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Review

Antisense-RNA regulation and RNA interference

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Abstract

For a long time, RNA has been merely regarded as a molecule that can either function as a messenger (mRNA) or as part of the translational machinery (tRNA, rRNA). Meanwhile, it became clear that RNAs are versatile molecules that do not only play key roles in many important biological processes like splicing, editing, protein export and others, but can also—like enzymes—act catalytically. Two important aspects of RNA function—antisense-RNA control and RNA interference (RNAi)—are emphasized in this review. Antisense-RNA control functions in all three kingdoms of life—although the majority of examples are known from bacteria. In contrast, RNAi, gene silencing triggered by double-stranded RNA, the oldest and most ubiquitous antiviral system, is exclusively found in eukaryotes. Our current knowledge about occurrence, biological roles and mechanisms of action of antisense RNAs as well as the recent findings about involved genes/enzymes and the putative mechanism of RNAi are summarized. An interesting intersection between both regulatory mechanisms is briefly discussed.

Keywords: RNA; Antisense-RNA regulation; RNA interference

1. Antisense-RNA regulation

1.1. Characteristics and occurrence of antisense RNAs

The first natural antisense RNAs were discovered in 1981 independently in Tomizawas and in Nordströms laboratories. These authors found that small plasmid-encoded RNA regulators control the copy numbers of the *Escherichia coli* plasmids ColE1 and R1, respectively [1,2]. Today, we know that these regulators are rather widespread. Antisense RNAs are small, diffusible, highly structured RNAs that act via sequence complementarity on target RNAs called sense RNAs. In eukaryotes, some processes like splicing or editing make use also of complementary small RNAs; however, these RNAs are not independent regulators, and are, therefore, not regarded as bona fide antisense RNAs. In the classical case, antisense RNAs are encoded in cis, i.e. they are transcribed from a promoter located on the opposite strand of the same DNA molecule, and are, therefore, fully complementary to their target RNAs. However, over the past years, a number of antisense RNAs were detected that are encoded in trans, reveal only partial complementarity to their target RNA and have more than one target. The sense RNAs are mostly mRNAs encoding proteins of important/essential functions. In the majority of cases, antisense-RNA action entails posttranscriptional inhibition of target RNA function. However, a few activating antisense RNAs have been found, too (see below). Naturally occurring antisense RNAs are between 35 and 150 nt long (one exception see below) and comprise between one and four stem-loops. Efficient antisense RNAs have 5–8 nt GC-rich loops. Stems that are important for metabolic stability are often (if >10 bp) interrupted by bulges to prevent dsRNase degradation and to facilitate melting upon antisense/sense RNA interaction [3,4]. Recognition loops of the antisense RNA or the complementary sense RNA often contain a YUNR motif forming a U-turn structure, a sharp bent in the RNA phosphate backbone, thus providing a scaffold for the rapid interaction with the complementary RNA [5,6]. Some antisense RNAs (those involved in plasmid copy number control and postsegregational killing) are unstable, others (most chromosomally encoded and a few phage and transposon antisense RNAs) are stable. In some cases, the degradation pathway has been studied (Hok/Sok of R1 [7]; CopA/CopT of R1 [8,9]; RNAI/RNAII of ColE1 [10]).

Abbreviations: aa, amino acids; bp, base pair; dsRNA, double-stranded RNA; kD, kilodalton; RdRp, RNA-dependent RNA polymerase; RBS, ribosome binding site; SD, Shine–Dalgarno sequence; ss, single-stranded; ssRNA, single-stranded RNA

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In this review, only naturally occurring antisense RNAs will be covered, and cases, where artificially designed antisense RNAs have been introduced to inhibit gene function will not be discussed. For the latter case, I refer to other reviews (e.g. Ref. [11]).

Almost all naturally occurring antisense-RNA regulated systems have been found so far and characterized in prokaryotes, and only a few systems are known from eukaryotes and one from archaea [12]. Other reviews, dealing with prokaryotic antisense RNAs [13,14], with some aspects of sense/antisense-RNA interaction [15,16], the kinetics of sense/antisense-RNA interaction [17,18] or with eukaryotic antisense RNAs [11,19,20] have been published recently, and the reader should refer to them for more details.

An overview will be given over the known prokaryotic and eukaryotic sense/antisense systems, and some of them will be discussed in more detail. The main focus will be the principal function of the system and the binding kinetics of both interacting molecules.

1.1.1. Antisense-RNA regulated systems in prokaryotes

In prokaryotes, antisense-RNA regulated systems have been mostly detected in so-called accessory DNA elements like plasmids, phages or transposons and only few (see below) have been found to be of chromosomal origin. However, during the past 4 years, some small chromosomally encoded RNAs with hitherto unknown function have been found to act as antisense RNAs, too, like OxyS, DsrA and 6S RNA [21–24]. Moreover, three recent publications report the detection of still uncharacterized 14, 17 or 11 chromosomally encoded small RNAs in *E. coli*, respectively, and at least some of them will turn out to be antisense RNAs, too [25–27].

Fig. 1 provides examples for seven well-studied prokaryotic and one eukaryotic antisense-RNA-mediated regulatory mechanisms.

1.1.1.1. Plasmid systems. In the case of plasmids, antisense RNAs are involved in the regulation of three different functions: replication, conjugation and segregation.

Control of plasmid replication. Antisense RNAs are synthesized constitutively and are metabolically unstable (exception: RNAIII of pIP501, see below). Antisense-RNA-mediated replication control works through a negative control circuit: any change in plasmid copy number is reflected by a change in antisense-RNA concentration. These changed concentrations are sensed and lead to altered replication frequencies. This scenario can be accomplished by the following different mechanisms.

Inhibition of primer formation (ColE1-related plasmids). The replication control of ColE1 has been investigated in great detail (reviewed in Ref. [28]). ColE1 requires the synthesis of a pre-primer, RNAII, of ≈550 nt length, but no plasmid encoded initiator protein for the regulation of its replication. For the formation of a persistent RNAII/DNA hybrid within the origin, RNAII must acquire specific secondary and tertiary structures, which form during RNAII synthesis. This is a well-characterized series of structural events. The RNA strand of the RNA/DNA hybrid is then cleaved by RNase H and the resulting mature primer is extended by DNA polymerase I. A 108-nt-long antisense RNA (RNAI) containing three stem-loops induces a change in the folding of the nascent primer so that primer maturation is prevented. Thereby, timing is crucial and binding of RNAI must occur within a short time-window. The plasmid encoded Rom protein of ColE1 strongly stabilizes kissing complex formation between RNAI and RNAII stem-loops. However, its effect on replication frequency is with two to fivefold relatively low, since the inhibition rate is primarily determined by the binding rate constant and not the binding affinity between loop–loop complexes.

Inhibition of synthesis of a leader peptide required for efficient rep translation (IncFII, IFc, FIII-type plasmids). The best studied example is IncFII plasmid R1, replicating in *E. coli* and closely related bacteria (reviewed in Refs. [13,14]). Two components control the synthesis of the rate-limiting replication initiator protein RepA: a small CopB protein acting as transcriptional repressor of the *repA* promoter and a 90-nt antisense RNA containing two stem-loops, CopA. CopA is complementary to its target in the *repA* mRNA leader, called CopT. Binding of CopA to CopT sterically blocks the initiation of translation of a 24-aa leader peptide, which is required for efficient *repA* translation because a stable secondary structure sequesters the *repA* RBS. Kinetics of CopA/CopT kissing complex and duplex formation have been studied (e.g. Refs. [29,30]), and the single steps of CopA–CopT interaction have been elucidated recently (see below).

Direct inhibition of rep translation by blockage of the rep RBS. This most trivial case for the action of an antisense RNA has been found for the pMV158 derivative pLS1, a broad host range streptococcal plasmid that also replicates in *E. coli*. Here, a small antisense RNA (RNAII) complementary to the *repB*-RBS directly inhibits ribosome loading [31]. A second control component, CopG (formerly RepA), represses transcription of the *repB* promoter and its own promoter. Both components act together to allow proper regulation [32]. A similar mechanism is suggested to be used by other related plasmids, such as pE194, etc.

Prevention of pseudoknot formation required for efficient Rep synthesis. Inhibition of rep expression in IncB, IncIα, IncK, IncL/M plasmids involves synthesis of a long-distance activator RNA pseudoknot [33,34]. As in R1, a leader peptide ORF, *repY* (in ColIB-P9), must be translated to allow RepZ synthesis (e.g. Ref. [35]). The main function of *repY* translation is the disruption of an inhibitory stem-loop at the *rep* RBS. This permits the formation of a short helix between the target loop and disrupted stem, located 100 nt apart. This long-distance pseudoknot activates *repZ* translation. A 70-nt antisense RNA, RNAI (Inc RNA), has a dual function: its interaction with the *repZ*-RNA blocks both leader peptide translation and pseudoknot formation [36,37].

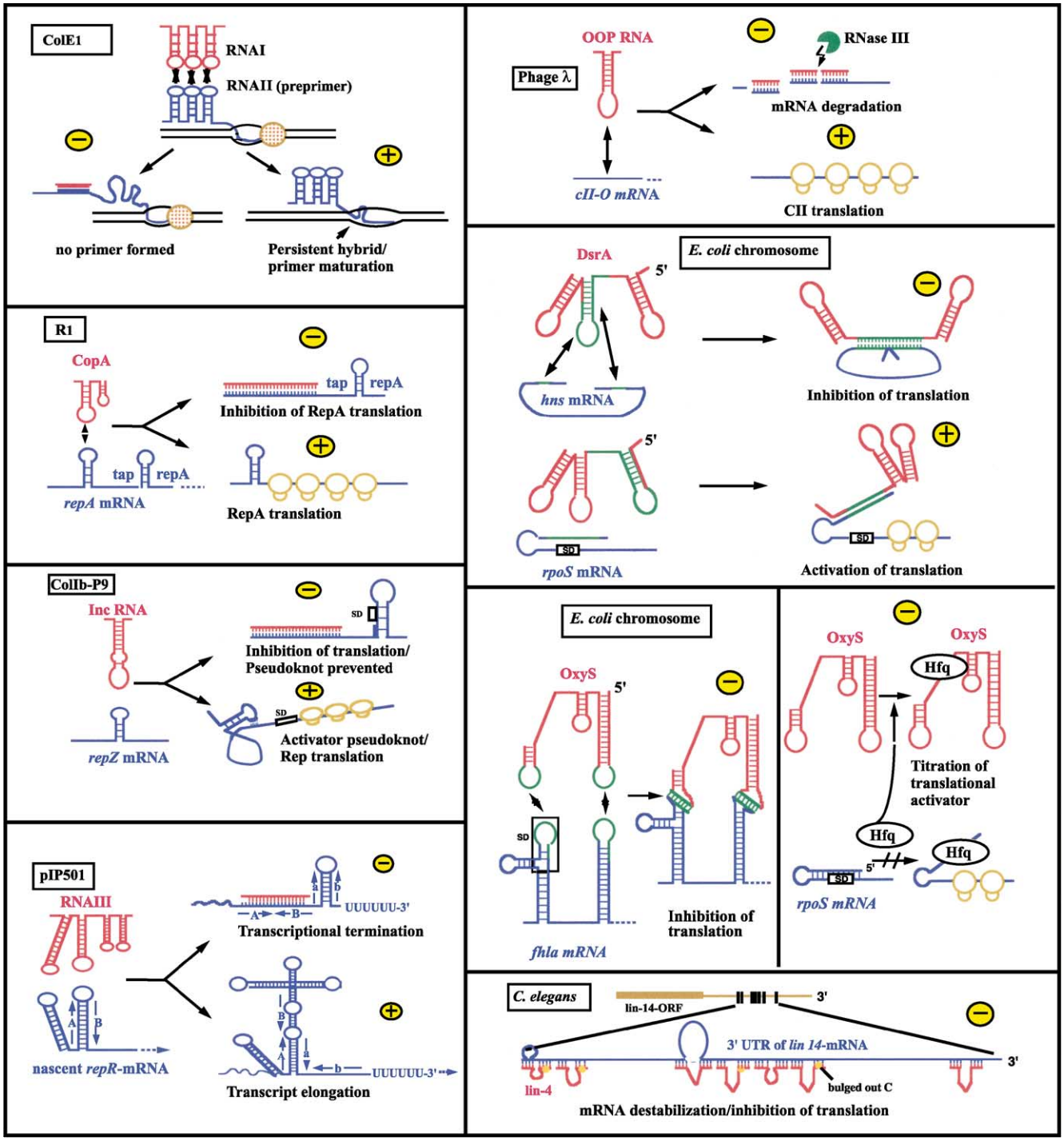

Fig. 1. Antisense RNA mediated regulatory mechanisms. Antisense RNAs are drawn in red, sense RNAs in blue. In the case of antisense RNAs that are only partially complementary to their targets, interacting sequences are highlighted in green. (+) and (−) stand for activation and inhibition, respectively. The left panel shows examples for four different control mechanisms found in plasmid replication, all exerted by cis-encoded antisense-RNAs. The right panel illustrates examples of phage and chromosomally trans-encoded antisense RNAs, among them is one example from eukaryotes. Details are described in the text. Based on Wagner and Altuvia [14].

Transcriptional attenuation (e.g. pT181, pIP501). Antisense-RNA-mediated transcriptional attenuation is a replication control mechanism first discovered for the staphylococcal plasmid pT181 [38] and later for the streptococcal plasmids pIP501 [39] and pAMβ1 [40]. This mechanism has, so far, not been detected in gram-negative bacteria. The nascent *rep*-mRNA can adopt two mutually exclusive conformations depending on the presence or absence of the

antisense RNA: in the presence of the antisense RNA (85 nt RNAI or 145 nt RNAII in pT181, 136 nt RNAIII in pIP501), a terminator stem-loop is induced in the nascent *rep*-mRNA. Consequently, premature termination of *rep* mRNA transcription upstream of the RBS occurs so that no RepR protein can be synthesized. In the absence of the antisense RNA, the *rep* mRNA can refold by complementary basepairing between two alternative segments, preventing terminator formation and allowing read-through (transcription of a full-length *rep*-RNA which then can be translated into the intact Rep protein). Only a short time-window exists, during which the antisense RNA can bind and exert its effect. No protein is necessary for sense/antisense-RNA interaction. The pIP501 system has been studied in detail in vitro and in vivo [41,42]. Since the antisense RNA (RNAIII) proved to be unusually long-living (half-life 30 min), a second control component is needed to allow proper regulation: the 10.6-kD CopR protein [43] which has a dual function. It represses *repR* transcription 10–20-fold and, additionally, prevents convergent transcription from sense- and antisense promoters pII and pIII [44]. The pT181 system which does not involve a repressor protein, but two antisense RNAs with the same function, has been analysed in vitro, and both differences and similarities with the pIP501 system have been found [45].

Control of plasmid maintenance (conjugation and postsegregational killing). The best studied examples for conjugational control are *E. coli* plasmids F and R1. The conjugal transfer operon comprises ≈30 genes whose transcription is activated by the TraJ protein. Translation of the *traJ* mRNA is regulated by the 79-nt antisense-RNA FinP (two stem-loops). Binding of FinP blocks the *traJ* RBS [46]. The 20-kD FinO protein promotes duplex formation between *traJ*-mRNA and FinP about fivefold [47] and prolongs the FinP half-life by protecting it against RNase E [48].

Many plasmids contain killer loci encoding both a stable mRNA that can be translated into a cytotoxic peptide and a short-living antisense RNA that prevents translation of the toxin mRNA. The prototype of these systems is the R1 hok/sok system [49]: Hok is the killertoxin-RNA that can, by complementary basepairing of 3′ and 5′ ends, adopt a fold-back inhibitory (fbi) structure that is slowly converted into a truncated translationally active structure. In plasmid-containing cells, the sok antisense RNA binds rapidly to the truncated hok-RNA and prevents cell killing. In plasmid-free cells, the unstable Sok RNA is rapidly degraded, leaving the stable hok-RNA behind, which—after truncation—is translated into the 52-aa killer toxin that kills the cell. The recently identified par system of the streptococcal plasmid pAD1 is composed of the *fst* gene (encoding a 33-aa killertoxin) and the *rnaII* (antisense RNA) gene. In contrast to the fully complementary hok and sok RNAs, the convergently transcribed Fst and RNAII have only two regions of complementarity: the bidirectional terminator region and two direct repeats at their 5′ ends. Interaction between Fst and RNAII blocks the *fst* RBS and, consequently, Fst translation. As in the hok/sok-system, *fst*-mRNA is stable, whereas RNAII is unstable [50,51].

1.1.1.2. Transposon systems. At present, two mobile elements are known, whose transposition is regulated by an antisense RNA: Tn10 and Tn30. In the case of Tn10 (IS10), the stable 70-nt RNA-OUT (antisense RNA) overlaps the RNA-IN (*tnp* mRNA) by 35 bp including the *tnp*-mRNA-RBS. Thus, ribosome binding and transposase translation are inhibited. Similar as with hok-mRNA, *tnp*-mRNA-translatability is further reduced by an fbi structure (reviewed in Ref. [13]). Since RNA-OUT acts in trans, but Tnpase preferentially in cis, antisense-RNA control can limit the accumulation of IS10-elements: there is little control with one element, but efficient control with multiple elements in one cell. In IS30, binding occurs within the coding region of *tnp*-mRNA, and duplex formation is proposed to block elongating ribosomes [52].

1.1.1.3. Phage systems. In the case of bacteriophages, control is mainly exerted by transcriptional repressors, and antisense RNAs are only used for fine-tuning in the decision between lysis or lysogeny (reviewed in Ref. [14]).

The 77-nt λ-OOP antisense RNA facilitates the decay of the *cII* mRNA by binding to its target: its binding site extends into the 3′-part of the cII region thus creating an RNase III substrate. The *ant*-mRNA (encoding the antirepressor) translation of phages P1 and P7 is inhibited by the 77-nt c4 antisense RNA, which is cotranscribed with ant and subsequently excised. C4 aids to maintain the lysigenic state. The cI RNA of P4 functions similarly. In the case of *Salmonella* phage P22, a 69-nt Sar antisense RNA acts on the *arc-ant*-target mRNA, and binding blocks the RBS. Superinfection by lambdoid phages can be inhibited by the P22-Sas-antisense RNA.

1.1.1.4. Chromosomally encoded systems. Bacterial antisense RNAs are mostly encoded in trans and reveal only partial complementarity to their—often multiple—targets.

Synthesis of the 93-nt **MicF** RNA, the first identified chromosomally encoded antisense RNA, is induced by stress conditions as elevated temperature, high osmolarity and redox stress. The target of MicF is *ompF*-RNA coding for a porin of the outer *E. coli* membrane whose expression is maximal at low temperature and low osmolarity. MicF–*ompF*-mRNA interaction leads to the formation of a partial duplex, which inhibits OmpF translation and promotes *ompF*-mRNA degradation [15]. **DicF** RNA (53 nt) inhibits cell division by forming a partial duplex with *ftsZ*-mRNA [53]. The first antisense RNA found to activate gene expression was the 514-nt **RNAIII** from the *agr* locus of *Staphylococcus aureus* [54]. The target for RNAIII is a self-inhibitory segment that sequesters the α-hemolysin-mRNA (*hla*-mRNA) RBS and inhibits translation. RNAIII action frees the SD, thus activating *hla*-mRNA translation. RNAIII is at the same time an mRNA encoding δ-hemolysin and a

regulator that activates or represses several genes. The 109-nt **OxyS-RNA** which is a pleitropic regulator for >40 genes is induced by oxidative stress. It inhibits translation of late stationary phase sigma factor RpoS by sequestering Hfq and *fhla*-translation by blocking the RBS [21,55]. The 87-nt **DsrA**-RNA also acts at different targets. It represses *hns*-mRNA translation by destabilization of the mRNA and blocking of the RBS and it activates *rpoS*-mRNA translation by resolving an inhibitory structure that normally sequesters the RBS. Two different stem-loops of DsrA are involved in either activity [22,23,56]. Consequently, DsrA is a riboregulator that can either activate or repress target genes. In *Clostridium acetobutylicum*, a 43-nt nitrogen-induced antisense RNA may regulate glutamine synthetase expression [57]. A number of other systems have been reviewed recently [14].

1.1.1.5. Kinetics of sense/antisense-RNA interaction. Whereas inhibition by a repressor protein depends on the K_D value, inhibition by an antisense RNA depends on the binding rate. The antisense-RNA structure is important for its binding rate and specificity.

The structures of many antisense RNAs and some target RNAs have been determined in solution (reviewed in Ref. [14]). All antisense RNAs contain one to four stem-loop structures interrupted by single-stranded regions. In some cases, recognition loops have been identified, where the interaction with the complementary loop of the sense RNA initiates. Point mutations in these loops alter the pairing specificity and can, e.g. in plasmid replication control, lead to copy-number up mutants or even new compatibility groups (reviewed in Refs. [13,14]). The target RNAs often contain complementary structures. Analysis of pairing rate-constants (complete duplex formation) yielded values of $\approx 10^6$ M^{-1} s^{-1}. When folding RNAs interact, simple helix progression in both directions is topologically impossible. Therefore, loop–loop initiating systems require a subsequent interaction at a distal site (e.g. single-stranded region) to overcome this limitation. These binding pathways are two-step pathways (ColE1, R1-CopA/T, IncIα). In alternative one-step pathways, the initial interaction involves an antisense-RNA loop and a sense-RNA-ss region (IS10), or a sense-RNA loop and an antisense-RNA-ss region (hok/sok). Both pathways were reviewed recently [14].

When helix formation is initiated, subsequent steps are unimpeded and rapid progression leads to stable complex formation. The rate of formation, rather than the affinity of a loop–loop complex, determines inhibition. In many systems complete duplex formation does not seem to be required for inhibition [16]. For example, for CopA/CopT of R1, the structure of the inhibitory complex has been determined recently and shown to contain a four-helix-junction with two intramolecular and three intermolecular helices [58,59]. Another example is pIP501, where the inhibition rate constant of RNAIII is with 1 to 2×10^6 M^{-1} s^{-1}, 10 times higher than the pairing rate constant indicating that steps preceding stable pairing with RNAII are sufficient for inhibition [41].

So far, very little is known about the binding kinetics of trans-acting antisense RNAs. The pairing rate-constant for OxyS/*fhla*—where total complementarity is only 7 and 9 nt restricted to two loop regions—was 5×10^5 M^{-1} s^{-1} [55]. This is in the range of cis-acting systems although no irreversible complex (complete duplex) can be formed.

The kinetics of other systems have been analyzed in detail, but cannot be discussed here [18,47,60–62].

1.1.2. Antisense-RNA regulated systems in eukaryotes

Eukaryotic antisense RNAs have been found only accidentally, and in most cases, their regulatory roles and the mechanism of action are still elusive. They seem to act preferentially via destabilization of the sense RNAs, but inhibition of splicing or translation has been suggested, too. Destabilization has been attributed to targeting of the antisense/sense-RNA duplex to dsRNase. Three cases are well studied.

In *Dictyostelium*, the stability of the constitutively transcribed *psvA*-mRNA encoding the prespore vesicle protein PSV-A is regulated by a differentially expressed 1800-nt antisense RNA originating from the same locus. Antisense-RNA-mediated mRNA destabilization occurs mostly in the cytoplasm [63]. In mammalian cells, the stability of the *eIF2α*-mRNA is regulated by a differentially expressed antisense transcript originating from a promoter located in the first intron of the *eIF2α* gene. In *Caenorhabditis elegans*, the *lin-4* antisense RNA displays partial complementarity to seven sequence elements in the 3′-UTR of the heterochronic genes *lin-14* and *lin-28*. Interaction of the short (22 nt) *lin-4* transcripts with mature *lin-14* mRNA yields imperfect duplexes, four of which contain a bulged C residue which is proposed to be a binding site for a protein. Sense–antisense-RNA interaction is required for translational down-regulation of LIN14-protein synthesis in the course of postembryonic development [64]. *Let7* encodes a 21-nt antisense RNA which is present in *C. elegans*, but also in *Drosophila* and vertebrates [65]. In *C. elegans*, it is complementary to 3′-UTRs of *lin-14*, *lin-28*, *lin-41* and *daf-12*. Sequential stage-specific expression of the *lin-4* and *let-7* regulatory RNAs triggers transitions in the complement of heterochronic regulatory proteins to coordinate developmental timing [66]. Further examples for eukaryotic antisense-RNA action include, e.g. bFGF in vertebrates, the *c-erbAα*-locus in mammals, or the control of transposition in the *Drosophila micropia* retrotransposon, where the antisense RNA inhibits RTase or RNase H (reviewed in Refs. [19,20]).

In plants, no naturally occurring antisense RNAs have been found so far. However, artificially introduced antisense transcripts are believed to target mRNA for degradation. Short antisense RNAs can be also generated by RdRp from aberrant sense RNAs. In addition to RNA interference (see below), such RNAs can mediate methylation of homologous DNA sequences in the plant genome, thus silencing gene

expression, a pathway which might be used by natural RNA regulators as well (reviewed in Refs. [67,68]).

Recently, a large number of small RNAs with probable regulatory functions have been discovered in *C. elegans* [69,70]. The expression of some of these miRNAs (micro-RNAs) varies during larval development, and the potential orthologs of several of these miRNA genes were identified in *Drosophila* and human genomes. These findings indicate that small regulatory RNAs may be ubiquitous in eukaryotes, too.

2. RNA interference

RNA interference (RNAi) is the induction of sequence-specific gene silencing by double-stranded RNA (dsRNA) (Fig. 2). It occurs posttranscriptionally and involves mRNA degradation. The term RNAi was coined after the discovery that the injection of dsRNA into *C. elegans* interferes with the expression of specific genes highly homologous in sequence to the delivered dsRNA [71]. Meanwhile, RNAi has been found to work in a broad variety of organisms including *Drosophila* [72,73], trypanosomes [74], planaria [75], hydra [76], zebrafish [77], frog [78] and mice [79] and is likely to also exist in human. This silencing mechanism only functions in eukaryotes, because in prokaryotes RNase III, a very potent and fast ds-specific RNase, degrades dsRNA substrates as short as 12 bp. There is one report that describes that parallel complementary RNA can function to inhibit gene expression in *E. coli* [80]. However, no experimental proof is provided that the parallel complementary RNA generated by

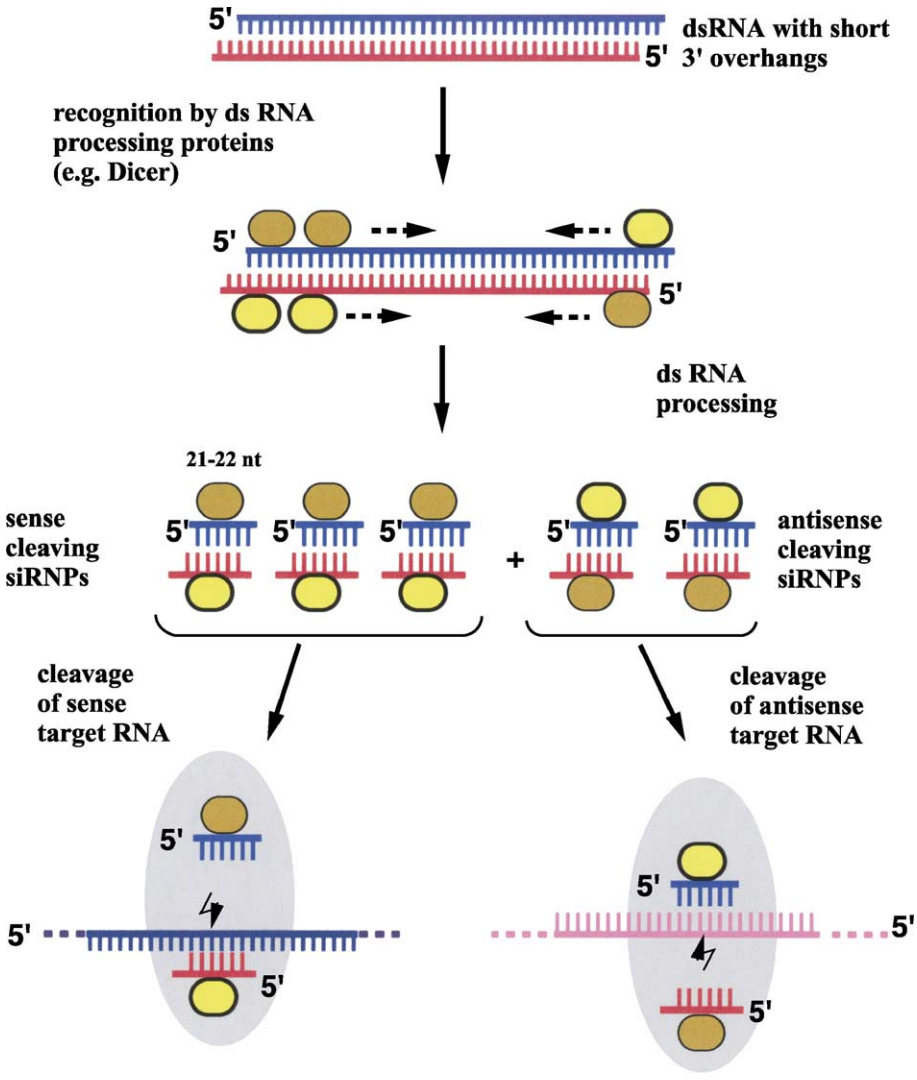

Fig. 2. Model for RNAi. Antisense RNA strands are drawn in red, sense RNA strands in blue. Sense target RNA is shown in dark purple, antisense target RNA in pink. The dsRNA processing proteins containing an RNA binding domain and a dsRNA-specific endonuclease domain are illustrated as brown and yellow ovals. The brown protein domain binds in the 5′–3′ direction, the yellow protein domain in the 3′–5′ direction. Only the siRNA associated with the yellow domain is able to guide target RNA cleavage. The RNA-induced silencing complex (RISC) is shown as large grey oval. A conformational change is proposed to occur in the RISC before target RNA cleavage because the cleavage site of the target RNA is displaced by 10–12 nt relative to the dsRNA processing site. The cleaved target RNA is directed into the processing pathway where it will be sequentially degraded. Based on Elbashir et al. [113].

artificial means in vitro is really formed in vivo. RNAi is related to the "posttranscriptional gene silencing" (PTGS) or "cosuppression" phenomena observed in plants [81–84] and "quelling" (silencing of an endogenous gene by the introduction of a transgenic copy of the gene) observed in *Neurospora* [85–87].

In the laboratory, RNAi is a powerful tool that makes gene inactivation possible in organisms that were not amenable to genetic analysis before. In nature, RNAi may both play an important biological role in protecting the genome against instabilities caused by transposons and repetitive sequences [88,89] and be an ancient antiviral response/protection mechanism in both animals and plants [84,90,91]. Recent genetic analyses provide evidence that RNAi may also have integral functions in the regulation of endogenous genes [92].

2.1. Genes and enzymes required for RNAi/PTGS

Genes and enzymes required for RNAi/PTGS were identified in *N. crassa*, *Dictoyostelium discoideum*, *C. elegans*, *Chlamydomonas reinhardtii*, *Arabidopsis thaliana* and *Drosophila*. The genes encode ds RNases, RdRPs, RNA-dependent helicases and proteins of unknown function. Some of them also have important roles in other cellular processes such as developmental control. In *N. crassa*, the *qde-1* gene (quelling defective) encodes the QDE-1 protein identified as a homologue of an RdRP characterized in tomatoes [93] and *qde-2* encodes a member of the Argonaute family (see below, [94]). RdRP might be associated with other proteins, such as helicases (like *qde-3*, [85]), that determine its template specificity in vivo.

In *Dictyostelium*, three RdRP homologs have been identified, two of them, *rrpA* and *rrpB*, differ by only ~3% in their amino acid sequence but only *rrpA* is required for RNAi [95].

In *C. elegans*, six genes called *rde-1* to *rde-4* (RNAi deficient), *mut-7* and *ego-1* were identified [88,89,96]. The RDE-1 protein is homologous to *N. crassa* QDE-2 and plant AGO-1, all members of the Argonaute family which also includes the rabbit eIF2C, a putative translation-initiation factor. Mutant *mut-7*, in which the transposition of transposons is activated in the germ line, is also RNAi defective. The gene was cloned and encodes a protein with homology to 3′–5′ exonucleases such as bacterial RNase D and others. The RNase D homology is interesting because the yeast RNase D homologue (Rrp6) probably functions as a 3′–5′ exoribonuclease that trims the 3′ end of specific 5.8 S RNA (see Ref. [97]). The *ego-1* gene encodes an RdRP homologue, and *ego-1* mutations disrupt RNAi for some germ-line genes and lead to defects in germ-line development. Furthermore, a subset of the *C. elegans smg* genes (*smg-2*, *smg-5*, *smg-6*) is important for the persistence of silencing by RNAi, and is also involved in nonsense mediated decay [98]. SMG-2 is thought to encode an ATP-dependent RNA helicase [99]. In *C. reinhardtii*, an RNA-dependent RNA helicase was shown to be important for RNAi or PTGS [100]. Mutations in its gene, *mut-6*, relieve silencing by a transgene and activate transposons. Perhaps Mut-6 unwinds dsRNA in some step of RNAi. In *A. thaliana*, SDE3, an RNA-dependent RNA helicase which is similar to *C. elegans* SMG-2 RNA helicase [101], was found to be involved in PTGS, whereas SDE1 seems to be important for gene silencing by transgenes, but not for PTGS [102]. Recently, Dicer, a dsRNA-specific RNase has been identified in *Drosophila* by scanning the genome for genes encoding proteins with RNase III signatures [103]. Dicer is needed for the production of guide RNAs in the first step of RNA silencing and is evolutionarily conserved in plants, worms, flies, fungi and mammals. The enzyme is ATP-dependent and has a distinctive structure, including an N-terminal helicase domain, two RNase III motifs and a C-terminal dsRNA binding motif. It also contains a region of homology to the Argonaute family, a so-called PAZ domain. Dicer can digest both 200- and 500-bp dsRNAs, but was significantly less active with shorter substrates, which may contribute to the size dependence of RNAi. In 2001, a null-mutation in *C. elegans* dicer-1 (*dcr-1*) was investigated [104,105]. The mutant animals had defects in RNAi under some, but not all, conditions indicating that RNAi can occur by multiple pathways, some require DCR-1 and some do not. Additionally observed germ-line defects in *dcr-1* mutants suggest that cleavage of dsRNA is a necessary event in normal development. The detection of all these genes raises the possibility that RNAi is—like RNA degradation—performed by a multiprotein complex [106] involving RNases and other proteins that can interact with RNA. In 2001, Hammond et al. [107] have indeed purified an RNP complex of ≈500 kD termed RNA-induced silencing complex (RISC) from cultured *Drosophila* cells. One constituent of this complex, a 130-kD protein termed AGO2, is a member of the Argonaute family (*rde-1*, *qde-2*) of proteins with yet unidentified functions [107]. Similarly, a 450-kD multiprotein complex with dsRNase activity was found in *Dictyostelium*. Since this complex degrades dsRNA to ~23mers in vitro, i.e. exactly the size of the in vivo processing products of RNAi, it has been suggested to be a homolog of the dicer complex [108].

2.2. Mechanism of RNAi

Biochemical analysis of RNAi became possible with the development of an in vitro Drosophila embryo lysate system for dsRNA-dependent gene silencing [109]. In this system, dsRNA—but not sense or antisense RNA—targets a corresponding mRNA for degradation without affecting the stability of noncognate mRNAs. A key finding was that small (21–23 nt) dsRNAs called short interfering RNAs (siRNAs) are generated from the input dsRNA during PTGS and RNAi [106,110–112]. These small RNAs have been detected in plants, *Drosophila* and *C. elegans* and have been suggested to serve as guide RNAs for target recognition. In *Drosophila* extracts, these siRNAs with their 3′-OH and 5′-phosphate termini resemble breakdown products of an

RNase III-like digestion [113]. Dicer (see above) can degrade large dsRNA (200 and 500 bp) to small 22-nt dsRNAs, and RNAi-triggered inhibition of this RNase reduced the efficiency of RNAi in *Drosophila* S2 cells [103]. Perhaps the 21-nt RNAs are present in a double-stranded form in the endonuclease complex, however, only one of the strands can be used for target-RNA recognition and cleavage. Naturally, 2–3-nt overhangs are found, and long (20 nt) overhangs at both 3′ ends of the dsRNA proved to be inhibitory in RNAi [113]. Probably, ssRNA-binding proteins could associate at these overhangs and interfere with the binding of one of the dsRNA-processing factors. Synthetic siRNAs can also induce gene-specific inhibition of expression in *Drosophila* extracts [109], in insect and mammalian cell lines [114,115] and in *C. elegans* indicating that the dsRNA-processing step and the targeting step can be uncoupled. In each case, the interference was superior to the inhibition mediated by ss antisense oligonucleotides [116]. Three models try to explain why the double-stranded trigger RNA is cleaved into small fragments: (1) Cleavage to segments of 21–23 nt might provide optimal specificity for a homology-based searching mechanism. Much shorter segments would leave insufficient specificity, while much longer segments might allow unwanted attacks on cellular genes with partial but extended identity to the trigger. (2) Cleavage of viral dsRNA would irreversibly break up the viral genome, so that the RNAi machinery would not contribute unwittingly to viral spread. (3) Fragmentation could increase the molarity of antiviral complexes within the cell and allow more efficient dissemination through the organism [115].

Using the *Drosophila* in vitro system, it has been shown that the direction of dsRNA processing determines whether a sense or an antisense target RNA can be cleaved by the siRNAP endonuclease complex [113].

The following model for dsRNA-directed mRNA cleavage is proposed (based on Refs. [103,111,113,116]): First, the dsRNA is cleaved to 21–23-nt-long fragments by Dicer or a Dicer homologue. Processing starts from the ends of the blunt-ended dsRNA or dsRNAs with short 3′ overhangs and proceeds in 21–23-nt steps. The resulting fragments (siRNAs) are bound by RNAi-specific enzymes possibly still including Dicer and could be incorporated into a distinct nuclease complex (RISC) that targets mRNA for degradation. In this complex, they pair with the target mRNA and cleave the mRNA in the center of the region recognized by the siRNA whereby the mRNA cleavage boundaries are determined by the sequence of the dsRNA. Either the same RNase that cleaves the dsRNA or another RNase that has to be recruited cleaves the target RNA, probably by temporarily displacing the passive siRNA strand not used for target recognition. The dsRNA-processing proteins or a subset of them remains associated with the siRNA duplex after the processing reaction. The orientation of the siRNA duplex relative to these proteins determines which of the two complementary strands functions in guiding target RNA degradation. (Chemically synthesized siRNA duplexes guide cleavage of sense as well as antisense target RNA, as they are able to associate with protein components in either possible orientation.) The recent finding in *C. elegans* that chemical modifications like 2′ -amino- or 5-iodouridine in the dsRNA are well tolerated at the sense, but not the cleavage-guiding antisense, strand [117] suggests a distinct role for both strands in RNAi. The antisense strand could, for instance, interact directly with the target RNA, whereas the sense strand could more indirectly participate in RNAi, e.g. by protecting the antisense strand against RNases. Since the center of target cleavage is displaced 10–12 nt relative to the dsRNA processing site, a conformational change must occur in the siRNPs before target RNA cleavage.

Additionally, an RdRp such as that encoded by the *ego-1* gene in *C. elegans* or the *qde-1* gene in Neurospora might amplify the dsRNA signal producing long-lasting posttranscriptional gene silencing in the absence of the dsRNA that initiated the effect. Sequence similarity between a purified RdRp from tomato [118] and the coding regions of several genes that can mutate to produce altered PTGS/RNAi responses [87,96,102,119] have supported an RdRp-dependent amplification of dsRNA. Thereby, RdRp might (1) convert an aberrant ssRNA population into dsRNA; (2) repeatedly copy the dsRNA to produce a population of ssRNAs that could then interact with target RNA; or (3) copy copies of the trigger thus generating a "self-replicating" trigger population. The data of Parrish et al. [117] were consistent with amplification model 1. Since a distinct requirement for the antisense trigger strand was observed which would be lost upon amplification, these authors ruled out models 2 and 3. However, Sijen et al. [120] detected recently in *C. elegans* somatic tissue a subpopulation of siRNAs (termed secondary siRNAs) that exhibited a distinct polarity (5′ to 3′ on the antisense strand) and appeared to derive from the action of a cellular RdRP encoded by the *rrf-1* gene. They propose a working model in which two factors—reuse of RNA-loaded RISC complexes and cyclic amplification by RdRP that is primed by existing siRNAs—enhance the potency of RNAi in *C. elegans* soma [120]. Long-term-RNAi in germ-line may also involve replication [121]. The absence of identified RdRP homologs in *Drosophila* and mammals suggests either that other RNA copying enzymes are used or that the primary siRNAs may suffice to produce detectable interference response.

Interestingly, in a *Dictyostelium* cell line where RNAi is impaired by knocking out the RdRp gene *rrpA*, ~23mers are not detected even though in vitro (in cell extracts) dsRNA is still "diced" to ~23mers. In addition, ~23mers are not found in a wild-type strain which contains the RNAi construct but not a target gene. Taken together, the authors concluded that RdRp is required for the amplification of the signal using the target as a template [95].

Regardless, whether the dsRNA signal is amplified in nature by an RdRp activity or not, it has been shown that RNAi works better when more dsRNA is used to initiate the

process. An excess of dsRNA would, on the one hand, allow to produce more 21–23-nt dsRNAs to survive dilution by cell division, and, on the other hand, outcompete other dsRNAs that could be also bound by RNAi-specific dsRNA binding proteins that have low sequence specificity in general. Furthermore, high dsRNA amounts could counteract ADARs which promiscuously deaminate adenosines in the long dsRNAs prior to processing and additionally change RNA structure (AU bp to IU mismatch), making a certain percentage of the dsRNA nonhomologous to the targeted mRNA [115]. The high ADAR activity in neuronal tissue might also explain why RNAi does only partially function in neurons.

ATP may be required for complex formation on the dsRNA, strand dissociation during or after dsRNA cleavage, pairing of the 21–23-nt RNAs with the target mRNA, mRNA cleavage and recycling of the targeting complex. Therefore, an RNA-dependent ATPase, or RNA helicase, is probably associated with the RISC.

Heritable RNAi in *C. elegans* requires the *rde-1* and *rde-4* genes to initiate but not to persist in subsequent generations. In contrast, the *rde-2*, *rde-3* and *mut-7* genes in *C. elegans* are required in the tissue where RNAi occurs but not for initiation of heritable RNAi [120]. These effector genes are likely to encode proteins functioning in the actual selection of mRNA targets in their subsequent cleavage.

2.3. Processes related to RNAi

Several processes, such as nonsense-mediated mRNA decay (NMD), regulation of transcription or translation or double-strand RNA directed methylation of DNA, can—based on the use of the same enzymes or a subset thereof—be linked to RNAi. These links have been reviewed recently [122]. Therefore, only one example, an intersection between RNAi and antisense-RNA regulation, should be mentioned here: An RNAi-like mechanism involving Dicer is used in *Drosophila* for the maturation of the 21-nt *let-7* RNA regulating developmental timing in probably all bilateral animals from a 72-nt hairpin precursor [123]. It is still unclear which properties of the precursor determine its asymmetric cleavage that results only in *let-7* and not its complement. If the stem of the hairpin were interrupted by distortions, possibly, a pair of 21–22-nt RNAs may be generated. Then, it might be possible to design stem-loop RNA precursors that produce a siRNA duplex in vivo in a specific cell type or developmental stage, which could target an mRNA for destruction by the RNAi machinery. Recently, Grishok et al. [105] have found that Dicer is required for both *lin-4* and *let-7* function in *C. elegans* indicating a broad role of this enzyme in the biogenesis of small regulatory RNAs. The Dicer-mediated processing of dsRNA into either stRNA or siRNA is directed by accessory proteins. Thus, RDE-1 may be responsible for the production of siRNAs, whereas the related ALG-1 and ALG-2 proteins were shown to be required for the maturation and activity of stRNAs [105].

In *Arabidopsis*, mutations in SIN-1/CAF, the homolog of Dicer, have dramatic developmental consequences suggesting that the plant enzyme might also process dsRNAs to small RNAs that regulate development [123].

In spite of the compelling similarities between RNAi and developmental regulation by stRNAs, there are several important differences: (1) Whereas dsRNAs used in RNAi contain long stretches of perfect basepairing, the stRNA precursors contain at the most 6 to 13 bp. (2) Whereas cleavage of dsRNAs in RNAi yields both sense and antisense siRNAs, only one strand of the stRNAs is detected indicating that the remaining sequences must undergo rapid degradation [105].

2.4. Procedure, practical aspects and problems with RNAi

Some practical aspects of RNAi as a tool in the laboratory should be summarized.

For dsRNA delivery, several methods can be applied: Electroporation is used in simpler organisms, whereas microinjection of dsRNA into germ line or early embryo is the method of choice in multicellular organisms. In *C. elegans*, injection into the intestine or pseudocoelom is almost as efficient as injection into the germ line. Even feeding worms with bacteria that express dsRNA, or soaking worms in dsRNA solutions has been applied with success [89,124,125]. In *C. elegans*, there may be an active mechanism for the transport of dsRNA across tissue and cellular boundaries. Source, length and modifications of the dsRNA need attention: Exonic sequences should be used as dsRNA, since dsRNA with exclusively intronic sequences does not affect development [71]. Because RNAi is homology-dependent, single basepair mismatches between siRNA and target RNA dramatically reduce silencing. Cross-interference between highly homologous (>90%) sequences [71,72] has to be considered upon choosing the target RNA. The length of the dsRNA can affect the RNAi efficiency [97]. Usually, dsRNA of at least 500 bp is applied but recently it has been found that perfectly matching duplexes as short as 21 bp suffice [115,117]. However, at least in *C. elegans*, much higher (250-fold) concentrations of the short ds RNAs have to be applied. Externally provided mixtures of sense and antisense RNA can be used, but duplex formation between the two trigger strands is required. No requirements for specific sequences or for A-, U- or C-residues in the targeted sequence were observed, however, certain modifications were found to be well tolerated on the sense, but not the antisense, strand (see above). Furthermore, substitution of inosine for guanine in either trigger strand inhibited RNAi at the dsRNA stage [117].

When dsRNA is injected into early embryos, it is diluted upon cell division. Therefore, early genes are more easily inactivated than late genes, which is especially a problem for higher organisms (in mouse, a construct was effective only until a 40–50-fold increase in cell mass [79]).

In *C. elegans*, the application of a plasmid with inducible (by heat-shock) promoter for the production of dsRNA

(sense RNA and antisense RNA expressed in the form of a hairpin) made inheritable RNAi possible [126]. With this approach inheritable transgenes are easily generated, large numbers of mutant organisms can be propagated delivering enough material for a broad variety of analyses and stage-specific RNAi can be performed. Furthermore, neurons, normally partially resistant to exogenous supply of dsRNA, became RNAi sensitive upon plasmid-derived in vivo supply of dsRNA [127]. The expression of dsRNA under the control of tissue-specific promoters instead of inducible ones is also conceivable.

The finding that synthetic duplexes of 21-bp siRNAs can be used in RNAi in mammals and insects cell lines raises the prospects of applying these duplexes as new tools for sequence-specific regulation of gene expression in functional genomics and biomedical studies. The siRNAs may be a new alternative to antisense or ribozyme therapeutics in mammalian systems, where long dsRNAs cannot be used because they activate the dsRNA-dependent protein kinase response [126].

Although experiments to elucidate the underlying mechanism progress rapidly, we are still at the beginning of our understanding of the molecular processes responsible for RNAi and the breadth of its function in biology. In contrast, practical applications have already allowed rapid surveys of gene functions (e.g. Refs. [128,129]) and will possibly result in new therapeutical interventions.

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