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Promoter protection by a transcription factor acting as a local topological homeostat

Mark Rochman[1,2], Michal Aviv[2], Gad Glaser[2] & Georgi Muskhelishvili[1,+]

[1]Max Planck Institute for terrestrial Microbiology, Karl-von-Frisch-Strasse, D-35043 Marburg, Germany and [2]Department of Cellular Biochemistry, The Hebrew University-Hadassah Medical School, Jerusalem 90101, Israel

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Binding of the *Escherichia coli* global transcription factor FIS to the upstream activating sequence (UAS) of stable RNA promoters activates transcription on the outgrowth of cells from stationary phase. Paradoxically, while these promoters require negative supercoiling of DNA for optimal activity, FIS counteracts the increase of negative superhelical density by DNA gyrase. We demonstrate that binding of FIS at the UAS protects the *rrnA* P1 promoter from inactivation at suboptimal superhelical densities. This effect is correlated with FIS-dependent constraint of writhe and facilitated untwisting of promoter DNA. We infer that FIS maintains stable RNA transcription by stabilizing local writhe in the UAS. These results suggest a novel mechanism of transcriptional regulation by a transcription factor acting as a local topological homeostat.

INTRODUCTION

The *Escherichia coli* global transcriptional regulator FIS activates the promoters of stable RNA (rRNA and tRNA) operons during the adjustment of cellular translation machinery to rapid growth conditions (for recent review, see Travers *et al.*, 2001). These promoters contain two *cis*-acting positive regulatory elements: the UP element involved in binding the α-CTD of RNA polymerase (RNAP) (Ross *et al.*, 1993); and the upstream activating sequence (UAS) containing multiple FIS-binding sites, which are usually arranged in helical register. The deletion of UAS strongly reduces promoter activity, whereas inactivation of the *fis* gene did not diminish transcription, suggesting a compensatory mechanism derepressing stable RNA promoters in cells lacking FIS (Lamond and Travers, 1983; Ross *et al.*, 1990; Lazarus and Travers, 1993). The activation of transcription by FIS requires the bending of UAS DNA (Nilsson *et al.*, 1990; Newlands *et al.*, 1991; Zacharias *et al.*, 1992; Lazarus and Travers, 1993). Notably, the UAS itself is anisotropically flexible and presumably forms a DNA microloop delimited by RNAP (Nachaliel *et al.*, 1989; Muskhelishvili *et al.*, 1997; I.K. Pemberton, G. Muskhelishvili, A.A. Travers and M. Buckle, submitted for publication). FIS is thought to stabilize this DNA microloop on binding at multiple phased sites in the UAS (Travers and Muskhelishvili, 1998). However, a recent study using the seven *E. coli rrn* P1 promoters demonstrated differences in the contribution of multiple FIS-binding sites to overall promoter activity (Hirvonen *et al.*, 2001). The input of distal FIS sites upstream of promoter-proximal site I is small at the *rrnB* P1 and *rrnG* P1 promoters but substantial at the *rrnA* P1 and *rrnE* P1 promoters, accounting at the former for ~75% of total activation.

Furthermore, the activity of stable RNA promoters strongly depends on negative superhelical density of DNA both *in vitro* and *in vivo* (Glaser *et al.*, 1983; Lamond, 1985; Ohlsen and Gralla, 1992a; Bowater *et al.*, 1994; Free and Dorman, 1994). In this class of promoter, the 'sensing' of superlelical density involves the GC-rich 'discriminator' sequence between the –10 hexamer and the startpoint of transcription (Travers, 1980). This structural element determines the unusually short lifetime of initial transcription complexes by acting as a barrier to promoter opening, which can be partially overcome by negative supercoiling of DNA (Figueroa-Bossi *et al.*, 1998; Pemberton *et al.*, 2000).

Whereas the activity of stable RNA promoters strongly depends on DNA superhelical density, FIS counteracts the elevation of negative superhelicity by DNA gyrase on the outgrowth of cells from stationary phase (Schneider *et al.*, 1999). This general effect of FIS is at variance with the maximal activity of stable RNA promoters observed at the same growth stage. We resolve this apparent paradox by providing evidence that stabilization of local writhe in UAS by FIS protects the *rrnA* P1 promoter from inactivation at suboptimal superhelical densities. This novel mechanism of transcriptional regulation reveals the

+Corresponding author. Tel: +49 6421 178 600; Fax: +49 6421 178 609; E-mail: muskheli@mailer.uni-marburg.de

property of a global transcription factor to act as a local topological homeostat maintaining selective gene expression.

RESULTS

To monitor stable RNA transcription under conditions of DNA relaxation, two different coumarin inhibitors of DNA gyrase—novobiocin and coumermycin (Maxwell, 1997)—were added to exponentially growing CSH50 wild-type and CSH50Δ*fis* cells at OD_{600} = 0.1. The cells were harvested after 5 min, total RNA isolated and the amount of chromosomal *rrnA* P1 transcripts measured by primer extension. Since the 5′ end of rRNA has been found to turn over rapidly, the relative amount of detected transcript essentially reflects the efficiency of transcription initiation (Sarmientos *et al.*, 1983; Aviv *et al.*, 1996). On addition of DNA relaxing agents, we observed a significant reduction in the amount of chromosomal *rrnA* P1 transcripts in *fis* mutant cells in comparison to wild-type cells (Figure 1A, lanes 1–3). Likewise, these agents reduced transcription of plasmid-borne *rrnA* P1 (pMRP1) on average to 40 and 80% in the wild-type and *fis* cells, respectively, whereas transcription of reference *bla* promoter was not reduced (Figure 1A, lanes 4–9). To exclude any role of toxic effects of drugs, we next used a set of isogenic strains containing different combinations of drug-sensitive and drug-resistant alleles of gyrase (*gyrA*^{L83}) and topoisomerase IV (*parC*^{K84}) genes. In these strains, the addition of a quinolone (norfloxacin) allows the variation of the overall superhelical density from almost fully relaxed (σ = –0.015, strain LZ41) to near physiological (σ = –0.073, strain LZ23) to hypernegatively supercoiled (σ = –0.095, strain LZ54) state. Without norfloxacin, these strains have a similar overall superhelical density of around –0.07 (Khodursky *et al.*, 1995; Zechiedrich *et al.*, 1997; Schneider *et al.*, 2000). We compared chromosomal *rrnA* P1 transcription in these strains and their *fis* derivatives grown to OD_{600} = 0.1. A significant reduction of transcripts on relaxation of DNA was observed only in *fis* mutant cells (Figure 1B). Hypernegative supercoiling of DNA also led to a reduction of *rrnA* P1 transcription in the *fis* mutant cells but not in the wild-type cells, whereas no significant differences were observed at near physiological levels of overall negative superhelicity (σ = –0.073).

Taken together, these results strongly suggest that *fis* counteracts either directly or indirectly the reduction of *rrnA* P1 transcription by any deviations from optimal superhelical densities. To distinguish between these possibilities, we measured *in vitro* transcription of the *rrnA* P1 promoter construct pMRP1 with and without FIS using topoisomers of different negative superhelical densities (Figure 2A). In the absence of FIS, the transcription of *rrnA* P1 demonstrated a clear optimum at a negative superhelical density of –0.076 (Figure 2B and C). This value is significantly higher than the expected unconstrained superhelicity *in vivo*, although similarly high *in vitro* optimum levels have been obtained for the supercoiling-dependent *fis* and *tyrT* promoters (Schneider *et al.*, 2000; H. Auner, M. Buckle, A. Deufel, T. Kutateladze, L. Lazarus, G. Muskhelishvili, I. Pemberton, R. Schneider and A. Travers, submitted for publication). Addition of FIS supported high levels of *rrnA* P1 transcription over a range of superhelical densities, including those at which the transcription by RNAP alone was suboptimal (Figure 2C). This result is consistent with the *in vivo* data and suggests that FIS compensates for the suboptimal template topology *in vitro*.

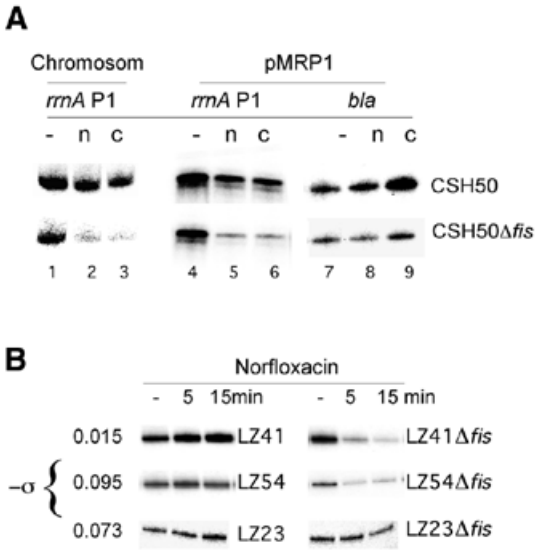

Fig. 1. FIS protects *rrnA* P1 transcription at suboptimal superhelical densities *in vivo*. (**A**) Relaxation of DNA by novobiocin (n) and coumermycin (c). Chromosomal *rrnA* P1 (lanes 1–3), pMRP1-borne *rrnA* P1 (lanes 4–6) and *bla* (lanes 7–9) transcripts detected in CSH50 and CSH50Δ*fis* cells. (**B**) Transcription of chromosomal *rrnA* P1 in LZ41, LZ54 and LZ23 strains and their *fis* derivatives 5 and 15 min after norfloxacin treatment. The overall negative superhelical densities (–σ) estimated in these strains after treatment with norfoxacin are indicated on the left.

To gain insight into the mechanism of this compensatory effect of FIS, we monitored open complex formation by permanganate reactivity assay using RNAP in excess of pMRP1 DNA and high salt conditions (230 mM NaCl) unfavourable for promoter opening (Ohlsen and Gralla, 1992a). Under these conditions, the permanganate reactivity signals in the –10 element and around the transcription startpoint indicative of RNAP open (initiation) complexes were detected only with templates of high negative σ levels (–0.076 and –0.1). Again, addition of FIS increased the range of superhelical densities at which promoter opening was detectable (Figure 2D). From these data, we infer that at suboptimal superhelical densities FIS maintains high levels of *rrnA* P1 transcription by facilitating initiation complex formation.

To prove the role of the UAS in the FIS-dependent protection of promoter activity, we compared the wild-type *rrnA* P1 with the deletion mutant *rrnA* P1Δ containing only the promoter-proximal FIS-binding site I (Figure 3A). The constructs pMSP1 and pMSP1Δ were transformed in the LZ41, LZ54 and LZ23 strains and the transcription of the wild-type and mutant promoters measured after norfloxacin treatment. As expected, FIS prevented the reduction of the plasmid-borne *rrnA* P1 activity under the conditions of both DNA relaxation and hypernegative supercoiling. In contrast, the transcription of the P1Δ mutant promoter could not be protected by FIS, indicating that an intact UAS is required (Figure 3B).

We next analysed promoter opening with supercoiled pMSP1 and pMSP1Δ in the presence of excess RNAP with or without FIS. We found that FIS facilitates open complex formation at both the *rrnA* P1 and *rrnA* P1Δ promoters. However, the effect of

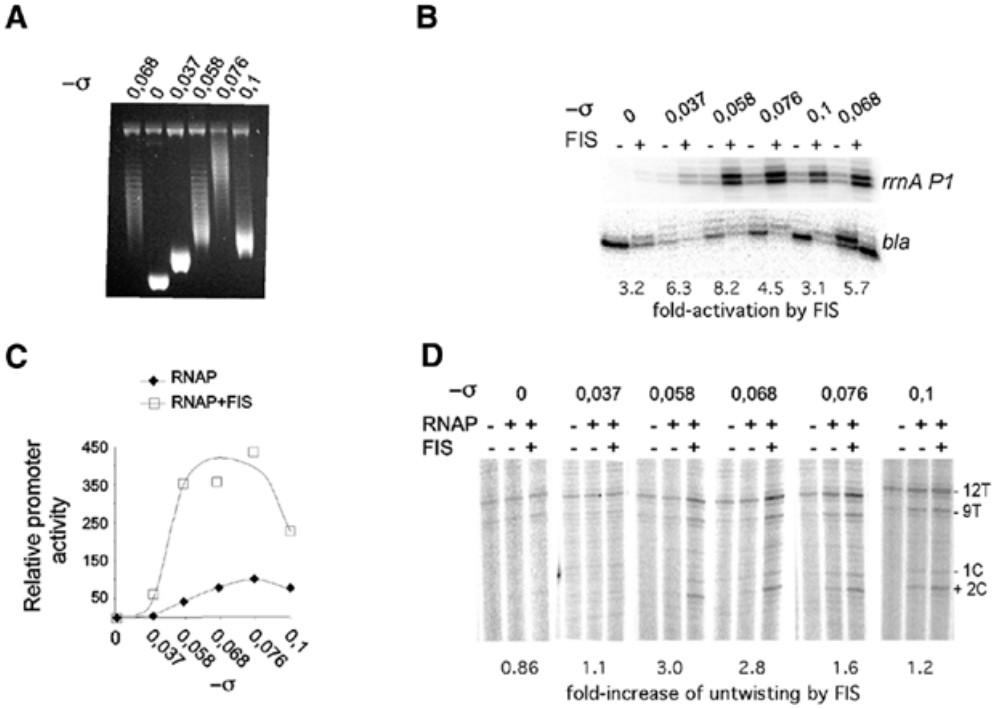

Fig. 2. FIS compensates for suboptimal superhelical densities *in vitro*. (**A**) The pMRP1 preparations used for *in vitro* experiments and the superhelical densities of each. (**B**) *In vitro* transcription from pMRP1 preparations shown in (A). Concentrations of DNA, RNAP and FIS were 10, 100 and 40 nM, respectively. Fold-activation by FIS for each σ is indicated below. The repression of the divergently oriented *bla* promoter on pMRP1 by FIS is presumably due to bending the UAS DNA towards *rrnA* P1. (**C**) Graphical representation of the experiment shown in (B). The relative promoter activities were normalized to the maximum *rrnA* P1 activity (100%) in the absence of FIS. (**D**) Permanganate footprinting of initiation complexes assembled with pMRP1. Concentration of DNA and proteins was as in (B). The reactive bases around the start and within the –10 region are indicated. The fold-increase of untwisting represents the ratio of summarized intensities of reactive bases in lanes with FIS divided by those without FIS.

FIS on DNA untwisting was more pronounced with the wild-type promoter, especially in the –10 region (Figure 3C and D). We infer that, at suboptimal superhelical densities, the FIS-binding site I is insufficient to rescue *rrnA* P1 transcription and that this failure is correlated with impaired initiation complex formation.

Finally, we investigated whether the binding of FIS at *rrnA* P1 UAS involves any changes in DNA writhe. For this purpose, RNAP initiation complexes were assembled with supercoiled pMSP1 and pMSP1Δ in the presence and absence of FIS and treated with topoisomerase I. Addition of FIS to RNAP initiation complexes changed the equilibrium distribution of topoisomers with pMSP1 but not with pMSP1Δ (Figure 3E and F). We infer that the constraint of writhe by initiation complexes requires an intact UAS DNA.

DISCUSSION

In this study we have demonstrated that FIS protects the *rrnA* P1 promoter from inactivation at suboptimal DNA negative superhelicities *in vivo*. Several lines of evidence suggest that this effect is mediated by direct binding of FIS at UAS. First, an intact UAS containing multiple FIS-binding sites is required to observe this effect *in vivo*. Secondly, FIS activates the *rrnA* P1 promoter about 3- to 8-fold at the lower than optimal levels of superhelicity (σ of 0 to –0.058) and about 3-fold under conditions of hypernegative supercoiling *in vitro*. Thirdly, at suboptimal superhelical densities, FIS facilitates open complex formation, a step thought to be rate-limiting in this class of promoter (Ohlsen and Gralla, 1992b; Figueroa-Bossi *et al.*, 1998). This latter result does not necessarily imply that FIS acts explicitly at the step of promoter opening. However, since we used a large excess of RNAP over DNA in all *in vitro* experiments, we infer that a step subsequent to initial complex formation is accelerated by FIS. This notion is supported by studies on *rrnD* P1, *rrnB* P1 and *tyrT* promoters (Sander *et al.*, 1993; Muskhelishvili *et al.*, 1997; Bartlett *et al.*, 2000; H. Auner, M. Buckle, A. Deufel, T. Kutateladze, L. Lazarus, G. Muskhelishvili, I. Pemberton, R. Schneider and A. Travers, submitted for publication).

We have not addressed the specific roles of the multiple FIS-binding sites of *rrnA* P1 UAS in the promoter protection mechanism described here. However, the proximal site I is not sufficient either to maintain transcription at suboptimal superhelical densities *in vivo* or for the constraint of writhe by initiation complexes and efficient open complex formation *in vitro*. These findings are consistent with the reported requirement of all FIS-binding sites for maximum *rrnA* P1 activation *in vivo* (Hirvonen *et al.*, 2001). We cannot exclude that other factors, in addition to FIS, may be involved in modulating the response of *rrnA* P1 to changes of supercoiling *in vivo*, but we consider it unlikely for two reasons: first, the putative factor should be able to activate the promoter under conditions of opposite changes in superhelical density, as we have demonstrated here for FIS; and, secondly, since the deletion of distal FIS-binding sites in UAS

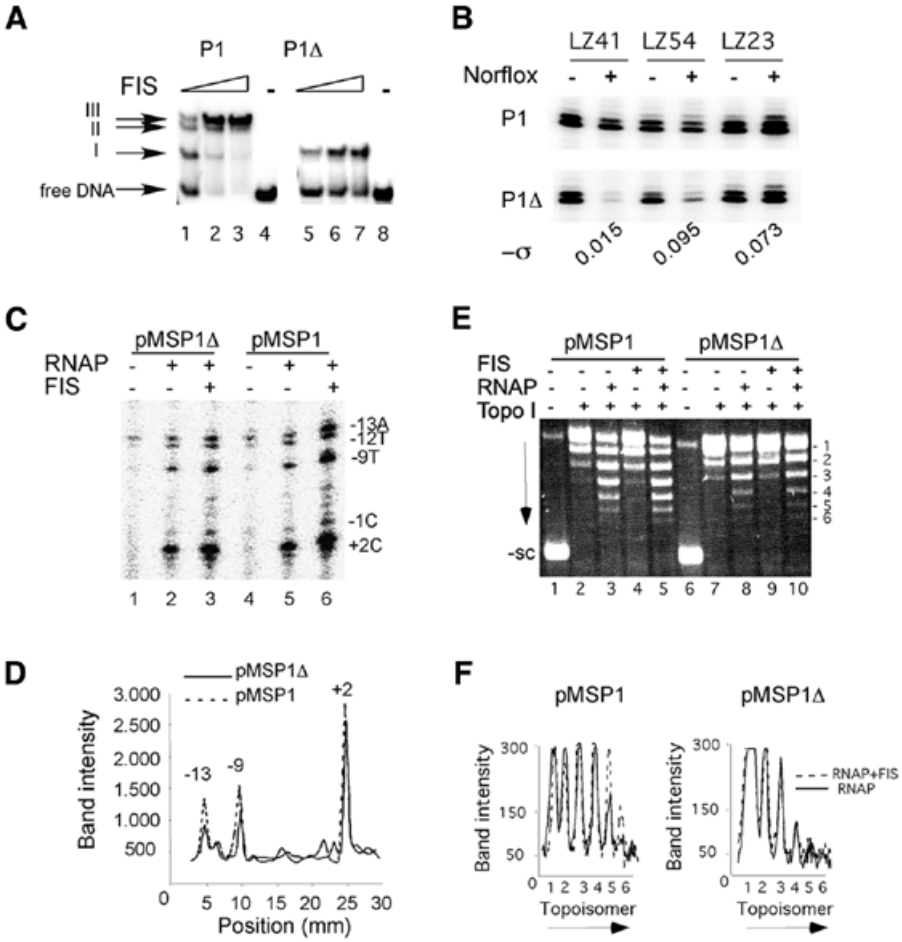

Fig. 3. The deletion mutant containing only proximal FIS-binding site I fails to protect *rrnA* P1 transcription. (**A**) Gel retardation analysis of the wild-type and mutant DNA fragments. The three complexes formed on binding FIS at the wild-type fragment are indicated. Note that the mutant forms only one complex, even at the highest FIS concentrations used. The concentration of FIS in lanes 1–3 and 5–7 was 4, 11 and 22 nM, respectively. (**B**) Transcription of *rrnA* P1 from pMRP1 and pMRP1Δ in LZ41, LZ54 and LZ23 strains after norfloxacin treatment. The overall negative superhelical densities estimated in these strains after norfoxacin treatment are indicated. (**C**) Permanganate footprinting of initiation complexes. The concentration of RNAP and FIS was 100 and 40 nM, respectively. The reactive bases are indicated. (**D**) Graphical representation of band intensities of lanes 3 and 6 shown in (C). Note the increased reactivity of bases –13 and –9 for wild-type *rrnA* P1. (**E**) Relaxation of pMSP1 DNA (10 nM) in the presence of RNAP (50 nM) with or without FIS. The concentration of FIS in lanes 4, 5, 9 and 10 was 33 nM. Untreated supercoiled plasmid (-sc). (**F**) Graphical representation of topoisomer distribution in lanes 3 and 5 (pMSP1, left panel) and 8 and 9 (pMSP1Δ, right panel) shown in (E). Note the increase in the abundance of pMSP1 topoisomers 5 and 6. Arrows indicate the direction of electrophoresis.

abolishes the protection effect, the putative factor would also require this same UAS region and, in addition, FIS bound at site I (see also Hirvonen *et al.*, 2001).

The position of FIS-binding site I (centred around –71) is highly conserved in all stable RNA promoters, whereas that of distal sites is more variable, although they are usually arranged in helical register (Verbeek *et al.*, 1990; Condon *et al.*, 1992; Lazarus and Travers, 1993; Hirvonen *et al.*, 2001). FIS bound at site I may interact with the α-CTD of RNAP (Bokal *et al.*, 1997), and this site has been shown to suffice for maximum activation of *rrnB* P1 *in vitro* (Gosink *et al.*, 1993). It is thus possible that, at least at some stable RNA promoters, the proximal site I is sufficient for high activity under optimal growth conditions, whereas the upstream sites are required to maintain promoter activity under conditions of suboptimal superhelicitiy caused by environmental stresses (Dorman, 1995; Tse-Dinh *et al.*, 1997). In UAS regions of stable RNA promoters so far characterized in detail, the local helical repeat separating the FIS-binding sites is lower than the intrinsic helical repeat of negatively supercoiled DNA (Herzel *et al.*, 1999), implying that bending of DNA by FIS would stabilize left-handed writhe (Travers and Muskhelishvili, 1998). Indeed, we observe FIS-dependent constraint of writhe in the ternary complexes assembled with wild-type promoter but not the UAS mutant *rrnA* P1Δ promoter. We estimate that the ternary complex assembled on *rrnA* P1 changes the linking number of pMSP1 DNA by 1. This correlates with facile initiation complex formation and argues for coupling between local writhe stabilized in *rrnA* P1 UAS and promoter untwisting. Such repartition of writhe and twist mediated by torsional transmission mechanism has been inferred for the closely related *tyrT* promoter, which also requires phased FIS-binding sites in UAS for maximal activity (Muskhelishvili *et al.*, 1997).

We thus infer that UAS is an important element involved in the physiological response of the *rrnA* P1 promoter to alterations in negative superhelicity. Notably, in contrast to the GC-rich discriminator and other core promoter elements implicated in sensing the supercoiling level (H. Auner, M. Buckle, A. Deufel, T. Kutateladze, L. Lazarus, G. Muskhelishvili, I. Pemberton, R. Schneider and A. Travers, submitted for publication), the UAS appears primarily involved in the adaptation to deviations of DNA superhelicity. This 'buffering' function of UAS requires binding of FIS at multiple phased sites and indicates, as proposed earlier (Travers and Muskhelishvili, 1998), that FIS acts as a local topological homeostat.

Expression of *fis* is activated by high levels of negative supercoiling (Schneider *et al.*, 2000). FIS is produced in large amounts on the outgrowth of cells from stationary phase and counteracts the elevation of negative superhelicity by gyrase (Schneider *et al.*, 1999). This FIS-dependent reduction of overall superhelicity is thought to prevent the deleterious effects of excessive supercoiling, but it would also limit the activity of the stable RNA as well as many other promoters that strongly depend on DNA superhelicity (Dorman, 1995; Tse-Dinh *et al.*, 1997). Our finding that FIS stabilizes a local DNA writhe (a microloop) in the upstream regions of stable RNA promoters resolves this apparent paradox and reveals a mechanism enabling a selective response of *rrn* operons, and hence of the translational machinery, to increased demands of protein synthesis on the commitment of cells to rapid growth. In *fis* cells, the overall level of negative superhelicity is elevated (Schneider *et al.*, 1997, 1999) and would thus derepress stable RNA promoters and compensate for the absence of FIS during the outgrowth from stationary phase.

METHODS

Bacterial strains and plasmids. Bacterial strains used in this study were *E. coli* K12 derivatives. The genotype of LZ41 is *parC*^{K84}*gyrA*$^{+}$*topA*10, that of LZ54 is *parC*$^{+}$*gyrA*^{L83}*topA*10 and that of LZ23 is *parC*$^{+}$*gyrA*^{L83}*topA*$^{+}$ (Khodursky *et al.*, 1995; Zechiedrich *et al.*, 1997; Schneider *et al.*, 2000). The *fis* derivatives of strains LZ41, LZ54 and LZ23 were obtained by phage P1 transduction from strain CSH50Δ*fis* described by González-Gil *et al.* (1998). All strains were grown in rich 2× YT medium (Schneider *et al.*, 2000).

pMRP1 contains the region ~300 bp upstream of the transcription startpoint of *rrnA* P1 and strong *rrnB* operon terminators ~250 bp downstream (Gafny *et al.*, 1994). pMRP1Δ was generated by PCR-aided internal deletion of the region between –94 and –147, removing all FIS-binding sites except the promoter-proximal site I. pMSP1 and pMSP1Δ were generated by internal deletion of a 3-kb fragment containing the *lacZ* gene from pMRP1 and pMRP1Δ, respectively.

Proteins. FIS was purified as described previously (Koch and Kahmann, 1986). *Escherichia coli* RNAP came from Boehringer-Mannheim, and Vaccinia topoisomerase was a kind gift of Karin Schnetz (Institute for Genetics, Cologne, Germany).

RNA analysis. Primer extension using total *E. coli* RNA was performed as described by Nasser *et al.* (2001). Extension of RNA templates transcribed from the chromosomal *rrnA* P1 promoter with the primer G11 (5′-GAGCAGTGCCGCTTCGC-3′) yields a 156-bp fragment. The extension products were resolved on a 6% sequencing gel and visualized by phosphoimaging. The length of the transcripts was identified by using corresponding dideoxy sequencing reactions as a reference.

High-resolution agarose gel electrophoresis. High-resolution agarose gel electrophoresis was carried out in 1× TBE buffer as described previously (Schneider *et al.*, 1997).

Preparation of topoisomer distibutions and determination of σ. Topoisomer distributions were prepared using Vaccinia topoisomerase as described by Schneider *et al.* (2000). σ was calculated according to the formula: $-\sigma = \Delta LK/LK_0$, where LK_0 for pMRP1 ~6.500-bp/10.5-bp/turn = 619. The error for all σ is 10–15%.

***In vitro* transcription from supercoiled templates.** *In vitro* transcription/coupled primer extension reactions using plasmids with distinct σ were carried out with reverse transcriptase (SUPERSCRIPT II) as described by Lazarus and Travers (1993). Extension of RNA templates transcribed from the plasmid-borne *rrnA* P1 promoter with the primer G11 yields a 65-bp fragment. The mRNA transcribed from the *bla* promoter located on the same plasmid yields a 95-bp fragment on extension with the primer bla3B4 (5′-CAGGAAGGCAAAATGCCGC-3′). The samples were analysed on 6% denaturing polyacrylamide gels, visualized by phosphorimaging and quantitated using ImageQuant software.

Potassium permanganate reactivity assay. The reactions for potassium permanganate reactivity assays were assembled as for *in vitro* transcription reactions, but only ATP and CTP (100 μM) were present in the incubation mixture to allow formation of the dinucleotide pppApC. Footprinting was performed as described by Nasser *et al.* (2001).

Topological analysis of transcription complexes. The pMSP1 DNA was relaxed in the presence of proteins as indicated for 1 h by calf thymus topoisomerase I (Amersham) according to the manufacturer's recommendations. The complexes were assembled as for potassium permanganate reactivity assays, and the samples were processed as described by Schneider *et al.* (1997) and run on 1% high-resolution agarose gels.

Gel-shift analysis. The ~130-bp fragments comprising the UAS regions of pMRP1 and pMRP1Δ were obtained by PCR amplification, radioactively end-labelled by [γ-^{32}P]ATP using T4 Polynucleotide kinase (Biolabs) and incubated at 37°C for 15 min in a buffer containing 10 mM Tris–HCl pH 7.9 and 100 mM NaCl with FIS as indicated. The samples were loaded on a 5% non-denaturing polyacrylamide gel and visualized on an X-ray film.

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