



SraL sRNA interaction regulates the terminator by preventing premature transcription termination of *rho* mRNA

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Transcription termination is a critical step in the control of gene expression. One of the major termination mechanisms is mediated by Rho factor that dissociates the complex mRNA-DNA-RNA polymerase upon binding with RNA polymerase. Rho promotes termination at the end of operons, but it can also terminate transcription within leader regions, performing regulatory functions and avoiding pervasive transcription. Transcription of *rho* is autoregulated through a Rho-dependent attenuation in the leader region of the transcript. In this study, we have included an additional player in this pathway. By performing MS2-affinity purification coupled with RNA sequencing (MAPS), *rho* transcript was shown to directly interact with the small noncoding RNA SraL. Using bioinformatic *in vivo* and *in vitro* experimental analyses, SraL was shown to base pair with the 5'-UTR of *rho* mRNA upregulating its expression in several growth conditions. This base pairing was shown to prevent the action of Rho over its own message. Moreover, the results obtained indicate that both ProQ and Hfq are associated with this regulation. We propose a model that contemplates the action of *Salmonella* SraL sRNA in the protection of *rho* mRNA from premature transcription termination by Rho. Note that since the interaction region between both RNAs corresponds to a very-well-conserved sequence, it is plausible to admit that this regulation also occurs in other enterobacteria.

sequence, leading to changes in translation and/or mRNA degradation (9–11). However, distinct mechanisms of action have been increasingly reported in the literature (11). For instance, the *Salmonella* sRNA ChiX was shown to induce premature transcription termination within the coding sequence of *chiP* as a result of its interaction with 5'-UTR of the operon *chiPQ*, thus affecting the expression of both genes of the operon (12). Conversely, it was also very recently established that some sRNAs are able to prevent premature transcription termination by interfering with Rho-mediated termination in the 5'-UTR of many genes (13). These and other studies seem to indicate that interference of sRNAs in Rho-dependent termination is a more common mechanism of regulation than previously envisioned.

SraL is a very-well-conserved *trans*-encoded sRNA that was initially identified in *E. coli* (14–16). Subsequently, the expression of this sRNA was also detected and studied in detail in *Salmonella enterica* serovar Typhimurium (15, 17). Although the transcriptional and posttranscriptional regulation of SraL sRNA have been described, only one target is known for this sRNA (15, 17). The ribosome-associated chaperone Trigger factor (TF), encoded by *tig* mRNA, is repressed by SraL binding to the *tig* 5'-UTR during late stationary phase of growth (15).

posttranscriptional control | functional regulatory RNA | prokaryotes | small noncoding RNA | transcription termination

In prokaryotes, two distinct mechanisms of transcription termination are known: intrinsic termination (or Rho-independent termination), which involves terminator sequences in the RNA indicating RNA polymerase where to stop, and Rho-dependent termination, which relies on the action of Rho factor to stop RNA synthesis at specific sites (1). Rho factor is a very-well-conserved protein across bacteria, and its corresponding gene is present in >90% of sequenced bacterial genomes (2). Rho is a helicase protein with RNA-dependent ATPase activity that catalyzes the disassociation of nascent mRNA from genomic DNA and RNA polymerase, promoting transcription termination. This protein is essential in many bacterial organisms, namely *Escherichia coli*, *Salmonella enterica*, *Shigella flexneri*, and *Pseudomonas aeruginosa* (3–6). In fact, Rho is responsible for termination of about half of the transcription events in *E. coli* (7). Rho-dependent termination plays a significant role even in organisms in which it is not essential (4). For instance, Rho inactivation in *Bacillus subtilis* affects gene expression of important pathways related to cell motility, biofilm formation, and sporulation (8).

The advent of the high-throughput techniques enabled the discovery of small noncoding RNAs (sRNAs), RNA molecules whose function and importance were underestimated. Since their discovery, sRNAs have been broadly described as important regulators of gene expression in both prokaryotes and eukaryotes. Most of the sRNAs known are *trans*-encoded and act by base pairing with their target(s) with short and imperfect complementary

Significance

Rho is an essential protein that promotes transcription termination at specific regions of the genome. Its activity is important not only at the end of genes, but also within leader regions where it has regulatory functions. This protein was shown to be involved in the regulation of *rho* mRNA promoting its premature transcription termination. In this study, we included an additional player in the complex pathway of regulation of this protein. Using *in vivo* and *in vitro* experiments, the small noncoding RNA SraL was shown to directly interact with a specific region in the 5'-UTR of *rho* mRNA protecting this transcript against the action of its own protein Rho.

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In this report, we aimed to identify new biological targets of *Salmonella* SraL. Notably, we have established the role of SraL in the regulation of expression of the important transcription termination factor Rho. Previously, *rho* mRNA expression was shown to be autogenously regulated by attenuation of transcription and consequent premature transcription termination (18–20). By mutational analysis, SraL was revealed to directly base pair with the 5'-UTR of *rho* mRNA in a region upstream of the previously reported attenuators. This interaction protects *rho* mRNA from premature transcription termination by Rho protein.

As mentioned above, sRNAs can base pair in 5'-UTR of transcripts to preclude premature transcription termination by Rho factor (13). It is noteworthy that the regulator can be also modulated by the same mechanism, since SraL sRNA is responsible for protection of *rho* mRNA from premature transcription termination. This finding adds one level of complexity to the network of control of gene expression by termination, showing that SraL sRNA acts upstream of a regulatory cascade and regulates the regulator.

Results

MS2 Affinity Purification Coupled with RNA Sequencing to Identify Targets of SraL. Several studies were performed regarding *Salmonella* SraL sRNA expression and transcriptional and post-transcriptional regulation (14, 15, 17, 21, 22). However, the only biological function known for this sRNA is the downregulation of chaperone Trigger factor (15).

To identify new targets of SraL, we used the recently developed *in vivo* technology MS2-affinity purification coupled with RNA sequencing (MAPS) (23–26). For this, SraL was fused to an MS2 RNA aptamer, which binds the MS2 coat protein with high affinity, enabling copurification of SraL with its mRNA(s) target(s). Two different conditions were selected for the application of this technology: late stationary phase of growth in LB medium (OD₆₀₀ of 2 plus 6 h), the condition in which this sRNA is more expressed (15, 17); and anaerobic shock, a condition reported to increase the expression of SraL sRNA (27) (*SI Appendix*, Fig. S1). MAPS was performed in an *sraL*⁻ background to avoid interference of native copies of the sRNA. After normalization of the read counts by coverage (28), enrichment of the putative RNA binding partners was determined by comparing the number of reads obtained from tagged MS2-SraL and untagged sRNA control. Enriched RNA partners are listed in [Dataset S1](#).

SraL sRNA Is a Positive Regulator of *rho* mRNA. One of the top candidates enriched in MS2-SraL sample was the transcription termination factor Rho. Rho is a well-conserved homohexameric protein ubiquitous throughout the bacterial domain (2, 29, 30), and it is essential for the viability of many bacterial species, including *Salmonella* (5, 31). Due to its major importance in regulation of gene expression we decided to pursue the validation of the result obtained by MAPS.

Expression of *rho* mRNA was analyzed using three strains with different SraL sRNA expression levels: a wild-type, a *sraL* null mutant, and a complemented *sraL* null mutant strain in which *sraL* was cloned into a constitutive expression plasmid (15). Since MAPS technology was applied in cells grown until stationary phase (STAT) and upon anaerobic shock (AS), expression level of *rho* mRNA was initially assessed in these conditions.

In contrast to what happens with the other known target of SraL (15), the absence of this sRNA in the cell produced a negative impact in *rho* mRNA expression. Quantification of *rho* transcripts using both Northern blots and quantitative real time-PCR (qRT-PCR) revealed a decrease of ~50% of *rho* mRNA in the absence of SraL, compared with the wild-type strain (Fig. 1 *A, I and II* and Fig. 1 *B, I and II*). Consistently, *rho* mRNA expression levels were restored in *sraL* null mutant upon ectopic expression of wild-type *sraL* from a constitutive promoter

(Fig. 1 *A, I and II* and Fig. 1 *B, I and II*). The low expression level of *rho* mRNA in late stationary phase did not allow a proper detection of this transcript by Northern blot analysis, but it was quantified by qRT-PCR.

Afterward, this regulation was also tested in middle exponential growth (MEP; OD₆₀₀ of 1), a condition in which SraL sRNA is only slightly expressed (15). Despite the relatively low expression level of SraL sRNA, the same type of regulation, as in the other conditions, was obtained (Fig. 1 *C, I and II*).

Hereupon, SraL sRNA positively regulates *rho* mRNA expression level in *Salmonella* in all conditions tested.

SraL sRNA Directly Interacts with *rho* mRNA. To study in more detail the role of SraL in the regulation of *rho* mRNA and verify whether pairing is direct or indirect, an *in silico* prediction of possible interaction regions between the sRNA and its target was performed using IntaRNA and RNA Hybrid software (32, 33). Both algorithms predicted the same interaction, corresponding to a region of 12 nucleotides in length located in the 5'-UTR of *rho* mRNA (Fig. 2*A*).

Since the effect of SraL sRNA over *rho* mRNA was observed in all of the aforementioned conditions, we decided to proceed using cells grown until middle exponential phase. To test whether the predicted region is required for SraL-*rho* interaction, three point mutations were introduced in *sraL* chromosomal region (SraLMUT) (Fig. 2*A*), which cause disruption of the predicted interaction between the RNAs (cf. *SI Appendix*, Fig. S2*A and C*). We tried to ensure that these mutations did not compromise the secondary structure of the sRNA by predicting the secondary structure of SraLMUT using the *Mfold* program (Fig. 2*B*). We verified by Northern blot and qRT-PCR analysis that point mutations introduced in SraL abolished the effect of the sRNA over *rho* mRNA (Fig. 2*C, Upper*, cf. lanes 1 and 3; Fig. 2*D*), presenting a similar effect to the one observed in *sraL* null mutant (Fig. 2*C, Upper*, cf. lanes 2 and 3; Fig. 2*D*). To validate this effect, point mutations were inserted in *rho* chromosomal region (*rho*MUT) at the positions corresponding to the mutations introduced in the sRNA (Fig. 2*A*). Full complementarity should be restored when using the mutated version of both RNAs. In this condition *rho* mRNA expression level should be similar to the one obtained when using the wild-type background. By Northern blot and qRT-PCR, we confirmed that the wild-type version of SraL (SraLWT) was only able to modulate the wild-type *rho* mRNA levels (*rho*WT) and not the mutated version (*rho*MUT) (Fig. 2*C, Upper*, cf. lanes 1 and 4; Fig. 2*D*). The same effect was observed when using the mutated version of SraL, which is only able to regulate *rho*MUT (Fig. 2*C, Upper*, cf. lanes 5 and 3; Fig. 2*D*). When comparing SraL expression levels between the constructed strains, there was a clear increase of SraL expression in the strain containing the SraL chromosomal mutations (Fig. 2*C, Lower*, lane 3). This result was a surprise since the secondary structure of the sRNA was maintained and did not change its promoter region. Thus, another level of regulation would have been affected by the point mutations, such as the inactivation of a ribonuclease cleavage site. However, this increase was not sufficient to increase the expression of *rho*WT, which reinforces the results that SraLMUT was only able to interact with *rho*MUT. Curiously, the increase in SraL expression levels was not detected when both SraL and *rho* chromosomal mutations were combined in the same strain (SraLMUT/*rho*MUT) (Fig. 2*C, Lower*, lane 5).

These results confirmed that in fact SraL sRNA is a positive regulator of *rho* mRNA and revealed that this regulation is mediated through base pairing between sRNA and mRNA.

SraL Does Not Affect *rho* mRNA Stability. So far, our study revealed that SraL sRNA somehow upregulates the mRNA expression of the crucial transcription termination factor Rho. Furthermore,

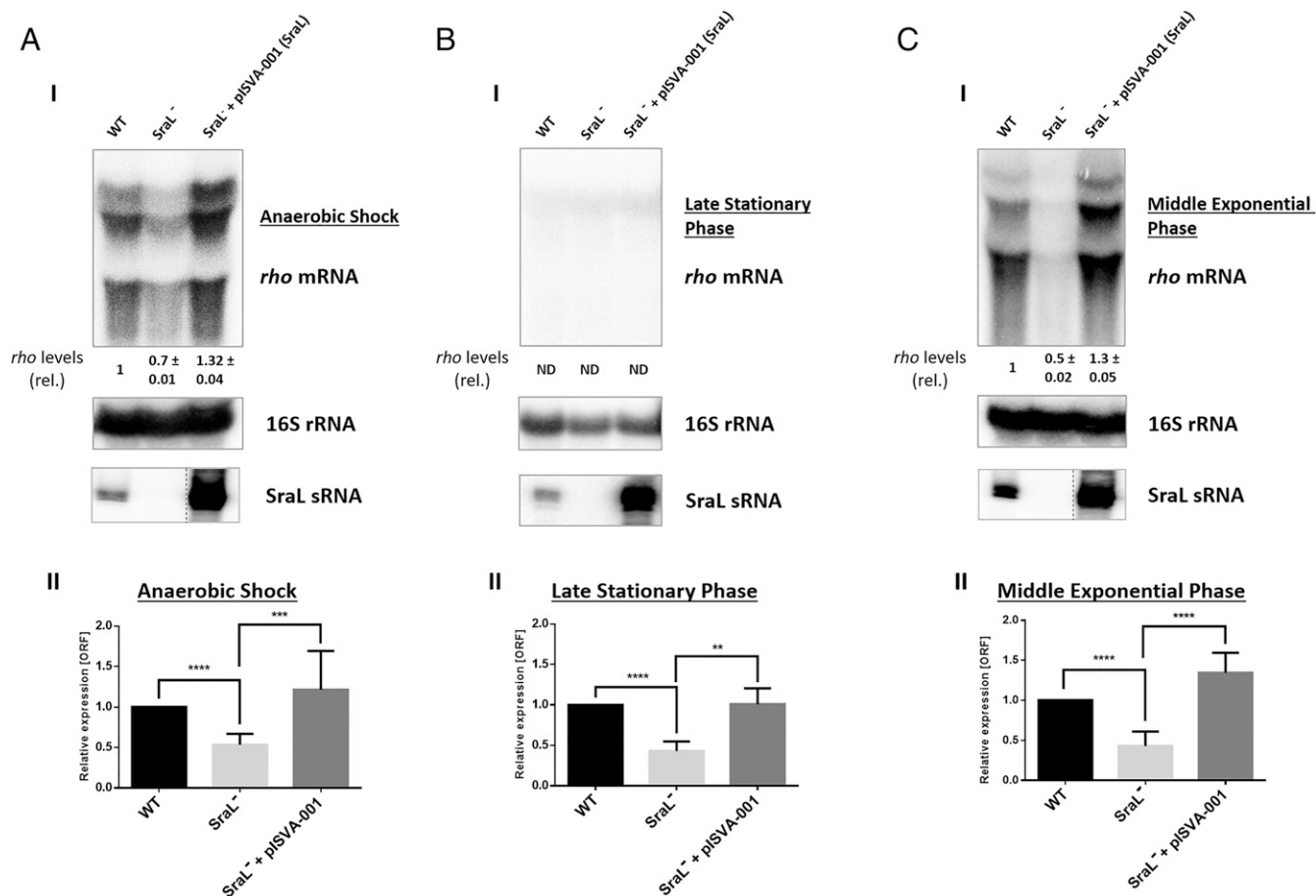


Fig. 1. *rho* mRNA regulation by SraL sRNA in different growth conditions. Total cellular RNA was extracted from *Salmonella* strains indicated in the figure. The following conditions were used: (A) Cells were grown in LB at 37 °C and 220 rpm to an OD₆₀₀ of 0.3 and then placed in a filled 50-mL Falcon tube and incubated at 37 °C without agitation during 30 min (AS). (B) Cells were grown in LB at 37 °C and 220 rpm until 6 h after OD₆₀₀ of 2 (late stationary phase). (C) Cells were grown in LB at 37 °C and 220 rpm until OD₆₀₀ of 1 (middle exponential phase). (I) (Upper) Twenty micrograms of total RNA were separated on a 1.3% formaldehyde/agarose gel. The gel was then blotted to a Hybond-N⁺ membrane and hybridized with the corresponding *rho* riboprobe. The transcripts were quantified using ImageQuant software. The amount of RNA in the wild type was set as one. The ratio between the amounts of RNA of each strain and the wild type is represented (relative levels). A representative membrane is shown, and the values indicated correspond to the average of several Northern blot experiments with RNAs from at least two independent extractions. The membrane was stripped and then probed with 16S rRNA as loading control. (Lower) Fifteen micrograms of total RNA were separated in a 6% PAA/8.3 M urea to determine the expression level of SraL; contrast of SraL⁻+pSVA-001 was adjusted separately as indicated by the dashed line. (II) The transcriptional level of *rho* was also determined by quantitative real time-PCR analysis using Rotor-Gene 3000 (Corbett) system and with cDNA synthesized from 1 μg of purified RNA. Values are shown relative to the expression levels in wild-type strain. Results were normalized with the expression of the housekeeping gene 23S rRNA and represent at least three independent experiments. *****P* < 0.0001, ****P* ≤ 0.0005, ***P* < 0.001 by Student's *t* test.

this regulation occurs through the base pairing between the sRNA and the 5'-UTR of *rho* mRNA. There are some mechanisms that might contribute to a regulation of this type (34). One of the most commonly described is related to the protective effect of sRNAs against ribonucleases, giving rise to an increased stability of the mRNA targets. To test if this is the case, stability of *rho* mRNA was accessed by measuring its half-life in the presence and absence of SraL sRNA. After addition of rifampicin, *rho* mRNA presented a fast decay rate of ~2.2 min in the wild-type strain (Fig. 3). Surprisingly, there was no significant difference in *rho* mRNA half-life with or without SraL sRNA expression, despite the already observed difference in steady levels (Fig. 3). These results indicate that SraL does not affect *rho* mRNA stability.

SraL Affects Rho-Dependent Termination at the 5'-UTR of *rho* mRNA.

It has been reported by several authors that the expression of *rho* is autogenously regulated at the transcriptional level, via at least five attenuators localized both in *rho* 5'-UTR and in the upstream region of its ORF (18–20) (Fig. 4A and B). This auto-

regulation results in premature transcription termination of *rho*, giving rise to the appearance of smaller mRNA transcripts containing only the 5' region of *rho* (20). Since SraL base pairs with *rho* in its 5'-UTR and near two of the already identified attenuators (Fig. 4A), we hypothesized that the sRNA could somehow be involved in this mechanism.

To test this hypothesis, three *rho* regions were selected to evaluate quantitatively the transcription termination and sRNA-mediated anti-termination by qRT-PCR: a segment in the 5'-UTR, upstream of the two predicted attenuators ("UTR" amplicon); the upstream region of the *rho* ORF ("5' ORF" amplicon); and a segment in the middle of *rho* ORF ("ORF" amplicon) (Fig. 4B). Using the "ORF" amplicon, qRT-PCR only detects transcripts which were not targeted by premature transcription termination (Fig. 4B). The qRT-PCR of the "UTR" amplicon detects not only transcripts subjected to premature transcription termination, but also the ones detected using the "ORF" amplicon (Fig. 4B). The internal normalization of the qRT-PCR values to the amount of "ORF" allowed us to discriminate the smaller transcripts mentioned above and to

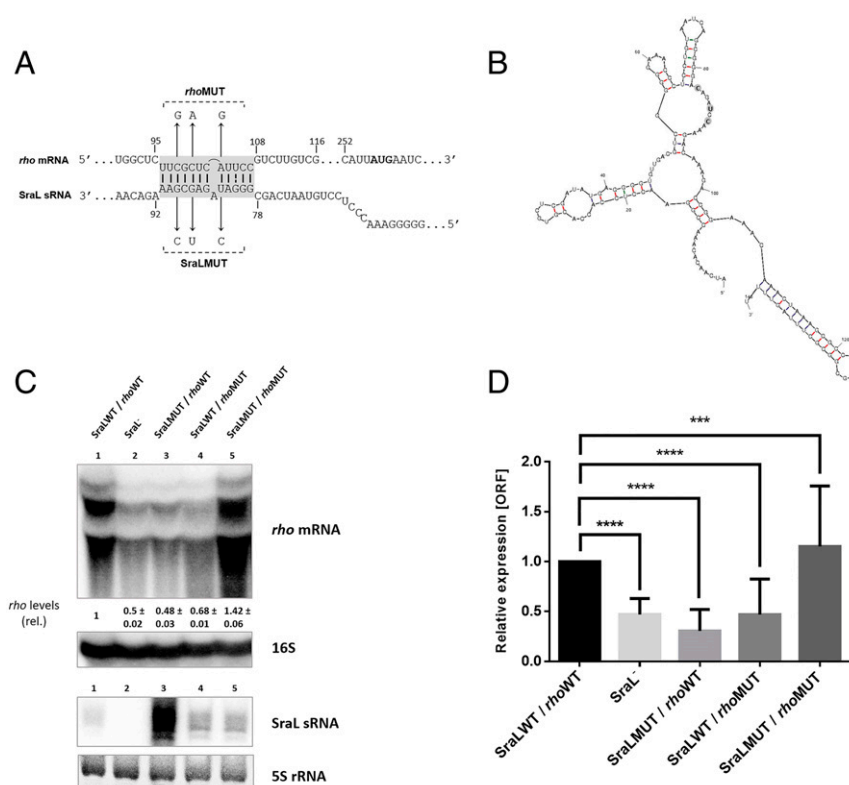


Fig. 2. Interaction between SraL sRNA and *rho* mRNA. (A) Predicted interaction region between *rho* mRNA and SraL sRNA by IntaRNA software (32) and RNA Hybrid (33). Chromosomal point mutations to generate *rho*MUT and SraLMUT alleles are indicated; *rho* mRNA AUG is also indicated in bold. (B) *S. Typhimurium* SraLMUT sRNA structure predicted by Mfold program (54). (C) Total cellular RNA was extracted from the *S. Typhimurium* strains indicated in the figure, grown in LB at 37 °C until OD₆₀₀ of 1. (Upper) The expression level of *rho* mRNA was determined by Northern blot using 20 µg of total RNA separated in a 1.3% formaldehyde/agarose gel. The amount of RNA in wild type was set as one. The ratio between the RNA amount of each strain and wild type is represented below each strain (relative levels). A representative membrane is shown, and values indicated correspond to the average of several Northern blot experiments with RNAs from at least two independent extractions. The membrane was stripped and then probed for 16S rRNA as loading control. (Lower) Fifteen micrograms of total RNA were separated in a 6% PAA/8.3 M urea to determine the expression level of both SraLWT and SraLMUT sRNAs; 5S rRNA was used as loading control. (D) The transcriptional level of *rho* was also determined by quantitative RT-PCR analysis with the Rotor-Gene 3000 (Corbett) system, using cDNA synthesized from 1 µg of purified RNA. Values are shown relative to the expression levels in wild-type strain. Results were normalized with the expression of the housekeeping gene 23S rRNA and represent at least three independent experiments. *****P* < 0.0001, ****P* < 0.0005 by Student's *t* test.

directly compare the termination efficiency within *rho* UTR among the different strains. The highest ratio of [UTR]/[ORF] detected in the *sraL* null mutant when comparing with the wild-type strain indicates a more efficient Rho-dependent termination in *rho* 5'-UTR in the absence of the sRNA (Fig. 4C). Curiously, the same tendency was obtained for [5'ORF]/[ORF] ratio (Fig. 4D). The effect obtained in the *sraL* null mutant was reversed to the wild-type situation when using the complemented *sraL* null mutant strain, corroborating the previous results (Fig. 4C and D).

Hereupon, the results obtained seem to indicate that SraL sRNA protects *rho* mRNA from premature transcription termination.

SraL sRNA Does Not Regulate *rho* mRNA in the Absence of Rho Activity. To determine whether SraL sRNA and Rho regulate *rho* mRNA expression in the same pathway, both wild-type and *sraL* null mutant strains were exposed to bicyclomycin (BCM). This antibiotic is highly specific to Rho and acts by disrupting its ATPase and translocase activity, making Rho an inactive protein (31). BCM has been used to mimic the absence of Rho in the cell, due to the impossibility of obtaining *rho* null mutants in *Salmonella* and other bacterial species (12, 13, 35, 36).

As expected, *rho* mRNA expression was increased more than threefold in the samples treated with BCM, reflecting the absence of Rho activity in its autoregulation (Fig. 5A, cf. WT -BCM and WT +BCM). When Rho was active (without BCM), a 50% reduction was obtained in *rho* mRNA expression in the

absence of SraL (Fig. 5A, cf. WT and SraL⁻ in -BCM). However, when Rho activity was inhibited by the addition of BCM this reduction was no longer observed (Fig. 5A, cf. WT and SraL⁻ in +BCM). Therefore, *rho* mRNA positive regulation by SraL seems to be dispensable in the absence of Rho activity.

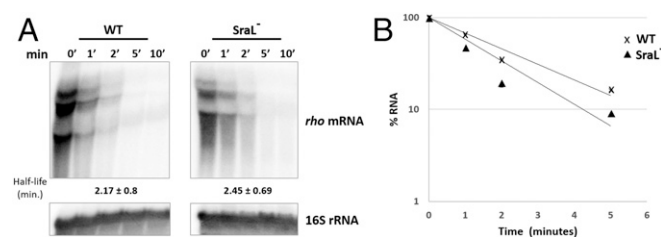


Fig. 3. Analysis of *rho* mRNA stability. Wild-type and SraL⁻ strains grown in LB medium at 37 °C and 220 rpm until OD₆₀₀ of 1. At this OD, a mixture of rifampicin and nalidixic acid was added to the cultures and samples were taken out at the indicated times. (A) Total RNA was extracted, and 20 µg of RNA was separated on a 1.3% formaldehyde/agarose gel. A representative membrane is shown, and the half-life values indicated correspond to the average of several Northern blot experiments with RNAs from at least two independent extractions. (B) The quantification of the transcripts was plotted versus time of extraction (in minutes) to calculate the half-life of the mRNA.

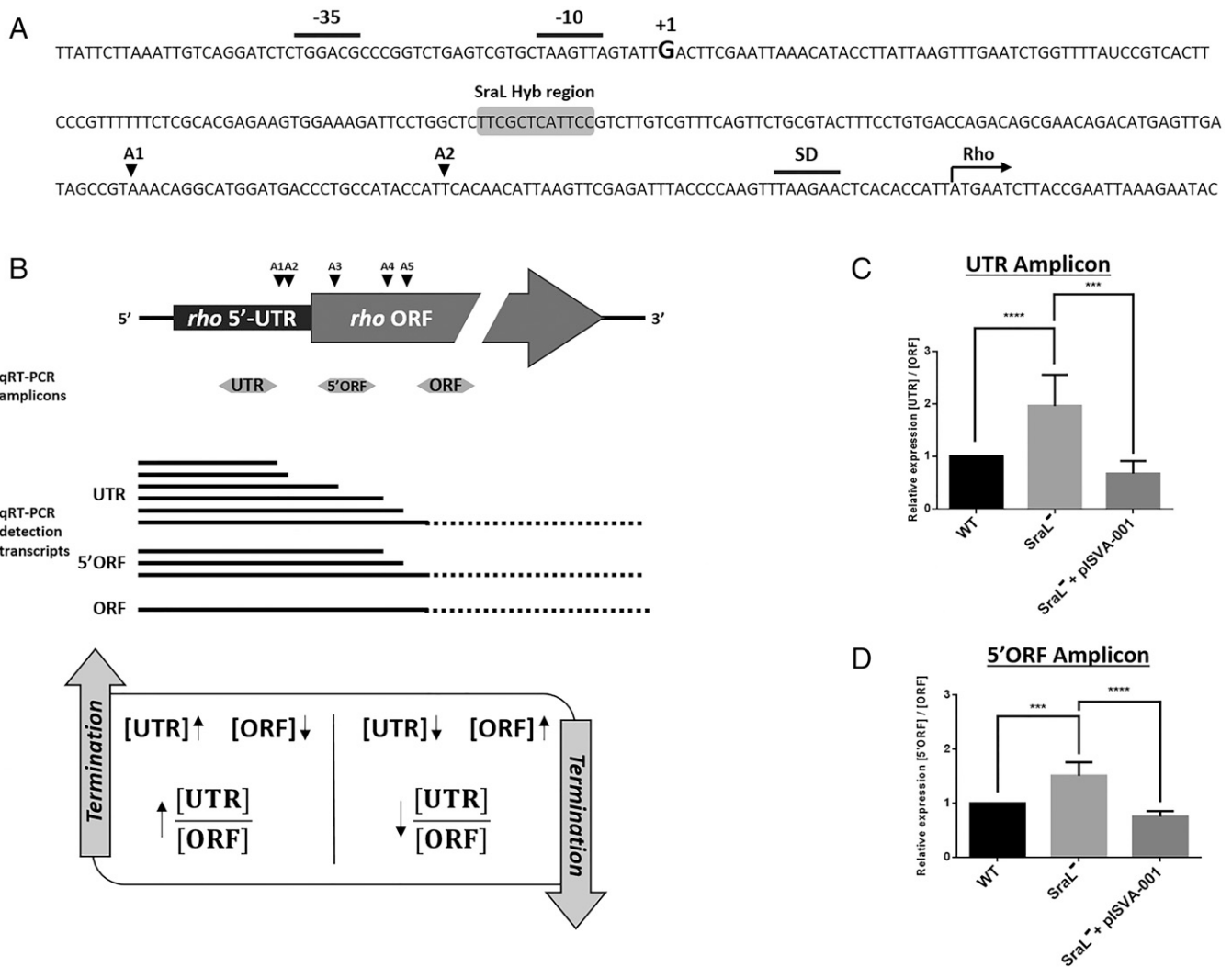


Fig. 4. SraL sRNA affects *rho* mRNA at the transcription termination level. (A) Nucleotide sequence of the upstream region of *rho* gene. The -35 and -10 sites, Shine–Dalgarno box (5), as well as the $+1$ (G) of the *rho* mRNA (20) and the interaction region where SraL sRNA hybridizes are indicated. A1 and A2 correspond to the attenuation sites described in ref. 20. (B, Upper) Scheme of *rho* 5'-UTR and ORF regions with the location of the five attenuation sites described in ref. 20. The locations of the three qRT-PCR amplicons are indicated below. (B, Middle) Transcripts detected with the different amplicons used in the qRT-PCR. (B, Lower) Diagram with the approach used for the estimation of Rho-dependent termination within *rho* leader. An increase in the [UTR]/[ORF] ratio corresponds to an increase in the termination efficiency and vice versa. An increase of the termination efficiency implies the increase of [UTR] and the decrease of [ORF] and vice versa. (C and D) Total cellular RNA was extracted from the *S. Typhimurium* strains indicated in the graphics, grown in LB at 37°C until OD_{600} of 1. Transcript levels of the [UTR] amplicon (C) and of the [5'ORF] amplicon (D) were quantified by qRT-PCR. The values were normalized to that of the ORF amplicon and represent at least three independent experiments. **** $P < 0.0001$, *** $P \leq 0.0005$ by Student's *t* test.

To further validate our hypothesis, we used the same approach as in the previous point to compare the termination efficiency within *rho* among the different strains, with and without BCM treatment. As expected, in the wild-type strain there was less premature transcription termination upon addition of BCM (inactivation of Rho), which was reflected by the decrease in the [UTR]/[ORF] ratio (Fig. 5B, cf. WT –BCM and WT +BCM). In agreement with the previous results, the difference obtained in [UTR]/[ORF] ratio between the wild-type and *sraL* null mutant strains when Rho was active (–BCM) was no longer observed when Rho was inhibited (Fig. 5B). Although there was a lower difference, the same tendency was obtained for [5'ORF]/[ORF] ratio (Fig. 5C). Thus, the protective effect of *rho* mRNA by SraL sRNA was only important when Rho is active in the cell.

These data indicate that SraL acts by base pairing with *rho* mRNA protecting it from the action of Rho in its 5'-UTR.

SraL sRNA Inhibits Rho Termination in Vitro. To further study the mechanism of action of SraL over *rho* mRNA, in vitro transcription termination experiments were performed using both *rho*WT and *rho*MUT as DNA templates, purified *Salmonella* Rho protein and purified SraL sRNA (Fig. 6). As expected, comparing reactions in the absence and presence of Rho protein (Fig. 6B, cf. lanes 1 and 2), it was possible to observe a decrease in the intensity of the runoff band (which corresponds to the transcription of the entire template) with concomitant appearance of smaller transcripts resultant from premature transcription termination. In agreement with in vivo results, upon addition of SraL sRNA there was an increment of the runoff transcript resultant from SraL protective effect against premature termination by Rho (Fig. 6B, Left gel, cf. lanes 2 and 3). Additionally, this effect was abolished when using *rho*MUT as a template (Fig. 6B, Right gel, cf. lanes 2 and 3), which corroborates the importance of the predicted interaction region in this

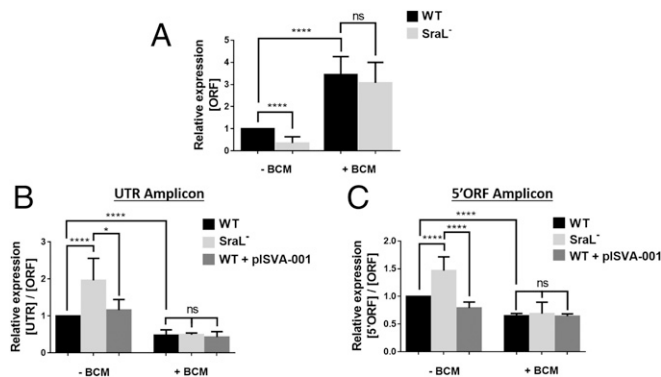


Fig. 5. SraL sRNA is not necessary for *rho* mRNA regulation in the absence of Rho. Total cellular RNA was extracted from the *S. Typhimurium* strains indicated in the graphics, grown in LB at 37 °C until an OD₆₀₀ of 1. Samples were taken before and after treatment with BCM (10 μg/mL, 15 min). (A) Transcript levels of the [ORF] amplicon were quantified by qRT-PCR. The values were normalized to that of the housekeeping 23S rRNA and represent at least three independent experiments. **** $P < 0.0001$, ns indicates non-significant by Student's *t* test. (B and C) Transcript levels of the [UTR] amplicon (B) and of the [5'ORF] amplicon (C) were quantified by qRT-PCR. The values were normalized to that of the ORF amplicon and represent at least three independent experiments. **** $P < 0.0001$, * $P \leq 0.05$, ns indicates non-significant by Student's *t* test.

regulation (Fig. 2). Two different controls were used in these experiments. DsrA sRNA was shown to be involved in the suppression of Rho-dependent transcription termination of *rhoS* (13); in this case it was used as a negative control (Fig. 6B, cf. lanes 2 and 4). Moreover, as a positive control we utilized a small oligonucleotide (Anti; 12 nucleotides) corresponding to the SraL sequence predicted to interact with *rho*WT mRNA (Fig. 2A). The result obtained (Fig. 6B, Left gel, cf. lanes 2 and 5) also confirmed the importance of this region since it was very effective in blocking Rho's action, leading to the increase of the runoff transcript's level and to the reduction of smaller transcripts' levels.

In conclusion, in vitro transcription termination experiments confirmed in vivo results obtained so far.

Influence of Chaperones in SraL-Rho Regulation. RNA-binding proteins that act in conjunction with sRNAs to regulate its targets have been widely studied (22, 37). In a previous study, chaperone Hfq was shown to be important for stabilization of SraL sRNA in stationary phase of growth (17). More recently, this sRNA was also shown to coimmunoprecipitate with ProQ, and this chaperone was also very important for the stability of SraL (22).

To study the importance of these chaperones for SraL-*rho* regulation Northern blot experiments were performed using strains lacking *proQ* and *hfq* (Fig. 7). Regarding the effect of ProQ, a 60% reduction was obtained in *rho* transcripts' level in *proQ* null mutant (Fig. 7A, Upper, cf. lanes 1 and 3) that was only partially restored when expressing *proQ* in trans (Fig. 7A, Upper, cf. lanes 3 and 4). To test if ProQ and SraL affect *rho* message through the same pathway, the effect of the double mutant SraL⁻/ProQ⁻ in *rho* transcripts level was evaluated. The level obtained was comparable to the one of *proQ* null mutant (Fig. 7A, Upper, cf. lanes 3 and 5). Moreover, *rho* mRNA expression levels were restored to the same values obtained with *sraL* null mutant upon ectopic expression of *proQ* from a constitutive promoter (Fig. 7A, Upper, cf. lanes 2 and 6). These results seem to indicate that even though SraL and ProQ affect *rho* mRNA through the same pathway, ProQ is also affecting *rho* mRNA through an additional pathway.

The absence of the other chaperone, Hfq, showed to be more critical since the level of *rho* transcript decreased 80% when

compared to the wild-type strain (Fig. 7B, Upper, cf. lanes 1 and 3). Contrarily to what happens in stationary phase (17), Hfq seems to somehow inhibit the expression of SraL in exponential phase (Fig. 7B, Lower, cf. lanes 1 and 3). Although SraL level was increased in *hfq* null mutant, the sRNA was not able to counteract the effect caused by the absence of *hfq*. Moreover, in the double mutant lacking both *sraL* and *hfq* the effect on *rho* mRNA level was not cumulative compared with the two single mutants (Fig. 7B, Upper, cf. lanes 2, 3 and 5). These results seem to indicate that SraL and Hfq are affecting this transcript through the same pathway. However, like ProQ, Hfq also seems to be affecting *rho* level through another pathway of regulation.

Impact of SraL in Rho Protein Level and Activity. So far, our results revealed the importance of the sRNA SraL for the protection of *rho* mRNA against the action of its own protein. The next step was to investigate whether the difference obtained for *rho* mRNA level in the absence of SraL would be also reflected at protein level. For that, Western blot analysis was performed using the same strains used in Northern blot analysis. Differences in Rho monomer expression level were assessed running total protein extracts in denaturing conditions (Fig. 8A). On the other hand, variations at the level of Rho hexamer were investigated using native conditions (Fig. 8B). In both cases, there was no

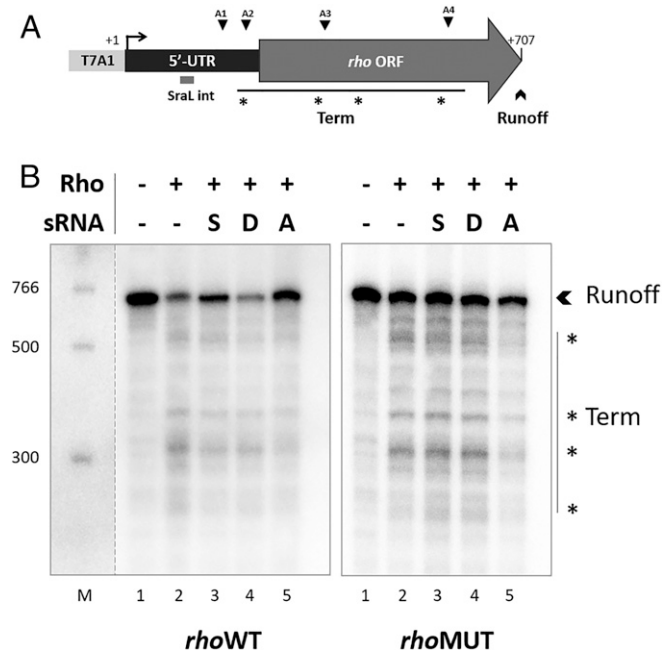


Fig. 6. *rho* in vitro transcription termination is also affected by SraL sRNA. (A) Scheme of *rho* DNA template used in the in vitro transcription termination assay. T7A1 corresponds to the promoter sequence recognized by *E. coli* RNA polymerase inserted in the templates by two consecutive PCRs. The approximate site where SraL interacts and the location of the four attenuation sites (A1–A4) described in ref. 20 are indicated. Termination products represented in B as the most prominent bands are indicated by asterisks (*). (B) In vitro transcription termination assay was performed using both *rho*WT (Left gel) and *rho*MUT (Right gel) as DNA template and in the absence (lanes 1 and 2) or presence of different sRNAs: SraL sRNA (S; lane 3), DsrA (D; lane 4; serving as a negative control), and Anti (A; lane 5; synthetic oligonucleotide fully complementary to *rho*WT mRNA sequence targeted by SraL, serving as a positive control). The runoff transcripts (Runoff) and the transcription termination region are indicated (Term). The bands identified by asterisks (*) represent the most prominent termination transcripts in this experiment. The contrast of the DNA ladder lane (M; PCR Marker, New England Biolabs) was adjusted separately as indicated by the dashed line. Representative gels are shown; the experiment was performed more than 3 times with each DNA template.

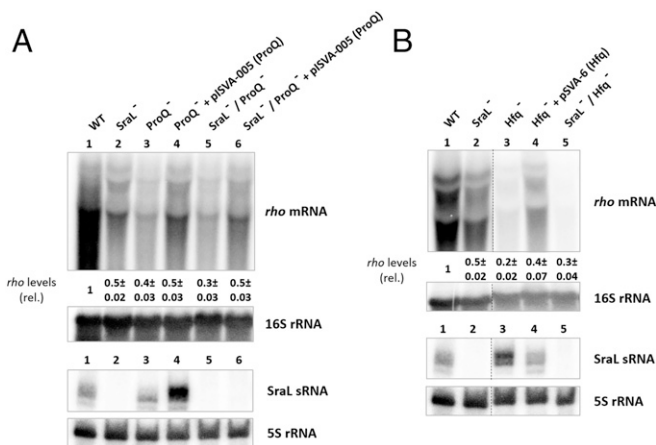


Fig. 7. Importance of the chaperones ProQ (A) and Hfq (B) in SraL-*rho* regulation. (A and B) Total cellular RNA was extracted from the *S. Typhimurium* strains, grown in LB at 37 °C until an OD_{600} of 1. (Upper) The *rho* mRNA expression level was determined by Northern blot using 20 μ g of total RNA separated in a 1.3% formaldehyde/agarose gel. The amount of RNA in wild type was set as one. The ratio between the RNA amount of each strain and wild type is represented below each strain (relative levels); 16S rRNA was used as loading control. (Lower) Thirty micrograms of total RNA were separated in a 6% PAA/8.3 M urea to determine the expression level of SraL sRNA; 5S rRNA was used as loading control. A representative membrane is shown, and values indicated correspond to the average of several Northern blot experiments with RNAs from at least two independent extractions. Dashed lines indicate noncontiguous lanes.

significant difference in Rho protein level between the strains used (Fig. 8 A and B).

The next step was to measure the activity of the protein. For that, we performed β -galactosidase activity assays using a *Salmonella* LT2 strain with *E. coli pgaABDC* promoter and *rut* site fused to *lacZ* reporter gene (MA11598). This construct was used since the existence of a Rho-dependent terminator in *pgaA* 5'-UTR was previously reported (38). Due to experimental constraints, the assay was only performed using the strains MA11598 and MA11598 overexpressing SraL *in trans* (MA11598 + pSVA001). As shown in Fig. 8C, there was a slight but significant decrease of β -galactosidase activity in the strain with high levels of SraL (MA11598 + pSVA-001) in all of the conditions tested. This decrease was a consequence of the increased transcription termination in *pgaA* Rho-dependent terminator mediated by Rho protein.

In conclusion, although we were not able to detect differences in Rho protein level by Western blot, the slight effect obtained in Rho activity upon overexpression of SraL shows that this regulation has a biological repercussion.

Discussion

There are several studies regarding SraL regulation in *Salmonella*, but only one biological function has been assigned for this sRNA as of yet. SraL was shown to downregulate the expression of the important chaperone Trigger factor in a mechanism that involves the base pairing of the sRNA with the 5'-UTR of *tig* mRNA (15). In the present study, we have uncovered the role of SraL as a positive regulator of the transcription termination factor Rho. SraL sRNA constitutes an example of the versatility of the sRNAs since it interacts with both of its targets via two different regions leading to negative or positive regulation (SI Appendix, Fig. S4) (15).

Rho is known to be crucial for the prokaryotic termination of transcription either at the end of genes and/or operons or in the leader sequence of several mRNAs, performing regulatory functions (3, 13, 31, 39). The transcription of *E. coli rho* was shown to be autogenously regulated, via several attenuators lo-

cated in its leader region (18–20). The existence of Rho-dependent termination within *rho* mRNA was very recently corroborated in *E. coli*. Using a very elegant experiment, the Rho termination score was calculated for several transcripts, and *rho* mRNA was used as a control (35). The high percentage of homology between *Salmonella* and *E. coli rho* sequence, namely in its 5'-UTR, suggests that the mechanism is similar in both organisms (SI Appendix, Fig. S3). In fact, the expression of *Salmonella rho* mRNA was shown to increase more than threefold upon the addition of the specific inhibitor of Rho protein BCM to the cell culture (Fig. 5A), revealing that in this pathogenic organism Rho also acts as a negative regulator of its own transcription.

The findings made in the current study suggest the existence of an additional player in the already described mechanism. Using the recently developed MAPS technology, *rho* mRNA was one of the top candidates present in the list of the RNAs predicted to base pair with the sRNA SraL (Dataset S1). Due to the high importance of this protein in transcription termination process, we decided to investigate this regulation in detail. Accordingly, SraL showed to be a positive regulator of *rho* mRNA expression not only when the sRNA is highly expressed (anaerobic shock and late stationary phase), but also in conditions where SraL is only slightly expressed (middle exponential phase) (Fig. 1). This fact corroborates the importance of the sRNA in *rho* mRNA regulation. The interaction region between SraL and the 5'-UTR of *rho* mRNA was predicted *in silico* and confirmed through the insertion of specific chromosomal mutations that abolish the interaction between both RNAs (Fig. 2). Curiously, analyzing the RNA-seq results obtained after MS2-SraL enrichment, the most enriched region of *rho* mRNA was indeed its 5'-UTR.

Since SraL was shown to directly base pair with the 5'-UTR of *rho* mRNA, we hypothesized that the sRNA would protect this mRNA from degradation by ribonucleases. However, the stability of

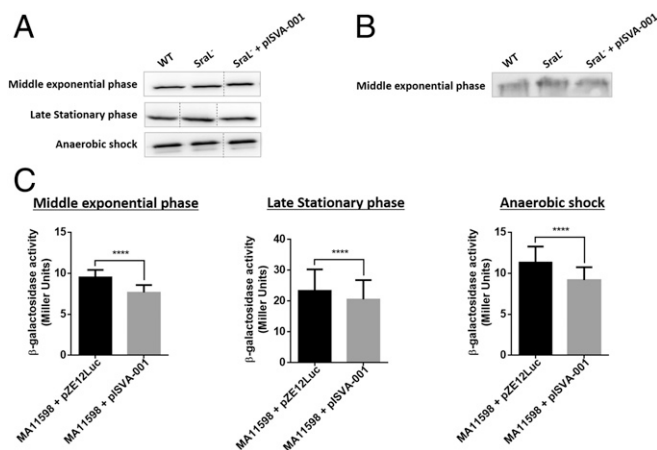


Fig. 8. The influence of SraL in Rho protein expression and activity. (A) Detection of Rho protein under denaturing conditions was performed by Western blot from 5 μ g of total protein extracts obtained in middle exponential phase, stationary phase, and upon anaerobic shock. Dashed lines indicate noncontiguous lanes. (B) Native Western blot to evaluate Rho protein expression in middle exponential phase was done from 40 μ g of total protein cellular extracts. For both denaturing and native conditions Rho expression was determined in strains expressing SraL sRNA at different levels using anti-Rho antibody (MyBioSource). (C) β -Galactosidase activity was accessed in a strain carrying a fusion of LacZ reporter under the control of *pgaABDC* operon promoter and *rut* site, using o-nitrophenyl- β -D-galactopyranoside (Sigma) as substrate. Rho protein activity in *pgaABDC* promoter was evaluated in middle exponential phase, stationary phase, and upon anaerobic shock. Results are expressed in Miller units and are representative of at least three biological replicates. Statistical analysis was carried out using GraphPad Prism 6 software. **** $P < 0.001$ (Paired *t* test).

rho mRNA was not affected by the absence of the sRNA (Fig. 3). Due to the previously described autogenous regulation of *rho* mRNA transcription, we decided to investigate the role of SraL in this mechanism. In the absence of the sRNA, we have obtained a more efficient Rho-dependent termination not only in the 5'-UTR, but also in the upstream region (5'ORF) of *rho* mRNA. Moreover, this difference was abolished when Rho factor activity was inhibited by the addition of BCM. Thus, SraL sRNA positively regulates the expression of *rho* mRNA by affecting Rho-dependent termination through base pairing with its 5'-UTR in a mechanism that is only important in the presence of an active Rho factor (Figs. 4 and 5).

There are five different attenuation regions described in *rho* mRNA that are positioned not only in its 5'-UTR, but also in its 5'ORF (Fig. 4B, Upper) (20). In the approach used to investigate in vivo Rho-dependent termination, when measuring the UTR amplicon by qRT-PCR not only was the full-length transcripts detected, but also the smaller transcripts resulting from premature transcription termination in all of the attenuators. However, only the two longer transcripts resulting from premature transcription termination (4, 5) were detected when measuring the 5'ORF. This fact explains the lower difference obtained in the wild-type strain for [5'ORF]/[ORF] ratio compared with the [UTR]/[ORF] ratio upon the addition of BCM and consequent inhibition of Rho activity (Fig. 5 B and C). The attenuation regions described for *E. coli rho* (20) were also identified in *Salmonella* by performing an in vitro transcription termination assay. In this experiment we were able to detect the appearance of most of the attenuation regions (A1 to A4) when comparing the samples without and with Rho protein (Fig. 6).

The attenuation region A5 is located downstream of the 3'-end of the DNA template used, and for that reason was not detected in this assay. In line with the in vivo results, the addition of SraL in the reaction gave rise to an increment of runoff transcripts and to a decrease in termination products, which confirms the protective effect of SraL over *rho* mRNA. In addition, this effect was specific to *rho*WT confirming in vitro the predicted interaction region between the two RNA species (Figs. 2 and 6).

The influence of chaperones in this type of riboregulation has been widely studied (22, 37). Indeed, SraL was already associated with two of the major RNA-binding proteins, Hfq and ProQ (17, 22). The results obtained seem to indicate that these chaperones are involved in the regulation of *rho* by SraL (Fig. 7). However, both chaperones seem to affect *rho* mRNA in an alternative pathway in which SraL is not involved. In fact, Hfq was reported to be directly associated with Rho protein, inhibiting its ATPase, helicase, and transcription termination activities (40). Since Rho protein negatively affects *rho* transcript, in the absence of *hfq*, the level of *rho* mRNA should be increased. In fact, this was not the case since in *hfq* null mutant *rho* mRNA level decreased 80%. These data, together with the results obtained regarding the level of Rho protein in the absence of *sraL* (Fig. 8), show the complexity behind the regulation of such an important protein like Rho.

With all of the results obtained in this study, we propose a model for *rho* mRNA regulation that contemplates the action of SraL sRNA (Fig. 9). When expressed in the cell, SraL binds to the 5'-UTR of *rho* mRNA, an interaction that can be mediated by ProQ and/or Hfq. The formation of sRNA/5'-UTR complex may cause various effects depending on the base-pairing region.

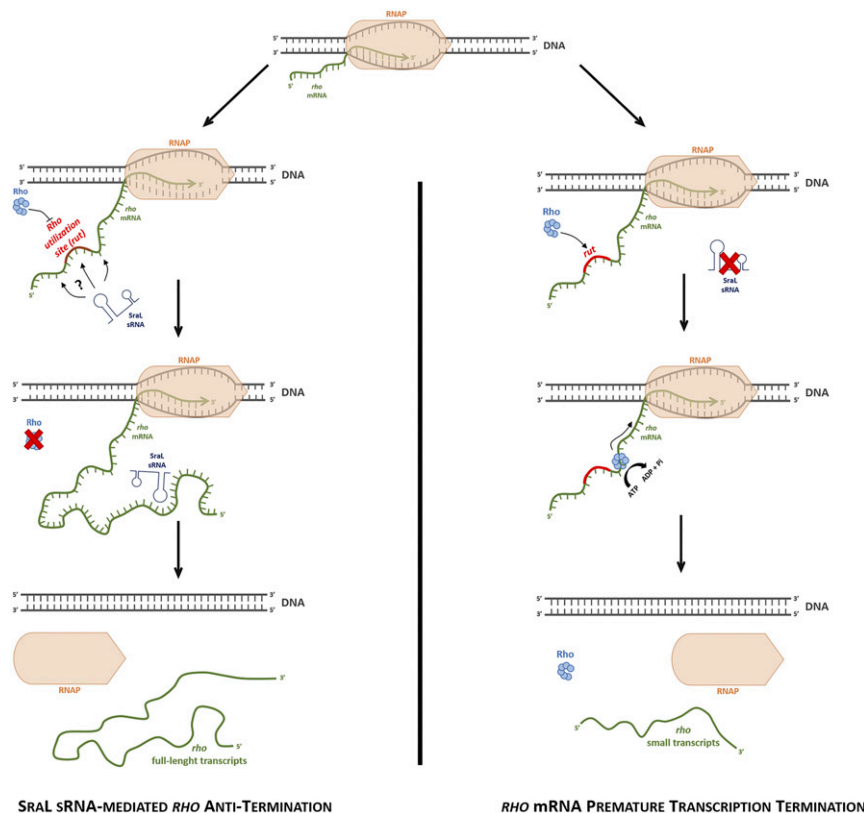


Fig. 9. Proposed model of transcription termination control of *rho* mRNA by SraL sRNA. (Left) Upon binding of the sRNA to the 5'-UTR of *rho* mRNA, *rut* site becomes inaccessible to Rho factor or Rho translocation is inhibited. This "protective" effect of SraL over *rho* mRNA allows the transcription to proceed until the complete synthesis of the full-length *rho* transcripts. (Right) In the absence of SraL sRNA, the *rut* sequence(s) localized in the upstream region of *rho* mRNA is recognized by Rho factor. After the loading onto the *rut* site, Rho translocates on RNA (in a reaction dependent on ATP) catching up to the elongating RNAP complex (the other factors involved in the transcription process are omitted in this model). Upon binding of Rho to RNAP, the unwinding of the RNA:DNA hybrid occurs and the release of the nascent transcripts from RNAP complex, and the premature transcription termination occurs.

If the binding between both RNAs occurs in the Rho utilization site (*rut*), Rho is not able to bind this region leading to the inhibition of Rho loading; the same effect is obtained if the sRNA interacts upstream or downstream of *rho rut* site leading to a rearrangement of the RNA secondary structure and consequent occlusion of *rut*. Additionally, the sRNA interaction can interfere with Rho translocation along RNA inhibiting also its action in *rho* mRNA autoregulation. Thus, by interacting with *rho* mRNA SraL protects this transcript from premature transcription termination (Fig. 9, *Left*). In the absence of *sraL* expression, Rho factor recognizes the *rut* site in the 5'-UTR of *rho* mRNA, and after loading and translocating along RNA, catches up to the elongating RNA polymerase (RNAP) causing premature transcription termination (Fig. 9, *Right*).

Interestingly, sRNA-mediated anti-termination was also recently described for the general stress σ^S subunit of RNA polymerase (13). The sRNAs DsrA, ArcZ, and RprA were previously known to relieve the translational inhibition by base pairing with the *rpoS* leader sequence, rendering the ribosome binding site (RBS) available to the ribosome (41–43). Beyond this mechanism, these three sRNAs also suppress premature Rho-dependent transcription termination stimulating the transcription of *rpoS* during the transition to the stationary phase of growth (13). By using the specific Rho inhibitor BCM and an RNA-seq approach, the authors also verified that Rho functions as a global attenuator of gene expression, and that sRNA-mediated anti-termination is a widespread mode of bacterial gene regulation (13). Curiously, most of the riboswitches from *E. coli* and *B. subtilis* were shown to control premature transcription termination. In *B. subtilis*, riboswitches modulate the formation Rho-independent terminators (44, 45). In turn, *E. coli* riboswitches control transcription termination by using Rho transcription factor (35, 46). This mechanism was also described for a *Salmonella* riboswitch (46). Taken together, all these studies indicate that the mechanism of anti-termination mediated by riboregulators seem to be widespread in several bacterial organisms.

Using a combination of experimental and bioinformatic approaches, we have described in detail the role of *Salmonella* SraL sRNA in the control of the expression of the important transcription termination factor Rho. This sRNA was shown to interact with the 5'-UTR of *rho* mRNA protecting the transcript against premature transcription termination by its own protein. Curiously, the interaction region in both RNA species corresponds to a very-well-conserved sequence in enterobacteria (*SI Appendix, Figs. S3 and S4*), which may indicate that this regulation also occurs in other bacterial organisms.

Materials and Methods

Oligonucleotides. The oligonucleotides used in this work are listed in *SI Appendix, Table S1* and were synthesized by STAB Vida (Portugal).

Bacterial Strains and Plasmids. All bacterial strains and plasmids used throughout this study are listed in *SI Appendix, Tables S2 and S3*, respectively. Unless stated, *Salmonella* strains used are isogenic derivatives of the wild-type *Salmonella enterica* serovar Typhimurium strain SL1344. Strains and plasmids constructions are detailed in *SI Appendix, Supplementary Materials and Methods*.

Bacterial Growth. All strains were grown in LB at 37 °C and 220 rpm. Electroporation was used for transformation of *S. Typhimurium*. The SOC (super optimal broth with catabolite repression) medium was used to recover transformants after electroporation procedure.

To apply anaerobic shock, cells were first grown at 37 °C and 220 rpm to an OD₆₀₀ of 0.3. Then, they were placed in a completely filled 50-mL Falcon tube and incubated at 37 °C without agitation for 30 min (27). For treatment with BCM, cultures were grown to middle exponential phase (OD₆₀₀ of 1), and then BCM was added to a final concentration of 10 µg/mL for 15 min before harvesting.

When appropriate, antibiotics were used at the following concentrations: 100 µg/mL ampicillin, 90 µg/mL streptomycin, 100 µg/mL kanamycin, and 25 µg/mL chloramphenicol.

MS2-Affinity Purification Coupled with RNA Sequencing. *Salmonella* Typhimurium SL1344 strains were grown in LB medium at 37 °C (100 mL). Cells were harvested in (i) exponential phase after anaerobic shock and (ii) late stationary phase. MAPS was performed as previously described in refs. 24 and 26 and are detailed in *SI Appendix, Supplementary Materials and Methods*. The cDNA libraries were prepared with ScriptSeq v3 RNA-Seq Library Preparation Kit (Illumina) and sequenced (MiSeq sequencing system; Illumina). We used Galaxy Project (47) to analyze data. The whole list of genes enriched is available in *Dataset S1*. The GEO accession number is GSE108234.

RNA Extraction and Northern Blot. Overnight cultures were diluted 1/100 in fresh medium and grown to the indicated cell densities (growth conditions are detailed in the respective figure legends). Culture samples were collected, mixed with 1 volume of stop solution (10 mM Tris pH 7.2, 25 mM NaNO₃, 5 mM MgCl₂, 500 µg/mL chloramphenicol), and harvested by centrifugation (10 min, 6,000 g, 4 °C) (48). For stability experiments, rifampicin (500 µg/mL) and nalidixic acid (20 µg/mL) were added to cells grown in LB at 37 °C, 220 rpm, until an OD₆₀₀ of 1. Incubation was continued, and culture aliquots were withdrawn at the times indicated in the respective figure.

RNA was isolated using the phenol/chloroform extraction method, precipitated with ethanol and resuspended in RNase free water (48). The integrity of the RNA samples was accessed by 1.5% Agarose gel and the samples were quantified on a Nanodrop 1000 machine (NanoDrop Technologies).

For Northern blot analysis, total RNA was separated under denaturing conditions either by 8.3 M urea/6% polyacrylamide gel in TBE (Tris/borate/EDTA) buffer or by 1.3% agarose Mops/formaldehyde gel. For polyacrylamide gels, transfer of RNA onto Hybond-N⁺ membranes (GE Healthcare) was performed by electroblotting in TAE (Tris/acetic acid/EDTA) buffer. For agarose gels, RNA was transferred to Hybond-N⁺ membranes by capillarity using 20× SSC as transfer buffer. In both cases, RNA was UV cross-linked to the membranes immediately after transfer. Membranes were then hybridized with PerfectHyb buffer (Sigma) at 68 °C for riboprobes and 43 °C for oligoprobes. Signals were visualized by PhosphorImaging (Fujifilm FLA-5100, FUJIFILM Life Science) and analyzed and quantified using ImageQuant software (Molecular Dynamics).

Hybridization Probes. Oligonucleotides for templates amplification are listed in *SI Appendix, Table S1*. Labeling of the probes was performed as previously described (17). The riboprobes were obtained using the primers pair pIS-021/pIS-022 for SraL riboprobe and pIS-049/pIS-051 for *rho* riboprobe; 5S rRNA and 16S rRNA were detected by the 5'-end-labeled oligonucleotides pIS-023 and pIS-024, respectively.

Quantitative Real Time-PCR Analysis. The transcriptional levels of transcripts were determined with the Rotor-Gene 3000 (Corbett) system, using SensiFast SYBR kit (Bioline) (according to the supplier's instructions), cDNA synthesized from 1 µg of purified RNA with SensiFAST cDNA synthesis kit (Bioline), and the primers pair pIS-050/pIS-051 for *rho* ORF, pIS-052/pIS-053 for *rho* 5'ORF, pIS-054/pIS-055 for *rho* UTR, and pIS-056/pIS-057 for 23S rRNA. For each pair of primers, the efficiency of real-time PCR amplification was estimated using the standard curve method in one color detection system (49). Relative quantification of gene expression was calculated using the threshold cycle ($\Delta\Delta CT$) method (50).

Rho Protein Expression and Purification. Protein purification was performed using a Novagen *E. coli* BL21 (DE3) strain carrying a pET28a plasmid containing Rho coding sequence (pISVA-004). Rho expression was induced in exponential phase using 1 mM of IPTG. After 2 h of induction, bacterial cells were harvested, and total protein extracts were obtained by lysis using French press cell. After clarification by centrifugation, the soluble extract was collected, and Rho protein purified with an imidazole gradient using AKTA purifier system (GE Healthcare) by histidine affinity chromatography with HiTrap HP columns (GE Healthcare). The fractions containing pure Rho protein were selected. To perform transcription termination assay, buffer exchange was done to Buffer 2 (100 mM KCl, 0.1 mM EDTA, 0.1 mM DTT, 10 mM Tris-HCl, 50% glycerol, pH 7.9), using PD-10 columns (GE Healthcare). Protein purity was accessed in a 10% SDS/PAGE gel (*SI Appendix, Fig. S5*). Quantification was performed according to Bradford method (51).

In Vitro Transcription Termination Assays. Transcription termination experiments were performed as described previously (52) and are detailed in *SI Appendix, Supplementary Materials and Methods*. Signals were visualized by PhosphorImaging (Fujifilm FLA-5100, FUJIFILM Life Science). The analysis and quantification were performed using ImageQuant software (Molecular Dynamics).

Western Blot. For Western blot analysis, total cellular extracts were collected in middle exponential phase, stationary phase and under anaerobic shock conditions. To determine Rho expression under denaturing conditions, total protein was obtained using Bugbuster reagent (Novagen) and denatured at 100 °C for 5 min. Protein quantification was performed according to Bradford method (51). Five micrograms of total protein were analyzed in a denaturing 10% SDS/PAGE gel and transferred to a nitrocellulose membrane (Hybond ECL; GE Healthcare). For native conditions, total protein extracts were obtained by cell resuspension in Lysis buffer [50 mM Tris-HCl pH 8, 150 mM NaCl, 0.03 mg of Lysozyme, 1 mM PMSF, 1× Halt Protease & Phosphatase Inhibitor Mixture (Thermo Scientific), and 125 U Benzamide (Sigma)] followed by freeze-thaw cycles. Forty micrograms of total protein were separated in a 10% native polyacrylamide gel and transferred to an Immoblot PVDF membrane (Biorad). In both conditions, membranes were probed with anti-Rho antibody (MyBioSource) with a dilution of 1:10,000 and anti-rabbit IgG with the same dilution as secondary antibody. Immunoblot signal was detected using Western Lightning Plus-ECL Reagents (PerkinElmer).

β-Galactosidase Activity Assays. β-Galactosidase activity was assayed as previously described by Miller (53) and is expressed in Miller units. The details of the assay are described in *SI Appendix, Supplementary Materials and*

Methods. Paired t test statistical analysis was carried out using GraphPad Prism 6 software.

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