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

Hongmarn Park¹, Yeongseong Yoon², Shinae Suk¹, Ji Young Lee¹ & Younghoon Lee¹,*

¹Department of Chemistry, KAIST, Daejeon 305-701, ²Korea Science Academy of KAIST, Busan 614-822, Korea

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.

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

Keywords: Artificial RNA, Antisense RNA, Double stem-loop, Enhancer, Up-regulation
Effects of different target sites on antisense RNA-mediated regulation of gene expression
Hongmarn Park, et al.

Fig. 1. Antisense RNAs and their effects on gene expression. (A) Secondary structure model of ARdSL6 RNA using the Mfold algorithm (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.

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, coding region, and 3' UTR of the fused lacZ mRNA are shown. The transcription start site, Shine-Dalgarno sequence (SD), 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.
Effects of different target sites on antisense RNA-mediated regulation of gene expression
Hongmarn Park, et al.

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 counter-part 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 ARdSL RNA 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.

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 EcoRI/Xbal 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 MiFold algorithm (36). Each selected antisense RNA sequence was expressed in *E. coli* as RNA species, embedded between the mpB 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 electrophoresis to a Hybond N+ membrane (Amersham Biosciences). Oligonucleotides were labeled with [γ-32P]ATP and T4 polynucleotide kinase (Takara), and the labeled oligonucleotides, ARlacZ+74R, lacZ+512R, and 55+90R (Supplementary Table 1) were used for probing antisense RNA species, lacZ mRNA and SS 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.

ACKNOWLEDGEMENTS

This study was supported by the National Research Foundation of Korea (NRF) Grant by the Korea government (MEST) (2010-0029167; 2011-0020322) and the Intelligent Foundation of Korea (NRF) Grant by the Korea government.
27. Nakashima, N., Tamura, T. and Good, L. (2006) Paired termini stabilize antisense RNAs and enhance conditional gene silencing in Escherichia coli. Nucleic Acids Res. 34, e138.

28. Morita, T. and Aiba, H. (2011) RNase E action at a distance: degradation of target mRNAs mediated by an Hfq-binding small RNA in bacteria. Genes Dev. 25, 294-298.

29. Georg, J. and Hess, W. R. (2011) cis-antisense RNA, another level of gene regulation in bacteria. Microbiol. Mol. Biol. Rev. 75, 286-300.

30. Thomason, M. K., Fontaine, F., De Lay, N. and Storz, G. (2012) A small RNA that regulates motility and biofilm formation in response to changes in nutrient availability in Escherichia coli. Mol. Microbiol. 84, 17-35.

31. Opdyke, J. A., Kang, J. G. and Storz, G. (2004) GadY, a small-RNA regulator of acid response genes in Escherichia coli. J. Bacteriol. 186, 6698-6705.

32. Opdyke, J. A., Fozo, E. M., Hemm, M. R. and Storz, G. (2011) RNase III participates in GadY-dependent cleavage of the gadX-gadW mRNA. J. Mol. Biol. 406, 29-43.

33. Thakur, N., Qureshi, A. and Kumar, M. (2011) VIRsiRNAdb: a curated database of experimentally validated viral siRNA/shRNA. Nucleic Acids Res. 40, D230-D236.

34. Matveeva, O., Nechipurenko, Y., Rossi, L., Moore, B., Saetrom, P., Ogurtsov, A. Y., Atkins, J. F. and Shabalina, S. A. (2007) Comparison of approaches for rational siRNA design leading to a new efficient and transparent method. Nucleic Acids Res. 35, e63.

35. Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W. S. and Khvorova, A. (2004) Rational siRNA design for RNA interference. Nat. Biotechnol. 22, 326-330.

36. Zuker, M. (2003) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31, 3406-3415.

37. Zhang, X. and Bremer, H. (1995) Control of the Escherichia coli rrnB P1 promoter strength by ppGpp. J. Biol. Chem. 270, 11181-11189.

38. Pedersen, S. and Reeh, S. (1978) Functional mRNA half lives in E. coli. Mol. Gen. Genet. 166, 329-336.

39. Calvori, C., Frontali, L., Leoni, L. and Tecce, G. (1965) Effect of rifamycin on protein synthesis. Nature 207, 417-418.