

3 Genes required for RNA interference

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Introduction

RNA interference (RNAi) is a recently discovered phenomenon in which double-stranded RNA (dsRNA) silences endogenous gene expression in a sequence-specific manner (Fire et al., 1998). Since its discovery, the use of RNAi has become widely employed in many organisms to specifically knock down gene function. RNAi shares a remarkable degree of similarity with silencing phenomena in other organisms (Cogoni et al., 1999a; Sharp, 1999). For instance, RNAi, post-transcriptional gene silencing in plants and cosuppression in fungi can all be activated by the presence of aberrant RNAs (Maine, 2000; Tijsterman et al., 2002a). Additionally, plant, worm, and fly cells or extracts undergoing RNA-mediated interference all contain small dsRNAs, around 25 nucleotides in length, identical to the sequences present in the silenced gene (Baulcombe, 1996; Hammond et al., 2000; Zamore et al., 2000; Catalanotto et al., 2000).

The high degree of similarity between these RNA-mediated silencing phenomena supports the notion that they were derived from an ancient and conserved pathway used to regulate gene expression, presumably to eliminate defective RNAs and to defend against viral infections and transposons. (Zamore, 2002). Components of RNAi have also been implicated in developmental processes, suggesting that RNAi may play a broader role in regulating gene expression (Smardon et al., 2000; Knight et al., 2001; et al., Ketting et al., 2001).

Although we have learned much about the general mechanisms underlying RNAi, a detailed understanding of how RNAi works remains to be elucidated. In this chapter we will discuss first the biology of RNAi, then the genes required for its function, and we will end with a discussion on recent findings that have implicated chromatin silencing in the mechanism of RNAi.

The biology of RNAi

Both genetic and biochemical analyses have significantly increased our understanding of how RNAi works. The RNAi mechanism involves an early step, in

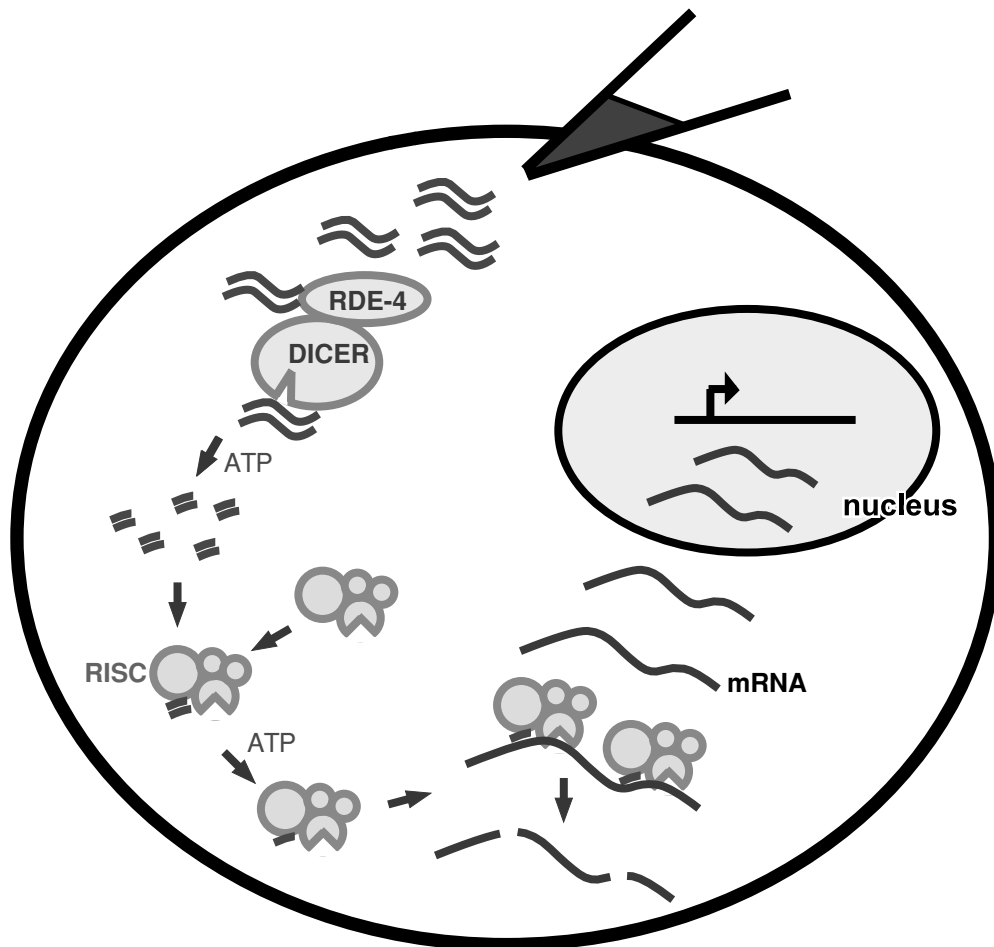


Figure 3.1. Model for mRNA degradation in the cytoplasm by RNAi. Introduced dsRNAs (red) are recognized by RDE-4/R2D2, a dsRNA binding protein. These dsRNAs are then processed by Dicer into 21-23nt duplexes that can associate with an enzyme complex called RISC. After unwinding of the siRNAs, RISC becomes competent to target homologous mRNA transcripts for degradation.

which the dsRNA is recognized and is targeted for RNase-dependent digestion, and a late step, which comprises the downstream events that lead to the silencing of the target gene.

Two-step model for RNAi

Figure 3.1 illustrates a current model by which introduction of dsRNAs into a cell can result in degradation of targeted mRNAs. Introduced dsRNAs are recognized by a dsRNA-binding protein, RDE-4/R2D2 (Tabara et al., 2002; Liu et al., 2003), that facilitates the subsequent dicing of these RNAs into small interfering RNAs (siRNAs) 21–25 nucleotides in length, with 2-nucleotide overhangs at both 3'-ends. Dicing is catalyzed by an RNase III enzyme named Dicer (Hammond et al., 2000; Bernstein et al., 2001; Hutvagner et al. 2002; Elbashir et al., 2001a). These siRNAs then act as guides in association with a protein complex to target

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homologous transcripts for degradation. This siRNA/protein complex, termed RISC, for RNA-Induced Silencing Complex (Hammond et al., 2000), becomes competent to target degradation of homologous mRNAs upon the ATP-dependent unwinding of the siRNAs (Nykanen et al., 2001).

siRNAs

siRNAs are complementary to both the sense and antisense strands of the targeted mRNA and have a distinct chemical polarity that is essential for their function (Zamore et al., 2000; Elbashir et al., 2001a; Nykanen et al., 2001; Schwarz et al., 2002; Schwarz et al., 2003). For instance, efficient siRNA mediated interference requires that the siRNAs contain 2 bp overhangs at their 3' ends as well as a 5' phosphate and, at least in *Drosophila*, a 3' hydroxyl group. Additionally, the base composition at the 5' end can influence which siRNA strand can initiate RNAi. It has also been shown that the sequence composition of the antisense strand is more important than that of the sense strand, as modifications on the antisense strand of the dsRNA trigger preferentially blocks RNAi (Elbashir et al., 2001b).

Long dsRNAs cannot be used for gene silencing in some organisms, including mammals, because of the presence of dsRNA defense mechanisms. In these organisms, the introduction of dsRNA leads to the activation of the protein kinase PKR and 2',5'-oligoadenylate synthetase. The activation of these two proteins leads to non-sequence-specific effects including the inhibition of translation and the degradation of mRNA (Sen et al., 1976; Stark et al., 1998). However, siRNAs alone can elicit a potent and effective RNAi response. It has been shown that chemically synthesized siRNAs, introduced into a variety of mammalian cell lines, could also specifically inhibit endogenous gene expression (Elbashir et al., 2001c). Moreover, RNAi has been shown to work in model organisms such as mice, *Drosophila*, *C. elegans*, and zebrafish, thus expanding the potential for its use in understanding gene function in a variety of model systems (Fire et al., 1998; Kennerdell and Carthew, 1998; Wargelius et al. 1999; Hunter, 1999; Wianny and Zernicka-Goetz, 2000).

Systemic nature of RNAi

One interesting aspect of RNAi, which was originally observed in plants, is that the effect can spread between tissues, in that gene silencing induced in one tissue can result in gene silencing in other tissues (Palauqui et al., 1997; Fire et al., 1998; Grishok et al., 2000). This suggests the existence of a mechanism to uptake and transport a silencing signal between cells.

Amplification of the RNAi response and transitive RNAi

Another intriguing aspect of RNAi is that the effect is surprisingly robust. Concentrations as low as a few molecules of dsRNA per cell can elicit a strong and persistent response (Fire et al., 1998), suggesting that an amplification step may occur within the RNAi pathway.

In some organisms, it is likely that the amplification mechanism involves RISC acting catalytically (Sijen et al., 2001) as amplification could be effected by

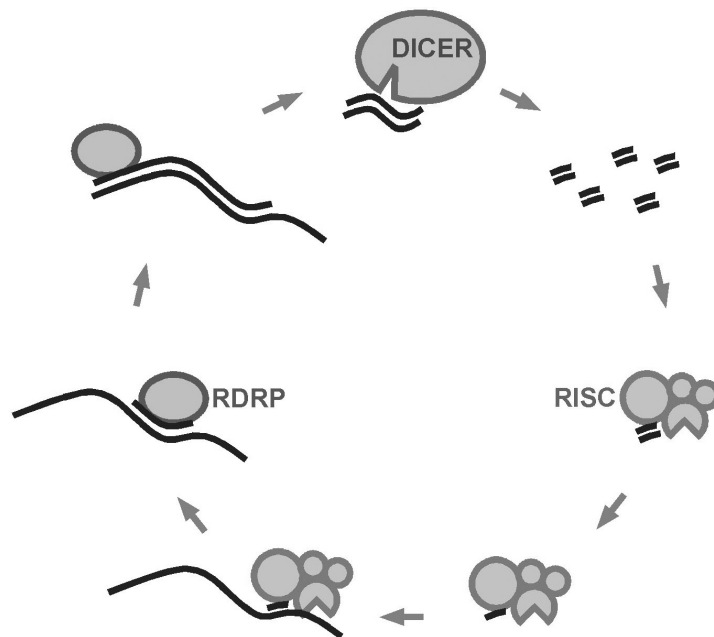


Figure 3.2. Amplification of dsRNA by an RNA-dependent RNA polymerase. In certain organisms, new dsRNAs can be generated by RDRPs, primed by siRNAs on mRNA targets. The new dsRNAs can be used subsequently by Dicer to create more siRNAs, which can lead to additional rounds of amplification.

multiple turnover of RISC (Hammond et al., 2001; Sharp, 2001). In other organisms, the activity of RNA-dependent RNA polymerases (RDRPs) has been implicated in the amplification mechanism (Smardon et al., 2000; Maine, 2000; Sijen et al., 2001; Simmer et al., 2002). The presence of siRNAs with sequences 5' to the initial trigger have been demonstrated in some animals undergoing RNAi (Figure 3.2). These siRNAs, called secondary siRNAs, correspond to sequences just upstream of targeted sequence on the same transcript and are produced by de novo RNA synthesis by an RDRP (Sijen et al., 2001). However, transitive RNAi does not appear to occur in cultured *Drosophila* cells (Celotto et al., 2002; Schwarz et al., 2002; Roignant et al., 2003).

These observations suggest that in certain organisms, mRNAs are not only targets of the RNAi machinery but can also be used to amplify the original signal. One consideration for researchers using RNAi as a gene silencing tool is that transitive RNAi may also lead to inactivation of mRNA species that were not originally targeted by the initial trigger sequence; for example, RNAi of Green Fluorescent Protein (GFP) in a strain bearing a GFP fusion transgene can in certain cases result in silencing of the gene to which GFP is fused (Sijen et al., 2001; Alder et al., 2003).

A link between RNAi and microRNAs

Although not a product of dsRNA-mediated interference, microRNAs (miRNAs) have also been shown to be key components of RNA-based gene regulation in

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Table 3.1. Proteins implicated in RNAi and related RNA silencing phenomena

	<i>C. elegans</i>	<i>Drosophila</i>	Humans	Plants	Fungi
Dicer RNase:	DCR-1	Dicer	Dicer	CAF/Sin-1	Dicer
RNA-dependent RNA polymerases:	EGO-1, RRF-1, RRF-3			SGS2/S DE1	QDE1, RDRP, RrpA
Proteins with PAZ/piwi domains:	RDE-1	AGO2, Piwi, Aubergine	elF2C1/2	AGO1, AGO4	QDE2, Ago1, Ago2
Nucleases:	MUT-7	Tudor-SN		WEX-1	
Helicases:	MUT-14, DRH-1/2	p68, Spindle-E		MUT6, SDE3	QDE3
Chromatin modifiers:	MES-3, -4, -6			DDM1, MET1	
dsRNA-binding protein:	RDE-4	R2D2			
Nonsense-mediated decay	SMG2, SMG-5, SMG-6				
Other proteins:	SID-1	FMRP, dFXR, VIG		SGS3, HEN1	

For references, see text and Cogoni and Macino, 1999a,b; Dalmay et al., 2000; Domeier et al., 2000; Mourrain et al., 2000; Wu-Scharf et al., 2000; Dalmay et al., 2001; Vaucheret et al., 2001; Kennerdell et al., 2002; Martens et al., 2002; Schauer et al., 2002; Bateman, 2002; Boutet et al., 2003; Doi et al., 2003; Glazov et al., 2003; Zilberman et al., 2003.

many organisms. In contrast to siRNAs, miRNAs are derived from the processing of endogenously encoded short hairpin RNAs. However, miRNAs are dependent on Dicer for processing (Carrington and Ambros, 2003) and associate with a complex that shares components present in RISC (Hutvagner and Zamore, 2002), suggesting a mechanistic link between the RNAi and miRNA pathways. Recent studies suggest that miRNAs play important roles in controlling development in both plants and animals (Carrington and Ambros, 2003). Anti-sense binding of miRNAs to target mRNA sequences can silence expression by either inhibiting translation (in animals) or directing the target mRNA for degradation [(in plants) (Carrington and Ambros, 2003)].

Genes required for RNAi

Genes required for RNAi (Table 3.1) have been identified by genetic screens aimed at isolating mutants that are defective in RNA-mediated silencing, by using RNAi knockdown of components of the RNAi machinery, and by biochemical methods. Determining how these proteins function in RNAi is an ongoing challenge. Here, we discuss the roles of genes with defined functions in RNAi.

Initiators

The *C. elegans* genes *rde-1* and *rde-4* (*rde* stands for “RNAi deficient”) are involved in the early step of RNAi. RDE-4 is a double-stranded RNA binding protein required for efficient recognition of the dsRNA trigger and, at least in *Drosophila*, for helping siRNAs transit from Dicer to RISC (Tabara et al., 2002; Liu et al., 2003). Consistent with RDE-4 acting early, *rde-4* mutant animals do not produce siRNAs, and the introduction of siRNAs can bypass the requirement for this gene (Parrish et al.,

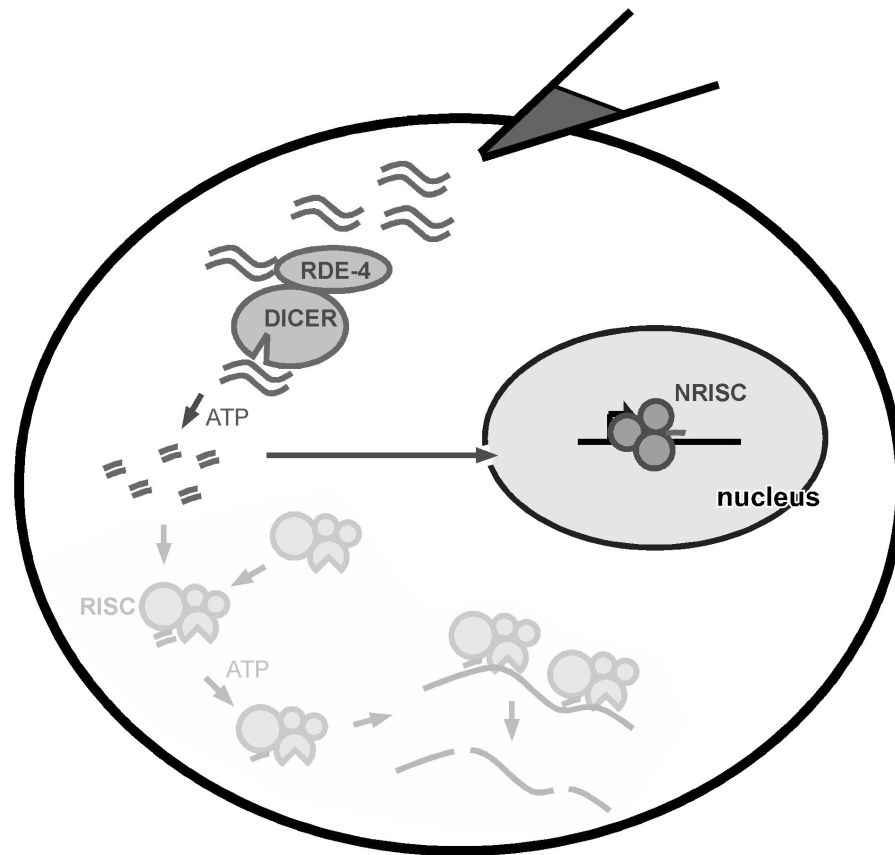


Figure 3.3. Model for gene silencing in the nucleus by RNAi. RNAi can also silence the transcription of targeted genes in certain organisms. In this model a signal can direct a putative nuclear RNAi silencing complex (NRISC), composed of chromatin modifying proteins, to the targeted locus, silencing gene expression at the level of transcription.

2001). RDE-4 has also been shown to physically associate with Dicer, RDE-1, and a Dicer-related helicase called DRH-1/2 [(Tabara et al., 2002) (Figure 3.3)].

The *rde-1* gene is a member of a large family (composed of over 20 genes in *C. elegans*), and has homologs in *Drosophila*, plants, fungi, and mammals (Tabara et al., 1999; Fagard et al., 2000). RDE-1 is a member of the PPD (PAZ and Piwi domain) family of proteins and can physically associate with RDE-4 (Tabara et al., 2002). However, unlike *rde-4*, siRNAs cannot bypass the requirement for *rde-1*, suggesting that *rde-1* is required downstream of siRNA production. Interestingly, secondary siRNAs are not produced in *rde-1* mutants (Parrish et al., 2001; Tijsterman et al., 2002b).

Effectors

Genes required in the late step of RNAi, after formation of siRNAs, include the *C. elegans* genes *rde-2* and *mut-7*. These genes were initially identified in screens for mutant animals unable to respond to RNAi and from screens designed to identify components that silence DNA transposition in the germline (Ketting et al., 1999).

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The fact that transposon silencing depends on factors required for RNAi suggests that silencing of transposons and RNAi may share related mechanisms. The *mut-7* gene encodes a putative 3'-to-5' exonuclease sharing homology to the nuclease domains of RNase D and a helicase implicated in Werner Syndrome, a human premature aging disorder (Ketting et al. 1999; Ketting and Plasterk, 2000). The *rde-2* gene product has not yet been identified.

Current research supports a scheme in which RDE-1 and RDE-4 are required to generate/stabilize siRNAs, whereas RDE-2 and MUT-7 are required to respond to this signal to silence the targeted gene.

RISC components

RISC is an siRNA/protein complex that directs the cleavage of targeted transcripts. There have been several RISC factors thus far identified. *Drosophila Argonaute-2* (AGO2, a homolog of *C. elegans* RDE-1, *Neurospora* QDE-2, and *Arabidopsis* AGO1) has been shown to associate with RISC during processing of miRNAs (Hammond et al., 2001b; Williams et al., 2002).

Other components found to be associated with RISC are two RNA-binding proteins: VIG (Vasa intronic gene), and FMR1 [(fragile X mental retardation syndrome protein) (Caudy et al., 2002)]. VIG is encoded within an intron of the *Vasa* gene in *Drosophila* and contains a protein motif able to bind RNA (Heaton et al. 2001). FMR1 is the *Drosophila* homolog of the mammalian fragile X mental retardation protein and encodes an RNA-binding protein that associates with translating ribosomes and is presumed to act as a negative regulator of translation (Ishizuka et al., 2002).

Drosophila Tudor-SN, a nuclease containing domains in common with Tudor and staphylococcal nuclease, also associates with RISC (Caudy et al., 2003), and Dicer has been shown to associate with RISC, although Dicer is not required for RISC activity (Bernstein et al., 2001).

RNA-dependent RNA polymerases

RDRPs have been implicated in RNAi in *C. elegans*. The *ego-1* gene encodes an RDRP required for RNAi specifically in the germline (Smardon et al., 2000; Sijen et al., 2001). The *C. elegans* genome contains three additional RDRP loci – *rrf-1*, *rrf-2*, and *rrf-3*. Mutations in *rrf-1* result in an RNAi-deficient phenotype, however, only somatic cells are RNAi-deficient. RRF-2 appears to have no role in RNAi, whereas mutations in *rrf-3* make *C. elegans* hypersensitive to RNAi suggesting that it may act as an antagonist to the RNAi pathway (Simmer et al., 2002; Sijen et al., 2001).

Certainly, the existence of an RDRP might explain the remarkable efficiency of dsRNA-induced silencing if it amplified either the dsRNA prior to cleavage or the siRNAs directly. RDRP activity has been reported in *Drosophila* embryo lysates. However, no homolog of an RDRP has been found in the *Drosophila* and human genomes (Lipardi et al., 2001). Additionally, siRNAs that have been modified at their 3' ends, which can no longer associate with an RDRP, can produce efficient RNAi in human tissue culture systems (Holen et al., 2002).

Novel component required for systemic RNAi

In *C. elegans*, a genetic screen was recently developed to identify components required for systemic RNAi (Winston et al., 2002; Feinberg et al., 2003). Over 200 mutants were identified and placed into five complementation groups: *sid-1*, *sid-2*, *sid-3*, *sid-4*, and *sid-5* (for systemic RNA interference deficient). To date, *sid-1* has been cloned. SID-1 localizes to the cell periphery and encodes a novel multi-pass transmembrane protein that is required cell autonomously and enables the passive uptake of dsRNA into cells. Interestingly, dsRNAs of approximately 100bp in length function as preferred substrates for *sid-1*-dependent RNAi over shorter dsRNAs (Winston et al., 2002; Feinberg et al., 2003).

Roles for chromatin-modifying proteins in RNAi

Until recently, most models predicted RNAi to act exclusively at the post-transcriptional level to target mRNA stability. However, recent work may have revealed an additional role for RNAi, a role in regulating transcription. Work in fission yeast has revealed that RNAi silences transcription of genes via chromatin modifications. Chromatin modification proteins have also been implicated in RNAi in *C. elegans*, and RNAi machinery components have been implicated in transcriptional silencing in *Drosophila*, suggesting that the mechanisms of gene silencing found in fission yeast might be operating in animal cells as well.

C. elegans: Using RNAi to identify genes required for RNAi

As discussed above, the molecular components of the RNAi machinery have been identified by screening for mutants that are RNAi-deficient, and by isolating proteins that interact with the known RNAi machinery. Recently an additional approach to identify RNAi components using RNAi itself was fortuitously developed out of a screen originally intended to identify genes required for early development. The screen, conducted in *C. elegans*, involved pooling together eight dsRNAs at a time, co-injecting these into hermaphrodites, and identifying pools containing genes essential for development by scoring progeny for embryonic lethality. For pools that resulted in lethality, sub-pooling was performed until the dsRNA responsible for the effect was isolated.

One such pool contained a dsRNA corresponding to the essential gene, *glp-1* (germ line proliferation 1). GLP-1 is a Notch-like molecule and loss of *glp-1* function results in a maternal-effect lethal phenotype. Surprisingly, no lethality was observed in the pool that included *glp-1* dsRNA. However, injecting *glp-1* dsRNA alone resulted in a high degree of lethality. In an attempt to identify the dsRNA responsible for suppressing *glp-1* (RNAi), each dsRNA in this pool was co-injected with *glp-1* dsRNA. It was found that only one of the dsRNAs was able to suppress *glp-1* (RNAi), and removing this dsRNA from the pool restored *glp-1* dsRNA-mediated lethality.

Interestingly, suppression was not limited to *glp-1* (RNAi), as the suppressing dsRNA was also able to suppress the lethality associated with other dsRNAs, suggesting that the suppressing dsRNA might work by affecting RNAi in general, and

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not just RNAi of *glp-1*. Consistent with this, known components of the RNAi machinery have been found to behave similarly (Hammond et al., 2000; Bernstein et al., 2001; Grishok et al., 2001; Dudley et al., 2002). These results suggested that co-injection of multiple dsRNAs could be used as an effective method to identify new genes required for RNAi (Dudley et al., 2002).

Surprisingly, several genes identified using this method in *C. elegans* encode proteins predicted to associate with chromatin, raising the possibility that RNAi in animal cells may also work at the level of chromatin, as it does in plants (Baulcombe, 1996; Morel et al., 2000). Some of the proteins identified form a complex that includes a protein homologous to a *Drosophila* Polycomb group protein, which in flies functions to repress gene expression through the modification of chromatin (Cao et al., 2002; Pirrotta, 2002), raising the possibility that repression of transcription by chromatin modifying proteins might play a role in RNAi-dependent gene silencing.

***Drosophila*: A piece of the RNAi machinery required for transgene-mediated transcriptional silencing**

Recent experiments have shown that polycomb-dependent transcriptional gene silencing is disrupted in an RNAi-defective background in *Drosophila*. (Pal-Bhadra et al., 2002). In mutants defective for *piwi*, a homolog of the *C. elegans* RNAi component RDE-1, transcriptional gene silencing was impaired alongside with posttranscriptional gene silencing. Transcription from an inserted transgene was monitored in both normal and *piwi* mutant flies. In normal flies, transcriptional gene silencing was induced by high copy numbers of the inserted transgene, while in the *piwi* mutants transcription was unaffected (Pal-Bhadra et al., 2002). These results show that an RNAi component is required for at least one aspect of transcriptional silencing.

Fission yeast: Silencing of centromeric DNA and the mat locus

Recent work in the fission yeast *Schizosaccharomyces pombe* implicates RNAi in both the initiation and the maintenance of centromeric silencing (Hall et al., 2002). Centromeric DNA is flanked on either side by repetitive sequences. It has recently been shown that a DNA fragment normally present within the centromere can be inserted into a transcriptionally active region of chromatin elsewhere and induce chromatin remodeling at the new site. Modifications such as the methylation of lysine 9 of histone H3 subsequently leads to gene silencing. Interestingly, methylation of lysine 9 on Histone H3 and silencing is impaired in yeast lacking functional RNAi components such as Dicer, RDRP and Argonaute (Volpe et al., 2002; Hall et al., 2003). Additionally, transcripts complementary to silenced centromeric sequences tend to accumulate in RNAi mutant backgrounds, suggesting that RNAi components respond to endogenous dsRNAs produced at centromeres, silencing centromeric sequences (Volpe et al., 2002). Indeed, it has been shown that non-coding overlapping centromeric sequences can produce transcripts that hybridize to form dsRNAs that are processed by the RNAi machinery. Additionally, ectopically expressed short hairpin RNAs can also induce heterochromatin

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formation at homologous loci. This process requires components of the RNAi machinery and a histone methyltransferase (Schramke et al., 2003). These data suggest that aberrant transcription within highly repetitive sequences can lead to the formation of hairpin RNAs, which are processed by the RNAi pathway, leading to heterochromatin formation and thus silencing of gene expression.

It was discovered recently that RNAi components are also required to initiate silencing at the yeast *mat* locus. Fission yeast can exist in a haploid state, or in a diploid state – which is formed when two haploid cells fuse. This fusion can only occur between two haploid cells that are of different mating types. The mating type of the haploid cells is determined by a single locus, termed the mating type locus (*mat*). The *mat* region consists of three loci, two of which are always silent and are required for the switching of the transcriptionally active locus. Repression of transcription from the *mat* locus is essential for mating and is accompanied by changes in chromatin structure (Kayne et al., 1988; Grewal et al., 1996).

RNAi components function to initiate heterochromatin formation at the *mat* locus. Mutants in yeast RNAi machinery failed to initiate heterochromatin assembly after removal of epigenetic markers by the use of the drug trichostatin A. Additionally, the levels of H3 lysine 9 methylation as well as Swi6, a heterochromatin protein, were greatly reduced at the *mat* locus in RNAi mutants (Hall et al., 2002).

Conclusion

RNA-mediated gene silencing has become an important tool to analyze gene function in many organisms as well as in mammalian tissue culture systems. As genome sequences become readily available, RNAi makes the ability to analyze the function of each and every gene an attainable goal. Moreover, the ability to readily silence specific genes holds promise in designing effective therapies to combat a range of illnesses. The continued identification of genes required for RNAi should further our understanding of the mechanisms of RNAi.

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