RESEARCH PAPER

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A *cis*-regulatory antisense RNA represses translation in *Vibrio cholerae* through extensive complementarity and proximity to the target locus

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As with all facultative pathogens, *Vibrio cholerae* must optimize its cellular processes to adapt to different environments with varying carbon sources and to environmental stresses. More specifically, in order to metabolize mannitol, *V. cholerae* must regulate the synthesis of MtlA, a mannitol transporter protein produced exclusively in the presence of mannitol. We previously showed that a *cis*-acting small RNA (sRNA) expressed by *V. cholerae,* MtlS, appears to post-transcriptionally downregulate the expression of *mtlA* and is produced in the absence of mannitol. We hypothesized that since it is complementary to the 5′ untranslated region (UTR) of *mtlA* mRNA, MtlS may affect synthesis of MtlA by forming an *mtlA*-MtlS complex that blocks translation of the mRNA through occlusion of its ribosome binding site. To test this hypothesis, we used in vitro translation assays in order to examine the role MtlS plays in *mtlA* regulation and found that MtlS is sufficient to suppress translation of transcripts harboring the 5′ UTR of *mtlA*. However, in a cellular context, the 5′ UTR of *mtlA* is not sufficient for targeted repression by endogenous MtlS; additional segments from the coding region of *mtlA* play a role in the ability of the sRNA to regulate translation of *mtlA* mRNA. Additionally, proximity of transcription sites between the sRNA and mRNA significantly affects the efficacy of MtlS.

Introduction

With an estimated 3–5 million cases a year, cholera represents a global public health concern as well as a paradigm model for known and emerging diseases.[1-3] *Vibrio cholerae*, the causative agent of cholera, is a bacterium that resides mostly in the marine habitat but also thrives in the human gastrointestinal (GI) tract and in freshwater environments endemic to cholera.[4-6] The life cycle of *V. cholerae* involves repetitive transitions between aquatic environments and the host GI tract. Genome-wide transcriptional changes occur as the bacteria transition between aquatic reservoir and host, suggesting that different sets of gene products allow *V. cholerae* to persist and thrive in environments with varying nutrients and carbon sources.[7-10] This characteristic is likely key to its fitness as a facultative pathogen.[11] Nevertheless, the molecular and cellular components involved in the ability of *V. cholerae* to adapt to different environments and to persist in its natural environment remain to be fully defined.[11]

In an effort to elucidate the molecular mechanisms by which *V. cholerae* adapts to changing carbon sources, we are investigating how *V. cholerae* regulates expression of *mtlA*, the gene encoding the mannitol-specific transporter, MtlA, of the phosphoenolpyruvate: sugar phosphotransferase system (PTS). Highly conserved in both Gram-negative and Gram-positive bacteria, the PTS catalyzes the uptake and concomitant phosphorylation of numerous carbohydrates.[12] In *V. cholerae*, the PTS is solely responsible for transport of several carbon sources, including mannitol.[13,14] *V. cholerae* lacking active MtlA is unable to ferment mannitol or survive in medium containing mannitol as the sole carbon source.[13,15,16] MtlA is up-regulated two-fold during colonization in the rabbit ileal loop, suggesting that *V. cholerae* may use MtlA and other components of the PTS to adapt appropriately to changing nutritional cues in the environment of the small intestine.[10] In addition, it was recently reported that the *V. cholerae* MtlA protein is capable of activating biofilm formation independent of its role in transporting mannitol.[17] In aquatic

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habitats, *V. cholerae* are known to form biofilms, a feature that enhances their environmental persistence.[5,11] It is hypothesized that mannitol may provide an extracellular cue to *V. cholerae* of hospitable environments to colonize via surface attachment and biofilm formation.[17] Collectively, these findings suggest that the regulation of *mtlA* expression may have implications throughout the life cycle of *V. cholerae*, affecting both the transmission and the persistence of the pathogen.

We recently reported that in *V. cholerae*, a small RNA (sRNA), MtlS, represses the expression of *mtlA* at the post-transcriptional level.[16,18] sRNAs are short, usually non-coding, strands of RNA that regulate gene expression, often by base pairing with mRNA targets. By binding to its mRNA target, the sRNA affects mRNA expression through a variety of mechanisms (for recent reviews see refs. 19–24). Broadly speaking, sRNAs can be classified as either *cis*- or *trans*-acting, depending on whether they are transcribed directly antisense or distal, respectively, to the genes they regulate.[19,24] It is common for *trans* sRNAs to be between 50 and 300 nt long, and to share imperfect complementarity with their gene target.[20] *Cis* sRNAs, on the other hand, can be anywhere from ~100 to over 1,000 nt long, share perfect complementarity with their target mRNA, and may overlap with the 5′-end, 3′-end or middle of the gene encoded opposite the sRNA.[19,25,26]

MtlS is a *cis*-acting sRNA. Intriguingly, a common theme among *cis*-acting sRNAs is that the best-characterized ones act by repressing the synthesis of a protein that is potentially toxic to the cell.[27-30] For example, the 67 nt Sok sRNA is part of the *hok/sok* system of plasmid R1, which mediates plasmid stabilization by post-segregational killing of plasmid-free cells.[31,32] The *hok* gene, encoding a toxic protein, is constitutively expressed from the R1 plasmid as a stable mRNA. Sok sRNA is also constitutively transcribed from the R1 plasmid, antisense to the *hok* mRNA leader region. In R1 plasmid-carrying cells, Sok sRNA forms a duplex with the 5′ untranslated region (UTR) of *hok* mRNA, preventing its translation.[32] Loss of plasmid R1 results in decreased cellular levels of Sok sRNA, allowing the stable *hok* mRNA to be translated and cell death to follow.[32] Although the two RNAs share 67 nt of complementarity, the working model suggests that Sok sRNA uses only 14 nt at its 5′ end to target *hok* mRNA.[32]

Like Sok sRNA, the 77 nt SymR *cis*-acting sRNA of *Escherichia coli* represses the translation of a toxic protein, SymE; however, the genes encoding the sRNA and its antisense target are encoded on the chromosome rather than on a plasmid.[33] SymR shares perfect complementarity with the majority of the 5′ UTR of *symE* mRNA, including the ribosome binding site (RBS).[33] Furthermore, in an *E. coli* Δ*symR* strain, SymE protein levels increase ~10-fold, while the *symE* mRNA levels only increase ~3-fold, suggesting that the sRNA regulates gene expression primarily through translational repression.[33] Similarly, the *E. coli* genome contains multiple Sib sRNAs, all of which are *cis*-acting regulators expected to prevent translation of the highly hydrophobic and toxic Ibs proteins through sRNA:mRNA duplex formation.[30] The SibC antitoxin RNA, for example, transcribed antisense to the gene encoding toxic IbsC, is 141 nt long and exhibits perfect complementarity with the entire coding sequence of *ibsC* and the majority of the *ibsC* 5′ UTR, including the RBS.[30,34]

It has been proposed that *cis* sRNAs may have several advantages over their *trans*-acting counterparts. First, the complete and extended complementarity between the sRNA and its target can lead to very stable duplexes, allowing regulation to be independent of proteins such as the RNA chaperone protein Hfq.[25] Indeed, we previously demonstrated that *cis*-acting MtlS sRNA represses MtlA synthesis in an Hfq-independent manner.[18] In addition, the *cis* position should increase effective molarity that may facilitate and enhance interactions between the regulator and target molecules.[25]

With regard to this current work, the *cis*-acting MtlS is 120 nt long and shares 71 nt of perfect complementarity with the 5′ UTR of *mtlA* (**Fig. 1**).[18] We previously observed an inverse relationship between MtlA protein and MtlS sRNA levels in *V. cholerae;* MtlS is expressed in cells grown in all carbon sources other than mannitol, while MtlA protein is only synthesized when mannitol is present.[18] We also demonstrated that MtlS sRNA represses MtlA synthesis without affecting *mtlA* mRNA levels.[18] These results led us to propose a model in which the MtlS sRNA represses MtlA synthesis by binding to the 5′ UTR of the *mtlA* transcript, blocking the translation machinery from binding the mRNA. While this mechanism is similar to that used by the SymR and Sib sRNAs, in this case, the target gene does not encode a toxin but a transporter protein.

In the present study, we tested our model by assessing whether the MtlS and *mtlA* RNAs are capable of binding to each other and whether duplex formation is sufficient for translational repression of *mtlA*. While the sense-antisense base pairing interactions for plasmid-transcribed RNAs have been extensively studied, the same cannot be said about chromosomal sense-antisense RNA pairs.[25,26] As MtlS represents a chromosomally encoded *cis*-acting RNA, and is also one of the few examples of an antisense sRNA that regulates a target other than a toxin-encoding gene, understanding the molecular mechanism by which MtlS affects *mtlA* expression could provide important insights into the capabilities of *cis* sRNAs in bacteria. Our work shows that MtlS and

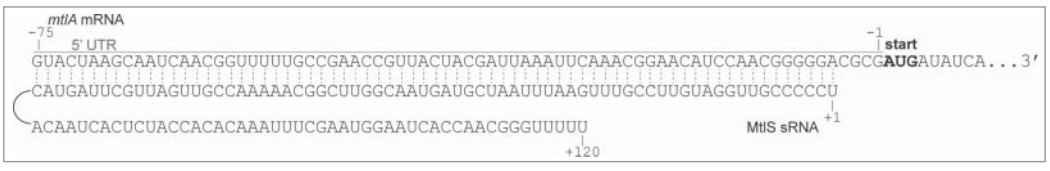

Figure 1. The *mtlA* mRNA and MtlS sRNA share 71 nt of perfect complementarity. The *mtlA* mRNA has a 75 nt long 5′ UTR, numbered -75 to -1 with respect to the start codon (bold type). The MtlS sRNA is 120 nt long. The predicted pairing between the two transcripts is shown with dashed lines.

mtlA RNAs can form a stable duplex that consequently represses the translation of *mtlA* mRNA. We also provide evidence that in the cellular context, MtlS is most effective at repressing *mtlA* mRNA translation when the sRNA is transcribed directly antisense to *mtlA*.

Results

MtlS binds specifically to the *mtlA* 5′ UTR and represses translation of downstream genes in vitro

To test directly whether MtlS binds to the *mtlA* mRNA, we investigated duplex formation between MtlS sRNA and the *mtlA* 5′ UTR RNA in vitro by gel mobility shift assays. First, a fixed concentration of biotin-labeled *mtlA* 5′ UTR RNA was incubated with increasing concentrations of unlabeled MtlS RNA for 30 min. Complex formation was analyzed by gel electrophoresis on a native polyacrylamide gel. We observed that MtlS is able to bind *mtlA* 5′ UTR RNA with an apparent K_d of ~ 70 nM and that the addition of unlabeled *mtlA* 5′ UTR RNA decreased complex formation (**Fig. 2A**, lane 6). As a control, we also tested binding between *mtlA* 5′ UTR RNA and the reverse complement of MtlS, antisense MtlS (asMtlS), which possesses no significant complementarity to the *mtlA* 5′ UTR. We observed no duplex formation between *mtlA* 5′ UTR and asMtlS (**Fig. 2B**). These experiments indicate that MtlS specifically binds the *mtlA* 5′ UTR in vitro with high affinity.

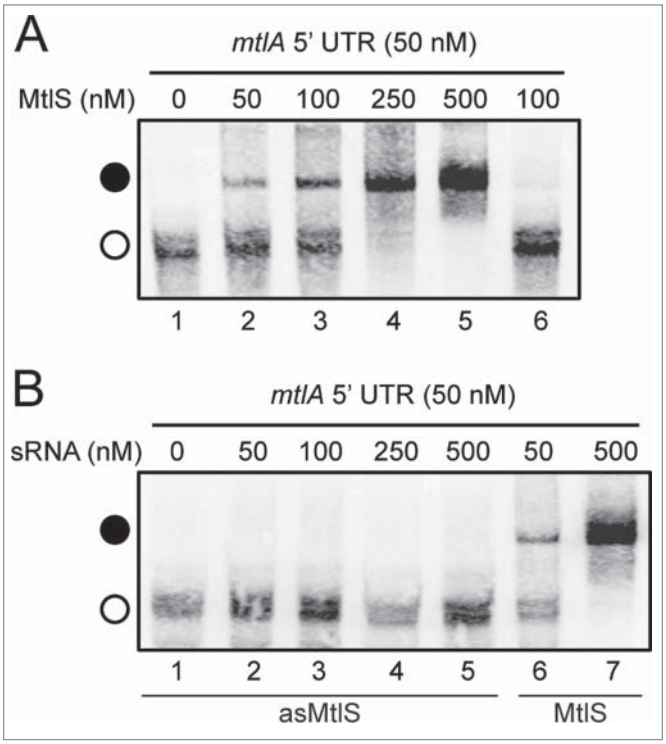

Figure 2. Duplex formation between *mtlA* RNA and MtlS RNA. (**A**) MtlS binds the 5′ UTR of *mtlA* mRNA in vitro. Electrophoretic mobility shift assay (EMSA) using a 3′-biotinylated *mtlA* 5′ UTR transcript (-75 rel. to AUG through start codon; 50 nM) and various concentrations of unlabeled MtlS. The sample run in lane 6 included an excess of unlabeled *mtlA* RNA (500 nM) that prevented MtlS RNA from binding labeled *mtlA* RNA. Positions of *mtlA* (open circle) or the MtlS/*mtlA* complex (filled circle) are indicated. (**B**) EMSA, performed as in (A) shows that unlike MtlS, the reverse complement of MtlS (antisense MtlS, asMtlS) is unable to bind the 5′ UTR of *mtlA* mRNA in vitro. In lanes 1–5, the sRNA mixed with the *mtlA* 5′ UTR was antisense MtlS; in lanes 6–7, the sRNA mixed with *mtlA* 5′ UTR was MtlS.

Next, we tested whether the binding of MtlS to the *mtlA* 5′ UTR is sufficient for repressing translation of the downstream coding sequence. Using in vitro translation assays,[35] we first combined an *mtlA* transcript with increasing amounts of MtlS RNA. The *mtlA* transcript included the 5′ UTR of *mtlA*, the first 378 nt of the *mtlA* coding sequence, and the sequence for a Flag epitope tag immediately prior to the stop codon (*mtlA'-flag*). This *mtlA* transcript was prepared by in vitro transcription and gel purification. The resulting RNA was mixed with increasing amounts of in vitro-transcribed, gel-purified MtlS RNA. The two RNAs were incubated at 37°C for 30 min and then added to an in vitro translation reaction mixture. The reaction was allowed to proceed for 60 min at 37°C at which point the reaction was stopped and the resulting proteins were quantified through western blot analysis. We observed that the addition of MtlS to the reaction mixture reduced translation of *mtlA* RNA in a dose-dependent manner with an IC_{50} ~ 200 nM (**Fig. 3A**). We considered the effect of MtlS on *mtlA* RNA translation to be specific because translation of a *gfp-flag* internal control RNA was largely unaffected under the same experimental conditions (**Fig. 3A**). Furthermore, the addition of asMtlS to the reaction mixture did not reduce *mtlA'-flag* translation, even when the asRNA was present in excess (**Fig. 3B**).

We also performed the in vitro translation assay with an RNA construct in which the 5′ UTR of *mtlA* was fused to the first 357 nt of the coding sequence of *gfp* and a C-terminal Flag epitope tag (*mtlA* 5′UTR-*gfp'*). Increasing amounts of MtlS reduced translation of the *mtlA* 5′UTR-*gfp'* transcript, while the amount of GFP synthesized from the *gfp-flag* internal control remained unchanged (**Fig. 3C**). Collectively, our in vitro data indicate that the *mtlA* 5′ UTR alone is sufficient for MtlS to repress the translation of downstream genes (either *mtlA* or *gfp*) in vitro.

Our proposed model suggests that occlusion of the *mtlA* RBS is responsible for translational repression of *mtlA* when MtlS sRNA is present. Thus, we expected that only the region of MtlS complementary to the RBS would be necessary to inhibit translation of *mtlA* mRNA. To test this, we constructed MtlS variants (**Fig. 3D**) and tested them using in vitro translation assays with *mtlA'-flag* RNA. Construct A includes only the 5′ end of MtlS (+1–45) and is expected to still bind the predicted *mtlA* RBS. Construct B (+1–71) served as a control in that it includes the region of MtlS predicted to bind the *mtlA* RBS along with the rest of the MtlS segment predicted to base pair with *mtlA* mRNA (**Fig. 1**). Initially, we observed that the full length MtlS was able to repress MtlA'-Flag synthesis in our in vitro assay, but Constructs A and B could not (data not shown). When we included denaturing and annealing steps with the RNA to improve binding, prior to adding the in vitro translation

reaction mixture, however, we consistently observed that both the full length MtlS and Construct B (+1–71) were able to repress MtlA'-Flag synthesis (**Fig. 3E**). The MtlS variant that included only the first 45 nt of MtlS (Construct A), however, was unable to repress MtlA-Flag synthesis (**Fig. 3E**). This difference in translational repression is not due to instability of Construct A, as we observed that all 3 sRNA constructs are stable after denaturing, annealing and incubation at 37°C for 30 minutes (**Fig. S1**). We also tested whether Construct A is able to bind the *mtlA* mRNA. Our observations suggest that this shortened MtlS transcript is not efficient in binding the *mtlA* mRNA 5′ UTR under the in vitro translation conditions (**Fig. S2**). Thus, in this in vitro setting, increased pairing between MtlS and *mtlA* mRNA allows for a more stable duplex to form, which is necessary for translational repression of the mRNA.

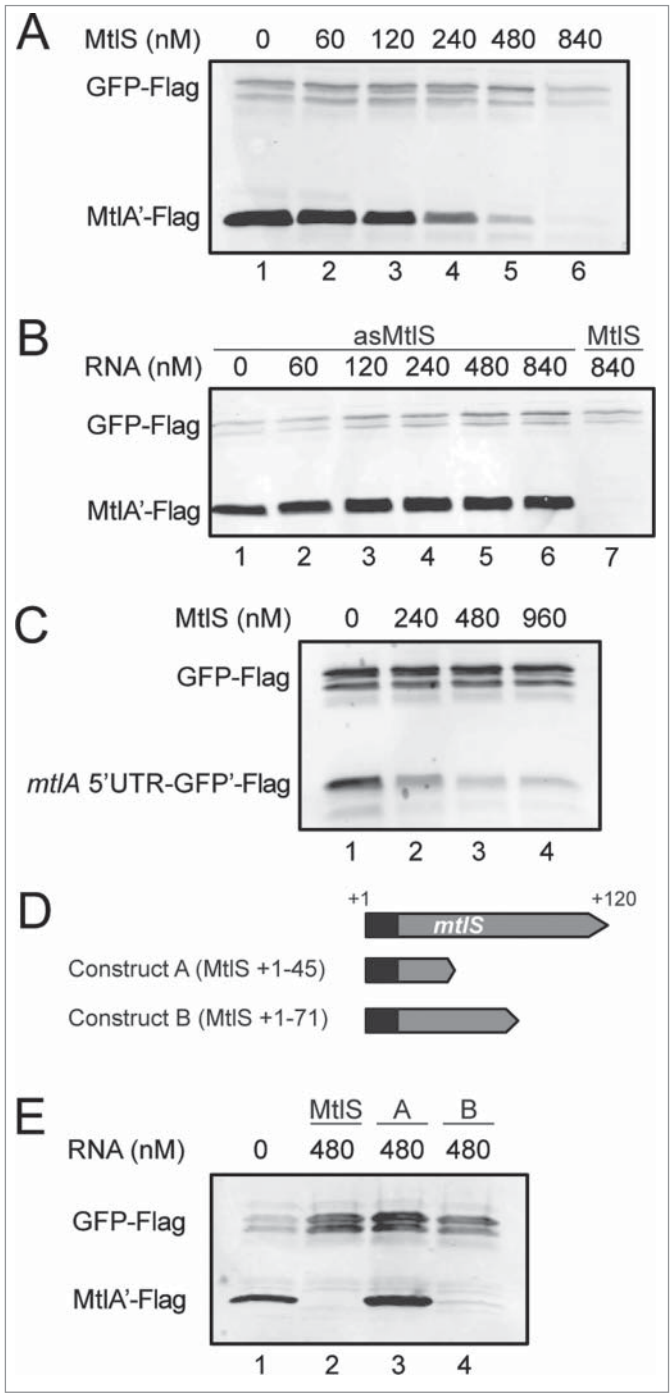

Figure 3. In vitro translation assays of *mtlA* mRNA. Unless otherwise specified, both *mtlA′-flag* RNA (120 nM) and *gfp-flag* RNA (320 nM) were translated together in a reaction mixture (37°C) with varying amounts of sRNA. After 60 minutes, 1/10 of the reaction mixture was removed and subjected to western blotting using an anti-Flag antibody. (**A**) Increasing the amount of MtlS represses translation of *mtlA′-flag* mRNA, but does not affect GFP-Flag synthesis. (**B**) A specific sRNA is required to repress *mtlA* translation. In lanes 1–6, the sRNA added to the reaction mixture was antisense MtlS (asMtlS); in lane 7, the sRNA added to the reaction mixture was MtlS. (**C**) The 5′ UTR of *mtlA* is sufficient for repression of downstream gene expression in vitro. An in vitro translation assay was performed, as in (A), replacing the *mtlA′-flag* RNA with *mtlA5′UTR-gfp′-flag* (5′ UTR of *mtlA* fused to the first 357 nt of the *gfp* coding sequence; 240 nM). (**D**) Schematic representation of MtlS, which is a 120 nt transcript. MtlS variants synthesized in this study are Construct A (nt +1-45 of MtlS) and Construct B (nt +1–71 of MtlS). The region of MtlS expected to overlap with the predicted RBS of *mtlA* is denoted by a black box. (**E**) An MtlS transcript containing only the first 45 nt is not sufficient for translational repression of *mtlA*. In lane 2, MtlS RNA was added to the reaction mixture. In lanes 3 and 4, Constructs A (+1–45) and B (+1–71) were added to the reaction mixtures, respectively.

MtlS overexpression represses translation of a reporter gene in *E. coli* and *V. cholerae*

To address whether the 5′ UTR of *mtlA* was also sufficient for translational repression in a cellular context, we cloned the *mtlA* 5′ UTR, along with the start codon, into a GFP reporter plasmid (pReporter5′UTR) and tested reporter repression by MtlS (**Fig. 4**). The *gfp* construct is transcribed from a constitutive promoter ($P_{LtetO-1}$), so changes in reporter activity indicate post-transcriptional regulation.[36] We first tested whether MtlS could repress translation of the *mtlA* 5′ UTR-*gfp* fusion in the absence of *Vibrio*-specific factors. Two plasmids, pReporter5′UTR and pMtlS, were introduced into *E. coli,* which lacks endogenous MtlS;[16] pMtlS expresses full-length MtlS from an arabinose-inducible promoter, allowing for tight, controlled overexpression of the sRNA.[18] As a control, pReporter5′UTR was also combined with a Vector plasmid, which expresses a ~250 nt long RNA that shares no extensive complementarity to *mtlA*. MtlS overexpression repressed *mtlA* 5′ UTR-*gfp* reporter activity by 70% in 3 separate growth conditions, as assayed by either fluorescence measurements or by western blot analysis (**Figs. 5A** and **S3**). These results support a model in which MtlS sRNA is able to repress translation of *mtlA* RNA through just the 5′ UTR of the messenger transcript.

We noted that a reporter plasmid containing a truncated *mtlA* 5′ UTR, consisting of only the last 48 of the 75 nt region, fused to the *gfp* coding sequence (pReporter5′Short; **Fig. 4**) was much less responsive to MtlS despite the fact that the MtlS and the shortened *mtlA* 5′ UTR were still able to bind to each other in vitro with a K_d of ~ 240 nM (**Figs. 5B and 5C**). Full length and shortened *mtlA* 5′ UTR constructs can both bind to MtlS during

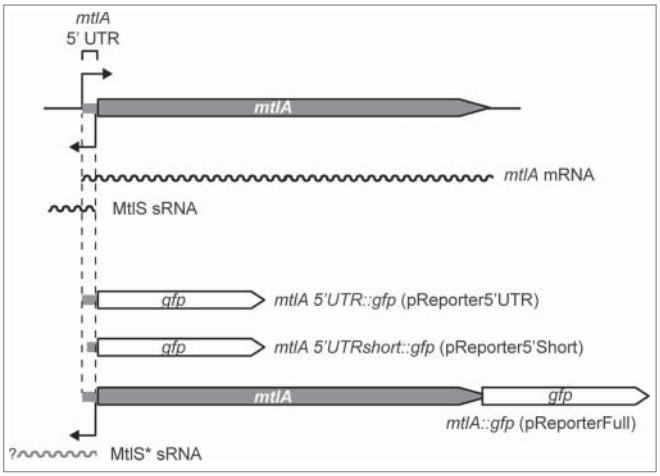

Figure 4. Schematic representation of the *mtlA* gene. The *mtlA* mRNA includes a 75 nt 5′ UTR. Translational *mtlA::gfp* fusions (under control of the constitutive $P_{LtetO-1}$ promoter) were constructed to include only the complete 5′ UTR of *mtlA* (pReporter5′UTR), only a shortened 5′ UTR (pReporter5′Short), or both the complete 5′ UTR and the entire coding sequence of *mtlA* (pReporterFull). The promoter for MtlS RNA lies within the coding region of *mtlA* resulting in transcription of an sRNA, MtlS, antisense to the *mtlA* 5′ UTR. MtlS shares 71 nt of perfect complementarity with the *mtlA* 5′ UTR. pReporterFull also harbors the *mtlS* promoter and may express MtlS*; the 3′ end of this transcript is unknown.

an in vitro gel shift assay with no other RNA binding macromolecules present, but the binding affinity for MtlS of the truncated *mtlA* 5′ UTR is 3 times lower than that of the full-length *mltA* 5′ UTR (compare **Figs. 2A** and **5C**). We postulate that, in a cellular context, the reduced affinity of the truncated *mtlA* 5′ UTR for MtlS may make the sRNA insufficient to out-compete the ribosome for binding the *mtlA* 5′ UTR and prevent translation. The very low levels of repression we observe with the pReporter5'-Short construct support this hypothesis (**Fig. 5B**). Decreased mRNA-sRNA pairing to compete with mRNA-ribosome pairing may also explain why the 45 nt Construct A (**Fig. 3D**) was unable to bind the *mtlA* 5′ UTR under in vitro translation conditions, which included ribosomes that could bind the *mtlA* 5′ UTR and prevent the truncated MtlS from binding (**Fig. S2**). These results suggest that in the cellular context, increased pairing between MtlS and *mtlA* mRNA (>45 bp) allows for more efficient translational repression of the mRNA.

To assess whether the repression of reporter activity observed upon overexpression of MtlS in *E. coli* cells could also occur in *V. cholerae*, we introduced both pReporter5′UTR and pMtlS into *V. cholerae*. It is important to note that the resulting strains harbor endogenous MtlS transcribed from the chromosome as well as any MtlS that may be expressed from pMtlS. Despite this additional source of MtlS and in contrast to our *E. coli* results, we found that the same experiment in *V. cholerae* resulted in only a 20–50 percent reduction in fluorescence relative to the Vector control when cells were grown in any of 3 different growth conditions (**Fig. 5D**).

These results are also in contrast to our previous observations that overexpression of MtlS under certain conditions is sufficient to completely repress MtlA synthesis from the chromosome within 60 minutes of inducing sRNA expression.[18] We have proposed that proteolysis of MtlA is also induced when MtlS levels increase.[18] GFP, however, is a stable protein,[37] and we would not expect MtlS to be able to repress its translation and lead to active degradation of already synthesized protein. This difference between GFP and MtlA protein stability after MtlS induction may explain the discrepancies we observe in how effectively MtlS knocks down MtlA synthesis versus GFP synthesis. The fact that we do observe down-regulation of *gfp* reporter activity in our cellular assays, however, suggests that this is an appropriate model to investigate MtlS activity on *mtlA* expression.

With regard to the differences between MtlS-mediated knockdown in *E. coli* vs. *V. cholerae*, we have observed similar overexpression levels of MtlS from pMtlS in both *E. coli* and in *V. cholerae* (data not shown). Thus, differences in MtlS levels in the different strains cannot necessarily explain our observations. Instead, these results suggest that while the *mtlA* 5′ UTR is sufficient for 70% repression of downstream gene expression by MtlS in *E. coli*, additional elements may be required to achieve similar repression levels in *V. cholerae*. Alternatively, in *V. cholerae*, MtlS may have multiple targets that may titrate the sRNA away from the *mtlA* 5′UTR-*gfp* target (see below).

The 5′ UTR of *mtlA* is not sufficient for reporter repression by endogenous MtlS in *V. cholerae*

The above experiments assayed reporter activity upon overexpression of the MtlS sRNA. However, to analyze the effects of natural MtlS levels on reporter activity, we assayed *V. cholerae* cells harboring only pReporter5′UTR and grown in either sucrose/glucose or mannitol media, conditions in which the bacteria express relatively high and low levels of MtlS, respectively.[16] The normal levels of MtlS in *V. cholerae* grown in sucrose or glucose media is low in comparison to strains that overexpress the sRNA from pMtlS.[18] Surprisingly, we did not observe any repression of reporter activity in *V. cholerae* harboring pReporter5′UTR when the bacteria were grown in sucrose (MtlS-high) medium (**Fig. 6A**). These results are directly in contrast to our in vitro translation data above which suggests that the 5′ UTR of *mtlA* should be sufficient for translational repression of any downstream gene by MtlS (**Fig. 3C**). One explanation for these results is that in the cellular context, the effective concentration of MtlS is low in the vicinity of the *mtlA* 5′UTR-*gfp* transcript. Endogenous *mtlA* transcripts, for example, could titrate away most of the MtlS, reducing the amount of sRNA available to effectively repress expression of *gfp* from the pReporter5′UTR plasmid. The multicopy reporter plasmid coupled with the constitutive $P_{LtetO-1}$ promoter could also cause excess production of *mtlA 5′UTR-gfp* mRNA, decreasing the relative concentration of MtlS compared to the chromosomal context and further exacerbating the situation.

However, we also hypothesized that additional segments within the coding sequence of *mtlA* may be necessary for repression of reporter activity by MtlS in *V. cholerae*. Thus, we created a *V. cholerae* strain harboring a single plasmid (pReporterFull) that constitutively expresses a fusion of the *mtlA* 5′ UTR, the

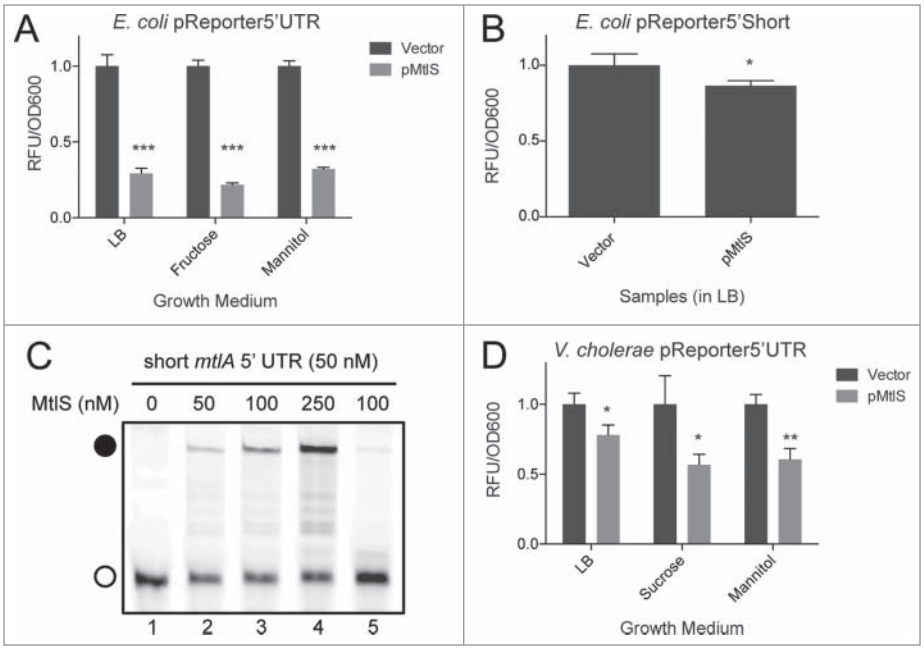

Figure 5. When overexpressed, MtlS can affect downstream gene expression through the full-length 5′ UTR of *mtlA*. (**A**) Regulation of GFP activity was monitored by fluorescence assays of *E. coli* TOP10 cells harboring pReporter5′UTR and either Vector or pMtlS. Cells were cultured in LB medium or M9 minimal medium with the indicated carbon source (0.4%), with 0.02% arabinose to induce *mtlS* expression from pMtlS, at 37 °C to an OD_{600} of 1; relative fluorescence units (RFU) were normalized to OD_{600}. All data points were normalized to the average RFU/OD_{600} of the Vector controls in each growth condition. (**B**) When the 5′ UTR is truncated (including only the last 48 nt of the 75 nt region), repression of reporter activity is largely abolished. GFP activity was monitored, as in (A), for *E. coli* TOP10 cells harboring pReporter5′Short and either Vector or pMtlS. (**C**) The truncated *mtlA* 5′ UTR used in (B) can still bind MtlS. EMSA using a 5′ TYE705-labeled, shortened *mtlA* 5′ UTR transcript and various concentrations of MtlS. The sample run in lane 5 included an excess of unlabeled *mtlA* RNA (500 nM) that prevented MtlS RNA from binding labeled *mtlA* RNA. Positions of *mtlA* (open circle) or the MtlS/*mtlA* complex (filled circle) are indicated. (**D**) Regulation of GFP activity was monitored, as in (A), for *V. cholerae* harboring pReporter5′UTR and either Vector or pMtlS. The bar graphs show the means and standard deviations from at least 3 independent samples. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$ by unpaired t-test.

entire *mtlA* coding sequence, and the coding sequence of *gfp* (**Fig. 4**). Initial fluorescence assays and western blot analysis indicated that this fusion is capable of producing full-length, fluorescent GFP (**Fig. S4 and S5**). When *V. cholerae* harboring pReporterFull were grown in glucose and mannitol media, we consistently observed a significant 30% repression of *gfp* expression when the bacteria were grown in glucose, as compared to mannitol medium (**Fig. 6A**). These results indicate that inclusion of the *mtlA* coding sequence confers translational repression of downstream gene expression by MtlS in *V. cholerae*, unlike when only the *mtlA* 5′ UTR is present (compare WT p5′UTR and WT pFull in **Fig. 6A**). Again, we do not observe complete knockdown of GFP levels, as we do for MtlA when MtlS is expressed;[18] however, this is most likely a result of the stability of GFP in these growth conditions compared to that of MtlA.

At this point, we reasoned that the *mtlA* coding sequence might contain essential elements for the post-transcriptional regulation of *mtlA-gfp* by MtlS. Although MtlS does not affect *mtlA* mRNA stability,[18] we postulated that MtlS might destabilize the *gfp* transcript, thereby repressing expression of the reporter. We tested this hypothesis by northern blot analysis of total RNA from bacteria harboring pReporterFull grown in both glucose and mannitol medium and observed no difference in *mtlA-gfp* RNA levels between the two growth conditions (**Fig. 6B**). Thus, the repression of reporter expression observed in **Figure 6A** (WT pFull) is not caused by differences in the abundance of the *mtlA-gfp* transcript in the different growth media.

Reporter activity is repressed by an antisense transcript expressed from the reporter plasmid

Previously, we mapped the precise +1 site of *mtlS* by sRNA-Seq,[16] which, as revealed by PromoterHunter,[38] is preceded by −10 and −35 elements separated by a 16-bp spacer. These putative promoter elements lie within the coding region of *mtlA*. Thus, we considered that inclusion of the *mtlA* coding sequence fused to *gfp* in the reporter plasmid might explain the greater translational repression of the encoded *mtlA-gfp* than the *mtlA* 5′ UTR alone, because the *mtlA* coding sequence enables transcription of an RNA in *cis* and antisense to the *mtlA-gfp* transcript. Hereafter, we refer to this putative transcript as MtlS* (**Fig. 4**). We consider MtlS* to be distinct from endogenous MtlS in that the plasmid-derived MtlS* is transcribed in close proximity to the *mtlA-gfp* fusion and could potentially bind more readily to the 5′ UTR of the reporter *mtlA-gfp* mRNA. We expect that since *mtlS** lacks the native transcriptional terminator of *mtlS*, the transcript may also include regions from the vector backbone of pReporterFull.

In order to test whether MtlS* is actually transcribed from the reporter plasmid, we used quantitative real-time PCR (qPCR) to analyze MtlS* levels in both the wild type and a Δ*mtlS* strain, each harboring pReporterFull. For the qPCR analysis, we used forward and reverse primers that anneal to sequences common to both MtlS and MtlS*. Thus, when analyzing RNA samples from the wild type strain, both MtlS and MtlS* are being measured. The Δ*mtlS* strain does not express any MtlS; however, we did observe MtlS* expression from the Δ*mtlS* strain harboring pReporterFull ($p < 0.01$, compared to Δ*mtlS* parent, by unpaired t-test), supporting a model in which an antisense transcript is expressed from the reporter plasmid (**Fig. 7**). We also attempted multiple northern blot experiments to observe MtlS* expressed from Δ*mtlS* bacteria harboring pReporterFull to determine the size of the transcript. However, after repeated attempts, we were unable to observe the *mtlS** transcript through northern blot

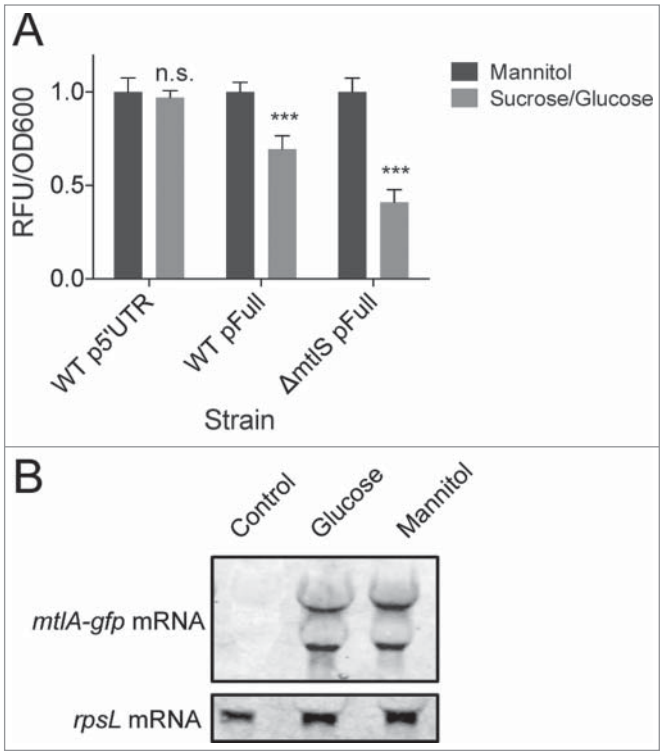

Figure 6. Including the *mtlA* coding sequence represses reporter activity in non-mannitol carbon sources. (**A**) Wild type (WT) *V. cholerae* harboring pReporter5′UTR or pReporterFull, and mutant *V. cholerae*, lacking the genomic *mtlS* locus (ΔmtlS) and harboring pReporterFull, were cultured in M9 minimal medium with the indicated carbon source (0.4%), at 37°C to an OD_{600} of 1; relative fluorescence units (RFU) were normalized to OD_{600}. All data points were normalized to the average RFU/OD_{600} of the mannitol samples. The bar graphs show the means and standard deviations from at least 3 independent samples. n.s., $p > 0.05$; ***$p < 0.001$ by unpaired t-test. (**B**) Northern analysis of RNA isolated from the same WT pFull cells used for the fluorescence assay in (A). *mtlA-gfp* RNA was detected with biotin-labeled RNA probes specific to *gfp*. The lower band may represent a processed version of the *mtlA-gfp* RNA. *rpsL* RNA was detected using an IR800-dye-labeled oligonucleotide specific to *rpsL* and was included as a loading control. The control lane was loaded with total RNA from wild type *V. cholerae* lacking *gfp*. This blot is representative of 3 independent trials.

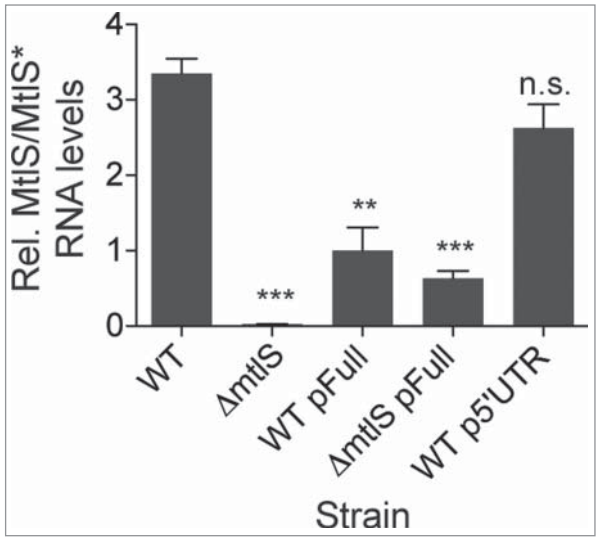

Figure 7. An antisense RNA is transcribed from pReporterFull. Strains of *V. cholerae* were cultured in M9 minimal medium with glucose (0.4%), at 37°C to an OD_{600} of 0.3. Expression from the *mtlS* promoter should be high under these conditions. qRT-PCR was used to measure the MtlS/MtlS* transcript levels of WT (wild type), Δ*mtlS*, WT harboring pFull (pReporterFull), Δ*mtlS* harboring pFull, and WT harboring p5′UTR (pReporter5′UTR) strains of *V. cholerae*. The relative abundance of MtlS/MtlS* transcript was determined as described in Materials and Methods. Shown are the means and standard deviations from at least 3 independent samples. n.s., $p > 0.05$; **$p < 0.01$; ***$p < 0.001$ by unpaired t-test comparing each sample to the WT sample.

analysis. It is possible that MtlS* is a very long transcript that does not enter the gel during electrophoresis, MtlS* is a relatively unstable transcript, and/or our northern blot method is not sensitive enough to detect this particular transcript.

Surprisingly, we consistently observed that wild type *V. cholerae* either harboring no reporter plasmid or pReporter5′UTR, neither of which should express MtlS*, had higher levels of MtlS than the MtlS/MtlS* levels detected in the strains carrying pReporterFull (**Fig.** 7). Not much is known regarding the regulation of *mtlS* expression.[18] It is likely, however, that transcription factors are required to regulate *mtlS* expression in response to available carbon sources. Thus, we theorize that the addition of pReporterFull provides additional binding sites for these transcription factors, thereby titrating these proteins away from the chromosomal locus and decreasing synthesis of MtlS sRNA.[39] If MtlS* is relatively unstable (see above), then the overall levels of MtlS/MtlS* RNA would be lower in cells harboring pReporterFull, which is consistent with our observations.

In order to evaluate the contributions of the plasmid-expressed MtlS* and chromosomally-derived MtlS, we tested reporter activity in the Δ*mtlS* strain harboring pReporterFull. The Δ*mtlS* pReporterFull strain exhibited a similar, if not greater, reduction in fluorescence as the wild type strain harboring pReporterFull when both strains were grown in glucose versus mannitol medium (compare WT pFull and ΔmtlS pFull in **Fig. 6A**). In the Δ*mtlS* mutants, MtlS* from the plasmid constitutes the only source of an RNA complementary to the *mtlA* 5′ UTR (**Fig.** 7, compare Δ*mtlS* and Δ*mtlS* pFull). Therefore, these results indicate that the plasmid-derived MtlS*, transcribed in close proximity to the *mtlA* 5′ UTR, is sufficient to repress *mtlA-gfp* expression.

We further reasoned that by truncating the *mtlA* coding sequence in pReporterFull, we could delineate the segment(s) of the *mtlA* coding sequence necessary for maximal expression of MtlS* and reveal potential regulatory elements of the *mtlS* promoter. We created truncated versions of pReporterFull that all contain the *mtlA* 5′ UTR followed by either the first 42, 102, or 1,011 nucleotides of the *mtlA* coding sequence fused to the 5′ end of *gfp* (creating pReporter42, pReporter102, or pReporter1011, respectively). These constructs were designed to incorporate only the predicted -10 and -35 elements of the predicted *mtlS* promoter (pReporter42), a short region beyond the

mtlS promoter (pReporter102), or a significant segment of *mtlA* extending beyond the *mtlS* promoter (pReporter1011).

Wild type strains harboring any of the 3 truncations exhibited some repression of reporter activity in glucose medium (**Fig. 8A**). However, the strains harboring pReporter102 and pReporter1011 were three-fold more effective than the strain harboring pReporter42 at repressing *gfp* expression. These results suggest that sequences between the +42 and +1011 nucleotides of the *mtlA* coding sequence contain important regulatory elements needed to repress translation of MtlA in *V. cholerae*. We postulated that these regulatory elements might include binding sites for transcriptional regulators that activate *mtlS* expression in the presence of glucose or the absence of mannitol. When we measured by qRT-PCR MtlS* levels expressed from Δ*mtlS* strains harboring pReporter42, pReporter102 and pReporter1011, however, we found that they all expressed similar levels of MtlS* (**Fig 8B**). As these measurements were taken in the Δ*mtlS* background, MtlS* is the only source of an RNA complementary to the *mtlA* 5′ UTR. We therefore reason that inclusion of nt +42–1011 of the *mtlA* coding sequence affects MtlA levels not by altering the total amount of *cis*-encoded antisense RNA, but by providing additional elements that contribute to the overall regulation of *mtlA* expression (see Discussion).

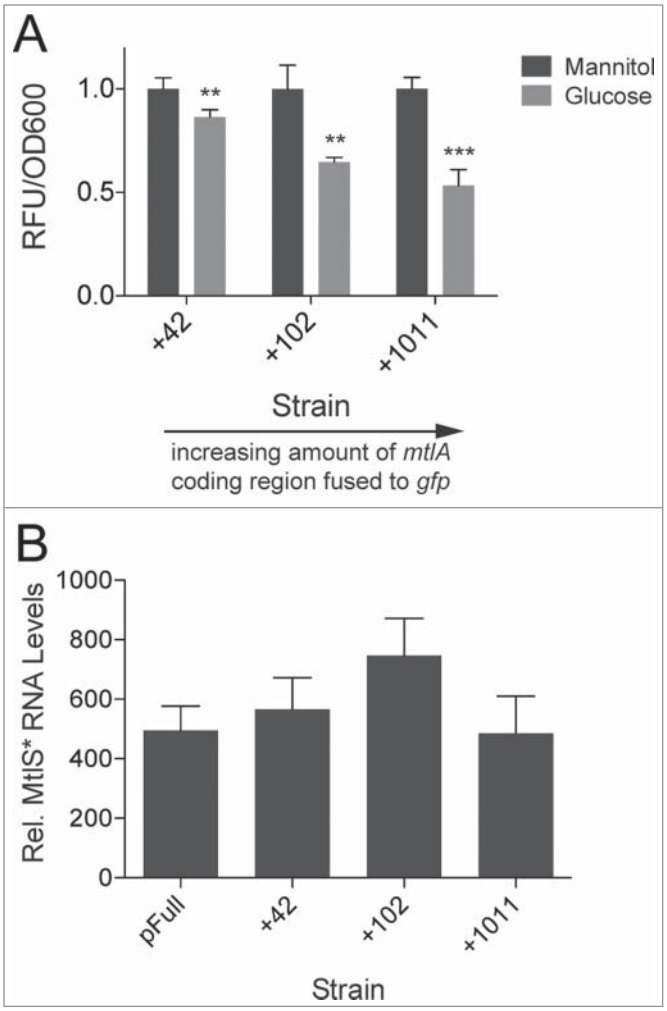

Figure 8. More than the first 42 nt of the *mtlA* coding region are required for maximal repression of *mtlA-gfp* mRNA translation. (**A**) Wild type *V. cholerae* harboring pReporter plasmids which fuse the 5′ UTR of *mtlA* and part of the *mtlA* coding sequence (+1–42, +1–102, or +1–1011) to *gfp* were cultured in M9 minimal medium with the indicated carbon source (0.4%), at 37°C to an OD_{600} of 1; relative fluorescence units (RFU) were normalized to OD_{600}. All data points were normalized to the average RFU/OD_{600} of the mannitol samples. Shown are the means and standard deviations from at least 3 independent samples. $^{**}p < 0.01$, $^{***}p < 0.001$ by unpaired t-test. (**B**) Strains of *V. cholerae* Δ*mtlS* harboring pReporter plasmids were cultured in M9 minimal medium with glucose (0.4%), at 37°C to an OD_{600} of 0.3. Expression from the *mtlS* promoter should be high under these conditions. qRT-PCR was used to measure the MtlS* transcript levels from pFull (pReporterFull), +42 (pReporter42), +102 (pReporter102), and +1011 (pReporter1011). The relative abundance of MtlS* transcript was determined as described in Materials and Methods. Shown are the means and standard deviations from 3 independent samples. No significant difference between the samples was observed by one-way ANOVA analysis, with reporter plasmid as the variable.

Effective molarity of MtlS affects translational repression of *mtlA* in *V. cholerae*

Our results above suggest that the proximal transcription of an RNA antisense to the 5′ UTR of *mtlA* is more effective at repressing MtlA synthesis than the expression of a complementary RNA distal to the *mtlA* locus (**Fig. 6A**). However, we questioned whether increasing the amount of distally transcribed MtlS could compensate for a lack of proximity between the sRNA and the *mtlA-gfp* loci. For this analysis, we created two strains of wild type *V. cholerae* that both harbored pReporterFull. Thus, both strains are able to transcribe MtlS from the chromosome and MtlS* from the reporter plasmid. One of these strains also carried pMtlS, allowing for overexpression of the sRNA. The other strain carried the Vector control. Consistent with our observations above, when the Vector strains were grown in mannitol (MtlS/MtlS*-low) and sucrose (MtlS/MtlS*-high) media, we observed an inverse relationship between MtlS levels and reporter activity; expression of MtlS/MtlS* was sufficient to decrease GFP fluorescence by >50 % (**Fig. 9A**, compare "Vector" samples). Upon overexpression of MtlS from pMtlS, however, there is a significant decrease in GFP fluorescence in both sucrose and mannitol medium, compared to the Vector controls (**Fig. 9A**). Thus, increasing the abundance of MtlS can further reduce *mtlA-gfp* expression, even if the sRNA is expressed distal to the *mtlA-gfp* locus.

When comparing the pMtlS strain grown in mannitol (pMtlS-Mtl) vs. the Vector strain grown in sucrose (Vector-Suc), we were able to further distinguish the contributions of MtlS and MtlS* in repressing *mtlA-gfp* expression. Both of these strains are expected to produce MtlS sRNA. The Vector-Suc strain transcribes MtlS because it is grown in non-mannitol growth conditions. Harboring the MtlS-overexpression plasmid, the MtlS levels in the pMtlS-Mtl bacteria were 75-fold higher than those in the Vector-Suc bacteria (**Fig. 9B**). However, the Vector-Suc bacteria still had lower fluorescence levels than the pMtlS-Mtl bacteria (**Fig. 9A**, $p < 0.05$, by unpaired t-test). MtlS* is expected to be present in the Vector-Suc sample but not in the pMtlS-Mtl bacteria because expression from the *mtlS* promoter is induced in

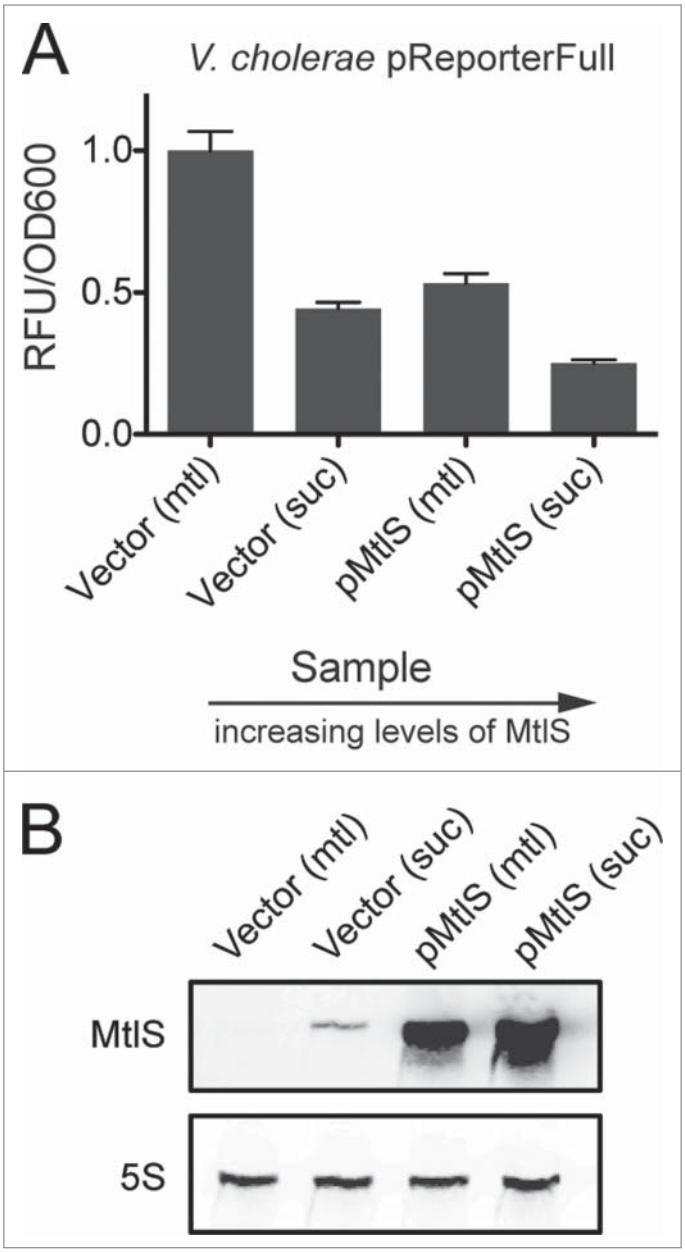

Figure 9. Overexpression of MtlS results in further repression of reporter activity in *V. cholerae* harboring pReporterFull. (**A**) Regulation of GFP activity was monitored by fluorescence assays of wild type *V. cholerae* harboring pReporterFull and either Vector or pMtlS. Cells were cultured in M9 minimal medium with the indicated carbon source (0.4%), with 0.02% arabinose to induce *mtlS* expression from pMtlS, at 37°C to an OD_{600} of 1; relative fluorescence units (RFU) were normalized to OD_{600}. All data points were normalized to the average RFU/OD_{600} of the Vector controls in mannitol medium. Shown are the means and standard deviations from at least 3 independent samples. $p < 0.001$ by one-way ANOVA analysis, with MtlS level as the variable. (**B**) Northern analysis of RNA isolated from the same cells used for the fluorescence assay in (A). MtlS RNA was detected with biotin-labeled RNA probes specific to MtlS. 5S RNA was detected using a 5′-end labeled DNA probe specific to 5S RNA and was included as a loading control.

non-mannitol growth conditions. Thus, although increasing the amount of distally transcribed MtlS can lead to further decreases in *mtlA* expression, the dominant factor that affects repression of *mtlA* would be the proximal expression of an antisense RNA. This is likely due to the high effective molarity that a regulatory RNA would have with respect to its mRNA target when it is expressed proximal to its target locus.[25,40]

Discussion

It is becoming increasingly apparent that bacteria use a variety of mechanisms for sRNA-mediated regulation of gene expression. One of the most extensively studied sRNAs is the *trans*-acting SgrS that negatively regulates the synthesis of PtsG, the glucose-specific transporter of the PTS in *E. coli* and *Salmonella*. Metabolic bottlenecks following sugar uptake by the PTS may cause toxic accumulation of intracellular sugar-phosphates.[41] In *E. coli* and *Salmonella*, SgrS is induced upon phosphosugar stress and represses translation of *ptsG* mRNA by base pairing with the 5′ UTR of the transcript through partial complementarity and the aid of Hfq.[42] Hfq can also bind to RNase E, a major endoribonuclease,[43] suggesting that upon pairing with *ptsG* mRNA, SgrS and Hfq ultimately target the *ptsG* message for rapid degradation by RNase E. The physiological outcome of SgrS induction is decreased glucose transport until phosphosugar stress is alleviated.

Whereas SgrS is thought to represent a canonical model of sRNA-mediated repression of mRNA translation, involving both RBS-occlusion and targeted mRNA degradation, there are also recent examples of sRNAs that activate targets by masking RNase E cleavage sites and stabilizing the mRNA.[44,45] In another example of sRNA-mediated gene activation, the *trans*-acting GlmY sRNA in *E. coli* acts as a molecular mimic, providing a decoy for an RNase adaptor protein to ultimately allow up-regulation of *glmS*, encoding glucosamine-6-phosphate synthase.[46]

With regard to *cis*-acting sRNAs, the best-studied class of these molecules is the anti-toxin sRNAs that are transcribed antisense to their targets, which encode proteins that are toxic to the cell.[30,33,34] As with most antisense sRNAs, these *cis*-acting RNAs downregulate translation of their targets by base pairing with the mRNA, blocking translation, and sometimes destabilizing the transcript as well.[47,48] In the present work, we investigated a *cis*-acting sRNA, MtlS, the target of which does not encode a toxin, but instead a transporter of the PTS in *V. cholerae*. Our in vitro data support a model in which MtlS is able to repress translation of *mtlA* mRNA by binding the 5′ UTR of the transcript. Surprisingly, however, both our in vitro data and cellular reporter assays suggest that extensive pairing (>45 bp) between MtlS and the *mtlA* 5′ UTR allows for the most efficient repression of *mtlA* translation. This was unexpected given that in numerous cases, small seed regions of an sRNA are sufficient for gene regulation. For example, although 31 nt of SgrS are capable of pairing with a 32 nt region of the *ptsG* mRNA, only a short portion (14 nt)

of SgrS is necessary for its regulatory activity; this minimal region of SgrS is complementary to the RBS of *ptsG*.[49] The SibC antitoxin sRNA exhibits perfect complementarity with most of the 5′ UTR and the entire coding sequence of *ibsC* mRNA.[30,34] Nevertheless, a small region of SibC, just 23 nt, is sufficient for initial base pairing and subsequent target repression.[34] Thus, we posit that the mechanism of MtlS-mediated regulation of *mtlA* is distinct from these prior examples and that a minor seed region for pairing the two RNAs may be insufficient to block translation of *mtlA* mRNA. Instead, an extensive duplex between the two RNAs is required for maximal repression, at least when MtlS is expressed distal to its *mtlA* mRNA target (see below).

Our results also suggest that the proximity of the *mtlA* and *mtlS* loci plays an important role in regulation of *mtlA* by the sRNA. We observed that the expression of a transcript perfectly complementary to the *mtlA* 5′ UTR is capable of repressing *mtlA* translation. However, it appears that expression of the sRNA from a locus directly antisense to *mtlA* results in the most efficient and effective repression of *mtlA* expression. This leads us to predict that the proximity of the two RNAs, transcribed from the same region of the genome, may positively contribute to the rapid formation of an extensive duplex that is necessary for maximal repression of *mtlA*. This is consistent with prior suggestions that *cis*-acting sRNAs, due to enhanced steric proximity, may be more efficient than their *trans*-acting counterparts at interacting with their targets and thereby affecting gene expression.[25] There is evidence in *E. coli* and *Caulobacter crescentus* that transcripts experience only limited diffusion from their sites of transcription;[40] thus, expression of the sRNA proximal to the target locus may be advantageous when fast and complete regulatory responses are required.

At the same time, we recognize that the coding sequence of *mtlA* may contain additional binding sites for MtlS or that MtlA, in non-mannitol conditions, may also undergo post-translational regulation. Either or both of these models would also be consistent with our observed results. With regard to the latter scenario, we previously reported that induction of *mtlS* expression in *V. cholerae* resulted in rapid turnover of MtlA, which is normally a highly stable protein.[18] We have proposed that active proteolysis may represent yet another mechanism by which MtlA levels are regulated in the cell. If the absence of mannitol and the presence of MtlS RNA does trigger rapid turnover of MtlA, one might expect the MtlA-GFP fusions to also be targets of activated proteases or protease adaptor proteins, thereby explaining the repressed reporter activity observed in our studies involving pReporterFull. We are actively investigating the post-translational regulation of MtlA.

In terms of the ability of MtlS to recognize additional regions within the *mtlA* coding sequence, it is possible that such binding events may contribute to translational repression of the mRNA in the cellular context. Although TargetRNA[50] does not predict any additional MtlS-binding sites within the *mtlA* gene, RNAHybrid[51] predicts extensive, but imperfect, base pairing between MtlS and nt +146–328 of the *mtlA* coding sequence. Thus, although in vitro binding of MtlS RNA to only the 5′ UTR of *mtlA* mRNA is sufficient to repress synthesis of MtlA, it is possible, in the cellular context, that additional pairing between MtlS and the *mtlA* coding sequence is necessary for maximal repression of *mtlA*. We are currently investigating the role of the +146–382 coding region of *mtlA* in MtlS-mediated regulation of *mtlA*.

Gaining a better understanding of how MtlS alters expression of *mtlA* will provide insight into how *V. cholerae* adapts to and thrives in a wide range of environments. We recognize that our understanding of the *mtlS-mtlA* circuit remains incomplete, as we have not identified the regulator that controls expression of *mtlS*. Our current observation that a plasmid containing the *mtlS* promoter region results in lower MtlS/MtlS* levels supports a model in which a transcription factor, available in limiting concentrations, is involved in regulating *mtlS* levels in response to carbon source availability. It is not uncommon for genes encoding transcriptional regulators to be found in proximity to the elements that they regulate.[39] The gene adjacent to *mtlA*, VCA1044, however, is annotated as encoding a hypothetical 47 aa protein with no known DNA-binding motifs and is not considered a likely candidate transcriptional regulator (KEGG Database). We also previously evaluated MtlR, synthesized from the *mtl* operon, as a candidate regulator of *mtlS*, but observed that it does not affect MtlS synthesis.[18] The reporter constructs used in this study provide tools for further identifying the transcription factor(s) responsible for regulating *mtlS* transcription and delineating its role in regulating MtlA levels in *V. cholerae*. Since this protein may be involved in the transmission, dissemination and persistence of the pathogen, uncovering the molecular mechanisms by which MtlA production is regulated will provide important insights into the *V. cholerae* life cycle.

Materials and Methods

Bacterial strains, plasmids and culture conditions

All strains and primers used in this study are described in **Tables S1** and **S2**. The *V. cholerae* strains used in this work were El Tor strain N16961 Δ*tcpA* and derivatives (**Table S1**). The *tcpA* mutant is highly attenuated for virulence[52] and was used for safety purposes. With regard to the expression of *mtlA* and *mtlS*, this mutant strain produces phenotypes identical to those of the wild type strain N16961;[16,18] throughout this manuscript, "wild type" refers to the N16961 Δ*tcpA* strain. A detailed description of plasmid and strain construction is provided in the Supplementary Materials.

All strains were grown at 37°C with aeration at 250 rpm and 37°C in LB broth or M9 minimal medium containing 0.4% w/v carbon source. The minimal medium for *E. coli* was also supplemented with 0.1% w/v casamino acids, 0.01% w/v thiamine, 2 mM $MgSO_4$, and 0.1 mM $CaCl_2$. The minimal medium for *V. cholerae* was supplemented with 0.1% trace metals (5% $MgSO_4$, 0.5% $MnCl_2$, 0.5% $FeCl_3$, 0.4% nitrilotriacetic acid), 2 mM $MgSO_4$ and 0.1 mM $CaCl_2$. When necessary, cultures were supplemented with 0.02% arabinose to induce the expression of genes inserted into pJML01 (**Table S1**). Antibiotics were used at the following concentrations: streptomycin, 50–100 μg/mL; carbenicillin, 50–100 μg/mL; chloramphenicol, 2.5–10 μg/mL.

DNA fragments

All primers used to construct DNA fragments are listed in **Table S2**. The *mtlS* and antisense *mtlS* DNA fragments containing either the entire *mtlS* gene (120 bp) or its reverse complement and preceded by a T7 promoter sequence were amplified by PCR from wild type *V. cholerae* chromosomal DNA using primer pairs LIU72/73 and T7mtlSfor/mtlSrev, respectively. In a similar manner, MtlS truncation Constructs A and B were generated using primer pairs LIU72/LIU171 and LIU72/LIU208, respectively. The *mtlA* 5′ UTR DNA fragment containing the entire *mtlA* 5′ UTR (-75 rel. to AUG through start codon, 78 bp) preceded by a T7 promoter sequence was amplified from wild type *V. cholerae* chromosomal DNA using primer pairs LIU94/93.

In order to produce the mRNA used for in vitro translation assays, a 2-step PCR was performed with chromosomal wild type *V. cholerae* DNA (primer pair LIU116/117, followed by LIU118/119), pXG-10 DNA (primer pair LIU120/121, followed by LIU118/119) or pReporter5′UTR DNA (primer pair LIU116/160, followed by LIU 118/119) to construct *mtlA'-flag*, *gfp-flag* and *mtlA5′UTR-gfp-flag*, respectively. In each case, the primers used added a T7 transcription promoter, a Flag epitope tag, and T7 transcription terminator.

In vitro RNA preparation

For sRNAs, in vitro transcription reactions were performed using T7 RNA polymerase (Promega, P2075) according to the manufacturer's instructions. Briefly, a DNA fragment was mixed in a 20 μL reaction with 500 μM rNTPs, 10 mM DTT, 1× T7 RNA polymerase buffer and 10–20 U T7 RNA polymerase. The reaction was incubated at 37°C for 2 hr and then treated with RQ1 DNase (Promega, M6101) at 37°C for 30 min. The RNA transcripts were purified on 10% TBE-urea polyacrylamide gels and eluted overnight at 37°C in buffer containing 20 mM Tris-HCl (pH 7.5), 0.5 M ammonium acetate, 1 mM EDTA, and 0.1% SDS, followed by phenol:chloroform extraction and ethanol precipitation.

For mRNAs, in vitro transcription assays were performed using the Ribomax RNA Large Scale Production Kit (Promega, P1300), following the manufacturer's instructions using 3–5 μg of template DNA in a 100 μL reaction mixture. The reaction was incubated at 37°C for 2 hr and then treated with RQ1 DNase at 37°C for 15 min. A small sample (3 μL) of the reaction was run on an 8% TBE-urea polyacrylamide gel to confirm that the correct-sized transcript had been generated. The remaining RNA was then purified using phenol:chloroform extraction and ethanol precipitation.

RNA was 3′-labeled with biotin using the RNA 3′ End Biotinylation Kit (Pierce, 20160) and following the manufacturer's instructions. Briefly, 50 pmol of RNA was mixed in a 30 μL reaction with 1× ligase reaction buffer, 40 U RNase inhibitor, 33.3 μM biotinylated cytidine (bis)phosphate, 40 U T4 RNA ligase, and 15% PEG-20,000. The reaction was incubated at 16°C for 2 hr and the biotinylated RNA was purified by phenol: chloroform extraction and ethanol precipitation.

Electrophoretic mobility shift assay

EMSAs were performed with labeled *mtlA* RNA in buffer containing 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 50 mM KCl, and 10 mM $MgCl_2$ in the presence or absence of sRNA in a 20 μL reaction mixture. The samples were heated to 90°C for 5 min and then slow-cooled to 37°C (0.1 °C/sec). After incubation at 37°C for 30 min, 2 μL of 10 x orange loading buffer (Licor, 927-10100) was added and the samples were loaded on a 10% TBE polyacrylamide gel. The electrophoresis was carried out at 4°C. With TYE705-labeled *mtlA* assays, the gel was directly visualized on an Odyssey Imager (Licor). With biotin-labeled *mtlA* assays, the RNA was transferred to a nylon membrane (GE Healthcare Life Sciences, RPN119B). The RNA was then visualized using a 1:10,000 dilution of IR680-conjugated streptavidin (Licor, 926-68031) in Blocking Buffer (Licor, 927-40000) with 1% SDS. Images were analyzed using Odyssey application software (Licor).

In vitro translation assay

Translation reactions were carried out using the PURExpress In vitro Protein Synthesis Kit (NEB, E6800S), following the manufacturer's instructions. RNA transcripts and an RNase inhibitor (NEB, M0314S) were mixed together and incubated at 37°C for 30 minutes. For assays with Constructs A and B, after the RNA transcript were mixed together, they were first heated to 85°C for 5 minutes, slow-cooled to 37°C (0.1°C/sec), and then incubated at 37°C for 30 minutes. Solutions A and B were then added to each sample to achieve final reaction volumes of 25 μL. The reaction mixtures were incubated at 37°C for 60 min. The reaction was terminated by placing the sample on ice; 2.5 μL of each reaction was used for western blotting analysis.

Western blotting

Total protein samples (from PURExpress reaction or from whole cells harvested from liquid cultures) were mixed 1:1 with sample buffer (250 mM Tris-HCl [pH 6.8], 10% SDS, 50% glycerol, 10% β-mercaptoethanol, 0.5% orange G). The samples were heated at 95°C for 10 min and separated on an SDS-containing polyacrylamide gel. Proteins were then transferred to a nitrocellulose membrane (Licor, 926–31090). The membranes were incubated with a dilution of primary antibody (1:2500 of rabbit anti-FLAG, AbCam, ab1162; 1:2000 of rabbit anti-GFP, AbCam, ab290; 1:10000 of mouse anti-RpoB, AbCam, ab12087) for 1 hr, followed by incubation with a 1:10000 dilution of an IR680-conjugated goat anti-rabbit and/or goat anti-mouse secondary antibody (Licor, 926–68021 and 926–32210) for 30 min. IR fluorescence was detected using Odyssey Imager (Licor) and images were analyzed using Odyssey application software (Licor).

Fluorescence measurements

Bacteria strains harboring *gfp* fusion plasmids were inoculated 1:100 from overnight cultures into 2 mL fresh medium in 13 mm glass test tubes. At least 3 independent overnight cultures were used throughout the study for each strain. At the indicated cell density, the bacteria were collected (1 mL, 8000 × *g*,

5 min), and resuspended in 1 mL PBS. Aliquots (200 μL) of each culture were transferred to a 96-well optical-bottom plate (Thermo Scientific Nunc, 165305), and relative fluorescence units (RFU; excitation 480 nm, emission 520 nm) and culture density (OD_{600}) were measured at room temperature in a Synergy 4 instrument (Biotek). Background fluorescence and OD_{600} from a blank (PBS) was subtracted from each sample. The background-corrected RFU was normalized to the background-corrected OD_{600} and the mean RFU/OD_{600} of biological replicates was determined. Strains harboring plasmids bearing *gfp* fusions were at least 4× more fluorescent than isotopic strains harboring a control plasmid that does not express *gfp* (**Fig. S5A**). Thus, the reported RFU measurements were all above the autofluorescence of the bacteria and reflect relative cellular GFP levels. We also noted that the various growth conditions used in this study did not affect RFU of wild type GFP (**Fig. S5B**). Statistical analysis was performed using GraphPad Prism 5 software.

RNA isolation

For analysis of mRNA, total RNA was isolated from 1.5 mL of bacterial culture. Bacteria were first pelleted at 6000 x *g* for 5 min and then resuspended in 500 μL of 1× M9 salts. Lysis buffer was then added to achieve a final mixture that consisted of 40 mM sodium acetate (pH 5.0), 1% SDS and 2 mM EDTA. An equal volume of acid phenol:chloroform (Life Technologies, AM9722) was added and the mixture was incubated at 65 °C for 5 min with vortexing every minute. The samples were then centrifuged at 16000 × *g* for 10 min. RNA in the aqueous layer was purified by acid phenol:chloroform extraction and ethanol precipitation. For sRNA analysis, total RNA from 1.5 mL of bacterial culture was isolated using the DirectZol RNA MiniPrep Kit (Zymo, R2051). Bacteria were first pelleted at 6000 × *g* for 5 min and then resuspended in 350 μL of TRI Reagent. RNA was isolated following the manufacturer's suggested protocol. For qRT-PCR analysis, DNA was removed from all samples using the TURBO DNA-free Kit (Life Technologies, AM1907), according to the manufacturer's instructions.

Northern blotting

For mRNA analysis, total RNA (15 μg) in glyoxal loading dye was run on a 1% TBE-agarose gel and then transferred to a nylon membrane using the NorthernMax-Gly Kit (Life Technologies, AM1946). For sRNA analysis, total RNA (2 μg) in Loading Buffer II (Life Technologies, AM8546G) was run on a 10% TBE-urea polyacrylamide gel and then transferred to a nylon membrane (GE Healthcare Life Sciences, RPN119B). For all blotting, RNA was subjected to UV cross-linking after transfer, the blot was pre-hybridized in ULTRAhyb-Oligo (Life Technologies, AM8663) at 65 °C for at least 30 min, and then hybridized overnight at 65°C. In some cases, hybridizations were performed using DNA probes (**Table S2**) 5′ end labeled with an IR dye. Otherwise, northern blotting was performed with RNA probes transcribed from PCR-derived templates (**Table S2**) with T7 promoters by using biotin-16-UTP and T7 RNA polymerase (Promega, P2075) according to the manufacturer's instructions (see above). All blots were washed and imaged as described in the Odyssey northern blot analysis protocol (Licor).

Quantitative real-time PCR analysis

qRT-PCR was carried out using a Stratagene MX3005P System and Brilliant II SYBR Green QRT-PCR Master Mix Kit (Agilent, 600835). The reactions contained 1× Brilliant SYBR Green QPCR Master Mix, 30 nM ROX reference dye, each primer at 100 nM (**Table S2**), 100 ng RNA, and 1 μL RT/RNase block enzyme mixture in a 25 μL reaction. All the reactions were carried out at the following conditions: 30 min at 50°C, 10 min at 95°C, and 40 cycles of 30 s at 95°C and 1 min at 60°C in 96-well optical reaction plates (Agilent, 401334). A dissociation curve analysis was carried out at the end of amplification to confirm PCR product specificity. Fluorescence data were collected at the end of the extension step. Technical replicates as well as no template and no RT negative controls were included and at least 3 biological replicates were studied in each case. No signals were detected in no-template controls and no-RT controls. Expression of RNA of interest was normalized to an endogenous control (4.5S RNA). Statistical analysis was performed using GraphPad Prism 5 software.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Materials

Supplemental data for this article can be accessed on the publisher's website.

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