Translational activation by the noncoding RNA DsrA involves alternative RNase III processing in the *rpoS* 5′-leader

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ABSTRACT

The intricate regulation of the *Escherichia coli rpoS* gene, which encodes the stationary phase sigma-factor σ^S, includes translational activation by the noncoding RNA DsrA. We observed that the stability of *rpoS* mRNA, and concomitantly the concentration of σ^S, were significantly higher in an RNase III-deficient mutant. As no decay intermediates corresponding to the in vitro mapped RNase III cleavage site in the *rpoS* leader could be detected in vivo, the initial RNase III cleavage appears to be decisive for the observed rapid inactivation of *rpoS* mRNA. In contrast, we show that base-pairing of DsrA with the *rpoS* leader creates an alternative RNase III cleavage site within the *rpoS*/DsrA duplex. This study provides new insights into regulation by small regulatory RNAs in that the molecular function of DsrA not only facilitates ribosome loading on *rpoS* mRNA, but additionally involves an alternative processing of the target.

Keywords: DsrA; mRNA stability; noncoding RNA; *rpoS*

INTRODUCTION

In response to adverse environmental conditions, bacteria launch specific stress responses (Storz and Hengge-Aronis 2000). Several of these mechanisms are modulated by small noncoding RNAs (ncRNAs) (Gottesman 2005; Kaberdin and Bläsi 2006). Many ncRNAs exert their regulatory role by base-pairing with target mRNAs, thereby affecting their translation and stability (Massé et al. 2003a; Morita et al. 2006). In contrast to classical antisense RNAs, stress-related ncRNAs usually form imperfect duplexes with their targets and generally require the RNA chaperone Hfq (Valentin-Hansen et al. 2004) to facilitate mRNA/ncRNA interactions. Several ncRNAs, including DsrA and RprA, have been shown to affect expression of the *rpoS* gene encoding the stationary phase/stress sigma-factor, σ^S (Repoila et al. 2003). σ^S is known to control transcription of a large number of bacterial genes that have stress-protective functions during carbon starvation, temperature downshifts, and exposure to high osmolarity, acidic pH, or denaturing reagents (Hengge-Aronis 2002a). The 5′-untranslated region of *Escherichia coli rpoS* mRNA forms a complex secondary structure, which results in weak accessibility of the ribosome binding site (rbs) and poor translation (Hengge-Aronis 2002b). Translation of *rpoS* mRNA can be activated by the ncRNA DsrA (Lease and Belfort 2000) through base-pairing with the *rpoS* leader, which leads to disruption of the inhibitory secondary structure (Lease and Woodson 2004).

The fate of intermediate ncRNA/mRNA complexes is still poorly understood. In *E. coli*, two RNase activities, RNase E and RNase III, have been implicated in decay of ncRNA/mRNA complexes upon translational silencing (Kaberdin and Bläsi 2006). Morita et al. (2005) have put forward a model wherein an Hfq–RNase E–ncRNA complex is targeted to mRNA, which is consistent with the previously observed RNase E-dependent degradation of ncRNA targets (Massé et al. 2003a; Kawamoto et al. 2005; Vanderpool and Gottesman 2005). Although endonucleolytic RNase E

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cleavage sites created by structural rearrangements upon ncRNA/mRNA duplex formation (Geissmann and Touati 2004) can play a role in generation of primary decay intermediates (Afonyushkin et al. 2005), it remains to be seen whether a sole RNase E-based pathway is generally valid for any ncRNA/mRNA intermediate. Other studies suggested a role for the double-strand-specific RNase III in turnover of target mRNAs upon translational silencing by ncRNAs (Vogel et al. 2004; Afonyushkin et al. 2005; Huntzinger et al. 2005). Hence, cleavage by RNase III within the ncRNA/mRNA duplex and the resulting subsequent decay of the mRNA intermediate by the *E. coli* RNA decay machinery could rather resemble the RNAi scenario in eukaryotic organisms (Agrawal et al. 2003).

In contrast to the mechanisms leading to disassembly and processing of the intermediate ncRNA/mRNA complexes during ncRNA-mediated translational silencing, the molecular events that determined the fate of such intermediate complexes upon functional activation of target mRNAs have not been studied. Here, we used the DsrA-dependent activation of *rpoS* expression as a model system to address this question. Our data revealed a central role of *E. coli* RNase III in controlling *rpoS* mRNA turnover. We found that the ncRNA DsrA, besides its established role in *rpoS* translational activation, stabilizes the *rpoS* transcript by redirecting RNase III cleavage in its 5′-UTR.

RESULTS AND DISCUSSION

RNase III affects *rpoS* mRNA stability and the level of RpoS

With the aim to identify factor(s) affecting *rpoS* regulation and stability, we tested for a possible role of RNase III in *rpoS* turnover. Northern blot analysis of total RNA isolated from an *E. coli* RNase III mutant strain revealed that the steady-state level and half-life of the full-length *rpoS* mRNA were increased when compared to the isogenic wild-type strain (Fig. 1). Correspondingly, the in vivo levels of σ^s were elevated in the *rnc* mutant (Fig. 1A,B), thereby suggesting that RNase III is involved in the control of *rpoS* mRNA stability, and hence in regulation of the level of RpoS. In addition to the full-length *rpoS* mRNA, we could also detect a processing intermediate (Fig. 1A) likely formed upon RNase III cleavage at G_{-112} (see below). The effect of RNase III inactivation on *rpoS* expression and stability was temperature-dependent and was more pronounced at 37°C when

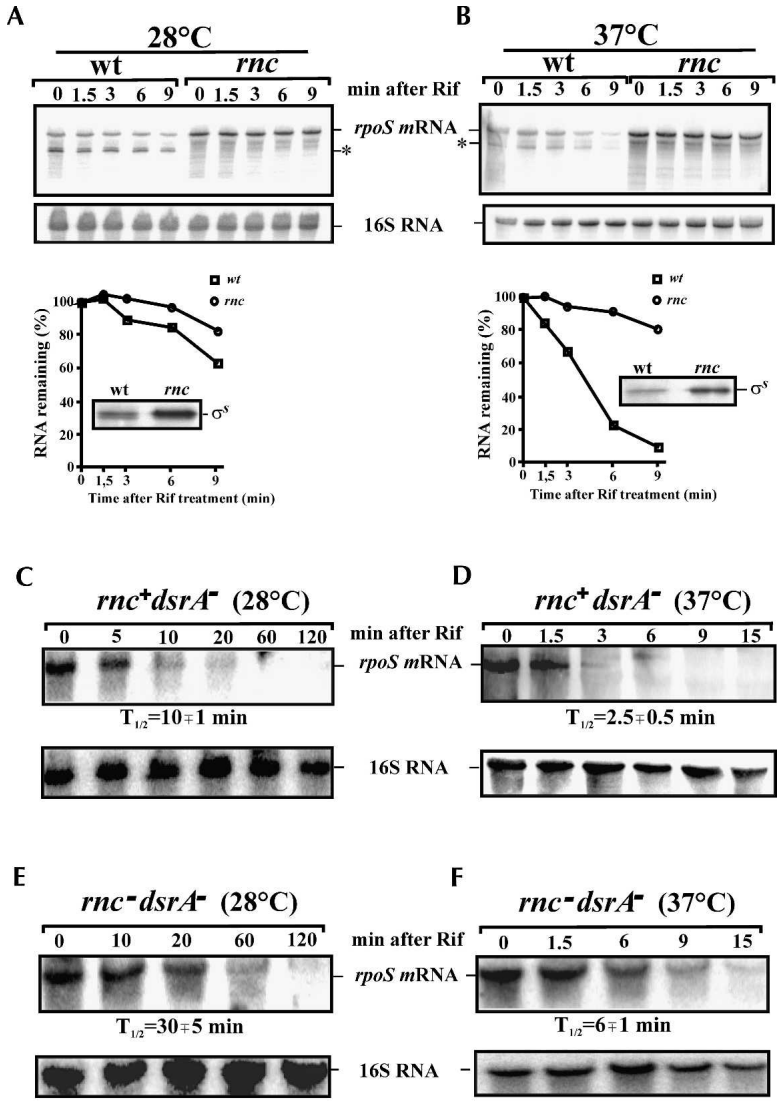

FIGURE 1. Effects of RNase III/DsrA inactivation on *rpoS* expression. The *E. coli* strain SDF204 (wt) and the isogenic RNase III (*rnc*) deficient strain SDF205 as well as the *dsrA* mutant (rnc^+dsrA^-) and its isogenic variant lacking functional RNase III (rnc^-dsrA^-) were grown to early log phase at (*A*,*C*,*E*) 28°C and (*B*,*D*,*F*) 37°C, respectively. RNA samples were prepared from each culture before and after rifampicin treatment at the times indicated on *top* of the autoradiograph and further analyzed by Northern blotting. (*A*,*B*) The corresponding graphs *below* show the relative amount of the full-length *rpoS* mRNA remaining at each time point as determined by PhosphorImaging and plotted as a function of time. The insets show the steady-state levels of σ^S determined by Western blotting in the wt strain and the isogenic *rnc* mutant. The positions of σ^s, *rpoS* mRNA, and its RNase III-dependent processing intermediate (*) are indicated. The half-lives of *rpoS* mRNA ($T_{1/2}$) were calculated using the data obtained in independent experiments.

compared to that at 28°C (Fig. 1). As the level of the ncRNA DsrA, one of the riboregulators stimulating *rpoS* translation, is known to be higher at 28°C than at 37°C (Sledjeski et al. 1996), our result suggested that the stabilizing effect of RNase III inactivation on *rpoS* mRNA is limited to mRNA molecules that are not involved in base-pairing with DsrA. Consistent with this idea, we observed a comparable half-life of *rpoS* mRNA in the wild-type strain and in the *dsrA*$^-$ mutant (Fig. 1B,D) and, when compared to the *dsrA*$^-$ strain (Fig. 1D), the stability of *rpoS* mRNA was found to be increased in the *rnc*$^-$ *dsrA*$^-$ double-mutant strain (Fig. 1F) at 37°C. The corresponding results were obtained with the isogenic strains at 28°C in the absence of DsrA (Fig. 1C,E).

RNase III cleavage sites in the *rpoS* leader

Next, we tested for RNase III cleavage sites in *rpoS* mRNA. RpoSII RNA (nucleotides −564 to +161 of *rpoS* mRNA) was incubated in the presence of increasing concentrations of RNase III, and the resulting products of cleavage were analyzed by primer extension. RNase III cleavage occurred with equal intensities within the double-stranded region of the *rpoS* leader at nucleotide positions −15 and −94, respectively (Fig. 2A). It seems possible that RNase III cleavage at this position results in a rapid decay of the *rpoS* transcript. Consistent with this hypothesis, primer extension analysis of total *E. coli* RNA isolated from the wild-type strain and the RNase III mutant (Fig. 3A) revealed no RNase III cleavage intermediates, which would argue for their instability in vivo. Although we cannot exclude the formal possibility that RNA-binding proteins inhibit RNase III cleavage, we consider this as less likely, because of the differences in *rpoS* mRNA stability observed in the wild-type and RNase III mutant strains (see Fig. 1). Further support for the decisive role of RNase III cleavage at positions −15/−94 in *rpoS* decay is provided by a recent mutational analysis of the *Salmonella enterica rpoS* leader. Mutations in stem II potentially interfering with RNase III cleavage were shown to increase expression of the corresponding *rpoS-lacZ* fusions (Brown and Elliott 1997; Jones et al. 2006). As the sequence of the three stem–loop structures (Fig. 2) including the mapped RNase III cleavage site upstream of the start codon is identical in *E. coli* and *S. enterica*, our results would suggest that the reported increase in expression of the mutant *rpoS-lacZ* fusions (Brown and Elliott 1997; Jones et al. 2006) results from inhibition or absence of RNase III cleavage in the altered *rpoS* leader(s).

An additional RNase III cleavage site was mapped in vivo at position G_{-112} (Fig. 3A). Moreover, cleavage at this position was detectable in wild-type cells but not in the corresponding isogenic *hfq*$^-$ mutant strain (Fig. 3A). As the absence of Hfq is known to impair efficient interactions of ncRNAs with their targets (Gottesman 2005), these data

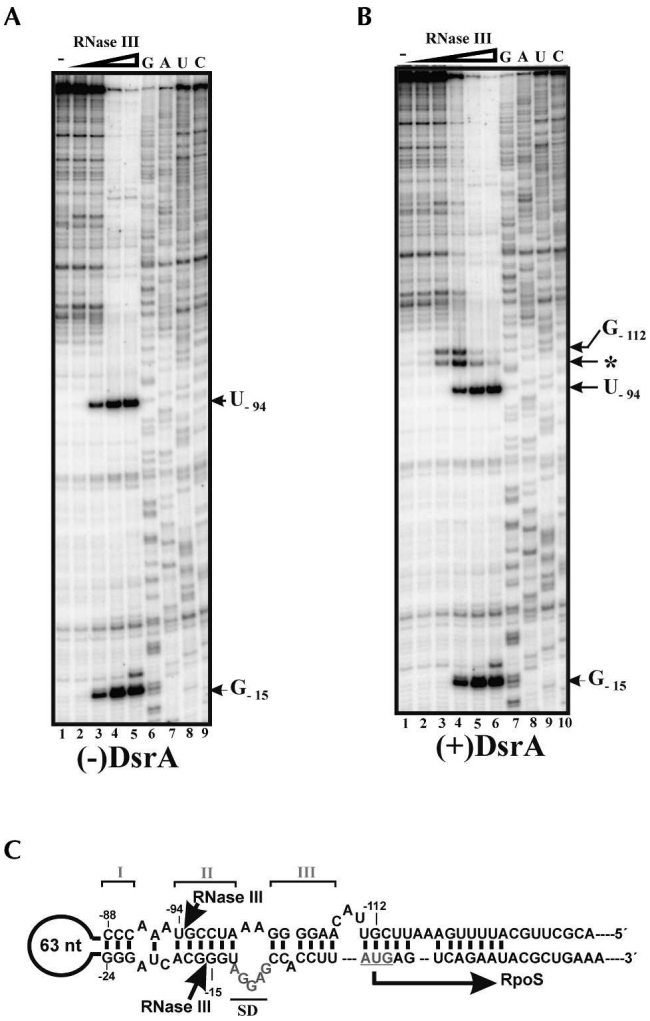

FIGURE 2. RNase III cleaves within the *rpoS* leader in vitro. In vitro transcribed RpoSII RNA (nucleotides −564 to +161) was incubated alone (lane *1*) or with increasing quantities of RNase III (lanes *2–6*) in the (*A*) absence or (*B*) presence of DsrA. The resulting products of cleavage were further analyzed by primer extension. (*C*) The RNA sequence of a segment of the *rpoS* 5′-leader is schematically depicted at the *bottom*. The elements of the inhibitory stem–loop structure (stems I, II, and III) and the ribosome-binding site (underlined) are indicated. The positions of DsrA-independent (−15/−94) and DsrA-dependent (−112/*) RNase III cleavage were determined using a concurrently run RNA sequencing ladder (lanes *7–10*) and are indicated by arrows.

suggested that RNase III cleavage at G_{-112} could result from *rpoS* interactions with small ncRNAs, for example, DsrA or RprA (Repoila et al. 2003). For verification, we compared the in vitro cleavage pattern generated by incubation of RpoSI RNA (nucleotides −146 to +55 of *rpoS* mRNA) with RNase III in the absence and in the presence of DsrA (Fig. 2B). This experiment showed that, in addition to the cleavage signals detected within the *rpoS* leader (Fig. 2A, positions −15/−94), two additional cleavage DsrA-dependent cleavage signals were observed at positions G_{-112} and at A_{-106} (Fig. 2B), the latter of which

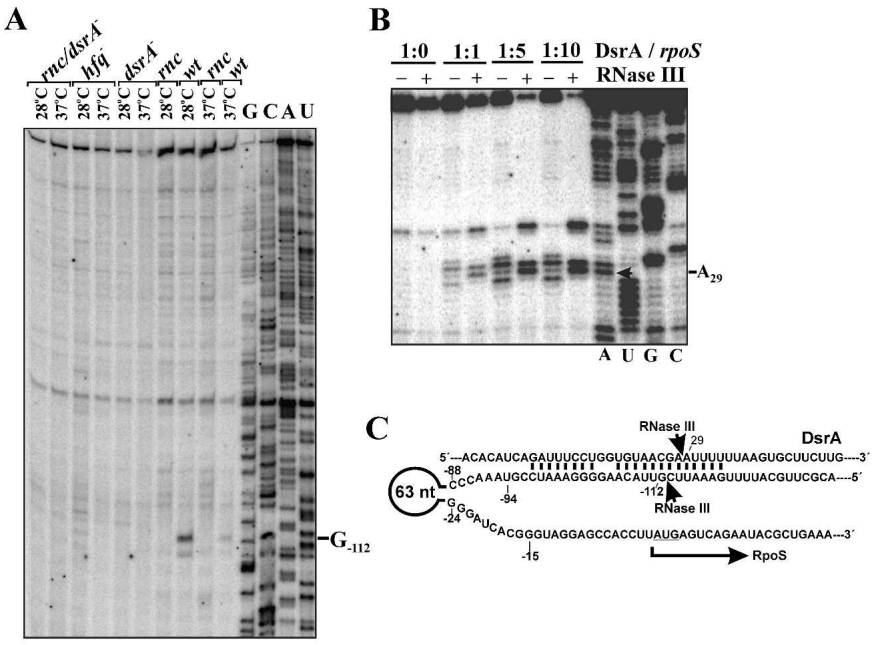

FIGURE 3. Base-pairing with DsrA induces RNase III cleavage in the *rpoS* 5′-UTR at an alternative site. (*A*) Primer extension analysis of total RNA isolated from the wild-type *E. coli* strain (wt) and isogenic RNase III (*rnc*), Hfq (*hfq*), DsrA (*dsrA*$^-$), and double (*rnc/dsrA*$^-$) mutants grown at 28°C and 37°C. The product of RNase III cleavage within the *rpoS* leader (position G_{-112}) accumulates only in the wild-type strain. (*B*) In vitro synthesized DsrA was incubated alone or with RNase III in the absence (−) or presence (+) of equimolar amount (1:1), fourfold, or ninefold molar excess of RpoSI for 10 min and further analyzed by primer extension. The arrow indicates the nucleotide (A_{28}) at which RNase III cleaves DsrA complexed with RpoSI. (*C*) Depicted is a part of the *rpoS*/DsrA complex. Arrows indicate the internucleotide bonds that are cleaved by RNase III. The coordinates of nucleotides in close vicinity to the scissile bonds (G_{-112} and A_{29}) were determined by primer extension (see Figs. 2B, 3B, respectively).

we consider to be an in vitro artifact. Taken together with the observation that the cleavage at G_{-112} cannot be detected in vivo in a *dsrA*$^-$ strain (Fig. 3A), our data show that RNase III cleavage at this position is dependent on DsrA and occurs only upon DsrA/*rpoS* base-pairing.

Figure 3B shows that DsrA alone is resistant to the nucleolytic activity of RNase III. However, it is efficiently cleaved between nucleotides A_{28} and A_{29} by this endoribonuclease in the presence of increasing amounts of RpoSI RNA containing the complementary *rpoS* sequence (Fig. 3B). Collectively, the results of these cleavage assays (Figs. 2B and 3B) demonstrate that RNase III cleaves both strands of the *rpoS* mRNA/DsrA duplex (Fig. 3C).

Alternative RNase III processing in the *rpoS* leader mediated by DsrA: An addition to the complex control of *rpoS* expression

Here, we have provided evidence that the decay of *rpoS* mRNA is retarded upon inactivation of RNase III in vivo (Fig. 1) and that RNase III cleaves within the 5′-UTR of this transcript in vitro (Fig. 2). Apparently, in the presence of the inhibitory secondary structure, the untranslated form of the *rpoS* is targeted for degradation via an RNase III-dependent mechanism, which, in turn, results in low levels of *rpoS* mRNA and diminishes σ^S synthesis (Fig. 4). In contrast, interactions of the *rpoS* 5′-UTR with DsrA and perhaps likewise with RprA not only disrupt the inhibitory secondary structure in the *rpoS* leader but also eliminate the original RNase III cleavage site and simultaneously trigger RNase III cleavage at an alternative site within the intermediate *rpoS* mRNA/DsrA duplex (Fig. 4). According to the results shown in Figure 1B, this cleavage does not significantly affect the stability of the translationally active form of *rpoS* mRNA, which may be attributed to its protection from nucleases by translating ribosomes. RNase III cleavage in the DsrA/*rpoS* duplex (see Fig. 3) apparently prevents recycling of DsrA. This outcome is analogous to the model of coupled degradation of an ncRNA and its mRNA target (Massé et al. 2003b) and could be important for bacterial cells to avoid further up-regulation of *rpoS* after the stress is relieved. In summary, our findings provide new insights into the molecular mechanisms by which riboregulators activate their target mRNAs and support the idea that not only RNase E but also RNase III plays a key role in bacterial stress responses mediated by *trans*-encoded ncRNAs.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions

The following *E. coli* strains were used in this study: SDF204 (W3110 *rnc*$^+$) (Dasgupta et al. 1998), SDF205 (*W3110 rnc105*) (Dasgupta et al. 1998), MC4100 (Tsui et al. 1994), AM111 (MC4100 *hfq*$^-$) (Tsui et al. 1994), SDF204DsrA (SDF204 *rnc*$^+$, *dsrA1::cat*), and SDF205DsrA (SDF205 *rnc105, dsrA1::cat*). SDF204DsrA and SDF205DsrA were constructed by P1 transduction of the *dsrA1::cat* mutation (Sledjeski et al. 1996) into SDF204 and SDF205 as previously described (Miller 1972). The *E. coli* strains were grown in Luria-Bertani (LB) medium supplemented with tetracycline (10 μg/mL), kanamycin (50 μg/mL), or chloramphenicol (20 μg/mL) where appropriate.

In vitro transcription and 5′-end labeling

RpoSI and RpoSII RNAs corresponding to *E. coli rpoS* mRNA nucleotides −146 to +55 and −564 to +161, respectively, as well as

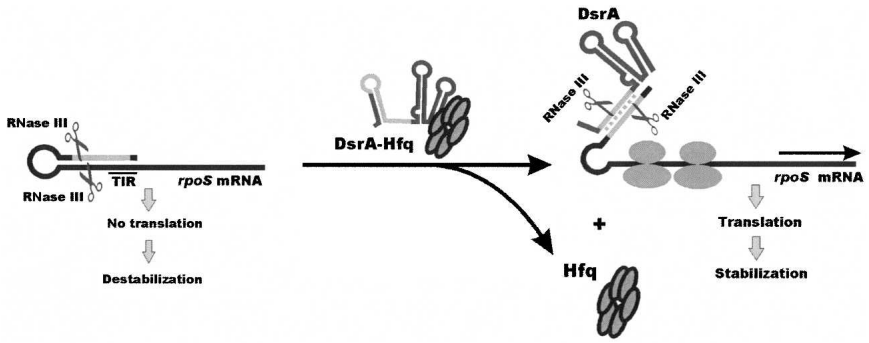

FIGURE 4. Model for post-transcriptional regulation of *rpoS* expression by RNase III. The translation initiation region (TIR) of the *E. coli rpoS* mRNA is embedded into a complex secondary structure, thereby preventing efficient ribosome binding. RNase III can cleave within the double-stranded segment of this structure, which results in destabilization of the transcript in the wild-type *E. coli* strain when compared to its isogenic counterpart lacking functional RNase III. In contrast to limited synthesis of RpoS under normal growth conditions, the level of RpoS is up-regulated in the presence of increasing amounts of DsrA, which accumulate at low temperature. By base-pairing with the complementary region of the *rpoS* leader, DsrA disrupts the inhibitory secondary structure, thereby facilitating ribosome loading and subsequent translation of RpoS (Repoila et al. 2003). DsrA/*rpoS* duplex formation, which is facilitated by the RNA chaperone Hfq, not only abrogates RNase III cleavage within the *rpoS* leader at positions (−94/−15), but also creates a new RNase III cleavage site within the DsrA/*rpoS* duplex. RNase III cleavage at this site prevents reuse of DsrA for multiple cycles of *rpoS* activation. Moreover, the main body of the DsrA-activated *rpoS* mRNA is covered by translating ribosome and therefore protected from degradation by *E. coli* ribonucleases.

DsrA were synthesized using a MEGAscript transcription kit (Ambion) and then gel-purified. The transcripts were dephosphorylated with bacterial alkaline phosphatase (MBI Fermentas), 5′-end-labeled with ^{32}P using T4 polynucleotide kinase (MBI Fermentas) together with an excess of [γ-^{32}P]ATP (Amersham Biosciences), and then again gel-purified as described previously (Kaberdin and Bizebard 2005). The DNA templates for transcription were generated by PCR amplification of *E. coli* genomic DNA using the oligonucleotide primers listed in Table 1.

Western blotting

Samples of bacterial cultures grown in LB medium at 28°C or 37°C were harvested in early log growth phase (OD_{600} of 0.4). Equimolar amounts of proteins were separated on 12% SDS polyacrylamide gels, electroblotted onto Immobilon-P (Millipore), and probed with anti-RpoS antibodies (kindly provided by F. Norel, Pasteur Institute, Paris), followed by development using the ECL detection kit (Amersham).

Northern blot and primer extension analysis

E. coli strains were grown at 28°C or 37°C to early log phase (OD_{600} of 0.4) when rifampicin (0.25 mg/mL) was added. Total RNA was isolated from aliquots of the cultures withdrawn at various times using the hot phenol method (Lin-Chao and Bremer 1986). RNA samples (5 μg each) were fractionated on 4%–8% denaturing polyacrylamide gels, transferred to Zeta-Probe membranes (Bio-Rad) using the Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad), and then hybridized to [^{32}P]-labeled antisense RNA complementary to the *E. coli rpoS* mRNA (nucleotides −130 to +105) according to the procedure described (Večerek et al. 2003). The DNA template for in vitro synthesis of the internally labeled anti-*rpoS* riboprobe was generated by PCR amplification of chromosomal *E. coli* DNA with primers rSriboF and rSriboR (Table 1). The relative amount of *rpoS* mRNA at each time point was calculated by normalizing their signals to the 16S rRNA signal.

Primer extension analysis was performed using aliquots of total RNA (15 μg) as described previously (Resch et al. 1996). Briefly, after annealing to 5′-[^{32}P]-labeled *rpoS*-specific oligonucleotide RpoSRT9 (or RpoSRT2) and addition of AMV-Reverse Transcriptase (Promega), cDNA synthesis was carried out for 60 min at 42°C. The products of extension along with a sequencing ladder prepared using the same primer and in vitro transcribed RpoSII (nucleotides −564 to +161) as a template were resolved on a 6% sequencing gel, and the resulting radioactive

TABLE 1. Primers used in this study

Name	Target gene	Sequence (5′ to 3′)
PCR amplification of DNA templates for in vitro transcription		
RpoSIfw	*rpoS*	TAATACGACTCACTATA**G**GGAACCAGTTCAACACGCT
RpoSIrev	*rpoS*	CATCAAATTCCGCATCTTCAT
RpoSIIfw	*rpoS*	GGGCTCTAGAGTAATACGACTCACTATA**GT**CGGGTGAACAGAGTGCTAACAAAATGTTGCCG
RpoSIIrev	*rpoS*	ACACGCTGTGTGGCTCCC
DsrAfw	*dsrA*	GGGTCTAGACGTAATACGACTCACTATAGAACACATCAGATTTCCTGGTGTAACGAATTTTTTAAGTG
DsrRev	*dsrA*	AAATCCCGACCCTGAGG
rSriboF	*rpoS*	TAATACGACTCACTATAGGGTTCCTGTTCTACTAAG
rSriboR	*rpoS*	GCTTGCATTTTGAAATTCGT
Primer extension analysis		
RpoSRT2	*rpoS*	TCCGTTCTCATCAAATTC
RpoSRT9	*rpoS*	TCCGTTCTCATCAAATTCCGCATC

The T7 promoter sequence is underlined.

signals were further visualized using a PhosphorImager (Molecular Dynamics).

RNase III cleavage assay

Reaction mixtures containing 0.08 pmol of in vitro transcribed RpoSII RNA pre-hybridized with 5′-end [^{32}P]-labeled RpoSRT2 primer were incubated alone or with increasing amounts of RNase III (Ambion) in the absence or presence of DsrA for 10 min at 37°C, and subsequently analyzed by primer extension (see Fig. 2) as described above, except that the reverse transcription was carried out for 10 min. The RNase III cleavage assay shown in Figure 3 was performed with 100 fmol of DsrA pre-hybridized with 5′-end [^{32}P]-labeled primer DsrRev. After pre-incubation in 1× RNase III buffer (Ambion) for 10 min at 37°C in the presence or absence of unlabeled complementary RNAs, RNase III (Ambion) was added. Following incubation for 10 min, aliquots were withdrawn, treated with phenol, ethanol-precipitated, and analyzed directly on 8% sequencing gels (Fig. 3B). The products of DsrA cleavage were analyzed by primer extension.

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