[Frontiers in Bioscience, Landmark, 20, 1-36, January 1, 2015]

Post-transcriptional inducible gene regulation by natural antisense RNA

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1. ABSTRACT

Accumulating data indicate the existence of natural antisense transcripts (asRNAs), frequently transcribed from eukaryotic genes and do not encode proteins in many cases. However, their importance has been overlooked due to their heterogeneity, low expression level, and unknown function. Genes induced in responses to various stimuli are transcriptionally regulated by the activation of a gene promoter and post-transcriptionally regulated by controlling mRNA stability and translatability. A low-copy-number asRNA may post-transcriptionally regulate gene expression with cis-controlling elements on the mRNA. The asRNA itself may act as regulatory RNA in concert with trans-acting factors, including various RNA-binding proteins that bind to cis-controlling elements, microRNAs, and drugs. A novel mechanism that regulates mRNA stability includes the interaction of asRNA with mRNA by hybridization to loops in secondary structures. Furthermore, recent studies have shown that the functional network of mRNAs, asRNAs, and microRNAs finely tunes the levels of mRNA expression. The post-transcriptional mechanisms via these RNA–RNA interactions may play pivotal roles to regulate inducible gene expression and present the possibility of the involvement of asRNAs in various diseases.

2. INTRODUCTION

The '*sense*' sequence of a gene is defined as the sequence of messenger RNA (mRNA) that encodes a protein, while the '*antisense*' sequence is complementary to the sense sequence. Thus, a gene (double-stranded DNA) consists of a sense strand that encodes mRNA and the complementary antisense strand. A '*natural antisense transcript*' (NAT), which is often called an '*antisense RNA*' (asRNA), is an endogenous transcript that is derived from the sequence of the antisense strand of a gene. Hereafter, we mainly use the term 'asRNA', and sometimes '*natural antisense transcript*' to avoid ambiguity. Genome-wide transcriptome analyses have indicated that three-quarters of the human genome can be transcribed and that the antisense strands of many mammalian genes are frequently transcribed (1–3). As described in our previous review, many natural antisense transcripts have been reported in eukaryotes (4), but their functions remain unclear. Here, we incorporated updated progress after our previous review was published.

Natural antisense transcripts are often classified as '*non-coding RNA*' (ncRNA) because many of them do not encode proteins or can only encode 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). MicroRNAs (miRNAs, miRs) were identified as small RNA molecules (of approximately 21 nucleotides (nts)) that regulate translation and interfere with mRNA (5). Genome-wide transcriptome analyses have demonstrated that an unexpectedly large number of ncRNAs and asRNAs are transcribed from the human and mouse genomes (1,6–8). Recent reports have shown that the human transcriptome includes approximately 9,000 small RNAs and approximately 10,000–32,000 long ncRNAs (9,10). It is generally difficult to characterize the functions of ncRNAs, including non-coding antisense transcripts. Therefore, many ncRNAs beyond the functionally identified ncRNA species are referred to as '*transcripts of unknown function*' (TUFs) (11). Various types of ncRNAs—*i.e.*, classical and recently characterized ncRNAs—are summarized in Table 1.

I has also been suggested that asRNAs may regulate gene expression at the post-transcriptional or translational levels (1,2,12). The physiological importance of asRNAs, however, has been overlooked due to their non-protein-coding feature, as well as their low expression level and high heterogeneity, which cause difficulty in performing the necessary functional studies.

Table 1. Various types of non-coding RNA

Name	Abbreviation	Size (nt)	Definition and features	Function	Examples	References
Ribosomal RNA*	rRNA	120–4,700	Classical non-coding RNA; cytoplasm	Translation	28S, 18S, 5.8S, 5S [mammalian]	
Transfer RNA*	tRNA	70–90	Classical non-coding RNA; cytoplasm	Translation		
Small nuclear RNA	snRNA	100–500	Regulatory RNA in the nucleus	Splicing and processing of precursor RNA	U1, U2, U5, U4, U6	147
Small nucleolar RNA	snoRNA	60–330	Regulatory RNA in the nucleolus	Modification of RNA		148
MicroRNA	miRNA, miR	20–23	Spliced from a precursor; functions in the cytoplasm	mRNA degradation and translation inhibition		5
Piwi-interacting RNA	piRNA	24–27	Interacts with Piwi proteins.	Involved in development, meiosis, and transposon repression	Repeat-associated short interfering RNA (rasiRNA)	149
Natural antisense transcript	NAT, asRNA, AS RNA	Long and variable	Transcripts harboring an antisense sequence with/without a cap and a poly(A) tail	mRNA stability, chromatin modification, etc.	mRNA-type ncRNA, lncRNA, lincRNA, ceRNA, TUF, etc.	This review
mRNA-type non-coding RNA	mRNA-type ncRNA	Long and variable	Spliced transcripts with a cap and a poly(A) tail; functions in the cytoplasm	mRNA stability, chromatin modification, etc.	X-inactivation RNA, steroid hormone RNA activator, asRNA, etc.	150,151
Long non-coding RNA	lncRNA	200–100,000	Long transcripts that do not encode proteins	mRNA stability, chromatin modification, ceRNA, etc.	asRNA, ceRNA, etc.	24,31,119
Long intergenic non-coding RNA	lincRNA	Long and variable	Long non-coding RNA that starts at the region between two genes; Type 3 antisense transcript	Epigenetic regulation, etc.	lncRNA, asRNA, etc.	21
Competitive endogenous RNA, competing endogenous RNA	ceRNA	Variable	Transcripts harboring microRNA-binding sites; competes for microRNA-binding sites located in the mRNA (miRNA sponge effect).	Competitively protects mRNA from microRNA; sequesters microRNAs and upregulates mRNA expression.	asRNA, ncRNA, lncRNA, lincRNA, circRNA, pseudogenes, mRNA	43,44,119, 138,152
Circular RNA	circRNA	Variable	Circular RNA harboring many microRNA-binding sites	Suppresses microRNA activity as a microRNA sponge.	ceRNA	153,154
Transcript of unknown function	TUF	Variable	Non-coding RNA whose functions are not identified	Unknown	asRNA, lncRNA, lincRNA, etc.	11

*Classified as classical non-coding RNA. nt, nucleotide.

Many recent studies have shed light on the functions of asRNAs, particularly the interactions among mRNA, asRNA, and microRNA. In this review, we focus on the effects of asRNAs on the expression of inducible genes and particularly on their ability to regulate mRNA stability, which is highly involved in inducible gene expression. We also discuss the functional

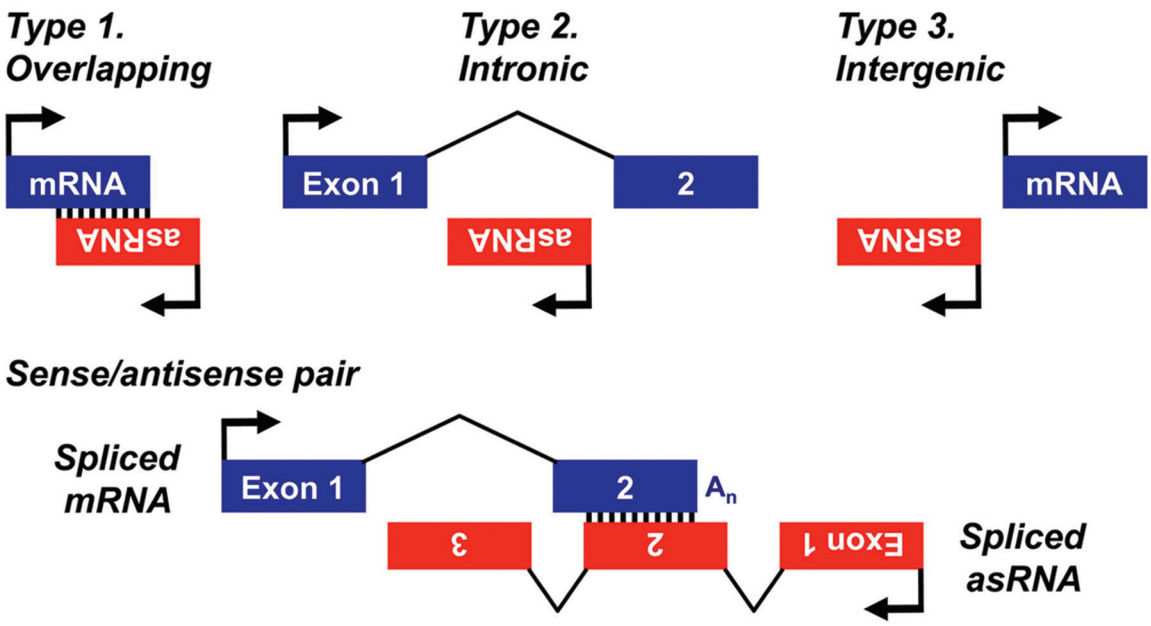

Figure 1. Classification of natural antisense transcripts by transcription initiation site. Natural antisense transcripts (asRNAs) are classified into three types according to their transcription initiation site. The transcription of asRNAs is shown schematically (upper). The transcriptional start is indicated with arrows. Boxes show transcribed regions (*i.e.*, first exons). A poly(A) tail is indicated by A_n. Type 1, overlapping start. Antisense transcription overlaps with sense transcription, which synthesizes mRNA. Both 3′-to-3′ and 5′-to-5′ overlapping are present, which may mutually interact. Intermolecular base-pairing is shown by small bars. Type 2, intronic start. The transcription starts at an intron of the gene that is also known as the intragenic start. Type 3, intergenic start. The transcription starts between two genes. An sense/antisense pair—*i.e.*, mRNA—and its antisense transcript with an intergenic transcription start are shown as an example (lower). This asRNA is spliced from three different types of exons: an intergenic start exon 1; an overlapping exon 2, which may interact with the mRNA exon 2; and an intronic exon 3.

network of gene regulation mediated by asRNA and microRNA.

3. CHARACTERIZATION OF NATURAL ANTISENSE TRANSCRIPTS

3.1. Classification of natural antisense transcripts

3.1.1. Transcription initiation sites and splicing

The features of asRNAs are variable. They are classified by several characteristics, such as by various transcriptional start sites, splicing, and modifications, including cap structure and polyadenylation. Many asRNAs do not encode proteins and are ncRNAs. The transcriptional initiation sites of asRNAs are classified into three types: (1) *overlapping*, (2) *intronic*, and (3) *intergenic* transcription start sites (7,13) (Figure 1).

In the case of type 1, the sequence of the asRNA partly overlaps with an mRNA sequence, particularly at the 3′ untranslated region (3′UTR) of an mRNA in many genes (14,15). Because the overlapping sequences are complementary, these regions may mutually interact by complete or partial hybridization; thus, the asRNA may function to regulate the expression of the overlapped mRNA. It has been reported that human and mouse asRNAs have a markedly preferential complementarity to mRNA 3′UTRs (16). In this review, we focus on the pairs of mRNA and this overlapping type of asRNA, such as Type 1 asRNAs. Because asRNAs are designated differently for each gene, Type 1 asRNAs that overlap with an mRNA of a gene are designated as '$asRNA_{gene}$' or '*gene* asRNA' in this review. If a specific name was used for the asRNA in the original paper, it is indicated in parentheses. For example, the asRNA transcribed from the hypoxia-induced factor (HIF) 1 alpha gene (human gene symbol, *HIF1A*; mouse or rat, *Hif1a*) was designated to 'aHIF-1alpha' by Bertozzi *et al.* (17); thus it is referred to as '$asRNA_{HIF1A}$ (aHIF-1alpha)' or 'HIF-1alpha asRNA' in this review.

The transcriptional starts and ends of asRNAs are sometimes variable. For example, transcription of asRNA$_{iNOS}$, an asRNA 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 (18). In addition, many asRNAs are spliced. For example, the asRNAs of the endothelial nitric oxide synthase (*eNOS*) and interferon alpha1 (*IFNA1*) genes are spliced and overlap with exon(s) (We also use '*exons*' for asRNA, although '*exons*' are originally defined as transcribed regions of mRNA, because there is no appropriate term for the regions of a non-coding transcript.) (19,20) As an example of splicing, an asRNA in Figure 1 consists of the following: an intergenic exon 1; an overlapping exon 2, which may interact with the overlapping mRNA; and an intronic exon 3.

In the case of Type 3, transcription starts at the region between the genes. It is reported that the mammalian genome encodes more than 1,000 *large intergenic non-coding RNAs* (lincRNAs) that are conserved across mammals (21). Many human lincRNAs are thought to associate with chromatin-modifying complexes and affect gene expression.

The sizes of asRNAs are variable and often produce a smear pattern, not a discrete band, on Northern blot analysis. These smeared bands are not generally caused by non-specific probe hybridization (2,18). By contrast, other asRNAs show discrete bands in Northern blot analysis: for example, asRNA$_{eNOS}$ (2.9. kilobases (kb)), asRNA$_{IFNA1}$ (approximately 4 kb), and asRNA$_{Tnf}$ (2.5. kb) that is transcribed from the rat tumor necrosis factor alpha (*Tnf*) gene (22). The asRNA transcribed from the gene encoding 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, is approximately 2 kb in length (23). Such long asRNAs are often designated as *long non-coding RNAs* (lncRNAs) (24).

3.1.2. Polyadenylation and sublocalization in the cell

Because asRNAs in this review are not classical ncRNAs, such as rRNA or tRNA, transcription of asRNAs may be driven by RNA polymerase II. To confirm the RNA polymerase type, the fungal toxin alpha-amanitin is generally used because it specifically inhibits RNA polymerase II activity in human and mouse cells, but not in rat cells (25).

After transcription in the nucleus, asRNAs sometimes are capped and polyadenylated and then exported 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 (7). Bimorphic transcripts are RNA transcribed as polyadenylated RNAs, which are processed to reduce or remove their poly(A) sequences under specific conditions (26). For example, the asRNAs of the rat *iNOS* gene and human *iNOS* pseudogene are non-polyadenylated (18,27), whereas asRNA$_{BACE1}$, asRNA$_{IFNA1}$, and asRNA$_{Tnf}$ possess poly(A) tails (20,22,23); thus, they are *mRNA-type antisense transcripts*.

Localization of the transcripts in the cell was also analyzed by Katinakis *et al*. Transcripts detected only in the nucleus or cytoplasm at a rate of 51.3.% or 10.2.%, respectively (26). Bertozzi *et al*. reported features and sublocalization of two HIF-1alpha asRNAs: 5′-HIF-1alpha asRNA, which possesses a cap and a poly(A) tail; and 3′-HIF-1alpha asRNA, which possesses neither (17). Both asRNAs are formed in the nucleus, and the 5′-HIF-1alpha asRNA accumulates at the perinuclear cellular compartment. However, polyadenylation of many other asRNAs and export of the asRNAs to the cytoplasm are not well studied. Although the Encyclopedia of DNA Elements (ENCODE) Project has been providing a clearer landscape of transcription (3,10), these data await future investigation.

3.2. Expression of natural antisense transcripts

3.2.1. Expression patterns of mRNAs and natural antisense transcripts

The expression patterns of many genes are classified into two types in response to various stimuli, constitutive or inducible expression. The former is regulated mainly at the transcriptional level by transcription factor binding to gene promoters, leading to promoter activation (*transcriptional regulation*). 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 (*post-transcriptional regulation*).

A typical example of inducible expression concerns the cytokine genes during inflammation that are induced by various adverse stimuli. In mouse fibroblasts, in response to the pro-inflammatory cytokine 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 (28). 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 post-transcriptional levels. The 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 (29).

Accordingly, asRNA-mediated post-transcriptional mechanisms in inducible expression are expected. Indeed, asRNAs are transcribed from many inducible genes, including those that encode TNF-alpha, interleukin-23 alpha subunit p19 (IL-23A), IFN-alpha1, chemokine (C-C motif) ligand 2 (CCL2), chemokine (C-X_3-C) motif ligand 1 (CX3CL1), iNOS, and nuclear factor-kappaB (NF-kappaB) p65 and p50 subunits (18,20,22,30). asRNA expression correlates to mRNA expression in many cases. It is expected that these asRNAs are involved in the regulation of mRNA stability.

3.2.2. *In vivo* expression of natural antisense transcripts

In the adult mouse brain, *in situ* hybridization analysis of 1,328 transcripts has revealed that 849 long ncRNAs (> 200 nts) are expressed and that most are associated with specific regions, cell types, or subcellular compartments (31). The *in vivo* expression of asRNAs has been demonstrated by several groups. The asRNAs 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 (32). This long mRNA/asRNA duplex interferes with translation of the nNOS protein via a Type A interaction (see *5.2.*). In human renal cell carcinomas, two types of $asRNA_{HIF1A}$ are overexpressed (17,33). The eNOS asRNAs ($asRNA_{eNOS}$) are expressed in the mouse uterus and placenta (19).

Both $asRNA_{iNOS}$ and $asRNA_{Tnf}$ have been detected in liver failure models of rats that were administered bacterial lipopolysaccharide (LPS) (22,34,35). Administration of many drugs, such as the free radical scavenger edaravone (36,37), the Na^+/H^+ exchange inhibitor FR183998 (34,38), and insulin-like growth factor 1 (IGF-I) (35), led to reduced levels of iNOS mRNA and $asRNA_{iNOS}$ in the liver of the model rats. Additionally, human $asRNA_{iNOS}$ has been detected in cancer tissues and cell lines (39).

Neurotrophins, including brain-derived neurotrophic factor (BDNF) and glial-derived neurotrophic factor (GDNF), are essential for neuronal growth, maturation, differentiation and maintenance. Both BDNF mRNA and $asRNA_{BDNF}$ (BDNF-AS) are expressed in the brain, muscle, and embryonic tissues (40). In humans, BDNF mRNA levels are generally 10- to 100-fold higher than $asRNA_{BDNF}$ levels, except in the testis, kidney and heart, which contain equal or higher $asRNA_{BDNF}$ levels. Inhibition of $asRNA_{BDNF}$ increases BDNF mRNA, alters chromatin marks at the *BDNF* locus, leads to increased BDNF protein levels, and finally induces neuronal outgrowth and differentiation both *in vitro* and *in vivo* (40). Together, these data demonstrate the *in vivo* expression of asRNAs and support the possibility that asRNAs play important physiological roles.

3.2.3. Natural antisense transcripts from pseudogenes

Many pseudogenes are present in eukaryotic genomes, and the ENCODE Project indicated that the human transcriptome includes 11,224 pseudogenes (10). Their biological roles are unclear because they do not encode functional, full-length proteins. Muro and Andrade-Navarro suggested that pseudogenes are an alternative source of asRNAs (41). They estimated that 80% of pseudogenes that arise from duplications are accompanied by asRNAs and presented several examples in support of this theory. The asRNAs transcribed from pseudogenes are classified as Type 3 asRNAs (Figure 1).

In one example, the snail neural nitric oxide synthase (nNOS) mRNA showed interference in *trans* from an asRNA that is transcribed from the *nNOS* pseudogene (32). The asRNA from the *nNOS* pseudogene forms an RNA duplex with nNOS mRNA, leading to the suppression of nNOS protein synthesis. In a second example, an asRNA was transcribed from the pseudogene *psiFGFR3* ($asRNA_{psiFGFR3}$), which seemed to be duplicated from the fibroblast growth factor receptor 3 (*FGFR3*) gene (42). In addition, a duplicated pseudogene

Table 2. Natural antisense transcripts involved in the regulation of mRNA levels

Name [name in papers]	Antisense encoded protein	Gene (mRNA)/ Gene (asRNA)	Overlapping region of mRNA	Correlation of mRNA and asRNA expression*	Species	References
asRNA$_{BACE1}$ [BACE1-AS]	(–)	*BACE1* / –	CDS	Positive/parallel	H, M	23
asRNA$_{BCL2\text{-}IGH}$	(–)	*BCL2* / *BCL2-IGH*	3′UTR	Positive/parallel	H	155
asRNA$_{BDNF}$ [BDNF-AS]	(–)	*BDNF* / *BDNF-AS*	CDS, (3′UTR)	Not tested	H, M	40
asRNA$_{Ccl2}$	(–)	*Ccl2* / –	3′UTR	Positive/parallel	Rat	30
asRNA$_{eNOS}$ [sONE]	(–)	*eNOS* / *eNOS(sONE)*	3′UTR	Negative/reciprocal	H, M, Rat	19
asRNA$_{GDNF}$ [GDNF-AS]	(–)	*GDNF* / *GDNF-AS*	?	Not tested	H	40
asRNA$_{HIF1A}$ [3′aHIF]	(–)	*HIF1A* / –	3′UTR	Negative/reciprocal	H, Rat	33
asRNA$_{HIF1A}$ [5′aHIF]	(–)	*HIF1A* / –	5′UTR	Not tested	H	17
asRNA$_{IFNA1}$ [IFN-alpha1 AS RNA]	(–)	*IFNA1* / –	CDS	Positive/parallel	H, Guinea pig	20
asRNA$_{iNOS}$ [iNOS asRNA]	(–)	*iNOS* / –	3′UTR	Positive/parallel	H, M, Rat	18,37,39
asRNA$_{psiNOS}$	(–)	– /*pseudo-iNOS***	CDS	Negative/reciprocal	H	27
asRNA$_{PTENpg1}$ [PTENpg1 asRNA, alpha and beta]	(–)	*PTENpg1*** / –	5′UTR, CDS	Negative/reciprocal	H	43
asRNA$_{Rela}$	(–)	*Rela* (*NF-kB p65*) / –	3′UTR	Positive/parallel	Rat	30
asRNA$_{psNOS}$	(–)	– / *pseudoNOS***	CDS	No (independent)	Snail	32
asRNA$_{P53}$ [Wrap53]	(+) [WD40]	*TP53* / *Wrap53*	5′UTR	Positive/parallel	H	122
asRNA$_{Tnf}$ [TNF-alpha asRNA]	(–)	*Tnf* / –	3′UTR	Positive/parallel	Rat	22

Abbreviations: CDS, coding sequence; UTR, untranslated region; H, human; M, mouse. *Positive, positively or concordantly correlated; negative, negatively or reciprocally correlated; **pseudogene.

of the human *iNOS* gene has been reported; a non-coding asRNA without a poly(A) tail has been transcribed from this pseudogene and overlapped with the coding sequence (CDS) of iNOS mRNA (27).

The expression of the tumor suppressor gene phosphatase and tensin homolog (PTEN) is post-transcriptionally regulated by the action of a *PTEN* pseudogene (PTENpg1, PTENP1) (43). A sense transcript transcribed at this pseudogene locus is PTENpg1 lncRNA, which sequesters numerous PTEN-targeting microRNAs by acting as a natural microRNA 'sponge' (44), resulting in increased PTEN mRNA stability and PTEN protein amounts. Furthermore, two isoforms (alpha and beta) of the asRNA transcribed from the *PTENpg1* locus have identified (43). asRNA$_{PTENpg1}$ alpha localizes to the *PTEN* promoter and epigenetically modulates *PTEN* transcription by chromatin remodeling. By contrast, asRNA$_{PTENpg1}$ beta interacts with PTENpg1 lncRNA through an RNA–RNA interaction that changes the stability and microRNA sponge activity of the PTENpg1 lncRNA to affect the PTEN protein level. Therefore, the network mediated by the sense and antisense RNAs from the *PTEN* pseudogene regulates the transcription and translation of PTEN mRNA.

3.2.4. Involvement of natural antisense transcripts in mRNA stability

It has been convincingly shown that large numbers of asRNAs are involved in the regulation of mRNA stability (45). Table 2 contains a list of known eukaryotic asRNAs that may affect mRNA levels. Many of them are classified as Type 1 asRNAs. When the expression profiles of many mRNA-asRNA (sense/antisense) pairs were analyzed, frequent concordant changes in their expression

Table 3. Methods for the detection of natural antisense transcripts

Method	Principles and procedures	Advantages and disadvantages
Northern blot analysis	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)	RNA transcripts are converted to cDNA by RT with strand-specific primers and amplified by PCR. Products are resolved by gel electrophoresis	High sensitivity. Small amount of RNA required. A semiquantitative method due to PCR bias. Genomic DNA may sometimes be amplified
Strand-specific reverse transcription and real-time PCR (quantitative PCR, qPCR)	RNA transcripts are converted to cDNA by RT 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 (RNase) mapping	After hybridization of a labeled RNA probe with mRNA/asRNA, the resultant RNA duplexes remain after RNase digestion	Start (5′) and stop (3′) sites of transcription are determined. When these sites are variable or splicing occurs, the results are unclear. Time-consuming
Rapid amplification of cDNA ends (RACE)	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 complementary DNA (cDNA)	Reverse-transcribed cDNA that are amplified by PCR or RACE is isolated and cloned into a vector	Structure of asRNA is confirmed by sequencing of its cDNA. Difficult to obtain the entire sequence. Time-consuming
Microarray analysis	Transcripts are fluorescently labeled, hybridized with oligonucleotides on a chip (microarray), and detected	High sensitivity with low bias. Not all the transcripts are analyzed. Expensive
RNA-seq transcriptome analysis	RNA transcripts are converted to cDNA by RT, ligated to adaptors, PCR-amplified, and sequenced by a next-generation DNA sequencer. Resultant short-read sequences are assembled and mapped on a genome	Complete transcriptome with gene organization is analyzed. Mining analyses of the huge sequence data require bioinformatics. Expensive

levels were detected (1). The correlation of the mRNA/asRNA expression levels is circumstantial evidence that asRNAs may affect mRNA function. For example, iNOS mRNA expression has been shown to increase after interleukin-1beta (IL-1beta) addition, peak at 6 hours, and then decrease (18). The 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 (18). At 4 hours after IL-1beta addition, both iNOS mRNA and $asRNA_{iNOS}$ were synthesized at the maximum rate.

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 (18). In this case, recognition sites for the transcription factors NF-kappaB and CCAAT/enhancer binding protein (C/EBP) beta 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. As shown in Table 2, the level of HIF-1alpha mRNA negatively correlates to that of $asRNA_{HIF1A}$ (46), and the expression of snail eNOS mRNA and $asRNA_{psNOS}$ is independent (19). It is important to analyze the promoter activity of the asRNA promoter. The correlation of mRNA and asRNA expression is insufficient to demonstrate the mRNA–asRNA interaction; direct evidence of the RNA–RNA interaction is required.

3.3. Methods to detect natural antisense transcripts

3.3.1. General view

The various methods for detecting asRNAs are summarized in Table 3. Northern blot analysis is the first choice for the detection of mRNA and

asRNA, and a discrete band corresponding to an asRNA is observed (19,20,22). However, a smeared band of asRNAs is often observed, which is not caused by RNA degradation or repetitive sequences (25,18). When the size of the asRNA is variable, broad or smeared bands may be detected by Northern blot analysis or 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. Due to the low abundance of many asRNAs, Northern blot analysis does not often work well.

3.3.2. Strand-specific reverse transcription-polymerase chain reaction

Strand-specific reverse transcription-polymerase chain reaction (RT-PCR), often combined with quantitative, real-time PCR (qPCR), is mainly performed to indicate the presence of asRNA (18,47). This method is the best alternative for Northern blot analysis with low-copy-number asRNAs. Strand-specific primers are used to synthesize complementary DNA (cDNA) by reverse transcription (RT) using a gene-specific sense primer for the asRNA and an antisense primer for the mRNA. Because the mRNA possesses a poly(A) tail, an oligo(dT) primer is used for RT of mRNA instead of a gene-specific antisense primer. The oligo(dT) primer may more efficiently hybridize to mRNA and prime cDNA synthesis, resulting in an increase in the mRNA:asRNA ratios in real-time PCR (30). The oligo(dT) primer can also prime cDNA synthesis for the asRNA when the asRNA 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, which is known as 'trans-splicing' (48). To avoid this problem, RT should be performed at a high temperature (47°C or higher) to disrupt secondary structures (47,48). For example, the asRNA transcribed from the *Caenorhabditis elegans* heat shock transcription factor 1 (*hsf1*) gene was reverse-transcribed at 53°C using an *hsf1* gene-specific sense primer (49). Second, to eliminate non-specific amplification, a sufficiently high temperature should be applied for the primer-annealing step during PCR. High-stringency protocols, such as a touchdown or step-down protocol with an anti-*Taq* DNA polymerase antibody (18,47), 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 genomic DNA contamination. Fourth, the size of the amplified cDNA should be confirmed using gel electrophoresis and DNA sequencing to exclude amplification of unrelated DNA or *trans*-spliced cDNA. Correctly amplified cDNA has an expected size and shows a single melting temperature in qPCR; if spliced *in vivo*, it has a sequence that follows the GT-AG rule at the exon-intron junctions (50).

Finally, cDNA synthesis during RT may be primed not only by a gene-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 can be easily assumed, the self-primed cDNA from mRNA (a small amount) may contaminate the sense primer-primed cDNA from the asRNA, and the self-primed cDNA from the asRNA (a small amount) may contaminate the AS primer-primed cDNA from the mRNA. To avoid this artifact, as mentioned above, RT at a higher temperature is recommended to disrupt snap-backs (51). 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 (M. Nishizawa and T. Kimura, unpublished data).

3.3.3. Microarray analysis

Similar to the oligonucleotide-based microarrays used for mRNA detection, microarrays for the detection of human and mouse asRNAs have been developed (2). However, commercially available microarray platforms are based on expressed sequence tags (ESTs) and reported ncRNAs and, thus, do not cover asRNAs that have not been yet identified. The progress being made in asRNA research will certainly lead to future improvements in microarrays to detect asRNAs and estimate their expression levels.

3.3.4. RNA-seq transcriptome analysis

The development of next-generation sequencing (NGS) provides a powerful technique, RNA-seq transcriptome analysis (52). When the expression level of asRNAs is low, and the cDNA

to the asRNA is difficult to be isolated, this short-read RNA sequencing method seems to be the best choice. Although this method is now expensive, the resultant data that cover the sequences of almost all the RNA transcripts in the cell ('*transcriptome*') are subjected to mapping on a reference genome. Gene organization of an asRNA, including splicing isoforms, is characterized by this method. Generally, low-copy-number asRNAs are unfavorable to be isolated by cDNA cloning because it is technically difficult to efficiently insert their cDNAs into vectors. RNA-seq analysis requires only small amounts of RNA and would provide a detailed landscape of asRNA transcription.

If RNA-seq analysis is combined with another method, such as antibody-mediated capture such as RNA immunoprecipitation, more informative data may be obtained, such as the identification of *Polycomb* protein-associated RNAs (53) and deciphering of N^6-methyladenosine RNA methylomes (54). Subtelny *et al*. performed poly(A)-tail length profiling by sequencing (PAL-seq), which provided accurate poly(A)-tail lengths of mRNAs isolated from various species (55). Poly(A)-tail lengths were coupled to translational efficiencies in early zebrafish and frog embryos, but this strong coupling diminished at gastrulation. Ribosome footprint profiling combined with RNA-seq was performed to measure the translational efficiencies from the embryonic samples used to measure the tail lengths, as well as to analyze the effects of a microRNA (miR-155) on translational repression and destabilization in zebrafish embryos (55).

4. STRUCTURAL FEATURES AND CORRELATION WITH NATURAL ANTISENSE TRANSCRIPT FUNCTIONS

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 asRNA. 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 (56). Furthermore, *cis*-controlling elements in mRNA are often the sites at which microRNAs and asRNAs interact (20,57). It is generally believed that RNA-binding proteins, in addition to microRNAs and asRNAs, may also distinguish among structural features of RNAs, such as the sequences of their *cis*-controlling elements and their secondary and tertiary structures, including 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 (58). AREs with more than 3 Us and other nonstandard ARE sequences can also be functional (59). 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 (60). A kinetic analysis of inducible genes in TNF-alpha-treated 3T3 cells and LPS-treated macrophages (28) has provided support for this idea. That 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 asRNAs were indeed transcribed from approximately 80% of these genes (30). Sequencing analysis of the cDNAs complementary to these asRNAs demonstrated that AREs were also present in asRNAs. Further studies are necessary to verify the hypothesis that an asRNA 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-nt ARE cluster) is highly conserved among mammals (61). When this ARE cluster was deleted in the mouse genomic *Tnf* gene, it caused misregulated TNF-alpha translation in macrophages, monocytes, and neutrophils. Furthermore, the mice harboring the *Tnf* 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 (62). 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 several mismatches and long single-stranded portions (bulge loops and internal loops) in the RNA molecules (62). 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 or three-dimensional structure. For example, the intramolecular loop–loop interaction of the stem loops causes pseudoknot formation. However, the intermolecular loop-loop interaction causes kissing loop interactions and an asRNA-mediated RNA–RNA interaction (see *5.*).

The secondary structure of RNA sequences can be predicted by free energy minimization without considering pseudoknots. Several prediction programs, such as mfold (63) and RNAfold (64), have been developed and are available on the internet although intramolecular interactions are predicted by these programs, intermolecular interactions between two RNA molecules are hardly predicted. 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 (18). These single-stranded loops of a domain are sites of mRNA–asRNA interactions (18,20,22,30).

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 (65–67). Recently, novel secondary structures that cause the nuclear export of mRNA to the cytoplasm have been found (68). These structures are formed by the CDS of the human IFN-alpha1 mRNA and are responsible for chromosome region maintenance 1 (CRM1)-dependent export of IFN-alpha1 mRNA. The core structure is the BSL domain that includes a bulge and a stem-loop structure, and the BSL domain interacts with not only $asRNA_{IFNA1}$ but also microRNA (20).

To analyze secondary structures, including stem-loop structures, of short RNA, melting analysis has generally been performed by measuring the melting temperatures of these structures (69). 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 (70). Using this method, structural analysis of over 3,000 transcripts of the yeast *Saccharomyces cerevisiae* has revealed the existence of more secondary structures in the coding regions than in the untranslated regions.

4.2.2. Secondary structures of natural antisense transcripts

The secondary structure of asRNA is a mirror image of that of mRNA. 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 asRNA to the above-mentioned domains A, B, C, and D, respectively, of the iNOS mRNA (18). The loops (each 5 to 10 nt long) of these domains are perfectly complementary to each other. As described later, these loops are involved in the intermolecular hybridization between iNOS mRNA and the asRNAs.

It is noteworthy that not only the nucleotide sequence but also the secondary structure (*e.g.*, stem-loop 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, mouse, and human (18,36,37). Our predictions of the 3′UTRs of several cytokine mRNAs, made using the mfold program, also support this hypothesis (30). Taken together with the mirror-image structure of the asRNAs, it is likely that both mRNA and asRNAs 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. RNA duplex formation may occur intermolecularly (*e.g.*, RNA:DNA duplex) and intramolecularly (inside RNA). Therefore, duplex conformation is another basis of the tertiary structure. Different duplexes, including RNA:RNA, DNA:DNA and DNA:RNA, produce different conformations, thereby altering the minor grooves, which indicate exactly where the interaction with a Na^+ ion is the most favorable (71).

In contrast to the B form of DNA:DNA duplexes (*i.e.*, double-stranded DNA), RNA:RNA duplexes take the A form (71). When microRNA binds to the 3′UTR of mRNA, a local RNA:RNA duplex (*i.e.*, double-stranded RNA) is formed, leading to the repression of translation (5,72). Because of an mRNA–asRNA interaction, an RNA:RNA duplex is assumed to be formed, a process that may cause torsion locally around the duplex. 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 (71). These subtle differences in conformational features define the key properties used by RNase H (73) to discriminate between the different duplexes.

Both RNA duplex conformation and RNA secondary structure may play a key role in RNA interactions with proteins, RNA, and DNA by changing their accessibility to RNA (see below).

4.3. RNA-binding proteins

4.3.1. Interactions with RNA-binding proteins

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 microRNA and asRNAs). When *trans*-acting factors are RNA-binding proteins, asRNAs, or microRNA, 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. By contrast, drugs are not thought to recognize specific nucleotide sequences, and drug–RNA interactions will be discussed later.

Many RNA-binding proteins are known, and their roles in post-transcriptional regulation have been investigated (56). RNA-binding proteins that bind to the 3′UTRs of inflammatory genes have been investigated in detail (29).

The RNA motifs recognized by RNA-binding proteins have also been studied in eukaryotic genes (74,75). When recognition sequences are present, these proteins may bind not only to mRNA but also to asRNAs. One notable group of proteins is 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. Human homolog R of the embryonic lethal-abnormal visual (HuR) protein stabilizes ARE-containing mRNAs that encode IL-2, IL-3, c-*fos*, and iNOS, among others (60,76–78).

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 (79), tristetraprolin (TTP) (80,81), and KH-type splicing regulatory protein (KSRP) (82,83). TTP is a Cys-Cys-Cys-His (CCCH)-type zinc-finger protein that destabilizes TNF-alpha mRNA (84). KSRP interacts not only with AREs but also with TTP to modulate the stability of iNOS mRNA (82,83). Butyrate response factor 1 (BRF-1), a zinc-finger protein homologous to TTP, also activates mRNA decay (85).

The RNA-binding protein polypyrimidine tract-binding protein (PTB)/hnRNP I is involved in interactions between RNA-binding proteins (79,86). PTB binds to hnRNP L, which binds to HuR (18,83). Additionally, HuR binds not only to iNOS mRNA but also to $asRNA_{iNOS}$ (18). These complicated interactions are involved in iNOS mRNA stability (29,83) and possibly in the function of the asRNA. Indeed, iNOS mRNA, asRNA, and various proteins form a stable RNA-protein complex (18).

Another example is the iron responsive element (IRE) and IRE-binding proteins. The IRE is present in the 3′UTR of the transferrin receptor mRNA and is recognized by IRE-binding proteins (87). 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. Another unique CCCH-type zinc-finger protein Zc3h12a (also known as regulatory ribonuclease-1, regnase-1) is a ribonuclease involved in the destabilization of IL-6 and IL-12 mRNA, as well as destabilization of

Zc3h12a mRNA itself (88). Regnase-1 recognizes the conserved stem-loop structure within their 3′UTRs and degrades these mRNAs.

Together with the data on RNA-binding proteins, evidence supports a role for the *cis*-controlling element as a scaffold for *trans*-acting factors that modulate mRNA stability.

4.3.2. Accessibility of RNA-binding proteins

RNA-binding proteins recognize specific nucleotide sequences—*i.e.*, *cis*-controlling elements. Accessibility of the RNA-binding protein may be determined not only by the recognition sequence but also within the context of the secondary structure. Meisner *et al.* characterized the binding of HuR protein to the ARE and identified secondary structure-dependent HuR recognition of mRN A (76). 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 (76). It is likely that this DNA:RNA hybrid-induced conformational change mimics an RNA:RNA hybrid-induced conformational change.

Furthermore, interactions of HuR protein with natural antisense transcripts in human HEK293 cells have been reported (89). The RNA-immunoprecipitation and exon microarray analyses revealed that HuR interacts not only with mRNAs but also with ncRNAs, including asRNAs, implying the involvement in the formation of mRNA-asRNA complexes. The authors also imply that secondary structures of 66–75 nts enhance HuR's recognition of its specific RNA targets composed of short primary sequence patterns.

Conversely, low-copy-number ncRNAs allosterically modify the RNA-binding protein TLS (for translocated in liposarcoma) to inhibit transcription (90). TLS protein specifically binds to CREB-binding protein (CBP) and p300 and inhibits CBP/p300 histone acetyltransferase activities on the human cyclin D1 (*CCND1*) gene. According to this evidence, interactions between an asRNA and an RNA-binding protein may cause allosteric changes in both the asRNA and protein. Therefore, the interaction of RNA (mRNA or asRNA) with an RNA-binding protein may alter the conformation to alter their functions or activities, threby affecting the mRNA stability or translatability.

4.4. Low-molecular-weight drugs and herbal medicines

4.4.1. Aspirin and acetylpromazine

Many chemically synthesized drugs have much lower molecular weights (< 1,000 Da) 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 up-regulates *TTP* gene expression to increase the destabilizing protein TTP, thereby reducing the stability of iNOS mRNA (91).

By contrast, several low-molecular-weight drugs bind directly to RNA and affect its structure and function. Aspirin (acetylsalicylic acid), a non-steroidal anti-inflammatory drug, intercalates RNA duplexes through both G:C and A:U base pairs and the backbone phosphate groups at a low concentration, whereas partial helix destabilization occurred at a high aspirin concentration (92). The donor groups (OCO) and ($OCOCH_3$) of aspirin are mainly involved in this aspirin–RNA interaction.

This partial helix destabilization (helix opening) may increase the chance of drug binding to different RNA donor sites that are locally melted (92). 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. Similarly, the synthetic estrogen diethylstilbestrol and RNA-staining dyes, such as acridine orange and methylene blue, also intercalate RNA duplexes (93–95). These data suggest a possibility that some low-molecular-weight drugs can intercalate RNA duplexes or externally bind to the phosphate groups of RNA, which causes a conformational change that may affect mutual recognition by mRNA and asRNAs. In support of this hypothesis, sodium salicylate (96) has been shown to reduce *iNOS* gene expression and destabilize iNOS mRNA by decreasing $asRNA_{iNOS}$ in hepatocytes, indicating that they can cause a conformational change in iNOS mRNA and/or $asRNA_{iNOS}$ to destabilize iNOS mRNA.

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 (97). Binding of acetylpromazine altered the three-dimensional structure of TAR and inhibited access of Tat protein to TAR (98). Dethoff *et al*. recently reported about nuclear magnetic resonance (NMR) visualization of RNA transitions toward invisible excited states, which result in RNA conformational changes (99). They applied this technique to characterize the TAR apical loop, as well as the kissing loops of the HIV-1 genome and ribosomal A-site internal loop. Such an approach may elucidate the detailed mechanism of RNA interactions in the future.

4.4.2. Other low-molecular-weight drugs

Many drugs and agents have been reported to destabilize iNOS mRNA, including the anti-inflammatory drug dexamethasone (100), neutrophil elastase inhibitor sivelestat (101), and antioxidant agent cysteamine (102). These substances have been shown to reduce the levels of both iNOS mRNA and $asRNA_{iNOS}$. By contrast, the anti-ulcer drugs rebamipide and 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) inhibitor pitavastatin have been shown to increase the levels of both iNOS mRNA and $asRNA_{iNOS}$ (103,104). Reporter assays performed in those studies have suggested that the drugs and agents that affect the levels of iNOS mRNA and $asRNA_{iNOS}$ regulate *iNOS* gene expression at the post-transcriptional level through the 3′UTR of the iNOS mRNA.

How does RNA recognize diverse small molecules, including drugs? The crystal structures of cobalamin (vitamin B_{12})-binding riboswitches, which regulate cobalamin biosynthesis in bacteria widely (105), may provide a hint to answer this question. The structures indicated that cobalamin recognizes RNA through shape complementarity between the RNA and cobalamin, with relatively few hydrogen bonding interactions. A composite cobalamin–RNA scaffold stabilizes a long-range intramolecular kissing-loop interaction that controls mRNA expression. The mechanisms of action of the low-molecular-weight drugs and any involvement of the asRNAs remain to be studied.

4.4.3. Herbal medicines and their constituents

Many herbal medicines and their constituents have been reported to destabilize iNOS mRNA. The levels of both iNOS mRNA and $asRNA_{iNOS}$ were reduced by hepatoprotective Kampo formulae *inchinkoto* (106) and *hochuekkito* (107) and by herbal medicines such as *kinginka* and its constituent chlorogenic acid (108), *gomishi* and its constituent gomisin N (109), and *chinpi* and its constituent nobiletin (110). 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.

In addition, functional foods, such as active hexose correlated compound (AHCC) and flavanol-rich lychee fruit extract (FRLFE), have been shown to reduce the levels of both iNOS mRNA and $asRNA_{iNOS}$ by regulation at the transcriptional and/or post-transcriptional levels through the 3′UTR of the iNOS mRNA (111–113). In *C. elegans*, AHCC increases the levels of hsf1 mRNA and $asRNA_{hsf1}$ to induce the transcription factor Hsf1, thereby enhancing the expression of several heat shock protein (*hsp*) genes (49).

The constituents of herbal medicines may interfere with the iNOS mRNA–asRNA interaction, thereby suppressing iNOS expression. It was recently reported that epigallocatechin gallate (*i.e.*, flavanol, catechin) directly binds to RNA (114) and that apigenin (*i.e.*, hydroxylated flavone) binds to RNA with high affinity (115). These results support the possibility that the flavonoids in herbal medicines interfere with mRNA–asRNA interactions by binding to the mRNA and/or asRNA, leading to the suppression of iNOS expression. Analyses of the effects of herbal constituents remain to be studied.

4.5. MicroRNAs

MicroRNA reduces mRNA stability and translatability by hybridizing with the 3′UTR of mRNA (5). This post-transcriptional mechanism is favorable to rapidly induce genes against various stimuli, such as infection and inflammation (29). Because microRNA preferentially targets mRNAs harboring ARE motifs (116), microRNA and ARE-binding proteins have a network to regulate mRNA expression. For example, a microRNA (miR-16) binds to an ARE motif in the 3′UTR of TNF-alpha mRNA and destabilizes the mRNA, which is involved in the regulation of translation (117,118).

Table 4. 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 and Ab / protein	The RNA–protein–Ab complex migrates more slowly than the RNA–protein complex in gel electrophoresis	RNA-binding proteins are identified using its Ab. 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 Ab, and the RNA–protein interaction is analyzed. RNA–RNA interaction is indirectly suggested	Ab is required. Formaldehyde crosslinking with protein may be performed before IP. 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
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 short interfering RNA (siRNA)	(–) /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, 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); Ab/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 Ab (IC)	Colocalization of RNA transcript and protein is demonstrated. Results provide indirect evidence of RNA-protein interaction

* Ab, antibody; asRNA, antisense RNA.

Both asRNA and microRNA possess an antisense sequence to the corresponding mRNA, and thus competition between the asRNA and microRNA is likely to occur, a process that may change the mRNA level. Indeed, the asRNA competed for a microRNA-binding site of BACE1 and IFN-alpha1 mRNA (20,57) (*see 5.4.*). Alternatively, if an asRNA shares many microRNA-binding sites with its mRNA, the asRNA may show a microRNA sponge effect by competing with the mRNA for microRNA binding (44,119), similar to the case of PTENpg1 lncRNA (*3.2.3.*).

4.6. Methods to analyze interactions with RNA

4.6.1. Methods to detect protein–RNA interactions

Various methods for the analysis of RNA interactions with protein and RNA are summarized in Table 4. Protein–RNA interactions can be detected using the yeast three-hybrid assay (Y3HA) (68,120). For example, an interaction between the IRE of transferrin mRNA and IRE-binding proteins was detected by Y3HA (87). Compared with protein–protein interactions detected by the yeast two-hybrid assay (Y2HA), protein–RNA interactions are relatively weaker (68).

Protein–RNA interactions can be detected by other methods, such as electrophoretic mobility shift assay (EMSA) with a labeled RNA probe and RNA-immunoprecipitation (RIP) assay with antibodies against RNA-binding proteins (18). In analyses of protein–RNA interactions, RNase activity in the protein preparation is problematic. RNase is abundant and is found in cellular and nuclear extracts, serum, and various protein

preparations, including antibodies. Inhibition of RNase activity is required throughout the entire experimental period.

4.6.2. Methods to detect RNA–RNA interactions

Compared with RNA–protein interactions, RNA–RNA interactions detected by the yeast RNA-hybrid assay (YRHA) (121) 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 asRNAs, are difficult to directly detect.

4.6.3. Natural antisense transcript-targeted regulation technology

A '*sense*' oligonucleotide can be used to examine the interactions between mRNA and asRNAs, as well as to determine the site of the interactions. Sense oligonucleotides are designed according to the mRNA sequence (3′UTR or CDS) and include at least one single-stranded loop. This loop of mRNA may hybridize with the corresponding loop of the asRNA. Sense oligonucleotides to mRNA have been shown to reduce the iNOS and IFN-alpha1 mRNA by inhibiting the mRNA–asRNA interaction and finally to reduce iNOS and IFN-alpha protein, respectively (18,20,39). A sense oligonucleotide can compete with mRNA and inhibit the mRNA–asRNA interaction (18). Alternatively, when the sense oligonucleotide (precisely oligodeoxyribonucleotides, ODNs; short DNAs) hybridizes with the asRNA, the resulting ODN:asRNA hybrid may be a substrate for RNase H (20). Our oligonucleotide-mediated natural antisense transcript-targeted regulation (NATRE; pronounced /nature/) technology also down-regulated the IL-23A mRNA level. Conversely, the NATRE technology up-regulated the mRNAs encoding several chemokines (*e.g.*, CCL2 and TNF-alpha) and the NF-kappaB p65 subunit (22,30).

By contrast, antisense oligoribonucleotides (ORNs; short antisense RNAs) can be used to attain the reverse effect to the sense ODN. For instance, the antisense ORNs that corresponded to the conserved secondary structure (BSL domain) of IFN-alpha1 mRNA augmented the IFN-alpha1 mRNA levels (20). These data suggest that the antisense ORN functionally mimics the $asRNA_{IFNA1}$, which stabilizes the IFN-alpha1 mRNA. The data also imply the possibility that the mRNA levels are controlled by either sense ODNs or antisense ORNs.

Similar approaches to the NATRE technology have been applied to the mRNA encoding tumor suppressor p53 to verify the interaction between p53 mRNA and its asRNA, $asRNA_{P53}$, which encodes the Wrap53 protein (122). Sense oligonucleotides blocked the p53 mRNA–$asRNA_{P53}$ interaction, resulting in a reduction in p53 mRNA. Modarresi *et al*. reported that inhibition of asRNAs by single-stranded oligonucleotides leads to increases in BDNF, GDNF and ephrin receptor B2 mRNA (40). Inhibition of $asRNA_{BDNF}$ up-regulated BDNF mRNA leads to increased protein levels and induces neuronal outgrowth and differentiation both *in vitro* and *in vivo*.

The NATRE technology is different from 'antisense technology,' a conventional mRNA knockdown method that uses single-stranded antisense oligonucleotides because the mRNA–asRNA interaction is not considered in antisense technology. The sense oligonucleotide in antisense technology is a negative control that does not change the mRNA levels, whereas the sense oligonucleotide in NATRE technology modulates the mRNA levels by interfering with the mRNA–asRNA interaction. Short interfering RNA (siRNA), which is a short double-stranded RNA, also reduces mRNA by binding to the 3'UTR, and an RNA-induced silencing complex (RISC) is involved in the degradation of mRNA by siRNA (123). The knockdown effects of the sense oligonucleotides on the iNOS mRNA levels are comparable to those of siRNA (39). When there is an asRNA sequence that does not overlap with the relevant mRNA, both the siRNA and sense oligonucleotide can selectively degrade the mRNA or asRNA.

4.6.4. Reporter assays

Reporter assays are another powerful method to analyze not only promoter activity (*i.e.*, transcriptional activity) but also mRNA stability and RNA–RNA interactions by measuring reporter protein activity or the half-life of a reporter mRNA. A reporter construct consists of the following: a promoter (inducible or constitutive), a reporter gene (*e.g.*, luciferase), a terminator or 3′UTR (including at least a poly(A) signal), and a vector. The activity of the reporter protein is affected by the promoter and 3′UTR. When a promoter of an mRNA or an asRNA

is used, the promoter activity can be estimated, implying the activity of *de novo* RNA synthesis. When a constitutive promoter (*e.g.*, elongation factor 1alpha promoter) is used, mRNA stability, as well as the effects of *cis*-controlling element(s) in the 3′UTR, can be estimated.

When the 3′UTR that harbors *cis*-controlling element(s) is ligated to a reporter gene, the stability of the reporter-UTR hybrid mRNA and reporter activity should change. When the firefly luciferase gene was ligated to the iNOS 3′UTR (124), which harbors a site for interaction with asRNA$_{iNOS}$, the half-lives of the luciferase mRNA and luciferase activity increased in the presence of asRNA$_{iNOS}$ (18). By contrast, when a construct with the luciferase-simian virus 40 late polyadenylation signal (SVLpA) (67) 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$_{iNOS}$ (18,124). SVLpA does not harbor *cis*-controlling elements that affect mRNA stability and, thus, is widely used for reporter plasmids. These constructs (luciferase–3′UTR and luciferase–SVLpA) were used to discriminate between the effects of the asRNAs and drugs on promoter activity and the effects on mRNA stability (100,104,111). Other genes, such as the beta-globin and green fluorescent protein genes, are also used as reporter genes to monitor mRNA stability (28,125).

The promoter activity of asRNA transcription can also be evaluated by the reporter assay with a construct harboring an asRNA promoter ligated to a reporter gene (see reference 18 for an example). It has been reported that the expression level of the asRNA is much lower than that of the mRNA. Generally, the mRNA:asRNA ratio correlates with the activity of promoters for the mRNA and asRNA, respectively. Therefore, it may be difficult to analyze the asRNA promoter by this assay.

To assess the effects of asRNA on mRNA expression, asRNA is either endogenously induced or exogenously over-expressed in reporter assays. The effects of microRNA over-expression can also be examined to evaluate the competition of microRNA and asRNA (see reference 20 for an example,). Genetically engineered mice (*e.g.*, knock-out or knock-in mice) may be finally required, when the regulation mechanism of the asRNA gene expression or physiological and pathophysiological roles of asRNA are studied *in vivo*, such as in a previous report on ARE-lacking mice (61).

Details of the above-mentioned methods and others for the analysis of RNA interactions have been reviewed elsewhere (126,127).

5. REGULATION OF MRNA STABILITY BY NATURAL ANTISENSE TRANSCRIPTS

5.1. mRNA stability and RNA–RNA interactions

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 (18,20,28,128), 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, revealing a half-life of 339 minutes in the presence of ActD (18). 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.

At various steps in gene expression, asRNAs may regulate the expression of inducible genes by interacting with *trans*-acting factor(s) and *cis*-controlling element(s). Among the many asRNAs that are involved in mRNA stability, only a few have been analyzed to demonstrate a direct interaction between the mRNA and asRNA (Table 2). Several putative mechanisms of regulation by asRNAs have been proposed (45,129). 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 2). In many cases, as described below, the initial interaction at loops involves the hybridization of several nucleotides (18,130), and the RNA–RNA interaction proceeds in concert with proteins. These interactions result in changes in the mRNA stability, and the half-life of mRNA is extended (Type B) or shortened (Type C).

5.2. Long RNA duplex formation

A Type A interaction starts with local hybridization of two long, complementary RNA molecules. During replication, dimerization of HIV-1

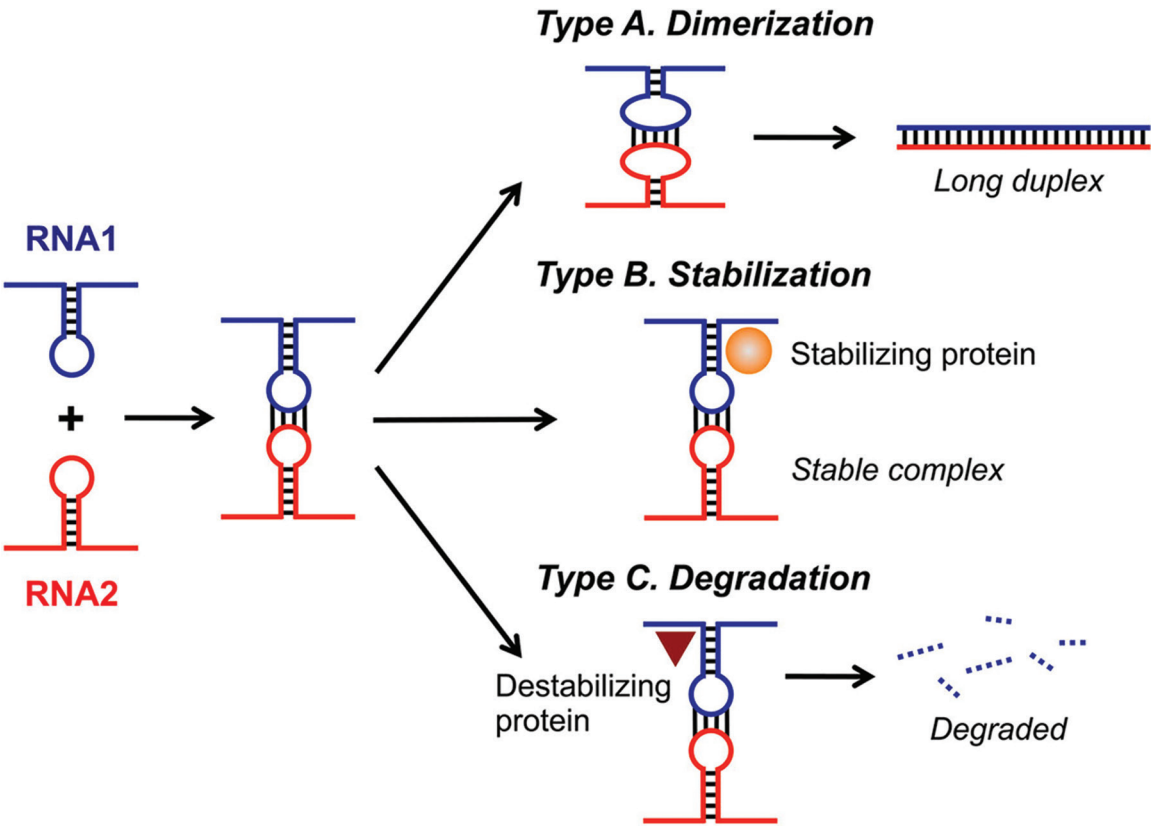

Figure 2. Types of intermolecular RNA–RNA interactions. When an RNA molecule interacts with another RNA molecule at loops by base-pairing hybridization (small bars), loop-loop interactions at stem-loop structures induce three different types of RNA–RNA interactions. Each stem-loop structure is depicted by two parallel bars and an open circle. Models are shown. Type A interaction (dimerization). A loop–loop interaction—*i.e.*, kissing loop interaction—triggers the hybridization of two RNA molecules to form a long RNA duplex, most likely in concert with dimerizing enzymes (*e.g.*, 131). Type B interaction (stabilization). Loop–loop interactions result in the recruitment of a stabilizing protein, such as HuR (60). RNA remains stable until the stabilizing protein detaches or a destabilizing protein attaches. Type C interaction (degradation). A loop–loop interaction results in the recruitment of a destabilizing protein (*e.g.*, tristetraprolin) (84) 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, such as stabilizing and destabilizing proteins, microRNA, and drugs.

genomic RNA occurs at its kissing loops (6 nt long); finally, a long RNA duplex of the genomes is formed (131). The resulting long RNA duplexes in virus-infected cells may be substrates for RNA editing or sources of endogenous siRNA to inhibit gene expression (132) and activate the signaling cascades that activate the IFN-alpha and beta (*IFNA* and *IFNB*) genes (133). Snail nNOS mRNA has been shown to hybridize with the asRNA from its pseudogene ($asRNA_{psNOS}$) to form a long RNA duplex in the CDS *in vivo* (32). This duplex of nNOS mRNA and $asRNA_{psNOS}$ was shown to prevent 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. Because type A interactions are reviewed elsewhere (129,130,132), we focus here on the following types of interactions.

5.3. mRNA–antisense transcript 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 2). 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 (134). The bcd mRNA dimerizes with the *trans*-acting factor Staufen at two kissing loops (6 nt long) in its 3′UTR to form stable ribonucleoprotein particles (130,134).

Several examples of interactions between mRNA and Type 1 asRNAs have been reported such as non-coding $asRNA_{iNOS}$ (18), $asRNA_{P53}$, which encodes Wrap53 protein (122), non-coding $asRNA_{IFNA1}$ (20), and non-coding $asRNA_{Tnf}$ (22) (Table 2). In all of the cases, direct evidence of the mRNA–asRNA interaction was supplied by sense oligonucleotide-mediated NATRE technology or a similar method. Reporter assays using luciferase gene–UTR constructs showed that the target of the asRNA was located in the mRNA UTR and was involved in the stability of the iNOS and p53 mRNAs (18,122).

The asRNA level may affect mRNA stability. When an asRNA is knocked down or over-expressed, the half-life of mRNA changes. For instance, sense oligonucleotides to the IFN-alpha1 mRNA degrade $asRNA_{IFNA1}$ with a 5-fold decrease in the half-life of the mRNA (20). By contrast, over-expression of $asRNA_{IFNA1}$ increased the half-life of IFN-alpha1 mRNA by 3-fold.

Based on the above-mentioned reports concerning RNA interactions, a model of asRNA-mediated regulation has been proposed (4). According to our '*recycling*' hypothesis, intermolecular interactions with mRNA and asRNAs occur at their loops (Figure 3). The short loop of the stem-loop structure of mRNA interacts with the corresponding loop of the asRNA (*4.2.*) as an initial interaction. The resultant short RNA:RNA duplex (several nts long) at the loops seems to be reversible and unstable *in vivo* due to the low melting temperature of the duplex. Loop-loop hybridization may occur between imperfectly complementary RNA molecules. When cross-homologies between the loop sequences exist, which are observed in the iNOS mRNA and $asRNA_{iNOS}$ (18), mRNA and asRNA may interact at the loops in various combinations.

Next, the asRNA is assumed to trigger a conformational change in the mRNA and partial destabilization at the stems (duplex), similar to the oligonucleotide-induced conformational change (*4.3.2.*) and drug–RNA interaction (*4.4.1.*). These changes may affect the accessibility of RNA-binding proteins, resulting in the recruitment of RNA-binding protein(s), such as stabilizing proteins (Type B interaction) or destabilizing proteins (Type C interaction). The stabilizing protein(s) can then promote protein–protein interactions to form an mRNA–asRNA–protein complex.

In a Type B interaction (Figure 2), this complex stabilizes the mRNA by prohibiting the access of deadenylation and decapping enzymes that degrade mRNA (135). The mRNA–asRNA–protein complex can be detected by the RIP assay with a specific antibody against an RNA-binding protein (for example, 18). Once a complex is formed and the mRNA is stabilized, the asRNA is no longer necessary. The asRNA is released from the complex and then recycled to stabilize another mRNA molecule. The mRNA gradually becomes unstable by the detachment of stabilizing protein(s) and/or binding of destabilizing protein(s), thereby leading to its degradation.

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. An example of this interaction was recently reported for the *Tnf* gene (22). According to our hypothesis (Figure 3), the initial loop-loop hybridization followed by conformational changes of RNA is the same as that of a Type B interaction. However, these changes most likely result in the recruitment of destabilizing protein(s) and/or detachment of stabilizing protein(s). It is possible that the destabilizing protein TTP (84) and stabilizing protein HuR (60) compete for ARE motifs. Furthermore, two ARE-binding proteins, TTP and its homolog BRF-1, are recruited and then activate mRNA decay, indicating that TTP and BRF-1 trigger the degradation of mRNA harboring ARE motif(s) (85).

Because many stabilizing and destabilizing proteins may interact with an mRNA, a balance of these proteins most likely determines the fate of each mRNA—*i.e.*, whether a Type B or C interaction occurs (30). When a destabilizing protein attaches to the mRNA, the asRNA is released from the complex and then recycled to degrade another mRNA molecule.

It has been reported that the expression level of the asRNA is much lower than that of the mRNA; for example, the mRNA/asRNA ratios are 7, 30, and 100 for *iNOS*, *IFNA1*, and *P53* genes, respectively (18,20,122). Similar results are reported

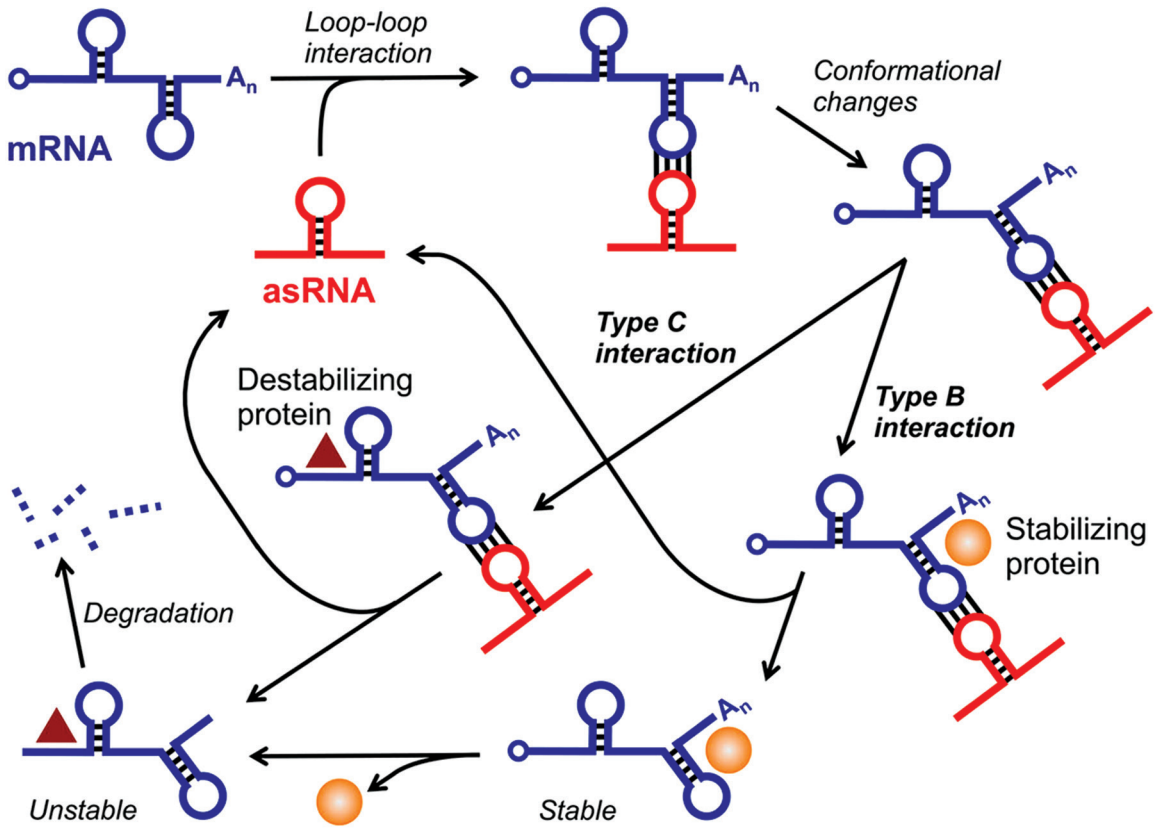

Figure 3. A model for the mechanism of asRNA-mediated post-transcriptional regulation. A '*recycling*' model for the mRNA–asRNA interaction, which is modified from the previous model (4), is indicated schematically. Both Type B and C interactions are indicated in this scheme. The 5' cap structure and 3' poly(A) tail of an mRNA are shown by a small circle and (A_n), 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 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. The asRNA triggers a conformational change in the mRNA to induce recruitment of RNA-binding proteins. As in a Type B interaction (Figure 2), a stabilizing protein is recruited, and this step further promotes protein–protein interactions to form a stable mRNA–asRNA–protein complex. Once the mRNA is stabilized, the asRNA is released from the complex and recycled to stabilize another mRNA molecule. The mRNA gradually becomes unstable and finally degraded. As in a Type C interaction, the conformational changes induce the recruitment of a destabilizing protein, leading to mRNA degradation. The asRNA is detached from the complex and then recycled. See details in the text.

in other genes (30). Because one of the major roles of the asRNA is thought to be a trigger to recruit proteins and form an mRNA–protein complex, our model (Figure 3) appears to be plausible in that a low-copy-number asRNA hits the mRNA and efficiently regulates mRNA stability during inducible expression. This hypothesis may explain the unsolved question concerning the stoichiometric mRNA–asRNA interactions (136)—*i.e.*, how a low-copy-number asRNA affects the functions of its mRNA. The asRNAs from many inducible genes that encode cytokines, chemokines, and transcription factors may mediate this novel post-transcriptional mechanism to modulate mRNA stability.

5.4. Interactions with microRNAs

Interactions of microRNA with mRNA and asRNA, as well as the above-mentioned mRNA–asRNA interactions, are important, in which *cis*-controlling elements (*e.g.*, AREs) and RNA-binding proteins (*e.g.*, ARE-binding proteins) are involved. Mainguy *et al*. performed transcriptional profiling of the *Hox* clusters, which play a crucial role in body patterning during animal development (137). The human and mouse *Hox* clusters encode both Hox transcription factors and microRNAs, as well as newly identified 14 non-coding transcriptional units antisense to *Hox* genes, 10 of which are conserved in the mouse clusters. Most of these asRNAs in both

human and mouse overlap with Hox mRNAs. These results provide a possibility that a network among the mRNAs, asRNAs, and microRNAs exists to coordinate *Hox* gene expression in development.

Although asRNA–microRNA interactions have been overlooked, a recent report showed that these two distinct groups of regulatory RNAs share a communal interface of action. Faghihi *et al*. demonstrated that the microRNA miR-485-5p and $asRNA_{BACE1}$ competed for a binding site in the BACE1 mRNA, which encodes BACE1 and is involved in the development of Alzheimer's disease (23). These findings support the existence of ncRNA-containing regulatory networks that may be implicated in Alzheimer's disease pathophysiology (57). In another case, IFN-alpha1 mRNA possesses a binding site for microRNA miR-1270 in a conserved secondary RNA structure with which $asRNA_{IFNA1}$ interacts (20). Various analyses have indicated that $asRNA_{IFNA1}$ masks the microRNA-binding site (also known as microRNA response element, MRE) to stabilize IFN-alpha1 mRNA. Because IFN-alpha1 induction is essential for the innate immune response against virus infection, it is likely that $asRNA_{IFNA1}$ and miR-1270 play pivotal roles in the rapid induction of IFN-alpha1 mRNA at the post-transcriptional level.

Recently, a '*competitive endogenous RNA'* (ceRNA) hypothesis in the regulatory network of the transcriptome was proposed (44). Like the PTENpg1 lncRNA (*3.2.3.*), a transcript that shares MREs with an mRNA sequesters many mRNA-targeting microRNAs by acting as a natural microRNA sponge by competing with the mRNA for microRNA binding, leading to the upregulation of the mRNA. Such a transcript (non-coding or protein-coding) is designated a ceRNA, and this ceRNA cross-talk has been discovered in many species and is assumed to be involved in transcription and translation (44,119). Indeed, Karreth *et al.* identified and validated several ceRNAs for PTEN mRNA and showed the relevance of the ceRNA networks for cancer biology (138). Because many ncRNAs that function as ceRNAs have been reported only recently (119), it is likely that there are long non-coding asRNAs (*i.e.*, asRNA ceRNAs) that share numerous MREs with the corresponding mRNAs.

5.5. Other mechanisms

The asRNAs may epigenetically regulate gene expression by modifying chromatin, such as in the *eNOS* (19) and progesterone receptor (139) genes, as well as the alpha isoform of PTENpg1 asRNA ($asRNA_{PTENpg1}$ alpha) (*3.2.3.*). In addition, Khalil *et al*. reported that lincRNAs associate with chromatin-modifying complexes and affect gene expression (21). Such epigenetic regulation by asRNA may be a new therapeutic target (140). Chromatin regulation remains to be studied in detail and warrants further investigation.

Recently, N^6-methyladenosine (m^6A)-dependent regulation of mRNA stability has been clarified (141). A modified RNA-seq analysis (m^6A-seq) confirmed that m^6A is the prevalent internal modification present in human and mouse mRNAs of all higher eukaryotes (54). Human YTH domain family 2 (YTHDF2) protein selectively recognizes m^6A present in both mRNA (particularly around the stop codon) and ncRNA to regulate mRNA degradation (141). Therefore, it is possible that the asRNA may include m^6A modification and that its stability is controlled by YTHDF2.

Nonsense-mediated mRNA decay (NMD) eliminates prematurely terminated transcripts and is another mechanism involved in mRNA stability (142). For example, activating transcription factor 5 (ATF5) mRNA stability is regulated by NMD factors through its 5′UTR (143). NMD is not discussed here.

Here, we reviewed various post-transcriptional mechanisms to regulate mRNA expression. Finally, we show an example of the regulation mechanisms for the expression of TNF-alpha mRNA, which is one of the best-studied mRNAs (Figure 4). The stability of TNF-alpha mRNA is assumed to be regulated by many factors in a hierarchy: *cis*-controlling elements in its 3′UTR (144,145); domains of secondary structure (22); *trans*-acting factors, including RNA-binding proteins (84,146), asRNA (22), and microRNA (117,118); and putative m^6A modification (54,141). Although an *in vivo* analysis using ARE-lacking mice was reported (61), the asRNA-mediated mechanisms and their implication in the pathophysiology of diseases remain not fully understood.

6. PERSPECTIVES

Inducible genes are involved in various diseases, such as inflammatory diseases, infectious diseases, autoimmune diseases, allergies, cancer, and neurodegenerative diseases. Many natural antisense transcripts seem to be transcribed and are involved in the regulation of post-transcriptional

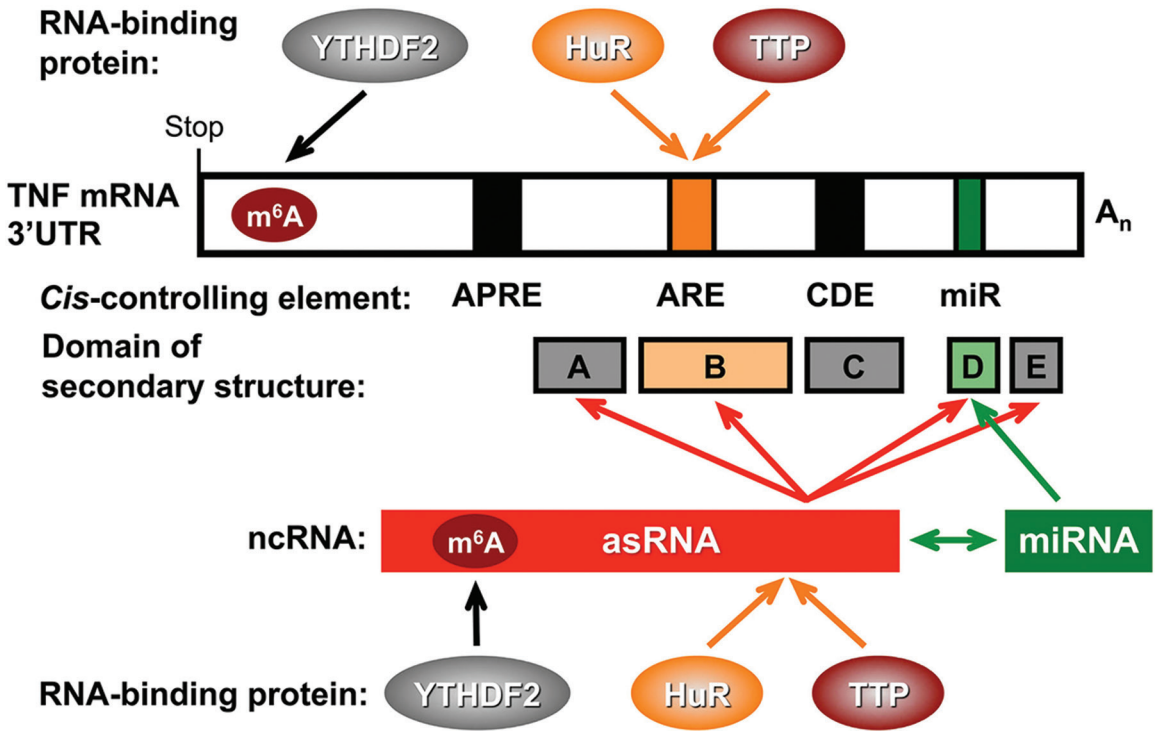

Figure 4. Various factors that affect the stability of TNF-alpha mRNA. The reported post-transcriptional mechanisms to regulate TNF-alpha mRNA expression are schematically shown. The 3' untranslated region (3'UTR; 831 nt) of rat TNF-alpha mRNA is indicated with its cis-controlling elements and *trans*-acting factors. The cis-controlling elements in 3'UTR are as follows: a cluster of AU-rich elements (ARE; 61); the 2-aminopurine-responsive element (APRE; 144); the constitutive decay element (CDE; 145); and a putative binding site (*i.e.*, an ARE motif) of the microRNA miR-16, which binds to this ARE motif and destabilizes TNF-alpha mRNA (117,118). These elements are primarily determined by reporter assays using mutated promoters of the *Tnf* gene. Domains of secondary structure (A to E) predicted by the mfold program (63) are shown beneath the cis-controlling elements. The TNF-alpha antisense transcript (asRNATnf) interacts with most of these domains to destabilize the TNF-alpha mRNA (22). The microRNA-binding site (miR) is located in domain D and may compete for this site with $asRNA_{Tnf}$ (22). The destabilizing protein tristetraprolin (TTP) (84) and stabilizing protein HuR (146) may compete for the ARE clusters in the TNF-alpha mRNA, as well as ARE motifs in $asRNA_{Tnf}$ (data not shown). YTH domain family 2 (YTHDF2) protein selectively recognizes N6-methyladenosine (m^6A) in the consensus motif [5'-(A/G)ACU-3'] that exists in the mRNA, as well as ncRNA, to regulate mRNA degradation (54,141). Because putative m^6A sites are present in both TNF-alpha mRNA and $asRNA_{Tnf}$ (data not shown), YTHDF2 may bind to both of them.

events, such as mRNA stability and translation. Accumulating data confirm that the asRNA-mediated post-transcriptional mechanisms via various RNA–RNA interactions are essential for mRNA expression of inducible genes. Furthermore, it is likely that the asRNA-mediated post-transcriptional mechanisms have a close relationship with the microRNA-based mechanism to form a transcriptome network. Therefore, functional studies performed *in vitro* and *in vivo* are necessary to elucidate the biological and physiological roles of asRNAs in detail.

Additionally, asRNAs, as well as asRNA-mediated post-transcriptional mechanisms, could be new therapeutic targets for many diseases, including cancers. Investigation of these mechanisms could have clinical implications to elucidate the pathophysiology of these diseases. Further studies should clarify the relevance of this new field of natural asRNAs.

7. ACKNOWLEDGMENTS

We wish to thank Ms. Noriko Kanazawa for secretarial assistance. This work was supported, in part, by research grants from the following: Amino Up Chemical Co., Ltd. (Sapporo, Japan) to all the authors; Lion Corporation (Tokyo, Japan) to M. N.; the Ritsumeikan Global Innovation Research Organization (R-GIRO) of the Ritsumeikan University to M. N., Y. I., T. O. and T. K.; the Japan Society for the Promotion of Science (JSPS) KAKENHI Grant

No. 24657121 to M. N. and No. 24510283 to T. K.; and the Japan Science and Technology Agency (JST) No. AS2511389Q to T. K.

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Abbreviations: mRNA: messenger RNA; asRNA: antisense transcript; ncRNA: non-coding RNA; rRNA: ribosomal RNA; tRNA: transfer RNA; miRNA, miR: microRNA; nt: nucleotide; 3′UTR: 3′ untranslated region; HIF: hypoxia-induced factor; iNOS: inducible nitric oxide synthase; eNOS: endothelial nitric oxide synthase; IFN: interferon; lincRNA: large intergenic non-coding RNA; TNF: tumor necrosis factor; BACE1: beta-site amyloid precursor protein-cleaving enzyme 1; lncRNA: long non-coding RNA; kb: kilobase; ERG: early response gene; COX-2: cyclooxygenase 2; IL: interleukin; NF-kappaB: nuclear factor-kappaB; LPS: lipopolysaccharide; BDNF: brain-derived neurotrophic factor; GDNF: glial-derived neurotrophic factor; nNOS: neural nitric oxide synthase; FGFR3: fibroblast growth factor receptor 3; CDS: coding sequence; PTEN: phosphatase and tensin homolog; RPA: ribonuclease protection assay; RT-PCR: reverse transcription-polymerase chain reaction; qPCR: quantitative PCR; cDNA: complementary DNA; RT: reverse transcription; NGS: next-generation sequencing; ARE : AU-rich element; CRM1: chromosome region maintenance 1; RNase: ribonuclease; HuR: human homolog R of the embryonic lethal-abnormal visual; AUF1: AU-binding factor 1; hnRNP: heterogeneous nuclear ribonucleoprotein; TTP: tristetraprolin; KSRP: KH-type splicing regulatory protein; CCCH: Cys-Cys-Cys-His; BRF-1: butyrate response factor 1; PTB: polypyrimidine tract-binding protein; IRE: iron responsive element; HIV-1: human immunodeficiency virus type I; Y3HA: yeast three-hybrid assay; EMSA: electrophoretic mobility shift assay; RIP: RNA-immunoprecipitation; ODN: oligodeoxyribonucleotide; NATRE: natural antisense transcript-targeted regulation; ORN: oligoribonucleotide; siRNA: short

interfering RNA; RISC: RNA-induced silencing complex; ActD: actinomycin D; bcd: bicoid; MRE: microRNA response element; ceRNA: competitive endogenous RNA; m^6A: N^6-methyladenosine; YTHDF2: YTH domain family 2; NMD: nonsense-mediated mRNA decay

Key Words: Natural Antisense Transcript, mRNA Stability, Secondary Structure, Non-Coding RNA, Herbal Medicine, Review

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