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Activation of prokaryotic translation by antisense oligonucleotides binding to coding region of mRNA

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ABSTRACT

A few examples of translational activation by antisense small noncoding RNAs (sRNAs) have already been discovered in prokaryotic cells, and all of them are through a sense–antisense interaction at the 5′-untranslated region (5′-UTR) of target mRNAs. Here, we report a novel phenomenon of translational activation of prokaryotic gene expression with *trans*-acting antisense oligonucleotides targeting the coding region of mRNA. Screening of antisense oligonucleotides complementary to the coding sequences of GFP or ZsGreen identified antisense sequences that activate translation of the mRNAs in a concentration-dependent manner. We also found that the translational activation highly depends on the hybridization positions of the antisense strands. Translation-activating antisense oligonucleotides (TAOs) tended to bind to the 5′-region rather than the 3′-region of the mRNA coding region. RNA folding simulation suggested that TAOs may disrupt the structured elements around the translation initiation region (TIR) by pairing with complementary sequences in the mRNA coding region, resulting in an increase in translation efficiency. Further, we demonstrate that number and position of locked nucleic acid (LNA) bases in the antisense strands govern the tendency of up- or down-regulation. Our findings described here may lead to the discovery of a new class of antisense sRNA and the development of a tool for activating desired gene expression in the future.

1. Introduction

Translation initiation is thought to be a rate-limiting step that determines the total efficiency of translation [1,2]. In the mechanism of prokaryotic translation initiation, TIR of mRNA first contacts with a small ribosomal subunit coupled to initiation factor 3 (pre-30S initiation complex (IC)), and then fMet-tRNA, initiation factors 1 and 2 assemble to the pre-30S IC to form the 30S initiation complex [3–5]. However, some nascent mRNAs are translationally inert because of their highly structured elements in the vicinity of TIR, and to become translated, they require subsequent refolding induced by environmental cues such as small metabolites [6–8], regulatory proteins [9], and sRNAs [9–11]. These *trans*-acting ligands most often bind to 5′-UTR, which has been taken into account as a regulatory element of gene expression, and alters the secondary structure of TIR, leading translational modulation. Thus, when a certain gene is positively or negatively regulated at translation level, alteration of the secondary and higher-order structure of TIR by some *trans*-acting molecules such as antisense nucleic acids is one of the most important events during the whole translation process.

Compared with repression mechanisms, the translational activation mechanism by antisense nucleic acids is less well understood. All of the previously reported sRNAs that activate mRNA translation target a 5′-UTR [12]. For example, RNAIII, which is the first sRNA to activate gene expression, disrupts an inhibitory hairpin structure around TIR by pairing to a 5′-UTR, and releasing an SD sequence, resulting in activation of translation [13]. *Escherichia coli* sRNAs, DsrA, and RprA interact with the same self-inhibitory hairpin in the 5′-UTR of the *rpoS* mRNA, facilitate the ribosome entry to TIR, and cause translational activation [14–18]. A translational activation mechanism via antisense nucleic acids binding to an mRNA coding region has not yet been discovered in living organisms. On the other hand, the involvement of an mRNA coding region in translation efficiency has been indicated [19], although the coding region had not been thought to engage in modulation of gene expression. The authors constructed a GFP gene library that varied randomly at synonymous sites, but all encoded the same amino acid sequences, and demonstrated that mRNA having weak 5′ coding sequence (5′-CDS) structure exhibits higher expression levels, suggesting that the mRNA structure of 5′-CDS can serve as a regulatory element in translational regulation as is the case for 5′-UTR.

Accordingly, we hypothesize that antisense oligonucleotides binding to an mRNA coding region could induce activation of

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translation by unfolding a stabilized structure around a TIR. In this study, coding region, we screened antisense single-stranded DNAs (ssDNAs) that have complementarity to the coding region of target mRNA in a cell-free translation system derived from *E. coli*, and identified several translation-activating antisense oligonucleotides (TAOs), which facilitate translation of their target mRNA. The characteristics of TAOs such as the length and binding positions were also investigated. The secondary structure prediction of mRNA suggests that TAOs could bind to inhibitory hairpin structure and alter the RNA conformation so that translation initiation proceeds more efficiently. In addition, the use of locked nucleic acid (LNA)-containing antisense oligonucleotides suggests that their affinity to the target mRNA should be moderate so that ribosome can peel off the antisense strands by its helicase activity.

2. Materials and methods

2.1. Preparation of DNA and RNA templates

Plasmids, pRSET (Invitrogen, Carlsbad, CA, USA) and pZsGreen (Clontech, Palo Alto, CA, USA) including GFP and ZsGreen gene, respectively, were purchased. From these plasmids, we amplified three linear DNA templates, GFPt, tagGFPt, and ZsGt by PCR for in vitro translation.

A primer pair, GFPt-F/GFP-R with pRSET plasmid pair yields GFPt containing only a GFP gene in the coding region. A tagGFPt-F/GFP-R pair with pRSET plasmid provides tagGFPt, which contains 6-His-tag, Xpress epitope, and EK cleavage site in the upstream of GFP gene. A ZsG-F/ZsG-R pair was used to amplify ZsG, a DNA template containing the ZsGreen gene in its coding region. Obtained PCR products were identified by agarose gel electrophoresis and then purified by isopropanol precipitation. Preparation of mRNA templates was performed by in vitro transcription using an Ampliscribe T7 Transcription Kit (Epicentre, Madison, WI, USA) according to the manufacturer's instructions. The transcripts were identified by agarose gel and purified by gel filtration on Microspin G-25 columns (GE Healthcare, UK).

2.2. Antisense oligonucleotides

All antisense DNA oligonucleotides were purchased from Invitrogen or JBioS (Saitama, Japan). LNA-containing oligonucleotides were purchased from Gene Design (Osaka, Japan). The sequences were designed to bind to a single site on the target mRNA by using Amplify 3X software. That is, the sequences that showed a possibility of hybridizing at more than two sites on target mRNA were excluded.

2.3. In vitro translation experiments and fluorescence measurements

A reconstituted cell-free translation system, PURE system (Fig. 1B, 2A, S1, and S2) and PURExpress (Fig. 1C, 2C, 2D, 3B, 3C, and S4) were purchased from the Post Genome Institute (Tokyo, Japan) and NEB, respectively. A total volume of 12 μl (50 μl for ZsGreen expression) of reaction mixture containing prepared DNA template (10 or 50 nM) or RNA template (1 μM), each antisense oligonucleotide (20 μM), and PURE system was incubated at 37 °C for 3 h. Fluorescence measurements of each reaction solution (10 or 45 μl) were performed using a fluorescence plate reader, Mithras LB 940 (Berthold Technologies, Bad Wildbad, Germany); excitation 485 nm, emission 535 nm, counting time 0.1 s, and lamp energy 7000.

2.4. RNA secondary structure prediction

RNA secondary structures of minimal free energy were predicted with mfold software (version 3.2) [20]. Truncated mRNAs (100, 150, 200, 250, and 300 mer) of GFPt, tagGFPt, or ZsGt were simulated. Illustrations of RNA structures were compiled by using Adobe Illustrator CS3.

2.5. RT-PCR with antisense ssDNAs

Full-length GFPt mRNA was reverse transcribed using antisense ssDNA, G4, G5, G8, or G10 as a primer. Reaction mixtures (10 μl) containing 5 units of AMV reverse transcriptase (Promega, Madison, USA), GFPt mRNA (1 μM), and each antisense ssDNA (20 μM) were incubated at 37 °C for 30 min and then heated at 99 °C for 5 min. The obtained solutions (0.5 μl) were mixed with PCR solution containing ExTaq (TaKaRa, Japan), forward primer, 5′-GGGA-GACCACAACGGTTTCC-3′, reverse primer (antisense). The obtained PCR products were analyzed by 8% PAGE.

3. Results

3.1. Screening of antisense oligonucleotides targeting mRNA coding region

Prior to screening of antisense ssDNAs, we designed and prepared two GFP-encoding DNA templates, GFPt, and tagGFPt (Fig. 1A). GFP gene was selected as a model gene because of its availability for direct and quantitative analysis of expression levels after in vitro translation. Whereas GFPt only encodes the GFP gene in the coding region, tagGFPt encodes an additional peptide tag sequence (40-aa) upstream of the GFP gene, which confers different mRNA structure around TIR from GFPt mRNA. Differences in the mRNA secondary structure should confer different stabilities and then translation initiation efficiencies. In fact, a preliminary in vitro translation experiment showed different absolute expression levels between these two templates; the absolute expression level of tagGFPt was approximately twofold higher than that of GFPt (data not shown). A cell-free reconstituted translation system, PURE system [21], which consists of about 30 purified enzymes necessary for transcription, translation and energy recycling was used throughout all in vitro gene expression experiments. The GFP expression levels were quantified by the fluorescence of each reaction solution. Sequences and hybridization positions of screened antisense ssDNAs are shown in Table 1. These sequences were selected so that each antisense strand binds to only a single site on the target mRNA CDS.

To confirm our hypothesis that mRNA coding regions participate in translational regulation involving antisense nucleic acids, we screened 15-mer antisense ssDNAs that bind to GFPt and tagGFPt mRNA coding regions. The screening revealed the existence of translation-activating antisense oligonucleotides (TAOs) that showed increased levels of gene expression (Fig. 1B). Downregulated gene expression with G4 in GFPt and G1 in tagGFPt is reasonable because G4 and G1 are designed to bind to the AUG start codon, producing a conventional antisense inhibition mechanism. Activated GFP levels were observed for TAOs binding to the region of mRNA upstream of the coding region rather than those binding downstream. This result is consistent with the fact that determinants of gene expression, namely regulatory RNA sequences are usually found in the upstream region of the mRNA coding region [19]. Furthermore, the antisense ssDNAs such as G4, G5, G6, G8, G9, and G10 activate GFPt expression, but not tagGFPt expression, although these ssDNAs can also hybridize to the upstream region of the coding region of tagGFPt mRNA (Fig. 1B). These screenings suggest that antisense strands targeting the mRNA coding region are able to up-regulate gene expression of their specific targets only when hybridization occurs at the appropriate position on mRNA.

There are a few examples of transcriptional activation in mammalian cells by promoter-targeted double-stranded RNAs that

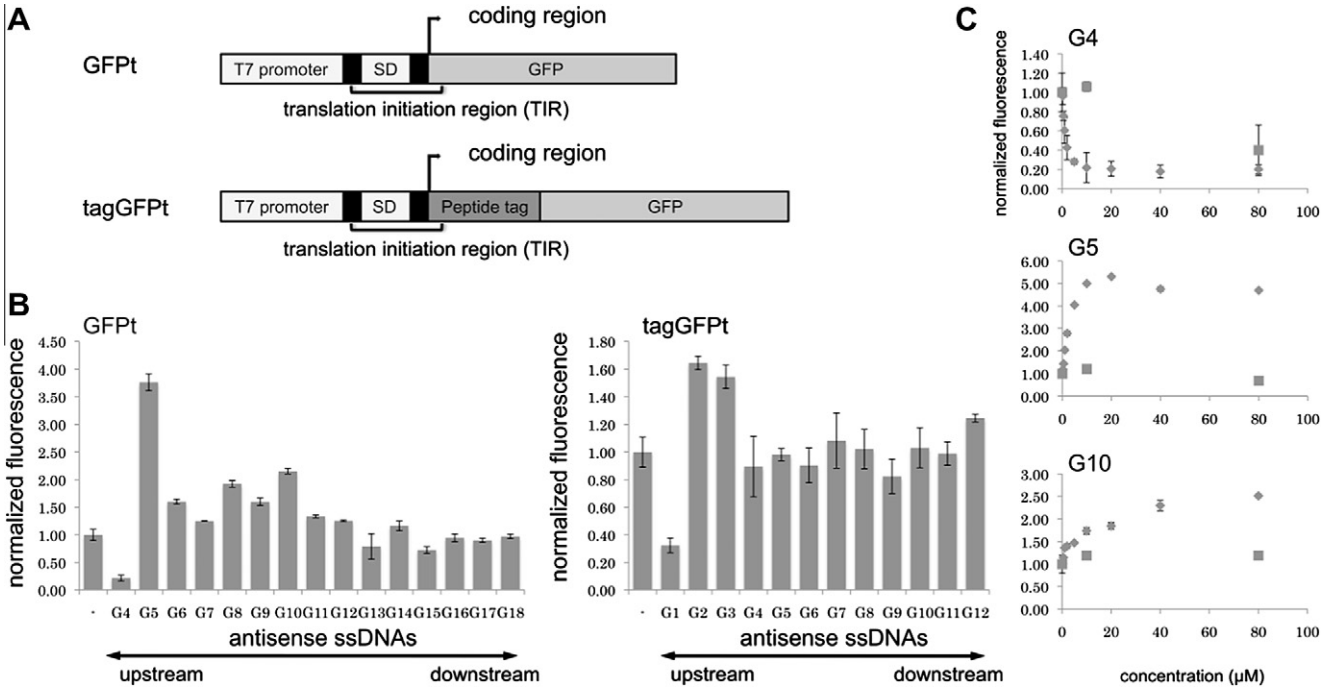

Fig. 1. Screening of antisense ssDNAs binding to the mRNA coding region. (A) Structures of two GFP-encoded DNA templates used in this study. GFPt contains only GFP gene sequence in the coding region, while tagGFPt has an additional peptide tag sequence upstream of the GFP gene. (B) Expression levels in in vitro gene expression experiments with GFPt and tagGFPt in the absence (–) and presence of antisense strands. Antisense ssDNAs are lined according to the hybridization position from upstream to downstream. Sequences and hybridization positions of antisense ssDNAs are summarized in Table 1. (C) Concentration dependency of the antisense strand G4, G5, and G10 in the expression of GFPt. Fluorescence measurements were performed in antisense concentrations of 0, 0.2, 0.5, 1.0, 2.0, 5.0, 10, 20, 40, and 80 μM (diamond). Scrambled controls were measured at 10 and 80 μM (square). The normalized fluorescence intensities were estimated by dividing each value by the value in the absence of antisense strand. The data indicate the mean value in each sample ($n = 3$). The error bars show the standard deviations obtained for three independent samples.

contain antisense sequence, although the mechanism is not clearly elucidated [22,23]. To demonstrate that antisense ssDNAs used in this study do not affect transcription, but do effect translation, we performed other in vitro translation experiments using pretranscribed mRNA as a template. Compared with the first screening, in which transcription and translation were coupled in the reaction solution, homologous expression patterns were observed (Fig. S1). These results confirm that TAOs found in our screening up-regulate the translation process, but not the transcription process.

We then looked for the TAOs toward another fluorescent protein gene, ZsGreen, which originates from the coral reef, Zoanthus [24,25], and has a nonhomologous amino acid sequence as compared with that of GFP. Thus, the secondary structure of ZsGreen mRNA is different from that of GFP. We examined several antisense ssDNAs complementary to the 5′-region of ZsGreen mRNA CDS (Table S1) with PURE system and identified several TAOs (Fig. S2). It is notable that, despite the fact that the two fluorescent genes used in this study are well optimized for reporter expression experiments, additional translational activation is achievable.

To confirm whether TAOs hybridize at the desired positions on target mRNA, we performed RT–PCR experiments in which each of the antisense ssDNAs G4, G5, G8, and G10 were used as primers of reverse transcription on GFPt mRNA. When these strands bind to proper sites, amplified products with G4, G5, G8, and G10 yield 78, 90, 167, and 242 bp dsDNA, respectively. Each RT–PCR product was observed at its expected position upon PAGE analysis (Fig. S3). These results confirmed that obtained TAOs function at the proper locations on their target mRNAs.

3.2. Characterization of translational activation by antisense oligonucleotides

Taking advantage of the cell-free translation system, we attempted to characterize the behavior of the translational activation induced by TAOs. First, GFPt expression levels were monitored with different concentrations of inhibitory antisense G4 and two TAOs, G5 and G10 (Fig. 1C). As the concentration of inhibitory antisense G4 increased, the expression level decreased, indicating that all transcribed mRNAs in the reaction mixture were masked by high concentrations of G4, leading to complete silencing of the translation. In contrast, an increase of the concentration of G5 and G10 resulted in increased expression levels. The requirement for an excess amount of antisense ssDNA (>10 μM) against transcribed mRNA (approximately 0.1–1.0 μM) deserves comment because formation of a full-matched 15-bp duplex should occur in the presence of equivalent amounts of complementary strands. This apparently low affinity of antisense strands to the target mRNA could be due to the competitive binding of antisense ssDNA to other mRNA sequences. We investigated GFP expression levels with mutated antisense ssDNAs, 1–4 M, which were designed to produce one–four mismatches when hybridized with the target sequence of G5 (Fig. 2A). As the number of mutations in antisense ssDNAs increased, the expression levels decreased and reached the same levels as in the absence of antisense ssDNAs. Higher concentration of 1 M (40 and 80 μM) became close to the fluorescence level of G5 (Fig. S4A), suggesting that the effect of 1 M was not saturated at 20 μM probably because of lower affinity to mRNA caused by single mutation. On the other hand, 2–4 M did not show enhancing behavior in the higher concentration.

The target sequence of the most effective TAO, G5, is located in CDS close to TIR where translational repression upon hybridization by antisense strands occurs. We investigated the boundary between activation and repression of translation by systematic coverage with antisense strands. Fifteen-mer antisense ssDNAs, G5−6, G5−5, G5−4, G5−3, G5−2, G5−1, G5, G5+1, G5+2, G5+3 and G5 + 4 (identical to G6), which are designed to hybridize from the nucleotide number 70 to 80 of GFPt mRNA, respectively, were used in GFPt translation experiments (Fig. 2B). As the hybridization

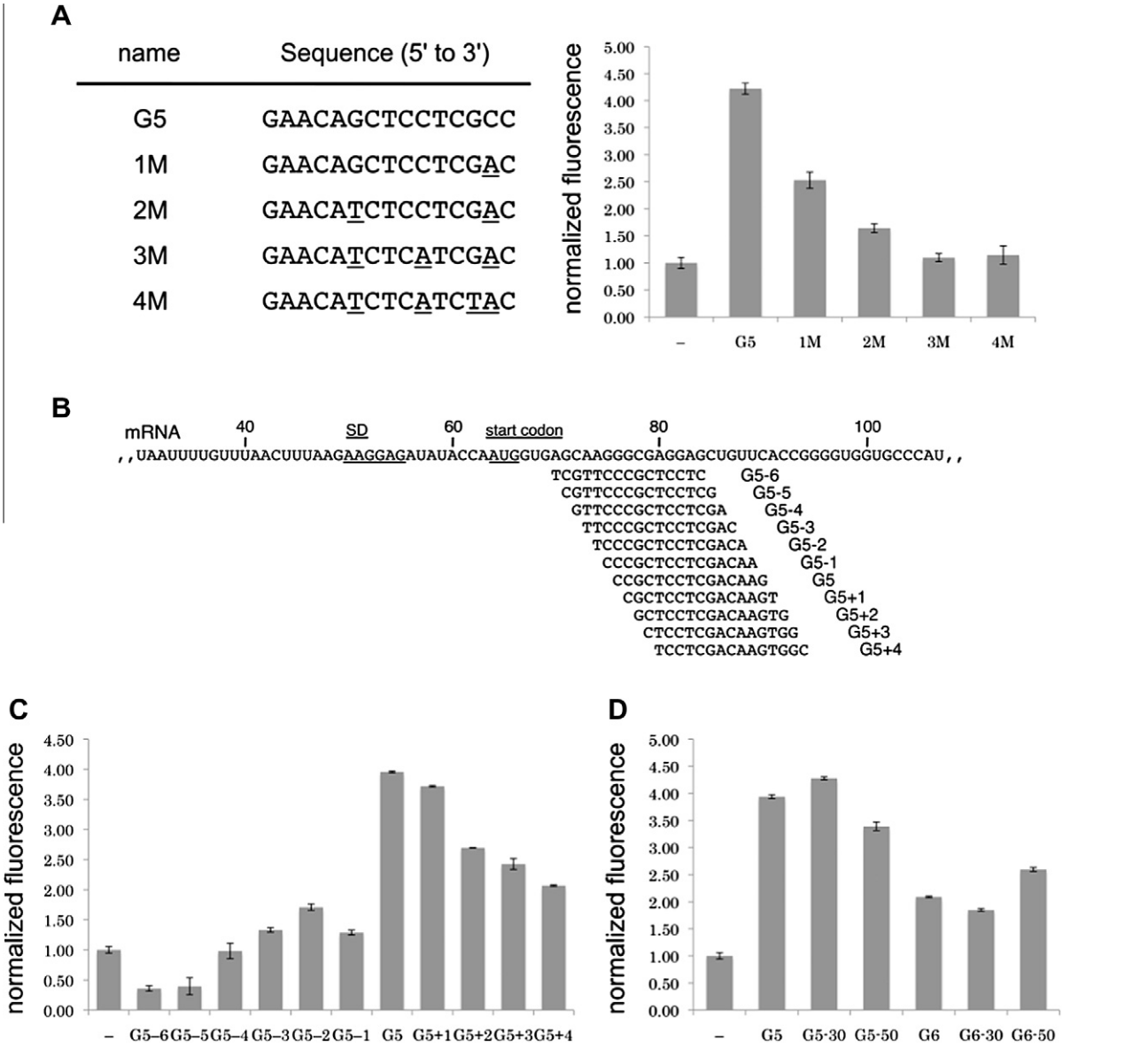

Fig. 2. Characteristics of TAOs. (A) Effects of mismatch bases introduced to antisense ssDNA G5. Antisense ssDNAs used in this experiment were shown. Underlined characters indicate mutated bases. (B) Illustration of mRNA sequence around TIR and hybridizing antisense strand sequences. (C) Effects of single base shifting of antisense ssDNAs hybridization and longer antisense sequences on GFPt expression. (D) GFP expression profiles of longer antisense. The normalized fluorescence intensities were estimated by dividing each value by the value in the absence of the antisense strand. The data indicate the mean value in each sample ($n = 3$). Error bars show the standard deviations obtained for three independent samples.

site of antisense strands is becoming close to the AUG start codon, expression levels of GFP decreased (Fig. 2C), indicating the presence of boundary between the region required for forming initiation complex and downstream CDS that ribosome goes through at the elongation step. Given that mRNA enters ribosome around position +13–15 in the initiation step [26], it is reasonable that G5 (hybridizing from +13 to +27) has potent enhancing activity whereas few upstream binding sequences have activating function probably due to failure of mRNA accommodation into ribosome. Additionally, increased concentration of G5−1 and G5−2 didn't affect the fluorescence level (Fig. S4B).

To investigate the relationship between the affinity of TAOs and the degree of activation, we prepared antisense sequences with different length. Based on the sequence of G5 and G6 (15 mer), 4 another antisense strands, G5−30, G6−30 (30 mer), G5−50 and G5−50 (50 mer) were designed to extend the hybridizing region to the downstream of mRNA. Activating effects of longer antisense molecules were not clearly different (Fig. 2D) indicating that mRNA structure of 5′ region sequestered by antisense strands, rather than the affinity of antisense, could be responsible for the degree of effect.

By using mfold for the simulation of RNA secondary structures [20], we attempted to predict the molecular mechanism of TAO activation with 3′-truncated mRNA sequences. The antisense target sequences of TAOs were found in the stem region that appears to diminish the efficiency of ribosomal initiation complex formation (Fig. S5). Therefore, it seems reasonable that G5 and G10 activate translation by disrupting stable intramolecular structured elements interfering with the formation of a translation initiation complex. G2 and Z11, which upregulate translation of GFPtag and ZsGreen mRNA, respectively, were also shown to bind similar stem structures (Fig. S6). However, as we have shown in Fig. 2C, a single or a few bases shifting of antisense binding provided substantial differences in expression levels. This could be explained

Table 1
Antisense ssDNAs used in this study. We use the mRNA numbering in which the first transcribed nucleotide is defined as position 1 and downstream nucleotides are numbered accordingly. Numbers shown in the "position" column indicate first and last nucleoside numbers to which each antisense strand binds.

Antisense ssDNA	Sequence (5′–3′)	Position (GFPt)	Position (tagGFPt)
G1	ATGAGAACCCCGCAT	No	64–78
G2	CGACCCATTTGCTGT	No	117–131
G3	GTACAGATCCCGACC	No	126–140
G4	GCCCTTGCTCACCAT	64–78	184–192
G5	GAACAGCTCCTCGCC	76–90	196–210
G6	CGGTGAACAGCTCCT	80–94	200–214
G7	ACTTGTGGCCGTTTA	131–145	251–265
G8	TCGCCCTCGCCGGAC	153–167	273–287
G9	GAACTTCAGGGTCAG	190–204	310–324
G10	GTGGGCCAGGGCACG	228–242	348–362
G11	GTAGGTCAAGGTGGT	250–264	370–384
G12	CTGCTTCATGTGGTC	292–304	412–426
G13	TAGCCTTCGGGCATG	327–341	447–461
G14	TTGAAGAAGATGGTG	354–368	474–488
G15	GTCTTGTAGTTGCCG	375–389	495–509
G16	GATGCGGTTCACCAG	421–435	541–555
G17	GGTGATATAGACCTT	511–525	631–645
G18	GTCCTCGATGTTGTG	571–585	691–705

by the consideration that translation initiation efficiency should depend on the 5′ region of mRNA sequestered by each antisense strand after it unwound the intrinsic mRNA structure.

Further, we investigated chemically modified antisense oligonucleotides containing LNA, which has higher affinity to the target mRNA than DNA or RNA antisense [27,28]. Fifteen-mer oligonucleotides composed of 8 LNA nucleotides were designed based on G5, G6, G8, and G10 (Fig. 3A). All of LNA-containing nucleotides showed inhibition of GFP expression (Fig. 3B), indicating that the interaction between antisense and mRNA would give insurmountable barrier for translating ribosome in elongation reaction (steric block). Then, we prepared 15-mer oligonucleotides with different number of LNA on their sequence to observe a boundary between up- and down-regulation. The antisense strands containing 2 LNA nucleotides (G5L2a and G5L2b) showed higher activation than original G5, while antisenses having 8 or 15 (all LNA) LNA nucleotides showed inhibition effect (Fig. 3C). Interestingly, antisenses including 4 LNA nucleotides showed both up- and down-regulation depending on the position of LNA. These results suggests that there might be a threshold of ribosome helicase activity, and only the antisense strands that don't have excess affinity to mRNA might have a chance to activate translation. And threshold of ribosome helicase activity seems to be provided by not total affinity (Tm) of antisense strand but partial affinity of the antisense molecules.

4. Discussion

The prokaryotic translation initiation efficiency highly depends on the complexity of mRNA structure around TIR. The recognition of structured mRNAs by a 30S ribosomal subunit can be divided into three phases: the docking of the folded mRNA onto the platform domain of a ribosome, the unfolding of a structured element, and the adaptation of mRNA into an mRNA path in a 30S subunit [29]. The delay time between docking and unfolding reflects the stability of the inhibitory hairpin structure. TAOs found in our study might relax the structured elements by hybridization from

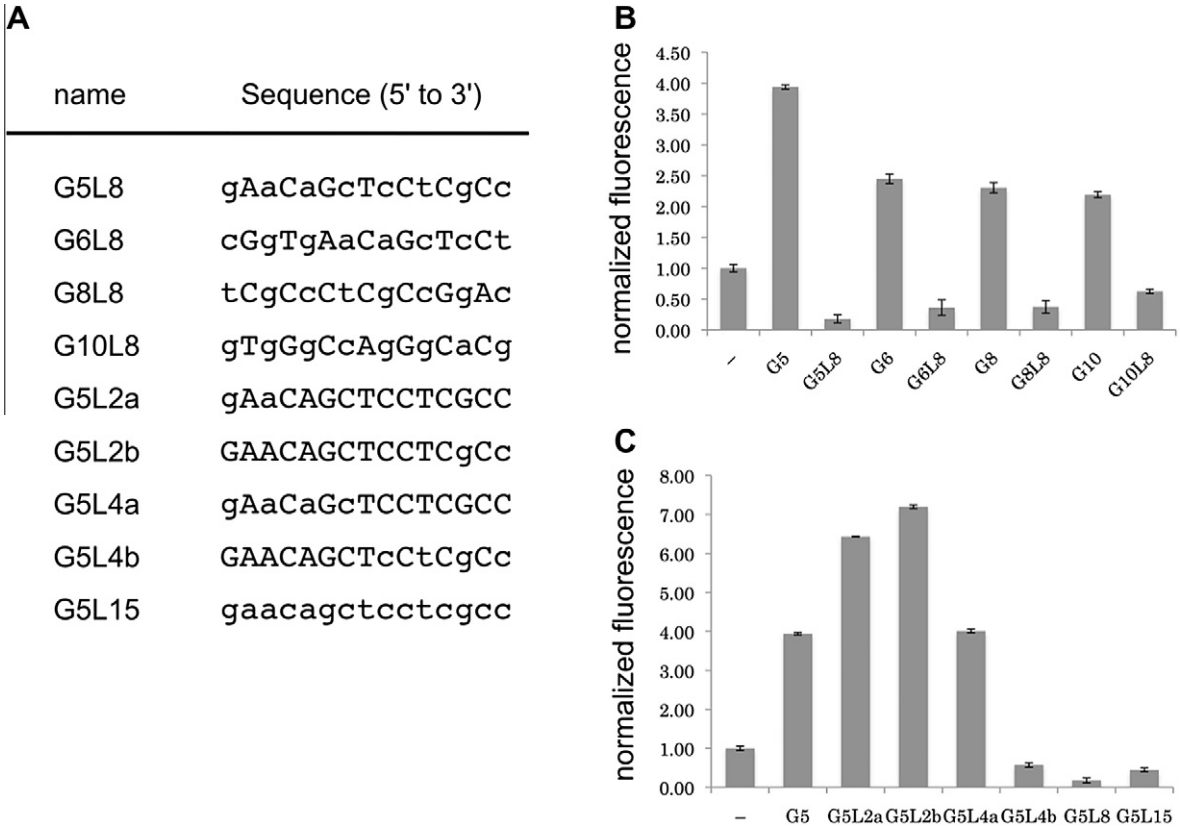

Fig. 3. Translational regulation by LNA-containing antisense oligonucleotides. (A) LNA-containing antisense sequences used in this study. Lower case, LNA monomers; Upper case, DNA monomers. (B) Antisense strands which have 8 LNA monomers alternately on their 15-mer bodies were used in the GFPt expression. (C) G5 sequences substituted by 2, 4, 8, or 15 LNA monomers were added to the reaction solutions. The normalized fluorescence intensities were estimated by dividing each value by the value in the absence of an antisense strand. The data indicate the mean value in each sample ($n = 3$). The error bars show the standard deviations obtained for three independent samples.

outside of TIR (indirect effect), while conventional repressive antisense ssDNAs decrease translation by forming another duplex structure around the SD or AUG start codon, or both.

Screening of 15-mer antisense ssDNAs that bind to CDS did not give the translation-inhibitory sequences. In the elongation step, ribosome needs to move on various mRNA sequences that may form extremely stable secondary structures. This is achieved by the RNA helicase activity of ribosomes that unwinds stable mRNA secondary structures during elongation [30]. Therefore, after antisense strands that bind to mRNA coding region facilitate the translation efficiency in the initiation step, hybridizing strands should be kicked off by a translating ribosome. On the other hand, antisense strands containing more than 4 LNA nucleotides caused significant inhibition of translation (Fig. 3). This is probably because steric block effect, which causes ribosomal elongation arrest, predominated over ribosome helicase ability, suggesting that TAOs need to have an appropriate affinity to the target mRNA to be peeled off by translating ribosome.

In conclusion, we were able to present a novel approach to activation of translation based on antisense nucleic acids complementary to 5′-CDS. Because a large number of sRNAs, whose function is unknown, have been discovered, some sRNAs might function using this mechanism. Moreover, by predicting more mRNAs whose expression levels are originally low because of their strict mRNA structures around TIR, we could be able to develop a technology that enables us to increase production of a target protein. Future studies on translational regulation should be conducted in view of the importance of the mRNA coding region as a regulatory element.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.10.072.

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