Cell. Mol. Life Sci. 63 (2006) 2102–2118
1420-682X/06/182102-17
DOI 10.1007/s00018-006-6070-2

Cellular and Molecular Life Sciences

Review

Overlapping transcripts, double-stranded RNA and antisense regulation: A genomic perspective

S. H. Munroe[a, *] and J. Zhu[b, *]

[a] Department of Biological Sciences, Marquette University, Wehr Life Sciences Building, PO Box 1881, Milwaukee, WI 53233 (USA), Fax: +1 414 288 7357, e-mail: munroes@marquette.edu
[b] Institute for Genome Sciences and Policy, Duke University, Durham, NC 27708 (USA), Fax: +1 919 668 0795, e-mail: jun.zhu@duke.edu

Received 13 February 2006; received after revision 19 May 2006; accepted 7 June 2006
Online First 17 July 2006

Abstract. Bioinformatic analysis of the transcriptomes of diverse eukaryotes has demonstrated the ubiquity and structural diversity of complementary antisense RNAs. These include both trans-encoded microRNAs and a large population of cis-encoded antisense RNAs that encompasses both coding and non-coding RNAs. Antisense regulation has previously been characterized primarily as a post-transcriptional response affecting RNA stability, nuclear processing, export, or translation. However, the formation of double-stranded (ds) RNAs by base-pairing between complementary RNAs may elicit regulatory responses at the transcriptional level as well. Analysis of antisense transcription at several imprinted loci has suggested a number of other mechanisms that may not require formation of dsRNA. Understanding the integration of transcriptional and post-transcriptional regulatory mechanisms represents a major challenge for understanding antisense regulation in eukaryotes. Such insight is also essential for understanding general principles of genetic regulation within the complex genomes characteristic of mouse and man as well as those of other eukaryotes.

Keywords. Antisense RNA, microRNA, non-coding RNA, RNA interference, RNA-RNA base pairing, double-stranded RNA.

Introduction

Although 5 years have passed since the first sequences of the human genome were published [1, 2], efforts to understand its large-scale organization are only now beginning to yield novel insights [3]. Perhaps the most remarkable and unexpected finding thus far is the discovery of a large class of small RNAs known as microRNAs (miRNAs) that directly modulate gene expression in many animals and plants. Within the human genome hundreds of miRNAs regulate expression of mRNAs coding for many important functions [4]. Stunning in its own right, this finding is best viewed as part of a larger emerging theme: the unexpected diversity of RNA-directed control mechanisms [5–8]. Ongoing studies of eukaryotic genomes have revealed surprising levels of transcriptional complexity. While the mammalian genome appears to comprise far fewer protein-coding genes than anticipated [9, 10], regions of the genome that previously appeared non-functional are now known to be actively transcribed to yield thousands of non-coding RNAs (ncRNAs) whose functions, if any, are unknown [11–13].

Here we focus on one particular characteristic of eukaryotic genomes, the presence of complementary RNAs known as antisense RNAs. Antisense RNAs are transcribed either from separate, non-overlapping loci that share complementary sequences (trans-antisense RNAs; Fig. 1) or from overlapping loci on opposite strands of the DNA (cis-antisense RNAs; Fig. 2). Each antisense RNA may potentially base-pair with its complementary 'sense'

* Corresponding authors.

RNA; the two together comprise a 'sense/antisense pair' or SAP. Cis-antisense pairs form extended regions of perfectly matched double-stranded RNAs (dsRNAs), while trans-antisense pairs, including miRNAs (Fig. 1a), usually form relatively short regions of base pairing that are frequently interrupted by mismatches. Although the distinction between antisense regulator and its sense target is sometimes unclear, we use the term antisense RNA generally to refer to the non-coding transcript when only one is an ncRNA. When both are either non-coding or coding, the distinction between antisense and sense is sometimes arbitrary [14], However, the antisense transcript is usually the presumptive regulatory RNA, while the sense RNA is more abundant, more widely expressed or has a better characterized or more direct function [15–18]. In this review we describe the properties and possible regulatory roles of antisense RNAs that are found in many diverse eukaryotes, focusing especially on recent results based on genome-wide surveys of mouse and human transcripts. A large number of other excellent reviews are available that discuss characteristics of many antisense RNAs that are not discussed here [15, 16, 19–23].

Historical overview

In their classic 1961 study outlining models for molecular regulation, Jacob and Monod [24] proposed that RNAs as well as proteins may directly regulate expression of individual genes. This and similar speculations that RNAs play a direct role in regulating gene expression [25, 26] were vindicated some years later by the discovery of endogenous antisense RNA regulation in 1981, with the important distinction that the regulatory RNA base-paired with the functional transcript of the gene and not with the gene itself. The first antisense RNAs to be rigorously

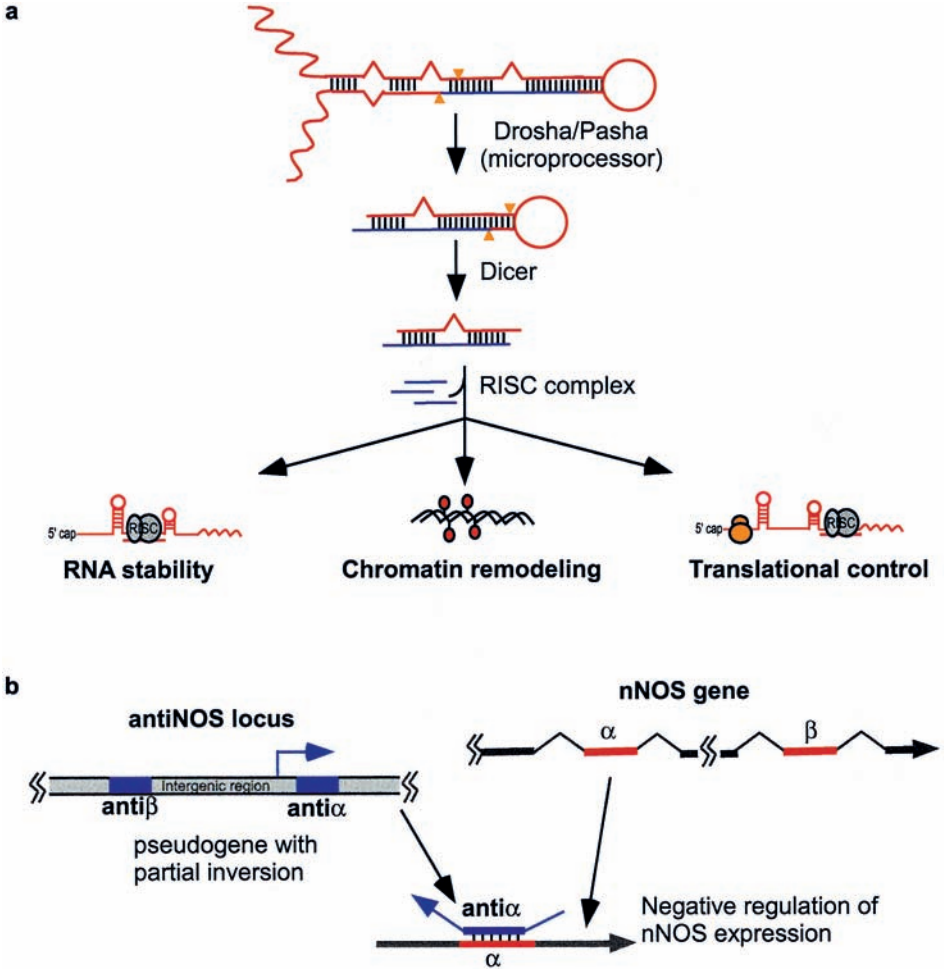

Figure 1. Examples of trans-sense/antisense pairs (trans-SAPs). (*a*) MicroRNAs. The biosynthesis of miRNAs requires the sequential action of two type III endoribonucleases. Drosha (or Pasha in *Drosophila*) cleaves the primary transcript yielding a pre-miRNA hairpin structure that is processed by Dicer. Three distinct antisense mechanisms are indicated: target cleavage mediated by perfect pairing of the miRNA with its target, translational repression mediated by mismatched pairing and chromatin remodeling. (*b*) Inverted region within a transcribed pseudogene yields trans-antisense RNA. Expression of neuronal nitric oxide synthase (nNOS) is blocked in specific neurons by an anti-α RNA transcribed from a partially inverted pseudogene [89, 207].

characterized were ones that regulated replication of bacterial plasmids, but other antisense RNAs were soon identified that controlled expression of endogenous bacterial mRNAs (reviewed in [7, 27, 28–30]). Further studies focused on potential antisense RNAs in various eukaryotic organisms. Subsequently, a detailed understanding of antisense-mediated gene expression regulation in bacteria emerged [7, 31], but the regulatory action of endogenous antisense RNAs in eukaryotes has remained unclear, with only a few notable exceptions [15, 16, 20, 23, 32, 33]. This remains the case despite the widespread use of artificial antisense RNA (and DNA) to block expression of targeted genes in eukaryotes, and even in such use the molecular mechanisms are often unclear [34, 35].

MicroRNAs represent a particularly important example of eukaryotic antisense regulation. In 1993 the laboratories of Victor Ambros and Gary Ruvkun reported the discovery of an unusual small RNA associated with the *lin-4* gene in the nematode, *C. elegans*. The *lin-4* gene product was only 21 nucleotides (nts) long, and was shown to repress translation of its mRNA target by base-pairing to a complementary element within the 3′ untranslated region (3′UTR) [36, 37]. It was not until 8 years later, following the discovery of a second miRNA, *let-7*, in *C. elegans* [38], that several groups demonstrated that these two RNAs were, in fact, prototypes for a large family of miRNAs [39–42] that appear to regulate expression of a significant fraction of mRNAs in many animals and plants.

MicroRNAs share a number of specific characteristics (reviewed in [4, 8]). They are typically 21–22 nts in length and are processed in two steps from a double-stranded hairpin structure located within a longer precursor, as shown in Figure 1a [43]. Although hundreds of miRNAs have been identified, only a relatively small number of targets have been confirmed to date. Other targets are the subject of intense ongoing investigations in many different physiological contexts, including cell differentiation, embryonic development [44, 45] and cancer cell biology [46].

In addition to the miRNAs, by 2002 approximately 40 pairs of eukaryotic cis-antisense RNAs had been identified in a variety of eukaryotic organisms [14]. Genome-level bioinformatics surveys [14, 19, 47] soon revealed that antisense transcription is exceedingly common in mammals and many other eukaryotes [48, 49]. These findings, together with a growing appreciation of the varied roles played by dsRNA in regulating gene expression, raise a number of important questions. For example, if a large fraction of chromosomal loci is bidirectionally transcribed, do the resulting complementary RNAs base-pair efficiently and activate various cytoplasmic or nuclear responses of the cell to dsRNA? If so, then how do these responses impact on other regulatory pathways? And if not, what factors, circumstances or competing pathways block base-pairing or otherwise prevent dsRNA from triggering such specific responses?

Cellular responses to dsRNA

Since dsRNAs are often associated with viral infection or transposable elements, eukaryotes possess a number of responses to control such challenges to normal cellular function [50–52]. One of these, the interferon-mediated response, involves the shutdown of protein synthesis and induction of interferon. This general, non-targeted response is triggered by the introduction of any dsRNA more than 30 base pairs into mammalian cells. This is a highly sensitive response that may be triggered by a single molecule of dsRNA [16, 51]. The interferon response ultimately culminates in programmed cell death, and thus does not regulate normal gene expression. However, individual components of the signaling pathways involved mediate responses to other physiological stressors.

In contrast to the interferon-mediated response, RNA interference, or RNAi, is a highly specific response in which dsRNA molecules are cleaved into discrete 21–23 nt dsRNA fragments known as small interfering RNAs (siRNAs). These siRNAs target the destruction of homologous single-stranded RNAs [53, 54]. RNAi is found in plants, fungi and animals. The processing of the *lin-4* miRNA from a dsRNA hairpin [36] was a critical observation regarding small RNAs in two respects. Not only is lin-4 processing representative of that of all known miRNAs [43, 55], it is also mechanistically related to other pathways that require the production of small dsRNAs [4, 56]. The common feature of these pathways is that they involve endonucleolytic processing of small dsRNA. In each pathway a type III endoribonuclease, known as Dicer, makes one or more staggered double-stranded cuts that determine the length of the miRNA or other small RNA product (cf. Fig. 1a). miRNAs are distinguished from siRNAs in that mature miRNAs are single-stranded RNAs formed by cleavage of intramolecular hairpins, and selected with a strand-specific bias. siRNAs are formed by cleavage of longer, intermolecular dsRNAs by Dicer into a series of short dsRNAs, also about 21 nts long, which in turn direct the cleavage and subsequent destruction of any homologous single-stranded RNAs (Fig. 1a) [57]. Thus, miRNAs, in contrast to siRNAs, are encoded by individual genes that give rise to defined singled-stranded antisense RNAs.

The formation of miRNAs and siRNAs are just two examples of a variety of regulatory pathways involving small RNAs. Components of the RNAi pathway are also required for other cellular processes, including chromatin-mediated silencing, DNA rearrangements and the processing of miRNAs [58, 59]. These pathways involve both transcriptional gene silencing (TGS) and post-tran-

scriptional gene silencing (PTGS) [60, 61]. PTGS includes siRNA- and miRNA-mediated regulation in which the small RNAs are associated with a specialized RNA-induced silencing complex (RISC complex). For centromeric heterochromatin formation in fission yeast, plants and *Drosophila*, TGS is mediated by repeat-associated small RNAs (rasRNA) that are produced by Dicer from transcripts of centromeric repetitive sequences. rasRNAs act in association with the RNA-induced transcriptional silencing complex (RITS complex) to direct chromatin modification and transcriptional silencing of centromeric DNA [62]. They may also act in mammals and other vertebrates where definitive evidence has proven elusive [63, 64]. The RISC and RITS complexes are linked not only by the involvement of dsRNA and Dicer, but also by the presence of Argonaute proteins, which are present in both eukaryotes and archaebacteria [65, 66].

A third response to dsRNA is the deamination of adenosine residues within a double-stranded region. This process is mediated by a small family of enzymes known as ADARs (adenosine deaminases acting on dsRNA) and results in the conversion of adenosine to inosine [67]. One apparent role of RNA editing is to modify the coding potential of the corresponding genes. In addition, A to I editing disrupts the pairing between adenine and uracil, since the purine base of inosine, like guanine, pairs with cytosine. In many instances ADARs modify intramolecular secondary structure, including inverted repeats embedded in the 3′UTRs of many mRNAs. In other instances, ADARs edit specific sites within mRNAs that are determined by formation of small intramolecular hairpins [68]. However, ADARs are also capable of non-specifically modifying larger intermolecular dsRNAs, a process referred to as hypermutation. Such promiscuous editing plays an important role in the replication cycle of polyomavirus in infected mouse cells. Recent studies from Carmichael's laboratory demonstrate that inosine-containing RNAs are specifically recognized in the cell nucleus, where they may trigger other signaling processes [50, 69–71]. Thus, ADAR modification, like RNAi, may play a role in mediating cell signaling events triggered by dsRNA, and there are indications that these two dsRNA-directed pathways may interact [72, 73]. ADAR activity may also be important for modifying endogenous dsRNAs that might trigger the interferon-mediated response and apoptosis [74].

Regulation by trans-antisense RNAs

miRNAs repress expression of their target RNAs by base-pairing with complementary elements, as shown in Figure 1a. In animal cells the target sites are usually located in the 3′UTRs of mRNAs, while in plants miRNA targets are often found within the coding region itself [75, 76]. In animal cells, imperfect base-pairing within the 3′UTR blocks translation, resulting in inactive but intact polysomes [77–80]. In plants, perfect or near-perfect base-pairing leads to miRNA-directed cleavage and inactivation of the target mRNA [75]. The latter pathway appears similar or identical to siRNA-mediated cleavage of RNA by homologous siRNA during RNAi. Although these two mechanisms represent the major pathways of miRNA action, it is clear that miRNAs are mechanistically versatile and can act through other mechanisms such as AU-rich element (ARE)-mediated destabilization of cytokine-related mRNAs [81].

While miRNAs represent a singularly well-studied class of trans-antisense RNA, they are by no means the only examples. Early studies described a specific translational repressor that apparently regulates myosin expression and muscle development in myoblasts [82]. A number of other small RNAs, including small nuclear RNAs (snRNA) and small nucleolar RNAs (snoRNAs), function primarily by base-pairing with target sites in larger RNAs. However, these trans-acting RNAs either form the catalytic core of RNA processing complexes (*e.g.* spliceosomal snRNAs) or serve as guide RNAs for site-specific editing or modification (*e.g.* snoRNAs) [7, 83] and hence are not considered further here.

A large number of trans-antisense RNAs may arise from bidirectional transcription of repetitive sequences that are especially abundant in the genomes of plants and vertebrates. Repetitive sequences include both simple repeats, such as those present at centromeres, and transposons, retroelements and pseudogenes [1]. In mammalian genomes, the most abundant retroelements are short and long interspersed elements (SINEs and LINEs) that are transcribed in either orientation when embedded in a larger transcript or transcribed independently from their own promoters. Transcripts of repetitive elements are the major physiological targets of the RNAi pathways [64]. Several examples of embedded SINEs were described in early studies of mammalian antisense RNA [84–86]. However, further evidence for a role in antisense regulation is lacking. Although pseudogenes are often transcriptionally silent, they can be transcribed in either sense or antisense directions if they become active. Pseudogene transcripts that include a partially inverted segment have been shown to serve as a functional antisense repressor that regulates expression of the parent gene [87–89]. One of these, the antisense RNA repressor of neuronal nitric oxide synthase in pond snails, is shown in Figure 1b.

The recent discoveries of miRNAs as a large conserved class of functionally unique RNAs has energized ongoing efforts to discover other types of structurally related ncRNAs through bioinformatics and biochemical studies [7, 90]. Given the complexity of eukaryotic genomes and the subtlety of RNA structure, it seems quite likely that such investigations will uncover other families of antisense RNAs dispersed within annotated and intergenic regions.

Table 1. Abundance of cis-overlapping transcription units in diverse eukaryotes.

Organism	Number of SAPs	Comments	Year	Reference
Mammals				
Human	2667	EST bioinformatics	2003	[18]
	2940	EST bioinformatics	2004	[17]
Mouse	4520	cDNA, exon-exon overlaps	2005	[49]
	4129	cDNA, intron-exon overlaps	2005	[49]
Plants				
A. thaliana	6598	Predicted genes (includes intron-exon overlaps)	2003	[94]
	957	Confirmed by FL cDNA	2005	[154]
Rice	601	Exon-exon overlaps	2004	[93]
	86	Intron-exon overlaps	2004	[93]
Fungi				
S. cerevisiae	369	Overlapping ORFs only	2005	[95]
	88	Exon-exon overlaps	2005	[96]
S. Pombe	20	Overlapping ORFs only	2005	[95]

SAPs refer to exon-exon overlaps only unless noted otherwise.

Abundance and ubiquity of cis-antisense transcripts

Large-scale sequencing and bioinformatics analysis has revealed that cis-antisense RNAs are abundant and ubiquitous in many diverse eukaryotic organisms, including mammals (*e.g.* humans and mouse [14, 17–19, 49, 91]), insects [92], plants [93, 94], fungi [95, 96], protozoans (*P. falciparum*) [97, 98] and diplomonads (*G. lamblia*) [99]. Representative studies depicting transcriptome-level analysis of cis-overlapping transcripts in plants, mammals and fungi are summarized in Table 1. Several different approaches are represented here, including large-scale sequencing of full-length cDNAs [12], bioinformatic analysis of multiple EST libraries [17, 18] and bioinformatic analysis of annotated protein-coding genes [95]. The FANTOM3 transcript dataset, from which the mouse antisense pairs are drawn, is comprised of more ncRNAs than coding transcripts, while the opposite is true of two surveys of human transcripts that include bioinformatic analysis of partially sequenced EST clones [12, 17, 49]. These differences largely reflect the different methods used to compile the databases, since the two mammalian transcriptomes are likely to be far more similar than these differences suggest.

Different organisms vary in their specific characteristics in several ways that are relevant to antisense expression. Plants, fission yeast (*S. pombe*) and nematodes have one or more RNA-dependent RNA polymerases (RdRPs), which are entirely lacking in mammals, drosophila and budding yeast (*S. cerevisiae*). Consequently, plants, fission yeast and nematodes can convert single-stranded RNA molecules into dsRNA using siRNAs as primers, and thereby amplify the initial RNAi response. Although both yeasts have very small genomes, they differ in other respects. Dicer, Argonaute proteins and other factors required for RNAi-related responses are present in *S. pombe* but missing in *S. cerevisiae* [62, 100, 101]. Nonetheless, as shown in Table 1, hundred of antisense transcripts have been identified in *S. cerevisiae* [95, 96, 102]. Co-expression of convergently arrayed genes has also been shown to lead to repression of both transcripts through transcriptional interference in *S. cerevisiae* [103, 104]. Since *S. cerevisiae* lacks some of the features associated with antisense transcription in other eukaryotes, while retaining others, this model organism may prove useful for selectively studying different possible roles of antisense transcription.

Since the RIKEN Institute's FANTOM3 database of full-length cDNAs provides the largest collection of unique transcripts, it provides an exceptionally comprehensive view of the mammalian transcriptome. The use of a large number of rigorously subtracted full-length cDNA libraries and multiple expression tag strategies is equivalent to about 12 million randomly selected cDNA clones [12, 49]. The FANTOM3 antisense collection includes 4520 pairs of overlapping full-length cDNA transcripts with exon-exon overlaps and another 4129 pairs that include only exon-intron overlaps [49]. These numbers refer to representative transcript pairs of full-length cDNAs. If transcripts represented by expression tags are also considered, 72% of the 43 553 identified transcription units have at least one overlapping antisense transcripts [49].

Analysis of the FANTOM antisense collection reveals that cis-antisense SAPs are found in every possible configuration (Fig. 2). Pairs containing coding and non-coding RNAs are most common, although overlaps between two mRNAs are also abundant [49, 105]. 3′ overlaps are the most common type of overlap found between two spliced mRNA molecules. In many compilations of SAPs these are the most frequent pairings [17, 18, 106]. However, the

preferred orientation of sense/antisense pairs may differ according to other features including the absence of introns or lack of coding potential. Among pairs containing unspliced ncRNAs in the FANTOM2 database [105], 5′ overlaps predominate.

Genomic context of cis-antisense RNAs

Although coding sequences comprise only a small fraction (1.2%) of the total genome in humans, this figure understates the likelihood of gene overlaps for several reasons. First, introns, not exons, comprise the greater part of most transcription units [12, 107]. Second, transcription units are often clustered, and these clusters may be separated by large transcriptional deserts [1, 12]. Within clusters adjacent transcripts are often co-regulated [108–111]. Regions of high gene density are often enriched for highly expressed genes [108, 112]. Individual transcription units themselves consist of clusters of overlapping transcripts differing in 3′ and 5′ ends and internal splicing [113, 114]. This hierarchical clustering of transcripts and transcription units greatly increases the possibilities for overlap between genes on opposite strands.

A third important feature of mammalian genomes is the abundance of ncRNAs and repetitive sequences. These sequences, sometimes dismissed as junk or transcriptional noise, may in fact play essential roles in regulating expression [12, 115]. Both human and mouse genomes contain substantially more non-coding transcription units than those that include at least one mRNA [10, 12]. Many ncRNAs are transcribed by RNA polymerase II, capped at their 5′ ends and polyadenylated. While the sequences of these mRNA-like ncRNAs are often not conserved between the human and mouse genomes [116], the rapid evolution of ncRNAs does not necessarily imply a lack of function [13, 117]. Transcription of ncRNAs is often regulated through conserved promoter elements [12, 111, 118]. Furthermore, the presence of such mRNA-like ncRNAs is supported by the comprehensive full-length cDNA libraries [12], expression tag libraries [12] and microarray tiling studies [49, 94, 111, 119, 120].

These abundant mRNA-like ncRNAs contribute to the transcriptional complexity of the mammalian genome. While some ncRNAs are transcribed from intergenic regions, others overlap other coding regions in the sense or antisense orientation (see Fig. 2c, d). A majority of the SAPs in the FANTOM3 antisense database consist of pairs of coding and non-coding RNAs, with relatively few overlaps between two ncRNAs [49]. Repetitive sequences, comprising, for instance, over 50% of the human genome, also contribute to the overall complexity of the genomes of mammals [121]. Some repetitive sequences, such as SINEs and LINES, are often embedded in the introns and exons of mRNAs and ncRNAs, while other elements are independently transcribed. In addition

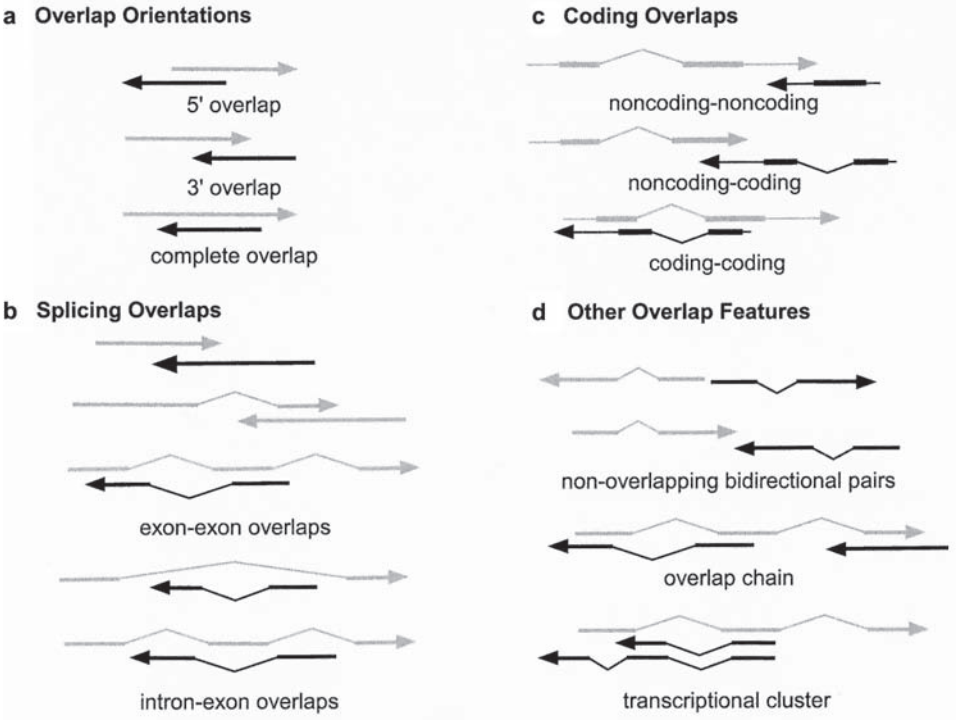

Figure 2. Examples of cis-sense/antisense pairs (cis-SAPs). (*a*) SAPs classified by overlap orientation. (*b*) Overlaps between spliced and unspliced RNAs. (*c*) Overlaps involving coding and untranslated regions of mRNAs. (*d*) Other features related to antisense overlaps: non-overlapping bidirectional pairs, chains of two or more different overlaps and transcriptal clusters (see [49, 128, 208]).

to providing a source of trans-encoded antisense (Fig. 1), repetitive elements influence the expression of host genes in many other ways [122–126].

Coordinate patterns of cis-antisense expression

The abundance, structural organization and other properties of overlapping transcripts suggest that they may play one or more central roles in formatting gene expression in the genomes of diverse eukaryotes. The nature of these roles, however, is unclear. In contrast to observations of many individual sense/antisense pairs in bacterial cells that display reciprocal patterns of expression under a variety of conditions, increased expression of one member of a sense/antisense pair in eukaryotic cells is frequently not accompanied by a reciprocal decrease the other, although such a relationship is seen in certain cases [49, 114]. In fact, recent studies strongly suggest that complementary transcripts from overlapping transcription units are most often co-expressed [49, 114, 127], as are many adjacent but non-overlapping transcription units [128]. Manipulating the expression of one member of a pair either by siRNA or transient overexpression gives various results. In only a few instances did a change in one RNA lead to a reciprocal change in the expression of the other complementary overlapping RNA [49].

The issue of co-expression *versus* reciprocal expression, however, is not straightforward. Although genes that are structurally adjacent tend to be co-regulated, it is well established that regulation occurs at many different levels, with one type of regulation often affecting another. Transcription itself is subject to both local and long-range controls. Expression patterns observed for complementary RNAs are probably subject to multiple regulatory inputs and ultimately dependent on the balance between synthesis and degradation.

Recent studies by Chen and his colleagues [106, 127] are important in demonstrating that transcripts sharing a complementary overlap exhibit statistically significant non-random patterns of co-expression, when evaluated in terms of tissue specificity and inverse expression and when a large number of paired conditions are examined. These patterns are apparent in a set of 1498 transcript pairs that are each represented at minimal threshold levels in SAGE tag libraries from 16 tissues. Co-expression of transcripts within a pair was substantially greater than that expected for randomly paired transcripts ($p < 10^{-5}$). The same 1498 pairs were then examined for evidence of reciprocal expression, defined as at least two instances (out of 43 pair-wise comparisons) of inverse expression, in which the change in the expression ratio of the two RNAs is greater than expected by chance. The comparison included normal tissues *versus* related tumors, two related tissue or tumor cell types, or the same cells cultured under different conditions. Although the significance of the inverse expression ($p < 0.005$) is smaller than that observed for co-expression, it is notable that many pairs of transcripts exhibit both co-expression with respect to tissue specificity and inverse expression with respect to the comparison cases. Most interestingly, cells displaying either co-expression or inverse expression patterns exhibited higher conservation between human and mouse than did most sense/antisense pairs [127].

In further studies, Chen and coworkers [129, 130] make two other significant observations regarding functionally relevant properties of antisense RNAs. First, the introns of antisense RNAs are much shorter than those of the sense targets. This appears to reflect a specific requirement for rapid expression of such potential regulatory RNAs, rather than a negative selection against longer primary transcripts. A second observation concerns the predominance of 3′ overlapping pairs among their SAP dataset [106] Careful analysis of such pairs shows that such pairing is non-random and therefore likely to be functionally important, and is consistent with the hypothesis that such overlaps impose functionally important evolutionary constraints [106, 131].

Microarray tiling studies provide an independent source of information on the distribution of antisense overlaps within the human genome [111, 119, 132–134]. In tiling studies, oligonucleotide probes are distributed at even intervals across the genome or chromosomal segment being studied. These studies permit a large-scale examination of RNA expression levels unbiased by selection of a particular library of sequences on which the probe design is based. Tiling arrays avoid many of the technical limitations of expression tag analysis [106, 127] and provide exon-by-exon expression data. Although the relationship between a series of presumptive exons or ‘transcribed fragments’ (transfrags) is usually unclear when they map to unannotated regions of chromosomes, tiling arrays provide an unbiased view of RNA expression across large regions of the genome [119, 133, 134].

When combined with RT-PCR to extend and clone novel transfrags, tiling arrays provide a direct method for the targeted discovery of novel transcription units [113, 119]. Gingeras and his colleagues [111] also used chromatin immunoprecipitation to demonstrate that expression of non-coding antisense RNAs is often regulated by many of the same transcription factors that are known to regulate protein coding genes. Other tiling studies from Gingeras’ group have shown that non-polyadenylated transcripts [poly(A)$^-$ RNA] comprise a substantial fraction of the total complexity of RNA in a human cell line, with about 30% attributable to nuclear RNA alone [119]. These data provide striking evidence for a large pool of poly(A)$^-$ RNA that may be independent of poly(A)$^+$ RNAs. Although poly(A)$^-$ RNAs are often overlooked, the RIKEN group has also identified many highly expressed

poly(A)⁻ RNAs among the antisense and ncRNAs in the FANTOM3 database. Tiling studies, together with novel tagging methodologies [12, 113, 119], promise to open a new door on the study of the mammalian transcriptome.

Models and mechanisms for antisense regulation

A fundamental question relating to antisense regulation concerns whether a given antisense transcript plays a direct role in mammalian gene expression. Given the diversity, ubiquity and regulated expression observed for antisense transcription, it seems clear that at least some antisense RNAs are actively involved in gene expression control. Although antisense regulation is often presumed to depend on base-pairing between complementary transcripts, it is important to consider both transcriptional and post-transcriptional mechanisms, as recently described for the expression of imprinted genes [135]. Some of the most fascinating examples of antisense transcription come from the study of X chromosome inactivation and imprinted genes in mammals, which share a common characteristic of monoallelic expression. Such studies are likely to hold general lessons for understanding the role of antisense RNA in regulating biallelically expressed genes. Below, we consider five possible models (Fig. 3). The first three involve transcriptional regulation and the last two post-transcriptional regulation by antisense RNA.

Class I model: Transcriptional regulation independent of overlapping antisense transcription

Cis-antisense transcription may regulate expression of overlapping genes by competing for (or sharing) transcriptional factors. This is especially apparent for SAP transcripts with juxtaposed promoters (*e.g.* 5′-5′ overlaps; Fig. 3a). It is also possible for 3′-3′ or fully overlapped transcripts to compete for or share common transcriptional factor binding sites, since genomic regions harboring regulatory elements can stretch as much as 1 Mb in either direction from the transcription unit [136]. For example, results from DNase I footprinting suggest that imprinted Igf2R and Air promoters appear to share common cis-regulatory elements [137]. In addition, one recent genome-scale study showed that about 4% of CREB binding sites are located at the 3′ end of the target genes, many of which have known antisense transcripts [138]. Sharing of common trans-acting factor may lead to co-expression of overlapping transcripts, whereas negative correlation is expected if distinct transcriptional factors compete for overlapping binding sites (Fig. 3a). This model is similar to that proposed for non-overlapping divergently transcribed gene pairs, which represent more than 10% of the genes in the human genome [128].

Class II model: Transcriptional regulation mediated by active transcription without direct involvement of antisense RNA

Concurrent transcription emanating from opposite strands may introduce topological constraint to DNA molecules [139], which in turn leads to transcription repression. RNA polymerases, on the other hand, may collide in convergent 3′ overlapping SAPs [103]. This model, shown in Figure 3b, has been invoked for inversely expressed genes at imprinted loci [140]. Conversely, the initiation of transcription of one strand may help activate transcription on the opposite strand, by altering local chromatin structure [141] or drawing adjacent promoters into an active transcriptional 'factory' [142]. Although the actual RNA transcripts are incidental to regulation, active transcription per se is instrumental and distinguishes the current model from the aforementioned sharing/competition model.

Class III model: Transcriptional regulation mediated by the antisense RNA transcript itself

There are several ways by which the antisense transcript itself may play a role in transcriptional level regulation. In principle, these may involve either single-stranded RNA or dsRNA. The nascent or mature antisense transcripts may feed back directly to the overlapping gene [24], or act indirectly by recruiting factors that promote or inhibit transcription of overlapping genes (Fig. 3d). In addition, emerging evidence supports the idea that duplex formation between sense and antisense transcripts may trigger epigenetic alteration through DNA methylation or chromatin remodeling. This can be achieved by a mechanism similar to heterochromatin formation at repetitive segments in the genomes (*e.g.* centromeric repeat), which depends on Dicer, an RNase III endonuclease, and RISC complex [64, 143, 144]. On the other hand, duplex RNAs can be recognized by RNA editing enzymes, known as ADARs, which convert adenosine residues to inosine. Edited dsRNA recruits several distinct protein complexes as shown in Figure 3d. One of them contains vigilin, which appears to induce heterochromatin formation by promoting HP1 phosphorylation [70]. Both models require dsRNA and postulate a reverse information flow to genomic DNA and may be related to those observed at imprinted loci [145].

Class IV model: Post-transcriptional regulation mediated by sense/antisense base-pairing that directly blocks binding of factors to the target transcript

Antisense RNA can act post-transcriptionally by blocking the binding of specific factors to the sense RNA (Fig. 3c), thereby masking sites required for its expression [20].

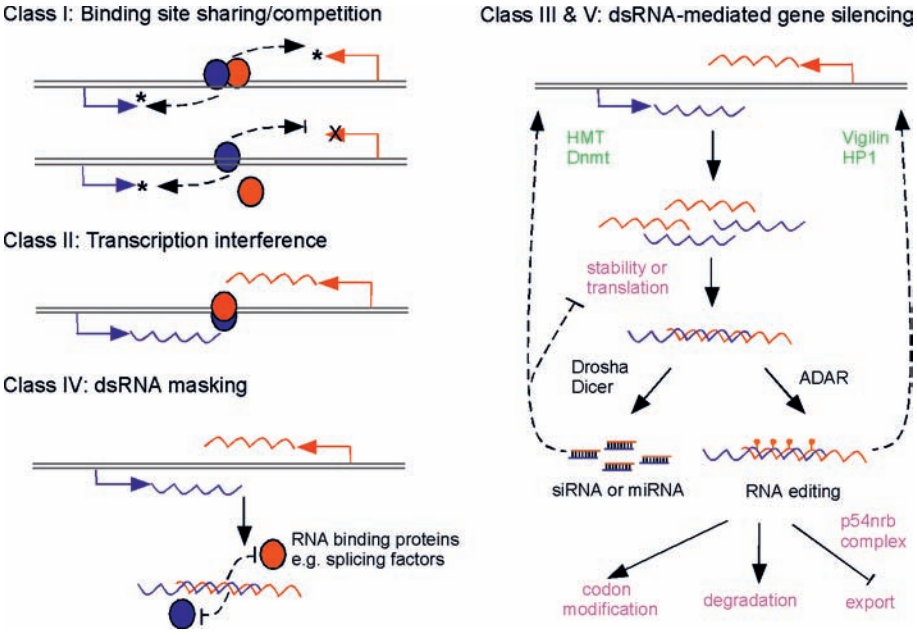

Figure 3. Models for antisense regulation. Key features for five classes of mechanisms similar to those proposed for antisense regulation at imprinted loci [135]. Class III and class V may involve similar components working at the level of transcriptional gene silencing (class III) or post-transcriptional events (Class V). These are indicated in green and violet for Class III and Class V, respectively. Core components that may be shared by both mechanisms are indicated in black.

This could happen at any step in the RNA life cycle, including splicing [146, 147], export [148], stability [149], and translational control [150]. In addition, pairing based on complementarity could effectively titrate out functional ncRNA, if there is any, within a sense-antisense pair [135]. This might explain why Tsix is in 10–100-fold molar excess over Xist RNA, and the overlap region coincides with the functional domain of the Xist gene [151, 152]. In both scenarios, antisense RNA acts stoichiometrically by forming a 1:1 complex with the complementary target site without triggering downstream signaling events.

Class V model: Post-transcriptional regulation mediated by antisense/sense base-pairing that recruits factors that alter downstream expression

In contrast to model IV, RNA duplex formation may also recruit factors that alter its expression (Fig. 3d). There are several examples of this type of post-transcriptional regulation, including ADAR modification of intermolecular dsRNA duplexes that are retained in the cell nucleus [71, 153]. In this case, the antisense regulator acts stoichiometrically in that both regulator and target are modified in the duplex. The formation of RNA duplex between sense-antisense transcripts might also induce PTGS through siRNA-related pathways [64]. In this case, an antisense RNA triggers the production of siRNA, resulting in the downstream cleavage of many homologous transcripts. Several siRNAs have been identified that originate from the overlapping transcripts in *Arabidopsis thaliana* [154]. siRNA formation from natural antisense transcript remains to be established in mammals.

The distinction between these models is not always clear. For example, miRNAs appear to act in several ways: stoichiometrically to block cap-dependent initiation of translation [78, 79]; catalytically to direct cleavage of complementary transcripts [155]; and stoichiometrically to promote degradation of transcripts through AREs [81]. Each of these trans-antisense mechanisms shares common mechanistic features, including the involvement of Argonaute proteins. However, translational inhibition is categorized as a Class IV mechanism, while cleavage and ARE-mediated degradation are Class V. Thus, multiple pathways might work in concert under physiological conditions to achieve coordinated gene expression.

Antisense regulation in imprinting and X inactivation

Genomic imprinting involves monoallelic expression of diploid genomes in a parent-of-origin-specific manner. Genome-wide prediction and large-scale microarray studies suggest that up to 2000 genes are imprinted in mammalian genomes [156, 157], although only about 100 imprinted transcription units are confirmed at present (http://www.mgu/har.mrc.ac.uk/research/ imprinting). As a special example of monoallelic expression, one of the two parental X chromosomes has to be silenced in female

cells to achieve dosage compensation. Sense-antisense transcripts are often found in imprinted loci [158], and they themselves are reciprocally expressed [135]. Some regions may consist of multiple SAPs, such as Rtl1/antiRtl1 and Dio3/anti-Dio3 in the Dlk/Gtl2 region [159]. In addition, a high proportion (30%) of imprinted transcription units are ncRNAs, including snoRNAs, microRNAs, pseudogenes and other RNAs of unknown function [12, 158, 160]. As a result, antisense ncRNAs appear to be a recurring theme in all well-characterized imprinted loci that are involved in human genetic disease. Among them, several antisense pairs have been shown to be required for silencing their reciprocally imprinted counterparts. These include Xist/Tsix expression during X inactivation and imprinting of Igf2r/Air and Kcnq1/Kcnq1ot1 on mouse chromosome 7 [161–163]. Other imprinted loci such as PWS/AS and Dlk/Gtl2 remain to be further characterized [164, 165].

Monoallelic expression of imprinted genes involves several epigenetic modifications, such as DNA methylation and chromatin remodeling, that differentially mark the paternal allele [166]. It further requires that communication between antisense RNA and genomic DNA is restricted in cis. Both differentially methylated regions (DMRs) within the 5′ CpG islands and antisense transcripts play an important role in these processes. Mice lacking DNA methyltransferase 1 (Dnmt1), an enzyme for methylation maintenance, show variable degrees of defects in imprinting. Although the SNRPN gene became biallelically expressed in Dnmt1-deficient mice, partial or full imprinting was retained at Igf2r and Kcnq1 loci [167, 168]. Apparently, DMRs may contain sequence features other than CpG islands that are critical for gene silencing, some of which presumably are able to communicate with antisense RNAs. Several studies have employed mouse genetics to uncouple DMR activity and antisense effects. Strategic knockouts and exogenous promoter insertions in Igf2r loci showed that the Air antisense transcript, other than Igf2r promoter, is essential for gene silencing at this locus [169]. In contrast, both DMR and antisense regulation are required for bidirectional silencing and methylation spreading at the Kcnq1/kcnq1ot locus. [163, 170]. These studies illustrate the complexity of underlying mechanisms by which imprinted genes are regulated, and suggest that partially redundant pathways may co-evolve during natural selection to ensure precise control of mammalian gene expression.

The role of two complementary overlapping ncRNAs, Xist and Tsix, in X chromosome inactivation has been investigated in some detail as shown in Figure 4a and b. Tsix repression is required at the onset of random X inactivation for the up-regulation of Xist RNA, which coats the future Xi [135, 171]. Targeted disruption of Tsix skews the Xi choice in cis, resulting in preferential inactivation (Fig. 4b) [161, 162]. However, overexpression of Tsix does not suffice to influence choice [172, 173], suggesting that other features at the X inactivation center may also required. Interestingly, early termination of Tsix transcript disrupts random X inactivation in heterozygous cells. Restoration of Tsix RNA in cis without concurrent transcription across Xist failed to rescue X choice [174]. These results suggest that antiparallel transcription between Tsix and Xist may serve as a feedback mechanism to 'lock' the X chromosome in an active state and restrict

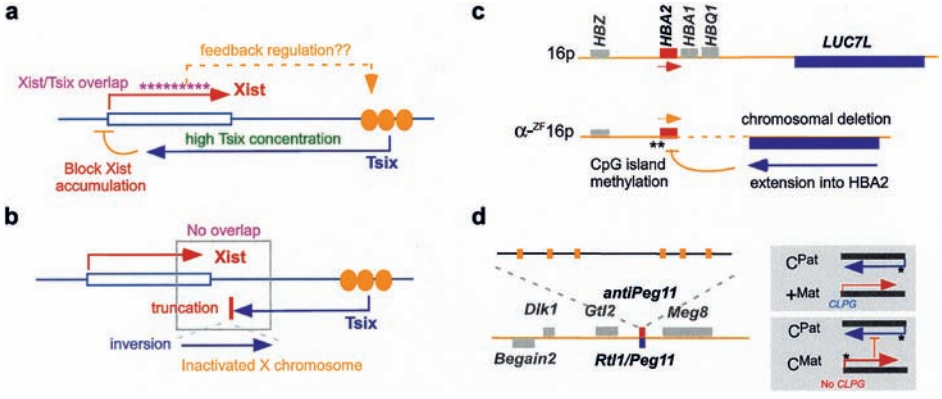

Figure 4. Examples of antisense-mediated gene regulation. (*a*) X chromosome inactivation. Transcription of Tsix (blue) from future active X chromosome blocks the accumulation of Xist (red) in cis. The overlap between Xist and Tsix transcripts (asterisks) is required for repression of Xist and may reinforce the X choice [174]. (*b*) Eliminating overlap with Xist by truncation of Tsix transcript with or without insertion of an inverted, but actively transcribed, Tsix cDNA into chromosome skews X chromosome choice [174]. (*c*) A deletion at the α-globin locus results in extension of transcription from Luc7L (blue) antisense to the HBA2 gene (red). This antisense overlap elicits DNA methylation (black asterisks) and repression of HBA2 [182]. (*d*) Left panel: The Dlk1/Gtl2 locus. The Rtl1/Peg11 (blue) and its antisense transcript, anti-Peg11 (red), are paternally and maternally imprinted, respectively. MicroRNAs are shown as orange boxes embedded in the anti-Peg11 gene. Right panel: (top) a paternally inherited (Pat) CLPG mutation results in overexpression of Rtl1/Peg11 and gives rise to the CLPG phenotype; (bottom) Both anti-Peg11 and Rtl1/Peg11 are overexpressed in CLPG homozygotes. MiRNAs from the maternally expressed anti-Peg11 transcripts inhibit the expression of Rtl1/Peg11 gene from the paternal allele carry CLPG mutation and produce a normal phenotype [209].

the antisense regulation locally. Although the molecular detail for cis-regulation is largely unknown, the spatiotemporal coordination of sense-antisense transcription is likely to be involved. Almost nothing is known regarding the cis/trans-relationship for antisense regulation at biallelically expressed genes. However, it is possible that some antisense RNAs transcribed in cis from non-imprinted loci may also act in cis through regulatory mechanisms similar to those found at imprinted genes.

Antisense regulation: development and disease

Given the ubiquity and diversity of antisense transcription, one would expect that such transcription is tightly regulated under physiological conditions. In addition to X inactivation and autosomal imprinting, antisense regulation has been demonstrated in a plethora of biological processes, such as the circadian clock [175], cardiac development [176], and brain and cognitive function [177, 178]. Defects in antisense regulation often lead to disease. Examples include spinocerebellar ataxia [179], hairy cell leukemia [180], and many additional loci that are discussed in recent reviews [20, 181]. Here we highlight two intriguing studies that suggest a widespread involvement of antisense transcripts in human disease.

Aberrant gene silencing mediated by antisense transcription provides an alternative mechanism underlying genetic disorders. Higgs and colleagues [182] showed that an 18-kb genomic deletion juxtaposes an otherwise distant Luc7 region to a structurally intact hemoglobin α2 (HBA2) gene. Abnormal overlap between these two genes leads to transcriptional silencing of HBA2 and ultimately α-thalassemia, as shown in Figure 4c. This phenomenon may be generalized to other inherited disorders, especially for those disease-related loci that lie in gene-dense regions. In addition, the mechanism may hold for acquired disorders as well. It is possible that hypomethylation in malignant cells could generate bulk aberrant antisense transcripts, some of which may randomly induce epigenetic alterations at key tumor suppressors or oncogenes [183].

Embedded miRNAs within antisense transcripts might provide an additional dimension of intricacy to fine tune host gene expression and related disease severity. For the DLK/GTL2 imprinted locus, at least five miRNAs are exclusively processed from maternally expressed anti-Peg11 precursor, but not from its complement, the Rtl1/Peg11 transcript (Fig. 4d). These miRNAs have been shown to inhibit Rtl1/Peg11 expression in trans via site-specific RNA cleavage [184]. This finding provides a molecular basis for the polar overdominant CLPG mutation encompassed in the DLK/GTL2 locus, which shows diseased phenotype only in paternally transmitted heterozygous individuals (Fig. 4d) [185]. Although intramolecular stem loops are the sole source of miRNA in this particular example, duplex formation between SAPs may act in a similar fashion and needs to be further characterized.

Requirement for dsRNA formation *in vivo*

Although regulation can be effected by processes in which antisense transcription is not directly involved (*e.g.* class I or class II mechanisms), the formation of dsRNA through sense/antisense duplex formation is central to known antisense regulatory mechanisms (*e.g.* classes III, IV and V). Thus, factors that affect intermolecular base pairing are likely to be important for antisense regulation.

Genetic and biochemical approaches have provided ample evidence for base-pairing between antisense and sense RNAs for a variety of small RNAs, including bacterial antisense regulators, miRNAs, rasRNAs, guide RNAs and specialized RNAs such as spliceosomal snRNAs. In each of these cases base-pairing is mediated by specific protein factors that are closely associated with the regulatory RNA. For example, miRNAs bind to their complementary sequences as part of the RISC complex, and rasRNAs as part of the RITS complex. However, surprisingly little is known about the formation of dsRNA from large, independently transcribed RNAs that comprise the bulk of antisense complexity in cells.

In some cases, the cellular response to dsRNA gives clear evidence for dsRNA formation. The identification of viral defense mechanisms against the interferon-mediated response, such as VA RNA in adenovirus, that thwart dsRNA-dependent responses of host cells provides further evidence for dsRNA *in vivo*, as does hypermodification by ADAR [16, 50, 67, 71]. An example of this is extensive ADAR editing of polyomavirus early mRNA during the early-late phase transition [16, 148] that indicates that a class V antisense mechanism is responsible for silencing early mRNA through nuclear retention. However, extensive analysis of EST sequences from mammalian sources provides little evidence for other intermolecular duplexes. Most ADAR modifications in EST sequences correspond to limited editing of intramolecular hairpin structures [186]. There are few examples of hypermodification of endogenous RNAs aside from those associated with repetitive sequences [71, 187].

Intriguing evidence for dsRNA formation comes from the characterization of endogenous siRNAs and rasRNAs in eukaryotes. One comprehensive study involves the use of massively parallel signature sequencing of small RNAs from *A. thaliana* [188]. Over 1.5 million small RNAs, 20–24 nts long, were sequenced, yielding about 75 000 distinct sequences, most of which may be siRNAs produced by Dicer. The distribution of these small RNAs within the genome is informative. RNAs that form dense clusters are disproportionately derived from transposons

and other repetitive sequences known to be associated with dsRNA production. Sparsely distributed RNAs map to possible miRNA genes. Although many of these small RNAs appear to reflect the formation of longer dsRNAs, there are other possible sources, including intramolecular duplexes formed by transcribing inverted repeat sequences and the amplification of repetitive transcripts by RdRPs in plants.

The study of Lu et al. [188] found no correlation between the presence of known SAPs and small RNA production and thus does not provide direct evidence for base-pairing between large independently transcribed non-repetitive RNA molecules. Another recent study in *A. thaliana*, however, provides just such evidence [189]. Under conditions of salt-induced stress, expression of pyrroline 5-carboxylation dehydrogenase (P5CDH) is silenced by expression of an overlapping cis-antisense mRNA. The silencing of P5CDH mRNA is accompanied by appearance of siRNA from the overlap region, suggesting a class V mechanism. Genetic analysis implicates RdRPs, Dicer and other RNAi proteins in this response [189].

Efforts have also been made to characterize dsRNA by single-strand specific nuclease digestion. These include a number of important studies of individual SAPs such as N-myc, myelin basic protein, neural nitric oxide synthase, troponin I and subunits of the neural nicotinic acetylcholine receptor [87, 89, 190–192]. Such approaches have also been adapted for qualitative screening and quantitative analysis [193, 194]. In each case the presence of RNase-resistant dsRNA was reported. However, thus far relatively few examples of dsRNA formation between sense and antisense RNAs have been obtained and further studies of the efficiency and stability of duplex formation are needed. RNAs that are transiently associated may be captured during isolation allowing more stable duplex to form, as proteins associated *in vivo* are removed during the course of isolation. Thus, while it is clear that dsRNA is present within cells, if only from specialized sequences such as transcripts of repetitive elements, inverted repeats and viruses, little at present is known regarding conditions that promote formation of intramolecular base-pairing between large transcripts such as found in most cis-SAPs.

Integration of transcriptional and post-transcriptional riboregulation

Accumulating evidence suggests that transcription is tightly associated with many post-transcription events in the nucleus, including splicing, polyadenylation, mRNP formation, export and turnover [195–198]. The co-transcriptional integration of nuclear RNA metabolism is mediated by interactions between the transcriptional apparatus and the processing, packaging, export and degradative machinery. The co-transcriptional deposition of packaging proteins associated with hnRNP and mRNP assembly is mediated by a highly conserved mRNA metabolism and export (THO-TREX) complex found in yeast, flies and mammals [196]. The formation of protein-RNA complexes may restrict or facilitate RNA-RNA interactions [199]. Other factors have been implicated in mediating RNA-RNA and RNA-protein interactions. Helicases are associated with many different steps in RNA metabolism and appear to play a catalytic role in remodeling both RNA and RNP structure [200–202]. Many RNA binding proteins also mediate or chaperone RNA-RNA interactions [203–205]. This coordinated network of activities may either restrict or facilitate intermolecular interactions between complementary transcripts. Further insight into mechanisms of antisense regulation will require a better understanding of each step in the biogenesis and metabolism of the sense and antisense strands, from the initiation of transcription to repression or degradation of each RNA [206]. In exploring the diverse mechanisms by which antisense RNAs regulate gene expression we will gain a far more comprehensive understanding of gene expression in complex genomes.

Acknowledgments. We would like to thank Huntington Willard, Beth Sullivan and Craig Struble for their comments on this manuscript. The authors' research is supported by grant GM069390 from the National Institutes of Health (S.H.M.) and a Basil O'Connor Starter Scholar Research Award from the March of Dimes (J.Z.).

1 Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W. et al. (2001) Initial sequencing and analysis of the human genome. Nature 409, 860–921.
2 Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., Smith, H. O., Yandell, M., Evans, C. A., Holt, R. A., Gocayne, J. D., Amanatides, P. et al. (2001) The sequence of the human genome. Science 291, 1304–1351.
3 The Encode Project Consortium (2004) The ENCODE (ENCyclopedia Of DNA Elements) Project. Science 306, 636–640.
4 Bartel, D. P. (2004) MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116, 281–297.
5 Mattick, J. S. (2004) RNA regulation: a new genetics? Nat. Rev. Genet. 5, 316–323.
6 Herbert, A. (2004) The four Rs of RNA-directed evolution. Nat. Genet. 36, 19–25.
7 Storz, G., Altuvia, S. and Wassarman, K. M. (2005) An abundance of RNA regulators. Annu. Rev. Biochem. 74, 199–217.
8 Eddy, S. R. (2001) Non-coding RNA genes and the modern RNA world. Nat. Rev. Genet. 2, 919–929.
9 Wong, G. K., Passey, D. A. and Yu, J. (2001) Most of the human genome is transcribed. Genome Res. 11, 1975–1977.
10 Claverie, J. M. (2005) Fewer genes, more noncoding RNA. Science 309, 1529–1530.
11 Vitali, P., Basyuk, E., Le Meur, E., Bertrand, E., Muscatelli, F., Cavaille, J. and Huttenhofer, A. (2005) ADAR2-mediated editing of RNA substrates in the nucleolus is inhibited by C/D small nucleolar RNAs. J Cell Biol 169, 745–753.
12 Carninci, P., Kasukawa, T., Katayama, S., Gough, J., Frith, M. C., Maeda, N., Oyama, R., Ravasi, T., Lenhard, B., Wells, C., Kodzius, R., Shimokawa, K. et al. (2005) The transcriptional landscape of the mammalian genome. Science 309, 1559–1563.

13 Costa, F. F. (2005) Non-coding RNAs: new players in eukaryotic biology. Gene 357, 83–94.
14 Shendure, J. and Church, G. M. (2002) Computational discovery of sense-antisense transcription in the human and mouse genomes. Genome Biol. 3, 4401–4414.
15 Vanhee-Brossollet, C. and Vaquero, C. (1998) Do natural antisense transcripts make sense in eukaryotes? Gene 211, 1–9.
16 Kumar, M. and Carmichael, G. G. (1998) Antisense RNA: function and fate of duplex RNA in cells of higher eukaryotes. Microbiol. Mol. Biol. Rev. 62, 1415–1434.
17 Chen, J., Sun, M., Kent, W. J., Huang, X., Xie, H., Wang, W., Zhou, G., Shi, R. Z. and Rowley, J. D. (2004) Over 20% of human transcripts might form sense-antisense pairs. Nucleic Acids Res. 32, 4812–4820.
18 Yelin, R., Dahary, D., Sorek, R., Levanon, E. Y., Goldstein, O., Shoshan, A., Diber, A., Biton, S., Tamir, Y., Khosravi, R., Nemzer, S., Pinner, E., Walach, S., Bernstein, J., Savitsky, K. and Rotman, G. (2003) Widespread occurrence of antisense transcription in the human genome. Nat. Biotechnol. 21, 379–386.
19 Lehner, B., Williams, G., Campbell, R. D. and Sanderson, C. M. (2002) Antisense transcripts in the human genome. Trends Genet. 18, 63–65.
20 Lavorgna, G., Dahary, D., Lehner, B., Sorek, R., Sanderson, C. M. and Casari, G. (2004) In search of antisense. Trends Biochem. Sci. 29, 88–94.
21 Crosthwaite, S. K. (2004) Circadian clocks and natural antisense RNA. FEBS Lett. 567, 49–54.
22 Makalowska, I., Lin, C. F. and Makalowski, W. (2005) Overlapping genes in vertebrate genomes. Comput. Biol. Chem. 29, 1–12.
23 Werner, A. and Berdal, A. (2005) Natural antisense transcripts: sound or silence? Physiol. Genomics 23, 125–131.
24 Jacob, F. and Monod, J. (1961) Genetic regulatory mechanism in the synhesis of proteins. J. Mol. Biol. 3, 318–356.
25 Spiegelman, W. G., Reichardt, L. F., Yaniv, M., Heinemann, S. F., Kaiser, A. D. and Eisen, H. (1972) Bidirectional transcription and the regulation of Phage lambda repressor synthesis. Proc. Natl. Acad. Sci. USA 69, 3156–3160.
26 Davidson, E. H. and Britten, R. J. (1979) Regulation of gene expression: possible role of repetitive sequences. Science 204, 1052–1059.
27 Eguchi, Y., Itoh, T. and Tomizawa, J. I. (1991) Antisense RNA. Annu. Rev. Biochem. 60, 631–652.
28 Itoh, T. and Tomizawa, J. (1980) Formation of an RNA primer for initiation of replication of ColE1 DNA by ribonuclease H. Proc. Natl. Acad. Sci. USA 77, 2450–2454.
29 Lacatena, R. M. and Cesareni, G. (1981) Base pairing of RNA I with its complementary sequence in the primer precursor inhibits ColE1 replication. Nature 294, 623–626.
30 Wagner, E. G. and Simons, R. W. (1994) Antisense RNA control in bacteria, phages, and plasmids. Annu. Rev. Microbiol. 48, 713–742.
31 Wagner, E. G., Altuvia, S. and Romby, P. (2002) Antisense RNAs in bacteria and their genetic elements. Adv. Genet. 46, 361–398.
32 Munroe, S. H. (2004) Diversity of antisense regulation in eukaryotes: multiple mechanisms, emerging patterns. J. Cell. Biochem. 93, 664–671.
33 Reis, E. M., Louro, R., Nakaya, H. I. and Verjovski-Almeida, S. (2005) As antisense RNA gets intronic. OMICS 9, 2–12.
34 Scherer, L. J. and Rossi, J. J. (2003) Approaches for the sequence-specific knockdown of mRNA. Nat. Biotechnol. 21, 1457–1465.
35 Crooke, S. T. (2000) Progress in antisense technology: the end of the beginning. Methods Enzymol. 313, 3–45.
36 Lee, R. C., Feinbaum, R. L. and Ambros, V. (1993) The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75, 843–854.
37 Wightman, B., Ha, I. and Ruvkun, G. (1993) Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*. Cell 75, 855–862.
38 Pasquinelli, A. E., Reinhart, B. J., Slack, F., Martindale, M. Q., Kuroda, M. I., Maller, B., Hayward, D. C., Ball, E. E., Degnan, B., Muller, P., Spring, J., Srinivasan, A., Fishman, M., Finnerty, J., Corbo, J., Levine, M., Leahy, P., Davidson, E. and Ruvkun, G. (2000) Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature 408, 86–89.
39 Lee, R. C. and Ambros, V. (2001) An extensive class of small RNAs in *Caenorhabditis elegans*. Science 294, 862–864.
40 Lau, N. C., Lim, L. P., Weinstein, E. G. and Bartel, D. P. (2001) An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. Science 294, 858–862.
41 Lagos-Quintana, M., Rauhut, R., Lendeckel, W. and Tuschl, T. (2001) Identification of novel genes coding for small expressed RNAs. Science 294, 853–858.
42 Wickens, M. and Takayama, K. (1994) RNA. Deviants – or emissaries. Nature 367, 17–18.
43 Kim, V. N. (2005) MicroRNA biogenesis: coordinated cropping and dicing. Nat. Rev. Mol. Cell. Biol. 6, 376–385.
44 Pasquinelli, A. E., Hunter, S. and Bracht, J. (2005) MicroRNAs: a developing story. Curr. Opin. Genet. Dev. 15, 200–205.
45 Kidner, C. A. and Martienssen, R. A. (2005) The developmental role of microRNA in plants. Curr. Opin. Plant Biol. 8, 38–44.
46 Meltzer, P. S. (2005) Cancer genomics: small RNAs with big impacts. Nature 435, 745–746.
47 Fahey, M. E., Moore, T. F. and Higgins, D. G. (2002) Overlapping antisense transcription in the human genome. Comp. Funct. Genomics 3, 244–253.
48 Carmichael, G. G. (2003) Antisense starts making more sense. Nat. Biotechnol. 21, 371–372.
49 Katayama, S., Tomaru, Y., Kasukawa, T., Waki, K., Nakanishi, M., Nakamura, M., Nishida, H., Yap, C. C., Suzuki, M., Kawai, J., Suzuki, H., Carninci, P. et al. (2005) Antisense transcription in the mammalian transcriptome. Science 309, 1564–1566.
50 Wang, Q. Q. and Carmichael, G. G. (2004) Effects of length and location on the cellular response to double-stranded RNA. Microbiol. Mol. Biol. Rev. 68, 432–452.
51 Williams, B. R. (1999) PKR; a sentinel kinase for cellular stress. Oncogene 18, 6112–6120.
52 Sledz, C. A. and Williams, B. R. (2004) RNA interference and double-stranded-RNA-activated pathways. Biochem. Soc. Trans. 32, 952–956.
53 Sharp, P. A. (1999) RNAi and double-strand RNA. Genes Dev. 13, 139–141.
54 Hannon, G. J. (2002) RNA interference. Nature 418, 244–251.
55 Hutvagner, G., McLachlan, J., Pasquinelli, A. E., Balint, E., Tuschl, T. and Zamore, P. D. (2001) A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science 293, 834–838.
56 Murchison, E. P. and Hannon, G. J. (2004) miRNAs on the move: miRNA biogenesis and the RNAi machinery. Curr. Opin. Cell Biol. 16, 223–229.
57 Zamore, P. D., Tuschl, T., Sharp, P. A. and Bartel, D. P. (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell 101, 25–33.
58 Zamore, P. D. (2002) Ancient pathways programmed by small RNAs. Science 296, 1265–1269.
59 Almeida, R. and Allshire, R. C. (2005) RNA silencing and genome regulation. Trends Cell Biol. 15, 251–258.
60 Sontheimer, E. J. and Carthew, R. W. (2005) Silence from within: endogenous siRNAs and miRNAs. Cell 122, 9–12.

61 Grewal, S. I. and Moazed, D. (2003) Heterochromatin and epigenetic control of gene expression. Science 301, 798–802.
62 Sontheimer, E. J. (2005) Assembly and function of RNA silencing complexes. Nat. Rev. Mol. Cell. Biol. 6, 127–138.
63 Fukagawa, T., Nogami, M., Yoshikawa, M., Ikeno, M., Okazaki, T., Takami, Y., Nakayama, T. and Oshimura, M. (2004) Dicer is essential for formation of the heterochromatin structure in vertebrate cells. Nat. Cell Biol. 6, 784–791.
64 Bernstein, E. and Allis, C. D. (2005) RNA meets chromatin. Genes Dev. 19, 1635–1655.
65 Liu, J., Carmell, M. A., Rivas, F. V., Marsden, C. G., Thomson, J. M., Song, J. J., Hammond, S. M., Joshua-Tor, L. and Hannon, G. J. (2004) Argonaute2 is the catalytic engine of mammalian RNAi. Science 305, 1437–1441.
66 Carmell, M. A., Xuan, Z., Zhang, M. Q. and Hannon, G. J. (2002) The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. Genes Dev. 16, 2733–2742.
67 Bass, B. L. (2002) RNA editing by adenosine deaminases that act on RNA. Annu. Rev. Biochem. 71, 817–846.
68 Maas, S., Rich, A. and Nishikura, K. (2003) A-I RNA editing: recent news and residual mysteries. J. Biol. Chem. 278, 1391–1394.
69 Zhang, Z. and Carmichael, G. G. (2001) The fate of dsRNA in the nucleus: a p54(nrb)-containing complex mediates the nuclear retention of promiscuously A-to-I edited RNAs. Cell 106, 465–475.
70 Wang, Q., Zhang, Z., Blackwell, K. and Carmichael, G. G. (2005) Vigilins bind to promiscuously A-to-I-edited RNAs and are involved in the formation of heterochromatin. Curr. Biol. 15, 384–391.
71 DeCerbo, J. and Carmichael, G. G. (2005) Retention and repression: fates of hyperedited RNAs in the nucleus. Curr. Opin. Chem. Biol. 17, 302–308.
72 Knight, S. W. and Bass, B. L. (2002) The role of RNA editing by ADARs in RNAi. Mol. Cell 10, 809–817.
73 Yang, W., Chendrimada, T. P., Wang, Q., Higuchi, M., Seeburg, P. H., Shiekhattar, R. and Nishikura, K. (2006) Modulation of microRNA processing and expression through RNA editing by ADAR deaminases. Nat. Struct. Mol. Biol. 13, 13–21.
74 Wang, Q., Miyakoda, M., Yang, W., Khillan, J., Stachura, D. L., Weiss, M. J. and Nishikura, K. (2004) Stress-induced apoptosis associated with null mutation of ADAR1 RNA editing deaminase gene. J. Biol. Chem. 279, 4952–4961.
75 Rhoades, M. W., Reinhart, B. J., Lim, L. P., Burge, C. B., Bartel, B. and Bartel, D. P. (2002) Prediction of plant microRNA targets. Cell 110, 513–520.
76 Lewis, B. P., Shih, I. H., Jones-Rhoades, M. W., Bartel, D. P. and Burge, C. B. (2003) Prediction of mammalian microRNA targets. Cell 115, 787–798.
77 Olsen, P. H. and Ambros, V. (1999) The lin-4 regulatory RNA controls developmental timing in *Caenorhabditis elegans* by blocking LIN-14 protein synthesis after the initiation of translation. Dev. Biol. 216, 671–680.
78 Humphreys, D. T., Westman, B. J., Martin, D. I. and Preiss, T. (2005) MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. Proc. Natl. Acad. Sci. USA 102, 16961–16966.
79 Pillai, R. S., Bhattacharyya, S. N., Artus, C. G., Zoller, T., Cougot, N., Basyuk, E., Bertrand, E. and Filipowicz, W. (2005) Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science 309, 1573–1576.
80 Petersen, C. P., Bordeleau, M. E., Pelletier, J. and Sharp, P. A. (2006) Short RNAs repress translation after initiation in mammalian cells. Mol. Cell 21, 533–542.
81 Jing, Q., Huang, S., Guth, S., Zarubin, T., Motoyama, A., Chen, J., Di Padova, F., Lin, S. C., Gram, H. and Han, J. (2005) Involvement of microRNA in AU-rich element-mediated mRNA instability. Cell 120, 623–634.
82 Heywood, S. M. (1986) tcRNA as a naturally occurring antisense RNA in eukaryotes. Nucleic Acids Res. 14, 6771–6772.
83 Kiss, T. (2002) Small nucleolar RNAs: an abundant group of noncoding RNAs with diverse cellular functions. Cell 109, 145–148.
84 Celano, P., Berchtold, C. M., Kizer, D. L., Weeraratna, A., Nelkin, B. D., Baylin, S. B. and Casero, R. A. Jr (1992) Characterization of an endogenous RNA transcript with homology to the antisense strand of the human c-myc gene. J. Biol. Chem. 267, 15092–15096.
85 Khochbin, S., Brocard, M. P., Grunwald, D. and Lawrence, J. J. (1992) Antisense RNA and p53 regulation in induced murine cell differentiation. Ann. N. Y. Acad. Sci. 660, 77–87.
86 Nepveu, A. and Marcu, K. B. (1986) Intragenic pausing and antisense transcription within the murine c-myc locus. EMBO J. 5, 2859–2865.
87 Okano, H., Aruga, J., Nakagawa, T., Shiota, C. and Mikoshiba, K. (1991) Myelin basic protein gene and the function of antisense RNA in its repression in myelin-deficient mutant mouse. J. Neurochem. 56, 560–567.
88 Tosic, M., Roach, A., de Rivaz, J. C., Dolivo, M. and Matthieu, J. M. (1990) Post-transcriptional events are responsible for low expression of myelin basic protein in myelin deficient mice: role of natural antisense RNA. EMBO J. 9, 401–406.
89 Korneev, S. A., Park, J. H. and O'Shea, M. (1999) Neuronal expression of neural nitric oxide synthase (nNOS) protein is suppressed by an antisense RNA transcribed from an NOS pseudogene. J. Neurosci. 19, 7711–7720.
90 Washietl, S., Hofacker, I. L., Lukasser, M., Huttenhofer, A. and Stadler, P. F. (2005) Mapping of conserved RNA secondary structures predicts thousands of functional noncoding RNAs in the human genome. Nat. Biotechnol. 23, 1383–1390.
91 Okazaki, Y., Furuno, M., Kasukawa, T., Adachi, J., Bono H, Kondo S, Nikaido I, Osato N, Saito R, Suzuki H. et al. (2002) Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. Nature 420, 563–573.
92 Lee, S., Bao, J., Zhou, G., Shapiro, J., Xu, J., Shi, R. Z., Lu, X., Clark, T., Johnson, D., Kim, Y. C., Wing, C., Tseng, C., Sun, M. I. N., Lin, W. E. I., Wang, J. U. N., Yang, H., Wang, J., Du, W. E. I., Wu, C.-I., Zhang, X. and Wang, S. M. (2005) Detecting novel low-abundant transcripts in *Drosophila*. RNA 11, 939–946.
93 Osato, N., Yamada, H., Satoh, K., Ooka, H., Yamamoto, M., Suzuki, K., Kawai, J., Carninci, P., Ohtomo, Y., Murakami, K., Matsubara, K., Kikuchi, S. and Hayashizaki, Y. (2004) Antisense transcripts with rice full-length cDNAs. Genome Biol. 5, R5.
94 Yamada, K., Lim, J., Dale, J. M., Chen, H., Shinn, P., Palm, C. J., Southwick, A. M., Wu, H. C., Kim, C., Nguyen, M., Pham, P., Cheuk, R. et al. (2003) Empirical analysis of transcriptional activity in the Arabidopsis genome. Science 302, 842–846.
95 Steigele, S. and Nieselt, K. (2005) Open reading frames provide a rich pool of potential natural antisense transcripts in fungal genomes. Nucleic Acids Res. 33, 5034–5044.
96 Havilio, M., Levanon, E. Y., Lerman, G., Kupiec, M. and Eisenberg, E. (2005) Evidence for abundant transcription of non-coding regions in the *Saccharomyces cerevisiae* genome. BMC Genomics 6, 93.
97 Gunasekera, A. M., Patankar, S., Schug, J., Eisen, G., Kissinger, J., Roos, D. and Wirth, D. F. (2004) Widespread distribution of antisense transcripts in the *Plasmodium falciparum* genome. Mol. Biochem. Parasitol. 136, 35–42.
98 Militello, K. T., Patel, V., Chessler, A.-D., Fisher, J. K., Kasper, J. M., Gunasekera, A. and Wirth, D. F. (2005) RNA polymerase II synthesizes antisense RNA in *Plasmodium falciparum*. RNA 11, 365–370.
99 Elmendorf, H. G., Singer, S. M. and Nash, T. E. (2001) The abundance of sterile transcripts in *Giardia lamblia*. Nucleic Acids Res. 29, 4674–4683.

100 Elgin, S. C. R. and Grewal, S. I. S. (2003) Heterochromatin: silence is golden. Curr. Biol. 13, R895–R898.
101 Aravind, L., Watanabe, H., Lipman, D. J. and Koonin, E. V. (2000) Lineage-specific loss and divergence of functionally linked genes in eukaryotes. Proc. Natl. Acad. Sci. USA 97, 11319–11324.
102 Kumar, A., Harrison, P. M., Cheung, K. H., Lan, N., Echols, N., Bertone, P., Miller, P., Gerstein, M. B. and Snyder, M. (2002) An integrated approach for finding overlooked genes in yeast. Nat. Biotechnol. 20, 58–63.
103 Prescott, E. M. and Proudfoot, N. J. (2002) Transcriptional collision between convergent genes in budding yeast. Proc. Natl. Acad. Sci. USA 99, 8796–8801.
104 Shearwin, K. E., Callen, B. P. and Egan, J. B. (2005) Transcriptional interference – a crash course. Trends Genet. 21, 339–345.
105 Kiyosawa, H., Yamanaka, I., Osato, N., Kondo, S. and Hayashizaki, Y. (2003) Antisense transcripts with FANTOM2 clone set and their implications for gene regulation. Genome Res. 13, 1324–1334.
106 Sun, M., Hurst, L. D., Carmichael, G. G. and Chen, J. (2005) Evidence for a preferential targeting of 3′-UTRs by cis-encoded natural antisense transcripts. Nucleic Acids Res. 33, 5533–5543.
107 The International Human Genome Consortium (2004) Finishing the euchromatic sequence of the human genome. Nature 431, 931–945.
108 Caron, H., van Schaik, B., van der Mee, M., Baas, F., Riggins, G., van Sluis, P., Hermus, M. C., van Asperen, R., Boon, K., Voute, P. A., Heisterkamp, S., van Kampen, A. and Versteeg, R. (2001) The human transcriptome map: clustering of highly expressed genes in chromosomal domains. Science 291, 1289–1292.
109 Chakalova, L., Debrand, E., Mitchell, J. A., Osborne, C. S. and Fraser, P. (2005) Replication and transcription: shaping the landscape of the genome. Nat. Rev. Genet. 6, 669–677.
110 Spellman, P. T. and Rubin, G. M. (2002) Evidence for large domains of similarly expressed genes in the *Drosophila* genome. J. Biol. 1, 5.
111 Cawley, S., Bekiranov, S., Ng, H. H., Kapranov, P., Sekinger, E. A., Kampa, D., Piccolboni, A., Sementchenko, V., Cheng, J., Williams, A. J., Wheeler, R., Wong, B., Drenkow, J., Yamanaka, M., Patel, S., Brubaker, S., Tammana, H., Helt, G., Struhl, K. and Gingeras, T. R. (2004) Unbiased mapping of transcription factor binding sites along human chromosomes 21 and 22 points to widespread regulation of noncoding RNAs. Cell 116, 499–509.
112 Lercher, M. J., Urrutia, A. O. and Hurst, L. D. (2002) Clustering of housekeeping genes provides a unified model of gene order in the human genome. Nat. Genet. 31, 180–183.
113 Kapranov, P., Drenkow, J., Cheng, J., Long, J., Helt, G., Dike, S. and Gingeras, T. R. (2005) Examples of the complex architecture of the human transcriptome revealed by RACE and high-density tiling arrays. Genome Res. 15, 987–997.
114 Kiyosawa, H., Mise, N., Iwase, S., Hayashizaki, Y. and Abe, K. (2005) Disclosing hidden transcripts: mouse natural sense-antisense transcripts tend to be poly(A) negative and nuclear localized. Genome Res. 15, 463–474.
115 Shapiro, J. A. and von Sternberg, R. (2005) Why repetitive DNA is essential to genome function. Biol. Rev. Camb. Philos. Soc. 80, 227–250.
116 Veeramachaneni, V., Makalowski, W., Galdzicki, M., Sood, R. and Makalowska, I. (2004) Mammalian overlapping genes: the comparative perspective. Genome Res. 14, 280–286.
117 Pang, K. C., Frith, M. C. and Mattick, J. S. (2006) Rapid evolution of noncoding RNAs: lack of conservation does not mean lack of function. Trends Genet. 22, 1–5.
118 Ravasi, T., Suzuki, H., Pang, K. C., Katayama, S., Furuno, M., Okunishi, R., Fukuda, S., Ru, K., Frith, M. C., Gongora, M. M., Grimmond, S. M., Hume, D. A., Hayashizaki, Y. and Mattick, J. S. (2006) Experimental validation of the regulated expression of large numbers of non-coding RNAs from the mouse genome. Genome Res. 16, 11–19.
119 Cheng, J., Kapranov, P., Drenkow, J., Dike, S., Brubaker, S., Patel, S., Long, J., Stern, D., Tammana, H., Helt, G., Sementchenko, V., Piccolboni, A., Bekiranov, S., Bailey, D. K., Ganesh, M., Ghosh, S., Bell, I., Gerhard, D. S. and Gingeras, T. R. (2005) Transcriptional maps of 10 human chromosomes at 5-nucleotide resolution. Science 308, 1149–1154.
120 Johnson, J. M., Edwards, S., Shoemaker, D. and Schadt, E. E. (2005) Dark matter in the genome: evidence of widespread transcription detected by microarray tiling experiments. Trends Genet. 21, 93–102.
121 Shapiro, J. A. (2005) A 21st century view of evolution: genome system architecture, repetitive DNA, and natural genetic engineering. Gene 345, 91–100.
122 Wassarman, K. M. (2004) RNA regulators of transcription. Nat. Struct. Mol. Biol. 11, 803–804.
123 Jurka, J. (2004) Evolutionary impact of human Alu repetitive elements. Curr. Opin. Genet. Dev. 14, 603–608.
124 Han, J. S. and Boeke, J. D. (2005) LINE-1 retrotransposons: modulators of quantity and quality of mammalian gene expression? Bioessays 27, 775–784.
125 Han, J. S., Szak, S. T. and Boeke, J. D. (2004) Transcriptional disruption by the L1 retrotransposon and implications for mammalian transcriptomes. Nature 429, 268–274.
126 Hirotsune, S., Yoshida, N., Chen, A., Garrett, L., Sugiyama, F., Takahashi, S., Yagami, K., Wynshaw-Boris, A. and Yoshiki, A. (2003) An expressed pseudogene regulates the messenger-RNA stability of its homologous coding gene. Nature 423, 91–96.
127 Chen, J., Sun, M., Hurst, L. D., Carmichael, G. G. and Rowley, J. D. (2005) Genome-wide analysis of coordinate expression and evolution of human cis-encoded sense-antisense transcripts. Trends Genet. 21, 326–329.
128 Trinklein, N. D., Aldred, S. F., Hartman, S. J., Schroeder, D. I., Otillar, R. P. and Myers, R. M. (2004) An abundance of bidirectional promoters in the human genome. Genome Res. 14, 62–66.
129 Chen, J., Sun, M., Hurst, L. D., Carmichael, G. G. and Rowley, J. D. (2005) Human antisense genes have unusually short introns: evidence for selection for rapid transcription. Trends Genet. 21, 203–207.
130 Chen, J., Sun, M., Rowley, J. D. and Hurst, L. D. (2005) The small introns of antisense genes are better explained by selection for rapid transcription than by 'genomic design'. Genetics 171, 2151–2155.
131 Dahary, D., Elroy-Stein, O. and Sorek, R. (2005) Naturally occurring antisense: transcriptional leakage or real overlap? Genome Res. 15, 364–368.
132 Kapranov, P., Cawley, S. E., Drenkow, J., Bekiranov, S., Strausberg, R. L., Fodor, S. P. and Gingeras, T. R. (2002) Large-scale transcriptional activity in chromosomes 21 and 22. Science 296, 916–919.
133 Kampa, D., Cheng, J., Kapranov, P., Yamanaka, M., Brubaker, S., Cawley, S., Drenkow, J., Piccolboni, A., Bekiranov, S., Helt, G., Tammana, H. and Gingeras, T. R. (2004) Novel RNAs identified from an in-depth analysis of the transcriptome of human chromosomes 21 and 22. Genome Res. 14, 331–342.
134 Bertone, P., Stolc, V., Royce, T. E., Rozowsky, J. S., Urban, A. E., Zhu, X., Rinn, J. L., Tongprasit, W., Samanta, M., Weissman, S., Gerstein, M. and Snyder, M. (2004) Global identification of human transcribed sequences with genome tiling arrays. Science 306, 2242–2246.
135 Ogawa, Y. and Lee, J. T. (2002) Antisense regulation in X inactivation and autosomal imprinting. Cytogenet. Genome Res. 99, 59–65.

136 Tommasi, S. and Pfeifer, G. P. (1999) *In vivo* structure of two divergent promoters at the human PCNA locus. Synthesis of antisense RNA and S phase-dependent binding of E2F complexes in intron 1. J. Biol. Chem. 274, 27829–27838.

137 Pauler, F. M., Stricker, S. H., Warczok, K. E. and Barlow, D. P. (2005) Long-range DNase I hypersensitivity mapping reveals the imprinted Igf2r and Air promoters share cis-regulatory elements. Genome Res 15, 1379–1387.

138 Impey, S., McCorkle, S. R., Cha-Molstad, H., Dwyer, J. M., Yochum, G. S., Boss, J. M., McWeeney, S., Dunn, J. J., Mandel, G. and Goodman, R. H. (2004) Defining the CREB regulon: a genome-wide analysis of transcription factor regulatory regions. Cell 119, 1041–1054.

139 Tsao, Y. P., Wu, H. Y. and Liu, L. F. (1989) Transcription-driven supercoiling of DNA: direct biochemical evidence from *in vitro* studies. Cell 56, 111–118.

140 Wang, Y., Joh, K., Masuko, S., Yatsuki, H., Soejima, H., Nabetani, A., Beechey, C. V., Okinami, S. and Mukai, T. (2004) The mouse Murr1 gene is imprinted in the adult brain, presumably due to transcriptional interference by the antisense-oriented U2af1-rs1 gene. Mol. Cell. Biol. 24, 270–279.

141 Navarro, P., Pichard, S., Ciaudo, C., Avner, P. and Rougeulle, C. (2005) Tsix transcription across the Xist gene alters chromatin conformation without affecting Xist transcription: implications for X-chromosome inactivation. Genes Dev. 19, 1474–1484.

142 Cook, P. R. (2003) Nongenic transcription, gene regulation and action at a distance. J. Cell Sci. 116, 4483–4491.

143 Volpe, T. A., Kidner, C., Hall, I. M., Teng, G., Grewal, S. I. and Martienssen, R. A. (2002) Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. Science 297, 1833–1837.

144 Saceda, M., Knabbe, C., Dickson, R. B., Lippman, M. E., Bronzert, D., Lindsey, R. K., Gottardis, M. M. and Martin, M. B. (1991) Post-transcriptional destabilization of estrogen receptor mRNA in MCF-7 cells by 12-O-tetradecanoylphorbol-13-acetate. J. Biol. Chem. 266, 17809–17814.

145 Verona, R. I. and Bartolomei, M. S. (2004) Role of H19 3′ sequences in controlling H19 and Igf2 imprinting and expression. Genomics 84, 59–68.

146 Hastings, M. L., Ingle, H. A., Lazar, M. A. and Munroe, S. H. (2000) Post-transcriptional regulation of thyroid hormone receptor expression by cis-acting sequences and a naturally occurring antisense RNA. J. Biol. Chem. 275, 11507–11513.

147 Lazar, M. A., Hodin, R. A., Cardona, G. and Chin, W. W. (1990) Gene expression from the c-erbA alpha/Rev-ErbA alpha genomic locus. Potential regulation of alternative splicing by opposite strand transcription. J. Biol. Chem. 265, 12859–12863.

148 Kumar, M. and Carmichael, G. G. (1997) Nuclear antisense RNA induces extensive adenosine modifications and nuclear retention of target transcripts. Proc. Natl. Acad. Sci. USA 94, 3542–3547.

149 Capaccioli, S., Quattrone, A., Schiavone, N., Calastretti, A., Copreni, E., Bevilacqua, A., Canti, G., Gong, L., Morelli, S. and Nicolin, A. (1996) A bcl-2/IgH antisense transcript deregulates bcl-2 gene expression in human follicular lymphoma t(14;18) cell lines. Oncogene 13, 105–115.

150 Li, A. W. and Murphy, P. R. (2000) Expression of alternatively spliced FGF-2 antisense RNA transcripts in the central nervous system: regulation of FGF-2 mRNA translation. Mol. Cell. Endocrinol. 170, 233–242.

151 Shibata, S. and Lee, J. T. (2003) Characterization and quantitation of differential Tsix transcripts: implications for Tsix function. Hum. Mol. Genet. 12, 125–136.

152 Wutz, A., Rasmussen, T. P. and Jaenisch, R. (2002) Chromosomal silencing and localization are mediated by different domains of Xist RNA. Nat. Genet. 30, 167–174.

153 Valente, L. and Nishikura, K. (2005) ADAR gene family and A-to-I RNA editing: diverse roles in posttranscriptional gene regulation. Prog. Nucleic Acid Res. Mol. Biol. 79, 299–338.

154 Wang, X. J., Gaasterland, T. and Chua, N. H. (2005) Genome-wide prediction and identification of cis-natural antisense transcripts in *Arabidopsis thaliana*. Genome Biol. 6, R30.

155 Hutvagner, G. and Zamore, P. D. (2002) A microRNA in a multiple-turnover RNAi enzyme complex. Science 297, 2056–2060.

156 Kawai, J., Shinagawa, A., Shibata, K., Yoshino, M., Itoh, M., Ishii, Y., Arakawa, T., Hara, A., Fukunishi, Y., Konno, H., Adachi, J., Fukuda, S. et al. (2001) Functional annotation of a full-length mouse cDNA collection. Nature 409, 685–690.

157 Luedi, P. P., Hartemink, A. J. and Jirtle, R. L. (2005) Genome-wide prediction of imprinted murine genes. Genome Res 15, 875–884.

158 Morison, I. M., Ramsay, J. P. and Spencer, H. G. (2005) A census of mammalian imprinting. Trends Genet. 21, 457–465.

159 Paulsen, M., Takada, S., Youngson, N. A., Benchaib, M., Charlier, C., Segers, K., Georges, M. and Ferguson-Smith, A. C. (2001) Comparative sequence analysis of the imprinted Dlk1-Gtl2 locus in three mammalian species reveals highly conserved genomic elements and refines comparison with the Igf2-H19 region. Genome Res. 11, 2085–2094.

160 Nicholls, R. D., Saitoh, S. and Horsthemke, B. (1998) Imprinting in Prader-Willi and Angelman syndromes. Trends Genet. 14, 194–200.

161 Lee, J. T. and Lu, N. (1999) Targeted mutagenesis of Tsix leads to nonrandom X inactivation. Cell 99, 47–57.

162 Sado, T., Wang, Z., Sasaki, H. and Li, E. (2001) Regulation of imprinted X-chromosome inactivation in mice by Tsix. Development 128, 1275–1286.

163 Thakur, N., Tiwari, V. K., Thomassin, H., Pandey, R. R., Kanduri, M., Gondor, A., Grange, T., Ohlsson, R. and Kanduri, C. (2004) An antisense RNA regulates the bidirectional silencing property of the Kcnq1 imprinting control region. Mol. Cell. Biol. 24, 7855–7862.

164 Stefan, M., Portis, T., Longnecker, R. and Nicholls, R. D. (2005) A nonimprinted Prader-Willi Syndrome (PWS)-region gene regulates a different chromosomal domain in trans but the imprinted pws loci do not alter genome-wide mRNA levels. Genomics 85, 630–640.

165 Bastepe, M., Frohlich, L. F., Linglart, A., Abu-Zahra, H. S., Tojo, K., Ward, L. M. and Juppner, H. (2005) Deletion of the NESP55 differentially methylated region causes loss of maternal GNAS imprints and pseudohypoparathyroidism type Ib. Nat. Genet. 37, 25–27.

166 Bartolomei, M. S. and Tilghman, S. M. (1997) Genomic imprinting in mammals. Annu. Rev. Genet. 31, 493–525.

167 Lewis, A., Mitsuya, K., Umlauf, D., Smith, P., Dean, W., Walter, J., Higgins, M., Feil, R. and Reik, W. (2004) Imprinting on distal chromosome 7 in the placenta involves repressive histone methylation independent of DNA methylation. Nat. Genet. 36, 1291–1295.

168 Santisteban, I., Arredondo-Vega, F. X., Kelly, S., Loubser, M., Meydan, N., Roifman, C., Howell, P. L., Bowen, T., Weinberg, K. I., Schroeder, M. L. et al. (1995) Three new adenosine deaminase mutations that define a splicing enhancer and cause severe and partial phenotypes: implications for evolution of a CpG hotspot and expression of a transduced ADA cDNA. Hum. Mol. Genet. 4, 2081–2087.

169 Sleutels, F., Zwart, R. and Barlow, D. P. (2002) The non-coding Air RNA is required for silencing autosomal imprinted genes. Nature 415, 810–813.

170 Fitzpatrick, G. V., Soloway, P. D. and Higgins, M. J. (2002) Regional loss of imprinting and growth deficiency in mice with a targeted deletion of KvDMR1. Nat. Genet. 32, 426–431.

171 Chow, J. C., Yen, Z., Ziesche, S. M. and Brown, C. J. (2005) Silencing of the mammalian X chromosome. Annu. Rev. Genomics Hum. Genet. 6, 69–92.

172 Luikenhuis, S., Wutz, A. and Jaenisch, R. (2001) Antisense transcription through the Xist locus mediates Tsix function in embryonic stem cells. Mol. Cell. Biol. 21, 8512–8520.

173 Stavropoulos, N., Lu, N. and Lee, J. T. (2001) A functional role for Tsix transcription in blocking Xist RNA accumulation but not in X-chromosome choice. Proc. Natl. Acad. Sci. USA 98, 10232–10237.
174 Shibata, S. and Lee, J. T. (2004) Tsix transcription- versus RNA-based mechanisms in Xist repression and epigenetic choice. Curr. Biol. 14, 1747–1754.
175 Kramer, C., Loros, J. J., Dunlap, J. C. and Crosthwaite, S. K. (2003) Role for antisense RNA in regulating circadian clock function in *Neurospora crassa*. Nature 421, 948–952.
176 Luther, H. P. (2005) Role of endogenous antisense RNA in cardiac gene regulation. J. Mol. Med. 83, 26–32.
177 Alfano, G., Vitiello, C., Caccioppoli, C., Caramico, T., Carola, A., Szego, M. J., McInnes, R. R., Auricchio, A. and Banfi, S. (2005) Natural antisense transcripts associated with genes involved in eye development. Hum. Mol. Genet. 14, 913–923.
178 Lipovich, L., Vanisri, R. R., Kong, S. L., Lin, C. Y. and Liu, E. T. (2005) Primate-specific endogenous cis-antisense transcription in the human 5q31 protocadherin gene cluster. J. Mol. Evol. 62,73–88.
179 Nemes, J. P., Benzow, K. A. and Koob, M. D. (2000) The SCA8 transcript is an antisense RNA to a brain-specific transcript encoding a novel actin-binding protein (KLHL1). Hum. Mol. Genet. 9, 1543–1551.
180 Hammarsund, M., Lerner, M., Zhu, C., Merup, M., Jansson, M., Gahrton, G., Kluin-Nelemans, H., Einhorn, S., Grander, D., Sangfelt, O. and Corcoran, M. (2004) Disruption of a novel ectodermal neural cortex 1 antisense gene, ENC-1AS and identification of ENC-1 overexpression in hairy cell leukemia. Hum. Mol. Genet. 13, 2925–2936.
181 O'Neill, M. J. (2005) The influence of non-coding RNAs on allele-specific gene expression in mammals. Hum. Mol. Genet. 14 Spec No 1, R113–120.
182 Tufarelli, C., Stanley, J. A., Garrick, D., Sharpe, J. A., Ayyub, H., Wood, W. G. and Higgs, D. R. (2003) Transcription of antisense RNA leading to gene silencing and methylation as a novel cause of human genetic disease. Nat. Genet. 34, 157–165.
183 Teixeira, A., Tahiri-Alaoui, A., West, S., Thomas, B., Ramadass, A., Martianov, I., Dye, M., James, W., Proudfoot, N. J. and Akoulitchev, A. (2004) Autocatalytic RNA cleavage in the human beta-globin pre-mRNA promotes transcription termination. Nature 432, 526–530.
184 Davis, E., Jensen, C. H., Schroder, H. D., Farnir, F., Shay-Hadfield, T., Kliem, A., Cockett, N., Georges, M. and Charlier, C. (2004) Ectopic expression of DLK1 protein in skeletal muscle of padumnal heterozygotes causes the callipyge phenotype. Curr. Biol. 14, 1858–1862.
185 Smit, M., Segers, K., Carrascosa, L. G., Shay, T., Baraldi, F., Gyapay, G., Snowder, G., Georges, M., Cockett, N. and Charlier, C. (2003) Mosaicism of Solid Gold supports the causality of a noncoding A-to-G transition in the determinism of the callipyge phenotype. Genetics 163, 453–456.
186 Neeman, Y., Dahary, D., Levanon, E. Y., Sorek, R. and Eisenberg, E. (2005) Is there any sense in antisense editing? Trends Genet. 21, 544–547.
187 Levanon, E. Y., Eisenberg, E., Yelin, R., Nemzer, S., Hallegger, M., Shemesh, R., Fligelman, Z. Y., Shoshan, A., Pollock, S. R., Sztybel, D., Olshansky, M., Rechavi, G. and Jantsch, M. F. (2004) Systematic identification of abundant A-to-I editing sites in the human transcriptome. Nat. Biotechnol. 22, 1001–1005.
188 Lu, C., Tej, S. S., Luo, S., Haudenschild, C. D., Meyers, B. C. and Green, P. J. (2005) Elucidation of the small RNA component of the transcriptome. Science 309, 1567–1569.
189 Borsani, O., Zhu, J., Verslues, P. E., Sunkar, R. and Zhu, J. K. (2005) Endogenous siRNAs derived from a pair of natural cis-antisense transcripts regulate salt tolerance in *Arabidopsis*. Cell 123, 1279–1291.
190 Krystal, G. W., Armstrong, B. C. and Battey, J. F. (1990) N-myc mRNA forms an RNA-RNA duplex with endogenous antisense transcripts. Mol. Cell. Biol. 10, 4180–4191.
191 Podlowski, S., Bramlage, P., Baumann, G., Morano, I. and Luther, H. P. (2002) Cardiac troponin I sense-antisense RNA duplexes in the myocardium. J. Cell. Biochem. 85, 198–207.
192 Solda, G., Boi, S., Duga, S., Fornasari, D., Benfante, R., Malcovati, M. and Tenchini, M. L. (2005) *In vivo* RNA-RNA duplexes from human alpha3 and alpha5 nicotinic receptor subunit mRNAs. Gene 345, 155–164.
193 Wang, S. and Dolnick, B. J. (1993) Quantitative evaluation of intracellular sense: antisense RNA hybrid duplexes. Nucleic Acids Res. 21, 4383–4391.
194 Rosok, O. and Sioud, M. (2005) Systematic search for natural antisense transcripts in eukaryotes. Int. J. Mol. Med. 15, 197–203.
195 Kornblihtt, A. R., de la Mata, M., Fededa, J. P., Munoz, M. J. and Nogues, G. (2004) Multiple links between transcription and splicing. RNA 10, 1489–1498.
196 Reed, R. and Cheng, H. (2005) TREX, SR proteins and export of mRNA. Curr. Opin. Cell Biol. 17, 269–273.
197 Aguilera, A. (2005) Cotranscriptional mRNP assembly: from the DNA to the nuclear pore. Curr. Opin. Cell Biol. 17, 242–250.
198 Beggs, J. D. and Tollervey, D. (2005) Crosstalk between RNA metabolic pathways: an RNOMICS approach. Nat. Rev. Mol. Cell. Biol. 6, 423–429.
199 Munroe, S. H. and Dong, X. F. (1992) Heterogeneous nuclear ribonucleoprotein A1 catalyzes RNA.RNA annealing. Proc. Natl. Acad. Sci. USA 89, 895–899.
200 Rocak, S. and Linder, P. (2004) DEAD-box proteins: the driving forces behind RNA metabolism. Nat. Rev. Mol. Cell. Biol. 5, 232–241.
201 Yang, Q. and Jankowsky, E. (2005) ATP- and ADP-dependent modulation of RNA unwinding and strand annealing activities by the DEAD-box protein DED1. Biochemistry 44, 13591–13601.
202 Cordin, O., Banroques, J., Tanner, N. K. and Linder, P. (2005) The DEAD-box protein family of RNA helicases. Gene 367, 17–37.
203 Wunsche, W. and Sczakiel, G. (2005) The activity of siRNA in mammalian cells is related to the kinetics of siRNA-target recognition *in vitro*: mechanistic implications. J. Mol. Biol. 345, 203–209.
204 Cristofari, G. and Darlix, J. L. (2002) The ubiquitous nature of RNA chaperone proteins. Prog. Nucleic Acid Res. Mol. Biol. 72, 223–268.
205 Herschlag, D. (1995) RNA chaperones and the RNA folding problem. J. Biol. Chem. 270, 20871–20874.
206 Moore, M. J. (2005) From birth to death: the complex lives of eukaryotic mRNAs. Science 309, 1514–1518.
207 Korneev, S. and O'Shea, M. (2002) Evolution of nitric oxide synthase regulatory genes by DNA inversion. Mol. Biol. Evol. 19, 1228–1233.
208 Lipovich, L. and King, M. C. (2003) Novel transcriptional units and unconventional gene pairs in the human genome: toward a sequence-level basis for primate-specific phenotypes? Cold Spring Harb. Symp. Quant. Biol. 68, 461–470.
209 Davis, E., Caiment, F., Tordoir, X., Cavaille, J., Ferguson-Smith, A., Cockett, N., Georges, M. and Charlier, C. (2005) RNAi-mediated allelic trans-interaction at the imprinted Rtl1/Peg11 locus. Curr. Biol. 15, 743–749.