNAP-rrnB P1 complexes (data not shown). At 200 mM potassium glutamate (Fig. 4), without Fis, RpoB3449 made a very small amount of the rrnB P1 transcripts when challenged with heparin, indicating that the RpoB3449-rrnB P1 preinitiation complexes were extremely unstable; the half-life of the complexes was estimated to be much less than 15 s. However, with Fis, RpoB3449 was able to make a noticeable amount of rrnB P1 transcripts in the presence of heparin, and the half-life of the complexes increased to ~3.5 min. It should be noted that although Fis exhibited a more dramatic effect on stabilizing the preinitiation complexes at rrnB P1 with the mutant RNAP than with the wild type enzyme (which is consistent with the *in vitro* transcription data (Fig. 1) where Fis activated transcription of rrnB P1 by the stringent RNAP to a greater extent than that by wild type RNAP), the overall stability of the rrnB P1 preinitiation complexes with the stringent RNAP in the presence of Fis was still low compared with that with wild type RNAP.

It has been shown that a G/C-rich region called a discriminator sequence (44) contributes to the instability of preinitiation complexes at several stringent promoters (45–47). To determine whether Fis overcomes the negative effect of the discriminator sequence on the initiation complexes of rrnB P1, we constructed a mutant rrnB P1 promoter in which the discriminator sequence was mutated and studied the effect of Fis on the transcription of the mutant promoter (Fig. 5). Compared with the wild type rrnB P1 promoter, the mutant promoter had high activity by both wild type RNAP and the stringent RNAP RpoB3449 in the absence of Fis. Moreover, Fis did not manifest transcriptional activation of the mutant promoter by either wild type or the stringent RNAP. As expected, the preinitiation complexes of the mutant rrnB P1 promoter were stable with a half-life of about 135 min either with or without Fis (data not shown). Our results indicate that Fis, the stringent RNAP, and the discriminator sequence of rrnB P1 affect the same step, which is likely to be a form of the open complex, but in opposite directions to modulate transcription at stringent promoters.

**Mutant Fis Proteins Defective in Activating Transcription of rrnB P1 Are Impaired in Their Ability to Stabilize RNAP-rrnB P1 Complexes**—The above results indicate that Fis-mediated transcriptional activation of rrnB P1 is the consequence of Fis-mediated stabilization of the preinitiation complexes. To provide further support for this argument, we took advantage of mutational analysis of Fis. Fis mutants that are defective in transcriptional activation at the rrnB P1 promoter and other functions have been described (29, 30, 32). We hypothesized that mutant Fis proteins defective in activating transcription of rrnB P1 would also be impaired for stabilization of RNAP-rrnB P1 complexes, whereas complexes formed with Fis proteins that are defective in other functions would be stabilized. To test this model, we used Fis mutant proteins that altered amino acid residues at the “activation patch” (Gln⁶⁸, Arg⁷¹, and Gly⁷²) important for transcriptional activation of rrnB P1 (30, 32). Also, as a control, we used a mutant Fis protein that altered residue Asp⁴⁰, whose mutation is not located in the putative interface between Fis and RNAP but is important for Hinc–catalyzed site-specific inversion (26, 27).

First, we determined the effects of different Fis proteins on transcriptional activation of rrnB P1 in the presence of 200 mM potassium glutamate (Fig. 6, A and B). Wild type Fis stimulated the synthesis of rrnB P1 about 4-fold when compared with levels generated in the absence of Fis under the conditions used. Two Fis mutant proteins, D20K and R71Y, behaved similarly to the wild type Fis protein. Two other Fis mutant proteins, Q68A and R71K, were partially defective in stimulating transcription of rrnB P1 and activated transcription of rrnB P1 only about 2.6- and 1.5-fold, respectively. The other three Fis mutant proteins, R71A, R71Q, and G72A, had minimal activity (~<30% of wild type Fis activity) in stimulating transcription of rrnB P1. Similar results were obtained when 150 mM NaCl was used in reactions (data not shown).

Second, we analyzed the effects of these mutant Fis proteins on the stability of preinitiation complexes at rrnB P1 (Fig. 6C). As expected from our model, there is a complete correlation between the ability of a Fis protein to activate transcription of rrnB P1 and its ability to stabilize RNAP-rrnB P1 complexes. For example, the two mutant Fis proteins (D20K and R71Y)
that were proficient for transcriptional activation behaved exactly like wild type Fis protein in stabilizing the RNAP-rrnB P1 complexes, with a value for the half-life of the complexes estimated to be 18 min. The three Fis mutant proteins (R71A, R71Q, and G72A) that exerted minimal transcriptional activation activity of rrnB P1 also had only a minimal effect on the stability of RNAP-rrnB P1 complexes in initiation. The value of the half-life of RNAP-rrnB P1 complexes in the presence of these Fis mutant proteins was about 8–9 min, a value very similar to that obtained in the absence of Fis. The other two Fis mutant proteins (Q68A and R71K) that only partially activated transcription of rrnB P1 also partially retained their abilities to

**Fig. 3.** Effect of Fis on the stability of preinitiation complexes of rrnB P1 with wild type RNAP in the presence of different salts. The experiment was performed as described under “Experimental Procedures.” The transcription reactions without heparin addition are indicated with a minus sign. A, autoradiogram of a representative in vitro transcription assay. B, the transcription activities of rrnB P1 and RNAI are plotted as a function of time (after the addition of heparin).

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**Fig. 4.** Effect of Fis on stability of preinitiation complexes of rrnB P1 with the mutant RNAP, RpoB3449. The experiment was performed with 200 mM potassium glutamate as described under “Experimental Procedures.” The transcription reactions without heparin addition are indicated with a minus sign. A, autoradiogram of a representative in vitro transcription assay. B, the transcription activities of rrnB P1 and RNAI are plotted as a function of time (after the addition of heparin).
Fis Stabilizes Interaction between RNAP and rRNAP P1

stabilize RNAP–rRNAP P1 complexes, with half-life values of 13 and 11 min, respectively. The complete agreement of the behaviors of these mutant Fis proteins in the two assays strongly suggests that the two activities of Fis in transcription of the ribosomal promoter (stabilization of the preinitiation complexes and activation of the transcription) are intimately related.

**Fis-mediated Transcriptional Activation of Ribosomal RNA**

**Synthesis Is Manifested during Early Log Phase in Vivo**—The in vitro studies described above would predict that Fis plays an important role in regulation of the synthesis of rRNA operons inside the cell (all seven rRNAP operons in *E. coli* have similar sequences in the P1 promoter/regulatory regions) under some physiological conditions. However, a fis null mutation has no or minimal effect on the expression of rRNAP P1 containing Fis sites in vivo (15, 37, 48). This lack of the in vivo phenotype of the fis mutant could be due to two particular conditions used in measuring the expression of rRNAP P1: 1) assays were indirect measurements for rRNA synthesis by following the β-galactosidase activity, which is very stable, from rRNAP P1-lacZ fusions; and 2) assays were from single point sampling rather than by following kinetics of cell growth.

We decided to determine the effect of fis on rRNA synthesis from the P1 promoters by following the expression of rRNA leader sequences directly as a function of cell growth (Fig. 7). Since the leader sequences of rRNAs are processed and degraded very rapidly in the cell (49–51), the levels of the leader sequences of rRNAs should reflect the rate of rRNA syntheses from the P1 promoters. The kinetic study is essential because the expression of the fis gene, and thus the level of Fis inside the cell is growth phase-dependent (22). The level of Fis peaks during early log phase (30,000–60,000 dimers/cell in rich media) and is minimal in stationary phase (≤100 dimers/cell). Thus, it is likely that the effect of the fis mutation on the synthesis of rRNA will be manifested most dramatically during early log growth phase, when the Fis level peaks inside wild type cells. Following a protocol as described (22), overnight cultures were diluted 20-fold with fresh prewarmed LB, and cell growth was followed (Fig. 7A). Total RNA was isolated from both wild type and fis mutant cells at different time points during cell growth and from overnight cultures. Synthesis of rRNA was determined by primer extension method using a primer that hybridizes to a conserved region of the leader
The expression of rRNA from the P1 promoters as a function of cell growth in wild type and an isogenic fis mutant. Overnight cultures of both wild type (MG1655) strain and the MG1655 fis null mutant (DJ2592) were diluted 20-fold with prewarmed LB and grown at 37 °C. Total RNA was extracted from cell cultures at the indicated intervals and overnight cultures (0 min). Equal amounts of RNA samples (~1 μg) were used in primer extension assays as described under “Experimental Procedures.” A, the growth curves of MG1655 (filled squares) and MG1655 fis (open circles) in LB at 37 °C after cell dilutions. B, an autoradiograph of a representative primer extension assay. The locations of cDNA products (119 bases for rRNA, 93 and 89 bases for ompA mRNA) are indicated. C, the relative amount of rRNA synthesized from the P1 promoters of rRNA operons, which is normalized to the level of ompA mRNA, is plotted as a function of cell growth in the MG1655 (filled circle) and MG1655 fis (open circle). The data represent an average of three independent experiments.

we did not know if the expression of ompA is affected by fis.

As shown in Fig. 7B, the expression of rRNAs was growth phase-dependent; in contrast, the levels of ompA transcripts were relatively constant during different growth phases (growth phase-independent) in both wild type and the fis mutant strains. Thus, the expression of rRNA (as ratios of the leader sequence transcripts over ompA transcripts) was quantified as a function of cell growth (Fig. 7C). Only a small amount of the leader sequences of rRNAs and ompA was detected in cultures grown overnight (time 0) for both strains, indicating that there was minimal transcription activity inside late stationary phase cells. However, significant amounts of the leader sequences of rRNAs were detected only 10 min after culture growth, and their expressions peaked around 30–45 min following the nutrient upshift. Afterward, the amounts of the leader sequences of rRNAs declined rapidly when cells growth slowed (60 min) and became minimal when cultures approached stationary phase (≥120 min) (Fig. 7, compare B with A).

Indeed, we found that the effect of the fis mutation on the expression of rRNAs was manifested most dramatically during the early log phase of cultures (10–45 min), parallel to the peak levels of the Fis protein in wild type cells (22). The amounts of the leader sequences of rRNAs in the fis mutant were reduced 2-fold compared with that in wild type cells. This significant difference in the amounts of the leader sequences of rRNAs between the two strains truly reflects the effect of fis on the synthesis of the transcripts, because we found that the half-life of the leader sequences of rRNAs to be the same (~55 s) in the two strains (Table I).

Interestingly, the temporal expression profiles of rRNA in early log phase were very similar in both wild type and the fis mutant strains, indicating that element(s) other than Fis are also involved in the activation of the rrnB P1 promoters of rRNA operons. Also, the amounts of rRNA in the later growth phases appeared to be higher in the fis mutant than that in wild type cells, suggesting that the regulation of rRNA synthesis is also altered after early log phase in the fis mutant.

The above experiments suggest that the effect of the fis mutation on cell growth is manifested primarily during early log phase, since it impairs the synthesis of rRNA most significantly during this period of growth compared with wild type cells. When we followed cell growth (as a population) in LB broth (Fig. 7A), we found that the fis mutant has only a slightly slower growth rate (~10% reduction) compared with wild type cells, similar to reports by others (15, 38, 48, 54). However, when we used a more sensitive assay to monitor the rate of single cell growth by following colony formation of both wild type and the fis mutant cells on solid LB media as a function of time, we observed the expected results (Fig. 8). Indeed, after 16 h of growth at 30 °C, there was a dramatic difference in colonies size between the two strains; whereas wild type cells formed recognizable colonies, the fis mutant cells only formed tiny colonies. The differences in growth between the two strains, however, were diminished upon prolonged incubation (after 40 and 64 h of growth). Significant differences in appar-

**TABLE I**

The half-life (seconds) of rRNA leader sequences in wild type and the fis mutant strains

|        | MG1655 | MG1655 fis |
|--------|--------|------------|
| s      | 55.0 ± 6.0 | 56.0 ± 3.0 |

Fig. 7. The expression of rRNA from the P1 promoters as a function of cell growth in wild type and an isogenic fis mutant. Overnight cultures of both wild type (MG1655) strain and the MG1655 fis null mutant (DJ2592) were diluted 20-fold with prewarmed LB and grown at 37 °C. Total RNA was extracted from cell cultures at the indicated intervals and overnight cultures (0 min). Equal amounts of RNA samples (~1 μg) were used in primer extension assays as described under “Experimental Procedures.” A, the growth curves of MG1655 (filled squares) and MG1655 fis (open circles) in LB at 37 °C after cell dilutions. B, an autoradiograph of a representative primer extension assay. The locations of cDNA products (119 bases for rRNA, 93 and 89 bases for ompA mRNA) are indicated. C, the relative amount of rRNA synthesized from the P1 promoters of rRNA operons, which is normalized to the level of ompA mRNA, is plotted as a function of cell growth in the MG1655 (filled circle) and MG1655 fis (open circle). The data represent an average of three independent experiments.

sequences initiated exclusively from the P1 promoters of four rRNA operons (rrnB, rrnA, rrnC, and rrnG). For comparison, we also determined the expression of the ompA gene in the same reactions. We chose ompA as a control, because its expression is known to be relatively abundant (52, 53), although...
ent growth rates on solid media are also observed when cells are plated from log phase liquid cultures (data not shown).

To further study the effect of fis on cell growth, we took advantage of the stringent RNAP mutant rpoB3449. Because Fis had a greater effect on the transcriptional activation of rrnB P1 by the stringent RNAP compared with wild type RNAP in vitro, it is possible that the fis rpoB3449 double mutant would manifest a greater effect on cell growth than the single rpoB3449 mutant. Thus, we constructed the fis rpoB3449 double mutant and followed the growth of the double mutant and the single rpoB3449 mutant strains after 24-h stationary phase cultures were diluted into fresh LB media. For comparison, wild type and the single fis mutant strains were also included in the assays. As shown in Fig. 9, the fis rpoB3449 double mutant had the slowest growth rate with a doubling time of about 40 min, whereas the single rpoB3449 mutant had a doubling time of about 30 min. Note that the fis rpoB3449 double mutant exhibited essentially only linear growth. Whereas the wild type strain started growth at the first time point (15 min), the single fis mutant had a prolonged lag phase of about 30–45 min. However, during the log phase growth, there was only a 10% difference in growth rate between the wild type (doubling time 22 min) and the fis mutant (doubling time 24 min). Note that the single rpoB3449 mutant also had a prolonged lag phase, similar to the single fis mutant. It is known that the fis promoter is a stringent promoter (55, 56); thus, it is likely that the expression of fis is reduced in the stringent RNAP mutant rpoB3449. Indeed, the expression of fis in the rpoB3449 mutant was reduced both kinetically (delayed about 60 min) and quantitatively (the peak level reduced about 3-fold) when compared with wild type cells under the same conditions (data not shown). Taken together, the above results are consistent with the notion that Fis plays an important role in the establishment of a robust growth when E. coli cells are emerging from a growth-arrested state (stationary phase) to a rapid growth phase (log growth).

**DISCUSSION**

In this study, we investigated the mechanism underlying the Fis-mediated transcriptional activation of the rrnB P1 promoter and the role of fis in rRNA synthesis as a function of cell growth in E. coli. Our results showed that Fis stabilizes the interaction between RNAP and rrnB P1, a regulatory step in transcription of the stringent promoter, thus leading to transcriptional activation of the promoter. Consistent with the in vitro data, our in vivo results demonstrate that Fis plays an important role in the establishment of robust rRNA synthesis when E. coli cells emerge from an arrested state in stationary phase to a rapid growth state in early log phase.

The Mode of Action of Fis in Transcriptional Activation of rrnB P1—Like other stringent promoters tested so far in vitro, the preinitiation complexes of rrnB P1 are intrinsically unstable because of the readily reversible nature of the steps prior to the formation of the first phosphodiester bond. Consequently, the interactions between RNAP and stringent promoters are unusually sensitive to salt concentration, DNA competitor, and superhelicity of the DNA template (4, 5, 57). The ATP concentration has been shown to be important for transcription of rrnB P1 (17, 58), probably by modulating the rate of the first phosphodiester bond (pppApC) formation. After the synthesis of pppApC, preinitiation complexes at rrnB P1 convert to an initial transcribing complex, which becomes very stable (4). This unique feature of stringent promoters (i.e., an intrinsically unstable open complex) is very likely the basis for the regulation of these genes and the stringent response. Thus, factors affecting this regulatory step of rrnB P1 would most effectively modulate the activity of the promoter. Previously, we found that the stringent RNAPs further destabilize preinitiation complexes at stringent promoters, leading to reduced RNA synthesis at rrnB P1 and other stable RNA operons (5, 9). Results from this study demonstrated that Fis also affects this regulatory step by stabilizing the preinitiation complexes at the rrnB P1 promoter, thus shifting the equilibrium toward the open complex. Our results argue that this mode of action may be largely responsible for Fis-mediated transcriptional activation of rrnB P1. In support of this model, mutational analysis of the Fis protein showed that there is a complete correlation between Fis-mediated transcription activation of rrnB P1 and Fis-mediated stabilization of preinitiation complexes of the promoter (Fig. 6), indicating that the two activities of Fis on the rrnB P1 promoter are coupled.

The fact that Fis stabilizes preinitiation complexes at rrnB P1 with both wild type and the stringent RNAPs indicates that this is a general function of Fis in transcription of the ribosomal promoter. Our results indicate that because the stringent RNAPs and the Fis protein both affect the same regulatory step...
in the initiation of \textit{rrnB} P1, but in opposite directions, the net outcome of the transcription of the ribosomal promoter by stringent RNAPs in the presence of Fis is likely to be determined by the competing effects of the two proteins. In our study, the effects of the stringent RNAPs on destabilizing the preinitiation complexes of \textit{rrnB} P1 are dominant over the effect of Fis in stabilizing the initiation complexes; thus, the mutant RNAPs are still defective in the synthesis of \textit{rrnB} P1 in the presence of Fis in vitro (37). As expected, these stringent \textit{rppB} mutants with wild type \textit{fis} still exhibit a stringent phenotype and have a very slow growth phenotype even in rich media (5, 8). Fis has also been found to almost completely compensate for the defects of other mutant RNAPs in \textit{rrnB} P1 transcription in vitro (37). Consequently, those RNAP mutants with wild type \textit{fis} have growth rates similar to wild type cells.

The mode of action of Fis in the initiation of \textit{rrnB} P1 described in this study is consistent with some of the effects of Fis on the promoter reported previously. For example, it has been shown that Fis reduces the apparent \textit{K}_o for ATP, the initial nucleotide for the \textit{rrnB} P1 transcript (37). It is likely that the stabilization of the open complex at \textit{rrnB} P1 by Fis is responsible for the decrease in the apparent \textit{K}_o for ATP at the ribosomal promoter. In addition, it might explain why Fis has reduced the equilibrium dissociation constant for RNAP binding to \textit{rrnB} P1 2.5-fold in DNase I footprinting assays (36). Also, it has been reported that Fis activates transcription of \textit{rrnB} P1 by wild type RNAP greatly at high salt concentrations (>100 mM NaCl) but exhibits only minimal or no effect at low salt concentrations (15). This is probably due to the fact that the interaction between RNAP and the \textit{rrnB} P1 promoter is relatively less stable at high salt concentrations but relatively more stable at low salt concentrations. For example, at salt concentrations of >50 mM NaCl or KCl, very few preinitiation complexes survive a challenge by heparin added at the same time as NTPs (data not shown), whereas most preinitiation complexes are stable enough to survive the heparin challenge at low salt concentration (30 mM) (Fig. 1). This also can explain why Fis exhibits greater transcriptional activation of \textit{rrnB} P1 by the stringent RNAPs at low salt concentration, since those mutant RNAPs form less stable preinitiation complexes at the promoter compared with wild type RNAP (Fig. 1). Our \textit{in vitro} results demonstrated that Fis exhibits a greater effect on relatively unstable open complex, indicating that Fis manifests its activity when preinitiation complexes at \textit{rrnB} P1 are in a metastable state.

It is likely that Fis also stabilizes preinitiation complexes from the P1 promoters of other ribosomal operons, because common features are shared in the regulatory regions of these promoters. For example, it has been reported that Fis stabilizes initially transcribing complexes in the presence of three nucleotides at \textit{rrnD} P1 (59). It is also possible that Fis behaves similarly on transcription of other stringent promoters, such as \textit{tyrT} and \textit{leuV} (60–62), which have Fis binding sites in their regulatory regions. Further experiments will be required to determine whether this is a common mode of action by Fis on these stringent promoters.

The Role of Fis in \textit{rRNA} Synthesis inside Cells—By determining the kinetic profiles of expression of the P1 promoters from several \textit{rRNA} operons directly, our results demonstrated that the \textit{rRNA} synthesis is reduced significantly (2-fold) in the \textit{fis} mutant compared with an isogenic wild type strain when cells emerge from an arrested state in late stationary phase (overnight cultures) to a rapid growing state in early log phase. The effect of \textit{fis} on the synthesis of \textit{rRNA} is parallel to the level of Fis inside cells, which also peaks at the first few doubling times after cell regrowth (22) and depends on growth conditions (38), indicating that Fis stimulates the robust synthesis of \textit{rRNA} during the transition in growth phases. It is known that stability of preinitiation complexes of stringent promoters are enhanced significantly by supercoiled DNA (5, 57). Moreover, it has been shown that DNA is relatively more relaxed in overnight cultures than in rapidly growing cultures (64, 65). Thus, it is likely that the interactions between RNAP and the \textit{rRNA} promoters are less stable in stationary phase cells compared with those in rapid growing cells, providing a basis for the modulation by Fis at the transition in cellular growth states. The potential role of Fis in transcription of \textit{rRNA} during the transition from stationary phase to early log phase has been proposed (15, 62) and challenged (37, 66). Also, it has been reported that Fis activates transcription of \textit{rrnB} P1 throughout exponential growth at low culture density (66). Other models on the role of Fis in the \textit{rRNA} synthesis have also been suggested (60, 67). It is interesting to note that the expression of wild type \textit{rrnB} P1 promoter is 2-fold higher than that of a mutant \textit{rrnB} P1 promoter that lacks the Fis binding site (66). Our study demonstrates that Fis plays an important role in establishing robust \textit{rRNA} synthesis when cells emerge from an arrested state into a rapid growing state. Consistent with the effect of \textit{fis} on the \textit{rRNA} synthesis, our results showed that Fis plays a critical role in the establishment of robust growth of \textit{E. coli} when cells are outgrowing from an arrested state (long term stationary phase) to a rapid growth state (log phase), primarily by reducing the lag time (Figs. 8 and 9).

It should be noted that although the expression of \textit{rRNA} is reduced in the \textit{fis} mutant during the early log growth compared with wild type strain, the overall shapes of expression profiles are parallel in the two strains. This indicates that element(s) other than Fis are also involved in the up-regulation of the P1 promoters of several \textit{rRNA} operons during the early log growth phase. Major candidates are the ATP/GTP concentration, which play an important role in the synthesis of \textit{rRNA} \textit{in vivo} and \textit{in vitro} (17, 58) and (pp)ppGpp, which is known to be at its lowest concentration in rapidly growing cells (68). In addition, other nucleoid-associated proteins such as H-NS, StpA, and HY, as well as DNA gyrase, directly or indirectly contribute to the control of \textit{rrnB} P1 activity (19, 63, 69, 70). Clearly, regulation of the ribosomal promoters is multivalent. Modulation of the transcription activity of the P1 promoters of \textit{rRNA} by Fis in early log growing cells is an integral element in the control of cell growth.

REFERENCES

1. Ingraham, J. L., Maalfe, O., and Neidhardt, F. C. (1983) \textit{Growth of the Bacterial Cell}, Sinauer, Sunderland, MA
2. Nomura, M., Gourse, R., and Baughman, G. (1984) \textit{Annu. Rev. Biochem.} 53, 75–117
3. Cashel, M., Gentry, D. R., Hernandez, V. J., and Vinella, D. (1996) in \textit{Escherichia coli and Salmonella typhimurium} (Neidhardt, F. C., ed) pp. 1458–1496, American Society for Microbiology Press, Washington, D.C.
4. Gourse, R. L. (1988) \textit{Nucleic Acids Res.} 16, 9789–9809
5. Zhou, Y. N., and Jin, D. J. (1997) \textit{Proc. Natl. Acad. Sci. U. S. A.} 94, 2908–2913
6. McClure, W. R. (1980) \textit{Annu. Rev. Microbiol.} 34, 523–553
7. Ross, W., Thompson, J. F., Newlands, J. T., and Gourse, R. L. (1990) \textit{Nucleic Acids Res.} 18, 6580–6585
8. Schroder, O., and Wagner, R. (2000) \textit{J. Mol. Biol.} 292, 445–446
Fis Stabilizes the Interaction between RNA Polymerase and the Ribosomal Promoter 
*rrnB P1*, Leading to Transcriptional Activation

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