Regulation of inducible gene expression by natural antisense transcripts

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1. ABSTRACT

Natural antisense transcripts are frequently transcribed from many genes in eukaryotes. Although natural antisense transcripts have been recognized for a long time, their importance has been overlooked due to their heterogeneity, low expression level, and unknown function. Genes induced in responses to various external stimuli are transcriptionally regulated by the activation of a gene promoter and post-transcriptionally regulated by controlling mRNA stability and translatability. Recent studies have shed light on the functions of natural antisense transcripts at the post-transcriptional level. An antisense transcript may regulate gene expression with *cis*-controlling elements on the mRNA, and the antisense transcript itself may act in concert with *trans*-acting factors, including various proteins that bind to *cis*-controlling elements, drugs, and microRNAs. A novel mechanism recently reported to regulate mRNA stability includes the interaction of the antisense transcript with mRNA by hybridization to single-stranded loops in secondary structures. This antisense transcript-mediated post-transcriptional regulation may be one of the general mechanisms for the regulation of inducible gene expression and presents the possibility of the involvement of natural antisense transcripts in disease.

2. INTRODUCTION

The '*sense*' sequence of a gene is defined as the sequence of messenger RNA (mRNA) that encodes a protein, while the '*antisense*' (AS) sequence is complementary to the sense sequence. Thus, a gene (double-stranded DNA) consists of a sense strand that encodes mRNA and the complementary AS strand. A '*natural AS transcript*' is an endogenous RNA that derives from the sequence of the AS strand of a gene. The transcript is called an '*antisense RNA*' (asRNA). Precisely speaking, the complementary strand of DNA is used as the template for synthesis of a transcript by RNA polymerase during transcription. The AS strand is used as the template for mRNA and the sense strand is used for the AS transcript. However, for simplicity, the synthesis of AS transcripts is often expressed as "antisense transcription" (1).

To date, many natural AS transcripts have been reported in eukaryotes, but their functions have been thus far unknown (2). For example, an AS transcript is transcribed from the *hypoxia-induced factor (HIF) 1alpha* gene (3). The expression level of the HIF-1alpha AS transcript has been shown to be reciprocal to that of the HIF-1alpha mRNA (4); therefore, the function of this AS transcript has been unclear. Recently, genome-wide transcriptome analyses have indicated that the AS strands of many mammalian genes are frequently transcribed (1, 5).

Natural AS transcripts are often classified as noncoding RNA (ncRNA) because many of them do not encode proteins or are able to encode only short peptides. The ncRNAs include various types of RNA species that do not encode proteins but have defined functions, such as ribosomal RNA (rRNA), transfer RNA (tRNA), small

nuclear RNA (snRNA), and small nucleolar RNA (snoRNA). However, many ncRNAs outside of these classical ncRNA species are considered 'transcripts of unknown function' (TUFs) (6). Among the heterologous TUFs found is microRNAs (miRNAs), which are small RNA molecules (of approximately 21 nucleotides) that regulate translation and interfere with mRNA (7). Several studies have demonstrated that an unexpectedly large number of ncRNAs and AS transcripts are transcribed from the human and mouse genomes (1, 8, 9, 10). It has also been suggested that AS transcripts may regulate gene expression at the post-transcriptional or translational levels (1, 5). The importance of AS transcripts, however, has been overlooked due to their low expression level and high heterogeneity, as well as the difficulty of performing the necessary functional studies.

Several recent studies have shed light on the functions of natural AS transcripts. In this review, we focus on the effects of natural AS transcripts on the expression of inducible genes and particularly on their ability to regulate mRNA stability. We also propose a model for AS transcript-mediated regulation of gene expression.

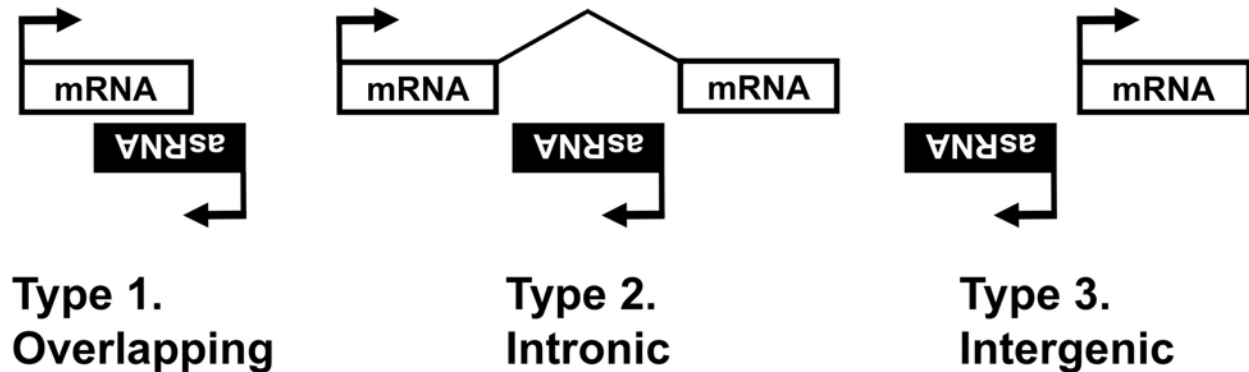# 3. CHARACTERIZATION OF NATURAL ANTISENSE (AS) TRANSCRIPTS

3.1. Classification of natural AS transcripts
3.1.1. Transcription initiation sites

The sizes and features of natural AS transcripts are variable. They are classified by several characteristics, such as variety of transcriptional start sites, splicing, and modifications including cap structure and polyadenylation. Many AS transcripts do not encode proteins and are ncRNAs. In regard to transcriptional initiation sites, AS transcripts are classified into three types: (1) *overlapping*, (2) *intronic*, and (3) *intergenic* AS transcription start sites (9, 11) (Figure 1). In the case of type 1, the sequence of the AS transcript partly overlaps an mRNA sequence, particularly at the 3'-untranslated region (3'UTR) of an mRNA in many genes (12, 13). Because the overlapping sequences are complementary, these regions may mutually interact by complete or partial hybridization; thus, the AS transcript may function to regulate the expression of the overlapped mRNA. In human and mouse transcripts, it has been reported that AS transcripts have a markedly preferential complementarity to mRNA 3'UTRs (14). For example, in the *eukaryotic initiation factor 2alpha (eIF-2alpha)* gene, AS transcription starts at intron 1 and overlaps the mRNA (15). In this review, we focus on the pairs of mRNA and this overlapping type of AS transcript, 'Type 1 AS transcripts'. Because AS transcripts are designated differently for each gene, Type 1 AS transcripts that overlap an mRNA of a gene are designated as 'asRNA$_{Gene}$' in this review. If a specific name was used in the original paper, it is indicated in parentheses. For example, the AS transcript of the *HIF-1alpha* gene (3) is expressed as 'asRNA$_{HIF1A}$ (aHIF).'

3.1.2. Sizes and splicing

The starts and ends of AS transcripts are sometimes variable. For example, transcription of

Figure 1. Classification of natural AS transcripts by transcription initiation sites. Natural AS transcripts (asRNAs) are classified into three types according to their transcription initiation sites. Transcription is shown schematically. Transcriptional initiation is indicated with arrows. Boxes show transcribed regions. Type 1, overlapping start. The AS transcription overlaps the sense transcription that synthesizes mRNA. Both 3'-to-3' and 5'-to-5' overlapping are present. Type 2, intragenic start. The AS transcription starts at an intron of the gene. Type 3, intergenic start. The AS transcription starts between two genes.

asRNA$_{iNOS}$, an AS transcript of the *inducible nitric oxide synthase (iNOS)* gene, starts at the end of the last exon (exon 27) of the *iNOS* gene, but terminates at various sites (16). In addition, some AS transcripts are spliced, and others are not. For example, the AS transcripts of the *endothelial nitric oxide synthase (eNOS)* gene, asRNA$_{eNOS}$ (sONE), are long and spliced (17, 18). According to the splicing pattern, these AS transcripts are assumed to be mixed type AS transcripts (Types 2 and 3). The sizes of AS transcripts are variable and often give a smear pattern, not a discrete band, on northern blot analysis. These smeared bands are not generally caused by non-specific probe hybridization (5, 16).

3.1.3. Polyadenylation

The AS transcripts discussed in this review are not classical ncRNAs such as rRNAs or tRNAs. Thus, transcription of AS transcripts may be driven by RNA polymerase II. To confirm the RNA polymerase type, the fungal toxin alpha-amanitin is generally used, which specifically inhibits RNA polymerase II activity in human and mouse cells, but not in rat cells (19).

After transcription in the nucleus, AS transcripts sometimes are capped and polyadenylated and then transported to the cytoplasm. When sites of transcription for 10 human chromosomes were mapped, 19.4, 43.7, and 36.9% of all transcribed sequences, *i.e.*, mRNA and ncRNA, were polyadenylated, nonpolyadenylated, and 'bimorphic', respectively (9). Bimorphic transcripts are RNA transcribed as polyadenylated RNAs, which are processed to reduce or remove their poly(A) sequences under specific conditions (20). Localization of the transcripts was also analyzed by this study. Transcripts detected only in the nucleus or cytoplasm were 51.3% and 10.2%, respectively. For example, the AS transcripts of the rat *iNOS* gene and human *iNOS* pseudogene are reported to be nonpolyadenylated (16, 21). Polyadenylation of the many other AS transcripts and transport of the AS transcripts to the cytoplasm are not well studied. They await future investigation.

3.2. Expression of natural AS transcripts
3.2.1. Expression patterns of mRNAs and natural AS transcripts

The expression patterns of many genes are classified into two types, constitutive or inducible expression, in response to various stimuli. The former is regulated mainly at the transcriptional level by transcription factor binding to gene promoters (promoter activation), and the latter is regulated not only at the transcriptional level, but also at the post-transcriptional level by regulation of mRNA stability or mRNA translatability. A typical example of inducible expression is the proinflammatory cytokine genes during inflammation, which are induced by various adverse stimuli. In mouse fibroblasts, in response to the pro-inflammatory cytokine tumor necrosis factor alpha (TNF-alpha), activated genes can be categorized into three groups, each of which has different induction kinetics: mRNA expressed at early, middle, and late time points (22). A so-called 'early response gene' (ERG) is defined as a gene expressed early in response to a stimulus, and strictly speaking, without *de novo* protein synthesis. Hence, almost all of the ERGs are classified into the first group, *i.e.*, early mRNA-expressing genes. ERGs and middle mRNA-expressing genes encode many cytokines and chemokines, as well as iNOS and cyclooxygenase 2 (COX-2). Such inducible genes may be regulated at both the transcriptional and the post-transcriptional levels. Post-transcriptional mechanisms that modify mRNA stability and translatability provide more rapid and flexible control of the inflammation process, and they are particularly important in coordinating the initiation and resolution of inflammation (23).

3.2.2. Involvement of natural AS transcripts in mRNA stability

It has been convincingly shown that large numbers of AS transcripts are involved in the regulation of mRNA stability (2, 24). Table 1 contains a list of known eukaryotic AS transcripts that are involved in mRNA stability. Most of them are classified as Type 1 AS

Natural antisense transcripts from inducible genes

Table 1. Natural antisense transcripts involved in mRNA stability.

Antisense transcript	Antisense encoded protein	Gene (mRNA)/ Gene (asRNA)	Overlapping region of mRNA	Correlation of mRNA and asRNA expression	Evidence for RNA-RNA interaction	Species	References
asRNA$_{BACE1}$	(−)	*BACE1 BACE1*	CDS[3]	Positive/ parallel	Yes	Human, Mouse	104
asRNA$_{Bcl2/IgH}$	(−)	*bcl-2 / bcl-2– IgH* [1]	3'UTR[4]	Positive/ parallel	Yes	Human	107
asRNA$_{CHRNA3}$	(−)	*CHRNA3 / CHRNA5*	CDS, 3'UTR	Not tested	Not tested	Human, Bovine	108
asRNA$_{eIF2α}$	(−)	*eIF2alpha / eIF2alpha*	5'UTR[5]	Not tested	Not tested	Human	15
asRNA$_{eNOS}$ (sONE)	(−)	*eNOS / eNOS(sONE)*	3'UTR	Negative/ reciprocal	Yes	Human, Mouse, Rat	17, 18
asRNA$_{EPOR}$ (asEPO-R)	(−)?	*EPO-R / EPO-R*	CDS, 3'UTR	Positive/ parallel	Not tested	Human, Canine	109
asRNA$_{ERCC1}$	(+)	*ERCC-1 / ERCC-1*	3'UTR	Not tested	Not tested	Human, yeast	110
asRNA$_{FADS1}$	(−)	*FADS1 / FADS1REV*	5'UTR	Positive/ parallel	Not tested	Human, Mouse, Rat	111
asRNA$_{FGF2}$ (GFG)	(+)	*FGF-2 / FGF-2(GFG)*	3'UTR	Not tested	Not tested	Human, Rat, *Xenopus*	112-115
asRNA$_{psiFGFR3}$	(−)	*FGFR-3 / psiFGFR-3* [2]	CDS	Not tested	Not tested	Mouse	27
asRNA$_{HIF1A}$ (aHIF)	(−)	*HIF-1alpha / HIF-1alpha*	3'UTR	Negative/ reciprocal	Not tested	Human, Mouse, Rat	3, 116
asRNA$_{IFNA1}$	(−)	*IFNA1 / IFNA1*	CDS	Positive/ parallel	Yes	Human	T. Kimura, unpublished data
asRNA$_{iNOS}$	(−)	*iNOS / iNOS*	3'UTR	Positive/ parallel	Yes	Human, Mouse, Rat	16, 33; M. Nishizawa, unpublished data
asRNA$_{psiNOS}$	(−)	*iNOS / pseudo-iNOS* [2]	CDS	Negative/ reciprocal	No	Human	21
asRNA$_{psNOS}$	(+)?	*nNOS / pseudoNOS* [2]	CDS	No (independent)	Yes	Snail	26
asRNA$_{p53}$ (Wrap53)	(+)	*p53 / p53(Wrap53)*	5'UTR	Positive/ parallel	Yes	Human	85

[1] A hybrid gene that consists of *bcl-2* and *IgH* genes, produced by chromosomal translocation; [2] pseudogene. Abbreviations: [3] coding sequence, [4] 3'-untranslated region, [5] 5'-untranslated region.

transcripts. When the expression profiles of many mRNA and AS transcript (mRNA/asRNA) pairs were analyzed, frequent concordant changes in their expression levels were detected (1). The correlation of mRNA/AS transcript expression levels is circumstantial evidence that the AS transcript may affect mRNA function. For example, iNOS mRNA expression has been shown to increase after interleukin (IL) 1beta addition, peak at 6 hours, and then decrease (16). Expression levels of asRNA$_{iNOS}$ showed a peak at 6 hours after IL-1beta addition and were positively correlated with the levels of iNOS mRNA (16). At 4 hours after IL-1beta addition, both the iNOS mRNA and the asRNA$_{iNOS}$ were synthesized at the maximum rate. Considerable degradation of iNOS mRNA and asRNA$_{iNOS}$ occurred at 7 hours.

Promoter analyses using the luciferase assay have shown that the promoter of asRNA$_{iNOS}$ is also IL-1beta-inducible, as is the promoter for iNOS mRNA (16). In this case, recognition sites for the transcription factors NF-kappaB and CCAAT/enhancer-binding protein (C/EBP) are present in both gene promoters. However, the transcription factor-binding sites in the mRNA promoter are not always the same as those in the asRNA promoter or in other genes. For example, the level of HIF-1alpha mRNA negatively correlates to that of asRNA$_{HIF1A}$, and expression of the snail eNOS mRNA and asRNA$_{psNOS}$ is independent (Table 1). It is important to analyze the promoter activity of the asRNA promoter. Correlation of mRNA and AS transcript expression is insufficient to demonstrate the mRNA-AS transcript interaction; direct evidence of the RNA-RNA interaction is required.

3.2.3. Natural AS transcripts from pseudogenes

Recently, Muro and Andrade-Navarro suggested that pseudogenes are an alternative source of AS transcripts (25). They estimated that 80% of pseudogenes that arise from duplications are accompanied by AS transcripts and presented several examples in support of this theory. AS transcripts transcribed from pseudogenes are classified as Type 3 AS transcripts (Figure 1).

In one example, the snail neural nitric oxide synthase (nNOS) mRNA showed interference in *trans* from an AS transcript that is transcribed from the *nNOS* pseudogene (26). The AS transcript from the *nNOS* pseudogene forms an RNA duplex with the nNOS mRNA, which leads to suppression of the nNOS protein. In a second example, an AS transcript was transcribed from the pseudogene *psiFGFR-3* (asRNA$_{psiFGFR3}$), which seemed to be duplicated from the *fibroblast growth factor receptor 3 (FGFR-3)* gene (27). In addition, a duplicated pseudogene of the human *iNOS* gene has been reported (21). A non-coding AS transcript without a poly(A) tail has been transcribed from this pseudogene and overlapped the coding sequence (CDS) of the iNOS mRNA. Rat AS transcripts have also been synthesized from a duplicated pseudogene that is located 35 kilobase pairs downstream of

Table 2. Methods for the detection of natural antisense transcripts.

Method	Target RNA(s)	Principles and procedures	Advantages and disadvantages
Northern blot analysis	mRNA, asRNA	RNA transcripts are resolved by gel electrophoresis, blotted on a filter membrane, and detected with a labeled, strand-specific RNA/DNA probe by hybridization.	Transcripts are detected without amplification bias. A time-consuming procedure. Difficult to detect transcripts expressed at low levels.
Strand-specific reverse transcription-polymerase chain reaction (RT-PCR)	mRNA, asRNA	RNA transcripts are converted to cDNA by reverse transcription with strand-specific primers and amplified by PCR. Products are resolved by gel electrophoresis.	High sensitivity. Small amount (less than a few microgram) of RNA required. A semiquantitative method due to PCR bias. Genomic DNA may sometimes be amplified.
Strand-specific reverse transcription and real-time PCR[1] (quantitative PCR, qPCR)	mRNA, asRNA	RNA transcripts are converted to cDNA by reverse transcription with strand-specific primers. Amplification of cDNA is monitored by real-time PCR. Threshold cycle (Ct) values are used to express RNA content.	Highly sensitive and quantitative method. A small amount of RNA is required. Genomic DNA may sometimes be amplified.
Ribonuclease protection assay (RPA), ribonuclease mapping (RNase mapping)	mRNA, asRNA	After hybridization of a labeled RNA probe with mRNA/asRNA, the resultant RNA duplexes remain after ribonuclease digestion.	Start (5′) and stop (3′) sites of transcription are determined. When these sites are variable or splicing occurs, the results are unclear.
Rapid amplification of cDNA[2] ends (RACE)	mRNA, asRNA	Reverse transcribed cDNA is ligated to linker-primers and amplified by PCR.	Start and stop sites of transcription are determined, even when these sites are variable. Long cDNAs are not always amplified.
Cloning of cDNA	mRNA, asRNA	Reverse-transcribed cDNA (PCR-amplified and RACE cDNA, cDNA in a library, etc.) is isolated and cloned into a vector.	Partial or entire structure of asRNA is confirmed by sequencing of its cDNA.
Microarray analysis	mRNA, (asRNA)	Transcripts are fluorescently labeled, hybridized with oligonucleotides on a chip (microarray), and detected.	High sensitivity with low bias. Expensive and time-consuming.

Abbreviations: [1] Polymerase chain reaction, [2] complementary DNA.

the *iNOS* gene (M. Nishizawa, T. Okumura, unpublished data). Sequence comparison indicated that this AS transcript originated from the *iNOS* pseudogene and was almost identical to that from the *iNOS* gene (asRNA$_{iNOS}$).

3.3. *In vivo* expression of natural AS transcripts

In the adult mouse brain, *in situ* hybridization analysis of 1,328 transcripts has revealed that 849 long ncRNAs (more than 200 nucleotides) are expressed and that the majority are associated with specific regions, cell types, or subcellular compartments (28). The *in vivo* expression of AS transcripts has been demonstrated by several groups. AS transcripts from the *NOS* pseudogene (asRNA$_{psNOS}$) have been detected in the neurons of the snail cerebral ganglion, and they form long RNA duplexes with the nNOS mRNA (26). This long mRNA/asRNA duplex interferes with translation of the eNOS protein. In human renal cell carcinomas, asRNA$_{HIF1A}$ is overexpressed (3). *In situ* hybridization has demonstrated that eNOS AS transcripts (asRNA$_{eNOS}$) are expressed in the mouse uterus and placenta (17). asRNA$_{iNOS}$ transcripts have been detected in rat sepsis models induced by D-galactosamine and bacterial lipopolysaccharide (29, 30) and in hepatic ischemia-reperfusion injuries in rats (31). Interestingly, administration of many drugs, such as the free radical scavenger edaravone (32, 33), the Na$^+$/H$^+$ exchanger inhibitor FR183998 (29, 31), and insulin-like growth factor 1 (IGF-I) (30), led to reduced levels of iNOS mRNA and asRNA$_{iNOS}$ in these rats. Human asRNA$_{iNOS}$ has been detected in cancer tissues and the placenta (M. Nishizawa, T. Okumura, unpublished data).

All of these data demonstrate the *in vivo* expression of AS transcripts and support the possibility that AS transcripts play physiological roles.

3.4. Methods to detect natural AS transcripts
3.4.1. General view

The various methods for detecting natural AS transcripts are summarized in Table 2. Northern blot analysis is the first choice for detection of mRNA and the AS transcript. A smeared band of AS transcripts is often observed, which is not caused by RNA degradation or repetitive sequences (5, 16). When the size of the AS transcript is variable, broad or smeared bands may be detected by northern blot or by a ribonuclease protection assay (RPA). In addition, it is generally difficult to detect RNA transcripts (mRNA and asRNA) when the expression level of the transcript is low.

3.4.2. Strand-specific reverse transcription-polymerase chain reaction

Strand-specific reverse transcription-polymerase chain reaction (RT-PCR) is mainly performed to indicate the presence of the AS transcript (16, 34). This method is the best alternative for when northern blot does not work due to the low level of the AS transcript. Strand-specific primers are used to synthesize complementary DNA (cDNA) by reverse transcription (RT) using a sense primer for the AS transcript and an AS primer for the mRNA. An oligo(dT) primer is used for RT of mRNA instead of an AS primer because the results with the AS primer are almost identical to those with oligo(dT) primer. The oligo(dT) primer can also prime cDNA synthesis for the AS transcript if the AS transcript is polyadenylated. Some artifacts may occur during strand-specific RT-PCR. These artifacts may be problematic when the expression level of the RNA transcript is low.

Several steps may be taken to ensure faithful detection. First, RT is sometimes inhibited by the secondary structure of RNA or the reverse transcriptase may sometimes override a stem-loop structure (*i.e.*, *trans-splicing* [35]). To avoid this problem, RT should be performed at high temperature (47°C or higher) to disrupt secondary structures (34, 35).

Second, to eliminate non-specific amplification, sufficiently high temperature should be applied to the

primer annealing during polymerase chain reaction (PCR) cycles. High-stringency protocols, such as a touchdown or step-down protocol (16, 34), are recommended for PCR.

Third, genomic DNA contaminating the RNA preparation may cause amplification of a genome sequence. Thus, deoxyribonuclease I (DNase I) treatment is essential. A negative control, such as RT(−), in which RNA is directly used for PCR without the RT step, is always required to monitor for genomic DNA contamination.

Forth, amplified cDNA should be confirmed by size using gel electrophoresis and DNA sequencing to rule out amplification of unrelated DNA or *trans*-spliced cDNA. Correctly amplified cDNA has an expected size, and, if spliced *in vivo*, it has a sequence that follows the GT-AG rule at the exon-intron junctions (36).

Last, cDNA synthesis during RT is primed not only by a specific primer and the oligo(dT) primer, but also by the 3'-end of RNA that is intramolecularly snap-backed. This primer-independent cDNA synthesis ('self-priming') occurs much less efficiently than priming by specific primers and is negligible in many cases. As is easily assumed, the self-primed cDNA from mRNA (a small amount) may contaminate to the sense primer-primed cDNA from the AS transcript, and the self-primed cDNA from the AS transcript (a small amount) may contaminate to the AS primer-primed cDNA from the mRNA. To avoid this artifact, as mentioned above, RT at higher temperature is recommended to disrupt snap-backs (37). When a biotinylated sense primer is used for RT and the resulting biotin-labeled cDNA is purified using streptavidin-conjugated beads, only the correctly primed cDNA can be amplified by PCR. This method was successfully applied to detect iNOS AS transcripts (M. Nishizawa and T. Kimura, unpublished data).

3.4.3. Stability of mRNA

The stability of mRNA is expressed as its half-life. The mRNA levels estimated by northern blot analysis or RT-PCR are dependent on the cumulative rate of *de novo* RNA synthesis and RNA degradation. If *de novo* RNA synthesis is blocked by actinomycin D (ActD) or 5,6-dichloro-1-beta-D-ribofuranosylbenzimidazole (16, 22, 38), an mRNA's half-life can be measured. For example, at 4 hours after IL-1beta addition, iNOS mRNA was shown to be synthesized at the maximum rate, which revealed a half-life of 339 minutes in the presence of ActD (16). Considerable degradation occurred at 7 hours after IL-1beta addition, and the half-lives of iNOS mRNA in the absence and presence of ActD were 84.9 and 60.2 minutes, respectively. In the presence of ActD, the half-life of iNOS mRNA before the peak was 5.6-fold longer than after the peak.

3.4.4. Microarray analysis

Similar to the oligonucleotide-based microarrays used for mRNA detection, microarrays for the detection of human and mouse AS transcripts have been developed (5). However, they are based on expressed sequence tags (ESTs) and thus do not cover all possible AS transcripts. The progress being made in AS transcript research will surely lead to future improvements in microarrays to detect AS transcripts.

4. Structural features and correlation to natural AS transcript function
4.1. *Cis*-controlling elements
4.1.1. General view

A *cis*-controlling element in RNA is defined as a nucleotide sequence that is present in the RNA molecule and regulates its features, including stability and translatability. This element may be present in both the mRNA and the AS transcript. The *cis*-controlling elements of RNA-binding proteins are their recognition sequences in mRNA, and the recognition sequences of many RNA-binding proteins have been identified (39). *Cis*-controlling elements in mRNA are often the sites at which miRNA and AS transcripts interact. It is generally believed that RNA-binding proteins, in addition to AS transcripts and miRNA, may also distinguish structural features of RNAs, such as the sequences of their *cis*-controlling elements and their secondary and tertiary structures, duplex conformation, and single/double-strandedness.

4.1.2. AU-rich elements

The most well-known *cis*-controlling element is an AU-rich element (ARE) that harbors a 5'-AUUUA-3' sequence (40). AREs with more than 3 Us and other nonstandard ARE sequences can also be functional (41). ARE motifs often appear in the 3'UTRs of inducible genes, including acute phase proteins involved in inflammation and infection, cytokines, iNOS, COX-2, and some proto-oncogenes. Therefore, it was suspected that AREs were involved in the regulation of mRNA stability and mRNA decay (42). A kinetic analysis of TNF-alpha-inducible genes (22) has provided support for this idea. This study revealed that mRNAs expressed early had abundant AREs in their 3'UTRs, whereas those expressed later had fewer. When we screened 30 IL-1beta-inducible genes from which mRNA harboring AREs were transcribed, we found that AS transcripts were indeed transcribed from approximately 80% of these genes (E. Yoshigai, M. Nishizawa, unpublished data). Sequencing analysis of the cDNAs complementary to these AS transcripts demonstrated that AREs were also present in AS transcripts. Further studies are necessary to verify the hypothesis that an AS transcript tends to be transcribed from an inducible gene that has ARE motif(s) in its mRNA.

In vivo roles for the ARE motif have been studied by Kontoyiannis *et al.* using the TNF-alpha mRNA, whose 3'UTR (with a 34-nucleotide ARE cluster) is highly conserved among mammals (43). When this ARE cluster was deleted in the mouse genomic *TNF-alpha* gene, it caused misregulated TNF-alpha translation in macrophages, monocytes, and neutrophils. Furthermore, the mice harboring the *TNF-alpha* gene that lacked the AREs showed chronic inflammatory arthritis and inflammatory bowel disease similar to Crohn's disease.

4.2. RNA secondary structure and RNA duplex conformations
4.2.1. RNA secondary structure

RNA is not simply a linear molecule. Both Watson-Crick type base pairing (A:U and C:G) and non-Watson-Crick type base pairing (G:U) inside the RNA molecule lead to the formation of secondary structures (44). This intramolecular base pairing often forms a stem-loop structure (also called a hairpin loop or hairpin structure). This structure consists of a single-stranded RNA loop and a double-stranded stem, which consists of a double helix (duplex). Other than the stem-loops, there are single-stranded and double-stranded regions that harbor a number of mismatches and long single-stranded portions (bulge loops and internal loops) in the RNA molecules (44). Intramolecular interactions among the single-stranded regions and loops (hairpins, bulges, and internal loops) located at different sites of the RNA molecule form the tertiary, *i.e.*, three-dimensional structure. For example, the intramolecular loop-loop interaction of the stem-loops causes pseudoknot formation. On the other hand, the intermolecular loop-loop interaction causes kissing loop interactions (see *4.6.*) and an AS transcript-mediated RNA-RNA interaction (see *4.7.*).

The secondary structure of RNA sequences can be predicted by free energy minimization without considering pseudoknots. Several software packages, such as mfold (45) and Centroidfold (46), have been developed and are available on the internet. These prediction methods are not always satisfactory for experimental applications. When using the mfold program, prediction of an RNA sequence provides many secondary structures. Practically, alignment of these structural predictions indicates that several conserved structural units ('domains') are usually found. For example, in the iNOS mRNA 3'UTR there are 4 common regions (domains A to D), each of which includes at least one stem-loop structure (16).

Tertiary structure is important for RNA biological function. Because secondary structure is the basis of tertiary structure, it contributes to various functions, such as the regulation of transcription, translation, and polyadenylation (47, 48, 49). Recently, novel secondary structures that cause the nuclear export of mRNA to the cytoplasm have been found (50). These structures are formed by the CDS of the human interferon alpha1 (IFN-alpha1) mRNA and are responsible for chromosome region maintenance 1 (CRM1)-dependent export of the IFN-alpha1 mRNA.

To analyze secondary structures, including stem-loops, of short RNA, melting analysis has generally been performed by measuring the melting temperatures of these structures (51). For longer RNA, RPA (also known as ribonuclease mapping) is performed on the premise that double-stranded RNAs are resistant to ribonuclease (RNase) A and T_1. A novel method for genome-scale measurement of secondary structure has recently been developed (52). Using this method, structural analysis of over 3,000 transcripts of the yeast *Saccharomyces cerevisiae* revealed the existence of more secondary structures in the coding regions than in the untranslated regions.

4.2.2. Secondary structures of natural AS transcripts

What is the secondary structure of an AS transcript? When an RNA molecule (RNA1) and its complementary RNA molecule (RNA2) are present, RNA2 has a nucleotide sequence complementary to that of RNA1. The above-mentioned prediction of secondary structure is performed using base pairing (A:U, C:G, and G:U). Given that the orientation of RNA is 5'-to-3', nucleotide complementarity suggests that the secondary structure of RNA2 is a mirror image of that of RNA1. It also suggests that stem-loop structures in RNA2 are formed at complementary sites in RNA1. For example, there were 4 corresponding loops (Aas, Bas, Cas, and Das) of the AS transcript to the above-mentioned domains A, B, C, and D, respectively, of the iNOS mRNA (16). The loops (each 5 to 10 nucleotides long) of these domains are perfectly complementary to each other. As described later, these loops are involved in the interaction between iNOS mRNA and the AS transcript (see *4.7.*).

It is noteworthy that not only the nucleotide sequence but also the secondary structure of the mRNA 3'UTR may be conserved in many species. For example, the nucleotide sequences, including AREs and the secondary structure of the iNOS mRNA 3'UTR, are conserved in the rat (16), mouse (33), and human (M. Nishizawa *et al.*, unpublished data). Our predictions of the 3'UTRs of several cytokine mRNAs, made using the mfold program, also support this hypothesis (M. Nishizawa *et al.*, unpublished data). Taken together with the mirror-image structure of the AS transcripts, it is likely that both mRNA and AS transcripts are structurally conserved in many species.

4.2.3. RNA duplex conformation

The conformation of the duplex formed by the interaction of RNA with other nucleotides can also be predicted. Different duplexes, including RNA:RNA, DNA:DNA and DNA:RNA, produce different conformations (Figure 2). The minor grooves (green zones) indicate exactly where the interaction with an Na^+ ion is the most favorable and reflect the different conformations produced (53). In contrast to the B form of DNA:DNA duplexes (*i.e.*, double-stranded DNA), RNA:RNA duplexes take the A form (53). When miRNA binds to the 3'UTR of mRNA, a local RNA:RNA duplex (*i.e.*, double-stranded RNA) is formed, which leads to the repression of translation (7, 54). As a result of an mRNA-asRNA interaction, an RNA:RNA duplex is assumed to be formed, which may cause torsion locally around the duplex (Figure 3). DNA:RNA hybrids, which may be formed by hybridization with an oligodeoxyribonucleotide, have an A/B conformation that reflects the presence of A form features with some B form-like features (53). These subtle differences in conformational features define the key properties used by RNase H (55) to discriminate between the different duplexes (Figure 3).
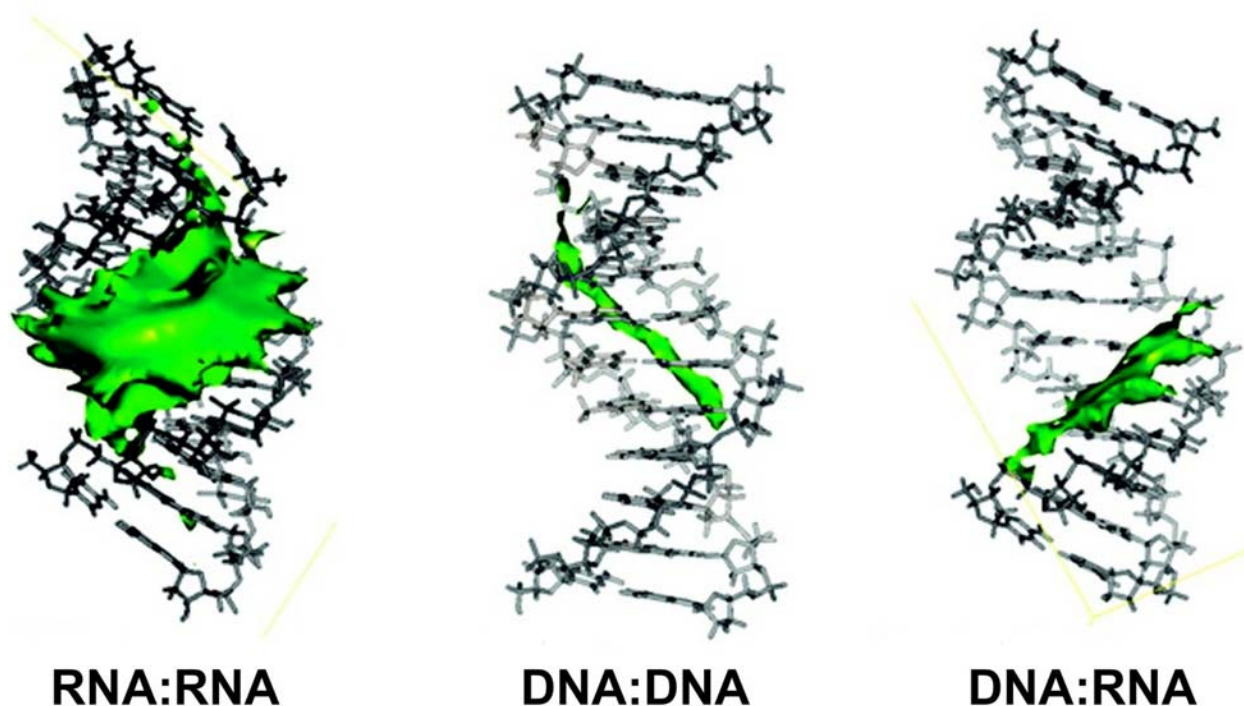
Figure 2. Various types of RNA duplex conformations. The classical molecular interaction potential (cMIP) isocontours (–3 kcal/mol) for interactions with an Na^+ probe at three different duplexes are shown (53). The sequence used is the Dickerson dodecamer, 5'-d/r(CGCGAATTCGCG)-3'. RNA:RNA and DNA:DNA duplexes show the A form and B forms, respectively. RNA:RNA duplexes may be formed by the hybridization of mRNA with asRNA or miRNA. In contrast, DNA:RNA duplexes have an A/B conformation and may be formed by the hybridization of an oligodeoxyribonucleotide with RNA. The green zones (minor groove) indicate exactly where the interaction with an Na^+ is more favorable than 3 kcal/mol. This reflects the different conformations because they depend on where the negative charges of the backbone point, whether toward the minor groove or toward the major groove. Reprinted in part with permission from American Chemical Society.

Both RNA duplex conformation and RNA secondary structure may mediate a variety of RNA interactions with proteins, RNA, and DNA (see below).

4.2.4. Accessibility of protein

RNA-binding proteins recognize specific nucleotide sequences, *i.e.*, *cis*-controlling elements. Accessibility of the RNA-binding protein may be determined by not only the recognition sequence but also the context of secondary structure. Meisner *et al.* (56) characterized the binding of human homolog R of the embryonic lethal-abnormal visual protein (HuR) (42, 57, 58) to the ARE and identified a secondary structure-dependent HuR recognition of mRNA. They used oligonucleotides complementary to the ARE motifs ('openers'), which form DNA:RNA hybrids at the AREs of IL-2 and TNF-alpha mRNAs. These oligonucleotides hybridized to the mRNAs and increased the affinity of HuR for the mRNAs, possibly by opener-induced rearrangement of the mRNA conformation. The authors proposed an 'accessibility hypothesis' that requires two factors for HuR-mRNA interaction: 1) a sequence match to the ARE motif and 2) the presentation of the ARE in a single-stranded conformation within the ARE secondary structure (56). It is likely that this DNA:RNA hybrid-induced conformational change mimics an RNA:RNA hybrid-induced conformational change (Figure 2).

4.3. *Trans*-acting factors
4.3.1. General view

A *trans*-acting factor for RNA is defined as a molecule that interacts with RNA and regulates its functions. *Trans*-acting factors include (but are not limited to) RNA-binding proteins, drugs, and RNAs (including miRNA and AS transcripts). When *trans*-acting factors are RNA-binding proteins, AS transcripts, or miRNA, they recognize and interact with specific nucleotide sequences (*i.e.*, *cis*-controlling elements). It is generally believed that they may also discriminate between structural features of RNA, including secondary and tertiary structures and duplex conformations. In contrast, drugs are not thought to recognize specific nucleotide sequences. Drug-RNA interactions will be discussed below (*4.4.*). RNAs, particularly AS transcripts, may also act as *trans*-acting factors that act on mRNA (see *4.6., 4.7.*).

4.3.2. RNA-binding proteins

Many RNA-binding proteins are known, and their roles in post-transcriptional regulation have been investigated (39). RNA-binding proteins that bind to the 3'UTRs of inflammatory genes have been investigated in

A. RNA-RNA

RNA

RNA

Protein

B. DNA-RNA

DNA

RNA

RNase H

C. Drug-RNA

Drug

RNA

Protein
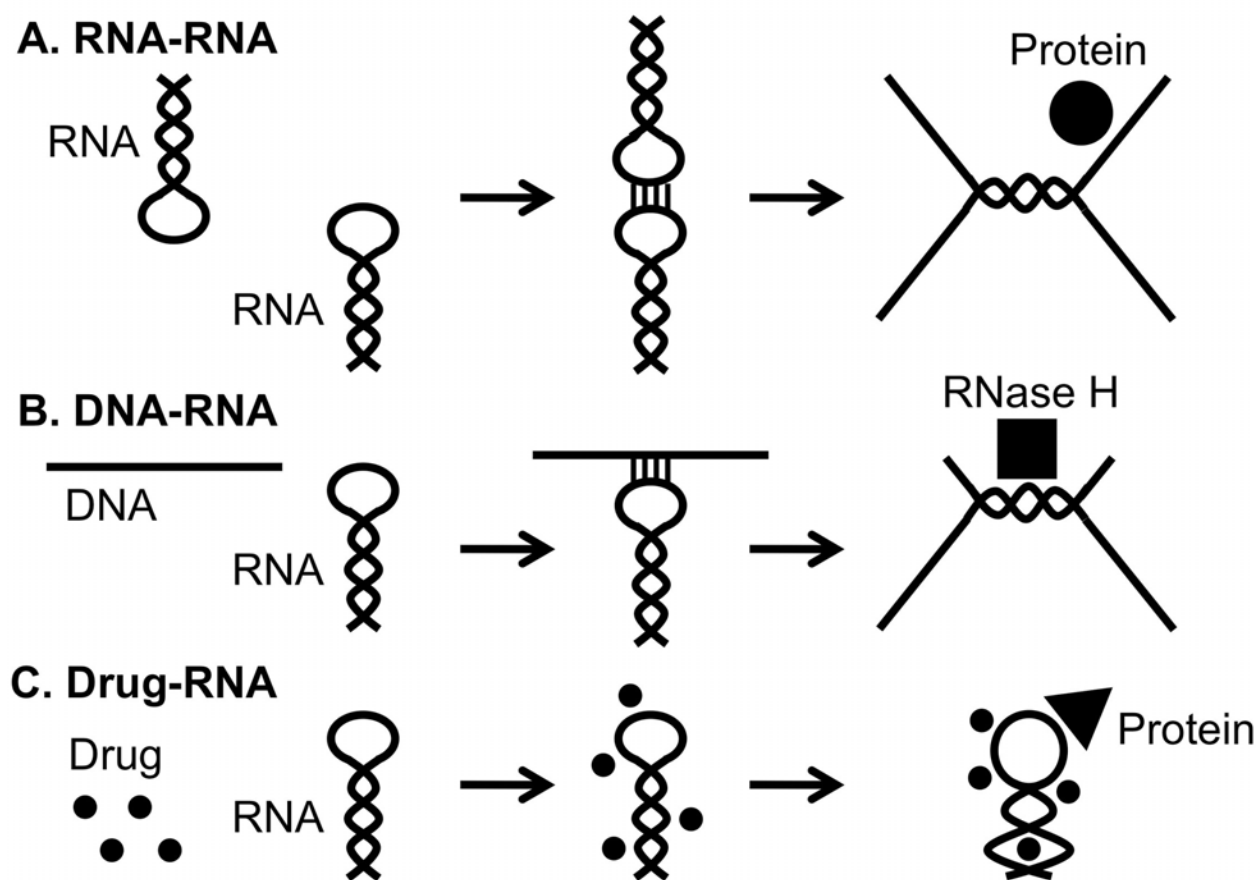
Figure 3. Structural changes induced by RNA interactions. Models of the structural changes induced by RNA interactions are depicted. RNA molecules with stem-loop structures are shown. Base pairing during the interaction is shown by short bars. (A) A structural change induced by an RNA-RNA interaction. Two RNA molecules that form stem-loop (hairpin) structures are shown. The stem regions of the RNA:RNA duplexes are in the A form. When the loops (several nucleotides long) of these molecules mutually interact, an intermolecular RNA:RNA duplex is formed. This induces local torsion around the duplex. To release torsion, a conformational change around the duplex is induced. This structural disruption may affect protein accessibility to RNA, such as the enhancement of binding of an RNA-binding protein (circle). This type of interaction is observed for the interaction of mRNA with asRNA or miRNA. (B) A structural change induced by a DNA-RNA interaction. A DNA molecule interacts with the loop of an RNA molecule harboring a stem-loop structure. The resultant DNA:RNA duplex showing A/B form induces local torsion and a conformational change around the duplex. This structural disruption may affect accessibility of an RNA-binding protein (triangle) and RNase H (square). This type is observed in the interaction of oligodeoxyribonucleotides with mRNA by antisense technology and with asRNA using NATRE technology (*4.5.*). (C) A structural change induced by a drug-RNA interaction. An RNA molecule forming a stem-loop structure interacts with drug molecules (small dots). The drug intercalates the RNA duplex (stem), externally binds to the phosphate groups of the RNA, and induces helix destabilization and a conformational change of the stem-loop structure. This structural disruption may affect the accessibility of an RNA-binding protein (triangle).

detail (23). When recognition sequences are present, these proteins may bind to not only mRNA but also AS transcripts. One notable group of proteins are ARE-binding proteins, which bind to the *cis*-controlling element ARE. Dozens of ARE-binding proteins have been reported, and many of them are mRNA-stabilizing proteins. AREs stabilize mRNA by interacting with ARE-binding proteins. HuR stabilizes ARE-containing mRNAs that encode IL-2, IL-3, c-*fos*, and iNOS, among others (42, 56, 57, 58).

In contrast to HuR, there are many ARE-binding proteins that destabilize iNOS mRNA, such as AU-binding factor 1 (AUF1)/heterogeneous nuclear ribonucleoprotein

(hnRNP) D (59), tristetraprolin (TTP) (60, 61), and KH-type splicing regulatory protein (KSRP) (62, 63). TTP is a Cys-Cys-Cys-His (CCCH) zinc-finger protein that destabilizes TNF-alpha mRNA (64). KSRP interacts with not only AREs but also TTP to regulate the stability of iNOS mRNA (62, 63). BRF-1, a homolog of TTP, also activates mRNA decay (65). Another CCCH-type zinc-finger protein, Zc3h12a, has RNase activity and also destabilizes IL-6 mRNA (66).

The RNA-binding protein polypyrimidine tract-binding protein (PTB)/hnRNP I is involved in interactions between RNA-binding proteins (59, 67). PTB binds to

hnRNP L, which binds to HuR (16, 63). Additionally, HuR binds to iNOS mRNA and its AS transcript (16). These complicated interactions are involved in iNOS mRNA stability (23, 63) and possibly in the function of the AS transcript. Indeed, the iNOS mRNA, AS transcript, and various proteins form a stable RNA-protein complex (see *4.7.*).

Another example of a *cis*-controlling element and its *trans*-acting factor is the iron responsive element (IRE) and IRE-binding proteins, respectively. The IRE is present in the 3'UTR of the transferrin receptor mRNA and is recognized by the IRE-binding proteins (68). The IRE forms a stem-loop structure that harbors A-form helical stem regions. Interaction with the IRE-binding proteins leads to specific inhibition of the degradation of the transferrin receptor mRNA. Together with the data on ARE-binding proteins, evidence supports a role for the *cis*-controlling element as a scaffold for *trans*-acting factors.

4.4. Low-molecular-weight drugs and drug-RNA interactions
4.4.1. Aspirin-RNA interactions

Many chemically synthesized drugs have much lower molecular weights (less than 1,000) than protein and RNA. Most low-molecular-weight drugs function by interacting with proteins, and several drugs are involved in mRNA stability. For example, the immunosuppressant drug rapamycin reduces the stability of the iNOS mRNA by up-regulation of *TTP* gene expression (69).

In contrast, several low-molecular-weight drugs bind directly to RNA and affect its structure and function. Aspirin (acetylsalicylic acid) is a non-steroidal anti-inflammatory drug that interacts with RNA (70). Fourier transform infrared (FTIR) difference spectroscopic data have shown that, at a low concentration, aspirin intercalated RNA duplexes through both G:C and A:U base pairs and the backbone phosphate groups. A partial helix destabilization occurred at a high aspirin concentration. The donor groups (O=C–O) and (O=C–OCH$_3$) of aspirin are mainly involved in this aspirin-RNA interaction (70). This partial helix destabilization (helix opening) may increase the chance of drug binding to different RNA donor sites that are locally melted (70). These drug-RNA interactions appear to be similar to the conformational change during RNA:RNA duplex formation and to rearrangements of mRNA conformation by oligonucleotides complementary to AREs (*4.2.*). Similarly, the synthetic estrogen diethylstilbestrol has been shown to induce intercalation through both G:C and A:U base pairs and a partial helix destabilization (71). RNA-staining dyes, such as acridine orange (72) and methylene blue (73), also intercalate RNA duplexes. These data suggest a possibility that some low-molecular-weight drugs can intercalate RNA duplexes or externally bind to the phosphate groups of RNA (Figure 3), which causes a conformational change that may affect mutual recognition by mRNA and AS transcripts. In support of this hypothesis, sodium salicylate (74) and aspirin (M. Nishizawa and T. Okumura, unpublished data) have been shown to reduce *iNOS* gene expression and destabilize iNOS mRNA by decreasing asRNA$_{iNOS}$ in

hepatocytes, which indicates that they can cause a conformational change in iNOS mRNA and/or asRNA$_{iNOS}$ to destabilize iNOS mRNA.

4.4.2. Drugs that affect levels of mRNA and AS transcripts

Many drugs and agents have been reported to destabilize iNOS mRNA, including the anti-inflammatory drug dexamethasone (75), the neutrophil elastase inhibitor sivelestat (76), the antioxidant agent cysteamine (77), the functional food active hexose correlated compound (AHCC) (78) and the flavanol-rich lychee fruit extract (FRLFE) (M. Nishizawa *et al.*, unpublished data). These substances have been shown to reduce the levels of both iNOS mRNA and asRNA$_{iNOS}$. In addition, reporter assays performed in these studies have suggested that these substances regulate *iNOS* gene expression at the transcriptional and/or post-transcriptional levels through the 3'UTR of the iNOS mRNA. Similarly, hepatoprotective Kampo medicines, such as *Hochu-ekkito*, *Daikenchuto*, and *Inchinkoto*, also reduced levels of both iNOS mRNA and asRNA$_{iNOS}$ (T. Okumura, Y. Ikeya and M. Nishizawa, unpublished data).

In contrast, the anti-ulcer drugs rebamipide and the 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) inhibitor pitavastatin have been shown to increase levels of both iNOS mRNA and asRNA$_{iNOS}$ (79, 80). Reporter assays performed in these studies have suggested that rebamipide and pitavastatin regulate *iNOS* gene expression at the post-transcriptional level through the 3'UTR of the iNOS mRNA. The mechanisms of action of these drugs and any involvement of the AS transcripts remain to be studied.

Meanwhile, several low-molecular-weight drugs that specifically recognize RNA structure have been reported. Acetylpromazine, a phenothiazine derivative psychotropic drug, specifically bound with high affinity to the unique bulge loop of the transactivation-responsive (TAR) RNA, to which the transactivating regulatory (Tat) protein of human immunodeficiency virus type I (HIV-1) binds (81). Binding of acetylpromazine altered the three-dimensional structure of TAR and inhibited access of Tat protein to TAR (82).

4.5. Methods to analyze interactions with RNA
4.5.1. Methods to detect protein-RNA interactions

Various methods for the analysis of RNA interactions with protein and RNA are summarized in Table 3. Protein-RNA interactions can be detected by the yeast three-hybrid assay (Y3HA) (50, 83). For example, an interaction between the IRE of transferrin mRNA and IRE-binding proteins was detected by Y3HA (68). Compared to protein-protein interactions detected by the yeast two-hybrid assay (Y2HA), protein-RNA interactions are relatively weaker (T. Kimura and M. Nishizawa, unpublished data).

Protein-RNA interactions can be detected by other methods, such as EMSA and the RNA-immunoprecipitation (RIP) assay with antibodies (16). In analyses of protein-RNA interactions, RNase activity in the

Table 3. Methods for the analysis of RNA interactions with protein and RNA.

Method	Probe/target	Principle and procedure	Advantages and disadvantages
Electrophoretic mobility shift assay (EMSA)	Labeled RNA / protein	The RNA-protein complex migrates more slowly than RNA alone in gel electrophoresis.	A sensitive method to detect RNA-binding proteins.
Supershift assay	RNA / protein and antibody	The RNA-protein- antibody complex migrates more slowly than the RNA-protein complex in gel electrophoresis.	RNA-binding proteins are identified using an antibody. This method is often performed with EMSA.
Yeast three-hybrid assay (Y3HA)	RNA / protein	Interactions between RNA and protein can be detected and measured in yeast.	A RNA-binding protein or RNA sequence bound to protein is essential for this assay.
RNA immunoprecipitation (RIP) assay	Protein / RNA	The protein-RNA complex is trapped with an antibody, and the RNA-protein interaction is analyzed. RNA-RNA interaction is indirectly suggested.	An antibody is required. Formaldehyde crosslinking with protein may be performed before immunoprecipitation. Relatively low signal-to-noise ratio.
Yeast RNA-hybrid assay (YRHA)	RNA1 / RNA2	Interaction between RNA1 and RNA2 can be detected and measured in yeast.	RNA-RNA interaction is weak. Relatively low signal-to-noise ratio.
Natural antisense transcript-targeted regulation (NATRE) technology	(–) / asRNA	Sense oligonucleotides block RNA-RNA interactions and affect mRNA stability. (They do not work in the absence of asRNA.)	Interaction between mRNA and asRNA is directly indicated. Sites of interaction in mRNA can be specified.
Knockdown by siRNA[1]	(–) / mRNA and asRNA	RNA transcripts are degraded by siRNA with RNA-induced silencing complexes (RISCs).	RNA transcripts are not always strand-specifically degraded by siRNA.
Reporter assay	(–) / mRNA (asRNA, effector)	When a target sequence (site of interaction) is present in a UTR[2], asRNA affects reporter-UTR mRNA stability and the reporter protein. Expression of asRNA (induced or overexpressed) is required as an effector.	Target sequence(s) in the UTR are essential to prepare reporter constructs. Deletion mutants without the target sequence can be constructed.
In situ hybridization (ISH); immunocytochemistry (IC)	Labeled DNA / RNA transcript (ISH); Antibody / protein (IC)	RNA transcripts in the cell or tissue are detected by a labeled DNA/RNA probe (ISH) and a protein is specifically detected by its antibody (IC).	Colocalization of RNA transcript and protein is demonstrated. Results provide indirect evidence of RNA-protein interaction.

Abbreviations: [1] Short interfering RNA, [2] untranslated region.

protein preparation is problematic. RNase is abundant and is found in cell and nuclear extracts, serum, and various protein preparations, including antibodies. Inhibition of RNase activity is required throughout entire experiments.

4.5.2. Methods to detect RNA-RNA interactions

Compared with RNA-protein interactions, RNA-RNA interactions detected by the yeast RNA-hybrid assay (YRHA) (84) are assumed to be even weaker and often transient *in vivo*. It is generally difficult to detect RNA-RNA interactions because the signal-to-noise (S/N) ratio is low in many assays. To improve the S/N ratio, several companies have developed new beads for the RIP assay, such as protein A- or streptavidin-conjugated beads, which reduce non-specific binding of RNA and protein. Short RNA duplexes, which are formed by interactions between mRNA and AS transcripts (see *4.7.*), are difficult to directly detect.

4.5.3. Natural AS transcript-targeted regulation technology

A '*sense*' oligonucleotide can be used to examine the interactions between mRNA and AS transcripts. Sense oligonucleotides are designed according to the mRNA sequence and include at least one single-stranded loop. This loop of mRNA may hybridize with the corresponding loop of the AS transcript (*4.2.*). A sense oligonucleotide can compete with mRNA and inhibit the interaction between mRNA and the AS transcript. Alternatively, when the sense oligonucleotide hybridizes with the AS transcript, the resulting oligonucleotide:asRNA hybrid may be a substrate for RNase H (Figure 3). Sense oligonucleotides to iNOS mRNA have been shown to reduce the level of iNOS mRNA by inhibiting the mRNA-asRNA interaction (16). Sense oligonucleotides to IFN-alpha1 mRNA digested the IFN-alpha1 AS transcript and reduced the level of IFN-alpha1 mRNA (T. Kimura and M. Nishizawa, unpublished

data). Thus, we designated this sense oligonucleotide-mediated method as natural antisense transcript-targeted regulation (NATRE; pronounced /nature/) technology. The NATRE technology produced successful results by reducing the mRNA levels of several other cytokine genes that generate AS transcripts (E. Yoshigai, M. Nishizawa, unpublished data).

A similar approach to the NATRE technology has been applied to the mRNA encoding tumor suppressor p53 to verify the interaction between p53 mRNA and its AS transcript, asRNA$_{p53}$, which encodes the Wrap53 protein (85). Sense oligonucleotides blocked the p53 mRNA-asRNA$_{p53}$ interaction, which resulted in a reduction in p53 mRNA.

The NATRE technology is totally different from 'antisense technology,' a conventional mRNA knockdown method that uses single-stranded AS oligonucleotides (86). Interaction of mRNA with the AS transcript is not considered in antisense technology. The sense oligonucleotide in antisense technology is a negative control that does not change mRNA levels, whereas the sense oligonucleotide in the NATRE technology reduces mRNA levels in the presence of the mRNA-AS transcript interaction. Short interfering RNA (siRNA), which is a short double-stranded RNA, also reduces mRNA by binding to the 3'UTR. An RNA-induced silencing complex (RISC) is involved in the degradation of mRNA by siRNA (87).

4.5.4. Reporter assays

Reporter assays are another powerful method to analyze not only promoter activity but also mRNA stability and RNA-RNA interactions by measuring reporter protein activity or the half-life of a reporter mRNA. When the untranslated region (UTR) that harbors *cis*-controlling
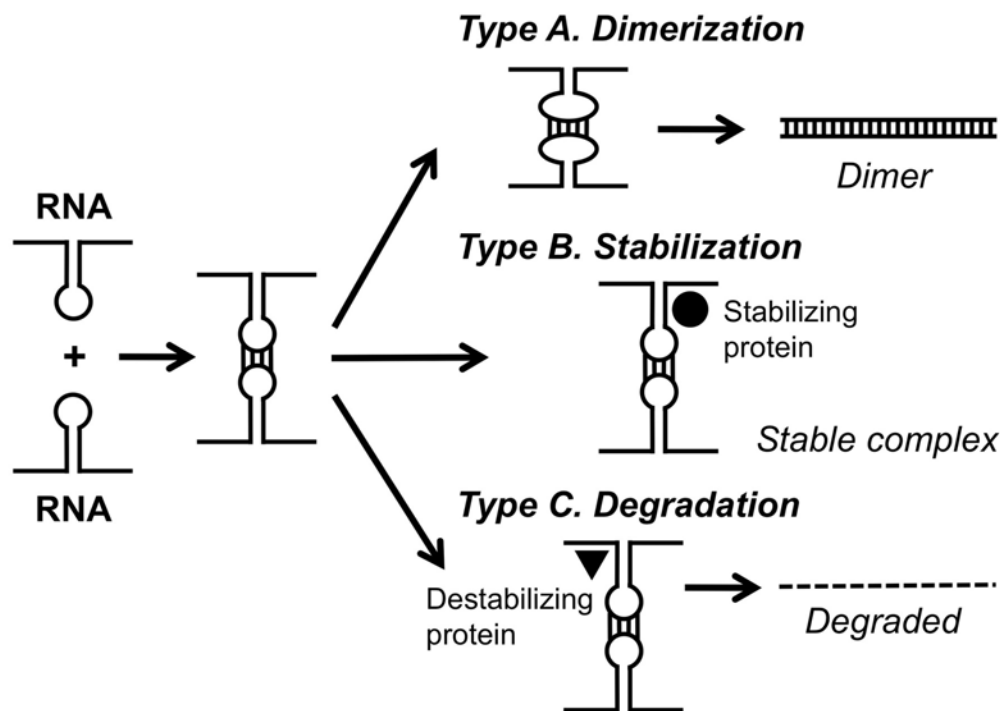
Figure 4. Types of intermolecular RNA-RNA interactions. When an RNA molecule interacts with another RNA molecule at loops by base pairing hybridization (small bars), loop-loop interactions at stem-loop structures induce three different types of RNA-RNA interactions. Each stem-loop structure is depicted by two parallel bars and an open circle. Models are shown. Type A, dimerization. Loop-loop interaction, *i.e.*, kissing loop interaction, triggers the hybridization of two RNA molecules to form a long RNA duplex, probably in concert with dimerizing enzymes. Type B, stabilization. Loop-loop interactions result in the recruitment of a stabilizing protein (closed circle). RNA remains stable until the stabilizing protein detaches. Type C, degradation. Loop-loop interaction results in recruitment of a destabilizing protein (triangle) and finally the degradation of the RNA (broken line). The fate of RNA after an RNA-RNA interaction is determined by several *trans*-acting factors, including stabilizing and destabilizing proteins, miRNA, and drugs.

element(s) is ligated to a reporter gene, the stability of the reporter–UTR hybrid mRNA should change. When the firefly *luciferase* gene was ligated to the iNOS 3'UTR, which harbors a site for interaction with asRNA$_{iNOS}$, the half-lives of the luciferase mRNA and the luciferase activity increased in the presence of asRNA$_{iNOS}$ (16). In contrast, when a construct with the *luciferase* gene–simian virus 40 late polyadenylation signal (SVLPA) (49) was used, which does not have target sites for asRNA$_{iNOS}$, the half-lives of the luciferase mRNA and luciferase activity did not change, irrespective of the presence of asRNA$_{iNOS}$ (32, 78, 80, 88). These constructs (luciferase–3'UTR and luciferase–SVLPA) were used to discriminate between the effects of the AS transcripts and drugs on promoter activity and the effects on mRNA stability (32, 78, 80, 88). Other genes, such as the *beta-globin* and *green fluorescent protein (GFP)* genes, are also used as reporter genes to monitor mRNA stability (22, 89). Expression of the AS transcripts, either endogenously induced or exogenously overexpressed, is essential for reporter assays to assess the effects of AS transcripts.

Details of the above-mentioned methods and others for the analysis of RNA interactions have been reviewed elsewhere (90, 91).

**4.6. Regulation of mRNA stability by natural AS transcripts**

4.6.1. General view of RNA-RNA interactions

At various steps in gene expression, AS transcripts may regulate the expression of inducible genes by interacting with *trans*-acting factor(s) and *cis*-controlling element(s). Among the many AS transcripts that are involved in mRNA stability, only a few AS transcripts have been analyzed to demonstrate a direct interaction between the mRNA and the AS transcript (indicated as 'Yes' in Table 1). Several putative mechanisms of regulation by AS transcripts have been proposed (2, 24, 92). In this review, we focus on complementarity-dependent mechanisms to regulate mRNA stability.

When two RNA molecules that harbor stem-loops interact with each other, the fate of the RNA differs in three ways, designated as types A to C (Figure 4). In many cases described below, the initial interaction at loops involves the hybridization of several nucleotides (16, 93), and the RNA-RNA interaction proceeds in concert with proteins.

4.6.2. Type A RNA-RNA interactions

A Type A interaction starts with local hybridization of two long, complementary RNA molecules.
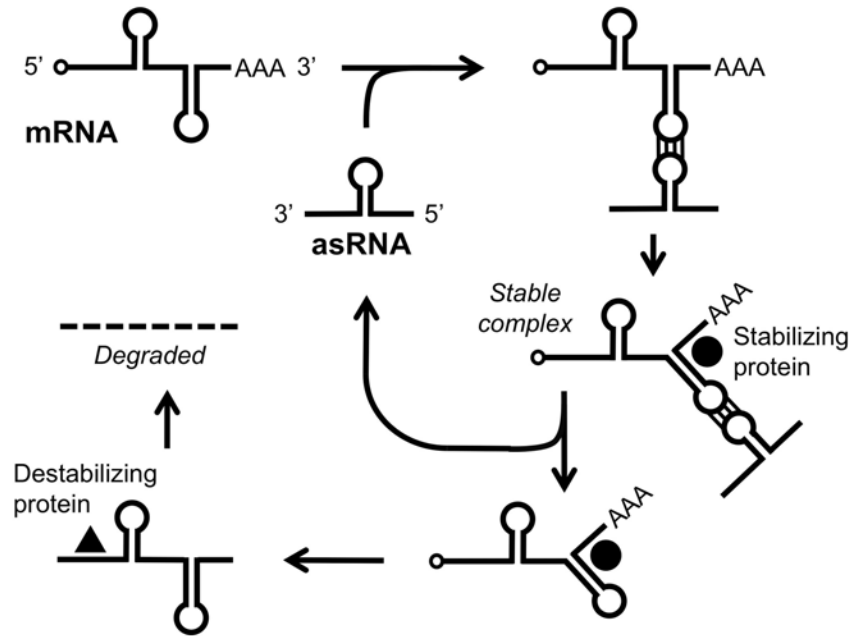
Figure 5. A model for the mechanism of AS transcript-mediated post-transcriptional regulation. A model for the interaction between mRNA and the AS transcript (asRNA) is indicated schematically. The 5'-cap structure and 3'-poly(A) tail of a mRNA are shown by a small circle and (AAA), respectively. Each stem-loop structure is indicated by two parallel bars and an open circle. Base pairing interactions are indicated by small bars. Initially, loop-loop interactions between the mRNA and the asRNA occur. The short loop of the stem-loop structure of the mRNA hybridizes with the corresponding loop of the asRNA. This loop-loop interaction is transient and reversible. As in a Type B interaction (Figure 4), the asRNA triggers a conformational change in the mRNA to induce recruitment of a stabilizing protein (closed circle). This step further promotes protein-protein interactions to form an mRNA-asRNA-protein complex, which stabilizes mRNA by inhibiting access of deadenylation and decapping enzymes. When the asRNA and the stabilizing protein detach from the complex, the mRNA is no longer stable and starts to be degraded by the binding of destabilizing protein (triangle) or degrading enzymes.

During replication, dimerization of HIV-1 genomic RNA occurs at its kissing loops (6 nucleotides long), and finally, a long RNA duplex of the genomes is formed (94). The resulting long RNA duplexes in virus-infected cells may be substrates for RNA editing or sources of endogenous siRNA to inhibit gene expression (95) and activate the signaling cascades that activate the *alpha-* and *beta-IFN* genes (96).

Type A interactions are similar to the formation of mRNA:AS transcript hybrids. Snail nNOS mRNA has been shown to hybridize with the AS transcript from its pseudogene (asRNA$_{psNOS}$) to form a long RNA duplex in the CDS *in vivo* (26). This duplex of nNOS mRNA and asRNA$_{psNOS}$ prevented the translation of the nNOS protein.

Type A interactions may undergo other double-stranded RNA-dependent mechanisms, including promoter interference, transcription interference, and epigenetic interference by masking protein-binding sites (92). Because Type A interactions are reviewed elsewhere (92, 93, 95), here we focus on the other types of interactions.

4.6.3. Type B RNA-RNA interactions

Type B interactions are triggered by RNA-RNA hybridization at the loops of two complementary RNA molecules, and the resultant RNA dimer is stabilized by

trans-acting factor(s) (Figure 4). Local hybridization at the loops is minimally required for this interaction. For example, *bicoid (bcd)* mRNA-Staufen ribonucleoprotein particles, which are essential for oogenesis and early embryogenesis in *Drosophila melanogaster*, are formed by *bcd* mRNA interactions (97). The *bcd* mRNA dimerizes with the *trans*-acting factor Staufen at two kissing loops (6 nucleotides long) in its 3'UTR to form stable ribonucleoprotein particles (93, 97).

Three examples of interactions between mRNA and Type 1 AS transcripts have been reported: noncoding asRNA$_{iNOS}$ (16); asRNA$_{p53}$, which encodes Wrap53 protein (85); and noncoding asRNA$_{IFNA1}$ (T. Kimura *et al.*, unpublished data) (Table 1). In all cases, direct evidence of the interaction between the mRNA and the AS transcript was supplied by mRNA knockdown by sense oligonucleotide-mediated NATRE technology. Reporter assays using *luciferase* gene–UTR constructs showed that the target of the AS transcript was located in the mRNA UTR and was involved in the stability of the iNOS and p53 mRNAs (16, 85).

Based on the mRNA-AS transcript interactions of the *iNOS* and *p53* genes (16, 85) and the above-mentioned reports, a model of AS transcript-mediated regulation has been proposed (Figure 5). According to our hypothesis,

intermolecular interactions with mRNA and AS transcripts occur at their loops. The short loop of the stem-loop structures of the mRNA interacts with the corresponding loop of the AS transcript (*4.2.*) as an initial interaction. The resultant short RNA:RNA duplex (several nucleotides long) at the loops seems to be reversible and unstable *in vivo* due to the low melting temperature of the duplex. Type B interactions may occur between imperfectly complementary RNA molecules, such as the interactions of AS transcripts from pseudogenes (*3.2.*). Alternatively, because cross-homologies between the loop sequences are observed in the iNOS mRNA and asRNA$_{iNOS}$, it is also possible that mRNA and asRNA interact at the loops in various combinations (16).

Similarly to the oligonucleotide-induced conformational change (*4.2.*) and the drug-RNA interaction (Figure 3), the AS transcript is assumed to trigger a conformational change of mRNA and a partial destabilization at the stems (duplex). These changes may affect the accessibility of RNA-binding proteins, resulting in the recruitment of a stabilizing protein. The stabilizing protein(s) can then promote protein-protein interactions to form an mRNA-asRNA-protein complex. This complex stabilizes the mRNA by prohibiting access of deadenylation and decapping enzymes that degrade mRNA (98). The mRNA-asRNA-protein complex can be detected by RIP assay with a specific antibody (*4.5.*).

According to our model, extremely short RNA:RNA duplexes of mRNA and AS transcripts may be transiently formed at the initial interaction to recruit stabilizing protein(s). Once an mRNA-asRNA-protein complex is formed, the mRNA remains stable until the AS transcript is released from the complex. The released AS transcript is assumed to be recycled. It has been reported that the expression level of the AS transcript is much lower than that of the mRNA; for example, the ratios are 1/7, 1/30, and 1/100 for iNOS, IFN-alpha1, and p53 mRNAs, respectively (16; T. Kimura *et al.*, unpublished data; 85). Because the major role of the AS transcript is thought to be a trigger to recruit proteins and form a stable complex, our model (Figure 5) appears to be plausible in that a small amount of the AS transcript efficiently regulates mRNA levels during inducible expression.

This mechanism is a novel post-transcriptional mechanism mediated by the AS transcript. It is likely that the AS transcripts from many inducible genes that encode cytokines, chemokines, and transcription factors may mediate this mechanism to regulate mRNA levels. A study to verify this hypothesis is in progress (E. Yoshigai, M. Nishizawa, unpublished data).

4.6.4. Type C RNA-RNA interactions

Type C interactions are also triggered by local RNA-RNA hybridization at loops of two complementary RNA molecules, but the RNA is degraded, in contrast to the fate of RNA in a Type B interaction (Figure 4). The initial loop-loop hybridization is the same as that of a Type B interaction. This probably results in the recruitment of destabilizing protein(s) or the detachment of stabilizing

protein(s). Indeed, there is a report that two ARE-binding proteins, TTP and its homolog BRF-1, are recruited and then activate mRNA decay (65). These data demonstrate that TTP and BRF-1 trigger degradation of mRNA harboring ARE motif(s). In support of this report, we have found a few examples of Type C interactions in cytokine mRNA/asRNA pairs (E. Yoshigai, M. Nishizawa, unpublished data).

Because many stabilizing and destabilizing proteins that bind to mRNAs are known, a balance of stabilizing and destabilizing proteins probably determines whether RNA is stabilized (Type B interaction) or degraded (Type C interaction).

4.7. Other mechanisms mediated by natural AS transcripts
4.7.1. Chromatin regulation

AS transcripts may also modify chromatin. For example, a long, spliced AS transcript is involved in the posttranscriptional regulation of the *eNOS* gene (17). Exceptionally, this AS transcript also encodes a protein. The increase of asRNA$_{eNOS}$ leads to the decrease of eNOS mRNA by modifying chromatin. Another example is the AS transcripts transcribed from the *PHO84* gene in *Saccharomyces cerevisiae* (99). These AS transcripts mediate silencing of the *PHO84* gene via the recruitment of histone deacetylases. Furthermore, expression of the *progesterone receptor (PR)* gene is post-transcriptionally regulated (100). Three transcripts are transcribed from this gene locus: PR mRNA, a Type 1 AS transcript that overlaps the 5'UTR; a noncoding sense transcript that overlaps the 3'UTR; and the 3′ end of the *PR* gene. These three transcripts, along with Argonaute 2, cooperatively induced chromatin changes at the *PR* gene promoter. In general, chromatin regulation has not been studied in detail and warrants further investigation.

4.7.2. Regulation by microRNAs

As another important mechanism of gene regulation, miRNA has been reported to regulate gene expression with AS transcripts. A computational analysis of many genes that regulate immunity revealed that the mRNAs encoding the ARE-binding proteins HuR, AUF1 and TTP were targets of miRNA (101). On the other hand, it has been reported that miRNA destabilizes TNF-alpha mRNA through AREs and is also involved in regulation of translation (102, 103). Thus, miRNA may also be involved in the regulation of mRNA stability. Together with these data, the post-transcriptional control seems to involve AREs, RNA-binding proteins (including ARE-binding proteins), AS transcripts, and miRNA.

Interactions between AS transcripts and miRNAs have been overlooked. However, a recent report showed that these two distinct groups of regulatory RNAs share a communal interface of action (104). Faghihi *et al.* demonstrated that an miRNA (miR-485-5p) and asRNA$_{BACE1}$ competed for a binding site in the BACE1 mRNA. This mRNA encodes beta-site amyloid precursor protein-cleaving enzyme 1 (BACE1; also known as beta-secretase 1), which is involved in the development of

Alzheimer's disease (105). These findings support the existence of noncoding RNA-containing regulatory networks that may be implicated in Alzheimer's disease pathophysiology (106).

5. PERSPECTIVE

Inducible genes are involved in various diseases, such as inflammatory diseases, infectious diseases, autoimmune diseases, allergies, and cancer, as well as Alzheimer's disease. For inducible genes, many natural AS transcripts seem to be transcribed and are involved in the regulation of post-transcriptional events, such as mRNA stability and translation. As described above, the AS transcript-mediated post-transcriptional mechanism via Type B or C RNA-RNA interactions may be a common mechanism to regulate inducible genes. Future functional studies on AS transcripts may elucidate whether this hypothesis is correct.

On the other hand, the AS transcript-mediated mechanism could be a potential drug target for many diseases. Investigation of this mechanism could have clinical implications. For example, regulation of mRNA levels by the NATRE technology is likely to be a new approach. Further studies should clarify the relevance of this new field of natural AS transcripts.

6. ACKNOWLEDGMENTS

7. REFERENCES

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Abbreviations: mRNA: messenger RNA; AS: antisense; asRNA: antisense RNA; HIF: hypoxia-induced factor; ncRNA: noncoding RNA; miRNA: microRNA; 3'UTR: 3'-untranslated region; iNOS: inducible nitric oxide synthase; eNOS: endothelial nitric oxide synthase; TNF-alpha: tumor necrosis factor alpha; ERG: early response gene; nNOS: neural nitric oxide synthase; FGFR-3: fibroblast growth factor receptor 3; CDS: coding sequence; RPA: ribonuclease protection assay; RT-PCR: reverse transcription-polymerase chain reaction; cDNA: complementary DNA; RT: reverse transcription; PCR: polymerase chain reaction; ActD: actinomycin D; ARE: AU-rich element; IFN-alpha1: interferon alpha1; IL: interleukin; HuR: human homolog R of the embryonic lethal-abnormal visual; hnRNP: heterogeneous nuclear ribonucleoprotein; TTP: tristetraprolin; RNase: ribonuclease; IRE: iron responsive element; TAR: transactivation-responsive element; Tat: transactivating regulatory protein; RIP: RNA-immunoprecipitation; NATRE: natural antisense transcript-targeted regulation; siRNA: short interfering RNA; UTR: untranslated

region; bcd: bicoid; BACE1: beta-site amyloid precursor protein-cleaving enzyme 1.

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