RNA Interference and Small Interfering RNAs

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1. Introduction

The term "RNA interference" (RNAi) was coined after the ground-breaking discovery that injection of double-stranded RNA (dsRNA) into the nematode *Caenorhabditis elegans* leads to specific silencing of genes highly homologous in sequence to the delivered dsRNA.[1] The RNAi phenotype is either identical to the genetic null mutant or resembles an allelic series of mutants. The dsRNA can also be delivered by feeding bacteria that express dsRNA from recombinant plasmids to the worm or by soaking the worm in a solution containing the dsRNA.[2, 3] In rapid sequence, RNAi was observed in other animals including mice,[4, 5] and therefore this process possibly exists also in humans.

RNAi appears to be related to the posttranscriptional gene silencing (PTGS) mechanism of cosuppression in plants and quelling in fungi.[6–12] Cosuppression is the ability of some transgenes to silence both themselves and homologous chromosomal loci simultaneously. The initiator molecule for cosuppression is believed to be aberrant RNA, possibly dsRNA, and some components of the RNAi machinery are required for posttranscriptional silencing by cosuppression.[7, 8, 13]

The natural function of RNAi and cosuppression is thought to be protection of the genome against invasion by mobile genetic elements such as transposons and viruses, which produce aberrant RNA or dsRNA in the host cell when they become active.[14–17] Thus, specific mRNA degradation is thought to prevent transposon and virus replication.

This minireview will highlight recent advances in understanding the molecular mechanism of RNAi and its biological function. The reader is also referred to a number of excellent reviews that have appeared recently (see refs. [18 – 24]).

2. Posttranscriptional gene silencing and RNA interference

Posttranscriptional gene silencing (PTGS) is a regulatory process in which the steady-state level of a specific messenger RNA (mRNA) is reduced by sequence-specific degradation of the transcribed, usually fully processed mRNA without an alteration in the rate of transcription of the target gene itself. If PTGS is explicitly mediated by dsRNA, the term RNA interference (RNAi) is preferred, but there may also be non-dsRNA sources, often termed *aberrant RNAs*, that may function as initiators of PTGS. Such aberrant RNAs may serve as templates for the putative

RNA-dependent RNA polymerases (RdRPs) which have been identified in plants,[10, 11, 25–27] fungi,[28] and *C. elegans*[9] and which are believed to produce dsRNA in order to initiate and maintain silencing. This idea was derived from the pioneering biochemical analysis of RdRP purified from tomato leaves.[25] Tomato RdRP synthesizes short RNAs from single-stranded RNA or DNA oligonucleotide templates and it initiates transcription near the 3′ end of an RNA template without the requirement for an oligonucleotide primer. It should, however, be noted, that RdRP activity has not yet been demonstrated for any other RdRP homologue.

Natural sources for aberrant RNAs or dsRNAs may be repetitive and mobile genetic elements such as transposons, or natural viruses. Integration of such elements nearby the promoters of endogenous genes is hypothesized to lead to unexpected antisense transcripts that at least partially anneal to the sense transcript to form dsRNA. Similarly, randomly integrated transgenes are responsible for activation of PTGS in plants. The probability of inducing PTGS by transgene integration is especially high if sense and antisense transcripts are expressed simultaneously,[29] or if inverted repeat genes are introduced, in which the RNA transcript can fold back on itself to produce a dsRNA hairpin.[30] It is also debated whether tandem or dispersed copies of genes, which are subject to transcriptional silencing, are capable of triggering posttranscriptional silencing.[31]

Calculations of the amount of dsRNA injected into *C. elegans* or *Drosophila melanogaster* suggest that only a few molecules of dsRNA per cell are sufficient to mount an RNAi response.[32, 33] It may therefore be extremely difficult to detect aberrant RNAs or dsRNAs that trigger cosuppression and RNAi in an organism. The extraordinary sensitivity of the cell towards aberrant RNAs or dsRNA is also illustrated by the success of earlier experiments in *C. elegans* in which silencing was observed after injection of what was thought to be single-stranded sense or antisense RNAs.[34] It was only realized later that the effect was due to the small amount of dsRNA that generally contaminates RNA transcribed in vitro.[1]

3. The mechanism of RNAi

3.1. Sequence-specific mRNA degradation and the role of siRNAs

It has long been thought that sequence-specific PTGS required a nucleic acid polymer to guide mRNA cleavage.[35] The presence of an antisense RNA species complementary to the target mRNA

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was anticipated, yet it was not detected by conventional RNA analysis. The breakthrough in the identification of the sequence-specific mediator came when an unexpectedly short (approximately 25 nucleotides) abundant RNA species was identified in cosuppressing or virus-infected plants. This RNA corresponded to both the sense and antisense sequences of the cosuppressed gene.[36] The 25-nt RNA species are absent from transgenic plants that do not show cosuppression. An improved protocol for the detection of such short RNAs in plants has been described recently.[37]

Biochemical analysis of the mechanism of RNAi became possible with the development of a *D. melanogaster* in vitro system that recapitulates many of the features of RNAi observed in vivo.[38] In this system, dsRNA is not only processed to an RNA species of 21 – 23 nt in length, but also some target mRNAs are cleaved in regular intervals of 21 – 23 nt only within the region spanned by the dsRNA.[39] This suggested that the dsRNA-derived 21 – 23-nt RNAs may function as the guide RNAs for target RNA degradation. These short RNAs were also detected in extracts from *D. melanogaster* Schneider 2 (S2) cells that had been transfected with dsRNA prior to cell lysis.[40] A sequence-specific nuclease activity was partially purified and it was shown that the active fractions contained 21 – 23-nt fragments, although some residual dsRNA was probably still present.[40] Formation of 21 – 23-nt fragments was also detected in vivo when radiolabeled dsRNA was injected into *D. melanogaster* embryos[41] or *C. elegans* adults.[42] The hypothesis that the 21 – 23-nt RNAs are indeed the mediators of sequence-specific mRNA degradation was only recently proven by showing that chemically synthesized 21- and 22-nt RNA duplexes are capable of guiding target RNA cleavage.[43] These short RNAs were therefore named siRNAs (short interfering RNAs) and the mRNA-cleaving RNA – protein complexes were referred to as siRNPs (small interfering ribonucleoprotein particles). It is interesting to note that dsRNAs of less than 38 bp are ineffecient mediators of RNAi because the reaction rate of siRNA formation is significantly reduced in comparison with longer dsRNAs.[43]

Chemical composition analysis of the 21 – 23-nt siRNAs, isolated from dsRNA processing in *D. melanogaster* embryo lysate, showed the presence of a 5′-monophosphate group and a free 3′-hydroxy group, and the absence of base or sugar – phosphate backbone modifications.[43] Sequence analysis further demonstrated that over 50 % of the siRNAs are exactly 21 nt in length, and that dsRNA processing occurs with no apparent sequence specificity for the 5′ and 3′ nucleotides flanking the cleavage site.[43] These observations support the idea that dsRNA may be processed by an RNase III-like reaction.[18] *Escherichia coli* RNase III cleaves both strands of the dsRNA and generates dsRNA fragments of about 15 bp in length with a 2-nt 3′ overhang.[44] Consistent with an RNase III-like cleavage reaction, it was observed that 21- and 22-nt siRNA duplexes with 3′ overhangs were more efficient in degrading target RNA than similar blunt-ended duplexes.[43] Taken together, these observations indicate that the mRNA targeting step can occur independently of the dsRNA processing reaction, and that 21- and 22-nt siRNA duplexes readily associate with the protein components required for the targeting step.

Two families of RNase III-like proteins are known in animals and plants.[45, 46] The first family is represented by the *D. melanogaster* protein Drosha (GenBank accession number AAD31170). It contains a conserved N terminus of unknown function, and in its C terminus, two RNase III motifs and one dsRNA-binding motif. The human homologue of Drosha RNase III has recently been characterized and was shown to be involved in ribosomal RNA processing.[47] The second family is represented by the *C. elegans* protein K12H4.8 (GenBank accession number S44849). It is composed of an N-terminal ATP-dependent RNA helicase domain, and the C terminus contains a repeated RNase III motif and a dsRNA-binding domain. A role of the K12H4.8 RNase III/helicase in dsRNA processing is further supported by the ATP requirement for RNAi in *D. melanogaster* lysate.[39] Lysate depleted of ATP showed a reduced rate of siRNA production and a 1-nt increase in the average size of the siRNAs. The dislocation of the dsRNA cleavage sites may be a consequence of insufficient dsRNA unwinding prior to cleavage. Recent biochemical evidence for a role of the RNase III/helicase protein (now termed "Dicer") in the production of siRNAs has just emerged.[96] RNA helicase activity may also be evoked in other steps of RNAi, including siRNP assembly and target RNA recognition.

The position of target mRNA cleavage relative to the guide siRNAs has been mapped.[43] The cleavage site is located near the center of the region covered by the 21- or 22-nt siRNA, 11 or 12 nt downstream of the first nucleotide opposite to the complementary siRNA. Because the target cleavage site is displaced 10 – 12 nt relative to the dsRNA-processing site, a conformational rearrangement or a change in the composition of an siRNP must occur prior to target RNA cleavage. One of the future key questions is whether the nuclease that cleaves dsRNA also cleaves the target RNA.

Another surprising result from the biochemical analysis of RNAi was the observation that the two strands of an siRNA duplex have distinct roles within an siRNP.[43] Depending upon the orientation of an siRNA duplex relative to the protein components of the siRNP, only one of the two strands is engaged in target RNA recognition. It also explains why certain chemical modifications (e.g., 2′-aminouridine, 2′-deoxythymidine, or 5-iodouridine) incorporated into dsRNA are well tolerated in the sense strand, but not in the cleavage-guiding antisense strand.[42] The relative orientation of the siRNA duplex within an siRNP is generally determined by the direction of dsRNA processing, presumably by proteins which are involved in the dsRNA processing and which remain associated with the released siRNA duplex. A model of the mechanism of RNAi is illustrated in Figure 1. Future questions that may need to be answered will concern the specificity of siRNPs in target RNA recognition, the identification of the protein components involved in RNAi, and the elucidation of their biochemical function.

3.2. Systemic spread of PTGS and inheritance of RNAi

One remarkable property of RNAi and cosuppression is that in both processes a signal appears to be generated, which travels through the organism to induce sequence-specific gene silenc-
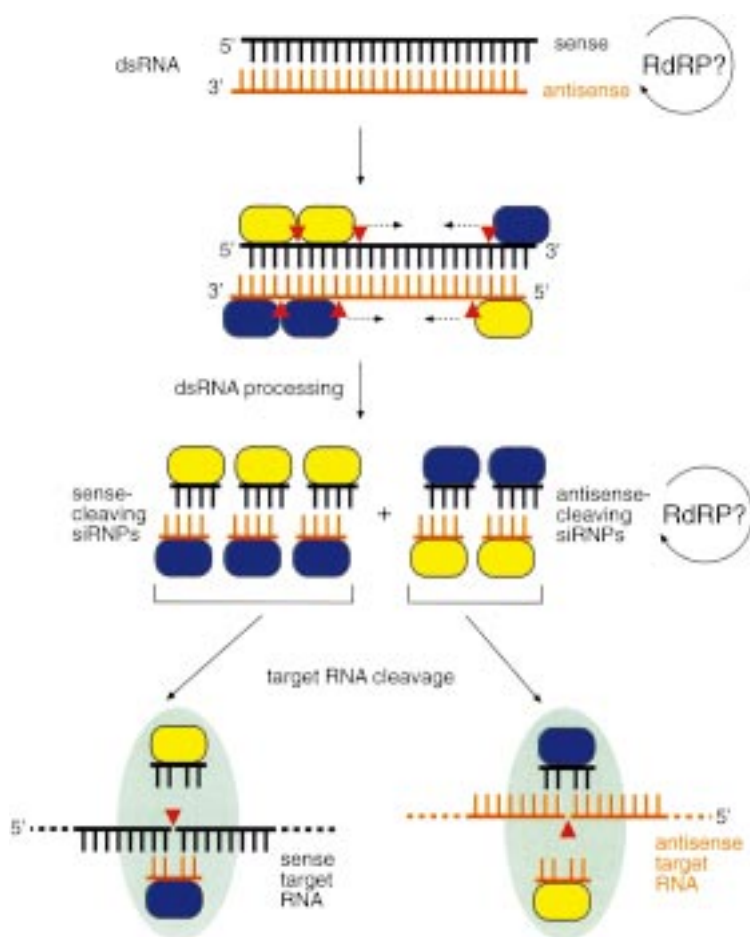
Figure 1. A model for the mechanism of RNA interference (RNAi). RNAi is initiated by the processing of dsRNA to siRNAs (21 – 23-nt fragments). The dsRNA-processing proteins (represented as yellow and blue ovals), which remain to be characterized, assemble on the dsRNA in an asymmetric fashion. These proteins (or a subset thereof) remain associated with the siRNA duplex and preserve the orientation as determined by the direction of dsRNA processing. Only the siRNA sequences associated with the hypothetical protein (blue) are able to guide target RNA cleavage. The siRNA duplex is thought to be temporarily disrupted during target recognition and the siRNA duplex is reformed after release of the cleaved mRNA. RNA cleavage sites are shown in red. The possible function of RdRP in replicating dsRNA or the siRNAs is indicated.

ing at a considerable distance. In plants, grafting experiments[48, 49] as well as the localized introduction of transgenes through bombardment of leaves with DNA-coated particles (biolistics)[50, 51] provide evidence for such a systemic process. The sequence-specific signals appear to spread from cell to cell via plasmodesmata until they reach the vascular system and spread through the entire plant. Even though the plant cell connections are different from those in animals, spreading of silencing signals is also observed in injected *C. elegans*.[1] The spreading signal may be the siRNAs itself, which could be continuously produced in cells that express dsRNA. It could also be envisioned that the siRNAs are replicated by the action of RdRPs.

It is remarkable that in *C. elegans* RNAi can be passed on to several consecutive generations without alterations of the genomic DNA sequence of the targeted gene.[32] Therefore, targeting of an essential gene not only compromises the viability of the dsRNA-exposed animal but also kills its developing

embryos.[1, 52] The ratio of dead to developing embryos is typically used to assess the essential character of a gene in *C. elegans*. Genes expressed in the germ line are particularly sensitive to RNAi and the respective phenotype can be observed for several generations, whereas the effect on other genes is less lasting.[53] Long-lasting RNAi is considered heritable and must require replication of a heritable agent, while RNAi that is only passed on to the next generation may still be explained by simple perdurance of the injected dsRNA.[53] In either case, the wild-type gene activity will finally be restored, probably due to dilution of the siRNAs through cell division and degradation of the siRNAs.

Heritable RNAi can be observed in the absence of the target gene locus,[53] suggesting the production of a dominant extragenic heritable agent produced from the once injected dsRNA. This agent may also be responsible for the systemic spreading of RNAi in the worm. It is interesting to note that the heritable agent is still maintained and most likely replicated in *rde-1* mutant worms (see Section 3.3) that can no longer initiate RNAi by injection of long dsRNAs.[53] This could suggest that dsRNA processing is not required for maintenance of RNAi, and that siRNAs rather than long dsRNAs are replicated.

In plants, in addition to PTGS, introduced transgenes can also lead to transcriptional gene silencing through RNA-directed DNA methylation of cytosines (see ref. [54] and references therein). Genomic targets as short as 30 bp are methylated in plants in an RNA-directed manner.[55] Together with the finding that a virus-encoded suppressor affects accumulation of siRNAs and genomic DNA methylation,[56] it is conceivable that siRNAs are also involved in directing DNA methylation and subsequent transcriptional silencing.

If RNAi is used as a genetic tool to mimic a gene knockout it is desirable to prevent the gradual loss of RNAi in the injected animal or its offspring. This is possible by the introduction of transgenes composed of inverted repeats which produce dsRNA hairpins after transcription. In *C. elegans*, for which targeted recombination-based gene knock-out techniques—unlike for the mouse model—are not available, it has been demonstrated that integrated inverted repeat genes confer potent and long-lasting specific gene inactivation, including neuronally expressed genes that otherwise appeared resistant to dsRNA injection.[15, 57] Stable RNAi by expression of dsRNA from transgenes has also been demonstrated in *D. melanogaster*,[58, 59] trypanosomes,[60] and plants.[61]

3.3. Genes involved in RNAi and cosuppression

Table 1 summarizes the genes identified in mutants defective for RNAi or cosuppression in the nematode *C. elegans*, the fungus *Neurospora crassa*, the plant *Arabidopsis thaliana*, and the green alga *Chlamydomonas reinhardtii*. It is tempting to assign a function to these genes according to the different steps of the

Table 1. *Genes essential for cosuppression and RNA interference.*

Gene	Accession code[a]	Organism	Protein domains	Putative function	Reference
rde-1	AAF06159	*C. elegans*	PAZ, piwi[b]	initiation of RNAi, not involved in cosuppression	[8, 13, 15, 53]
rde-2	not cloned	*C. elegans*	–	RNAi, cosuppression, inhibition of transposon jumping	[13, 15, 53]
rde-3	not cloned	*C. elegans*	–	RNAi, inhibition of transposon jumping	[15, 53]
rde-4	not cloned	*C. elegans*	–	initiation of RNAi	[15, 53]
mut-2	not cloned	*C. elegans*	–	RNAi, cosuppression, inhibition of transposon jumping	[8, 14]
mut-7	CAA80137	*C. elegans*	3′,5′-exonuclease	RNAi, cosuppression, inhibition of transposon jumping	[13, 14]
mut-8	not cloned	*C. elegans*	–	RNAi, cosuppression, inhibition of transposon jumping	[8, 14]
mut-9	not cloned	*C. elegans*	–	RNAi, cosuppression, inhibition of transposon jumping	[8, 14]
smg-2	AAC26789	*C. elegans*	group I RNA helicase	nonsense-mediated mRNA decay, persistence of RNAi	[62]
smg-5	Q94994	*C. elegans*	–	nonsense-mediated mRNA decay, persistence of RNAi	[62]
smg-6	not cloned	*C. elegans*	–	nonsense-mediated mRNA decay, persistence of RNAi	[62]
ego-1	AAF80367	*C. elegans*	RdRP	cosuppression, dsRNA synthesis, germ line development	[9]
qde-1	CAB42634	*N. crassa*	RdRP	cosuppression, dsRNA synthesis	[28]
qde-2	AAF43641	*N. crassa*	PAZ, piwi[b]	cosuppression	[7]
qde-3	AAF31695	*N. crassa*	similar to RecQ DNA helicase	cosuppression	[63]
sgs-1	not cloned	*A. thaliana*	–	cosuppression	[64]
sgs-2/sde-1	AAF73959/AAF74208	*A. thaliana*	RdRP	cosuppression, dsRNA synthesis	[10, 11]
sgs-3	AAF73960	*A. thaliana*	no homologue in animals	cosuppression	[11]
ago-1	AAC18440	*A. thaliana*	PAZ, piwi[b]	cosuppression, development	[12]
mut-6	AAG33228	*C. reinhardtii*	DEAH RNA helicase	cosuppression, inhibition of retrotransposition	[65]

[a] Accession codes for sequence retrieval in the GenBank database (www.ncbi.nlm.nih.gov). [b] The PAZ domain is named after the proteins Piwi, Argonaute, and Zwille. In these proteins, the PAZ domain is typically followed by a second domain which has been termed piwi domain (Pfam 5.4 database, St. Louis (pfam.wustl.edu)). The functions of these domains are unknown. Interestingly, an isolated PAZ domain is found in the class of RNA helicase/RNase III homologues represented by the *C. elegans* protein K12H4.8 (accession code S44849).[66]

silencing process: production of dsRNA, processing of dsRNA to siRNAs and concomitant formation of siRNPs, degradation of target mRNA by siRNPs, and maintenance and systemic spread of silencing. However, because biochemical systems that recapitulate RNAi or cosuppression in vitro are not established for the above organisms, the function of the gene products could not be tested directly and was often only inferred from database homology searches. At the same time, the proteins that mediate RNAi in the *D. melanogaster* biochemical systems remain to be identified and *D. melanogaster* RNAi mutants are not available. The gap between biochemical and genetic studies is expected to be closed in the near future.

Genetic studies in *C. elegans* indicate that cosuppression and RNAi have overlapping but distinct genetic requirements (Figure 2). The *rde-1* and *rde-4* mutants are only defective in RNAi, but not in cosuppression or transposon silencing, while mutants in *rde-2*, *rde-3*, *mut-2*, *mut-7*, *mut-8*, and *mut-9* are defective in all processes.[8, 13–15, 53] Genes required for all forms of silencing are most likely involved in dsRNA processing and mRNA targeting. The *rde-1* gene product is only required for the initial formation of the heritable interfering agent from injected dsRNA, and not needed for interference thereafter, while *rde-2* and *mut-7* are dispensable for initial formation of the interfering agent, but are required at a later step to achieve interference.[53] The *rde-1* gene is a member of a large *piwi/argonaute/zwille* gene family with 22 homologues in *C. elegans*, as well as numerous homologues in plants, animals, and fungi. It was suggested that other homologues of *rde-1* may be involved in mediating silencing by recognizing stimuli distinct from dsRNA.[53] Indeed, members of this gene family, *ago-1* in *A. thaliana*[12] and *qde-2* in *N. crassa*,[7] are required for transgene-mediated PTGS and may contribute in some unknown manner to formation, stabilization, or local-
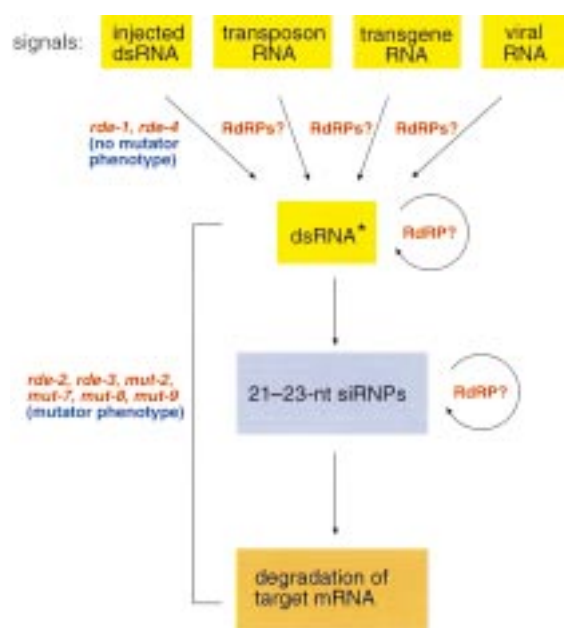
Figure 2. *Assignment of gene function to the steps involved in RNAi and cosuppression of* C. elegans. *Injected dsRNA or RNA from aberrant transcripts of transposons, viruses, or cosuppressing transgenes is converted into dsRNA*, a hypothetical form of dsRNA or dsRNA – protein complex, which is committed to dsRNA processing. Processing of dsRNA* leads to the formation of siRNAs or siRNPs, which mediate the degradation of target mRNA. Genes that affect the individual steps when mutated and the respective effect on transposon activity (mutator phenotype) are indicated. Steps at which RdRPs may generate or amplify the initiator molecules or siRNAs are also shown.*

ization of dsRNA prior to siRNA and siRNP formation. The Mut-7 protein contains a 3′,5′-exonuclease motif similar to DNA polymerases, WRN protein, and *E. coli* ribonuclease D and was

therefore suggested to play a role in mRNA degradation.[14] The other *mut* genes as well as *rde-2*, *rde-3*, and *rde-4* remain to be characterized.

Some members of the eukaryotic multigene family of putative RdRPs are also required for RNAi and cosuppression: *ego-1* in *C. elegans*,[9] *qde-1* in *N. crassa*,[28] and *sgs-2/sde-1* in *A. thaliana*.[10, 11] RdRPs could be required for replication and maintenance of silencing signals or for triggering of silencing by synthesizing dsRNA from aberrant RNAs. Interestingly, there is no obvious homologue of an RdRP in the recently completed genome sequence of *D. melanogaster*, which may indicate the absence of autonomous replication of siRNAs or dsRNAs in this organism.

Cosuppression in *N. crassa* also requires the *qde-3* gene, which encodes a homologue of the RecQ DNA helicase family.[63] It has been proposed that *qde-3* is involved in sensing repetitive DNA elements and thereby contributes to the initiation of gene silencing. This may also point to a link between transcriptional and posttranscriptional silencing.

Some of the genes involved in nonsense-mediated mRNA decay in *C. elegans*, *smg-2*, *smg-5*, and *smg-6*, have also an effect on RNAi.[62] Mutants of these particular *smg* genes recover from the effect of injected dsRNA more rapidly than wild-type animals, suggesting that the corresponding gene products contribute in some manner to the persistence of RNAi.

Finally, the *mut-6* gene from the unicellular green alga *C. reinhardtii* is required for the silencing of a transgene and two transposon families.[65] The *mut-6* gene encodes a protein that is highly homologous to RNA helicases of the DEAH box family and closely related to the splicing factor Prp16. It may be envisioned that RNA helicase activity may be required during dsRNA processing or target RNA recognition.

Genetic analysis is far from complete and the identification of new genes involved in RNAi and cosuppression will continue to provide hints for the understanding of the process of PTGS. Direct biochemical roles for the genetically identified factors have yet to be assigned.

4. The biological function of RNAi

One natural function of RNAi seems to be protection of the genome against endogenous transposable elements.[14, 15] Transposons are present in many copies (ten to tens of thousands) in a cell and transposon dsRNA could be produced when a transposon copy integrates near an endogenous promoter in the antisense direction. Similar to transposition, integration of transgenes into the genome is a rather random event and can activate PTGS of sequences similar to those of the introduced transgenes. In plants, cytoplasmically replicating RNA viruses also act as both targets and inducers of PTGS, thereby suggesting an additional function of PTGS as an antiviral defense mechanism.[67] Consistent with this hypothesis, it has been found that certain plant viruses encode proteins that suppress PTGS.[56, 68–72]

In *D. melanogaster*, bursts of transposon mobilization are observed in the daughters from crosses between males of a strain containing active transposons (inducer strain, I) and

females of a strain devoid of active transposons (reactive strain, R) (for a review, see ref. [73]). The uncontrolled transposon activity leads to a syndrome of female sterility: the daughters lay normal amounts of eggs, but most of them fail to hatch. Reciprocal crosses, I mothers with R fathers, do not show a fertility defect of their daughters, suggesting that a repressor of transposon mobilization is transferred with the egg of the I strain and is not present in the egg of the R strain or in sperm. There is mounting evidence that the repressor molecules may be produced from aberrant RNA transcripts in the I strain as a consequence of transposition in the germ line.[16, 74, 75] The I – R hybrid dysgenesis syndrome is abolished when transgenes expressing transposon fragments from the I element are introduced into the R strain prior to mating with the I strain.[16, 75, 76] The inhibition of hybrid dysgenesis requires only transcription of the transgenic I element fragment but no protein synthesis and is reminiscent of cosuppression. Because any transgene is able to induce cosuppression of its homologous genes, it yet remains to be demonstrated that transposons are indeed silenced by cosuppression or RNAi in the absence of an expressed transgene. It should be noted that for at least some transposons, transposon-encoded proteins also act as repressors that are maternally inherited[77] and that regulation of transposable elements occurs at many other levels including their expression and insertional specificity. When dsRNA is used to target an endogenous, single-copy gene for silencing, the machinery underlying this sequence-specific control of transposons is hijacked and redirected towards destruction of the endogenous mRNA.

5. Biomedical and functional genomics applications of RNAi

The extraordinary sequence-specificity of RNAi and the simplicity of administering dsRNA to organisms whose genomes have already been sequenced will make RNAi a first choice in studying genome function. RNAi has already proven to be an efficient and robust tool for functional genomics studies in *C. elegans*[78, 79] although possibly some of its genes, for example those specifically expressed in neurons, are difficult to silence by dsRNA microinjection.[57] In *D. melanogaster*, targeted degradation of maternal and early zygotic mRNAs is efficient by dsRNA injection in the fertilized egg,[33] yet targeting of genes in tissues that give rise to adult structures such as the wings, legs, eyes, and brain has been difficult, and it appears only possible when dsRNA is expressed from transgenes in the form of an extended hairpin loop RNA.[58, 59] This could be due to the lack of an efficient amplification mechanism in this organism (see Section 2). Heritable RNAi due to transgenic expression of RNA hairpins has also been established in trypanosomes[60] and plants.[61] Injection of dsRNA in *Xenopus laevis* embryos specifically interferes with target gene expression,[80, 81] but RNAi is only moderately efficient in zebrafish embryos,[82, 83] in which the specific phenotype may be obscured by nonspecific effects.[84] In mice, RNAi is active in the oocyte and the preimplantation embryo[4, 5] and persists for several rounds of cell divisions after microinjection of the dsRNA.

RNAi is not believed to function in later stages of mammalian development or in adult mammals, because dsRNA will activate a nonspecific viral defense mechanism, the interferon response, that leads to an arrest of protein synthesis and nonspecific mRNA degradation in the affected cells (for a review, see ref. [85]). Interferons are a group of signaling molecules which are induced and secreted when cells are infected by RNA viruses or exposed to dsRNA. The interferon response is sequence-unspecific and generally activated by dsRNAs greater than 80 bp in length. The most potent inducers of interferons are duplexes of the homopolymers of inosine and cytidine. Interferons trigger the expression of many genes[86] which may all contribute to the arrest of viral replication and the establishment of the antiviral state of the cell. So far, only the most abundantly expressed interferon-induced genes have been characterized. It has recently been shown that cultured embryonic fibroblasts of knock-out mice deficient in RNase L and PKR (the RNA-activated protein kinase that phosphorylates eukaryotic initiation factor 2 (eIF2)), the major player in the sequence-nonspecific interferon response, were still able to mount a substantial antiviral response after interferon treatment.[87] Perhaps the residual antiviral response is RNAi, which is normally hidden under the major interferon response. Knock-out animals or cell lines defective in the interferon-regulated pathways are certainly interesting objects in the future search for RNAi in mammals.

Genetic analysis with RNAi is particularly valuable in studying organisms for which only a limited number of genetic tools are available such as the milkweed bug (*Oncopeltus fasciatus*),[88] the red flour beetle (*Tribolium castaneum*),[89, 90] the planarian *Schmidtea mediterranea*,[91] or the freshwater polyp (*Hydra magnipapillata*).[92] RNAi will therefore significantly advance comparative evolutionary biology aimed at understanding the morphological and developmental variability between species.

The use of RNAi in cultured cells should dramatically facilitate the dissection of signaling pathways and the study of cell growth and division in order to understand the biology of cancer. Currently, the applications are restricted to *D. melanogaster* S2 cells, into which dsRNA is introduced by transient transfection or by direct addition to the culture medium.[40, 93–95] A screen of mammalian cells from three different species showed no evidence for the specific down-regulation of gene expression by dsRNA.[94] Also, rabbit reticulocyte lysate, in contrast to *D. melanogaster* lysates, does not support RNAi in vitro.[38] For cultured CHO-K1 cells (CHO = Chinese hamster ovary), it seems possible to down-regulate a reporter gene by dsRNA transfection, but this requires at least a 2500-fold higher concentration of dsRNA when compared to S2 cells.[95] It is unclear whether CHO-K1 cells are defective in their interferon response, as exposure of mammalian cells to dsRNA generally blocks protein synthesis and can also lead to apoptosis. It may, however, be envisioned that a combination of dsRNA and inhibitors of the interferon response, or cells deficient in components of the interferon pathway[87] may enable the use of RNAi for the study of mammalian gene function in tissue culture.

Due to the danger of activating the interferon response, it is difficult to imagine the application of RNAi to cure genetic diseases or viral infections in humans. Yet, it may be possible to discover dsRNA analogues that do not activate the interferon response but mediate RNAi. Alternatively, it is conceivable that by administering short, maybe chemically modified siRNAs, one might be able to reconstitute a functional siRNP complex in vivo. Independent of a biomedical application, sequence-specific tools that interfere with gene expression will be of great demand as tools for functional genomics and as therapeutics, and RNAi will undoubtedly grow to one of the leading methodologies in the field.

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