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Effects of different target sites on antisense RNA-mediated regulation of gene expression

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Antisense RNA is a type of noncoding RNA (ncRNA) that binds to complementary mRNA sequences and induces gene repression by inhibiting translation or degrading mRNA. Recently, several small ncRNAs (sRNAs) have been identified in *Escherichia coli* that act as antisense RNA mainly via base pairing with mRNA. The base pairing predominantly leads to gene repression, and in some cases, gene activation. In the current study, we examined how the location of target sites affects sRNA-mediated gene regulation. An efficient antisense RNA expression system was developed, and the effects of antisense RNAs on various target sites in a model mRNA were examined. The target sites of antisense RNAs suppressing gene expression were identified, not only in the translation initiation region (TIR) of mRNA, but also at the junction between the coding region and 3' untranslated region. Surprisingly, an antisense RNA recognizing the upstream region of TIR enhanced gene expression through increasing mRNA stability. [BMB Reports 2014; 47(11): 619-624]

INTRODUCTION

Half a century ago, Singer *et al*. (1) reported that translation of polyuridylic acid is blocked by antisense polyadenylic acid. In 1977, Paterson *et al*. (2) showed that a DNA-RNA hybrid arrests mRNA translation *in vitro*, using rabbit β-globin mRNA and cDNA as the template and antisense oligomers, respectively. Over the years, antisense oligomers have been diversified with DNA, RNA, and various modified versions of nucleic acids (3) to effectively repress translation of mRNA. Antisense RNA is believed to inhibit translation or induce degradation of mRNA through base pairing. Recently, many small noncoding RNAs (sRNAs) have been identified in *E. coli* (4-6). The majority of these RNA molecules function as antisense oligomers through base pairing with mRNAs (7-11). These base pairing leads to gene repression, but in some cases, it can trigger gene activation. The majority of sRNA target sites are located in the mRNA translation initiation region (TIR) (7-14). The base pairing in this region affects ribosome binding, which consequently affects on translation. Alternatively, the TIR ready to accommodate ribosomes is the most accessible site, so sRNA binding inhibits ribosome interactions and/or induces degradation of sRNA-bound target mRNA. However, target sites for some sRNAs have been identified in the regions other than TIR of mRNA, such as the coding region or 3' untranslated region (UTR) (15, 16). In the current study, we examined whether antisense RNAs physiologically behave like sRNAs in the cell by repressing and activating translation. If this is the case, it would be interesting to establish how the location of the target site affects sRNA-mediated gene regulation. For this purpose, we designed and constructed an expression system generating metabolically stable antisense RNAs with easier cloning. The antisense sequences recognizing various target sites on mRNA were incorporated into the antisense RNA expression system. Notably, target sequences of the mRNA suppressed by the majority of antisense RNAs were located in TIR. Mild repression was observed upon binding to target sites located at the junction of the C-terminal coding sequence and 3' UTR. Interestingly, an antisense RNA recognizing a sequence in the 5' UTR region induced gene activation.

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RESULTS AND DISCUSSION

Antisense RNA expression

Antisense RNA for expression in *E. coli* was designed considering two factors: metabolic stability of antisense RNA and single-strandedness of both antisense sequences and target mRNA sites. Two different stable RNA stem-loop structures were tethered to the 5' and 3' ends of a defined antisense sequence, including a 3 nt linker sequence for blunt-end cloning. The P1 stem, a crucial factor for the metabolic stability of M1 RNA (17, 18), was added to the 5' end, and the transcription terminator hairpin of *SibC* was placed at the 3' end (Fig. 1A) (19). In this construct, an antisense sequence would hardly interact with sequences within the P1 or terminator stem, since both stem structures are very stable. The target was the fused *lacZ*

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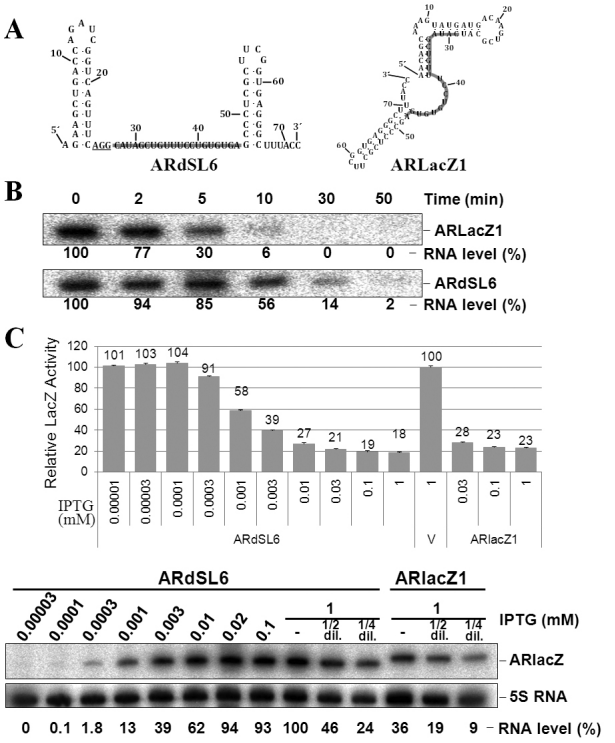

Fig. 1. Antisense RNAs and their effects on gene expression. (A) Secondary structure model of ARdSL6 RNA using the Mfold algorism (36). The antisense sequence to fused *lacZ* mRNA is presented in gray. Three extra nucleotides (AGG) were added during the cloning of antisense RNA, which are underlined. Another type of artificial sRNA, ARlacZ1 (24), carrying the same antisense sequence is presented for comparison. (B) Half-lives of antisense RNAs. Total cellular RNA was prepared from ARlacZ1 or ARdSL6-expressing cells with 1 mM IPTG at the indicated times after rifampicin treatment. Cellular levels of antisense RNA were analyzed via Northern blot. Relative RNA levels are presented in comparison to RNA levels from cells before rifampicin treatment. (C) Cells expressing ARdSL6 RNA were treated with IPTG at increasing concentrations from 0 to 1 mM, and β-galactosidase activities from differently regulated *LacZ* proteins were measured. For comparison, cells expressing ARlacZ1 RNA were additionally analyzed. β-Galactosidase activities were expressed as LacZ activities in relative to the control cells containing the vector and treated with 1 mM IPTG. The actual activity of the control cells was 4,790 Miller units. Cellular levels of ARdSL6 and ARlacZ1 RNA were measured using Northern blot analysis. RNA levels were expressed relative to ARdSL1 in cells induced with 1 mM IPTG, using a standard curve by serial dilutions denoted with 1/2 dil. and 1/4 dil., after normalization to 5S RNA. V, cells containing the plasmid vector.

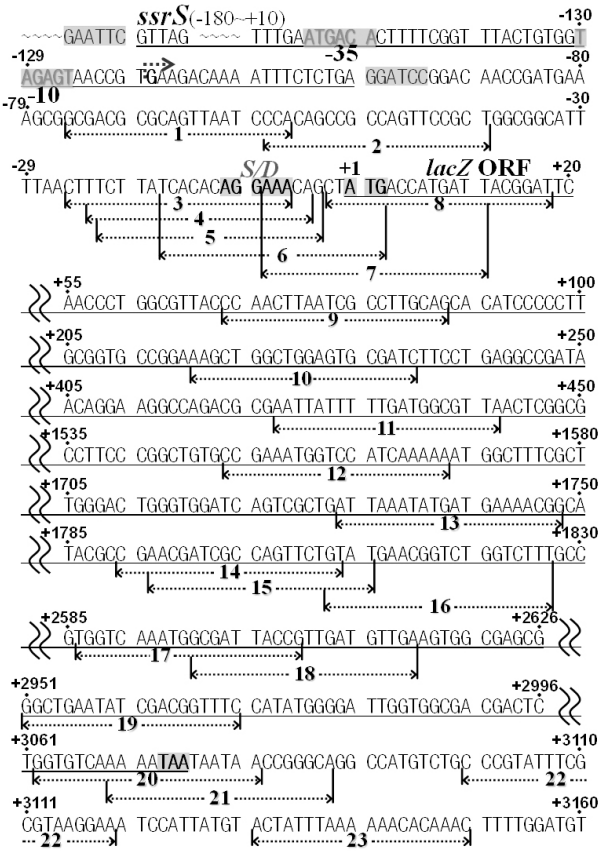

Fig. 2. Gene structure of the *ssrS-lacZ* lysogen. Schematic map of the *ssrS* P1-*lacZ* fusion in the *ssrS-lacZ* lysogen. DNA sequences encoding 5' UTR, coding region, and 3' UTR of the fused *lacZ* mRNA are shown. The transcription start site, Shine-Dalgarno sequence (S/D), translation initiation site, termination codon, and target regions are indicated. The target region recognized by each ARdSL RNA is indicated by the number of the corresponding ARdSL RNA. The antisense sequence for each target region was embedded in the ARdSL RNA scaffold.

mRNA expressed from the *ssrS* P1 promoter (20) in a lysogen carrying the *ssrS-lacZ* transcriptional fusion, in which the *ssrS* P1 promoter was fused to the TIR of *lacZ* followed by its coding sequence (Fig. 2). The *ssrS* P1 promoter is constitutively active during the exponential phase (21). The antisense sequences of 20 nt in length were designed based on the fused *lacZ* mRNA sequence (Fig. 2), and their effects on *lacZ* expression was monitored via measurement of β-galactosidase activity. We subdivided the fused *lacZ* mRNA sequence into five regions: 5' UTR, TIR, coding region, junction between coding region and 3' UTR, and 3' UTR (Fig. 2). With the aid of siRNA Section Server, one of siRNA search tools (22, 23), twenty-three 20 nt antisense sequences against *lacZ* mRNA were selected, which were assumed to satisfy the general requirements for effectiveness of antisense RNA, including highly accessible target sites and single-strandedness of the sequences. The designed antisense sequences were cloned into the RNA expression vector, pHM-tac (24), and a transcription was induced with IPTG to generate artificial double stem-loop small RNAs (ARdSLRNAs). ARdSL6, which is one of those RNAs, was examined for metabolic stability with a ri-

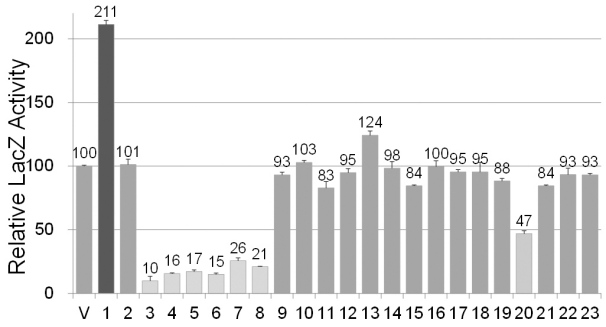

Fig. 3. Effects of target sequence location on gene expression. Relative LacZ activities of cells expressing ARdSL RNA (numbered) in the presence of 1 mM IPTG were measured. β-Galactosidase activities were expressed as relative LacZ activities in Fig. 1C. In this experiment, the actual activity of the control cells containing the vector and treated with 1 mM IPTG, was 3,360 Miller units. The indicated values are calculated from at least three independent experiments.

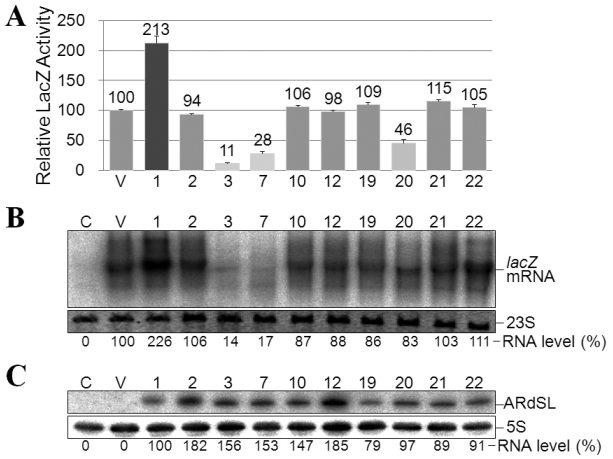

Fig. 4. Detailed analysis of selected ARdSL RNAs. Antisense RNAs with distinct effects on gene expression as well as their counterpart controls were selected. Effects of antisense RNAs on gene expression were evaluated by measuring the relative LacZ activity, as shown in Fig. 1C. In this experiment, the actual activity of control cells containing the vector and treated with 1 mM IPTG was 3,090 Miller units (A). Levels of *lacZ* mRNA and ARdSLRNA were analyzed via Northern blot using total RNA separated on a 1.0% agarose gel containing formaldehyde (B) and 5% polyacrylamide gel containing 7 M urea (C), respectively. 23S, 23S RNA bands stained with ethidium bromide. 5S, 5S RNA signals analyzed by Northern blot. C, cells without plasmid DNA. RNA levels of *lacZ* mRNA and ARdSL relative to those of control cells containing the vector and treated with 1 mM IPTG after normalization to 23S and 5S RNA, respectively, are presented in the bottom.

fampicin chase experiment and was compared with that of ARlacZ1 (24), an artificial small RNA (afsRNA) previously constructed from a scaffold of SibC (Fig. 1B). ARdSL6 and ARlacZ1 had the same target recognition sequence. ARdSL6 had a half-life of 11 min, which was two-fold longer than that of ARlacZ1, resulting in a two-fold higher concentration of ARdSL6 than ARlacZ1 in *E. coli* (Fig. 1C). ARdSL6 RNA caused more repression of β-galactosidase expression in *ssrS-lacZ* lysogen cells than did ARlacZ1 (Fig. 1C), possibly due to its higher cellular level. In the subsequent experiments, various ARdSL RNAs recognizing different targets were induced with 1 mM IPTG in the same lysogen cells containing the corresponding RNA expression plasmids, and their effects on *lacZ* expression were examined (Fig. 3).

5' UTR and TIR regions

All TIR-targeting antisense RNAs induced significant repression of original β-galactosidase activity with 74 to 90% inhibition (Fig. 3). This region spanned positions -25 to +18, relative to +1 of the initiation codon. Among these, ARdSL3 suppressed activity to the most significant extent (90% inhibition). The ARdSL3 containing complementary sequences from positions -25 to -6 was capable of base pairing with the region upstream of S/D and the S/D sequence itself, but not the AUG translation codon. The ARdSL7 and ARdSL8 targeting only the AUG codon but not the S/D sequence exerted slightly lower gene silencing effects (less than 80% inhibition) than antisense RNAs including ARdSL3 to 6, which caused more than 80% inhibition. The *lacZ* mRNA levels were also decreased in proportion to β-galactosidase activity (Fig. 4), suggesting that the gene silencing effects of these antisense RNAs result from degradation of *lacZ* mRNA. Previously, the ribosome binding site of *lacZ* mRNA was mapped from positions -14 to +21 (24, 25). Therefore, TIR-targeting antisense RNAs appear to interfere with ribosome binding to *lacZ* mRNA. When the target sequence was shifted upstream spanning positions -58 and -39, the repression effect disappeared in ARdSL2. Surprisingly, ARdSL1 targeting a further upstream sequence between positions -75 and -56 increased *lacZ* expression by more than 2-fold. The RNA analysis further revealed that the *lacZ* mRNA level is increased by ARdSL1, suggesting that ARdSL1 stabilizes *lacZ* mRNA. However, the mechanism by which ARdSL1 enhances the metabolic stability of *lacZ* mRNA remains to be established. Since the presence of a stable base-paired region at the 5' end of RNA increases its metabolic stability (26, 27), ARdSL1 may provide this type of base pairing for *lacZ* mRNA. Alternatively, base pairing of the target site with antisense RNA may block a site(s) vulnerable to cleavage by ribonucleases, such as RNase E (28).

Internal coding region

The effects of antisense RNAs targeting sequences in the internal coding region were examined. In total, 11 sites (3 in the N-terminal coding region, 5 in the central coding region, and 3 in the C-terminal region) in ARdSL9 to 19 were selected (Fig. 2). All antisense RNAs examined did not affect *lacZ* gene expression (Fig. 3). This result indicates that the antisense RNAs

are not effective for this region in the mRNA (Fig. 4). This may be attributed to the complex two-dimensional or three-dimensional structures of the coding region, along with elongating ribosomes that obstruct the access of antisense RNA. Even in the case of binding to mRNA, the elongating ribosome may remove already bound antisense RNA (16, 29). The results clearly indicate that the coding region is not an effective target of antisense RNA, which may explain why the majority of natural sRNAs interact with TIR of mRNAs (7-14).

Junction between the coding region and 3' UTR

The two antisense RNAs binding to the junction between the coding region and 3' UTR were examined. ARdSL20 recognizing the *lacZ* mRNA region encompassing 11 nt before the UAA stop codon (Fig. 2) led to 53% of inhibition (Fig. 3). On the other hand, no repression was evident with ARdSL21 containing only 4 nt of the coding sequence (Figs. 2 and 3). Northern analysis further revealed that the *lacZ* mRNA level is not significantly reduced (Fig. 4), suggesting that the repression by ARdSL20 is not due to degradation of *lacZ* mRNA but inhibition of translation. Previously, ArcZ was shown to induce degradation of *arcB* mRNA by binding to 3' UTR, including the coding sequence 6 nt upstream of the *arcB* stop codon (15). The mode of action of ARdSL20 may differ from that of ArcZ, although the mechanism by which the binding of ArcZ to the junction affects gene silencing of *arcB* remains to be determined.

3' UTR

Earlier research has shown that McaS RNA, a *cis*-encoded sRNA specifically overlapping the 3' UTR region of its cognate mRNA, has no effect on antisense *abgR* mRNA expression (30). Other *cis*-encoded sRNAs such as GadY RNA have been shown to stabilize target *gadX* mRNA (31). In our experiments, the two antisense RNAs, ARdSL22 and ARdSL23, recognized the 3' UTR region (Fig. 2) and had no effects on *LacZ* expression (Fig. 3). In the case of GadY, specific machinery may be required to induce a positive effect (31, 32).

CONCLUSION

Here, we constructed an efficient antisense RNA expression system to generate metabolically stable and functionally competent RNAs, and examined their effects on various target sites in the fused *lacZ* mRNA. The majority of antisense RNAs that induced suppression of gene expression recognized target sites within TIR of mRNA. In one case, the target site was identified at the junction between the coding region and 3' UTR. Interestingly, when a target site upstream of TIR was recognized by antisense RNA, gene expression was activated via induction of increased cellular mRNA levels. Our results suggest that antisense RNAs physiologically behave as sRNAs in the cell, by not only repressing but also activating the gene expressions, supporting their utility as gene enhancers as well as silencers.

MATERIALS AND METHODS

Bacterial strains, plasmids, and oligonucleotides

The *E. coli* K-12 strain, DH5α, was used for plasmid construction. A previously constructed lysogen containing an *ssrS::lacZ* transcriptional fusion was used in assays to evaluate the effects of antisense RNAs (24).

The pHM-tac plasmid, an IPTG-inducible vector (24), was used for RNA expression. RNA-coding sequences were amplified using PCR, and were cloned into the *EcoR*I/*Xba*I sites of pHM-tac for generating artificial double stem-loop small RNAs (ARdSLRNAs). Each ARdSLRNA carried the P1 stem of M1 RNA at the 5' end and the *sibC* terminator. The primers or oligonucleotides employed are listed in Supplementary Table 1.

Design of antisense RNAs

Antisense RNAs complementary to the fused *lacZ* mRNA sequence with a length of 20 nt, mimicking the length of eukaryotic siRNA, were selected utilizing siRNA selection web programs (33-35). Web-based siRNA search tools can be used to effectively select antisense RNAs through consideration of the thermodynamic stability of binding between sRNA and mRNA and the accessibility of target mRNA, using the Mfold algorithm (36). Each selected antisense RNA sequence was expressed in *E. coli* as RNA species, embedded between the *rnpB* P1 and *sibC* terminator stem loops.

β-Galactosidase assay

Three colonies for each strain were pooled and grown overnight in LB containing ampicillin (50 μg/ml). After 1:100 dilution of overnight culture in fresh LB, cells were grown at 37°C for 2 h in the presence of IPTG. Relative β-galactosidase activities were determined, as described previously (37). At least three independent measurements were performed for each strain.

Northern blot analysis

Total cellular RNA was prepared from the same cultures used for β-galactosidase assays with hot phenol extraction, as described previously (18). The RNA samples were separated on a 5% polyacrylamide gel containing 7 M urea for sRNA or 1.0% agarose gel for *lacZ* mRNA, followed by electrotransfer to a Hybond N+ membrane (Amersham Biosciences). Oligonucleotides were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase (Takara), and the labeled oligonucleotides, ARlacZ+74R, lacZ+512R, and 5S+90R (Supplementary Table 1) were used for probing antisense RNA species, *lacZ* mRNA and 5S RNA, respectively. Subsequently, hybridization was performed according to the manufacturer's instructions. Membranes were visualized and quantified using Image Analyzer FLA 7000 (Fuji).

RNA stability assay

RNA stability assay was performed as described previously (38).

Briefly, cells cultured overnight were diluted (1:100) into fresh medium and were grown at 37°C for 2 h in the presence of 1 mM IPTG. Rifampicin (39) was added to the culture at a final concentration of 250 μg/ml to terminate further transcription. Cultures were obtained at different time intervals, and the total RNA prepared were subjected to Northern blot analysis.

SUPPLEMENTARY DATA

Supplementary Data are available at BMB Reports online: Supplementary Table 1.

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