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PROSPECTS

Diversity of Antisense Regulation in Eukaryotes: Multiple Mechanisms, Emerging Patterns

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Abstract High-throughput analysis of RNA molecules in multicellular eukaryotes has revealed an abundance of complementary antisense RNAs that are transcribed from separate or overlapping genes. In mammals these include many novel non-coding RNAs of unknown function. This unexpected complexity of the mammalian transcriptome suggests that expression of many genes is regulated post-transcriptionally by mechanisms mediated by RNA–RNA base pairing. The recent discovery of the widespread expression of microRNAs in animals and plants provides a prototypic example of such regulation in eukaryotes. However, there are likely to be numerous other types of antisense regulation in eukaryotes, many as yet uncharacterized. J. Cell. Biochem. 93: 664–671, 2004. © 2004 Wiley-Liss, Inc.

Key words: antisense RNA; RNA–RNA base pairing; double-stranded RNA; microRNA; non-coding RNA; RNA interference

The role of antisense RNA in regulating gene expression is well established in prokaryotic systems. Complementary RNAs transcribed from overlapping genes were first shown to regulate the replication of plasmids in 1981 [Eguchi et al., 1991]. The first cellular antisense RNA, micF, was identified in *E. coli* in 1984 and was shown to repress translation of its target, OmpF mRNA, through base pairing with its 5′ end. Since then other antisense RNAs have been described in bacteria and have been shown to repress (and in some cases activate) gene expression through RNA–RNA base pairing [Wagner and Flardh, 2001].

Antisense regulation has proven more difficult to demonstrate in eukaryotes despite the widespread use of artificial antisense molecules to target repression of selected genes. Recently, a large number of overlapping transcripts have been described in mice and humans [Okazaki et al., 2003; Yelin et al., 2003]. These pairs of transcripts may be best regarded as candidate systems for antisense regulation, though in most cases the existence of a regulatory interaction has not yet been demonstrated.

One exciting front in the exploration of antisense regulation involves the discovery of hundreds of small microRNAs (miRNAs) in diverse multicellular organisms. This work builds on pioneering studies in the laboratories of Victor Ambros and Gary Ruvkun [reviewed by Bartel, 2004]. In animals, miRNAs act by pairing to complementary elements in the 3′ UTR of target mRNAs and blocking their translation. The mechanism of this inhibition is not well understood, in part because the target sequences for most miRNAs are unknown. The discovery of miRNAs definitively demonstrates a role for endogenous antisense RNA in regulating gene expression in eukaryotes. Although the specific regulatory function of most miRNAs remains to be established, it appears that many play important roles in development, differentiation and other key processes [Bartel and Chen, 2004].

The discovery of RNA interference (RNAi) and related phenomena directed by double-stranded RNA (dsRNA) is yet another area in which recent developments have contributed to

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a growing interest in antisense regulation. Although dsRNAs were previously known to provoke a non-targeted, interferon-mediated response in mammals, RNAi represents a specifically targeted response to dsRNA that results in destruction of homologous transcripts. RNAi is mediated by a highly conserved molecular apparatus that also mediates a wide range of other genetic processes, including heterochromatin formation, DNA elimination, and miRNA biogenesis [Hannon, 2002; Grewal and Moazed, 2003]. The unexpected abundance of complementary pairs of sense/antisense transcripts coupled with the RNAi and other dsRNA-induced responses poses major challenges to achieving a comprehensive understanding of gene expression at the genomic level.

In this article, I will examine the evidence for widespread regulation mediated by RNA–RNA base pairing. The possible role of antisense regulation mediated by diverse classes of complementary RNAs has broad implications for understanding the regulation of complex genomes.

DIVERSITY OF ANTISENSE REGULATION

Antisense transcripts in eukaryotes are remarkably diverse. They can be distinguished by size, coding potential, and orientation with respect to complementary target sequences (Fig. 1 and Table I). Other properties that are likely to be important for regulatory functioning, such as copy number, stability, and secondary structure, also vary widely. Since most RNAs function via base pairing with other RNA molecules, the term antisense regulation generally refers to regulation of expression of an RNA target through direct base pairing with a complementary RNA. Such regulation is usually negative, but in some cases an antisense RNA facilitates expression of its target. For example, base pairing of DsrA antisense RNA with OxyS mRNA in *E. coli* promotes the translation of OxyS by disrupting intramolecular secondary structure that prevents ribosome binding [Lease and Belfort, 2000]. Similarly, positive antisense regulation is also found in eukaryotes [Tasheva and Roufa, 1995].

The most important distinction for purposes of classifying different antisense RNAs is that between *cis*-encoded and *trans*-encoded RNAs. *trans*-antisense RNAs are those encoded at a locus separate from that of the target gene,

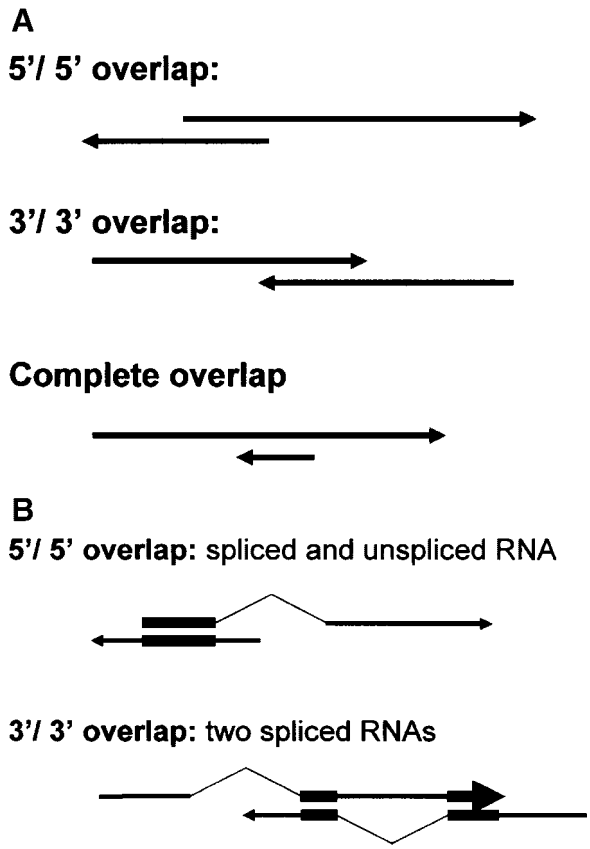

Fig. 1. Patterns of overlaps between *cis*-encoded RNAs. **A**: The three possible patterns for overlapping transcription units. **B**: Patterns of exon–exon overlaps between spliced and unspliced transcripts. Representative patterns of overlaps are shown to illustrate alterations of overlap and non-overlap regions in pairs including one or two spliced RNAs. Exons are indicated with horizontal lines; complementary regions within exons are indicated in bold.

while *cis*-antisense RNAs are those that are transcribed from two overlapping genes located on opposite strands of the DNA. *cis*- and *trans*-antisense RNAs differ in at least two functionally important properties. First, while

TABLE I. Diversity of Mouse Antisense Pairs in the FANTOM2 Collection

	5′/5′	Complete	3′/3′	Total
ncRNA/ncRNA	97	83	118	298
ncRNA/mRNA	506	421	372	1,299
mRNA/mRNA	303	170	361	834
Total	906	674	851	2,431

Analysis of 2,431 pairs of complementary RNAs that overlap by 20 or more base pairs in the mouse genome [Okazaki et al., 2002; Kiyosawa et al., 2003]. Pairs are classified by coding (mRNA) or non-coding (ncRNA) properties of component RNAs and the overlap orientation.

cis-antisense RNAs form perfectly matched pairs with their complementary targets, the pairing of *trans*-antisense RNAs with their targets is usually interrupted by multiple mismatches. Second, *cis*-antisense RNAs have a unique relationship to their overlapping transcripts. In contrast, a given *trans*-antisense RNA may pair equally well with multiple targets.

Direct demonstration of base pairing between sense and antisense RNA through genetic or biochemical analysis is rare. The best-characterized examples of eukaryotic antisense regulation involve *trans*-encoded miRNAs. At 21-22 nucleotides in length, miRNAs are among the smallest naturally occurring RNAs. The sequence of the miRNA and its target may be independently altered to assess requirements for their interaction. At the other extreme, some antisense RNAs are exceptionally large, *cis*-encoded transcripts. These include several imprinted RNAs, such as the *Ube3a* antisense transcript, which is about 460,000 nucleotides long and overlaps multiple transcripts [Runte et al., 2001]. For *cis*-encoded RNAs it is not possible to independently alter the sequences of regulator and target. The large size of the complementary overlap also makes it difficult to identify specific regions of sequences critical for regulation.

VERSATILITY OF *trans*-ANTISENSE RNAs

The first miRNAs to be described, *lin-4* and *let-7* mi RNAs in *C. elegans*, are still the best-characterized eukaryotic antisense RNAs. *lin-4* and *let-7* regulate larval development in the nematode by blocking translation of target mRNA through base pairing with target sequences located in the 3′ UTRs of *lin-14* and *lin-41* mRNAs, respectively [reviewed by Bartel, 2004]. In each case translation of the target mRNA is inhibited but both the miRNA and mRNA remain stably associated with polysomes. *lin-4* and *let-7*, like other miRNAs, are transcribed as part of longer precursors. The processing of miRNAs and their effects on target expression are mediated by factors also required for RNAi.

Antisense regulation by miRNAs in plants is different from that established for *lin-4* and *let-7*. Plant miRNAs form nearly perfectly paired duplex throughout the length of their target sequences, which are typically located within the coding region [Bartel, 2004]. The result of these interactions is cleavage of the mRNA target rather than translational silencing. This mechanism closely resembles that observed in RNAi.

Other types of small, *trans*-antisense RNAs may also exist. Small nucleolar RNAs (snoRNAs) represent another distinct class of small RNAs. Like miRNAs, snoRNAs base pair with complementary target sites [Kiss, 2002]. However, most snoRNAs act as guide sequences and direct site-specific modification of nucleotides at the target sites, as opposed to directly regulating their expression. The function of certain snoRNAs is not yet understood, and some may act to directly regulate target expression [Cavaille et al., 2000].

Pseudogene transcripts represent another potential source of antisense RNAs. Although generally considered non-functional gene copies, many pseudogenes are actively transcribed. In several instances antisense copies of duplicated genes or processed pseudogenes post-transcriptionally block expression of the functional parental gene. For example, the expression of nitric oxide synthase is down regulated in giant neurons of the pond snail by a pseudogene transcript that contains a 150-nucleotide inversion [Korneev et al., 1999]. Inverted duplications are also a source of *trans*-antisense transcripts that inhibit expression of the parent gene [Okano et al., 1991]. Given the abundance of pseudogenes and growing evidence for their transcription, antisense pseudogene transcripts may be present in many organisms [Hirotsune et al., 2003].

Further examples of *trans*-antisense RNAs have been identified within mRNAs transcribed from disparate loci on the human and mouse genome. A significant amount of such complementarity can be attributed to copies of short repetitive retroelement sequences (SINEs) incorporated into mRNA of the human. Even when complementary repetitive sequences are excluded, pairs of *trans*-encoded mRNAs represent almost half of the complementary mRNAs found in the human RefSeq library [Lehner et al., 2002]. These results suggest that other trans-encoded complementary transcripts may occur frequently in the human genome.

ABUNDANCE OF *cis*-ANTISENSE RNAs

Until recently fewer than 50 pairs of complementary *cis*-encoded transcripts had been

identified in eukaryotes, and such overlapping RNAs were considered rarities [Shendure and Church, 2002]. In the last 3 years this perception has changed dramatically with the identification of thousands of overlapping RNAs in the genomes of animals and plants [Okazaki et al., 2002; Yelin et al., 2003; Osato et al., 2003]. The FANTOM2 consortium identified a total of 2,431 pairs of complementary transcripts that overlap by a minimum of 20 base pairs within a set of 37,000 clusters of full-length mouse cDNAs [Okazaki et al., 2002]. This collection was expanded by an additional 899 pair of overlapping transcripts in which exons of one transcript are complementary to introns of the other [Kiyosawa et al., 2003]. Thus, about 15% of the FANTOM2 transcript clusters are paired with overlapping transcription units on the opposite strand of the DNA. Compugen, Inc., (Tel Aviv, Israel) compiled a similar collection of 2667 pairs of overlapping human RNAs from EST sequences [Yelin et al., 2003]. Both the mouse and human cDNA collections in which these antisense pairs were identified include more than 15,000 non-coding RNAs. The number of sense/antisense pairs in the mammalian genome is probably significantly larger than that found by either study. The total number of transcription units in mouse is estimated at nearly twice that found by the FANTOM2 consortium [Okazaki et al., 2002]. Microarray analysis of transcripts from human chromosomes 21 and 22 also indicates the presence of many unannotated non-coding RNAs and antisense RNAs [Kampa et al., 2004].

On the basis of orientation and coding sequences, many different configurations of overlaps might be expected and most, indeed, have been found. Pairs of transcripts can be classified according to the coding potential and the presence or absence of introns in each of the complementary transcripts, and by overlap orientation (Table I). The three possible overlap orientations include 5′/5′ overlaps where both promoters are located within the transcriptional unit on the opposite strand, 3′/3′ overlaps where overlapping RNAs are convergently transcribed from promoters external to the overlap region; and complete overlaps where one transcript is initiated upstream and terminated downstream of the other (Fig. 1A). In pairs of two spliced mRNAs, 3′ overlaps are the most common orientation. On the other hand, noncoding RNAs show a preference for 5′/5′ overlaps with mRNAs [Table I; also Yelin et al., 2003]. Promoters of 5′/5′ overlapping genes are often located opposite introns on the complementary strand. Less frequently both the promoters are located within the 5′ exons of the transcripts. Such closely spaced promoters may be coregulated by overlapping promoter elements.

If one or both transcripts are spliced, each may be a mosaic of alternating overlapping and non-overlapping segments (Fig. 1B). The effective length of the overlap will depend on whether sense and antisense pair before or after splicing. For spliced RNAs, only exon–exon overlaps would yield complementary transcripts, while overlaps between both exons and introns may result from base pairing between unspliced pre-mRNAs.

FUNCTIONAL CORRELATES OF *cis*-ANTISENSE REGULATION

Several recent reviews describe the diversity of sense/antisense RNAs in different species [Kumar and Carmichael, 1998; Lehner et al., 2002; Shendure and Church, 2002]. Here, I will describe three examples that illustrate the diverse structural organization and potential regulatory interactions between pairs of complementary overlapping RNAs.

Alternative processing of α-thyroid hormone receptor transcripts in mammals yields mRNAs encoding functionally antagonistic nuclear receptor proteins, TRα1 and TRα2. TRα2 mRNA, encoding a non-hormone-binding variant, is formed by use of an alternative 3′ terminal exon located downstream of the 3′ exon specific for TRα1 [refs. in Hastings et al., 2000]. A gene for a third nuclear receptor protein, Rev-Erbα, is located on the opposite strand of the genomic DNA, where its 3′ exon overlaps TRα2 but not TRα1 mRNA. Several lines of evidence suggest that expression of the complementary Rev-Erbα mRNA negatively regulates TRα2 processing, resulting in a shift in the ratio between TRα1 and TRα2. Levels of Rev-Erbα and TRα2 mRNAs vary in a reciprocal fashion in many cells and tissues. Where Rev-Erbα mRNA levels are high, TRα2 mRNAs levels are low relative to alternatively spliced TRα1 mRNA [Lazar et al., 1990; Hastings et al., 2000]. Overexpression of Rev-Erbα in vivo results in a shift in the TRα1/TRα2 ratio, and Rev-Erbα RNA efficiently blocks TRα2 splicing in vitro [Munroe and Lazar, 1991]. Finally, the constant stability of

TRα1 and TRα2 mRNAs with increased expression of Rev-Erbα provides evidence that the co-regulation of these genes is post-transcriptional, consistent with an effect on mRNA splicing [Lazar et al., 1990].

Since TRα1 and TRα2 are functionally antagonistic transcription factors small changes in their relative expression may have significant physiological effects. Recent studies have shown that Rev-Erbα is a core component of the circadian oscillator in mammals and that its mRNA levels show cyclic fluctuations in many cell types within a 24 h period [Preitner et al., 2002]. These findings suggest a model in which circadian fluctuations in Rev-Erbα mRNA may modulate the cellular response to thyroid hormone by altering the balance between TRα1 and TRα2 expression [Crosthwaite, 2004]. In this model, Rev-Erbα's previously defined role as a negative transcriptional regulator in the mammalian clock [Preitner et al., 2002] would be augmented by its role as an antisense regulator of TRα2.

The second example of antisense regulation involves the post-transcriptional regulation of the translational initiation factor eIF-2α during T cell proliferation [Noguchi et al., 1994]. eIF-2α levels are low in quiescent T cells and become rapidly elevated upon mitogenic stimulation. The increase in eIF-2α mRNA primarily reflects its stabilization and is accompanied by a parallel decrease in an antisense transcript initiated at a promoter within the first intron of the eIF-2α gene. The activity of the antisense promoter correlates inversely with the accumulation of eIF-2α. The antisense transcript itself has been detected only by RT-PCR due to its low levels and apparent instability [Noguchi et al., 1994]. The location of an antisense promoter within an exon of a regulated mRNA may prove a common feature of antisense regulation in mammalian cells.

A final example of possible antisense regulation involves two large non-coding RNAs that regulate X chromosome inactivation. In mammals, X chromosome inactivation depends on the expression *in cis* of a 17 kb non-coding RNA transcript designated *Xist*. The expression of *Xist* RNA on an inactive X chromosome depends on the shutdown of a large, completely overlapping antisense transcript, *Tsix*, that negatively regulates *Xist* expression, also *in cis*. Both *Xist* and *Tsix* are multiply spliced but neither codes for protein. Several models have been proposed by Ogawa and Lee [2002] to account for the allele-specific regulation of *Xist* expression by *Tsix*. Negative regulation of *Xist* by *Tsix* on the active X chromosome may reflect an effect of *Tsix* transcription per se that shuts down transcription of the *Xist* gene, either through epigenetic alterations in chromatin structure or by transcriptional interference. Alternatively, *Tsix* RNA may be required to titrate out the *Xist* transcript by base pairing. The large excess of *Tsix* over *Xist* just prior to shutdown of *Xist* on the active X is consistent with such an antisense mechanism.

The allele-specific interactions between *Tsix* and *Xist* resemble interactions between complementary RNAs at a number of other imprinted loci in mammalian genomes [Runte et al., 2001; Kiyosawa et al., 2003]. At these loci genes expressed from either the maternal-specific or paternal-specific allele are paired with reciprocally expressed non-coding antisense RNAs [Ogawa and Lee, 2002]. Since double-stranded RNAs have been shown to be involved in remodeling of chromatin through a Dicer-dependent mechanism, a requirement for RNA-RNA base pairing may reflect either direct antisense regulation or an RNAi dependent pathway [Grewal and Moazed, 2003].

These examples illustrate the diverse roles antisense RNAs play in cellular regulation and represent all three possible overlap orientations and all combinations of coding and non-coding sequences. Although there is significant evidence for antisense regulation in each instance, rigorous proof of an antisense mechanism requires that regulation in each case be tightly linked to the base pairing between complementary RNAs.

MECHANISMS OF ANTISENSE REGULATION

In principle an antisense regulator may act at any step required for the biogenesis or mobilization of the target RNA, including transcription, processing, transport, translation, or storage. Key questions involve determining the stage in expression at which the antisense regulator and its target pair, the sites on each molecule that are critical for efficient pairing, and consequences of pairing for the activity of the targeted RNA.

Translational regulation is the most common mechanism observed for antisense regulation. In prokaryotes antisense RNAs target initiation

by pairing with the ribosomal binding site and the initiation codon. microRNAs also block translation in animals but do so by pairing with complementary sequence elements near the 3′ end. Several other examples of translational inhibition by *cis*- or *trans*-acting antisense RNAs have also been proposed [reviewed by Kumar and Carmichael, 1998].

Since formation of a base-paired intermediate is a key feature of all antisense mechanisms, a thorough mechanistic understanding will require an analysis of the factors that promote formation of base pairing and those that determine the specific regulatory effect. Base pairing may be demonstrated in *trans*-antisense RNAs by examining the effects on regulation of complementary mutations in the antisense and target sequences. The direct characterization of base pairing in vivo is difficult for several reasons. Base pairing between two complementary RNAs may be constrained by intramolecular folding. Also, base pairs may form only transiently or the dsRNA formed may be rapidly degraded or modified, and thereby escape detection [Eguchi et al., 1991; Wagner and Brantl, 1998].

In many eukaryotes dsRNAs constitute a potent signal that provokes several responses at the cellular level: a non-targeted interferon-mediated response in mammals, which results in a general shutdown of translation culminating in apoptosis, and two types of targeted responses, namely, Dicer-dependent RNAi-related responses and ADAR-directed modification of dsRNA. Dicer is an RNase III-like endonuclease that cleaves dsRNA into miRNA-sized fragments of small interfering RNAs (siRNA) [Hannon, 2002]. ADARs are a family of dsRNA deaminases that convert adenosine to inosine within the double-stranded portion of the molecule [Bass, 2002]. Both Dicer and ADAR produce characteristic products that provide strong evidence for the presence of dsRNA.

An important example of ADAR-modified dsRNA formation is observed in cells infected with polyomavirus during the transition from early to late phase replication and. In this system high levels of read through transcription of late viral genes suppress expression of complementary early mRNA [Kumar and Carmichael, 1997]. The early and late RNAs form long, perfectly matched duplex structures that are extensively modified by ADAR. The modified inosine-containing RNAs are retained in the nucleus, thereby resulting in the rapid shutdown of early gene expression [Zhang and Carmichael, 2001]. This extensive modification of viral RNA further demonstrates that long, *cis*-encoded mRNAs form long dsRNAs in vivo.

The extreme sensitivity of the interferon response in mammals and the absence of high levels of endogenous siRNAs or ADAR-modified transcripts indicate that most endogenous dsRNAs are either unstable or sequestered within the cell. The efficacy of antisense regulation depends on the annealing and unwinding proteins, helicases and chaperones that direct and restrict RNA–RNA pairing in vivo [Herschlag, 1995; Lorsch, 2002]. The involvement of dsRNA and RNAi machinery in heterochromatin formation in fungi, flies and plants provide further evidence for a complex response to antisense RNA in eukaryotic cells.

PROSPECTS

The genomes of multicellular organisms are far more complex than anticipated, and there is accumulating evidence that non-coding RNAs are involved in novel regulatory roles. Three lines of research are particularly important for understanding the roles of diverse antisense RNAs in regulating gene expression in eukaryotes.

First, the systematic identification, characterization, and analysis of novel antisense RNAs provides a promising approach similar to that used in the characterization of snoRNAs and miRNAs. Since antisense RNAs represent a significant fraction of the total transcriptome of many organisms, identification of both *cis*- and *trans*-antisense RNAs will contribute substantially to our overall understanding of transcriptional activity. One particularly important goal is to identify new classes of *trans*-antisense RNAs that share well-defined sequence or structural motifs. Characterization of the expression and turnover of novel antisense RNAs represents an essential first step in identifying their roles in regulation.

Second, it is important to develop additional methods for characterizing intermolecular RNA–RNA base pairing in order to determine requirements for RNA–RNA base pairing in vivo. This approach will facilitate identification of RNA structural features or protein binding sites that are associated with nucleation sites for base pairing on different transcripts. Such

cis- and *trans*-acting factors are likely to be involved in other aspects of eukaryotic RNA metabolism. RNA helicases, chaperones, and annealing proteins have been shown to play a role in certain aspects of RNA metabolism but other roles remain to be discovered [Lorsch, 2002].

Finally, the identification and characterization of pathways impacted by the formation of double-stranded RNA represents a third critical area of research. As different classes of complementary interacting RNAs are discovered, it is likely that new pathways will be found that mediate general and targeted responses to these interactions. Intersecting pathways mediating RNAi and regulation via miRNAs are only now beginning to be explored.

Each of these lines of attack is likely to yield new insights into mechanisms of gene regulation. Although elucidation of the pathways involved in eukaryotic antisense regulation is challenging, characterization of antisense RNAs and the complementary interactions between antisense RNAs and their targets is likely to provide continuing insights into novel aspects of gene regulation.

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