Translational regulation by bacterial small RNAs via an unusual Hfq-dependent mechanism

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ABSTRACT

In bacteria, the canonical mechanism of translational repression by small RNAs (sRNAs) involves sRNA-mRNA base pairing that occludes the ribosome binding site (RBS), directly preventing translation. In this mechanism, the sRNA is the direct regulator, while the RNA chaperone Hfq plays a supporting role by stabilizing the sRNA. There are a few examples where the sRNA does not directly interfere with ribosome binding, yet translation of the target mRNA is still inhibited. Mechanistically, this non-canonical regulation by sRNAs is very poorly understood. Our previous work demonstrated repression of the mannose transporter *manX* mRNA by the sRNA SgrS, but the regulatory mechanism was unknown. Here, we report that *manX* translation is controlled by a molecular role-reversal mechanism where Hfq, not the sRNA, is the direct repressor. Hfq binding adjacent to the *manX* RBS is required for sRNA-mediated translational repression. Translation of *manX* is also regulated by another sRNA, DicF, via the non-canonical Hfq-dependent mechanism. We posit that the sRNAs act to recruit Hfq to the appropriate binding site or stabilize the sRNA-mRNA-Hfq complex. This work adds to the growing number of examples of diverse mechanisms of translational regulation by sRNAs in bacteria.

INTRODUCTION

Almost 35 years ago, the first non-coding RNA, RNA I, was discovered in bacteria (1). Since then, in all three domains of life, a surprisingly diverse yet poorly characterized pool of regulatory RNAs has been discovered. Bacterial sRNAs are small transcripts, most in the range of 50-250 nucleotides, that act by imperfect, non-contiguous base pairing with mRNA targets to control translation or mRNA stability. Examples of positive regulation by sRNAs are less numerous compared to negative regulation, but sRNAs have been shown to pair with the 5´-UTR of an mRNA to prevent formation of a translation-inhibitory secondary structure (2,3) or to pair with ribonuclease recognition sequences, thus stabilizing the mRNA (4). For negative regulation, sRNAs often, but not always, operate as translational repressors by directly pairing with sequences overlapping the RBS, sequestering it from the incoming ribosome (5-7). To perform any of these regulatory tasks, sRNAs frequently depend on the chaperone protein Hfq. Hfq was initially discovered as the host factor for the replication of bacteriophage Qβ, but over the last few decades, its pleiotropic role in cellular physiology has reignited the interest of the research community (8). Hfq has emerged as a key factor in sRNA-mediated gene regulation, and in control of stability of mRNAs and sRNAs (9,10). Hfq is thought of as a matchmaking chaperone that promotes interaction between the sRNA and the target by binding to both RNAs. Another key role of Hfq is protection of sRNAs from RNase E-mediated degradation (9,11,12). Hfq is a donut-shaped homohexameric protein belonging to the large family of Sm- and Sm-like proteins that are present in all three domains of life (9,13,14).

In this study, we explored the regulatory mechanism of two Hfq-dependent sRNAs, SgrS and DicF, that negatively regulate *manX* translation by an unconventional mechanism. The physiological condition that triggers expression of DicF is unknown, but SgrS is expressed during a metabolic state called glucose-phosphate stress (15). Sugars are critical nutrients that fuel central metabolic pathways: glycolysis, the pentose phosphate pathway, and the TCA cycle, to generate precursor metabolites needed to synthesize nucleotides, amino acids, and fatty acids. Nonetheless, accumulation of excess phosphorylated sugar intermediates, and their non-metabolizable derivatives can be growth inhibitory (16,17). For instance, when cells are grown in the presence of non-metabolizable sugar analogs, such as α-methyl glucoside (αMG) or 2-deoxy-D-glucose (2DG), it creates glucose-phosphate stress; glycolysis is inhibited, and cells stop growing (15,18). Under these conditions, *E. coli* induces expression of SgrS, an Hfq-dependent small RNA with regulatory activities that restore cell growth. When produced under glucose-phosphate stress conditions, SgrS base pairs with mRNA targets to regulate their translation and

stability (15,19-21). One of the key activities of SgrS during glucose-phosphate stress is repression of mRNAs encoding phosphotransferase system (PTS) sugar transporters, *ptsG* (15) and *manXYZ* (19,22). This repression inhibits new synthesis of PTS transporters and reduces uptake of sugars that are not being efficiently metabolized during stress. We have shown that base pairing-dependent repression of transporter synthesis by SgrS is required for continued growth under stress conditions (23).

Many sRNAs that repress gene expression do so by inhibiting translation initiation by preventing ribosome binding to target mRNAs. Since bacterial translation initiation requires RNA-RNA base pairing between the 16S rRNA and the SD region, sRNAs typically base pair with a site close to the SD region and compete with the 30S ribosomal subunit (24). Initial research on the interaction between mRNA and the ribosome suggested that initiating ribosomes occupy ~40 nucleotides, from -20 nt in the 5´ UTR to +19 nt into the coding region, where numbering is with respect to the start codon (5,25). A study using synthetic antisense oligonucleotides tested the boundaries to define the region where a base pairing interaction could prevent formation of the translation initiation complex (TIC). This study indicated that oligos base pairing within 15 nucleotides downstream of the start codon can inhibit TIC formation (26). This led to the "five codon window" hypothesis that proposed that if an sRNA base pairs with nucleotides comprising the first five codons of the mRNA, it can directly inhibit binding of the 30S ribosomal subunit and repress translation initiation. Interestingly, some studies have uncovered apparent exceptions to this hypothesis where sRNAs repress translation, either directly or indirectly, by base pairing outside of the five-codon window (20,27). For example, the Massé group found that binding of the sRNA Spot 42 at a site ~50 nt upstream of the *sdhC* start codon. Their evidence suggested that the Spot 42 itself does not directly compete with the initiating ribosome, but instead may recruit the RNA chaperone Hfq to bind near the *sdhC* ribosome binding site (RBS) and act as the primary repressor (27).

In this study, we investigated the mechanism by which SgrS regulates the first cistron of the *manXYZ* operon, *manX*. We observed previously that regulation of *manX* mRNA by SgrS involves base pairing 20 nt downstream of the start codon, which lies outside the 5 codon window (19). We also characterized regulation of *manX* translation by another sRNA regulator, DicF (28), a 53-nt long Hfq-dependent sRNA (29). The DicF binding site on *manX* mRNA is even further downstream than the SgrS binding site. We hypothesized that each of these sRNAs regulates *manX* translation by a "non-canonical" mechanism, since their binding sites are positioned too far downstream for sRNA-mRNA base pairing to directly occlude ribosome binding. To test this

hypothesis, we addressed several questions. Does sRNA-mRNA duplex formation directly inhibit translation by preventing formation of the translation initiation complex? If not, then is Hfq required for translational repression? Does Hfq bind to the *manX* mRNA near the ribosome binding site?

Our results demonstrate that Hfq is absolutely required for translational repression mediated by SgrS and DicF in vivo. In vitro, Hfq, but not the sRNAs, can specifically inhibit formation of the TIC on *manX* mRNA. RNA footprints confirmed that SgrS and DicF have distinct binding sites in the *manX* coding region, and both sRNAs facilitate Hfq binding at a site close to the RBS. Taken together, our data demonstrate sRNAs mediate regulation of *manX* translation by a non-canonical mechanism involving recruitment or stabilization of Hfq binding at a site where it can directly interfere with translation.

MATERIALS AND METHODS

Strains and plasmids

The strains, plasmids, and oligonucleotides used in this study are listed in Supplementary Tables 1 and 2. Derivatives of *E. coli* K-12 MG1655 were used for all experiments. Alleles were moved between strains using P1 transduction (30) or λ-red recombination (31). Translational LacZ fusions, under the control of an arabinose-inducible P_{BAD} promoter, were constructed by PCR amplifying DNA fragments using primers with 5´-homologies with the promoter and to the 9th codon of *lacZ* (Table S2). These fragments were integrated into the chromosome by λ-red recombination using counterselection against *sacB* as described previously (32).

SA1328, a strain with tet-Cp19-115nt-*manX*`-`*lacZ, ΔsgrS, lacI*^{q}, kan^{R}, *Δhfq* genotype was constructed in two steps. First, *Δhfq::FRT-kan-FRT* was transduced into JH111 and pCP20 was used to flip out the kan^{R} cassette. The tet-Cp19-115nt-*manX*`-`*lacZ* cassette was then transduced into the latter strain.

Strains containing truncated *manX* translational fusions under the control of a P_{BAD} promoter, were constructed in strain PM1205 (32). The P_{BAD} -22nt-*manX'-'lacZ* and P_{BAD} -25nt-*manX'-'lacZ* fusions were generated by PCR amplifying DNA fragments with primer pairs O-SA178/O-SA176 and O-SA177/O-SA176 primer pairs respectively, containing 5´ homologies to pBAD and *lacZ* (Table S2). The PCR products were recombined into PM1205 using λ-red homologous recombination as described previously. The same fusions with mutations in the Hfq binding site (in strains SA1522 and SA1620), were created using the method above, but using oligonucleotides O-SA177/O-SA176 and O-SA177/O-SA433 to obtain the PCR products.

Media and regents

Unless otherwise stated, bacteria were cultured in LB broth or on LB agar plates at 37°C. TB medium was used for β-Galactosidase assays. To induce Lac promoters, 0.1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) was used. L-arabinose was used at concentrations of 0.001%, for solid media, and 0.002%, for liquid media, to induce P_{BAD} promoters. Antibiotics were used at following concentrations: 100 µg/ml ampicillin, 25 µg/ml chloramphenicol, 50 µg/ml spectinomycin, and 25 µg/ml kanamycin.

β-Galactosidase assays

Strains with *lacZ* fusions were grown overnight in TB medium and subcultured 1:100 to a fresh medium containing Amp and 0.002% L-arabinose (for P_{BAD} promoters). Cultures were grown at 37°C with shaking to OD_{600}~0.2, and 0.1 mM IPTG was added to induce expression of SgrS or DicF. Cells were grown for another hour to OD_{600}~0.5. β-Galactosidase assays were performed on these cells according to the previously published protocol (33).

In vitro transcription

For in vitro transcription, template DNA was generated by PCR using gene specific oligonucleotides with a T7 promoter sequence at the 5´ end of the forward primer. The following oligonucleotides were used to generate templates for RNA footprinting and gel shift assays: O-JH219/O-JH119 and O-JH218/O-JH169 to generate *manX* and SgrS template DNA. DNA template for DicF transcription was generated by hybridizing two oligos, DicFW and DicFC, in TE buffer. Transcription of these DNA templates was performed using the MEGAscript T7 kit (Ambion) following manufacturer's instructions.

Purification of His-tagged Hfq

Hfq-His protein was purified following a previously published protocol (34). BL21(DE3) cells harboring pET21b-Hfq-His_6 was cultured in 400 ml LB medium. At OD_{600}~0.3, 1mM IPTG was added to the culture and incubation was continued for 2 hrs. The cells were washed with STE buffer (100 mM NaCl; 10 mM Tris·HCl, pH 8.0; 1 mM EDTA) and resuspended in 10 ml Equilibration buffer (50 mM Na_2HPO_4–NaH_2PO_4, 300 mM NaCl, and 10 mM imidazole). The suspension was treated with 25 mg lysozyme, incubated on ice for 10 minutes and sonicated. The supernatant was collected after centrifugation at 16,000 × g for 10 min at 4°C followed by incubation at 80°C for 10 minutes. The sample was centrifuged again, at 16,000 × g for 10 min at 4°C. The supernatant was fractionated using a Ni^{2+}NTA agarose column following manufacturer's

instructions (Roche) and checked by SDS-PAGE electrophoresis. The fractions containing Hfq were pooled, dialyzed, and stored in a storage buffer (20 mM Tris·HCl pH 8.0, 0.1 M KCl; 5 mM $MgCl_2$, 50% glycerol, 0.1% Tween 20, and 1 mM DTT) at −20°C.

Toeprinting assays

Toeprinting assays were performed using unlabeled $manX_{ATG}$ and P^{32}-end-labeled primer in the presence and absence of Hfq and SgrS following the previously published protocol (35). For each reaction, 2 pmol of *manX* RNA and 1.6 pmol of end-labeled primer (O-JH119) were heated for one min at 95°C in toeprint buffer (20 mM Tris-HCl pH 7.5, 50 mM KCl, 0.1 mM EDTA, and 1mM DTT). The mixture was chilled in ice for five minutes, and 10 mM $MgCl_2$ and 1 mM of dNTPs were added. Purified Hfq and in vitro synthesized SgrS RNA were added to the appropriate reaction mixtures and incubated at 37°C for 10 minutes. Next, ribosomes (1.3 pmol, NEB) were added to this reaction mixture, and the incubation was continued at 37°C for five minutes. Thirteen picomoles of fMet-tRNA (Sigma) was added to this reaction and cDNAs were synthesized using SuperScript III reverse transcriptase (Invitrogen). The reaction was stopped by adding 10 µl of loading buffer II (Ambion). The reaction products were analyzed on an 8% polyacrylamide-urea gel. Sequencing ladders were generated using Sequenase 2.0 DNA sequencing kit (Affymetrix).

Footprinting assays

In vitro RNA-footprinting reactions were performed as described previously (36) with some modifications. 0.1 mol of 5´-end labeled *manX* mRNA was incubated at 37°C for 30 minutes in structure buffer (Ambion) containing 1 ng of yeast RNA (Ambion), in the presence or absence of 100 pmoles of unlabeled SgrS and 3.7 pmoles of Hfq. Lead acetate (Sigma) was added to perform the cleavage reaction (2.5 µM) and incubated at 37°C for two minutes. Reactions were stopped by adding 12 µL of loading buffer (Ambion). The alkaline ladder was generated by incubating 5´-end labeled *manX* mRNA at 90°C for 5 minutes in alkaline buffer (Ambion). RNase T1 was used for 5 minutes at 37°C to generate the G ladder. The samples were resolved on an 8% polyacrylamide/urea gel.

Electrophoretic mobility shift assay

RNA-RNA and RNA-protein gel electrophoretic mobility shift assays were performed using 0.01 pmol of P^{32}-labeled denatured *manX* RNA and the indicated amounts of SgrS (denatured at 95°C for 1 min) or Hfq in binding buffer (20mM Tris-HCl pH 8.0, 1mM DTT, 1mM $MgCl_2$, 20mM KCl, 10mM Na_2HPO_4–NaH_2PO_4 pH 8.0). The mixture was incubated at 37°C for 30 minutes, and

non-denaturing loading buffer (50% glycerol and 0.1% bromophenol blue) was added. The samples were resolved on a 4.6% native polyacrylamide gel for 1.5 hours at 10 mA. The fraction of *manX* RNA bound was determined using Fluorescent Image Analyzer FLA-3000 (FUJIFILM) to quantitate the intensities of the bands. The data were fit into Sigmaplot software to obtain the K_D value.

RESULTS

Hfq is essential for translational repression of manX

Previously, we identified *manXYZ* mRNA, which encodes a mannose and (secondary) glucose transporter (EII^{Man}), as a target of SgrS (19,22). The polycistronic *manXYZ* mRNA is negatively regulated post-transcriptionally via two independent SgrS-*manXYZ* mRNA base pairing interactions (22). The physiological outcome of this regulation is repression of EII^{Man} transporter synthesis, which helps rescue cell growth during glucose-phosphate stress (19). One of the SgrS binding sites was mapped to the coding region of *manX*, and this binding site was shown to be necessary for translational repression of *manX* (19,22). Although we have identified the base pairing sites for SgrS on the *manXYZ* transcript and established that translational regulation of this target is important for cell growth during glucose-phosphate stress, the exact mechanism of SgrS-mediated *manX* translational repression is unknown. The SgrS binding sites on the *manXYZ* mRNA are too far from the RBS of the *manX* and *manY* cistrons (22) to directly occlude ribosome binding (Fig. S1). We hypothesized that *manX* translation might be repressed by a non-canonical mechanism where Hfq serves as the direct repressor of translation while SgrS plays an accessory role, perhaps recruiting Hfq to bind stably near the SD.

To test this hypothesis, we first investigated whether translational repression of two different SgrS targets, *ptsG* and *manX,* was dependent on Hfq in vivo. Aiba and coworkers have already shown that SgrS can inhibit translation of *ptsG* in the absence of Hfq (34), consistent with the canonical model for repression where the sRNA pairs near the TIR and directly occludes ribosomes. Here, we utilized *manX′-′lacZ* and *ptsG′-′lacZ* translational fusions that we have already demonstrated are good reporters for SgrS-dependent regulation of these targets (19). We monitored fusion activities after SgrS production was induced in wild-type and Δ*hfq* backgrounds. Since stability of *E. coli* SgrS ($SgrS_{Eco}$) is greatly reduced in the absence of Hfq (37), we utilized the *Salmonella* SgrS allele ($SgrS_{Sal}$) which is more stable than $SgrS_{Eco}$ in the Δ*hfq* background and has very similar seed region that allows it to efficiently regulate *E. coli* SgrS target mRNAs

((38) and Fig. S2). In agreement with our previous study, we found that $SgrS_{Sal}$ can complement a Δ*sgrS* mutant for regulation of *manX* (Fig. 1A) (38). Consistent with the direct ribosome occlusion mechanism for translational regulation of *ptsG,* $SgrS_{Sal}$ efficiently repressed the *ptsG′-′lacZ* fusion in both the wild-type and Δ*hfq* backgrounds (Fig. 1B). In contrast, while SgrS efficiently repressed *manX* in a wild-type background, it failed to repress the fusion in an *hfq* mutant background, even at high levels of inducer (Fig. 1C). These data indicate that SgrS cannot regulate *manX* in vivo in the absence of Hfq.

If the role of SgrS in *manX* regulation is to recruit or enhance binding by the putative primary or direct regulator Hfq, it follows that Hfq should bind close to the *manX* TIR. To determine if there were elements in the 5′ UTR, such as a putative Hfq binding site, that were required for translational regulation, we constructed a series of *manX* translational fusions with truncations in the 5′ UTR (Fig. 2A). Activity of these fusions was measured in the presence and absence of ectopically expressed *sgrS*. The constructs contained 65, 30, 25, and 22 nt of the *manX* 5′ UTR region, and in the hfq^{+} background, all four fusions showed a similar pattern of regulation compared to the construct containing the full-length 115-nt *manX* 5′ UTR (Fig. 1A). Further truncation of the *manX* 5′ UTR was not possible without interfering with the RBS. Nevertheless, the fact that all truncated fusions were regulated similarly to wild-type suggested that the putative Hfq binding site resided downstream of the 5′ boundary defined by the 22-nt fusion (Fig. 2A). An A/U-rich motif just upstream of the *manX* RBS is similar to the motif that was shown in other studies to be preferentially bound by Hfq (39,40). To test the role of this motif in the regulation of *manX* translation, we constructed a mutant *manX* fusion where the A/U-rich motif was converted to a C/G-rich motif (Fig. 2B). In contrast with the wild-type *manX* fusion, which was efficiently repressed when SgrS was ectopically expressed, SgrS failed to alter translation of the mutated fusion (Fig. 2B). It is important to note that the A/U-rich putative Hfq binding motif is far upstream from the known SgrS base pairing site (Fig. 2B). Thus, the loss of regulation in the mutant suggests that regulation of *manX* translation is not directly mediated by SgrS base pairing, but may instead involve Hfq binding to a site near the *manX* RBS.

Hfq alone inhibits formation of the translation initiation complex on manX mRNA

Regardless of whether the direct repressor of *manX* translation is Hfq or SgrS, we predicted that competition for binding of the repressor and the ribosome at the RBS leads to the formation of mutually exclusive macromolecular complexes, *i.e.*, mRNA-ribosome-$tRNA^{fMet}$ or mRNA-SgrS-Hfq. For sRNA-mediated translational repression, sRNAs generally inhibit the initiation stage of translation (27,34). We used toeprinting assays (35) to assess whether Hfq or

SgrS could directly inhibit translation initiation. In the toeprinting assay, stable binding of the 30S ribosomal subunit and $tRNA^{fMet}$ to the RBS blocks a primer extension reaction and produces a product with a characteristic size. Since native *manX* has a weak GTG start codon that does not stably associate with commercially available preparations of 30S ribosomes, we changed the start codon to the canonical ATG to ensure strong initiation complex formation *in vitro*. We showed previously that this construct, $manX_{ATG}$, was efficiently repressed by SgrS (19). The toeprint assay was performed by mixing $manX_{ATG}$ RNA, P^{32} end-labeled primer, ribosomes and $tRNA^{fMet}$ in the presence and absence of Hfq. Reverse transcriptase was then added to begin the primer extension reaction. In the positive control reaction containing only $manX_{ATG}$ RNA, P^{32} end-labeled primer, ribosomes and $tRNA^{fMet}$, we saw the characteristic toeprint signal caused by termination of reverse transcription at position +15/+16 relative to the start codon (Fig. 3A). With the addition of increasing concentrations of Hfq, the formation of the TIC was completely inhibited (Fig. 3A, 3B). However, when increasing concentrations of SgrS were added in the absence of Hfq, TIC formation was unperturbed and we saw strong toeprints (Fig. 3B). These results are consistent with the in vivo studies, and add further evidence supporting our hypothesis that Hfq itself directly inhibits *manX* translation at the initiation stage.

manX *is regulated by DicF sRNA*

In a previous study, *manX* was identified as a putative target of another sRNA, DicF (28). To further investigate regulation of *manX* by DicF and determine the regulatory mechanism, we monitored activity of a *manX´-´lacZ* translational fusion (under the control of a constitutive promoter to rule out indirect effects on *manX* transcription) in control cells and cells where DicF was ectopically expressed. Cells expressing *dicF* showed ~40% reduced β-galactosidase activity compared to control cells (Fig. 4A). Compared to SgrS, which reduces *manX* translation by about 70% (Fig. 2B), DicF is a rather weak regulator.

Previous studies have demonstrated that sequences at either the 5′ or the 3′ end of DicF can base pair with mRNA targets (28,41). We identified a potential base pairing interaction between the 3′ end of DicF and the coding region of *manX* just downstream of the known SgrS binding site (Fig. 4B). To test this base pairing prediction, we made a mutation in nucleotides of DicF that should disrupt the base pairing interaction (Fig. 4B). This mutant allele, $dicF_{20}$, lost the ability to regulate the *manX´-´lacZ* translational fusion (Fig. 4C), consistent with the base pairing prediction. If DicF also base pairs within the *manX* coding sequence, well outside the window that would allow direct interference with ribosome binding, then like SgrS, DicF may also repress *manX* translation by influencing Hfq binding in the *manX* TIR. To test whether DicF-mediated

regulation requires the putative Hfq binding site near the RBS, we constructed a mutant version of the *manX´-´lacZ* fusion (containing the putative DicF base pairing site) where the A/U-rich region next to the RBS is changed to G/C-rich (as in Fig. 2B). In contrast with the wild-type *manX* fusion, which was repressed upon *dicF* expression, activity of the fusion with the mutation in the putative Hfq binding site was not substantially altered by DicF (Fig. 2A). This observation is consistent with the model that DicF-mediated regulation of *manX* is similar to SgrS in that it requires Hfq binding proximal to the RBS where it acts as the direct translational repressor.

Hfq binds next to the manX *ribosome binding site*

We predicted that DicF base pairs at a site just downstream of the SgrS binding site, from residues G145 to C162 (Fig. 4B). Our genetic analyses suggest that Hfq binds in the 5' UTR just upstream of the RBS to act as the direct repressor of *manX* translation for both SgrS- and DicF-mediated regulation (Figs. 2B and 4A). To further test this prediction, we performed in vitro footprinting experiments with labeled *manX* RNA to identify the Hfq binding site(s) occupied in the presence of each individual sRNA. As we showed previously, SgrS protects its binding site from C139 to G152 on *manX* mRNA even in the absence of Hfq, and addition of Hfq does not change the footprint (Fig. 5A) (19). Notably, SgrS alone does not affect the structure around the RBS or start codon. Consistent with our prediction (Fig. 4B), DicF protects *manX* mRNA from G150 to C167 in the absence and presence of Hfq (Fig. 5B). Again, DicF only impacted the reactivity of nucleotides comprising its binding site in the *manX* coding region, and the structure upstream in the TIR was unaffected. In the presence of either sRNA, Hfq clearly protected *manX* mRNA nucleotides A97-A103 (Fig. 5A). Note that this region is the same A/U-rich region that we predicted as the Hfq binding site (Fig. 2B), and that when mutated, prevented SgrS- and DicF-dependent regulation (Figs. 2B and 4A, respectively). Additionally, we observe some weak protections for residues U91-C95, which are likely to be participating in a stem loop structure (Fig. 5B). These findings demonstrate that Hfq binds at precisely the same location on *manX* mRNA, adjacent to the RBS, regardless of which sRNA is present in the sRNA-mRNA-Hfq ternary complex.

DicF is a weak regulator of manX

Compared to DicF, SgrS is a stronger repressor of *manX* translation (compare repression in Fig. 2B to Fig. 4A). Our data suggest that each of these sRNAs mediates translational regulation indirectly, via facilitating or stabilizing Hfq binding to a site in the 5′ UTR adjacent to the RBS (Fig. 5A). To explore the basis for the different efficiencies of regulation, we conducted experiments to measure the affinity of sRNA-mRNA interactions and sRNA-Hfq interactions. We reasoned that

differences in the binding affinity of the sRNAs *for manX* mRNA and/or Hfq, could be important determinants of regulatory efficiency for each sRNA-mRNA-Hfq interaction. Electrophoretic mobility shift assays (EMSAs) were used to measure the specific binding of SgrS and DicF individually to *manX* mRNA in vitro. We found that SgrS base paired with *manX* mRNA, with a dissociation constant K_D of 4.53 μM. DicF interacted less strongly with *manX* mRNA, with a K_D of 21.8 μM. The higher K_D for DicF-*manX* mRNA is consistent with our observation that compared to SgrS, DicF is a weak repressor of *manX* translation. In a previous study, we found that in vivo, the K_D for SgrS binding to full-length *manXYZ* mRNA (with both *manX* and *manY* binding sites) was 2.3 μM (42). Thus, our in vitro measurement is in good agreement with the in vivo data for SgrS.

EMSAs to monitor interactions of each sRNA with Hfq also revealed differences between SgrS and DicF. The Hfq-SgrS interaction was relatively strong, with a calculated K_D of 3.37 nM. Hfq bound DicF less tightly with a calculated K_D of 22.0 nM. The dissociation constant values we calculated for Hfq and SgrS or DicF are in a similar range as those reported previously for the binding of Hfq to OxyS, RyhB, DsrA, and Spot 42 sRNAs (9,43-45). Taken together, our data suggest that SgrS is a more efficient regulator of *manX* translation than DicF and that differences in sRNA-mRNA binding interactions and sRNA-Hfq interactions could influence the efficiency of regulation.

DISCUSSION

Our study shows that two sRNAs, SgrS and DicF, base pair with *manX* mRNA at distinct sites in the coding region, outside the window that would allow translational repression via a direct ribosome occlusion mechanism. Instead, we propose that Hfq acts as the direct regulator of *manX* translation and that the sRNAs play an accessory or secondary role. This model is supported by multiple lines of evidence presented in this study. In vivo, SgrS cannot repress *manX* translation in the absence of Hfq (Fig. 1C). This is in contrast with regulation of another SgrS target, *ptsG*, which is known to occur via the canonical (direct) mechanism of translational repression (15,46). SgrS efficiently represses *ptsG* translation in an *hfq* mutant background (Fig. 1B). Loss of regulation by both sRNAs is seen in an *hfq*+ background when the Hfq binding site upstream of the *manX* RBS is mutated (Figs. 2B, 4A). Structural analyses clearly demonstrate that SgrS and DicF bind to sites in the coding region of *manX* mRNA and have no impact on the structure near the TIR. In contrast, in the presence of either SgrS or DicF, Hfq binds to the same site on *manX* mRNA, directly adjacent to the RBS (Fig. 5A). Differences in the relative strength of *manX*

translational regulation promoted by SgrS and DicF were correlated with the strength of sRNA-mRNA and sRNA-Hfq interactions (Fig. 6A, B). Collectively, our data are consistent with a non-canonical mechanism of regulation where the sRNAs play a secondary or accessory role in regulation by promoting Hfq binding to a site near the *manX* RBS so that Hfq itself directly interferes with ribosome binding (Fig. 7). This model is in stark contrast to the canonical model of bacterial sRNA-mediated translational repression where sRNAs are the direct competitors of ribosome binding, while the chaperone Hfq assumes the secondary role. Here, the chaperone Hfq swaps its role with the RNA partner.

A recent study reported a non-canonical mode of translation regulation where the sRNA Spot 42 binds its target *sdhC* mRNA far upstream of the RBS. Spot 42 itself cannot compete with the incoming ribosome for binding to *sdhC* mRNA, but instead seems to facilitate binding of Hfq to a site near the TIR in order to block translation initiation (27). Here, we show that sRNAs can employ similar mechanism and recruit Hfq to bind near the RBS even if they base pair downstream of the start codon in the mRNA coding region. Footprinting of *manX* mRNA with SgrS, DicF, and Hfq revealed that SgrS base pairs with nucleotide +24 to 36 on the *manX* transcript, while DicF base pairs little downstream, with nucleotides +34 to +51, but both sRNAs recruit Hfq at the same location near RBS (Fig 6). In other words, the identity and the location of base pairing sites do not seem to be the determining factor for non-canonical sRNA-based regulation effected by Hfq binding near the TIR. We propose that the sRNAs carry out the task of substrate recognition that subsequently allows the protein partner to be recruited at a particular binding site. Similar mechanisms are widely utilized by eukaryotic non-coding RNAs, including small interfering RNAs, microRNAs, and small nucleolar RNAs. All of these types of eukaryotic small RNAs act as a part of a ribonucleoprotein complex where the RNA component recognizes the substrate nucleic acid while the protein component performs catalysis. In bacteria, we have yet to uncover the mechanistic details of regulation carried out by the vast majority of sRNAs, but of those for which mechanisms have been established, sRNAs are typically the primary effectors of regulation. This raises some intriguing questions. Is the “canonical” mechanism of sRNA-mediated regulation with sRNA as primary regulator really the most common, or have computational and experimental approaches used to study sRNAs been biased toward discovery of these mechanisms because they were the first type described? Regardless of the prevalence of each of these two different mechanisms, what features distinguish them and make one or the other more favorable for regulation of a given mRNA target?

One advantage of sRNA-mediated regulation that involves base pairing interactions outside the TIR could be that it provides a larger and more diverse sequence space to evolve new regulatory interactions. We have found that regulation of *ptsG*, the primary glucose transporter, by SgrS is conserved among *E. coli* relatives where SgrS orthologs were found (47,48). The SgrS-*ptsG* mRNA interaction involves pairing between the most highly conserved seed region of SgrS and the *ptsG* RBS, a region where the sequence is highly conserved for ribosome binding. In contrast, SgrS-dependent regulation of *manX* involves a less well-conserved portion of SgrS (adjacent to the conserved seed) and the coding sequence of *manX,* and this interaction is not entirely conserved among enteric species. For instance, *E. carotovora* SgrS and *Y. pestis* SgrS do not regulate their cognate *manX* orthologs (19) because the SgrS sequences that pair with *manX* mRNA are not conserved in these organisms. Analyses by Peer and Margalit (49) indicate that the SgrS-*ptsG* mRNA interaction evolved first, with the binding sites on both mRNA and sRNA co-appearing in evolutionary time. Their data suggest that SgrS-*manX* mRNA interaction evolved much later. So, SgrS first established a regulatory interaction with *ptsG* in an ancestral organism of the order *Enterobacteriales,* which established this sRNA regulator in the genome and allowed evolution of interactions with additional targets. Other recent work on sRNA evolution suggests similar target acquisition mechanisms where sRNAs establish one target and gradually establish other interactions with the concurrent evolution of Hfq (49,50). Perhaps flexibility in regulatory mechanisms, *e.g.,* where the sRNA can act as either a primary or accessory regulator along with Hfq, facilitates rapid evolution of additional sRNA-target interactions.

Regulation of *manX* translation by DicF, an sRNA encoded on the cryptic prophage Qin on the *E. coli* chromosome, was confirmed in this study. Like other small RNAs encoded on horizontally acquired genetic elements like prophages and pathogenicity islands, DicF is poorly characterized. However, research over the last decade, suggests that horizontally-acquired sRNAs are crucial regulators of bacterial physiology, growth, and stress responses. For instance, the sRNA InvR, encoded in *Salmonella* pathogenicity island 1, is a major regulator of outer membrane porin OmpD (51). IpeX, an sRNA encoded on the cryptic prophage DLP12 in *E. coli*, is a regulator of outer membrane porins, OmpC and OmpF (52). DicF was identified in the 1980s when it was observed to cause a filamentation phenotype when expressed from a multi-copy plasmid (53). We recently demonstrated that DicF directly regulates translation of mRNA targets encoding diverse products involved in cell division and metabolism (28). These include mRNAs encoding the tubulin homolog FtsZ, xylose uptake regulator XylR, and pyruvate kinase PykA (28,54). Our current study extends the DicF targetome to include *manX*. Though SgrS and DicF share a common target in *manX*, these sRNAs are not expressed under the same conditions. We

did not see DicF expression when cells were challenged with αMG or 2DG (data not shown). Under standard laboratory growth conditions, the *dicBF* operon is not expressed, and we do not yet know the signal that triggers the expression of this operon. Further research aimed at uncovering the physiological conditions stimulating DicF production may provide insight into the biological role of DicF-mediated *manX* regulation.

A long-held notion about sRNA-mediated gene regulation in eukaryotes is that the primary role of sRNAs is target recognition, while the associated protein partners perform the primary regulatory function of gene silencing or translational repression. In bacteria, the prevailing model has been the opposite—that the sRNA is the primary regulator and associated proteins play secondary roles in promoting RNA stability or making the regulation irreversible (in the case of mechanisms involving mRNA degradation). Our findings, along with one other recent report on a similar non-canonical mechanism of regulation in bacteria (27) suggest that bacteria can utilize a broader range of sRNA-mediated regulatory strategies than previously suspected. So, while there are considerable differences among the domains in terms of the mechanisms of translation initiation, sites of sRNA binding, and the nature of the ribonucleoprotein complexes carrying out regulation, sRNA-directed recruitment of regulatory proteins to mRNA targets appears to be a common mode of regulation in all three domains of life.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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FIGURE LEGENDS

Figure 1. SgrS-mediated translational repression of *manX* is Hfq-dependent. A) Strain JH175 has an in locus *manX′-′lacZ* translational fusion under the control of Cp19, a constitutive promoter. This strain was transformed with a vector control or plasmids with SgrS homologs from *Salmonella* ($SgrS_{Sal}$) or *E. coli* ($SgrS_{Eco}$), expressed after induction with 0.1 mM IPTG, and assayed for β-galactosidase activity after 60 min. Units of activity in the experimental samples were normalized to the levels the vector control strains to yield percent relative activity. **B)** The same vector control and $SgrS_{Sal}$ plasmids described in A were transformed to strain JH184 (*hfq*+) or DB151 (Δ*hfq*), containing a Cp19-*ptsG′-′lacZ* fusion. Induction of SgrS and β-Galactosidase assays were conducted and analyzed as in A. **C)** Strains JH175 (*hfq*+) and SA1328 (Δ*hfq*) with the Cp19- *manX′-′lacZ* fusion were transformed with vector control or $SgrS_{Sal}$ plasmids. SgrS was induced using the indicated concentrations of IPTG and β-Galactosidase assays were conducted and analyzed as in A.

Figure 2. Genetic analysis of a putative Hfq binding site in the *manX* 5′ UTR. A) The full-length *manX* UTR in *E. coli* is 115 nt. Four translation fusions with decreasing lengths of UTR (as indicated) were constructed by moving the heterologous promoter closer to the TIR. Vector control and SgrS plasmids were transformed into the resulting strains (JH178, JH181, SA1404 and SA1403) and β-Galactosidase assays were conducted and analyzed as described for Fig. 1A. **B)** An A/U rich motif upstream of the *manX* RBS, was mutated in the context of the 25-nt *manX* translational fusion. The positions of the putative Hfq binding site and confirmed SgrS binding site are indicated with gray boxes. The RBS is in blue letters and the *manX* start codon is in green. The activity of SgrS on wild-type (strain SA1404) and mutant (strain SA1522) fusions was assessed after induction as described for Fig. 1A.

Figure 3. Toeprint assays reveal that Hfq, but not SgrS, can prevent ribosome binding to *manX* mRNA. A) Toeprint assays were conducted as described in Materials and Methods. Ribosomes, $tRNA^{fMet}$ and Hfq were added to *manX* mRNA as indicated above the gel image. In lanes 4-6, Hfq concentrations were 0.15 μM, 0.5 μM, and 1 μM, respectively. The sequencing ladder is indicated by "T – G – C – A", and was generated with the same oligo (OJH119) used for reverse transcription. The toeprint signal is indicated at +15/16 relative to the start codon. **B)** Lanes 1-6 on the left represent the same reactions as described in part A. Lanes 1-6 on the right represent similar reactions, except SgrS was added at concentrations of 100 nM, 250 nM, and 500 nM (lanes 10-12).

Figure 4. DicF, a prophage-encoded sRNA, also regulates *manX* translation. A) Strains with *manX'-'lacZ* fusions with a wild-type (JH175) or mutant (putative) Hfq binding site (SA1620) (as shown in Fig. 2B) were transformed with vector control or DicF-expressing plasmids. Expression of DicF was induced with 0.1 mM IPTG, and β-Galactosidase assays were conducted and analyzed as described for Fig. 1A. **B)** Base pairing interactions for *manX* mRNA (middle sequence) and SgrS (top sequence) or DicF (bottom sequence). The position of the DicF20 mutation is illustrated in green. **C)** Strain JH175, containing the wild-type *manX'-'lacZ* fusion, was transformed with vector control or plasmids expressing wild-type DicF or mutant DicF20. Expression of DicF was induced with 0.1 mM IPTG, and β-Galactosidase assays were conducted and analyzed as described for Fig. 1A.

Figure 5. Footprinting maps SgrS, DicF and Hfq binding sites on *manX* RNA. In vitro transcribed *manX* mRNA containing the full-length 115-nt UTR and a portion of the coding region extending 51 nt downstream of the predicted DicF base pairing region was end labeled with ^{32}P and incubated with and without unlabeled SgrS, DicF and Hfq to perform footprinting reactions. Samples were treated as follows: "T1," RNase T1; "OH," alkaline ladder; "PbAc," lead acetate. Positions of G residues are indicated to the left of each gel image and nucleotides numbered as indicated in B. Positions of the GUG start codon and RBS are indicated to the left of each image. **A)** Footprinting SgrS and Hfq (left image) or DicF and Hfq (right image) binding sites on *manX* mRNA. **B)** Sequence and putative structure of *manX* following interaction with Hfq and SgrS or DicF. Positions of SgrS and DicF binding are indicated (from residues 139-167). The start codon is indicated by orange nucleotides. The Hfq binding site is highlighted in orange and the RBS is highlighted in green.

Figure 6. In vitro analyses of sRNA binding to *manX* mRNA and Hfq. A) Native gel electrophoresis was used to examine binding of *manX* mRNA with SgrS and DicF sRNAs. In vitro transcribed ^{32}P-labeled *manX* mRNA (0.01 pmol) was mixed with indicated amounts of cold SgrS and incubated at 37°C for 30 min. The reaction mixture was resolved on a chilled native acrylamide gel. Bands were quantified and the fraction of *manX* mRNA bound was calculated and plotted to calculate K_D. **B)** Gel mobility shift assay for SgrS (right) or DicF (left) and Hfq. Measured band densities (n replicates, top left) were plotted to determine the dissociation constants.

Figure 7. A model for the non-canonical roles played by two distinct sRNAs, SgrS and DicF, to repress *manX* translation via an Hfq-dependent mechanism.

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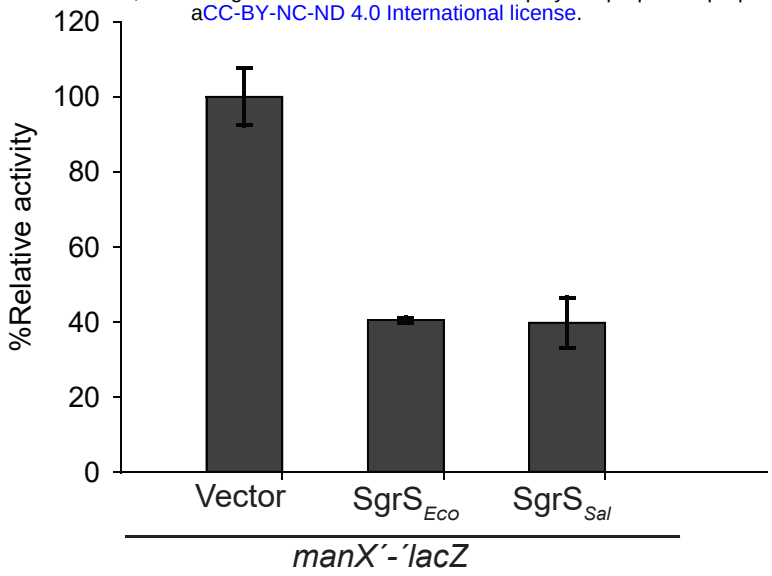
A)



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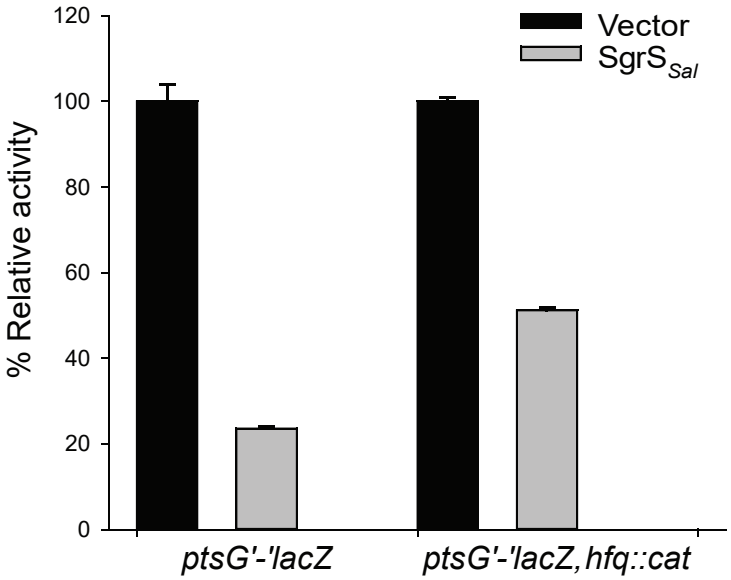

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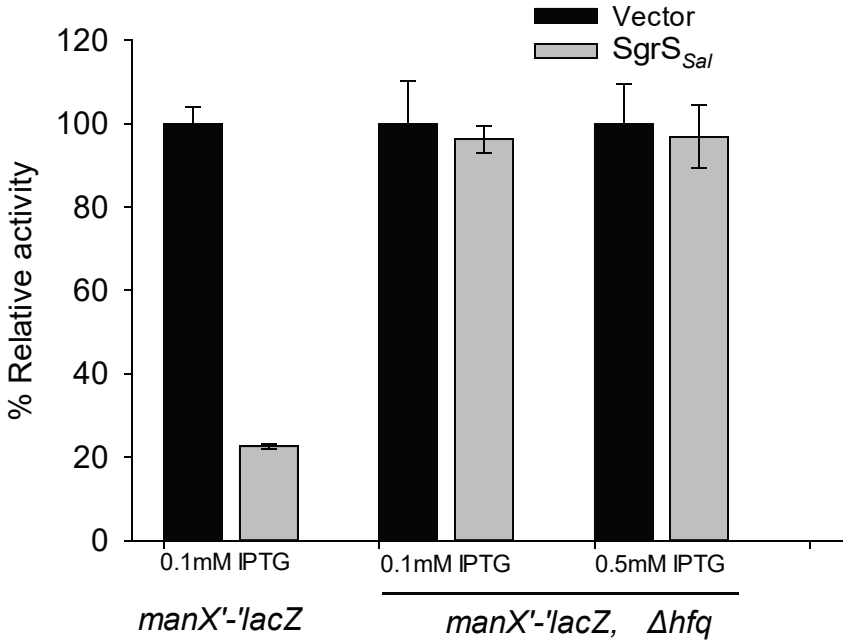

Fig 1

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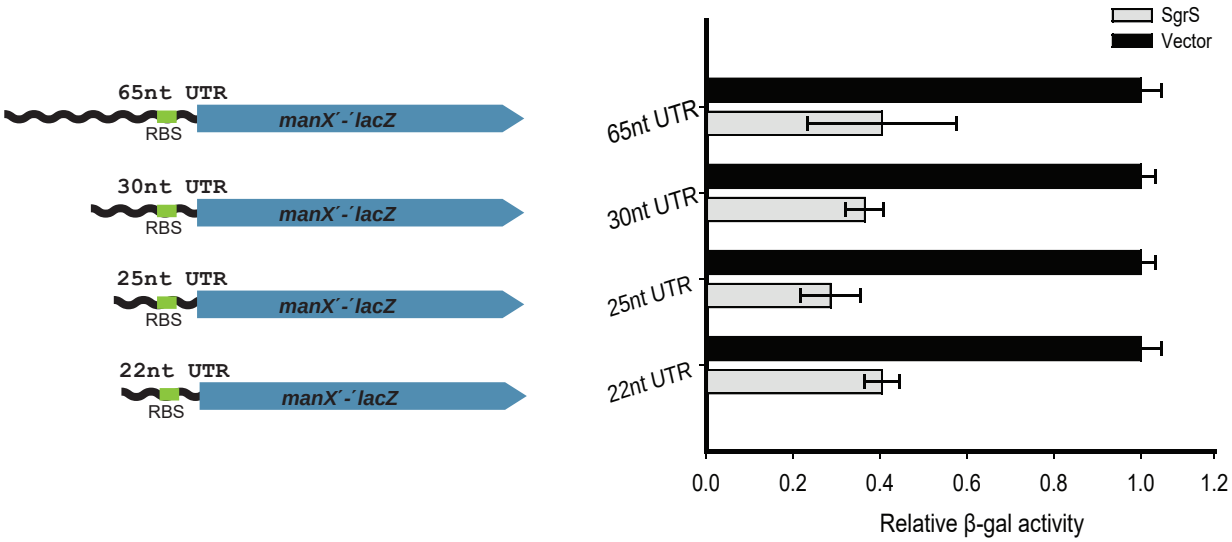

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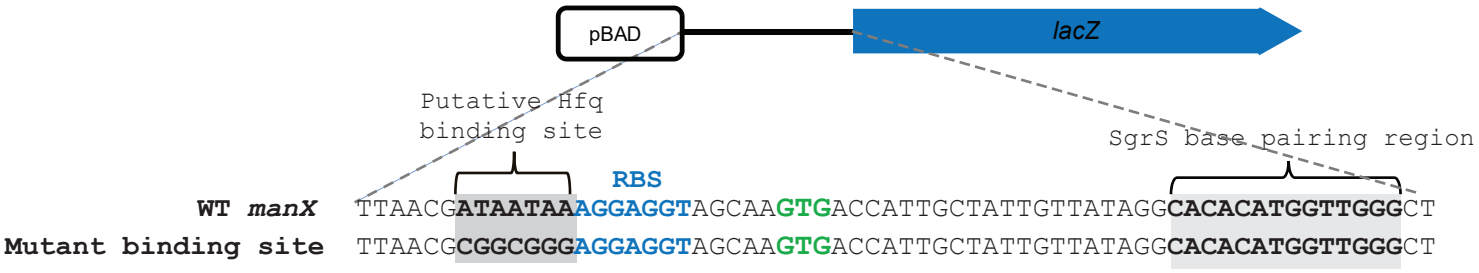


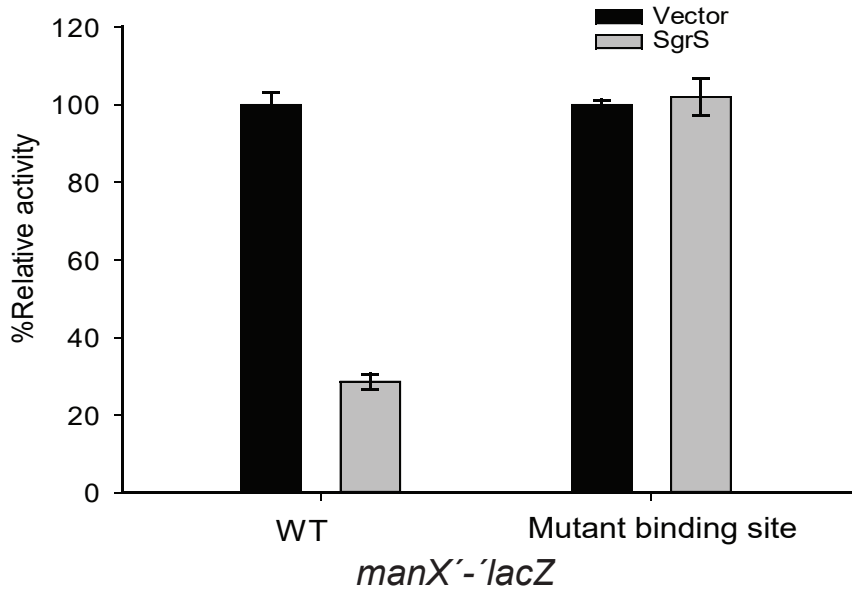


Fig 2

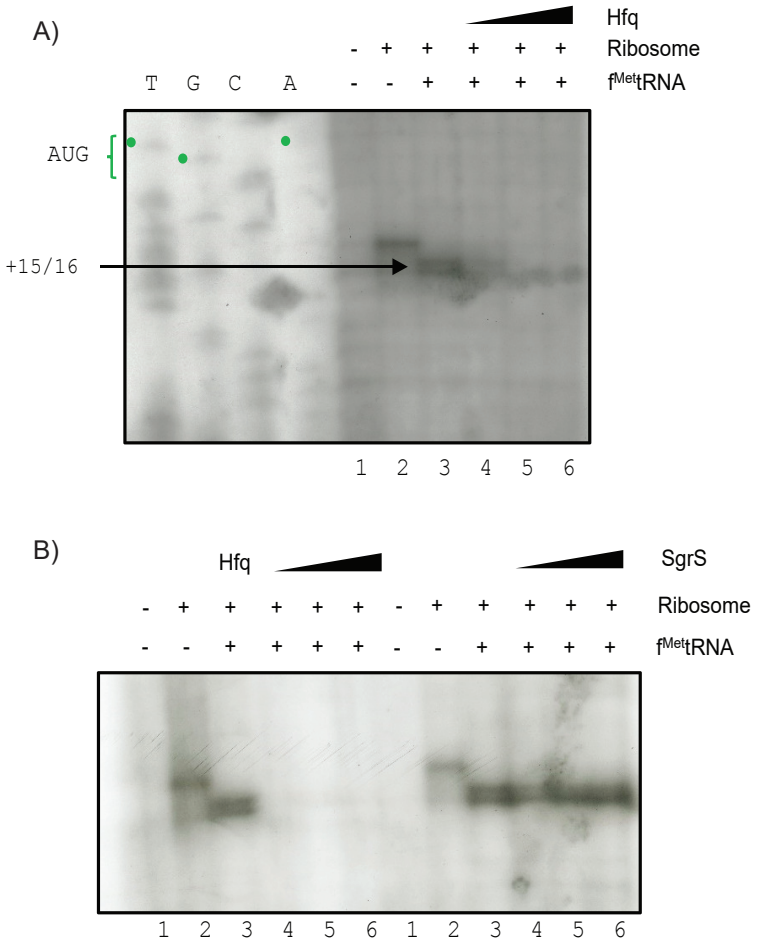
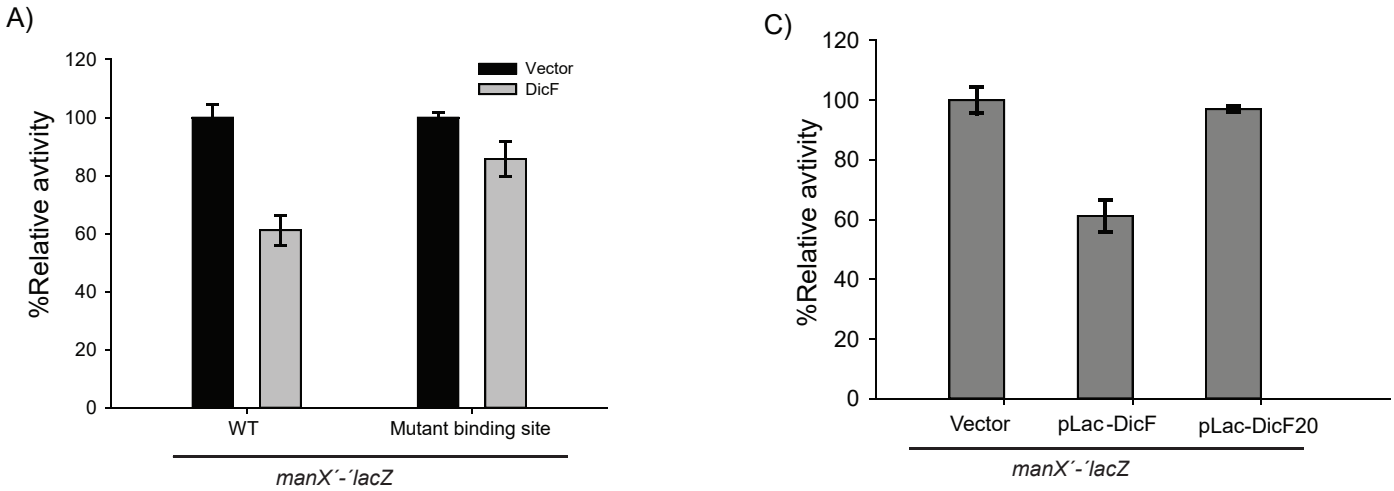


Fig 3



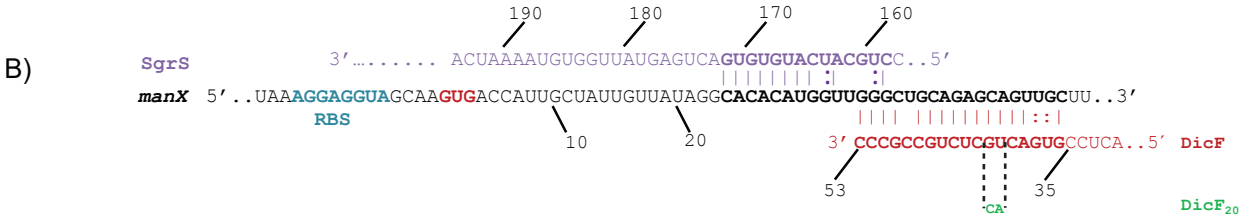


Fig 4

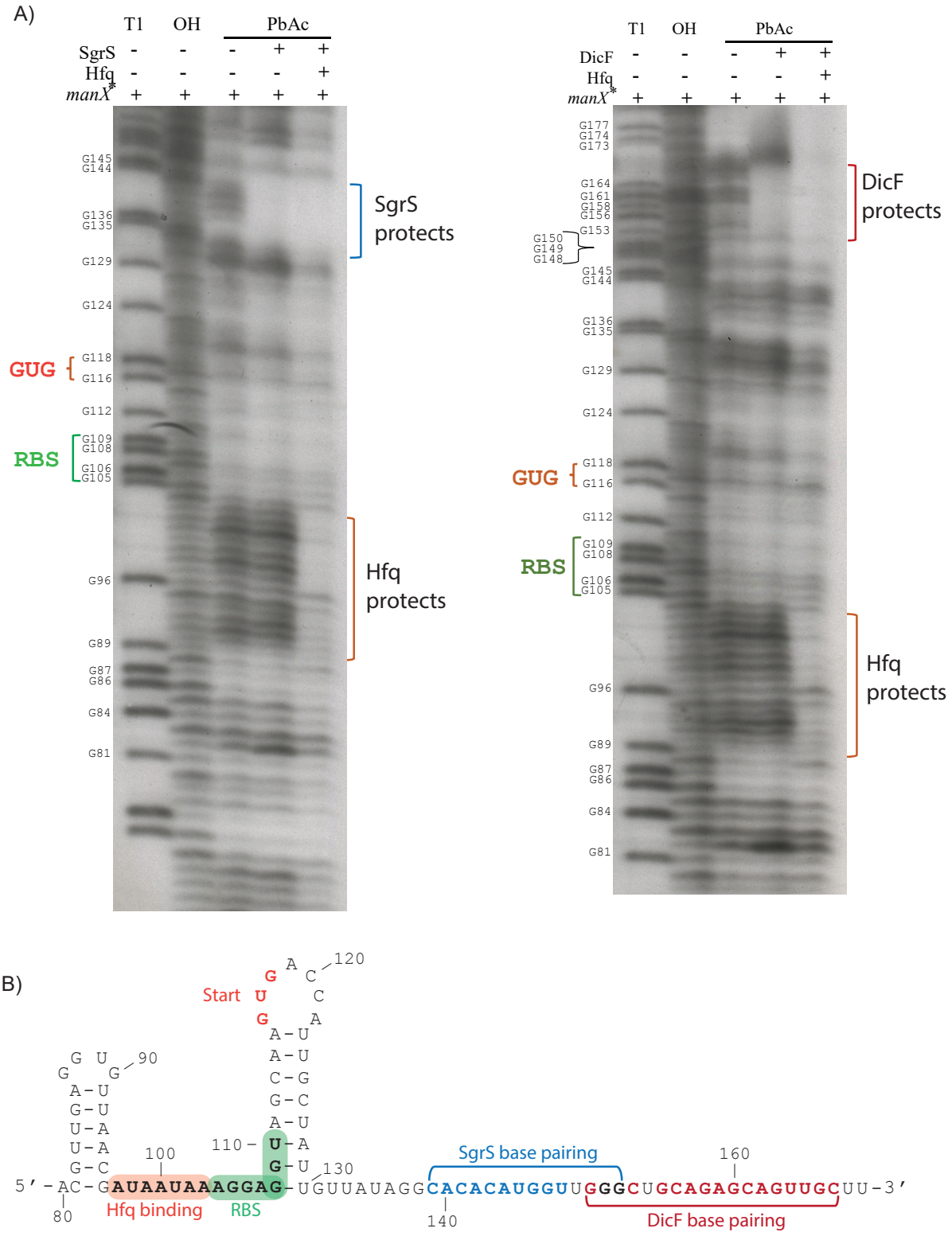


Fig 5

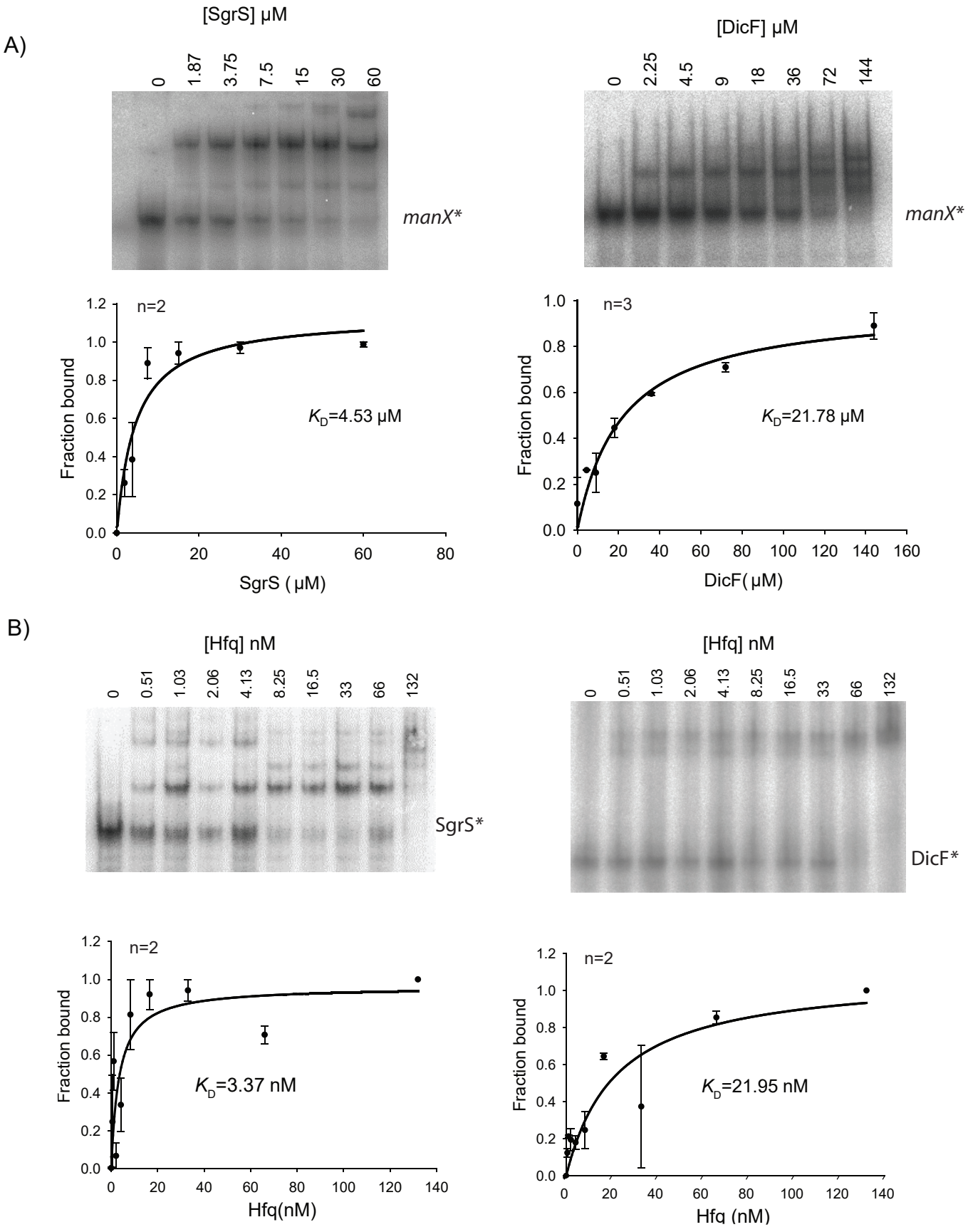


Fig 6

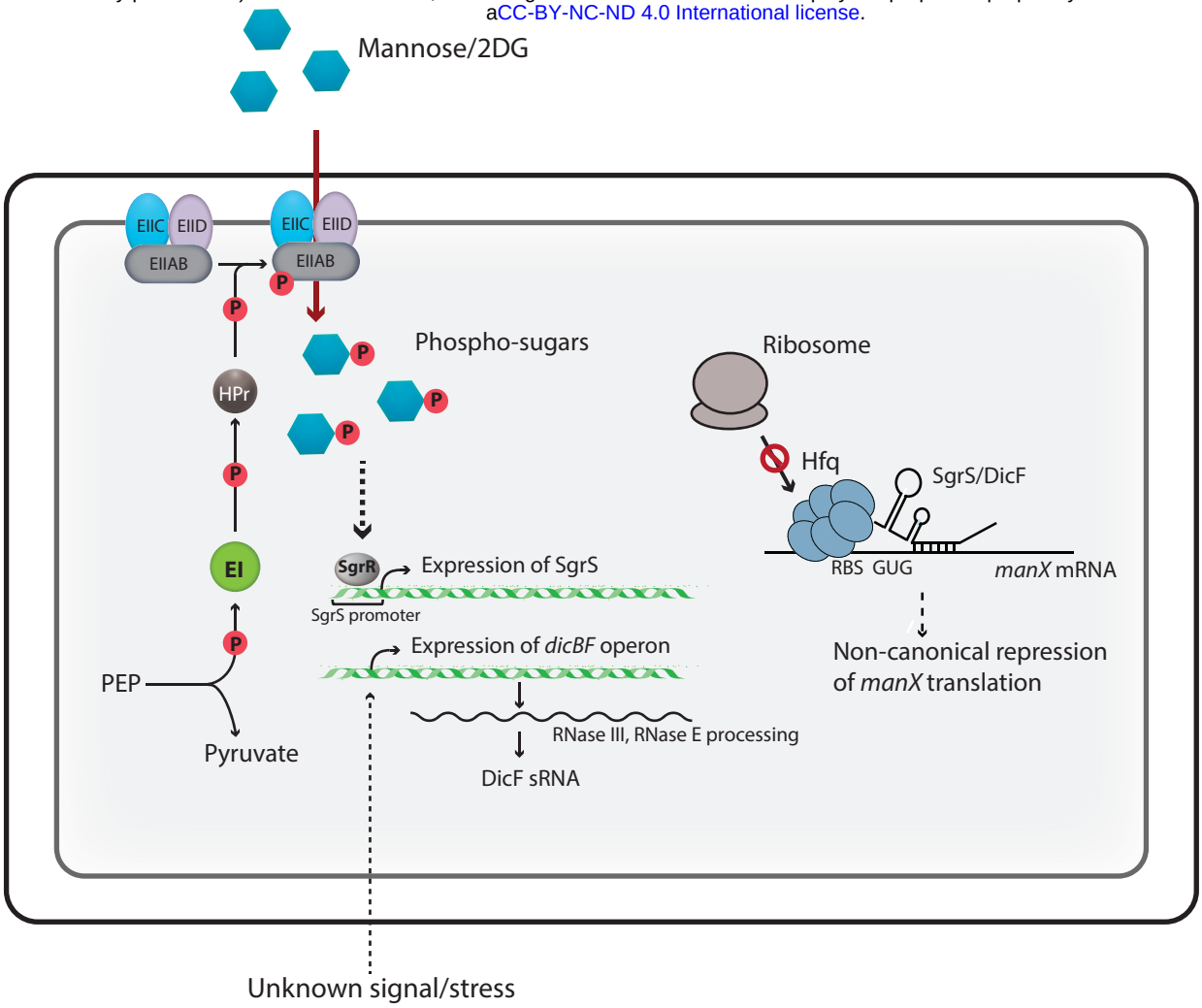


Fig 7