RNA Interference in *Caenorhabditis elegans*

Mechanism and Application

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RNA Interferentie in *Caenorhabditis elegans*
Mechanisme en Applicatie
(met een samenvatting in het Nederlands)

Proefschrift

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Femke Simmer
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Promotor: Prof.dr. R.H.A. Plasterk
Universiteit Utrecht
Hubrecht Laboratorium, KNAW

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Contents

Chapter 1
Introduction

Gene Silencing

Gene silencing phenomena were first observed by scientists working with plants (Jorgensen, 1990; Matzke et al., 1989; Napoli et al., 1990; Smith et al., 1990; van der Krol et al., 1990). They found that transgenes, introduced into plants for overexpression of a certain gene, induced the opposite phenotype of what was expected. This was called cosuppression; a transgene is suppressed and induces suppression of the endogenous gene. In follow-up experiments it was shown that cosuppression can be the consequence of transcriptional and posttranscriptional mechanisms. Transcriptional gene silencing (TGS) involves changes at the DNA level that cause loss of transcription, e.g. methylation and chromatin remodeling (Matzke and Matzke, 1991; Meyer et al., 1993). Posttranscriptional gene silencing (PTGS) does not affect transcription but involves sequence specific mRNA degradation (de Carvalho et al., 1992; van Blokland et al., 1994). Transgene silencing can also occur between related transgenes independent of an endogenous homologous locus (Dehio and Schell, 1994; Ingelbrecht et al., 1994). Transgene silencing was subsequently shown in *C. elegans*, *Neurospora crassa*, and *Drosophila melanogaster* (Cogoni et al., 1996; Cogoni and Macino, 1997; Gaudet et al., 1996; Jensen et al., 1999b; Jones and Schedl, 1995; Kelly et al., 1997; Pal-Bhadra et al., 1997). In addition, other targets of gene silencing in plants and *Drosophila* are viruses (Li et al., 2002; Lindbo and Dougherty, 1992; van der Vlugt et al., 1992).

Posttranscriptional Gene Silencing

RNA interference (RNAi) was discovered in *C. elegans* (Fire et al., 1998) and subsequently in many different organisms of other kingdoms. This is PTGS directly induced by double-stranded RNA (dsRNA) designed to target mRNA of a specific gene. When dsRNA corresponding to the sequence of an endogenous mRNA is introduced, the cognate mRNA is degraded. As mentioned above, other triggers of PTGS are transgenes. In addition, viruses can also induce PTGS (Ruiz et al., 1998).

Much effort is used to unravel the mechanisms underlying RNAi/PTGS. Common aspects such as homologous genes, dsRNA and small interfering RNAs (siRNAs) (discussed in detail below) were shown to be required for the PTGS phenomena in the different organisms. This implies a conserved mechanism between kingdoms. However, differences in the mechanisms between organisms and between the different triggers are also observed. For instance, some genes needed for PTGS are organism specific or specific for a subset of organisms. These genes could be involved in organism specific fine-tuning of the mechanism. Alternatively, these genes are dispensable in some organisms because other genes or processes full-fill their task.

PTGS can be used in directed gene silencing, in experimental biology, agriculture and also possibly in disease therapy. In experimental biology PTGS is currently mainly used for analysis of gene function. Food products modified by PTGS are also on the market; PTGS is used to enhance crop production or product quality. One of the first products was a tomato in which transgenes reduced the expression of a gene required for softening of ripening fruit, which keeps the tomatoes firm after ripening and protected against damage by handling. Therefore, they can be left to ripen on the vine and taste better than conventional tomatoes that are harvested green (Baulcombe, 2002). Investigators in Japan are currently trying to make decaffeinated coffee plants using PTGS that could replace industrially decaffeinated coffee, because the process to remove the caffeine is expensive and the taste of industrially decaffeinated coffee is poor (Ogita et al., 2003). Attention is now turning to assessing the potential applications of PTGS in disease treatment. PTGS could be used to inhibit the production of proteins involved in the initiation or progression of many diseases. Two key clinical areas where PTGS can have a great impact are cancer and infectious disease (Brisibe et al., 2003; Caplen, 2003). PTGS could inhibit the production of proteins involved in virus or parasite-host interactions, pathogen replication, oncogenesis or cell toxicity. Promising is the increasing list of genes successfully knocked-down by RNAi in mammalian cells and improvements in the delivery of siRNAs to cells, including *in vivo* delivery to mice (McCaffrey et al., 2002; Wianny and Zernicka-Goetz, 2000).

Working Model for RNAi

RNAi is induced by dsRNA. dsRNA can be made *in vitro* and applied to organisms or it can be made from transgenes, cellular genes and by RNA viruses. To produce dsRNA transgenes can be designed. For instance, transgenes that produce transcripts that fold back to generate a hairpin. Transgenic

arrays can generate dsRNA due to their repetitive structure. From single-copy or dispersed elements dsRNA can be produced by read-through or fortuitous transcription.

Studies of *Drosophila* extracts showed an activity capable of processing long dsRNA substrates into small RNAs (Zamore et al., 2000). Immunoprecipitates of a candidate protein degraded dsRNA into the same kind of small RNAs. These were called small interfering RNAs or siRNAs. The candidate protein was an RNase III/helicase enzyme that was called DICER. Decreased DICER levels *in vivo* correlated with decreased gene silencing strengthening the central role of the DICER enzyme in RNAi. Similar *in vitro* experiments were done with *C. elegans* extracts. Immunoprecipitates with antiserum to the *C. elegans* ortholog of DICER (DCR-1) showed processing of long dsRNA into siRNAs. DCR-1, like *Drosophila* DICER, required ATP for efficient cleavage (Bernstein et al., 2001), and ATP hydrolysis further enhanced siRNA production (Ketting et al., 2001). This may indicate that unwinding of siRNAs by a helicase is required for DICER to act catalytically and/or that DICER converts dsRNA into siRNAs through a processive cleavage reaction, extracting energy for translocation from ATP hydrolysis. To test the involvement of DCR-1 in RNAi *in vivo*, *C. elegans* mutants of *dcr-1* were analyzed (Ketting et al., 2001; Knight and Bass, 2001). The *dcr-1* mutants in *C. elegans* were shown to be defective in RNAi (Table 1 lists the proteins that are based on genetic mutants implicated in RNAi/PTGS in *C. elegans*). Thus, RNAi triggered by long dsRNA is initiated by DICER, which processes dsRNA into siRNAs (Figure 1a).

The features of siRNAs are: 21-25 nt length, a double-stranded structure of 19-23 bp with 2 nt 3' overhangs, and 5' phosphates (Elbashir et al., 2001b). Interestingly, in *C. elegans*, plants, mammalian cells, *Drosophila* extracts, and *Drosophila* embryos it was demonstrated that the upstream part of RNAi can be bypassed by the direct administration of siRNAs (Boutla et al., 2001; Caplen et al., 2001; Elbashir et al., 2001a; Elbashir et al., 2001b; Klahre et al., 2002; Parrish et al., 2000; Williams and Rubin, 2002). This is very useful for the studies on gene function, especially in mammalian cells where long dsRNAs induce responses that are not sequence-specific (such as interferon responses) (Kaufman, 1999; Levy and Garcia-Sastre, 2001; Majde, 2000; Williams, 1999).

RNAi is sequence specific mRNA degradation. It is thought that sequence specific cleavage is brought about by the siRNAs. They can hybridize to the mRNA and in this way tag the mRNA for degradation. Experiments in *Drosophila* showed that treatment with dsRNA induced the assembly of a nuclease activity that specifically degraded transcripts homologous to transfected dsRNA. Partial purification of the enzyme complex revealed that siRNAs co-fractionate (Hammond et al., 2000; Nykanen et al., 2001). This indicates that siRNAs are bound to specific proteins, which together form a multicomponent nuclease. This complex is termed RNA-induced silencing complex (RISC). For base-pairing with the substrate the siRNA duplex must be unwound. This is most likely done by an RNA helicase. There are indications that each individual active RISC contains only one siRNA strand (Martinez et al., 2002; Schwarz et al., 2002). Cleavage of the mRNA was shown to occur in the centre of the region spanned by the siRNA and with approximate 21 nt intervals (Elbashir et al., 2001b; Zamore et al., 2000).

Several protein subunits of *Drosophila* RISC are identified such as AGO2, VIG (Vasa intronic gene), dFXR (the *Drosophila* homolog of the fragile X mental retardation protein (FMRP)) and Tudor-SN (Tudor Staphylococcal Nuclease) (Caudy et al., 2003; Caudy et al., 2002; Hammond et al., 2001; Ishizuka et al., 2002). AGO2 is a member of the PAZ/Piwi family, a protein family conserved in many species of which several are implicated in RNAi/PTGS. Suppression of AGO2 expression in *Drosophila* culture cells by RNAi correlated with a pronounced reduction in the ability to silence an exogenous reporter RNA. This shows that AGO2 is important for RNAi. AGO2 can also be co-immunoprecipitated with DICER. Possibly, during this interaction between DICER and AGO2 the siRNAs are transferred to RISC (Hammond et al., 2001). VIG and dFXR are two putative RNA-binding proteins (Caudy et al., 2002; Ishizuka et al., 2002). Tudor-SN is a protein containing five staphylococcal/micrococcal nuclease domains and a Tudor domain. Purified Tudor-SN exhibits nuclease activity and could contribute to the mRNA degradation by RISC (Caudy et al., 2003).

RISC is conserved in several organisms. There are similar complexes detected in mammalian cells and RISC activity is demonstrated in HeLa cell extracts (Hutvagner and Zamore, 2002; Martinez et al., 2002; Mourelatos et al., 2002; Schwarz et al., 2002). In plants there is also evidence of RISC activity. In *Arabidopsis* plants and wheat germ extracts endogenous small RNAs and cleavage products from mRNAs with homology to the small RNA are detected (Llave et al., 2002b; Tang et al., 2003). Several experiments with *C. elegans* extracts could not show RISC activity, in part due to aspecific RNases (Ketting and Plasterk, personal communication). However, VIG-1 specific antibodies can co-immunoprecipitate TSN-1, suggesting a similar association of the *C. elegans* orthologs of

Drosophila RISC proteins (Caudy et al., 2003). Thus, in the general RNAi working model RISC brings about the mRNA degradation step (Figure 1a).

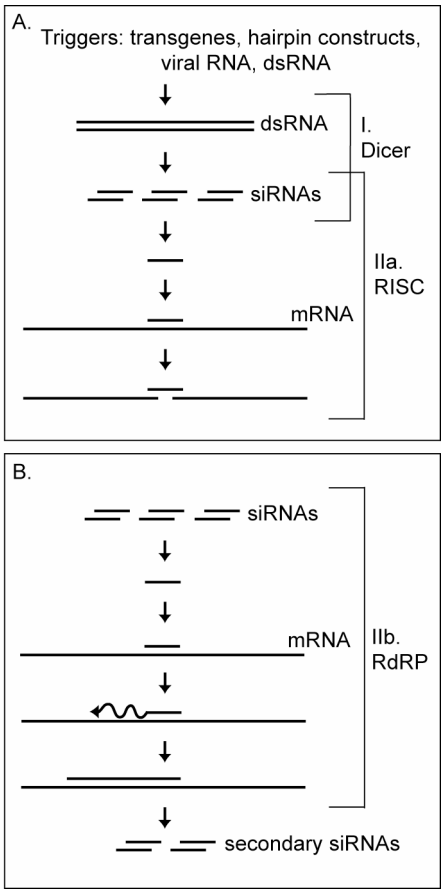

Figure 1. Working Model for RNAi
(A) Two mayor enzyme activities are required for sequence specific mRNA degradation. I) dsRNA is cleaved by an ATP-dependent ribonuclease, DICER, into siRNAs. IIa) The siRNAs are transferred to RISC, which also contains an endoribonuclease. The siRNAs base-pair with the target mRNA, in this way they guide the endonuclease to the correct target. The mRNA is cleaved near the center of the sequence covered by the siRNA. The mRNA is subsequently degraded and the siRNAs are recycled.
(B) Alternatively, siRNAs that are annealed to target mRNA can prime RNA synthesis by an RdRP. The dsRNA that is formed can be used as new substrate for DICER. This results in destruction of the mRNA and amplification of the siRNA population. The new/secondary siRNAs can start more rounds of mRNA degradation.

RNA Amplification by RNA Directed RNA Polymerases

In *Neurospora*, *Arabidopsis*, *C. elegans* and *Dictyostelium* RNA directed RNA polymerases (RdRPs) are implicated in RNAi/PTGS. Mutations in the putative RdRP genes *qde-1* of *Neurospora*, *ego-1*, *rrf-1* of *C. elegans*, *rrpA* of *Dictyostelium*, and *sgs2/sde1* of *Arabidopsis* cause defects in PTGS (Cogoni and Macino, 1999; Dalmay et al., 2000; Martens et al., 2002; Mourrain et al., 2000; Sijen et al., 2001a; Smardon et al., 2000). The first member of this RdRP family was identified in tomato leaves. This tomato RdRP was purified from viroid-infected leaves and shown to catalyze RNA synthesis *in vitro* (Schiebel et al., 1993a; Schiebel et al., 1993b). However, the recombinant enzyme produced in bacteria was inactive (Schiebel et al., 1998).

Several models have been proposed for the role of RdRP in PTGS. For instance, an RdRP might be required for the synthesis of the initial dsRNA triggers (Cogoni and Macino, 2000; Dougherty and Parks, 1995; Vaucheret and Fagard, 2001; Wassenegger and Pelissier, 1998). Secondly, an RdRP could replicate dsRNA templates, thus intensifying the silencing (e.g.Waterhouse et al., 1998). Alternatively, an RdRP could produce new dsRNA using the target mRNA as a template (Sijen et al., 2001a; Sijen and Kooter, 2000).

In *Neurospora*, the first PTGS mutant mutated in a putative RdRP gene (*qde-1*) was identified. Mutations in *qde-1* cause loss of the transgene induced loss-of-function phenotype and at the RNA level abolish the accumulation of siRNAs and degradation of target mRNA (Catalanotto et al., 2002; Cogoni and Macino, 1999). Soluble recombinant QDE-1 protein was isolated (Makeyev and Bamford, 2002). The protein catalyzed RNA-dependent RNA polymerization on different single stranded RNA (ssRNA) templates using either primer-dependent and primer-independent initiation modes. The products that were made without a primer were produced either by *de novo* synthesis or back-priming (back-folding of 3' end template). These assays resulted in full-length extension products. In addition, a large amount of 9-21 mer RNA oligonucleotides were formed when QDE-1 was assayed with ssRNA

templates without primers. Importantly, dsRNA substrates were not recognized by QDE-1 *in vitro*. These studies together give genetic and biochemical evidence for a role of an RdRP in PTGS in *Neurospora*.

C. elegans has four putative RdRP genes (*ego-1*, *rrf-1*, *rrf-2*, *rrf-3*). Work described in the second chapter of this thesis (Sijen et al., 2001a) demonstrate that in the RNAi pathway of *C. elegans* new dsRNA is produced using mRNA as a template. Firstly, we showed that siRNAs corresponding to sequence outside of the inducing dsRNA occurred. More specifically, siRNAs were detected that corresponded to sequence of the target mRNA upstream of the sequence corresponding to the trigger dsRNA. These siRNAs were termed secondary siRNAs. Secondly, *rrf-1* and *ego-1* mutants are defective in RNAi. In *rrf-1* mutants RNAi for genes expressed in somatic tissues is lost, while interference is retained for genes expressed in the germline. In *ego-1* mutants the reverse situation occurs; they have normal sensitivity to dsRNA corresponding to genes predominantly expressed in the soma, yet little or no sensitivity to dsRNAs corresponding to genes predominantly expressed in the germline (Smardon et al., 2000). In addition, *rrf-1* mutants are defective in a process called transitive RNAi. This is spreading of the mRNA targeting from the sequence corresponding to the trigger dsRNA to sequence of the mRNA outside of this region. Analysis of the siRNAs in *rrf-1* animals showed no secondary siRNAs, but a small population of siRNAs corresponding to the original dsRNA trigger could be detected. These siRNAs may represent the primary siRNAs that are produced from the dsRNA trigger independent from the RdRP. We proposed that primary siRNAs serve as primers for the RdRPs *rrf-1* or *ego-1* to produce new dsRNAs using mRNA as a template. The newly generated dsRNA could be cleaved by DICER, which results in secondary siRNAs that could reinitiate the amplification cycle or mediate mRNA degradation by RISC (Sijen et al., 2001a). The proposed action of an RdRP extends the model for RNAi depicted on figure 1a from essentially DICER followed by RISC to DICER followed by RISC and/or amplification (Figure 1b). Additional support for the RdRP action on the target mRNA in *C. elegans* RNAi comes from the observation that short RNA of antisense polarity can also trigger silencing when administrated in close proximity to the target mRNA (Tijsterman et al., 2002a). The antisense RNAs (asRNAs) might directly act as primers allowing extension of the 3' end by an RdRP. The fact that *rrf-1* mutants are defective in RNAi suggests that the primary siRNAs do not result in significant mRNA degradation and that amplification is essential for effective RNAi (Sijen et al., 2001a).

In *Dictyostelium* the knock-out of *rrpA* results in a loss of PTGS induced by hairpin constructs. Simultaneously there is a loss of detectable siRNAs. Besides RrpA, the target gene was also required for the production of detectable amounts of siRNAs *in vivo*. However, primary siRNAs were generated *in vitro* when labeled dsRNA was incubated with extracts from *rrpA* mutants. This indicates that the putative RdRP RrpA and target sequences are involved in siRNA amplification (Martens et al., 2002). Possibly, RrpA in Dictyostelium is involved in making new dsRNA from the target mRNA, like in *C. elegans*.

In *Arabidopsis* PTGS initiated by transgenes that overexpress an endogenous mRNA also requires a putative RdRP, SGS2/SDE1 (Dalmay et al., 2000; Mourrain et al., 2000), but transgenes designed to generate dsRNA or silencing induced by viruses replicating through a dsRNA bypass this requirement (Beclin et al., 2002; Dalmay et al., 2000). It was proposed that silencing by sense transgenes might require the putative RdRP SGS2/SDE1 to produce the dsRNA trigger, which then enters the (potentially RdRP-independent) PTGS pathway. Alternatively, the RdRP is involved in amplification of small amounts of dsRNA that are formed from sense transgenes. In this scenario, production of large amounts of dsRNA by viruses or dsRNA expressing transgenes is sufficient for significant target mRNA break-down without replication.

Interestingly, the persistence of PTGS and the movement of PTGS over large distances throughout *Arabidopsis* are dependent on SGS2/SDE1 (Dalmay et al., 2001; Himber et al., 2003). In addition, different experiments showed that there was a strict correlation between these aspects of PTGS and the efficiency of spreading of RNA targeting (Vaistij et al., 2002; Voinnet et al., 1998). This indicates that SGS2/SDE1 is in some aspects of PTGS involved in the production of new dsRNA using the target mRNA as template. Studies on siRNAs showed that SGS2/SDE1 were not necessary for the production of primary siRNAs (Dalmay et al., 2000), but were necessary for the accumulation of secondary siRNAs (Vaistij et al., 2002). An important difference between *Arabidopsis* and *C. elegans* is that the secondary siRNAs of plants corresponded to both up- and downstream regions of the target mRNA compared to the trigger dsRNA. This suggests that new dsRNAs are produced by SGS2/SDE1 using the full-length target RNA as a template. Most likely the RNA is synthesized by unprimed RdRP activity. To keep sequence specificity for the target mRNA, the primary siRNAs could be used to tag

the mRNA instead of functioning as a primer. The tag could recruit the RdRP for de novo synthesis, starting at the 3' end of the mRNA.

A role for an RdRP in PTGS in plants is supported by *in vitro* studies in wheat germ extracts. Tang et al (2003) showed that wheat extracts contain an RdRP activity that can synthesize dsRNA using exogenous single-stranded RNA as a template with and also without an exogenous primer. The resulting dsRNA could be converted into siRNAs.

It is striking that most data on RNAi in *Drosophila* indicate that RdRPs are not necessarily involved in the *Drosophila* RNAi pathway. There is no homolog of the cellular RdRP family in the *Drosophila* genome. *In vitro* experiments showed that relatively low concentrations of ATP are sufficient for target RNA degradation in *Drosophila* embryo lysates, which are likely to be insufficient to support synthesis of new dsRNA by an RdRP (Nykanen et al., 2001). An experiment designed to monitor the *in vitro* degradation of labeled mRNA did not reveal any cleavage upstream of the target sequence present in the trigger dsRNA (Zamore et al., 2000), which would have been expected if an RdRP was involved in spreading of mRNA targeting by mRNA dependent synthesis of new dsRNA. In addition, the requirement for 3'-hydroxyl groups on siRNAs would be expected if the synthesis of new dsRNA was primer dependent. However, it was shown that the 3'-hydroxyl group of siRNAs is not required for RNAi in *Drosophila* embryo lysates (Schwarz et al., 2002). One study by Lipardi et al (2001) carried out in *Drosophila* embryo extracts was contradicting to all other *in vitro* experiments. They showed primer-dependent synthesis of secondary dsRNAs. In this study labeled siRNAs were incorporated into a full-size dsRNA product dependent on the use of complementary template RNA. Subsequently, the new full-length siRNA-labeled dsRNA could be processed into new siRNAs. In addition, they showed that siRNAs were only active in mRNA degradation with 3'-hydroxyl groups (Lipardi et al., 2001). However, *in vivo* experiments showed that dsRNA triggers only target the corresponding region of the mRNA without transitive effect directed to sequences downstream from or upstream of the initial trigger region (Celotto and Graveley, 2002; Roignant et al., 2003). Like most *in vitro* results, this indicates that in *Drosophila* mRNA template dependent synthesis of new dsRNA is most likely not conserved.

In mammals, there are also several indications that an RdRP is not an integral component of the RNAi pathway. Stein et al. (2003) showed that Cordycepin, an inhibitor of RNA synthesis, did not prevent mRNA degradation upon injection of corresponding dsRNA in mouse oocytes. In addition, targeting a chimeric mRNA with dsRNA corresponding to the right part of the chimeric mRNA does not reduce the endogenous mRNA corresponding to the left part of the chimeric mRNA, but does target the chimeric mRNA. This implies that no new dsRNA/siRNAs corresponding to sequences upstream of the trigger dsRNA were formed (Stein et al., 2003). Finally, it was shown that the 3'-hydroxyl group of siRNAs were not required for RNAi in cultured human cells (Chiu and Rana, 2002; Martinez et al., 2002).

In summary, at least in *C. elegans*, *Dictyostelium* and *Arabidopsis* there is evidence that an RdRP can produce new dsRNA in the RNAi/PTGS pathway using the target mRNA as a template. Recently, another example of spreading of RNA targeting was shown in the fungus *Mucor circinelloides* (Nicolas et al., 2003). Together with data on QDE-1 from *Neurospora* this suggests that RdRPs in fungi are also involved in mRNA dependent amplification of dsRNA. In *Drosophila* and mammals there is no clear evidence for a role of an RdRP in RNAi. The dsRNA synthesis activity observed *in vitro* by Lipardi et al (2001) could be entirely unrelated to the mechanism of RNAi. It is possible that other enzymes without homology to the cellular RdRP family are involved in production of dsRNA in a non transitive-way. For instance, the dsRNA trigger could be directly copied. Alternatively, dsRNA synthesis could be involved in transgene induced silencing. In that case dsRNA would be synthesized from transgenes transcripts.

PAZ/Piwi Proteins

Members of the PAZ/Piwi family are implicated in RNAi/PTGS in most organisms. These proteins have a PAZ and a C-terminal PIWI domain. *Drosophila* contains in total four characterized PAZ/Piwi proteins (Piwi, Aubergine, AGO1 and AGO2). In addition, DICER also contains a PAZ domain. AGO2 is discussed above. AGO1 was shown to be required for efficient RNAi in embryos. Mutations in AGO1 result in late embryonic/early larval lethality and a reduced RNAi response when injected with either long dsRNA or siRNA (Williams and Rubin, 2002). However, AGO1 appears to have little effect on the efficiency of RNAi in culture cells (Caudy et al., 2002). The failure to detect a significant role for

AGO1 in RNAi in culture cells could be because the function of AGO1 was tested using RNAi; this could have resulted in only partial loss of AGO1. Another possibility is that different AGO family members function in different developmental stages. *In vitro* experiments with embryo extracts showed that AGO1 is required for the degradation of targeted mRNA but not required for the DICER-mediated cleavage of dsRNA into siRNAs (Williams and Rubin, 2002). This suggests that AGO1 functions somewhere between siRNA production and target mRNA degradation. Other *in vitro* experiments with culture cell extracts showed co-fractionation of AGO1 with siRNAs, no detectable association of AGO1 with VIG or dFXR (members of RISC), and no significant nuclease activity in AGO1 complexes (Caudy et al., 2002). This could mean that AGO1 is involved in the stabilization of siRNA, before the mRNA degradation.

Mutations in *ago1* in *Arabidopsis* impaire transgene induced PTGS and also cause developmental defects (Fagard et al., 2000; Morel et al., 2002). In *Neurospora* QDE2 is essential for transgene induced PTGS. QDE2 was found to copurify with siRNAs, but was not necessary for siRNA accumulation. This suggests that the role of QDE2 is downstream of the production of siRNAs. QDE2 could be a component of the siRNA-directed nuclease complex in *Neurospora*, like AGO2 in *Drosophila* (Catalanotto et al., 2002).

The *C. elegans* genome encodes 24 putative PAZ/Piwi proteins. At least two proteins, RDE-1 and PPW-1 are involved in RNAi. *rde-1* mutant animals are fully defective in RNAi and *ppw-1* mutants have a reduced efficiency in RNAi against germline expressed genes (Tabara et al., 1999; Tijsterman et al., 2002b). Genetic experiments indicated that RDE-1 functions together with RDE-4 (a dsRNA binding protein) in the initiation of RNAi (Grishok et al., 2000). Using tagged protein versions of RDE-1 and RDE-4 it was shown that they interact *in vivo*. It was also shown that RDE-4 binds the long trigger dsRNA and that RDE-1 is required for the accumulation of this dsRNA bound to RDE-4 (Tabara et al., 2002). In addition, upon induction of RNAi wild-type levels of siRNAs directly produced from the trigger dsRNA were detected in *rde-1* mutants, whereas *rde-4* mutants showed reduced levels of these primary siRNAs. Finally, introduction of synthetic siRNAs could bypass the RNAi defect in *rde-4* mutants, but not in the *rde-1* mutants (Parrish and Fire, 2001). These findings suggest that RDE-4 is involved prior to or during the initial cleavage of the dsRNA trigger to produce siRNAs, while RDE-1 is involved later in the pathway. The interaction between the two proteins can be explained by a multi-protein/multi-RNA complex that changes during RNAi. There is another PAZ/Piwi protein, PPW-2, in *C. elegans* that is implicated in transgene induced PTGS of genes expressed in the germline and transposon silencing in the germline (Vastenhouw et al., 2003). One could speculate that it replaces RDE-1 that functions in RNAi induced by dsRNA, in PTGS induced by transgenes and transposons.

RNA Helicases

Different RNA helicases are required for RNAi/PTGS in several organisms. In addition, DICER and the putative RdRP RrpA of *Dictyostelium* have a helicase domain. Until now, for most helicases the actual step in the silencing pathway in which they act is not known. RNA helicases may mediate the ATP-dependent unwinding of the siRNA duplex. Another possibility is that they change confirmation of the long dsRNAs that initiate the PTGS process. However, in general many RNA helicases are not involved in unwinding of dsRNA. Instead they bind RNA and help in the formation of different interactions in multistep reactions.

Recently it was found that in *Drosophila* culture cells AGO2 can occur in a complex with Dmp68. In addition, RNAi targeting *dmp68* resulted in inhibition of RNAi. Dmp68 is a *Drosophila* ortholog of human p68, which has been demonstrated to unwind short but not long dsRNAs in an ATP-dependent manner. Therefore, Dmp68 may actually be the helicase that does the unwinding of the siRNA duplex that activates RISC (Ishizuka et al., 2002). Another RNA helicase called Spindle-E influences RNAi in *Drosophila* oocytes. This is a DE-H box RNA helicase. Mutations in *spindle-E* perturb translation control during oogenesis and block RNAi activation during egg maturation. Spindle-E could have a role in both processes or maybe only in translation control and indirectly cause an effect on RNAi. Successful translation is possibly necessary to link a transcript to RNAi mediated degradation (Kennerdell et al., 2002).

SDE3 is an *Arabidopsis* RNA-helicase protein with limited similarity to DexD/H RNA helicases. *Arabidopsis* plants carrying mutations at the *sde3* locus are partially defective in PTGS mediated by transgenes. However, PTGS mediated by a virus was not affected. It is suggested that SDE3 could act at a step in the mechanism that is not absolutely necessary when PTGS is mediated by viruses, for instance where ssRNA is converted into a ds form by an RdRP (Dalmay et al., 2001).

In *Chlamydomonas reinhardtii* a gene (*mut6*) was cloned that is required for PTGS of a transgene. *mut6* encodes a protein that is highly homologous to RNA helicases of the DEAH-family. The detection of aberrant RNAs in the mutant suggest that the helicase is directly or indirectly involved in the degradation of abnormal RNAs and could be a component of the PTGS machinery in *Chlamydomonas* (Wu-Scharf et al., 2000).

In *C. elegans* three putative DexD/H RNA helicases are implicated in RNAi. A RNA helicase-related protein DRH-1 is found in a complex with RDE-4. In addition, RNAi targeting *drh-1* results in inhibition of RNAi. RDE-4 is a dsRNA binding protein that is thought to play a role in the recognition of foreign dsRNA and interacts with DICER. The DRH-1 protein could be involved in changing the confirmation of the dsRNA or may facilitate transfer of the dsRNA from RDE-4 to DICER (Tabara et al., 2002).

C. elegans mut-14 mutants are mutated in a putative DEAD-Box RNA helicases and are defective in RNAi that targets genes expressed in the germline and PTGS induced by transgenes corresponding to germline expressed genes. MUT-14 is also important for gene silencing triggered by asRNAs, which are assumed to function as primers for RNA synthesis on mRNA. The dsRNA that is formed can be used in DICER cleavage. In addition, primary siRNAs are efficiently produced *in vitro*, but secondary siRNAs seem to be reduced *in vivo*. Taken together, this helicase might be involved in facilitating the RNA synthesis (Tijsterman et al., 2002a).

smg-2 is a RNA helicase important for nonsense mediated mRNA decay (NMD) in *C. elegans*. NMD is an evolutionarily conserved “mRNA surveillance” mechanism, which protects cells from the potential deleterious effects of truncated proteins. Both NMD and RNAi involve turnover of RNA. A link between NMD and RNAi has been demonstrated for three of the seven *smg* genes that are required for NMD in *C. elegans*. Mutations in *smg-2*, *smg-5* and *smg-6* resulted in non-persistence of the phenotype produced by RNAi. Recovery only occurred when the target mRNA was continuously transcribed and not essential. Thus, RNAi against a maternally transcribed gene that is essential for embryogenesis did not result in recovery in *smg* mutants. One idea is that the three SMG proteins facilitate the amplification step in RNAi (Domeier et al., 2000).

Exonuclease

The *C. elegans* MUT-7 protein and the *Arabidopsis* WEX protein are both defective in RNAi/PTGS and share a 3’ to 5’ exonuclease domain. Mutations in *mut-7* result in animals that are defective in RNAi against genes expressed in the germline (Ketting et al., 1999). In addition, MUT-7 is also important for gene silencing triggered by asRNAs (Tijsterman et al., 2002a). Other experiments showed that the introduction of long dsRNA to *mut-7* mutants does not result in detectable levels of siRNAs. However, *in vitro* analysis showed that siRNA production directly from the introduced dsRNA is not dependent on MUT-7. Interestingly, accumulation of siRNAs *in vivo* is also shown to be dependent on the presence of target RNA (Tijsterman et al., 2002a). This suggested that MUT-7 functions downstream the cutting of long dsRNA into siRNAs by DICER and possibly upstream of or in a step involving the target mRNA.

MUT-7 forms a complex with several components. One of these components is RDE-2 (also known as MUT-8). The MUT-7/RDE-2 containing complex increases in molecular weight upon induction of RNAi by long dsRNA. This shift is dependent on RDE-1 and RDE-4, but not dependent on the presence of target mRNA (Tops et al., submitted). This is consistent with earlier genetic experiments that placed *mut-7* and *rde-2* downstream of *rde-1* and *rde-4* (Grishok et al., 2000). All results together indicate that MUT-7 functions after siRNA generation and before siRNA mediated target recognition.

By reverse genetics a gene encoding a protein with an RNaseD-like domain, *wex*, was knocked-out in *Arabidopsis*. Loss-of-function mutants of *wex* are defective in PTGS induced by a transgene consisting of a direct tandem inverted repeat. This is consistent with the RNAi resistance phenotype of *mut-7* in *C. elegans* (Glazov et al., 2003).

Posttranscriptional and Transcriptional Gene Silencing

PTGS and TGS should not be considered unrelated mechanisms. They can both be induced by dsRNA. For TGS this was shown in plants; dsRNA corresponding to promoter regions could induce TGS by DNA methylation of the promoters (Mette et al., 2000; Sijen et al., 2001b). In the fission yeast *Schizosaccharomyces pombe* siRNAs were found to be important for chromatin-based gene silencing, which indicates more links between TGS and PTGS. In addition, the *S. pombe* DICER, RdRP and a

PAZ/PIWI protein were implicated in this TGS process (Hall et al., 2002; Reinhart and Bartel, 2002; Schramke and Allshire, 2003; Volpe et al., 2002). In *Drosophila* it was shown that a member of the PAZ/Piwi gene family, *piwi*, is important for both PTGS and TGS (Pal-Bhadra et al., 2002). Remarkably, the modifications at the DNA and/or chromatin level found with TGS are also associated with PTGS. There are several reports about PTGS in plants where there is associated methylation of the transgenes. In addition, partial loss of methylation due to mutations or drugs can cause a partial loss of PTGS (Jones et al., 2001; Kovarik et al., 2000; Morel et al., 2000) and mutations that inhibit PTGS can also have an effect on the associated DNA methylation of the transgenes in plants (Dalmay et al., 2000; Elmayan et al., 1998; Fagard et al., 2000; Mourrain et al., 2000). In *C. elegans* RNAi-based screens to find genes required for RNAi identified several ORFs that are predicted to encode chromatin-associated proteins (Dudley et al., 2002). Furthermore, transgenic arrays in the *C. elegans* germline appear to be less condensed in mutants deficient in RNAi (Dernburg et al., 2000). Other pleiotrophic effects such as chromosome missegregation are also seen in these mutants (Ketting et al., 1999; Tabara et al., 1999). Maybe TGS and PTGS are the nuclear and cytoplasmatic branches respectively of gene silencing that in some cases reinforce each other and in other cases function alone.

Biological Function of PTGS

It is thought that a biological function of PTGS is resistance against parasitic nucleic acids; viruses and transposons. Indications for a role in defense against viruses in plants are threefold: 1) viruses are potent inducers of PTGS in plants (Ratcliff et al., 1999), 2) viruses encode factors inhibiting this silencing response (Li and Ding, 2001; Voinnet et al., 1999), 3) mutants that lose the ability to mount a PTGS response are hypersensitive to virus infection (Dalmay et al., 2001; Mourrain et al., 2000). In other organisms there are several indications for a role of PTGS in transposon silencing. In *C. elegans* several PTGS deficient strains (RNAi and transgene induced PTGS) show high rates of transposition (Ketting et al., 1999; Tabara et al., 1999). Examples are *mut-7*, *mut-8*, and *mut-14* (these are discussed above; see also table 1). siRNAs corresponding to transposon sequences were detected in *C. elegans* (Ambros et al., 2003), and recently dsRNA corresponding to transposon sequences were detected in *C. elegans*. In addition, it was shown that transgenes with transposon sequence are silenced .(Sijen and Plasterk, in press) In *Chlamydomonas reinhardtii* a mutation in a DEAH-box RNA helicase (Mut6) causes desilencing of transgene induced PTGS and derepression of two transposons (Wu-Scharf et al., 2000). In *Drosophila* it was shown that transgenes can silence transposition and the other way round that transposons can silence transgenes (Jensen et al., 2002; Jensen et al., 1999a; Jensen et al., 1999b; Malinsky et al., 2000; Robin et al., 2003). In addition, mutations in the *spindle-E* gene encoding an RNA helicase relieve a PTGS-like silencing of endogenous *Stellate* genes, which also results in an increase of the amount of transcripts produced by some transposable elements. The silencing of *Stellate* genes occurs by paralogous genomic *Su(Ste)* tandem repeats and is associated with Su(Ste) dsRNA and small RNA species (Aravin et al., 2001). Finally, siRNAs corresponding to different kinds of transposons were found not only in *C. elegans* but also in for instance *Drosophila*, *Trypanosoma brucei*, and *Arabidopsis* (Djikeng et al., 2001; Elbashir et al., 2001b; Llave et al., 2002a).

PTGS Components Functional in Other Regulatory Processes

Investigators have demonstrated a role for components of PTGS in the regulation of gene expression influencing development. In *C. elegans* small temporal RNAs (stRNAs) *lin-4* and *let-7* regulate development (Lee et al., 1993; Moss et al., 1997; Olsen and Ambros, 1999; Reinhart et al., 2000; Slack et al., 2000; Wightman et al., 1993). Larger transcripts corresponding to these stRNAs are also present, which are predicted to form hairpin structures (Grishok et al., 2001; Lee et al., 1993). In *C. elegans* Dicer mutants the stRNAs are not present, which results in a number of developmental abnormalities (Grishok et al., 2001; Ketting et al., 2001; Knight and Bass, 2001). Furthermore, it was shown that *in vitro* wild-type DICER produces these stRNAs from the hairpin precursor RNAs (Ketting et al., 2001). Thus, DICER is important for both the RNAi and the stRNA pathway. Proteins of the PAZ/Piwi family are also required in the stRNA pathway suggesting a broader similarity between the RNAi and stRNA pathways (Grishok et al., 2001). Despite the overlap and similarity, the RNAi and stRNA pathway have different outcomes. In RNAi the target mRNA is rapidly degraded (Montgomery et al., 1998; Tuschl et al., 1999), whereas the stRNA pathway inhibits translation. The proteins regulated by the stRNAs are encoded by mRNAs whose 3' untranslated region (3' UTR) contains sites complementary to the stRNAs. Upon expression of stRNAs the levels of the proteins rapidly decline

but the mRNA levels remain constant due to basepairing of the stRNAs to sequences in the 3' UTRs (Olsen and Ambros, 1999; Wightman et al., 1993).

In *Drosophila* and mammals *let-7* stRNAs and their precursors are also present (Hutvagner et al., 2001; Pasquinelli et al., 2000; Slack et al., 2000). Interestingly, it was shown that in *Drosophila* and HeLa cells stRNAs are associated with RISC. This indicates that the overlap between the two pathways is more than the DICER cleavage step (Caudy et al., 2002; Hutvagner and Zamore, 2002; Ishizuka et al., 2002; Mourelatos et al., 2002).

To analyse whether more *let-7* like small RNAs exist cloning and bioinformatics methods were developed to identify more small RNAs. This resulted in the identification of many small RNAs in fly, worm, plants, mouse and human cells (Elbashir et al., 2001b; Lagos-Quintana et al., 2001; Lagos-Quintana et al., 2003; Lagos-Quintana et al., 2002; Lau et al., 2001; Lee and Ambros, 2001; Lim et al., 2003a; Lim et al., 2003b; Llave et al., 2002a; Mourelatos et al., 2002; Park et al., 2002; Reinhart et al., 2002). For most small RNAs that were identified it was checked whether they originate from a hairpin-like structure. These small RNAs were called microRNAs (miRNAs). Given the number and diversity of different miRNAs it is possible that miRNAs not only function in development but in many more processes and maybe not only via translational repression. To find out the possible functions of miRNA investigators are now focusing on expression patterns of the miRNAs, identification of targets and isolation of mutants of the miRNAs and their targets.

Recently, new genetic evidence supporting the biological importance of miRNAs and their diverse regulatory functions was published. Mutations in two miRNAs, *mir-14* and *bantam*, were identified and both of these miRNAs were implicated in programmed cell death in *Drosophila* (Brennecke et al., 2003; Xu et al., 2003). The expression of Hid, a apoptosis regulator, was shown to be repressed by *bantam*, most likely by blocking translation of the *hid* mRNA that has 5 potential binding sites for *bantam* (Brennecke et al., 2003).

The miRNAs in plants seem to function predominantly like siRNAs. They correspond mostly to coding regions of mRNAs and guide sequence specific cleavage in development and/or tissue specific manner (Llave et al., 2002b; Rhoades et al., 2002). Examples of *Arabidopsis* miRNAs are *miR39/miR171* and *miR165/166*. *miR39/171* functionally interacts with mRNA targets encoding several members of the *Scarecrow*-like (*SCL*) family of putative transcription factors (Llave et al., 2002b). *miR165/166* has been proposed to down-regulate *PHV* and *PHB* mRNA expression. *PHV* and *PHB* encode homeodomain-leucine zipper transcription factors (Rhoades et al., 2002; Tang et al., 2003). Both kinds of transcription factors are implicated in developmental processes. In *Arabidopsis* a mutation in *hen1* affects both miRNA and transgene siRNA accumulation again suggesting common features between the stRNA/miRNA pathway and PTGS (Boutet et al., 2003). Interestingly, there is also a report about miRNAs in *Arabidopsis* with homology to promoters suggesting regulation of gene expression by miRNAs at the DNA level (Park et al., 2002).

The miRNAs or small RNAs really appear to represent a previously unsuspected layer of regulation in many organisms. As mentioned before, *Su(Ste)* tandem repeats, dsRNA and small RNA intermediates are involved in the silencing of *Stellate* genes in *Drosophila*. The *Stellate* genes are testis-expressed genes involved in the maintenance of male fertility. Hyperexpression of *Stellate* causes meiotic abnormalities and defects in the primary spermatocytes (Aravin et al., 2001).

miRNAs are now also implicated in an important human disease. In *Drosophila* components of RISC and miRNAs are found associated with a protein FMRP that in humans is involved in the fragile X syndrome (Caudy et al., 2002; Ishizuka et al., 2002). The FMRP protein is implicated in neuronal synapse formation a function. Biochemical studies suggest that FMRP acts as negative regulator of translation, although it is unclear how (Bardoni and Mandel, 2002). Possibly the miRNAs function as translation inhibitors.

In *S. pombe* small RNAs that match the centromeres are thought to be DICER cleavage products. The colocalization of these RNAs with the sites of dsRNA transcription, transgene and chromatin modifications suggests that they guide heterochromatic silencing (Reinhart and Bartel, 2002). In addition, mutants of DICER, an RNA-directed RNA polymerase (RdRP) and the PAZ/Piwi of *S. pombe* ARGOUNAUTE, all components of PTGS, are defective in this process (Hall et al., 2002; Volpe et al., 2002).

Another potential function for small RNAs has been found in *Tetrahymena thermophila*. In this organism they direct DNA deletion (Mochizuki et al., 2002). *Tetrahymena* have two nuclei; each cell contains a germline micronucleus and a somatic macronucleus. The micronucleus is functional during sexual conjugation, whereas the macronucleus is transcriptionally active during vegetative growth. The macronucleus lacks a large part of the DNA sequences that are found in the micronucleus. This is

due to sequence elimination that occurs in developing the macronucleus during the late stages of conjugation. dsRNA and short regulatory RNAs that correspond to the deleted DNA have been implicated in these chromosome rearrangements (Chalker and Yao, 2001; Mochizuki et al., 2002; Yao et al., 2003). These RNAs have the characteristics of processing by DICER-like enzymes. In addition, a Piwi homolog (TWI1) is required in this process (Mochizuki et al., 2002). These observations suggest that this process also overlaps in part with RNAi/PTGS.

Conclusion

RNAi/PTGS is a very intriguing process. It is an ancient defense mechanism against viruses and transposons, which also has links to other regulatory processes in many different organisms. In addition, it can be a useful phenomenon for many applications. Currently, a relative simple working model can be drawn for the pathway of PTGS, with DICER, RISC and in several organisms a role for an RdRP. There are also many components implicated in the pathway that do not have a clear position yet.

Outline of this Thesis

The main focus of the study was the genetic analysis of components in the RNAi pathway in *C. elegans*. In addition, RNAi was used to identify new gene functions.

Chapter 2 describes experiments that investigate an amplification step in RNAi. The experiments indicate that in *C. elegans* the RNAi pathway contains an amplification step mediated by an RdRP. A population of siRNAs is detected, which has characteristics that suggest they were generated upon RdRP activity. In addition, a deletion in a putative RdRP gene, *rrf-1*, results in resistance to certain RNAi triggers and loss of this siRNA production. This led to a model that proposes the synthesizes of new dsRNAs by *rrf-1*, using targeted mRNAs as template and primary siRNAs as primers, and subsequent steps that involve the destruction of the mRNA and amplification of the siRNA population.

Chapter 3 shows that mutations in another member of the RdRP-like family, *rrf-3*, cause hypersensitive to RNAi. It will require more experimental analysis to understand the nature of this effect. However, the *rrf-3* strain can be useful as a tool to increase the RNAi effect in many different RNAi experiments. This detailed study of the RNAi sensitivity of *rrf-3* animals shows that *rrf-3* is generally more sensitive to RNAi than wild-type worms. Most interestingly, RNAi for neuronally expressed genes, which often has no effect in wild-type animals, was enhanced.

Chapter 4 discusses a genome-wide RNAi screen using the hypersensitive *rrf-3* strain. An RNAi library that consists of bacterial clones expressing dsRNA corresponding to practically all predicted genes of *C. elegans* was previously screened using the wild-type strain. To increase the functional information on the *C. elegans* genome, *rrf-3* was used. The RNAi data can be a starting point for many new experiments. In addition, we studied the variability between RNAi experiments, and found persistent levels of false negatives. This is an important feature to take in account when RNAi experiments are conducted and/or interpreted.

Chapter 5 concerns the analysis of systemic RNAi in *C. elegans*. The worm produces a systemic response to the localized introduction of dsRNA. This lead to the assumption that an uptake mechanism exists that functions to transport the dsRNA or a related RNA product from one cell or tissue to another. We obtained a set of mutants with defects in the response to dsRNA. These mutants do respond to dsRNA delivered by injection but fail to respond to dsRNA expressed in the food. Therefore, they could have defects in cellular uptake of dsRNA or defects in systemic spread of RNA silencing signals. One of the mutated genes encodes a protein with an ENTH domain, which suggests that vesicle transport might be involved in systemic RNAi.

Table 1. Proteins implicated in RNAi/PTGS in *C. elegans*

Protein	Domain structure	Trigger	Mutant Phenotype	Putative Function	References
DCR-1	RNA helicase, PAZ, RNase III, and dsRNA binding	dsRNA	RNAi resistant (this was difficult to assess since homozygous *dcr-1* worms are sterile and maternal protein seems to be contributed that can rescue RNAi for some genes)	Production of siRNAs from long dsRNA.	(Grishok et al., 2001; Ketting et al., 2001; Knight and Bass, 2001)
RDE-1	PAZ and Piwi	dsRNA, not transgenes	RNAi resistant	Initiation of RNAi, downstream of siRNA production. Associates with RDE-4.	(Grishok et al., 2000; Parrish and Fire, 2001; Tabara et al., 1999; Tabara et al., 2002)
PPW-1	PAZ and Piwi	dsRNA and asRNAs corresponding to germline expressed genes	Partially impaired in RNAi for germline expressed genes	Efficiency factor in germline tissue.	(Tijsterman et al., 2002b)
PPW-2	PAZ and Piwi	Transgenes corresponding to germline expressed genes	PTGS resistant for germline expressed genes	Maybe the substitute of RDE-1 in transgene induced PTGS; different PAZ/Piwi proteins with different triggers.	(Vastenhouw et al., 2003)
RDE-4	dsRNA binding	dsRNA, not transgenes	RNAi resistant	Initiation of RNAi. Binds to the long dsRNA, DICER and RDE-1.	(Grishok et al., 2000; Parrish and Fire, 2001; Tabara et al., 1999; Tabara et al., 2002)
MUT-7	3' to 5' exonuclease	dsRNA, transgenes and asRNAs corresponding to germline expressed genes	RNAi/PTGS resistant for germline expressed genes	Downstream of siRNA production, upstream of mRNA recognition.	(Ketting et al., 1999; Ketting and Plasterk, 2000; Tabara et al., 1999; Tijsterman et al., 2002a; Tops et al., submitted)
RDE-2/MUT-8		dsRNA and transgenes corresponding to germline expressed genes	RNAi/PTGS resistant for germline expressed genes	Part of a complex with MUT-7.	(Ketting et al., 1999; Ketting and Plasterk, 2000; Tabara et al., 1999; Tops et al., submitted)
MUT-14	RNA helicase	dsRNA, transgenes and asRNAs corresponding to germline expressed genes	RNAi/PTGS resistant for germline expressed genes	Downstream of siRNA production, could make the mRNA accessible for an RdRP.	(Tijsterman et al., 2002a)
MUT-16		dsRNA corresponding to germline and somatically expressed genes and transgenes corresponding to germline expressed genes	RNAi resistant for germline expressed genes, partially defective in RNAi for somatically expressed genes, and defective in PTGS for germline expressed genes		(Vastenhouw et al., 2003)

RRF-1	RNA directed RNA Polymerase	dsRNA	RNAi defective for somatically expressed genes	Amplification of the RNAi effect; production of additional dsRNA.	(Sijen et al., 2001a)
EGO-1	RNA directed RNA Polymerase	dsRNA	RNAi defective for germline expressed genes	Amplification of the RNAi effect; production of additional dsRNA.	(Smardon et al., 2000)
RRF-3	RNA directed RNA Polymerase	dsRNA and transgenes	Increased sensitivity to RNAi and transgene induced silencing	Negative regulator/ inhibitor of RNAi/PTGS.	(Sijen and Kooter, 2000; Simmer et al., 2002b)
MES-3		dsRNA and transgenes corresponding to germline expressed genes	RNAi defective for germline expressed genes upon introduction of high levels of dsRNA and defective in transgene induced silencing of germline expressed genes	Possibly affects transcription of the target gene, resulting in production dsRNA that amplifies the RNAi/PTGS effect. Alternatively, regulates transcription of unrelated genes that can produce dsRNA that could saturate the RNAi/PTGS mechanism.	(Dudley et al., 2002; Tabara et al., 1999)
MES-4	SET	dsRNA and transgenes corresponding to germline expressed genes	RNAi defective for germline expressed genes upon introduction of high levels of dsRNA and defective in transgene induced silencing of germline expressed genes	Possibly affects transcription of the target gene, resulting in production dsRNA that amplifies the RNAi/PTGS effect. Alternatively, regulates transcription of unrelated genes that can produce dsRNA that could saturate the RNAi/PTGS mechanism.	(Dudley et al., 2002; Tabara et al., 1999)
MES-6	WD40 repeats	dsRNA and transgenes corresponding to germline expressed genes	RNAi defective for germline expressed genes upon introduction of high levels of dsRNA and defective in transgene induced silencing of germline expressed genes	Possibly affects transcription of the target gene, resulting in production dsRNA that amplifies the RNAi/PTGS effect. Alternatively, regulates transcription of unrelated genes that can produce dsRNA that could saturate the RNAi/PTGS mechanism.	(Dudley et al., 2002; Tabara et al., 1999)
SMG-2	RNA helicase	dsRNA	Initially sensitive to RNAi, but rapid recovery	Involved in persistence of the phenotype produced by RNAi. Facilitate the amplification step in RNAi?	(Domeier et al., 2000)
SMG-5	PINc; nucleotide binding	dsRNA	Initially sensitive to RNAi, but rapid recovery	Involved in persistence of the phenotype produced by RNAi. Facilitate the amplification step in RNAi?	(Domeier et al., 2000)
ADR-1	Adenosine deaminase	Transgenes corresponding to somatically expressed genes (transgenes with inverted repeats or simple arrays)	Increases the sensitivity to transgene induced PTGS of somatically expressed genes	dsRNA editing in the nucleus; the adenosines of dsRNA are deaminated creating inosines (A:U to I:U). This could result in dsRNA that is not completely base-paired and fails to trigger PTGS.	(Knight and Bass, 2002)
ADR-2	Adenosine deaminase	Transgenes corresponding to somatically expressed genes	Increases the sensitivity to transgene induced PTGS of somatically	dsRNA editing in the nucleus; the adenosines of dsRNA are deaminated creating inosines (A:U to	(Knight and Bass, 2002)

		(transgenes with inverted repeats or simple arrays)	expressed genes	I:U). This could result in dsRNA that is not completely base-paired and fails to trigger PTGS.	
SID-1/RSD-8	Transmembrane (11x)	dsRNA	Defective in RNAi when the dsRNA is not delivered into the target tissue	Could be involved in the uptake of the RNA intermediate that causes spreading of RNAi throughout the animal.	(Winston et al., 2002)
RSD-3	Epsin N-terminal homology (ENTH)	dsRNA introduced via the food of the animal	Defective in RNAi when worms are fed bacteria expressing dsRNA; not defective in RNAi when the dsRNA is introduced by injection	Spreading of RNAi throughout the animal? The ENTH domain frequently occurs in vesicle trafficking proteins.	(Tijsterman et al., submitted)
RSD-2		dsRNA corresponding to germline expressed genes introduced via the food of the animal	Defective in RNAi when worms are fed bacteria expressing dsRNA corresponding to germline expressed genes	Spreading of RNAi throughout the animal? RSD-2 interacts with RSD-6 in a yeast 2-hybrid screen.	(Tijsterman et al., submitted)
RSD-6	Tudor	dsRNA corresponding to germline expressed genes introduced via the food of the animal	Defective in RNAi when worms are fed bacteria expressing dsRNA corresponding to germline expressed genes	Spreading of RNAi throughout the animal? RSD-2 interacts with RSD-6 in a yeast 2-hybrid screen. Tudor domains are frequently found in RNA-binding proteins.	(Tijsterman et al., submitted)

In this table the proteins that are implicated based on genetic mutants in RNAi or PTGS induced by other triggers in *C. elegans* are listed. Only the RNAi/PTGS mutant phenotype is listed. The mutants can have additional phenotypes, for example phenotypes concerning development, sterility, and/or transposition. The putative function is in some case more speculative then in others.

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Chapter 2

On the role of RNA Amplification in dsRNA-Triggered Gene Silencing

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On the Role of RNA Amplification in dsRNA-Triggered Gene Silencing

Titia Sijen,[1,4] Jamie Fleenor,[3,4] Femke Simmer,[1,4] Karen L. Thijssen,[1] Susan Parrish,[2,3] Lisa Timmons,[3] Ronald H.A. Plasterk,[1,5] and Andrew Fire[3,5]

[1] Hubrecht Laboratory
Center for Biomedical Genetics
Uppsalalaan 8, 3584 CT
Utrecht
The Netherlands

[2] Biology Graduate Program
Johns Hopkins University
Baltimore, Maryland 21218

[3] Department of Embryology
Carnegie Institution of Washington
Baltimore, Maryland 21210

Summary

We have investigated the role of trigger RNA amplification during RNA interference (RNAi) in *Caenorhabditis elegans*. Analysis of small interfering RNAs (siRNAs) produced during RNAi in *C. elegans* revealed a substantial fraction that cannot derive directly from input dsRNA. Instead, a population of siRNAs (termed secondary siRNAs) appeared to derive from the action of a cellular RNA-directed RNA polymerase (RdRP) on mRNAs that are being targeted by the RNAi mechanism. The distribution of secondary siRNAs exhibited a distinct polarity (5′→3′ on the antisense strand), suggesting a cyclic amplification process in which RdRP is primed by existing siRNAs. This amplification mechanism substantially augments the potency of RNAi-based surveillance, while ensuring that the RNAi machinery will focus on expressed mRNAs.

Introduction

RNA-mediated interference (RNAi) is a conserved gene silencing mechanism that recognizes double-stranded RNA (dsRNA) as a signal to trigger the sequence-specific degradation of homologous mRNA (see Sharp, 2001 for a recent review). Analyses of RNAi and related processes in diverse systems have uncovered several surprising properties, including the double-stranded character of the trigger RNA and a catalytic aspect of the interference reaction. Indeed, a few molecules of dsRNA are sufficient in *C. elegans* or *Drosophila* cells to trigger the decay of a much larger population of target mRNAs (Fire et al., 1998; Kennerdell and Carthew, 1998).

Several features of the RNAi mechanism have been proposed to contribute to the remarkable potency of the reaction. Some degree of amplification is likely to derive from cleavage of the dsRNA trigger into short pieces of 21–25 nt (called siRNAs) by the RNaseIII-like nuclease DICER (e.g., Zamore et al., 2000; Bernstein et al., 2001). For the most commonly used dsRNA triggers (500–1000 bp), this would result in a 20- to 40-fold increase in the molar ratio of trigger to target. A simple (single-use) utilization of the siRNAs would be sufficient to explain the molar efficiency of RNAi in extracts of *Drosophila*, but would be insufficient to account for in vivo potency in *C. elegans*. A multiround mechanism (use of a single siRNA for hundreds or thousands of rounds of target degradation) would be much more efficient.

An additional contribution to the potency of RNA-triggered gene silencing has been proposed to involve physical amplification of an aberrant RNA population through an RNA-directed RNA polymerase (RdRP) activity (Dougherty and Parks, 1995). By producing a large number of copies of a triggering RNA, an RdRP activity might dramatically increase the effectiveness of RNAi. The possibility of RdRP involvement in posttranscriptional gene silencing has been supported by the isolation of an endogenous RdRP activity from tomato (Schiebel et al., 1993a, 1993b, 1998), followed by subsequent demonstrations that factors with protein sequence homology to this RdRP were required for efficient silencing in fungal, nematode, and plant systems (Cogoni and Macino, 1999; Smardon et al., 2000; Dalmay et al., 2000; Mourrain et al., 2000).

A number of apparent constraints on the roles of RdRP activity in RNAi are suggested by experimental observations. Embryonic extracts from *Drosophila* with no measurable RdRP activity can carry out a complete RNAi reaction (Zamore et al., 2000; P. Zamore, personal communication). This, combined with the absence in available *Drosophila* or mammalian genomic sequences of a clear homolog of the RdRP-like genes implicated in other systems, argues that an RNAi reaction can proceed without RdRP. It should be noted, however, that formation of unstable (transient) copy RNAs during the in vitro reaction might be difficult to detect, and that additional enzymes (such as RNA polymerase II and retroviral type reverse transcriptases) are capable of polymerizing RNA in response to certain RNA templates (e.g., Diener, 1991; Filipovska and Konarska, 2000; Modahl et al., 2000). A more limited constraint on possible roles for RdRP in RNAi comes from experiments in which the two trigger strands have been modified differentially prior to injection into *C. elegans* or *Drosophila* (Parrish et al., 2000; Yang et al., 2000). These experiments showed a more stringent requirement for structure and sequence of the antisense strand of the original trigger, as compared to the sense strand. These "strand-preference" experiments do not rule out a role for RdRP in the interference reaction, but do severely limit models in which the RdRP carries out a multiround replication of a double-stranded trigger (e.g., Waterhouse et al., 1998) to produce exponential amplification: this type of exponential amplification would result in loss of memory of the difference between the original two strands and would thus be incompatible with the observed effects of strand-specific modification.

[4] These authors made equal contributions to this work.

[5] Correspondence: plasterk@niob.knaw.nl; fire@ciwemb.edu

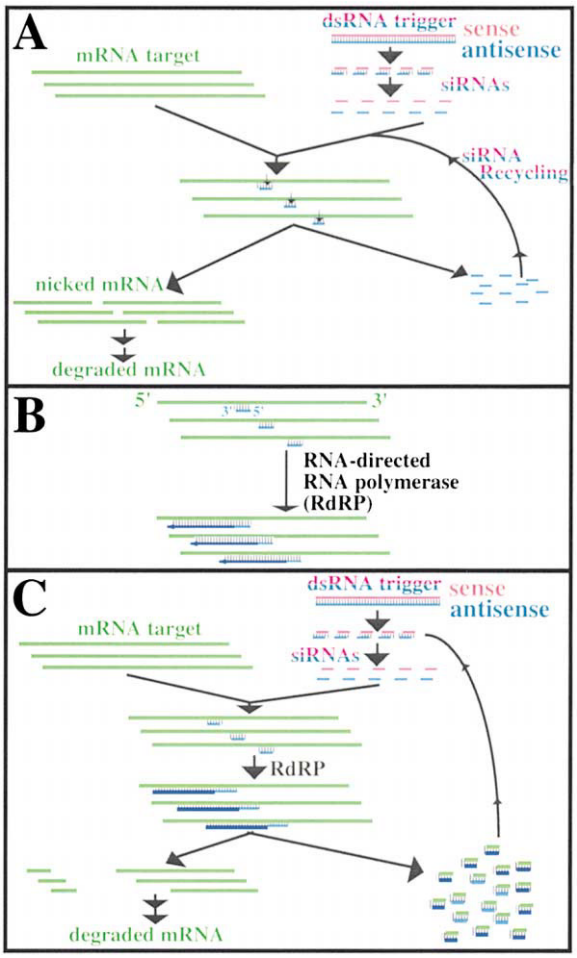


Figure 1. Could siRNA-Primed Copying of Target RNAs by an RNA-Directed RNA Polymerase Contribute to RNAi?

(A) A current model of the nucleic acid alterations during RNA interference based primarily on in vitro studies of RNAi in *Drosophila* extracts (e.g., Zamore et al., 2000; Hammond et al., 2001; Bernstein et al., 2001; Elbashir et al., 2001). After cleavage of the dsRNA trigger into short siRNA segments, the individual antisense siRNAs pair with complementary mRNAs, with degradation of mRNA and (eventual) recycling of siRNAs.

(B) shows that at the heart of the working model is an intermediate with the antisense strand of an siRNA hybridized to an mRNA target. Since the siRNAs possess a 3′-terminal hydroxyl group, the resulting intermediate might function as a template for elongation by an RdRP activity.

(C) shows a possible consequence of the reactions proposed in (A) and (B), with the sequential activity of RdRP and a dsRNA-specific nuclease (e.g., DICER) leading to a target-dependent amplification of the siRNA population.

Of the numerous roles proposed for RdRP during gene silencing, we were most intrigued by the possibility (Figure 1) that antisense siRNAs that have annealed to a ssRNA target might be elongated by RdRP to produce longer stretches of dsRNA (Sijen and Kooter, 2000). This model is particularly attractive in that (1) siRNAs are known to have a 3′ hydroxyl group (Elbashir et al., 2001), which would be poised for elongation by an RNA polymerase, (2) cleavage of the RdRP-elongated regions of dsRNA to produce short siRNAs would result in a net amplification of the initial population of siRNAs at the expense of target transcripts, and (3) this mode of amplification utilizes the two input strands of the RNA trigger differentially; thus, there is no inconsistency with earlier results which had shown more stringent chemical requirements for the antisense strand of the initial trigger RNA (Parrish et al., 2000; Yang et al., 2000).

The model in Figure 1C leads to a number of testable predictions; in particular, we would expect to observe a population of secondary siRNAs after RdRP-mediated synthesis of duplex RNAs followed by cleavage by RNaseIII/DICER activity. These secondary triggers would be derived primarily from sequences upstream of the initial trigger region on the target mRNA and would be expected to induce a secondary RNA interference reaction directed to any homologous target RNA.

In this paper, we demonstrate the production and biological activity of RdRP-dependent secondary triggers during RNA interference in *C. elegans*.

Results

Biochemical Evidence for Secondary siRNAs

We first sought to demonstrate the existence of secondary siRNAs through direct analysis of RNA populations. Although the appearance of short RNAs in the 21–25 nt range has universally been observed in studies of RNA-triggered gene silencing, the abundance of such RNAs varies considerably between systems. In particular, siRNAs observed during RNAi are apparently much less abundant in *C. elegans* than in plants and *Drosophila* (e.g., Hamilton and Baulcombe, 1999; Parrish et al., 2000; Yang et al., 2000). In order to characterize populations of siRNA from *C. elegans* in detail, we used RNase protection assays. ^{32}P labeled ssRNA molecules (used as probes) were hybridized to denatured cellular RNA, and the resulting material treated with ssRNA-specific ribonucleases to degrade any unhybridized probe. We used single-stranded probes from the sense strand in order to detect the siRNA signal while avoiding a background due to breakdown products of the cellular mRNA target. To generate a large mass of *C. elegans* actively performing RNAi, we used a procedure in which animals are grown on bacteria engineered to express high levels of a specific dsRNA (Timmons and Fire, 1998; Fraser et al., 2000).

Each RNase-protection experiment involves two segments: a dsRNA trigger produced in bacteria and a probe RNA used to detect siRNA molecules. Figure 2 shows results for two target genes: the muscle-specific gene *unc-22* and the germline-specific gene *pos-1*. In each case, the strongest siRNA signals were obtained when the trigger and probe sequences corresponded. This population of siRNAs would be expected from models in which a dsRNA-specific nuclease cleaves the original dsRNA trigger to produce siRNA segments. In addition to the trigger-coincident siRNAs, we also detected populations of small antisense RNAs that correspond to regions of the target gene outside the original trigger. We tentatively refer to these as secondary siRNAs. The secondary siRNAs were generally detected at levels substantially below those of the trigger-coincident siRNAs, but were reproducibly observed using several

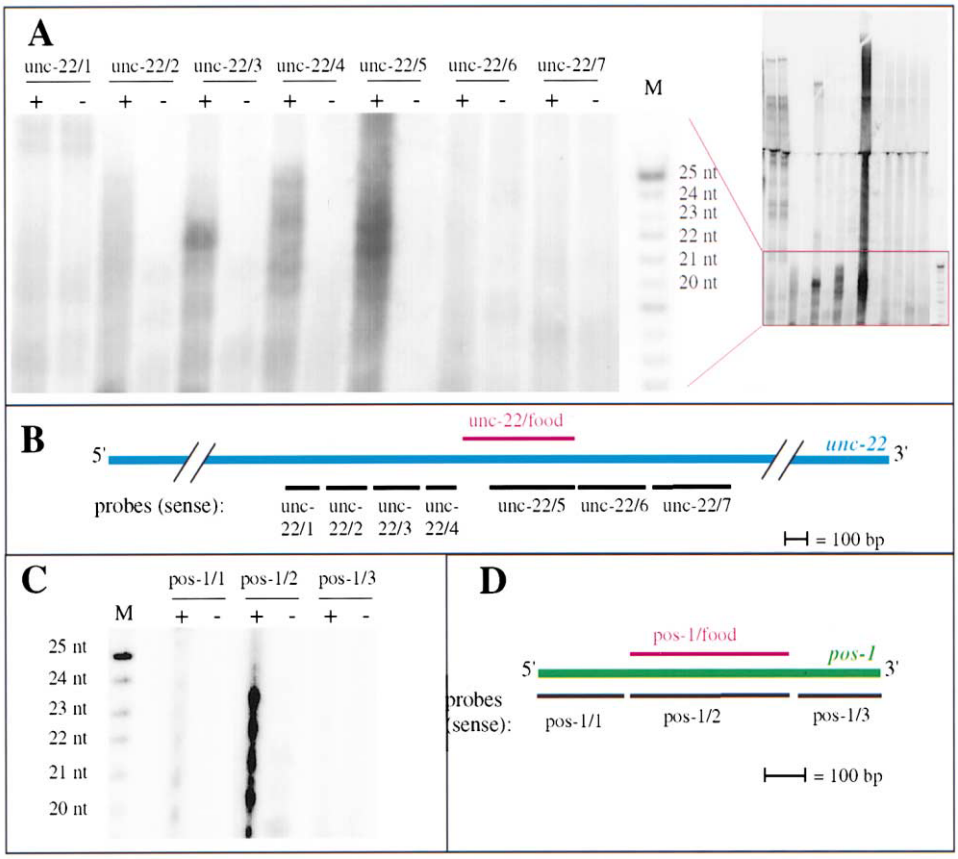


Figure 2. Biochemical Detection of Secondary siRNAs

Analysis of small RNAs from wild-type animals grown on *E. coli* expressing dsRNA segments of *unc-22* or *pos-1*. Total RNA was isolated and RNase protection assays were performed using various *unc-22* or *pos-1* specific probes (all of sense polarity).

(A) Products of RNase protection assay (right: protected fragments of probe resolved on polyacrylamide-urea gel; left: detail of 16–30 nt portion of gel). Feeding on *unc-22* dsRNA yielded siRNAs from the dsRNA segment comprising the food, but also produced siRNAs mapping upstream of this region. Lanes designated "+": RNA from animals fed *unc-22* dsRNA. To determine levels of probe-derived background, negative controls ("−") were carried out by performing RNase protections with yeast tRNA as input RNA. A similar background in the siRNA size range was observed in RNase protection assays on RNA from animals grown on induced bacteria containing the feeding vector L4440 with no insert (data not shown). RNase protection assays have also been carried out using RNA from IPTG-induced *E. coli* producing *unc-22* dsRNA; these showed some level of probe protection but no protected fragments in the siRNA size range (data not shown). Labels above the lanes indicate probes. "M": ^{32}P-labeled 25 nt RNA oligonucleotide marker.

(B) Map of *unc-22* mRNA with positions of probes and bacterially produced dsRNA.

(C) Secondary siRNAs are also produced upon feeding with *E. coli* producing *pos-1* dsRNA. Since *pos-1* is a germline-specific gene, RNA was isolated from egg preparations. "+": *C. elegans* populations fed with *E. coli* producing *pos-1* dsRNA; "−": equivalent RNA preparations from animals grown on *E. coli* containing the empty L4440 vector.

(D) Map of *pos-1* mRNA with positions of probes and bacterially produced dsRNA.

different combinations of trigger and probe sequences. Although the detection limits of the system preclude a definitive measurement of siRNA levels for each trigger/probe combination, two points emerge rather clearly from the analysis. First, occurrence of a detectable secondary antisense population was limited to cases in which the probe sequence was upstream (closer to the 5′ end of the target mRNA) as compared with the trigger sequence. Second, the abundance of secondary siRNA molecules appeared to decrease as a function of distance from the primary trigger.

Transitive RNAi

Secondary siRNAs might be expected to act as functional RNAi triggers, targeting any homologous mRNA sequences for degradation. To test this hypothesis, it is necessary to distinguish between targeting by the initial dsRNA trigger and by the secondary siRNAs. This is most conveniently carried out by means of a "transitive RNAi" assay. Essentially, such an assay entails a cell with two populations of target RNA: the first population (primary target) has a segment which matches the dsRNA trigger; the second population has no homology to the initial dsRNA trigger, but has a segment which is identical to the primary target.

Figure 3 shows an example of transitive RNAi in which both primary and secondary target RNAs are transgene-derived transcripts carrying *gfp*. The primary target in this experiment encodes a nuclear-targeted GFP-LACZ fusion protein (NLS-GFP-LACZ), while the secondary

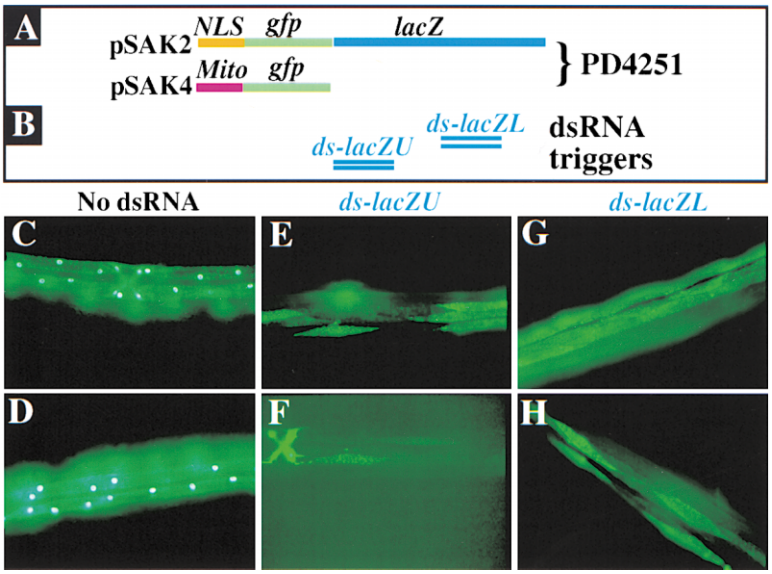


Figure 3. Assays for Transitive RNAi Using Distinct *gfp* Transgenes

The transgenic line used for this assay (PD4251) carries two different *gfp* reporter constructs (A). pSAK2 produces nuclear-localized GFP fused at the C terminus to additional sequences encoding *E. coli* β-galactosidase (*lacZ*). pSAK4 produces mitochondrially localized GFP with no additional sequences at the C terminus. PD4251 animals express both nuclear and mitochondrial GFP forms in all cells of the body musculature (Fire et al., 1998). Young adult progeny of adult animals injected with specific dsRNA segments (B) were examined to determine the level of interference with nuclear- and mitochondrial-targeted *gfp*s.

(C and D) Mock injected control animals with both GFP isoforms expressed in each muscle cell.

(E and F) Progeny of animals injected with ds-*lacZU.* This injection produced a strong transitive RNAi effect, interfering in a majority of cells not only with the nuclear targeted *gfp*::*lacZ* transgene, but also with the mitochondrial-targeted *gfp*. (A bright "X" shape in [F] shows vulval muscles fortuitously included in the photo; these cells are generally nonresponsive to parentally injected dsRNA; Fire et al., 1998)

(G and H) Progeny of animals injected with ds-*lacZL*. This segment had only a modest effect on the expression of mitochondrially targeted *gfp*, so that the majority of cells continue to produce GFP in mitochondria but not nuclei. (F) and (H) are representative of the strongest transitive RNAi response in each population, while (E) and (G) are representative of the weakest effect. As negative controls, PD4251 animals injected with a variety of unrelated dsRNA segments (*unc-22*A, *unc-22*B, *lin-26*IVS3) showed no evident decrease in either nuclear or mitochondrial GFP. Animals injected with *gfp* dsRNA show near-complete (98%) loss of both nuclear and mitochondrial GFP (Fire et al., 1998).

target encodes a mitochondrially targeted GFP (MtGFP) which has no sequences from *lacZ* (both transgene mRNAs are driven by the *myo-3* promoter). As a control, animals carrying only one of the two transgene constructs show the expected effects: both GFPs are dramatically reduced in progeny of animals injected with dsRNA corresponding to GFP, while only the NLS-GFP-LACZ construct is affected by dsRNAs corresponding to *lacZ* (data not shown). A line carrying both transgene constructs produces both nuclear LACZ-GFP and mitochondrial GFP (PD4251; Figures 3C and 3D). Injection of dsRNA segments from *lacZ* into the line carrying both transgenes produces a transitive effect: reduction of both nuclear GFP-*lacZ* and mitochondrial GFP. Of two different *lacZ* segments tested, a trigger that was located just 3′ to the *gfp*::*lacZ* junction (*ds-lacZU*) was most potent in the transitive RNAi assay, producing reduction of mitochondrial GFP to background in 60% of targeted cells, while a dsRNA trigger located further downstream (*ds-lacZL*) produced a more modest effect (reduction of GFP in 28% of cells) (Figure 3 and data not shown).

A second example of transitive RNAi is presented in Figure 4. In this case, the primary target is an *unc-22*::*gfp* fusion transgene (Figure 4C), while the secondary target is an endogenous gene (*unc-22*; Brenner, 1974; Moerman et al., 1988). Injection of dsRNA corresponding to *gfp* into wild-type animals (no transgene) produced no phenotype; injection of ds*gfp* RNA into animals carrying a transgene expressing GFP alone produced a decrease in GFP but no *unc-22* phenotype. Injection of ds*gfp* RNA into animals expressing the *unc-22*::*gfp* transgene produced the twitching phenotype that is characteristic of loss of *unc-22* expression (e.g., *ds-gfpA*; Figure 4C).

To test whether transitive RNAi could proceed with endogenous genes as targets, we carried out the two experiments shown in Figure 5. In-frame deletion alleles of *unc-22* and *unc-52* provide a useful genetic tool: these alleles each produce proteins that lose a fraction of the coding region (658 amino acids for *unc-22*(*st528*); 150 amino acids for *unc-52*(*ra511*)) but retain full wild-type function (Kiff et al., 1988; Fire et al., 1991; Rogalski et al., 1993; Mullen et al., 1999). As expected, dsRNAs corresponding to the deleted regions produced strong gene-specific RNAi effects in wild-type animals, but no effect in animals homozygous for the corresponding deletion alleles. The test for transitive RNAi in each case consists of introducing these trigger RNAs into heterozygous animals carrying both wild-type and mutant alleles. In each case, we found a strong transitive RNAi effect: heterozygotes exhibited interference with both deletion and wild-type alleles. These experiments demonstrate that transitive RNAi is not limited to transgene targets, but can also target physiological expression of cellular genes.

Structural Requirements for Triggering of Transitive RNAi

Certain features of transitive RNAi are illuminated by the requirements for structure and dose of the primary trigger. A prediction of the model in Figure 1C is that the effect should exhibit a defined polarity, with interference depending on the order of the two segments in the primary target mRNA. This was the case, as shown by the lack of sensitivity to transitive RNA when the order of segments in the transgene construct was reversed (Figure 4E).

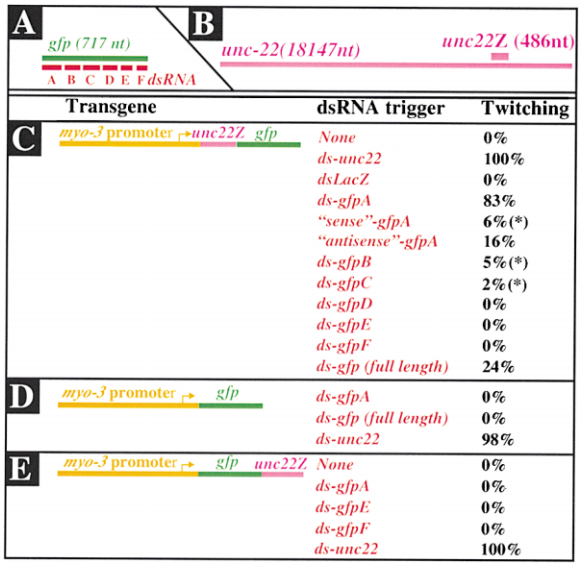


Figure 4. Assays for Transitive RNAi Using a Chimeric *unc-22*::*gfp* Transgene

Transgenic lines used for these assays carry the *C. elegans myo-3* promoter driving the indicated combinations of the *gfp* coding region (717 nt) and a segment within the *unc-22* gene (*unc-22z*; 486 nt). Following propagation of clonal transgenic lines for several generations, transitive RNAi was assayed by injecting adults with a variety of dsRNAs. After ~3.5 days, injected animals and postinjection progeny (>50 animals derived from 5–20 injected parents) were scored for twitching in levamisole. Assays marked with an "*" showed twitching predominantly in the injected adults; the remaining positive assays showed twitching in both injected adults and progeny, while negative assays showed twitching in neither injected adults nor progeny.

(A and B) Segments used in this analysis. mRNA structures are shown; the *gfp* coding region is interrupted in each DNA construct by three 51 nt introns. The *gfp*-derived dsRNAs (Parrish et al., 2000) were each functional in primary RNAi, as assayed by reduction of GFP in injected adults and progeny.

(C) A twitching phenotype was observed when the injected dsRNA corresponded to sequences from *gfp* downstream of the *unc-22*::*gfp* junction. Note that *ds-gfpA* produced the most effective twitching response, presumably by producing the highest molar concentration of siRNAs immediately downstream of the *unc-22*::*gfp* junction.

(D and E) Transitive RNA was specific to the structure and arrangement of the initial dsRNA trigger and transgene.

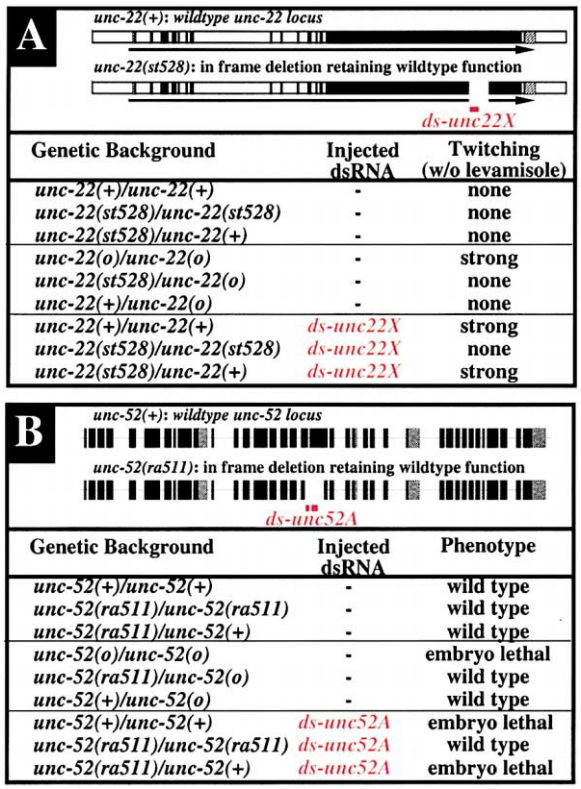


Figure 5. Transitive RNAi Can Operate on Native Chromosomal Genes

(A) Maps of wild-type *unc-22* and an in-frame deletion (*st528*) that retains wild-type function (Moerman et al., 1988; Benian et al., 1993; Kiff et al., 1988; black, exons; white, introns). *unc-22* null mutants exhibit a strong twitching behavior in the absence of levamisole (we used *unc-22*(*e66*) as a canonical null for this analysis; Brenner, 1974). The strong twitching phenotype is not seen with animals that have a single functional dose of the wild-type or *st528* allele. Following injection of *ds-unc22X* RNA, twitching without levamisole was observed in 100% of *unc-22*(+) animals, 0% of *unc-22*(*st528*)/*unc-22*(*st528*) animals, and 60% of *unc-22*(*st528*)/+ animals.

(B) Maps of wild-type *unc-52* and a deletion allele that removes nonessential sequences (*unc-52*(*ra511*); Mullen et al., 1999; black, exons; white, introns; hatched, alternatively spliced exons). The null phenotype for *unc-52* is a zygotic-effect embryonic lethality with paralysis (Williams and Waterston, 1994; Rogalski et al., 1993). A chromosomal deficiency (mnT11; Herman et al., 1982) was used to definitively determine *unc-52*(+)/*unc-52*(o) and *unc-52*(*ra511*)/*unc-52*(o) phenotypes. Animals that have a single functional dose of the wild-type or *ra511* allele show no lethal or visible phenotype. Following injection of *ds-unc52A* RNA, embryonic lethality with paralysis was observed in 100% of *unc-52*(+) animals, 0% of *unc-52*(*ra511*)/*unc-52*(*ra511*) animals, and 100% of *unc-52*(*ra511*)/+ animals.

Interference showed a dose response to the concentration of primary trigger, with a modest interference response observed at doses as low as 1.5×10^6 molecules per injected parent (data not shown). Given the expression levels of *unc-22* (Fire et al., 1991), and assuming equal dispersion of trigger RNA among the cells of the affected progeny, this corresponds to a stoichiometry on the order of ~100 molecules of trigger RNA for ~5000 molecules of target mRNA in each muscle cell of the affected animals. Triggering also appeared to be structure-specific: although some interference was observed with sense or antisense RNA preparations alone, there was a dramatic stimulation upon mixing the two preparations. As with previous studies (e.g., Fire et al., 1998), it was not straightforward to distinguish whether residual activity of our ssRNA preparations was due to low levels of dsRNA contamination even after purification. In any case, these data indicate that the initial triggering reaction is either fully dependent on, or greatly stimulated by, delivery of a trigger RNA with double-stranded character.

Not all potential trigger RNAs were capable of producing transitive interference. For each target RNA, we observed a graded effect as a function of distance between primary and secondary target sequences. The precise relationship between distance and effectiveness appeared to depend on the details of the experiment (compare positional dependence in Figures 3E–3H with that

in Figure 4C), but in each case, the effect decreased with increasing distance between the segments.

The Cellular RdRP Homolog *rrf-1* Is Required in Somatic Cells for Production of Secondary siRNA Triggers and for Transitive RNAi

Genetic screens for factors responsible for RNA-triggered silencing phenomena in diverse organisms have identified (among many other components) factors with substantial homology to a cellular RdRP isolated from viroid-infected tomato leaves (Schiebel et al., 1998; Cogoni and Macino, 1999; Smardon et al., 2000; Dalmay et al., 2000; Mourrain et al., 2000). *C. elegans* has four members of this gene family (*ego-1*, *rrf-1*, *rrf-2*, and *rrf-3*) (Smardon et al., 2000). Two of these genes, *ego-1* and *rrf-1*, are closely linked (0.9 kb apart in tandem orientation), while *rrf-2* and *rrf-3* map to distinct loci. *ego-1* is an essential gene required for fertility: adult *ego-1* homozygotes can only be derived as progeny of heterozygous mothers, thus it is not possible to carry out RNAi assays in the complete absence of maternal and zygotic *ego-1* product (Smardon et al., 2000). Despite this limitation, Smardon et al. (2000) were able to demonstrate a requirement for *ego-1* in producing an efficient RNAi response in the adult germline; no role for *ego-1* during RNAi in somatic tissue has been detected.

To extend our understanding of the RdRP gene family in *C. elegans*, we produced deletion alleles of the *rrf-1*, *rrf-2*, and *rrf-3* genes through PCR-based screening of a chemical deletion library (Figure 6A; protocol from Jansen et al., 1997). We obtained single deletion alleles for each *rrf-* gene: *rrf-1*(*pk1417*) deletes 401 aa, including the majority of the residues conserved in the RdRP family; *rrf-2*(*pk2040*) deletes the presumed promoter region and the first five exons; *rrf-3*(*pk1426*) produces an out-of-frame truncation after the fourth exon, effectively removing most or all of the RdRP domain. These three deletions would be predicted to behave as genetic nulls. Each of the three *rrf* deletions was viable and fertile; none showed any obvious morphological or growth defects (the *rrf-3*(*pk1426*) strain produces a slightly higher incidence of male progeny than wild-type; the source of this effect has not been characterized). While this work was being carried out, an additional transposon (Tc1)-induced allele of *rrf-3* was obtained (F.S. and R.P., unpublished data; protocol from Zwaal et al., 1993). Although the majority of our analysis was carried out with the three deletion alleles, the transitive RNAi properties of *rrf-3* (see below) were confirmed with the Tc1 allele.

As shown in Figure 6B, the *rrf-2* and *rrf-3* deletion strains were sensitive to RNA interference in all tissues (soma and germline) and for all assays performed (both standard RNAi assays and transitive RNAi assays). For *rrf-2*(*pk2040*), we observed no differences from wild-type in any of the RNAi assays. These results indicate either a redundant role for RRF-2 in RNAi or (alternatively) a role in a distinct cellular process. Interestingly, the *rrf-3* deletion and Tc1 insertion strains both showed reproducible increases in sensitivity to RNAi when compared to wild-type animals. This increase in sensitivity is evident for several different target genes and for both standard and transitive RNAi assays (Figure 6B and data not shown). While it is interesting to speculate on possible negative roles for *rrf-3* in the RNAi response (e.g., loss of *rrf-3* function might release specific RdRP cofactors for use in RNAi), the nature of the effect will require further experimental analysis; the major conclusion that we can draw at this point is that RRF-3 is nonessential for the RNAi responses tested.

By contrast to the RNAi sensitivity observed in *rrf-2* and *rrf-3* mutants, we observed complete resistance of the *rrf-1* deletion allele to certain RNAi triggers. As shown in Figure 6B, there was a strong correlation between site (tissue) of function for the target gene and the efficacy of interference: interference for genes expressed in somatic tissue was lost in *rrf-1* deletion mutants, while interference was retained for genes expressed in the germline. Consistent with our analysis of *rrf-1(pk1426)*, D. Conte and C. Mello (personal communication) have observed loss of RNAi in soma but not germline tissue in an independently isolated set of *rrf-1* missense mutations.

We used two assays to address the production of secondary siRNAs in the RdRP mutants. These assays were carried out for somatic targets, since infertility of *ego-1* mutants (likely to affect germline RdRP; Smardon et al., 2000) would confound our biochemical and genetic assays. We first transformed each *rrf* deletion mutation with a DNA construct (*myo-3*::*unc-22Z*::*gfp*, as shown in Figure 4) that allows a functional test for transitive interference. In these assays, we observed no loss of transitive interference in *rrf-2*(*pk2040*) and *rrf-3*(*pk1426*), while *rrf-1*(*pk1417*) completely blocked the transitive interference. In parallel, we assayed directly for physical production of secondary trigger molecules (Figure 6C). By this assay, we failed to detect upstream (secondary) siRNAs in *rrf-1*(*pk1417*) animals. *rrf-2*(*pk2040*) and *rrf-3*(*pk1426*) retained the ability to produce the secondary triggers. Interestingly, *rrf-1*(*pk1417*) mutants retain the ability to produce a small population of siRNA molecules corresponding to the original trigger RNA. The siRNAs produced in *rrf-1*(*pk1417*) may represent the primary trigger RNAs. These results are consistent with an RdRP-independent cleavage of the initial dsRNA trigger, followed by RdRP- and target-dependent amplification of the trigger population.

A variety of genes have been shown to play essential or contributory roles in RNAi in *C. elegans*. To identify additional genetic requirements for transitive RNAi, we first assayed two genes for which the most straightforward genetic tools were available. *rde-1* and *rde-4* are the only *C. elegans* genes known to be essential for RNAi in all tissues. Since both genes are dispensable for organismal viability and fertility, the assays for transitive RNAi were straightforward. We found that both genes were required for the transitive RNAi assay (Figure 6B).

We note an ambiguity that is inherent in both siRNA and transitive RNAi assays: since both assays depend on early steps in the RNAi pathway, the results with *rrf-1*, *rde-1*, and *rde-4* mutants do not distinguish between (1) a specific loss of secondary siRNAs and (2) a decrease in secondary siRNAs as a result of inefficiency in earlier stages in the RNAi pathway (e.g., primary siRNA production). For *rde-1*, this ambiguity is addressed by previous results. Extracts of *rde-1* mutant animals are comparable to wild-type extracts in cleavage of labeled dsRNA into short siRNA fragments (Ketting et al., 2001). This initial cleavage process also proceeds in vivo: after injection of a ^{32}P-labeled dsRNA trigger into the syncytial

germline, *rde-1*(*ne300*) null mutants are comparable to wild-type in the production of ^{32}P-labeled siRNAs (Parrish and Fire, 2001). *rde-4* mutants have also been analyzed in the in vivo assay; *rde-4* shows a decreased primary siRNA production, suggesting a possible defect in primary siRNA production (Parrish and Fire, 2001). For the RdRP products, the straightforward assay for cleavage of labeled dsRNA after germline injection (or extract preparation) is not available: since *ego-1* is an essential gene, we have no source of healthy RdRP(−) animals for direct assays of siRNA production.

A second test that has been used to address mutational effects on the role of siRNAs in the interference reaction involves injection of a large population of synthetic siRNAs directed at a specific target sequence. The siRNAs are prepared with the characteristic duplex structure and 2-base 3′ overhang (Elbashir et al., 2001). For *C. elegans*, synthetic siRNAs of 24–25 bp yield robust interference in wild-type animals and partially bypass the RNAi defect in *rde-4* mutants (but not in *rde-1* mutants) (Caplen et al., 2001; Parrish and Fire, 2001). When tested in the *rrf-1* mutant backgrounds (point or deletion), we observed no interference by preformed siRNAs, even at concentrations 10-fold above those required for interference in a wild-type background (Figure 6B and data not shown).

An Essential Role for Secondary siRNAs and RdRP Activity in the RNAi Mechanism

The insensitivity of *rrf-1* mutants to phenotypic interference in the soma suggested that the initial siRNA:target interaction might be insufficient to produce a phenotypically significant effect on gene expression. This was particularly surprising with an *unc-22* target, since a relatively modest decrease in gene expression (on the order of 30%–40%) is detectable using the assays employed. Additional experiments were carried out using quantitative RNase protection in attempts to detect small decreases in *unc-22* expression in the *rrf-1*(*pk1426*) mutant animals; no decrease in mRNA level was observed (Figure 6E).

Discussion

A Working Model for RNA Interference in the *C. elegans* Soma

We have demonstrated that RNA interference in *C. elegans* involves the production of at least two populations of siRNA molecules. One group of siRNA molecules had been previously described and is derived from the initial injected dsRNA. A second group of siRNAs has sequence, structural, and biological characteristics that indicate formation by an RdRP, potentially following priming of target RNAs by the antisense strand of primary siRNAs. Based on the results of this work and of the many studies of RNAi in diverse biological systems over the last several years, we present a working model for RNA interference and related pathways in the *C. elegans* soma (Figure 7).

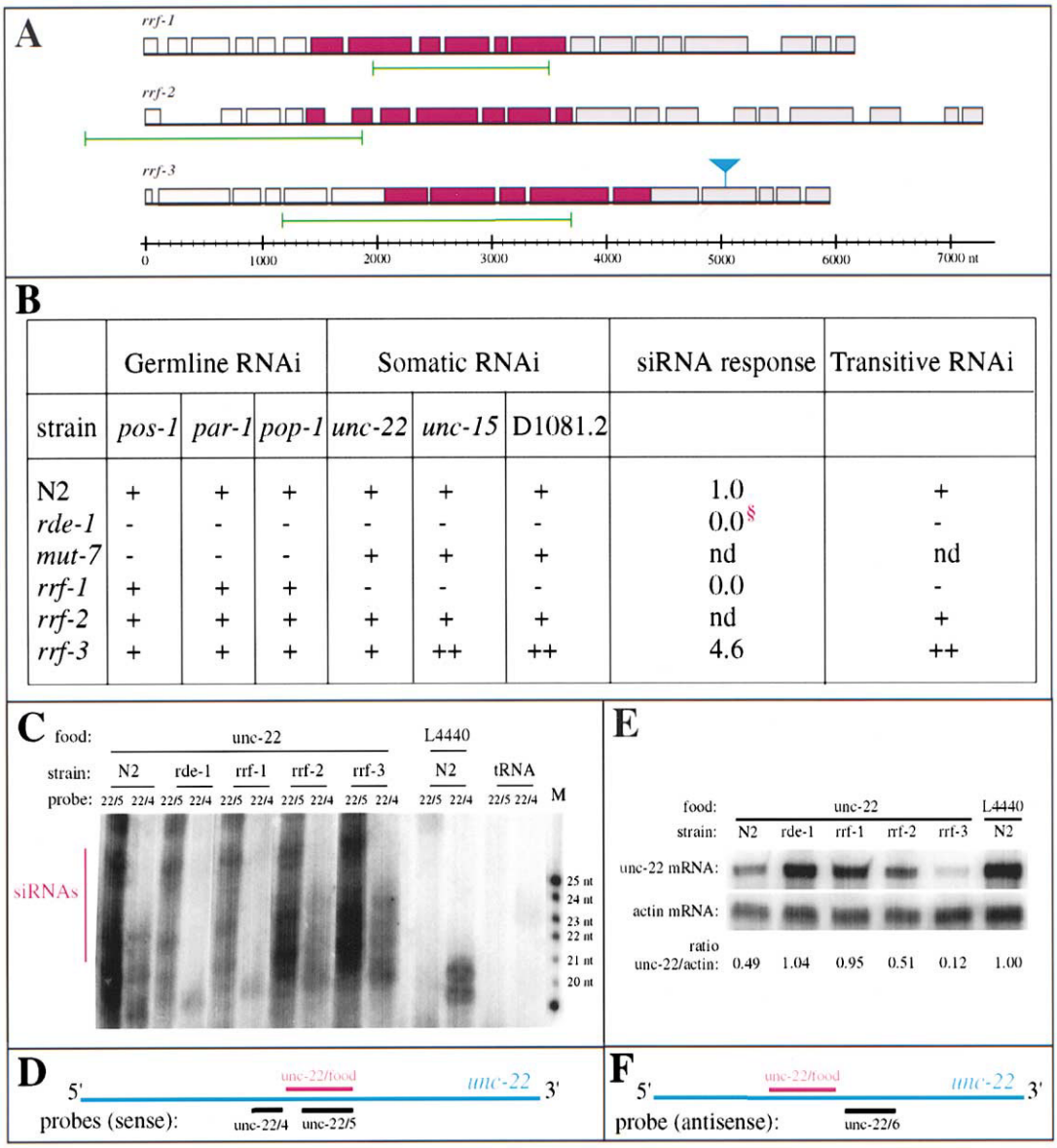
The first steps in the RNAi pathway involve uptake of dsRNA by cells and an inefficient cleavage of the original trigger RNA into short fragments. The cleavage reaction has been studied in detail in extracts of *Drosophila* and *C. elegans* (Bernstein et al., 2001; Zamore et al., 2000; Elbashir et al., 2001; Ketting et al., 2001), and has been shown to be mediated by the RNaseIII-like factor DICER; genetic experiments in *C. elegans* suggest, in addition, the involvement of RDE-4 (Tabara et al., 1999; Parrish and Fire, 2001). These initial siRNAs are apparently not numerous enough (or not of the proper structure) to effect an efficient interference response in vivo. They must, however, have an appropriate structure to allow interaction in vivo with complementary sequences on the target mRNA. Two possible consequences could follow this initial interaction: the siRNA might prime synthesis of longer antisense RNA; alternatively, cleavage of the target mRNA in the region of siRNA homology might produce an end structure which signals RdRP to initiate de novo synthesis of antisense RNA on the cleaved mRNA template. Interestingly, the biochemical analysis of plant RdRP is consistent with either model: the tomato RdRP activity is capable of both primed and unprimed synthesis (Schiebel et al., 1993a, 1993b). Whatever the mechanism by which synthesis of new antisense RNA is primed, the subsequent activity of DICER or another dsRNA-specific nuclease could function both (1) to destroy the mRNA and (2) to amplify the population of siRNA triggers.

At some point in the RNAi process, there is an absolute requirement for a member of the Argonaute superfamily. Although there are 24 Argonaute homologs in *C. elegans*, RDE-1 is completely required for specific interference responses to exogenous dsRNA (Tabara et al., 1999). Potential roles for RDE-1 would be to stabilize the primary siRNAs (M. Tijsterman et al., submitted) and/or to facilitate scanning of potential target RNAs for regions of homology. Consistent with these proposals are recent studies by Hammond et al. (2001) showing that *Drosophila* Argonaute-2 forms a tight complex with siRNAs during RNAi in *Drosophila* cultured cells.

Certain biochemical features of RdRP-derived amplification are suggested from our in vivo observations. In particular, our analyses of positional dependence showed a loss of the transitivity and secondary siRNA signals at distances greater than several hundred base pairs from the original trigger. Given that this distance may reflect multiple rounds of elongation and reduction to siRNAs, these data suggest that only relatively short transcripts are produced by RdRP in our assays. Several different aspects of the reaction might limit the extent of dsRNA formed: (1) the processivity of the enzyme in vivo may be very limited; (2) the enzyme may be blocked from producing large dsRNAs by secondary structure or protein factors bound to target RNA, or (3) templates available for RdRP may be of limited length (perhaps short segments of sense RNA that are derived through partial degradation of the target mRNA). Given the ability of the RdRP enzyme to initiate RNA synthesis at the end of a short RNA segment (Schiebel et al., 1993a, 1993b), it is certainly possible that the RdRP would carry out an additional reaction of copying sense segments of the input siRNA.

A Diversity of Roles RdRP and Amplification Processes in Gene Silencing?

One surprising aspect of our data was the lack of measurable RNAi response in *rrf-1* mutant soma. Given that some siRNAs are produced in the mutant, and given



	Germline RNAi			Somatic RNAi			siRNA response	Transitive RNAi
strain	*pos-1*	*par-1*	*pop-1*	*unc-22*	*unc-15*	D1081.2		
N2	+	+	+	+	+	+	1.0	+
rde-1	-	-	-	-	-	-	0.0§	-
mut-7	-	-	-	+	+	+	nd	nd
rrf-1	+	+	+	-	-	-	0.0	-
rrf-2	+	+	+	+	+	+	nd	+
rrf-3	+	+	+	+	++	++	4.6	++

Figure 6. Contributions of the RdRP-Homologous Genes *rrf-1*, *rrf-2*, and *rrf-3* to RNAi and Secondary siRNA Production

(A) Predicted structures of *rrf* genes and mutant alleles. Boxes represent exons. Red boxes: RdRP-related segments (*rrf-1*: nt 1413–3837/aa 471–1279; *rrf-2*: nt 1362–3771/aa 454–1257; *rrf-3*: nt 2049–4383/aa 683–1461). Green lines: *rrf-1*(*pk1417*), *rrf-2*(*pk2040*), and *rrf-3*(*pk1426*) remove nt 1991–3407, 572–1878, and 1190–4205, respectively. Blue triangle: *rrf-3*(*pk2042*) has a Tc1 transposon inserted between nt 5016 and 5017.

(B) RNAi sensitivity assays. Animals were fed *E. coli* producing different dsRNAs and progeny scored for survival (germline-expressed genes) or uncoordinated or paralyzed phenotype (somatically expressed genes). "−": resistance to RNAi (full survival or normal movement). "+": sensitivity to RNAi (no survival or uncoordinated movement; effects comparable to those in wild-type animals). "++": hypersensitivity to RNAi (greater sensitivity to RNAi than observed in wild-type; this was only testable for the *unc-15* and *D1081.2* genes for which the dsRNA-producing bacteria yielded a partially penetrant phenotype in wild-type animals). "siRNA response": twitching behavior for progeny of animals injected with 5 mg/ml of a synthetic 25 nt siRNA from *unc-22* (23 bp duplex with 2 base 3′ overhangs; Caplen et al., 2001). Percentages of animals twitching in levamisole are normalized to fractions observed in wild-type. "§": data from Parrish and Fire (2001). "Transitive RNAi" refers to the assay in Figure 4C: mutant strains were transformed with the *myo-3*::*unc-22*::*gfp* fusion construct to generate several independent transgenic lines, and animals from these lines injected with dsRNA for segment *gfpA*. No twitching in levamisole (i.e., no transitive RNAi) was observed in *rrf-1*(*pk1417*) (two lines), *rde-1*(*ne300*) (two lines), or *rde-4*(*ne299*) (one line). For *rde-1* and *rde-4* (where fewer lines were derived), efficacy of each transgene as a substrate for transitive RNAi was confirmed by crossing out of the *rde* background and assaying in a wild-type background. For *rrf-3*(*pk1426*) (two lines) and *rrf-3*(*pk2042*) (two lines), we observed apparent increases in transitive RNAi, as evidenced by an increase of 10- to 15-fold in twitching response to a dsRNA segment located further downstream of the *unc-22*::*gfp* junction (*gfpB*).

(C) RNase protection assays of total RNA from animals raised on *E. coli* containing the *unc-22* dsRNA expression construct pTS302, or the empty vector (L4440); lanes labeled "tRNA" show RNase protection assays carried out on yeast tRNA. Probes (all of sense polarity) are indicated above the lanes. The putative siRNA region of the gel (22–26 nt RNAs) is noted; lower bands in the gel (in the 20–21 nt region,

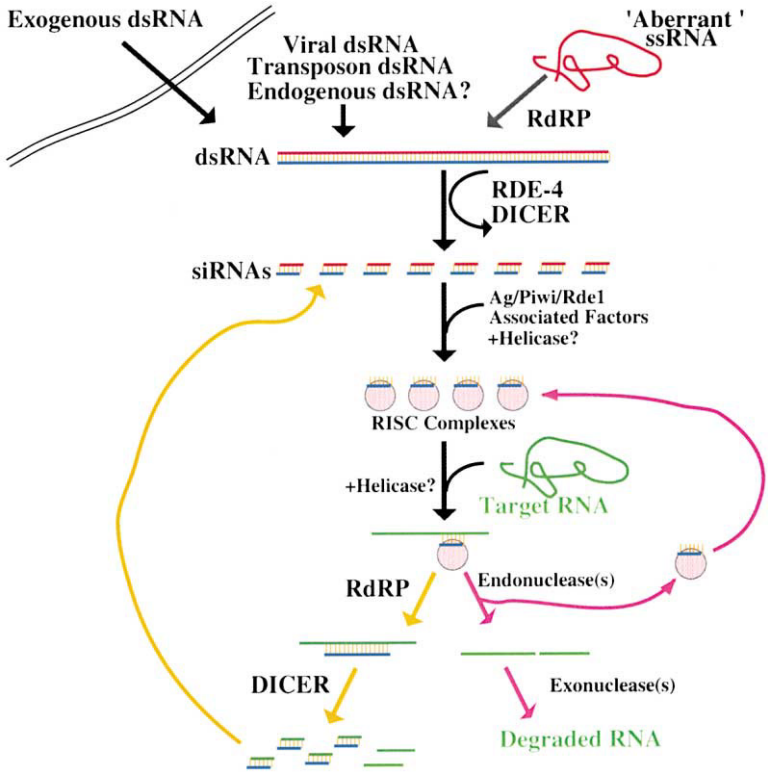


Figure 7. A Working Model for RNA Interference

Two different aspects of the model enhance the potency of the RNAi reaction. Reuse of RNA-loaded RISC complexes (magenta arrows) should provide the reaction with a catalytic component, while physical amplification by RdRP (orange arrows) provides a physical amplification of the initial trigger RNA.

that siRNAs can be injected at high concentrations, we might have expected at least a modest interference response. The lack of such an effect suggests one of three possibilities. The first would be a quantitative insufficiency: it is conceivable that the levels of primary siRNAs (even following the injection of preformed siRNA at high concentration) are insufficient for a measurable response (perhaps incorporation of injected siRNAs into RISC complexes [Hammond et al., 2001] is much less efficient than the incorporation of secondary siRNAs formed in vivo). Alternatively, the initial siRNA::mRNA interaction may be relatively transient or unstable in vivo and may require stabilization through the polymerization of additional bases on the end of the duplex. Under such circumstances, the formation of a region of duplex by RdRP may be sufficient to block gene expression even before (or in the absence) or further cleavage by DICER/RNaseIII. A third possibility is perhaps mechanistically most intriguing: RRF-1 and other RdRP-like factors could have an additional biochemical role in the RNAi reaction. Since these factors must be capable of interacting with dsRNA, their binding could promote or stabilize interactions between siRNAs and target RNA. More speculatively, RdRP-like factors might catalyze phosphorolysis reactions in addition to template-dependent nucleotide polymerization, perhaps breaking the target mRNA or tagging it for destruction.

Genetic analysis in plants of RdRP function during silencing and pathogen defense has suggested both commonality and diversity of roles. One of the *Arabidopsis* RdRP homologs, SDE-1/SGS-2, is required for RNA-triggered silencing by a variety of sense transgenes and for RNA-triggered defense against some but not all viral genomes (Mourrain et al., 2000; Dalmay et al., 2000). Dalmay and colleagues proposed that silencing by sense transgenes might require RdRP to produce a dsRNA trigger, which then enters a (potentially RdRP-independent) RNAi pathway. Alternatively, a central role for RdRP in RNAi might be obviated during certain viral infections by unwitting amplification of specific trigger RNAs by viral replicase. Xie et al. (2001) describe the involvement of a distinct RdRP homolog in tobacco viral resistance; it is not clear whether this factor has a role in RNAi.

Given the complexity of RNAi and other gene silencing responses, it seems likely that multiple amplification processes cooperate to provide a highly sensitive and selective response. The absence of an identified RdRP homolog in *Drosophila* and mammals suggests either (1) that other RNA copying enzymes are used in these

particularly with the 22/4 probe) represent background hybridization that is observed in the absence of ongoing RNAi (e.g., L4440 lanes).
(D) *unc-22* mRNA with positions of *E. coli* produced dsRNA and probes.
(E) RNase protection assay on total RNA isolated from animals fed with *E. coli* producing *unc-22* dsRNA. *unc-22* and an actin (*act-1*) probes, both of antisense polarity, were both added during hybridization. *act-1* and *unc-22* steady-state mRNA levels were quantitated and the ratio *unc-22*/*act-1* mRNA determined.
(F) Relative positions of probes and bacterially produced dsRNAs for (E).

systems for amplification or (2) that the primary siRNAs may be sufficient to produce a detectable interference response (as is observed in *Drosophila* extracts). With or without an RNA copying process, a variety of additional amplification mechanisms may contribute to silencing. In this regard, it is of interest to note two previous examples of transitive silencing: Pal-Bhadra et al. (1999) observed examples of transitive silencing in *Drosophila*, while Voinnet et al. (1998) reported transitive silencing with a GFP transgene target in plants. These examples may reflect different underlying processes than we have reported; in particular, neither study noted a specific polarity in the transitivity, and the biological systems that were used are known to enforce silencing both at a posttranscriptional level and at the level of chromosome modification (methylation in plants [Wassenegger, 2000]; polycomb-group binding in animals [Pal-Bhadra et al., 1997]). A number of extant models for gene silencing in plants propose an amplification step relying on such chromosome-targeted effects (e.g., Bender, 2001). It will be of interest in the future to understand the breadth of different amplification events operating in gene silencing and their biological roles.

Experimental Procedures

dsRNAs

Previously described plasmids were used to produce dsRNA segments for *gfp* (*gfp*A–*gfp*F: L5051, L5108, L5058, L5050, L5059, L5052; Parrish et al., 2000), full-length *gfp* (*gfp*G; Fire et al., 1998), *unc-22* (*unc-22A*, *unc-22B*; Fire et al., 1998), and *lacZ* (*lacZ*L; Fire et al., 1998). Additional dsRNAs were from pRP1245 and *unc22X* (nt 16219–17207 and 10687–10861 of the spliced *unc-22* coding sequence), *ds-lacZU* (nt 158–1957 from the *lacZ* coding region), and *ds-unc52A* (nt 12002–12349 from *unc-52* (GenBank: CELUNC52X; Rogalski et al., 1993). *ds-lin26ivs3* (used for some negative controls) was identical in sequence to that described by Bosher et al. (1999); in our hands, injection of a highly purified and concentrated preparation of *ds-lin26ivs3* dsRNA produced no lethality or other phenotypes.

Plasmids for dsRNA production in *E. coli* were derivatives of vector L4440 (Timmons and Fire, 1998): pTS302 contained nt 11139–11728 of the spliced unc-22 coding region; pTS301 contained nt 183–620 of the spliced pos-1 coding region; pRP1251 contained the full pos-1 cDNA from pCCM114 (provided by H. Tabara; Tabara et al., 1999). dsRNA expression plasmids for *unc-15*, *D1081.2*, *pop-1*, and *par-1* (Fraser et al., 2000) were from J. Ahringer. Bacterial feeding was performed as described (Fraser et al., 2000); L4 hermaphrodites fed on dsRNA-producing bacteria (20–22°C) for approximately 24 hr were transferred to a second dsRNA-bacteria plate for >48 hr and progeny scored for behavior (both plates) or survival (second plate).

RNase Protection Assays

RNase protection assays were chosen to provide maximally sensitive detection of small RNAs. By comparison with Northern blot procedures (e.g., Hamilton and Baulcombe, 1999), the avoidance of a filter transfer allows a substantial increase in fraction of siRNAs detected. A disadvantage of RNase protection assays is that the measured size (length of protected probe) is subject to possible imprecision of a few nucleotides due to frayed or overhanging ends produced by RNase. The siRNA size heterogeneity of several nucleotides seen in Figures 2 and 6 is likely to reflect (at least in part) this aspect of the assay.

To isolate RNA, animals or eggs lysed in protease K were extracted with phenol/chloroform, precipitated with isopropanol (1:1), and resuspended in 1 ml 1.5× STE (1× STE = 0.1 M Tris [pH 7.5], 0.1 M NaCl, and 10 mM EDTA). 100 mg cellulose powder (MN 301, Macherey-Nagel) was added, samples were mixed 10 min at room temperature, two aliquots of 290 μl 96% ethanol were added (mixing each time), and RNA was allowed to bind for one hour. After brief centrifugation, pellets were washed 3 times with 1× STE/35% ethanol, and RNA was eluted with 2 ml 1× STE, precipitated with ethanol, treated with RNase-free DNaseI, extracted with phenol/chloroform, and precipitated with ethanol.

^{32}P-labeled RNA probes were generated from cloned fragments by in vitro transcription with T3 or T7 polymerase followed by gel purification. Probes used were: *unc-22*/1 (10452–10562), *unc-22*/2 (10557–10798), *unc-22*/3 (10807–11004), *unc-22*/4 (10999–11138), *unc-22*/5 (11206–11728), *unc-22*/6 (11729–12075), *unc-22*/7 (12228–12564), *pos-1*/1 (1–188), *pos-1*/2 (183–620), *pos-1*/3 (615–795), *act-1* (199–390) (numbers from spliced coding sequences).

RNase protection assays were performed essentially as described (Sijen et al., 2001) with minor modifications: after hybridization, samples were treated with 20 μg/ml RNaseA, 10 U/ml RNaseT1, and 10 U/ml RNaseOne (45 min at 30°C followed by 45 min at 37°C). For each sample, 20 μg of total RNA was used.

Derivation of Transgenic Lines

Derivation of transgenic lines using the markers *pha-1*(+) or pRF4 was as described (Granato et al., 1994; Mello and Fire, 1995). Some transgenic lines exhibit cosuppression in the absence of injected RNA (e.g., Fire et al., 1991), possibly reflecting unintended antisense products of the transgene that would complicate the subsequent analysis of polarity for transitive RNAi. We sought to minimize this problem in two ways: (1) to improve transport and stability of sense transcripts (thereby maximizing steady-state ratios of sense/antisense), our transgene structures were similar to native *C. elegans* genes in having short 5′ and 3′ UTR sequences and internal punctuation by introns; and (2) we screened lines to eliminate those with detectable cosuppression: the *gfp* transgenic line in Figure 3 (PD4251) was chosen from several similar lines based on uniformity of expression and lack of sporadic silencing. The *unc-22*::*gfp* and *gfp*::*unc-22* constructs (Figure 4) were used to make numerous independent lines; 10%–20% of these lines showed cosuppression and were eliminated. Of the remainder, 5–10 lines were tested for each construct and yielded essentially identical results.

Rescue of *rrf-1*(*pk1417*)

A PCR product containing the wild-type *rrf-1* gene (1226 bp of upstream sequence, 568 bp downstream sequence) was injected (20 ng/ml, with 100 ng/ml pRF4) into *pk1417*. Transgenic animals showed a normal RNAi response to bacterially produced *unc-22* dsRNA.

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Chapter 3

Loss of the Putative RNA-Directed RNA Polymerase RRF-3 Makes *C. elegans* Hypersensitive to RNAi

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Loss of the Putative RNA-Directed RNA Polymerase RRF-3 Makes *C. elegans* Hypersensitive to RNAi

Femke Simmer,[1] Marcel Tijsterman,[1] Susan Parrish,[2,3] Sandhya P. Koushika,[4] Michael L. Nonet,[4] Andrew Fire,[3] Julie Ahringer,[5] and Ronald H.A. Plasterk[1,6]

[1]Hubrecht Laboratory
Center for Biomedical Genetics
Uppsalalaan 8
3584 CT, Utrecht
The Netherlands

[2]Biology Graduate Program
Johns Hopkins University
Baltimore, Maryland 21218

[3]Department of Embryology
Carnegie Institution of Washington
Baltimore, Maryland 21210

[4]Department of Anatomy and Neurobiology
Washington University School of Medicine
660 S. Euclid Avenue
Saint Louis, Missouri 63110

[5]WellcomeTrust
Cancer Research UK Institute
University of Cambridge
Tennis Court Road
CB2 1QR Cambridge
United Kingdom

Summary

RNA interference (RNAi) is a broadly used reverse genetics method in *C. elegans* [1]. Unfortunately, RNAi does not inhibit all genes [2, 3]. We show that loss of function of a putative RNA-directed RNA polymerase (RdRP) of *C. elegans*, RRF-3, results in a substantial enhancement of sensitivity to RNAi in diverse tissues. This is particularly striking in the nervous system; neurons that are generally refractory to RNAi in a wild-type genetic background can respond effectively to interference in an *rrf-3* mutant background. These data provide the first indication of physiological negative modulation of the RNAi response and implicate an RdRP-related factor in this effect. The *rrf-3* strain can be useful to study genes that, in wild-type, do not show a phenotype after RNAi, and it is probably the strain of choice for genome-wide RNAi screens.

Results and Discussion

A loss-of-function mutation in *rrf-3* (*pk1426*) [4] does not result in any obvious morphological defects but does cause a high incidence of males (7–10 times higher than wild-type) and a temperature-sensitive decrease in brood size; *rrf-3* animals grown at 25°C produce few progeny (10 ± 2 compared to 95 ± 8 for wild-type at 25°C). An independently isolated transposon insertion allele (*pk2042*) [4] displays identical phenotypes. Recent analysis of the *rrf* genes of *C. elegans* (a family of putative RNA-directed RNA polymerases [RdRP]) hinted that mutations in the *rrf-3* gene cause increased sensitivity to RNAi [4]. To investigate this, we first assayed bacteria expressing 80 distinct dsRNAs chosen from a genome-wide library designed to induce RNAi when fed to *C. elegans* ([2] and R.S. Kamath et al., submitted). We primarily selected dsRNA segments that do not produce a phenotype when fed to wild-type *C. elegans. rrf-3* (*pk1426*) and wild-type animals were fed as described by Kamath et al. [5]. We scored the percentage of embryonic lethality and assayed sterility, developmental delay, and postembryonic phenotypes. Of the 80 dsRNAs tested, we found 26 that induced phenotypes in a wild-type genetic background. In an *rrf-3* genetic background, we found phenotypes for an additional 23 dsRNAs. Several dsRNAs induced more than one phenotype in the set tested; in total, we detected 45 phenotypes in a wild-type background and 75 in an *rrf-3* background. A large fraction of the dsRNA segments were chosen to correspond to genes with known mutant phenotypes so that we could compare the RNAi phenotypes to known mutant phenotypes. For *rrf-3*, we detected 15 known phenotypes that we could not detect for wild-type animals (Figure 1A). *unc-73* and *lin-1* are two genes that nicely illustrate the increased sensitivity of *rrf-3* to RNAi (Figure 1B). The other independently derived allele of *rrf-3* (*pk2042*) confirmed the enhanced sensitivity to RNAi and showed that the mutations in *rrf-3* cause the increased sensitivity to RNAi.

Previous results have shown that both endogenous genes and transgenic reporter genes can show partial resistance to RNAi in the nervous system [5, 6]. For a GFP reporter (Figure 1C), we see that a wild-type strain is almost fully resistant to RNAi in the neurons in the head region, while the *rrf-3* strain shows clear loss of GFP expression, indicating an enhancement in neuronal sensitivity of *rrf-3* mutant animals to feeding-induced RNAi. Several endogenous genes that are specifically expressed in neurons show a similar effect. *rrf-3* animals that were fed dsRNA for *unc-30*, *unc-33*, or *unc-86* showed a clear uncoordinated movement phenotype, while no phenotype was detected in wild-type animals. We tested five additional neuronally expressed genes by injection of dsRNA into gonads. As shown in Figure 1D, we see a clear enhancement for four genes. Together, these data show that *rrf-3* animals are more sensitive to RNAi for a broad set of *C. elegans* genes.

The applicability of *rrf-3* mutants for functional analysis in the nervous system requires that the nervous system itself is "normal" in such mutants. No behavioral defects were evident in *rrf-3* animals; the wiring of the nervous system also appeared normal, as visualized by staining with antibodies to synaptic components SNT-1, UNC-10, and UNC-64.

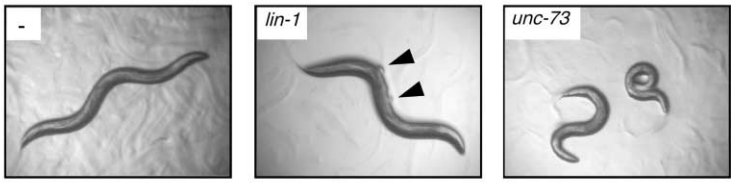
In addition to the effects on RNAi, we have also observed that *rrf-3* animals are more sensitive to transgene silencing. Although transgene silencing in *C. elegans* has been found to occur most dramatically in germline

[6]Correspondence: plasterk@niob.knaw.nl

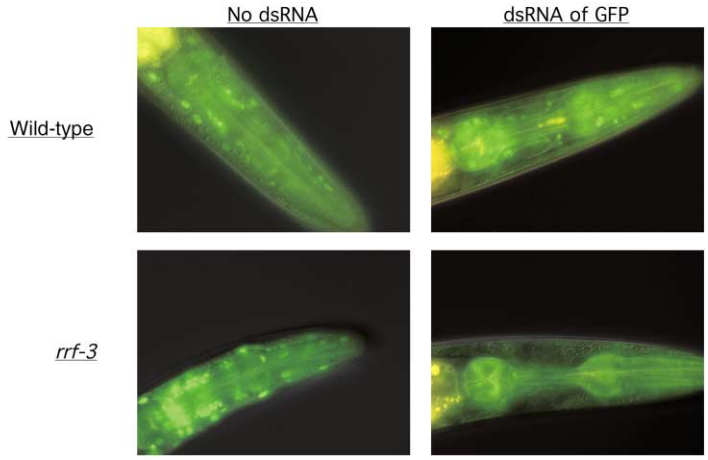
A

Phenotype	Published mutant phenotypes	Published phenotype detected	
		N2	*rrf-3*
All	52	11	26
Emb	10	6	7
Ste	1	0	0
Lvl	7	1	1
Post Emb	37	4	18

B



C



D

Gene	N2	*rrf-3*	RNAi Phenotype
unc-119	0% (372)	82% (324)	very slow curlers
unc-18	0% (128)	68% (374)	slow, thin kinkers
unc-25	0% (180)	46% (168)	spastic response in reverse direction
unc-104	0% (327)	32% (186)	slow, thin kinkers
unc-13	0% (300)	0% (250)	no (wild-type)
gfp	0% (744)	0% (744)	no (wild-type)

Figure 1. RNAi in the *rrf-3* (*pk1426*) Mutant

(A) Detection of published mutant phenotypes by RNAi (Emb, embryonic lethality; Ste, sterility; Lvl, larval lethality; and Post Emb, postembryonic phenotype). We targeted the following genes: *apr-1*, *cel-1*, *clr-1*, *cye-1*, *daf-2*, *dpy-14*, *dpy-18*, *eft-3*, *egl-30*, *etr-1*, *gon-4*, *gpc-2*, *gsa-1*, *him-3*, *hlh-1*, *hmr-1*, *lag-2*, *let-502*, *lin-1*, *lin-31*, *lin-49*, *mom-5*, *par-1*, *pha-1*, *pop-1*, *pos-1*, *ptr-2*, *rec-8*, *ric-19*, *spo-11*, *unc-3*, *unc-4*, *unc-5*, *unc-6*, *unc-11*, *unc-13*, *unc-14*, *unc-15*, *unc-17*, *unc-22*, *unc-29*, *unc-30*, *unc-33*, *unc-36*, *unc-37*, *unc-38*, *unc-40*, *unc-47*, *unc-73*, *unc-76*, *unc-86*, *unc-87*, *unc-89*, *unc-93*, *unc-101*, *unc-104*, *unc-130*, *zyg-1*, C01A2.3, C17E4.9, C32E8.1, C32E8.2, C32E8.3, C32E8.4, C32E8.5, C32E8.6, C32E8.9, C32E8.11, D1081.2, F09F7.4, F20H11.2, F54C4.3, F56F3.1, K04G7.12, PAR2.4, R11A5.1, T23D8.5, Y39A1A.B, Y52B11A.9, and ZK1098.5 (detailed data available upon request).

(B) *rrf-3* animals were fed on food without dsRNA (−) and on food with dsRNA of *lin-1* or *unc-73*. *rrf-3* animals that were fed on *lin-1* dsRNA have multiple protruding vulvae (arrowheads). Animals that were fed on dsRNA of *unc-73* are uncoordinated and dumpyish. These phenotypes are expected based on the described *lin-1* and *unc-73* mutants, but they are not detected for wild-type animals fed on the dsRNAs.

(C) Transgenic wild-type (N2) and *rrf-3* animals that broadly express GFP (*let-858::GFP*) were fed with dsRNA for GFP: (nuclear) expression (small dots) is silenced only in the mutant.

(D) RNAi of neuronally expressed genes by injection of dsRNA into the gonad.

tissue, there have also been examples of silencing in somatic tissue [7, 8]. Transgene arrays carrying the dominant marker gene *rol-6*(*su1006*) cause rolling movement in wild-type; in at least one case, such an array shows wild-type movement in an *rrf-3* mutant background (Figure 2). This failure to display the rolling phenotype depends on the action of the RNAi/mutator genes *mut-7* and *mut-16*, which are also required for cosuppression in the *C. elegans* germline. In contrast, genes that are required specifically for RNAi, i.e., *rde-1* and *rde-4*, are not needed, indicating that the genetic requirements for germline cosuppression in wild-type animals and somatic silencing of this transgene are similar [9, 10].

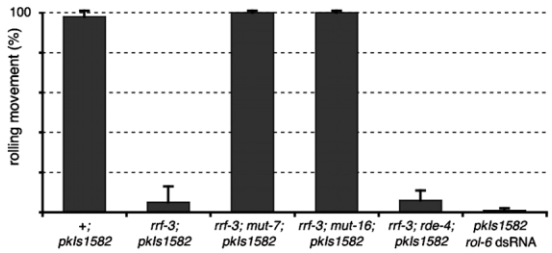


Figure 2. Hyperactive Somatic Transgene Silencing in *rrf-3* Animals

An integrated transgenic array that expresses the dominant *rol-6*(*su1006*) marker is assayed for its ability to induce a rolling phenotype in wild-type (+), *rrf-3*, *rrf-3; mut-7*, *rrf-3; mut-16*, and *rrf-3; rde-4* genetic backgrounds at 20°C. The activity of *rol-6*(*su1006*) can also be silenced by exposing these transgenic animals to *rol-6* dsRNA.

In summary, we here describe that two different loss-of-function alleles of *rrf-3* make *C. elegans* supersensitive to RNAi. This is seen both in the number of genes for which a phenotype is detected as well as the severity and penetrance of some phenotypes. A working hypothesis is that the RRF-3 protein might compete with RRF-1 and EGO-1 for components or intermediates in the RNAi reaction [4, 11]; this indicates that RNAi in wild-type *C. elegans* is under negative regulation.

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Chapter 4

Genome-Wide RNAi of *C. elegans* Using the Hypersensitive *rrf-3* Strain Reveals Novel Gene Functions

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Genome-Wide RNAi of *C. elegans* Using the Hypersensitive *rrf-3* Strain Reveals Novel Gene Functions

Femke Simmer[1☯], Celine Moorman[1☯], Alexander M. van der Linden[1☯], Ewart Kuijk[1], Peter V. E. van den Berghe[1], Ravi S. Kamath[2], Andrew G. Fraser[2], Julie Ahringer[2], Ronald H. A. Plasterk[1*]

1 Hubrecht Laboratory, Centre for Biomedical Genetics, Utrecht, The Netherlands, **2** University of Cambridge, Wellcome Trust/Cancer Research Institute and Department of Genetics, Cambridge, United Kingdom

RNA-mediated interference (RNAi) is a method to inhibit gene function by introduction of double-stranded RNA (dsRNA). Recently, an RNAi library was constructed that consists of bacterial clones expressing dsRNA, corresponding to nearly 90% of the 19,427 predicted genes of *C. elegans*. Feeding of this RNAi library to the standard wild-type laboratory strain Bristol N2 detected phenotypes for approximately 10% of the corresponding genes. To increase the number of genes for which a loss-of-function phenotype can be detected, we undertook a genome-wide RNAi screen using the *rrf-3* mutant strain, which we found to be hypersensitive to RNAi. Feeding of the RNAi library to *rrf-3* mutants resulted in additional loss-of-function phenotypes for 393 genes, increasing the number of genes with a phenotype by 23%. These additional phenotypes are distributed over different phenotypic classes. We also studied interexperimental variability in RNAi results and found persistent levels of false negatives. In addition, we used the RNAi phenotypes obtained with the genome-wide screens to systematically clone seven existing genetic mutants with visible phenotypes. The genome-wide RNAi screen using *rrf-3* significantly increased the functional data on the *C. elegans* genome. The resulting dataset will be valuable in conjunction with other functional genomics approaches, as well as in other model organisms.

Introduction

RNA interference (RNAi) is targeted gene silencing via double-stranded RNA (dsRNA); a gene is inactivated by specific breakdown of the mRNA (Fire et al. 1998; Montgomery et al. 1998). It is an ideal method for rapid identification of in vivo gene function. Initial studies on RNAi used microinjection to deliver dsRNA (Fire et al. 1998), but it was subsequently shown that dsRNA can be introduced very easily by feeding worms with bacteria that express dsRNA (Timmons and Fire 1998). Using this technique on a global scale, an RNAi feeding library consisting of 16,757 bacterial clones that correspond to 87% of the predicted genes in *Caenorhabditis elegans* was constructed (Fraser et al. 2000; Kamath et al. 2003). Upon feeding to worms, these clones will give transient loss-of-function phenotypes for many genes by inactivating the target genes via RNAi. By feeding the clones in this library to wild-type Bristol N2 worms, loss-of-function phenotypes were assigned to about 10% of genes. However, RNAi phenotypes were missed for about 30% of essential genes and 60% of genes required for postembryonic development, probably because RNAi is not completely effective (Kamath et al. 2003). Other global RNAi screens have been recently performed in *C. elegans* using this RNAi library or other techniques (Gönczy et al. 2000; Maeda et al. 2001; Dillin et al. 2002; Piano et al. 2002; Ashrafi et al. 2003; Lee et al. 2003; Pothof et al. 2003). These screens were done using wild-type worms.

We have already shown that mutation of *rrf-3*, a putative RNA-directed RNA polymerase (RdRP), resulted in increased sensitivity to RNAi (Sijen et al. 2001; Simmer et al. 2002). There are four RdRP-like genes in *C. elegans*. Two of these, *ego-1* and *rrf-1*, are required for efficient RNAi, as apparent from the fact that these mutants are resistant to RNAi against germline or somatically expressed genes, respectively (Smardon et al. 2000; Sijen et al. 2001). A third gene, *rrf-2*, appears to have no role in RNAi. The *rrf-3* strain, mutated in the fourth RdRP homolog, shows an opposite response to dsRNA; this mutant has increased sensitivity to RNAi (Sijen et al. 2001).

A more detailed study of RNAi sensitivity of *rrf-3* mutants using a set of 80 genes showed that *rrf-3* is generally more sensitive to RNAi than wild-type worms (Simmer et al. 2002). RNAi phenotypes in *rrf-3* animals are often stronger, and they more closely approximate a null phenotype, when compared to wild-type. In addition, loss-of-function RNAi phenotypes were detected for a number of genes using *rrf-3* that were missed in a wild-type background. For example, known

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Abbreviations: Adl, adult lethal; Bli, blistering of cuticle; Bmd, body morphological defects; Brd, low broodsize; Clr, clear; Dpy, dumpy; dsRNA, double-stranded RNA; Egl, egg-laying defective; Emb, embryonic lethal; Gro, growth defect/slow postembryonic growth; IPTG, isopropylthio-β-D-galactoside; Lon, long; Lva, larval arrest; Lvl, larval lethality; Mlt, molt defects; Muv, multivulva; Prz, paralyzed; Pvl, protruding vulva; RdRP, RNA-directed RNA polymerase; RNAi, RNA interference; Rol, roller; Rup, ruptured; Sck, sick; Ste, sterile; Stp, sterile progeny; Unc, uncoordinated

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*To whom correspondence should be addressed. E-mail: plasterk@niob.knaw.nl

☯These authors contributed equally to this work.

phenotypes were detected for many more neuronally expressed genes in the *rrf-3* background. These features suggest that the *rrf-3* strain could be used to improve and extend functional information associated with *C. elegans* genes.

We have conducted a genome-wide RNAi screen using the *rrf-3* strain. In total, we found reproducible RNAi phenotypes for 423 clones that previously did not induce a phenotype (corresponding to 393 additional genes). To explore the variability of global RNAi screens, we performed the *rrf-3* screen twice for Chromosome I and carried out a Chromosome I screen with wild-type. These were cross-compared and also compared to the results of the wild-type screen of Fraser et al. (2000). From this, we find that *rrf-3* consistently allowed detection of more phenotypes than wild-type. In addition, we found that there is a significant screen-to-screen variability (10%–30%).

Results

Comparative Analysis of RNAi for Chromosome I with Wild-Type and *rrf-3*

We first conducted a pilot screen of Chromosome I using *rrf-3* and found RNAi phenotypes for 456 bacterial clones. We compared these data to those obtained by Fraser et al. (2000) for a screen in the wild-type Bristol N2 strain. For 153 of these 456 clones, no phenotypes were reported by Fraser et al. (2000) and phenotypes were observed for 303 clones in both screens. The N2 screen done by Fraser et al. (2000) resulted in RNAi phenotypes for 40 clones for which no phenotypes were found using *rrf-3* (Figure 1A). These results indicate that *rrf-3* can be used in a global screen to identify loss-of-function phenotypes for additional genes. However, some phenotypes were missed in the *rrf-3* screen. To explore the reproducibility and variability of RNAi screens, we next screened the clones of Chromosome I using N2 and *rrf-3* side by side. We detected phenotypes for 447 clones: 140 were found only in *rrf-3*, 11 only in N2, and 296 in both strains (Figure 1B). These data confirm that *rrf-3* is more sensitive to RNAi and, in addition, these data indicate that global RNAi screens with *rrf-3* will result in more clones with a detectable phenotype.

Variability of the RNAi Effect

When we compared the RNAi results that we obtained using N2 with the Fraser et al. (2000) data, we were surprised to find significant differences: we only detected phenotypes for 75% of the clones that gave a phenotype in Fraser et al. (2000), and these researchers reported phenotypes for 84% of clones for which we found a phenotype (Figure 1C). The differences do not appear to be due to false positives. For example, Fraser et al. (2000) detected the predicted phenotype for *goa-1* and *unc-73*, whereas we did not detect a mutant phenotype. Similarly, we detected the known mutant phenotype for *egl-30* and *cdc-25.1*, which were not detected by Fraser et al. (2000). In addition, we found that the false-positive rate is negligible (see below).

It is possible that different laboratories or investigators have slightly different results. However, when we compare the results that we obtained with two independent screens of Chromosome I using *rrf-3* in our laboratory, we also see differences. For 394 clones we detected a phenotype in both experiments, 54 are specific for the first experiment, and 34 for the second (Figure 1D). Among the clones that only gave

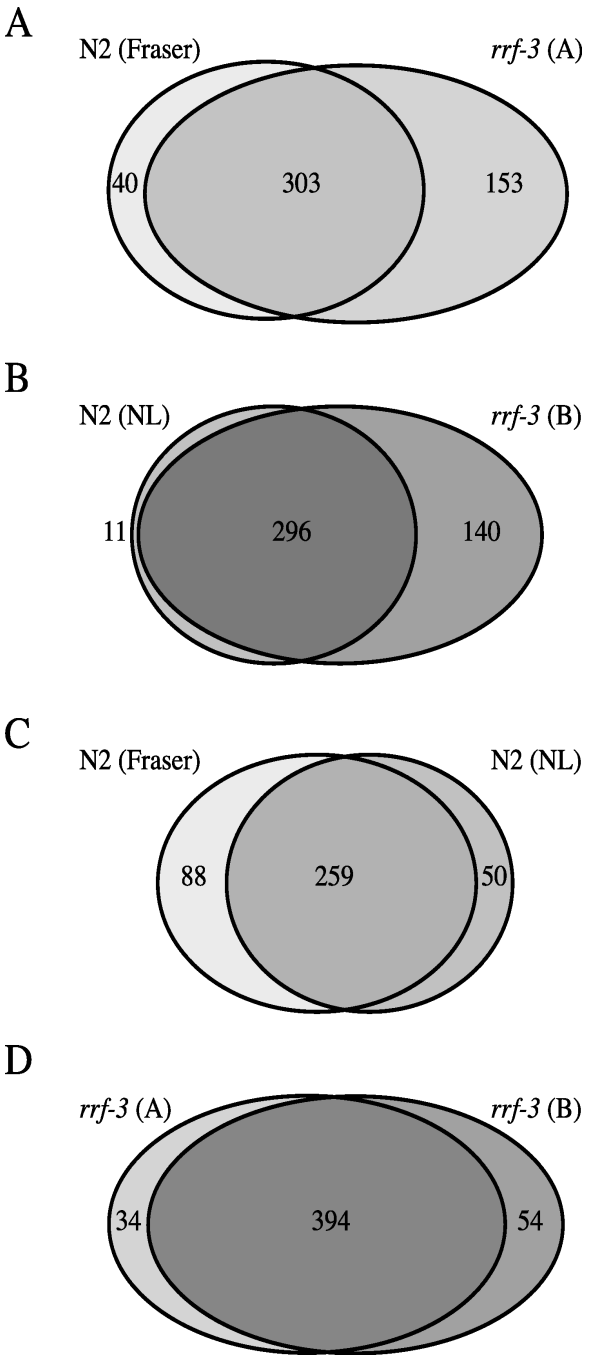

Figure 1. Comparison of Different RNAi Experiments of Chromosome I Using Wild-Type Bristol N2 and *rrf-3*

Differences between different laboratories or investigators and between experiments done within the same laboratory and by the same investigators are observed. Ovals represent the amount of bacterial clones that gave an RNAi phenotype in an experiment. Areas that overlap represent clones for which in both experiments an RNAi phenotype was detected. Differences and overlap between an RNAi experiment done with the *rrf-3* mutant strain and the data obtained by Fraser et al. (2000) done with the standard laboratory strain, Bristol N2 (A); N2 and *rrf-3* tested at the same time within our laboratory (B); experiments done with N2 in two different laboratories: this study ('NL') and Fraser et al. (2000) (C); two experiments done with the same strain, *rrf-3*, within our laboratory (D).

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Table 1. Variable RNAi Effects

GenePairs Name (Predicted Gene)	Locus	Known Mutant Phenotype	Experiment	RNAi Phenotype
F53G12.5	*mex-3*	Emb, Lvl	N2 (Fraser)	100% Emb
			N2 (NL)	100% Emb
			rrf-3 (A)	100% Emb
			rrf-3 (B)	100% Emb
M01D7.7	*egl-30*	Egl, Unc	N2 (Fraser)	o
			N2 (NL)	Egl
			rrf-3 (A)	Egl, Prz
			rrf-3 (B)	Egl, Prz
F55C7.4 (*F55C7.7*)	*unc-73*	Emb, Unc/Prz	N2 (Fraser)	20%–40% Emb, Bmd
			N2 (NL)	o
			rrf-3 (A)	Prz, Egl
			rrf-3 (B)	Slu
F54C1.3	*mes-3*	Stp	N2 (Fraser)	Stp
			N2 (NL)	o
			rrf-3 (A)	o
			rrf-3 (B)	Stp
F08B6.4	*unc-87*	Unc/Prz	N2 (Fraser)	o
			N2 (NL)	o
			rrf-3 (A)	Unc
			rrf-3 (B)	Unc
M05B5.5	*hlh-2*	Emb	N2 (Fraser)	100% Emb, 6–10 Brd, Unc, Pvl
			N2 (NL)	o
			rrf-3 (A)	o
			rrf-3 (B)	o
F08B6.2	*gpc-2*	Emb	N2 (Fraser)	o
			N2 (NL)	o
			rrf-3 (A)	o
			rrf-3 (B)	20%–40% Emb

Selection of clones that induced variable RNAi results in this study ('NL') and or in the study by Fraser et al. (2000). In this subset of bacterial clones, each corresponds to a gene for which a mutant phenotype is known. The expected phenotypes are detected with RNAi, but not in each experiment, indicating false-negative results. The bacterial clones are indicated by 'GenePairs Name' (name of genepair used to PCR-amplify a genomic fragment) and 'Predicted Gene' (predicted gene targeted by the named genepair). 'Locus' gives the genetic locus; 'Known Mutant Phenotype' gives the mutant phenotype for the indicated gene described in the literature. The RNAi phenotypes are defined in the Materials and Methods section.

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an RNAi phenotype in one of the experiments are again clones that induced the predicted phenotype based on the phenotypes of genetic mutants (*unc-40*, *gpc-2*, and *sur-2*). These data show that large-scale RNAi screens done within the same laboratory and by the same investigators also give variable results. A few examples of variable RNAi results are shown in Table 1.

In conclusion, we find that RNAi results from different laboratories and from experiments done in the same laboratory vary from 10% to 30%. This appears to be due to a high frequency of false negatives in each RNAi screen, even when the same method is used in the same laboratory.

The Genome-Wide RNAi Screen

Based on the positive results of the Chromosome I screen using the *rrf-3* strain, we next screened the complete RNAi library with *rrf-3* mutant animals. We obtained results for 16,401 clones and detected phenotypes for 2,079 (12.7%). Of these, we identified phenotypes for 625 clones for which no phenotype was reported in the Fraser et al. (2000) or Kamath et al. (2003) screens using N2, with the remaining 1,454 generating phenotypes in both screens (Table S1, found at http://dx.doi.org/.1371/journal.pbio.0000012.st001). In addition, there are 287 clones for which only Fraser et al. (2000) or Kamath et al. (2003) found phenotypes (23 of these were not done in our screen).

The clones for which we only detected an RNAi phenotype once and that were specific for the *rrf-3* screen were retested. Subsequently, the phenotypes of the clones corresponding to Chromosomes II to X that were not confirmed by this repetition were tested once more. In this way, the clones specific for the *rrf-3* screen had two chances to be confirmed. Of the 625 clones for which no phenotype was found in the Fraser et al. (2000) and Kamath et al. (2003) N2 screens, the phenotypes of 423 clones were confirmed and 202 remained unconfirmed (Table 2; see Table S1). Combining the N2 screens and these 423 clones, the percentage of clones with a phenotype increases from 10.3% to 12.8%.

Some of the RNAi phenotypes only found with *rrf-3* that remained unconfirmed could be confirmed by RNAi pheno-

Table 2. Genome-Wide RNAi

Chromosome	Positive Clones			
	Clones	*rrf-3*	Overlap	$N2^{(F/K)}$
I	2,402	135	314	37
II	2,866	54	261	85
III	2,115	54	356	40
IV	2,595	66	247	39
V	4,092	62	187	25
X	2,331	52	89	38
Total	16,401	423	1,454	264

Summary of the bacterial clones that induced detectable RNAi phenotypes ('Positive Clones'). For 423 clones, RNAi phenotypes were reproducibly detected in our laboratory using *rrf-3*, but no RNAi phenotypes were reported in the N2 screens; 1,454 clones induced phenotypes in both laboratories; 264 were specifically detected by Fraser et al. (2000) or Kamath et al. (2003). For 202 clones, RNAi phenotypes were detected with *rrf-3* and no RNAi phenotypes were reported in the N2 screens, but this result could not be repeated. In addition, there are 23 clones for which we did not obtain results that gave a phenotype with N2. In the column with the overlapping clones, the *rrf-3* data are mainly from one experiment, whereas the N2 data reported by Fraser et al. (2000) and Kamath et al. (2003) are from repeated experiments. The phenotypes that were scored are described in the Materials and Methods section.

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types detected with other clones of the RNAi library corresponding to the same gene or by other laboratories using different RNAi methods. For example, for the clones corresponding to the predicted genes *F56D1.1* (a member of the zinc finger C2H2-type protein family) and *F27C8.6* (a member of the esterase-like protein family), we detected sterile progeny (Stp) and embryonic lethality (Emb), respectively; these were also found by Piano et al. (2002). In addition, some unconfirmed RNAi phenotypes are confirmed by comparing to phenotypes of genetic mutants such as *gpc-2*, *hlh-8*, and *unc-84*. This suggests that many of the unconfirmed phenotypes reflect true gene functions.

Analysis of the *rrf-3* Results

To validate the results obtained using *rrf-3*, we first assayed the rate of false positives in the total dataset (all RNAi results obtained with *rrf-3* for the 16,401 clones tested). In the assay used by Kamath et al. (2003), a set of genes for which it is known that genetic mutants display no lethality was selected. A false positive in the RNAi data is then defined as detecting a lethal RNAi phenotype for any of these genes. In the N2 screen, the false-positive rate was 0.4%. We find that the false-positive rate in the *rrf-3* data is similarly low (0 of 152 genes).

To further determine the effectiveness of the screen, we compared the RNAi phenotypes with loss-of-function phenotypes of genetic mutants. For all chromosomes except for Chromosome I, the *rrf-3* data were confirmed by refeeding only if there was no phenotype detected in the N2 screens by Fraser et al. (2000) or Kamath et al. (2003). Therefore, to compare the difference in detection of known phenotypes between the *rrf-3* and the N2 screens, we used the Chromosome I datasets, where phenotypes were confirmed independently for the two strains. Of 75 genetic loci on Chromosome I, Fraser et al. (2000) detected 48% of published phenotypes, compared to 59% for *rrf-3* (Table S2, found at http://dx.doi.org/.1371/journal.pbio.0000012.st002). Using the genome-wide *rrf-3* dataset (excluding the 202 unconfirmed phenotypes), we detected the published phenotype for 54% of 397 selected loci, compared to 52% for N2 (Table 3; see Table S2).

We next asked whether using the *rrf-3* strain improved general phenotype detection or whether certain types of phenotypes were particularly increased compared to the N2 screens by Fraser et al. (2000) and Kamath et al. (2003). To do this, we analysed the detection rate of different types of Chromosome I loci. First, we looked at a set of 23 loci with nonlethal postembryonic mutant phenotypes. Using *rrf-3*, we reproducibly detected the published phenotype for 11 of these compared to only two for N2. Of 50 loci required for viability (essential genes), we detected 31 using *rrf-3*, compared to 33 for N2. Thus, detection of essential genes was similar in the two strains, but detection of postembryonic phenotypes was improved with *rrf-3*. Finally, for the whole genome using *rrf-3*, we reproducibly detected the published phenotypes for 34 genetic mutants for which no RNAi phenotype was reported in the N2 screens (nine essential genes, 21 with postembryonic mutant phenotypes, and four with a slow-growth mutant phenotype). By comparison, published phenotypes were detected for 23 loci only with N2 (16 essential genes and seven with postembryonic mutant phenotypes) (see Table S2). We conclude that *rrf-3* particularly improves detection of genes with postembryonic mutant phenotypes, a class that is poorly detected using wild-type N2.

A striking feature of the *rrf-3* dataset is the high number of clones where a slow or arrested growth (Gro/Lva) defect was induced, without associated embryonic lethality or sterility. Overall, 619 clones induced a Gro/Lva defect using *rrf-3*, compared to 276 for N2, whereas the number of essential genes detected was similar (1,040 versus 1,170, respectively). In addition, in the confirmed set of 423 clones with *rrf-3*-specific phenotypes, Gro/Lva defects are the largest category (42%), whereas this is only 18% for N2, with the largest category being essential genes (49%). These data suggest that *rrf-3* might particularly enhance detection of genes that mutate to a slow-growth phenotype; we cannot easily test this hypothesis, as there are currently few known loci with this mutant phenotype. In some cases, a Gro/Lva phenotype was seen in *rrf-3*, whereas a different phenotype was seen in N2 (e.g., either lethality or a weak postembryonic phenotype). This suggests that some of the Gro/Lva phenotypes detected are due to incomplete RNAi of an essential gene (where lethality was seen in N2) or by a stronger RNAi effect (where no growth defect was seen in N2). In addition, it is possible that some of the Gro/Lva phenotypes detected are synthetic effects of using the *rrf-3* mutant strain.

To summarise, using the *rrf-3* RNAi supersensitive strain in large-scale screens increases the percentage of clones for which it is possible to detect a phenotype. Detection of postembryonic phenotypes is particularly increased, whereas detection of essential genes is similar in *rrf-3* and N2. In addition, using *rrf-3*, there is a high rate of induction of Gro/Lva defects.

Positional Cloning of Genetic Mutants with Visible Phenotypes

Despite the advantages of RNAi, genetic mutants remain indispensable for many experiments. In the past decades, forward genetic screens identified a large number of genetic

Table 3. Effectiveness of the *rrf-3* Screen

Chromosome	Total Genetic Loci Scored		RNAi Phenotype Detected		Published Phenotype Detected	
	rrf-3	$N2^{(F/K)}$	*rrf-3*	$N2^{(F/K)}$	*rrf-3*	$N2^{(F/K)}$
I	75	76	54	44	45	35
II	62	62	40	40	37	39
III	86	87	61	64	58	60
IV	61	66	29	34	24	31
V	49	50	28	23	24	19
X	64	64	32	27	25	25
Total	397	405	244	232	213	209
Percentage	100%	100%	61%	57%	54%	52%

RNAi phenotypes obtained with *rrf-3* (confirmed using N2 data or *rrf-3* refeeding), and the N2 screens by Fraser et al. (2000) or Kamath et al. (2003) were compared with those of genes that have known loss-of-function phenotypes. 'Total Genetic Loci Scored' denotes the number of genes that were analysed by RNAi. All loci have a loss-of-function phenotype that was detectable in our screen. 'RNAi Phenotype Detected' gives the number of genes for which a phenotype was identified. 'Published Phenotype Detected' gives the number of genes for which the RNAi phenotype matched the phenotype described in the literature.

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mutants, many of which are not yet linked to the physical map. We used the RNAi phenotypes obtained with the genome-wide screens to test whether we could systematically clone genes that are mutated in existing genetic mutants. First, the genetic map positions of all uncloned genetic mutants with visible phenotypes were checked using WormBase (http://www.wormbase.org, the Internet site for the genetics, genomics, and biology of *C. elegans*). Second, we searched for clones near the defined map positions that, when fed to N2, *rrf-3*, or both, gave phenotypes corresponding to the phenotypes of the genetic mutants. For most genetic mutants, more than ten clones with a similar phenotype were found in the interval to which the genetic mutant was mapped. However, for 21 genetic mutants, only one or a few candidate clones were found. The genes corresponding to these clones were subsequently sequenced in the genetic mutant to determine whether a mutation was present. In total, we sequenced 42 predicted genes for the 21 genetic mutants (Table S3, found at http://dx.doi.org/.1371/journal.pbio.0000012.st003). For seven of these—*bli-3*, *bli-5*, *dpy-4*, *dpy-6*, *dpy-9*, *rol-3*, and *unc-108*—we found a mutation in one of the sequenced genes (Table 4). The mutated gene was confirmed by sequencing the same gene in a second or third allele (or both) of these genetic mutants (Table 4).

The identification of mutations in *unc-108* encoding the homolog of the small GTPase Rab2 is of particular interest. The RNAi phenotype of this gene gives a clue about the genetic property of the mutations in the mutants of *unc-108*. With *rrf-3*, we find that inactivation of Rab2 (F53F10.4) by RNAi causes uncoordinated movement (Table 4). Mutations in *unc-108* were isolated in a screen for dominant effects on behaviour; heterozygous *unc-108* mutants display dominant movement defects and are indistinguishable from homozygous mutants (Park and Horvitz 1986). RNAi phenocopies a loss-of-function phenotype, suggesting that the dominant movement defects of *unc-108* mutants may be due to haploinsufficiency. In eukaryotes, Rab2 is involved in regulating vesicular trafficking between the endoplasmic reticulum and Golgi. Based on the movement defects of *unc-108* mutants, UNC-108 might be involved in vesicle transport in neurons that regulate locomotion. Thus, the RNAi data are a powerful tool to facilitate rapid cloning of the genes identified by genetic mutants and will provide important starting points for further studies of their function.

Discussion

With this genome-wide RNAi screen using the hypersensitive strain *rrf-3*, we have significantly increased the functional information on the *C. elegans* genome, and we confirmed many RNAi phenotypes observed previously. We have assigned RNAi phenotypes for 406 genes (corresponding to the 423 extra clones) using *rrf-3*. For 13 genes, Kamath et al. (2003) or Fraser et al. (2000) had already found a phenotype using a different clone from the RNAi library that targeted the same gene, and for at least 44 genes a genetic mutant exists (see Table S2). Other investigators have also found RNAi phenotypes for some of the genes using different methods. However, for most genes our result is to our knowledge the first hint about their biological function.

Although we have identified new RNAi phenotypes for a substantial number of genes, others will have been missed in our screen for the following reasons. First, besides its increased sensitivity to RNAi, the *rrf-3* strain has an increased incidence of males (Him) and displays slightly increased embryonic lethality and a reduced brood size (Simmer et al. 2002). In our *rrf-3* experiments, we therefore made some minor adaptations to the original RNAi protocol described by Fraser et al. (2000). We did not score for the Him phenotype and had more stringent criteria for embryonic lethality and sterility. This may have reduced the number of extra clones identified with a phenotype. Moreover, the changes in the protocol can also account for some differences in the detection of RNAi phenotypes between *rrf-3* and N2. Second, when an RNAi phenotype is detected with N2 and not with *rrf-3*, the lack of a detectable phenotype may be the result of variability in the efficiency of RNAi. This is consistent with the fact that we observe differences between experiments done with the same strain.

When an RNAi phenotype is detected with *rrf-3* and not

Table 4. Properties of the Genetic Mutants Cloned Using the RNAi Phenotypic Data

Gene Name	Allele	Genetic Map Position	Chromosome	Mutated Gene	Description	Mutation	Change	RNAi Phenotype Using *rrf-3*	RNAi Phenotype Using N2
bli-3	*e767* *n259*	−18.97	I	*F56C11.1*	Protein with similarity to NADPH-oxidases, homolog of human Duox1	GGT→GAT GAT→AAT	G246D D392N	Bli, Lva, Lvl, Mlt	Bli, Lvl, Mlt
unc-108	*n501* *n777*	−2.0	I	*F53F10.4*	GTP-binding protein of the Rab family, homolog of human Rab2	GAC→AAC TCT→TTT	D122N S149F	Unc	Wild-type
bli-5	*e518* *s277*	21.52	III	*F45G2.5*	EB module Kunitz bovine pancreatic trypsin inhibitor domain family member	TCA→TTA GTG→ATG	S56L Splice donor site, intron 2	Bli, Unc, Lvl, Adl	Bli
dpy-9	*e12* *e1164*	−27.27	IV	*T21D12.2*	Cuticular collagen family member, has similarity to human COL9A1, α1 collagen, type IX	GGA→GAA CAA→TAA	G149E Q253stop	Dpy	Dpy, Unc
dpy-4	*e1158* *e1166*	12.61	IV	*Y41E3.2*	Member of the collagen triple-helix repeat family, has strong similarity to *C. elegans* DPY-13	CCCC→CCCCC CCCC→CCCCC	Frameshift at position 569 Frameshift at position 392	Dpy, Unc, Lvl	Dpy
rol-3	*e754* *s1040*	1.27	V	*C16D9.2*	Putative tyrosine-protein kinase, has similarity to *Drosophila* Evenless	GAG→AAG GAA→AAA	E1782K E1127K	Rol, Unc	Wild-type
dpy-6	*e14* *e2762* *f11*	−0.17	X	*F16F9.2*	Contains actin-interacting protein domain, has similarity to human Mucin-2 precursor	TGG→TGA 60 bp deletion[a] Multiple[b]	W5stop 6 aa deleted of exon 8 Frameshift at position 2792	Dpy	Dpy

Genetic mutants were linked to the physical map using RNAi phenotypes. The 'Genetic Map Position' is based on WormBase annotation. 'Mutated Gene' denotes the predicted gene, which is mutated in the genetic mutant. 'RNAi Phenotype' gives the loss-of-function phenotype either using *rrf-3* or N2 (the latter is based on findings of Kamath et al. [2003]). The phenotypes that were scored are described in the Materials and Methods section.

[a] *dpy-6(e2762)* has a deletion that removes the first six amino acid residues (aa) of the eighth exon and part of the seventh intron.

[b] Multiple mutations in *dpy-6(f11)* (5′-tcgAaaa[G/T]tt[C/A]aaccccacgccaact[G/T]cc); the AAA→AAAA mutation at position 2792 bp of the *F16F9.2* coding sequence causes a frameshift that results in a premature stop in the fifth exon.

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with N2, this can be due to the increased sensitivity to RNAi of *rrf-3*. However, besides the higher sensitivity, we may also be observing synthetic effects with *rrf-3* (e.g., embryonic lethality, sterility, or developmental delay). In particular, a large number of clones induced a developmental delay phenotype using *rrf-3*. Synthetic effects cannot be excluded without investigating genetic mutants. Again, variability in the efficiency of RNAi will also contribute to these differences, and a small portion may be false positives. In general, the few false positives that occur in the screen are most likely due to experimental errors, whereas the false negatives are due to reduced efficiency of the RNAi. Finally, differences between *rrf-3* and N2 do not only involve the absence and presence of an RNAi phenotype, but also differences in the phenotypes for clones that did induce phenotypes in both screens (e.g., embryonic lethal in one screen and a postembryonic phenotype in the other). For example, we detected for *unc-112* a 100% embryonic lethal (Emb) phenotype with *rrf-3*, whereas Kamath et al. (2003) detected an adult lethal (Adl), uncoordinated (Unc), and paralyzed (Prz) phenotype

with N2. Conversely, Kamath et al. (2003) detected for *gon-1* a 100% Emb phenotype and other phenotypes with N2, while we did not detect an Emb phenotype with *rrf-3*.

What could be the source of the interexperimental variation of RNAi? Different phenotypes for the same gene can possibly occur owing to slight differences in the developmental stage at which the animals are exposed to dsRNA and owing to changes in temperature during the experiment. However, this probably does not account for the differences we see, as we always used animals of the same larval stage (L3/L4) and used incubators for constant temperature. It was shown previously that the level of induction of dsRNA production by isopropylthio-β-D-galactoside (IPTG) can modify the penetrance of the RNAi phenotype (Kamath et al. 2000). Therefore, differences in the induction of the dsRNA either by changes in the concentration of IPTG, temperature, timing, or the bacteria may be an important source of the variation in the outcome of RNAi. RNAi is starting to be used extensively in other systems experimentally, as well as therapeutically and agriculturally. The relative variability of the RNAi effect is an important fact to take in account also for the use of RNAi in other systems.

The RNAi data can be a useful starting point for many new experiments, such as positional cloning of genetic mutants. By sequencing candidate genes based on the RNAi phenotypes, we identified the causal mutation in seven genetic mutants. Identification of these mutated genes gives insight into the biological process in which they are involved. In addition, cloning of these genes increases the resolution of the genetic map of *C. elegans*, since these mutants have been extensively used as visible markers in linkage studies.

The complete set of RNAi phenotypes detected for the 2,079 clones using *rrf-3* will be submitted to WormBase, annotated as confirmed or unconfirmed. There the data can be evaluated in the context of information on gene structure, expression profiles, and other RNAi results.

Materials and Methods

Nematode strains. We used the following *C. elegans* strains: Bristol N2, NL4256 *rrf-3(pk1426)*, CB767 *bli-3(e767)*, MT1141 *bli-3(n259)*, CB518 *bli-5(e518)*, BC649 *bli-5(s277)*, CB1158 *dpy-4(e1158)*, CB1166 *dpy-4(e1166)*, CB14 *dpy-6(e14)*, CB4452, *dpy-6(e2762)*, F11 *dpy-6(f11)*, CB12 *dpy-9(e12)*, CB1164 *dpy-9(e1164)*, BC119 *dpy-24(s71)*, CB3497 *dpy-25(e817)*, MT1222 *egl-6(n592)*, MT1179 *egl-14(n549)*, MT1067 *egl-31(n472)*, MT151 *egl-33(n151)*, MT171 *egl-34(n171)*, *egl-34(e1452)*, MQ210 *mau-4(qm45)*, CB754 *rol-3(e754)*, BC3134 *srl-2(s2507dpy-18(e364)*; *unc-46(e177)rol-3(s1040)*, CB713 *unc-67(e713)*, CB950 *unc-75 (e950)*, HE177 *unc-94(su177)*, HE33 *unc-95(su33)*, HE151 *unc-96(su151)*, *unc-96(r291)*, HE115 *unc-100(su115)*, MT1093 *unc-108(n501)*, and MT1656 *unc-108(n777)*.

RNAi by feeding. RNAi was performed as described elsewhere (Fraser et al. 2000; Kamath et al. 2000) with minor adaptations when the *rrf-3* strain was used: after transferring L3- to L4-staged hermaphrodites onto the first plate, we left them for 48 h at 15°C instead of 72 h and then plated single adults onto other plates seeded with the same bacteria. Furthermore, we did not remove the mothers from the second plates. The phenotypes assayed are these: Emb (embryonic lethal), Ste (sterile), Stp (sterile progeny), Brd (low broodsize), Gro (slow postembryonic growth), Lva (larval arrest), Lvl (larval lethality), Adl (adult lethal), Bli (blistering of cuticle), Bmd (body morphological defects), Clr (clear), Dpy (dumpy), Egl (egg-laying defective), Lon (long), Mlt (molt defects), Muv (multivulva), Prz (paralyzed), Pvl (protruding vulva), Rol (roller), Rup (ruptured), Sck (sick), Unc (uncoordinated) Thin and Pale. Emb was defined as greater than 10% dead embryos for N2 and greater than 30% dead embryos for *rrf-3*. Ste required a brood size of fewer than ten among fed N2 worms and fewer than five among *rrf-3*. Each postembryonic phenotype was required to be present among at least 10% of the analysed worms.

Sequencing of genetic mutants. The coding sequence and the 5′- and 3′-untranslated region (about 500 bp upstream and downstream of the coding sequence) of the predicted genes, as annotated in WormBase, was analysed for mutations by sequencing amplified genomic DNA of the genetic mutants (see Table S3). Nested primers were designed using a modification of the Primer3 program available on our website (http://primers.niob.knaw.nl/). Sequence reactions were done using the ABI PRISM Big Dye terminator sequencing kit (Applied Biosystems, Foster City, California, United States) and were analysed on the ABI 3700 DNA analyser.

Sequences were compared to the genomic sequence of *C. elegans* using the BLAST program (http://www.sanger.ac.uk/Projects/C_elegans/blast_server.shtml) or analysed using the PolyPhred program (available from http://droog.mbt.washington.edu/PolyPhred.html).

Supporting Information

Table S1. RNAi Phenotypes for Bacterial Clones Using *rrf-3*

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Table S2. Detailed Comparison of RNAi Phenotypes with Those of Known Loci

View online at DOI: 10.1371/journal.pbio.0000012.st002 (188 KB PDF).

Table S3. Summary of Genes Sequenced in Several Genetic Mutants

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Accession Numbers

RNAi data from this study will be submitted to WormBase (http://www.wormbase.org).

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Chapter 5

Genes Required for Systemic RNA Interference in *Caenorhabditis elegans*

Submitted for publication

Genes required for systemic RNA interference in *Caenorhabditis elegans*

Marcel Tijsterman*, Robin C. May*, Femke Simmer, Kristy L. Okihara & Ronald H.A. Plasterk[+].
Hubrecht Laboratory, Centre for Biomedical Genetics, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands.
+ Correspondence: plasterk@niob.knaw.nl
** These authors contributed equally to this work*

Summary

RNA interference (RNAi) in the nematode worm, *Caenorhabditis elegans*, occurs systemically. Double-stranded RNA (dsRNA) provided in the diet can be absorbed from the gut lumen and distributed throughout the body, triggering RNAi in tissues that are not exposed to the initial dsRNA trigger (Timmons and Fire, 1998). This is in marked contrast to other animals, in which RNAi does not spread from targeted tissues to neighbouring cells (Roignant et al., 2003). Here we report the characterisation of mutants defective in the systemic aspect of RNAi, but not in the core RNAi process itself. Analysis of these mutants suggests that dsRNA uptake is a specific process involving several unique proteins.

Results and Discussion

RNAi is a powerful tool to silence gene expression post-transcriptionally. In *C. elegans* RNAi acts systemically – the local application of dsRNA triggers silencing of homologous sequences throughout the organism and not merely in the region exposed to the initial trigger. In contrast, systemic spread of RNAi does not appear to occur in other animals such as *Drosophila* (Roignant et al., 2003) or mammals. Understanding how systemic RNAi occurs in *C. elegans* – and why this does not occur in humans - will be critical to the future clinical application of RNAi.

To investigate the molecular basis of systemic RNA we conducted a genetic screen to identify mutants defective in this process (Figure 1a). By selecting for animals that were resistant to RNAi induced by dietary dsRNA but sensitive to RNAi induced by injection of the same dsRNA into the gonad, we isolated mutants in which RNAi can no longer spread systemically, although the RNAi mechanism itself remains unimpaired. These mutants we termed *rsd*, for RNAi spreading defective.

From this screen we isolated thirty mutants that fall into at least five complementation groups. We could divide these mutants into two classes based on phenotype. *rsd-4* and *rsd-8* showed no RNAi phenotype when fed dsRNA for germline-expressed genes (such as *pos-1*) or somatic genes (such as *unc-22*), although injection of either dsRNA was able to generate the corresponding RNAi phenotype (Table 1). The second class, composed of *rsd-2, rsd-3* and *rsd-6*, also showed no phenotype when fed on dsRNA against a germline gene such as *pos-1*. However, RNAi directed against somatic genes was still able to produce somatic phenotypes in these mutants. This effect was reproducible for a number of different somatic and germline genes (Table 1). In addition, this effect was also seen when transgenic animals expressing GFP in all tissues were fed on RNAi food targeting GFP (Figure 1b, 1c). Thus *rsd-4* and *rsd-8* mutants appear to have a complete defect in the cellular uptake of dsRNA. In contrast, *rsd-2*, *rsd-3* and *rsd-6* mutants appear not defective in the initial uptake of dsRNA from the gut into somatic tissues but are unable to further distribute this dsRNA to the germline.

The absence of an established *in vitro* assay for RNAi activity in *C. elegans* means that we cannot formally exclude the possibility that the *rsd* genes affect the efficiency of the RNAi mechanism itself. However, in contrast to mechanistic RNAi mutants (such as *rde-1* (Tabara et al., 1999)) all the *rsd* mutants are capable of performing RNAi when dsRNA is injected into the germ line even at low concentration (0.5 ng μl^{-1}, data not shown), suggesting that they do not function in modulating the efficiency of the core RNAi machinery within the cell.

dsRNA uptake could occur either via a specific mechanism or by 'piggy-backing' on a more general mechanism for transporting molecules across cell membranes. There is no apparent nutritional defect in any of the *rsd* mutant animals, nor are lifespan, mating efficiency or brood size different to wild-type (data not shown), suggesting that the deficiency in dsRNA uptake does not reflect

a general defect in nutrient absorption. We also tested whether the *rsd* mutants were specifically defective in nucleic acid uptake. In the absence of endogenous nucleotide synthesis, *C. elegans* is able to meet its nucleotide requirement by absorbing nucleic acids from the gut. Inhibition of endogenous nucleic acid synthesis by the folic acid antagonist aminopterin does not affect the growth rate of wild-type worms, whereas mutants that are impaired in dietary nucleic acid uptake (e.g. *nuc-1* (Sulston, 1976)) show severe developmental retardation under the same conditions. Neither *rsd-2*, *-3, -4, -6* or *–8* animals showed a reduction in growth rate or increased lethality when exposed to 10 mM, 50 mM or 100 mM aminopterin in the growth medium (n ≥ 10 per mutant, per treatment) whereas *nuc-1* animals showed severe growth reduction even at the lowest concentration. Thus the dsRNA uptake pathway leading to systemic RNAi is distinct from the general nucleic acid scavenging pathway.

Table 1 The *rsd* mutants fall into two classes based on phenotype

	Gene tested by RNAi	Wildtype (N2)	Class I (*rsd-4, rsd-8*)	Class II (*rsd-2, rsd-3 , rsd-6*)
	unc-22	Twitch	-	Twitch
Somatic genes	*unc-15*	Unc	-	Unc
	D1081.2	Unc	-	Unc
	pos-1	Emb	-	-
	par-1	Emb	-	-
	F26H9.6	Emb	-	-
Germline genes	*par-6*	Emb	-	-
	ZK858.4	Emb	-	-
	F36F2.3	Emb	-	-
	rab-5	Emb	-	-
	ZK1014.1	Ste	-	-

The *rsd* genes can be divided into two classes based on phenotype. Class I mutants are not susceptible to RNAi via feeding of either germline or somatic genes, whereas Class II mutants are sensitive to RNAi of somatic genes, but not of germline genes. Phenotypes based on that used by Wormbase (www.wormbase.org): Twitch, uncontrollable body muscle twitching; Unc, uncoordinated motion; Emb, embryonic lethality; Ste, sterility.

We wondered whether general endocytic/exocytic pathways might be responsible for the uptake and distribution of dsRNA that is necessary for systemic RNAi. We tested several genetic mutants (*dyn-1* and *ehs-1*, components of the clathrin endocytosis pathway; *cav-1* and *cav-2,* components of the caveolin pathway*; rme-1 and rme-8*, proteins required for receptor mediated endocytosis – see (Fares and Grant, 2002; Salcini et al., 2001) and references therein) and numerous additional endocytosis genes by RNAi but none showed any defect in systemic RNAi (although previously reported endocytosis defects were observed as expected). Similarly, the *rsd* mutants do not show nutritional or neuronal phenotypes, as might be expected if vesicle trafficking was aberrant in these animals, nor are there defects in the uptake of fluorescent dextran from the medium (data not shown), in the behaviour of highly endocytic cells such as coelomocytes (data not shown) or in the endocytic uptake of yolk proteins into the oocyte (data not shown). Taken together, these results indicate that the uptake and cell-to-cell spread of dsRNA does not occur by “piggy-backing” on a general endocytosis mechanism.

Via positional cloning, candidate sequencing and cosmid rescue, we identified four of the five genes isolated from our genetic screen (*rsd-4* has not yet been cloned but maps to the far end of chromosome III). The first gene to be cloned in this way was *rsd-8*, identified as C04F5.1 on chromosome V (Figure 2a). Non-complementation testing and candidate sequencing identified four alleles of *rsd-8* (Figure 2b). RSD-8 is predicted to be a multi-pass transmembrane protein with a large extracellular portion, suggestive of a role as a dsRNA receptor or channel. During the course of this work *rsd-8* was independently cloned and reported by another group (Winston et al., 2002) as *sid-1* (systemic interference defective). We therefore focussed our subsequent work on the other *rsd* genes.

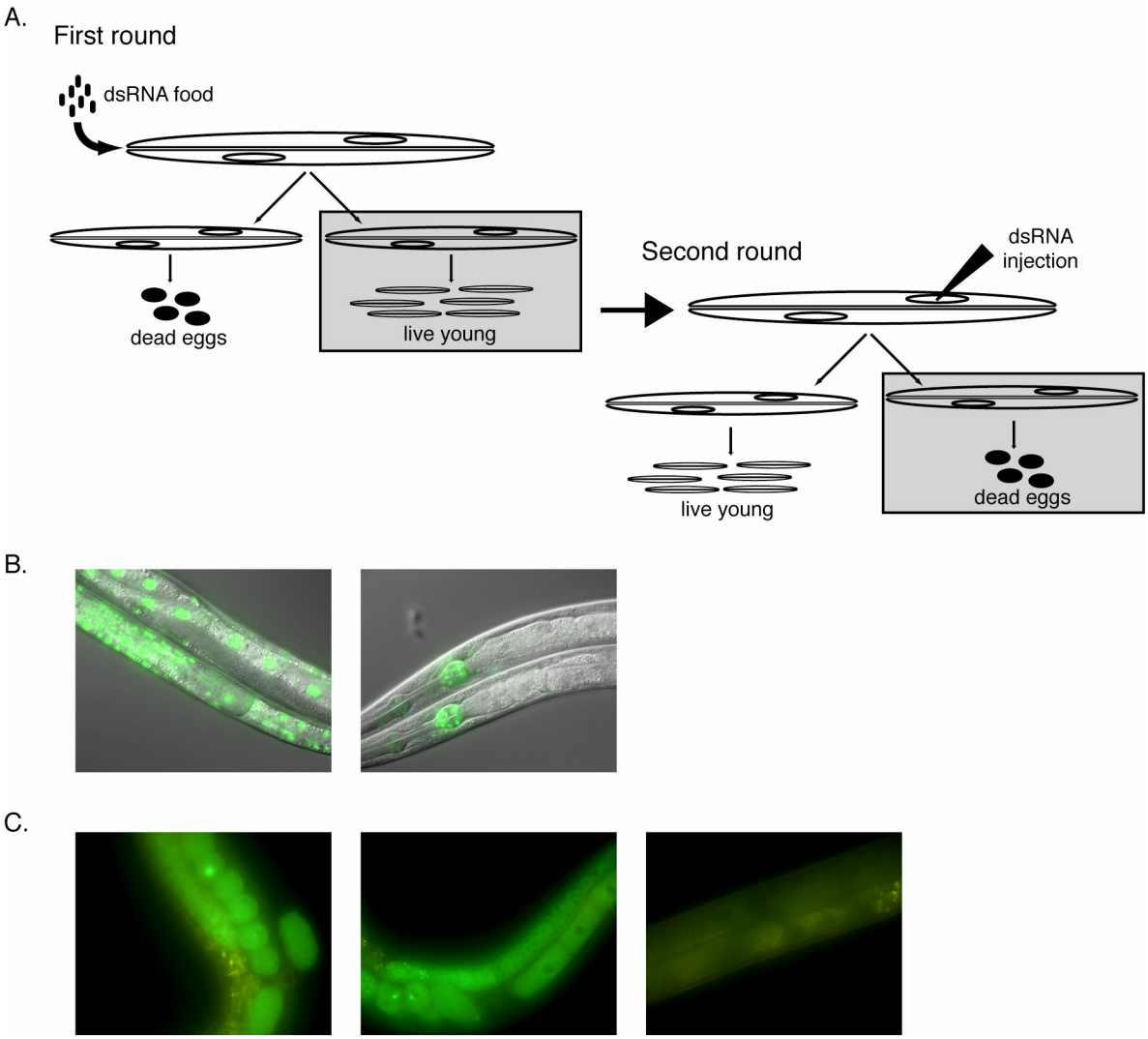


Figure 1. Screen for Mutants in Systemic RNAi

(A) A genetic screen for mutants defective in systemic RNAi. First round: Mutagenised L4 animals were fed with bacteria expressing dsRNA for a portion of the *pos-1* or *par-1* gene. Wild-type worms grown on these foods show complete embryonic lethality, laying only dead eggs. We isolated surviving F1 worms from this screen to select for mutants with a defect at some point in the RNAi pathway (grey box). Second round: To exclude mutants with a defect in the core RNAi processing mechanism, we injected the progeny of these mutants with *in vitro* synthesised dsRNA for the same portion of the *pos-1* or *par-1* gene and then selected for mutants that now produced dead eggs (grey box). Mutants that laid viable eggs when dsRNA was delivered in the diet but only dead eggs when injected with the same dsRNA are thus impaired in systemic RNAi, but not in the RNAi mechanism itself.

(B) One group of *rsd* mutants (*rsd-2*, *rsd-3* and *rsd-6*) remain sensitive to RNAi against somatic genes. Left: *rsd-3* animals show widespread somatic expression of GFP under control of an *rpl-5* promoter. Right: Feeding these animals on bacteria expressing dsRNA for GFP triggers silencing of somatic GFP expression except in some neuronal cells (reflecting the fact that neuronal cells are frequently recalcitrant to RNAi). This effect is also seen for *rsd-2* and *rsd-6* animals, whilst *rsd-4* and *rsd-8* show no silencing of somatic GFP expression under the same conditions (data not shown).

(C) *rsd* animals do not show silencing of germline genes following ingestion of dsRNA. Left: The *pie-1* promoter drives expression of GFP in the germline of *rsd-3* animals. Middle: This expression is not reduced by feeding these animals on dsRNA against GFP. Right: In contrast, germline GFP expression is lost upon feeding GFP dsRNA to wildtype (N2) animals. Germline expression of GFP is also not silenced by feeding dsRNA in any of the other *rsd* mutants (data not shown).

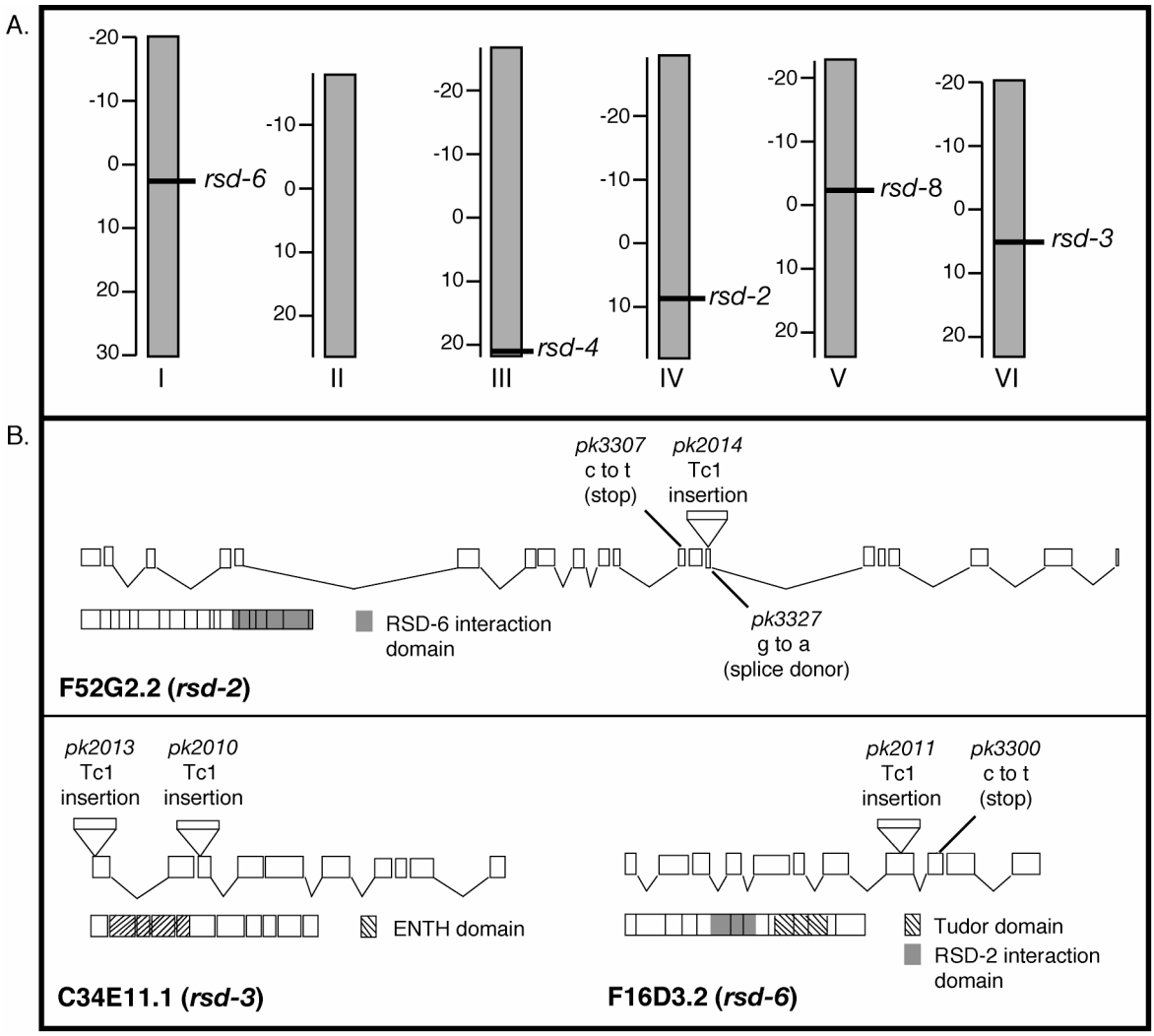


Figure 2. Gene Identification

(A) The location of the *rsd* genes in the *C. elegans* genome. Roman numerals designate the six different *C. elegans* chromosomes, vertical measurements indicate position along chromosome in centimorgans.

(B) Diagrammatic representation of the novel *rsd* alleles isolated from the screen. Each gene is shown as linked exons (above) and a complete cDNA (below), with conserved domains highlighted and the nature of the different alleles shown.

Two independent alleles of *rsd-3* were mapped to locus C34E11.1 (chromosome X, Figure 2a, 2b). C34E11.1 encodes a protein with an epsin N-terminal homology (ENTH) domain. ENTH domains bind phosphoinositides and frequently occur in vesicle trafficking proteins (De Camilli et al., 2002), suggesting that RSD-3 may play a vesicle-trafficking role during systemic RNAi. In support of this, two independent transgenic lines expressing RSD-3 under its own promoter showed widespread expression but with particularly high expression in the coelomocytes (Figure 3a), highly endocytic cells that perpetually 'sample' fluid within the body cavity of the worm (Fares and Grant, 2002; Fares and Greenwald, 2001). By reciprocal BLAST analysis, RSD-3 is the homologue of a recently identified human protein, enthoprotin, that has been implicated in vesicle trafficking in mammals (Wasiak et al., 2002) (Figure 3b). We analysed the evolutionary relationship of all *C. elegans* and human proteins containing ENTH domains (Figure 3c). RSD-3 and enthoprotin cluster together and are not closely associated with ENTH-domain proteins known to play more general roles in endocytosis (Figure 3c), in agreement with our observation that *rsd-3* mutants do not have a general endocytic defect. Thus RSD-3 may act to regulate vesicle trafficking in a pathway specific for systemic RNAi.

We mapped *rsd-2* and *rsd-6* to loci F52G2.2 (chromosome IV) and F16D3.2 (chromosome I), respectively, and identified two (*rsd-6*) and three (*rsd-2*) independent alleles of each (Figure 2a, 2b). RSD-2 is a large protein with no discernible motifs or close homologues in other organisms that may be informative of its molecular function. In contrast, RSD-6 contains a Tudor domain, a structure

frequently found in RNA-binding proteins although not itself believed to be an RNA-binding motif (Selenko et al., 2001), implicating RSD-6 in RNA binding. To probe the function of RSD-6 we performed a yeast 2-hybrid screen against a complete *C. elegans* cDNA library. Surprisingly, the protein most frequently found to interact with RSD-6 was RSD-2. By serial truncation of the two proteins we mapped the interacting interface to amino acids 280 to 411 of RSD-6 and residues 818 to 1266 of RSD-2 (Figure 2b). Thus systemic RNAi requires a complex of RSD-2 and RSD-6.

Finally, we sought to visualise systemic RNAi at the cellular level by soaking animals in a solution of fluorescently-labelled dsRNA overnight (Maeda et al., 2001). Soaking in fluorescent dsRNA against *unc-22* or *pos-1* was able to trigger the corresponding RNAi phenotype (twitching or embryonic lethality, respectively), showing that the dsRNA was entering and spreading throughout the animal. However, we were unable to observe specific accumulation of fluorescent dsRNA at the cellular level and thus conclude that the quantities of dsRNA taken up to induce systemic RNAi are too small to be visualised in this way. In a second strategy, we sought to follow systemic RNAi indirectly, by observing cell-to-cell spread of the RNAi effect in transgenic animals. Animals were generated in which widespread GFP expression was silenced in some intestinal cells (the likely entry point of dietary dsRNA) by expression of dsRNA against GFP from a hairpin construct (see methods). However, although cells containing the hairpin construct showed full silencing of GFP (indicating that dsRNA against GFP is being produced by the hairpin and is able to trigger an RNAi effect) we never observed a systemic spread of this RNAi effect from the hairpin-containing cell to other tissues or even to neighbouring intestinal cells that do not contain the hairpin (Figure 4). There is one report in the literature of systemic RNAi triggered by a hairpin construct (Winston et al., 2002), but the construct used in this case is likely to express dsRNA to extremely high levels (Timmons et al., 2003), and the extent of systemic spreading observed is weak. In addition, another group has recently reported a lack of systemic spreading from hairpin constructs (Timmons et al., 2003). This suggests that dsRNA expressed from a hairpin within a cell is unable to trigger systemic RNAi, whilst dsRNA against the same gene that is delivered from the environment (by feeding or soaking) can trigger a systemic effect. This may reflect intrinsic differences between hairpin-derived dsRNA and exogenously supplied dsRNA. For example, perhaps dsRNA molecules must be ‘packaged’ into endocytic vesicles before being distributed systemically and this packaging step does not occur with endogenously produced dsRNAs.

RNAi is an evolutionarily widespread phenomenon, occurring in fungi, protozoa, plants and animals. However, the ability of RNAi to be induced systemically following a local trigger appears to be highly restricted - only *C. elegans*, plants and, recently, planarians (Newmark et al., 2003), have been reported to show systemic RNAi. We report here the identification of proteins required for systemic RNAi in *C. elegans*. Of the four genes described, only one (*rsd-3*) has a close homologue in other animals. This may explain the absence of systemic RNAi in mammals and suggests a possible means of producing mammalian cells capable of performing systemic RNAi.

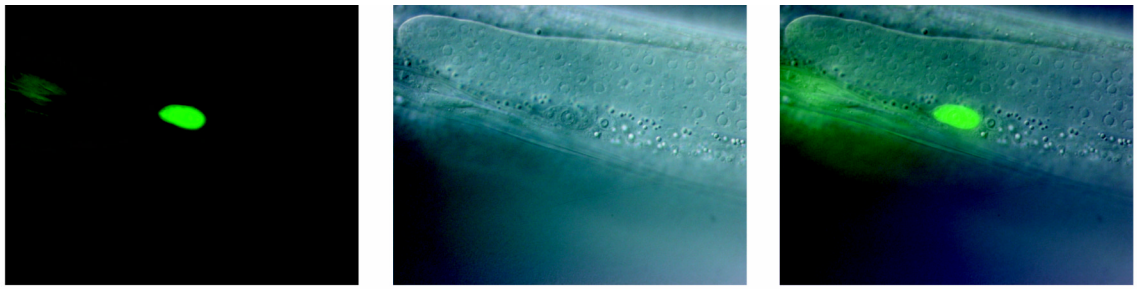
Figure 3. RSD-3 Encodes a Protein with an ENTH Domain

(A) *rsd-3* is highly expressed in endocytic cells. A transgenic animal with GFP fused to exon V of the *rsd-3* gene shows high expression in the coelomocytes. An identical expression pattern was observed for transgenic animals in which GFP was fused to the end of the protein (data not shown). Left: Fluorescent image, showing RSD-3 expression. Midle: Nomarski image of the same area of the animal. Right: Merged image.

(B) Alignment of *C. elegans* RSD-3 and human enthoprotin. The ENTH domain is shaded grey and residues highlighted in red (high consensus), blue (low consensus) or black (no consensus). The two proteins show 37% identity and 51% similarity over the entire length of RSD-3.

(C) A rooted tree showing the evolutionary relationship of *C. elegans* and human ENTH domain-containing proteins. Enthoprotin and RSD-3 do not cluster together with ENTH-domain proteins that act in the clathrin-endocytosis pathway. Characterised proteins are named, all other ENTH-domain containing proteins are denoted by their ENSEMBL reference (human proteins) or their Wormbase gene name (*C. elegans* proteins).

A.



B.

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                1       10        20        30        40        50        60        70        80        90       100       110       120       130
                |-------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
RSD-3                    MSDLLAGITTSIKSTANAITKNEY-VRKVTESMNDAIMNYPKAMMDVREATNEDPWGPTGPQMKKICEYTRSRYMEDFYNVYTPLFQRMLENNKDAWRRVYKSLILLDYLLKKNGSERFV
ENTHOPROTIN PRPGTAPVRKCSGVRGEQEREAADRPARGTMLNMAKVRELVDKATNVYMNYSEIESKVREATNDDPWGPSGQLMGEIAKATFM--YEQFPELMNMLWSRMLKDNKKNWRRVYKSLLLLAYLIRNGSERVV
Consensus       ..........mgdrgaqereaadrpAraimlNew.YRelt#kan#a!MNYpeaemdVREATN#DPWGPsGqqMgeIaeaIrm..mE#Fp#lmnmLfqRMLe#NKdaWRRVYKSLiLLaYLirNGSERfV

                131     140       150       160       170       180       190       200       210       220       230       240       250       260
                |-------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
RSD-3           QEARERKAYELRRLESYKYIDEKGKDQQGINIRHRVKQILEMMNDDELLQAERQKANSDDKSKYRGFDQYDM---KMSGSSFKSSNSGYDNKWDKEDSFSSSVTK--KYDDQYKSKEVSNFSFSSNVNRSPS
ENTHOPROTIN TSAREHIYDLRSLENYHFVDEHGKDQQGINIRQKVKELVEFAQDDDRLREERKKAKKN-KDKYVGVSSDSVGGFRYSERYDPEPKSKWDEEWDKNKSAFPFSDKLGELSDKIGSTIDDTISKFRRKDRELDS
Consensus       qeAREhaY#LRrLEnYh%!DEhGKDQQGINIRqrVK#ilEma#DD#rLraERqKAnk#.KdKYrGfdqddm...rnSersdkepnSgwD#eWDK#dSafpfsdK..eldDqigSkeddniSkfrrk#RedS

                261     270       280       290       300       310       320       330       340       350       360       370       380       390
                |-------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
RSD-3           PELGFVDDSKKK-----DDDDGFGDFVSSRSSQPAATNKSKPNPSKSFFDDPIPAIPPPSGAPSNYVAPPLSPVSKNAPRNMDLLFDVSAPTPAAP----------QNNGAPIDLFGKL----DAPVVN
ENTHOPROTIN PERCSDSDEEKKARRGRSPKGEFKDEEETVTTKHIHITQATETTTTRHKRTANPSKTIDLGAAAHYTGDKASPDQNASTHTPQSSVKTSVPSSKSSGDLVDLFDGTSQSTGGSADLFGGFADFGSAAASG
Consensus       PErcfddDeeKK.....ddddeFgDeeesrssqhaainqakenpskrhkrdanPaipidlGAaanYtadkaSPdqnaaprnm#llfdtSaPspaap..........QnnGapaDLFGgl....dAaasn

                391     400       410       420       430       440       450       460       470       480       490       500       510       520
                |-------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
RSD-3           AAPA-VAPSNGGLDLFAFGAAPMAQSIPMAT---------PVAAPIN---------TASNGSFNGFDFGGSFGSTNNSTPSYGGAAPLQPMSGLSQPQAQPAQSNLGGPSPKIGNSFAGLDQFMSLSLG
ENTHOPROTIN SFPSQVTATSGGNGDFGDWSAFNQAPSGPVASSGEFFGSASQPAVELVSGSQSALGPPPAASNSSDLFDLMGSSQATMTSSQSMNFSMMSTNTVGLGLPMSRSQNTDMVQKSVSKTLPSTWSDPSVNISLD
Consensus       aaPa.YaasnGngDlgafgAanqAqSgPnAs..........Paaal!n.........pAannSf#gFDlggSfqaTnnSsqSmngaamlqnmsGLgqPqarpa#s#$gqkSpkignpfaglDqfmniSLd

                521     530       540       550       560       570       580       590       600       610       620       630       640       650
                |-------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------+---------|
RSD-3           ST----NSNAPKAKTLNQMMGGNN
ENTHOPROTIN NLLPGMQPSKPQQPSLNTMIQQQNMQQPMNVMTQSFGAVNLSSPSNMLPVRPQTNALIGGPMPMSMPNVMTGTMGMAPLGNTPMMNQSMMGMNMNIGMSAAGMGLTGTMGMGMPNIAMTSGTVQPKQDAF
Consensus       nl....#pnaPqaksLNqMiqq#N.....................................................................................................................

                651  658
                |-----|
RSD-3
ENTHOPROTIN ANFANFSK
Consensus       ........
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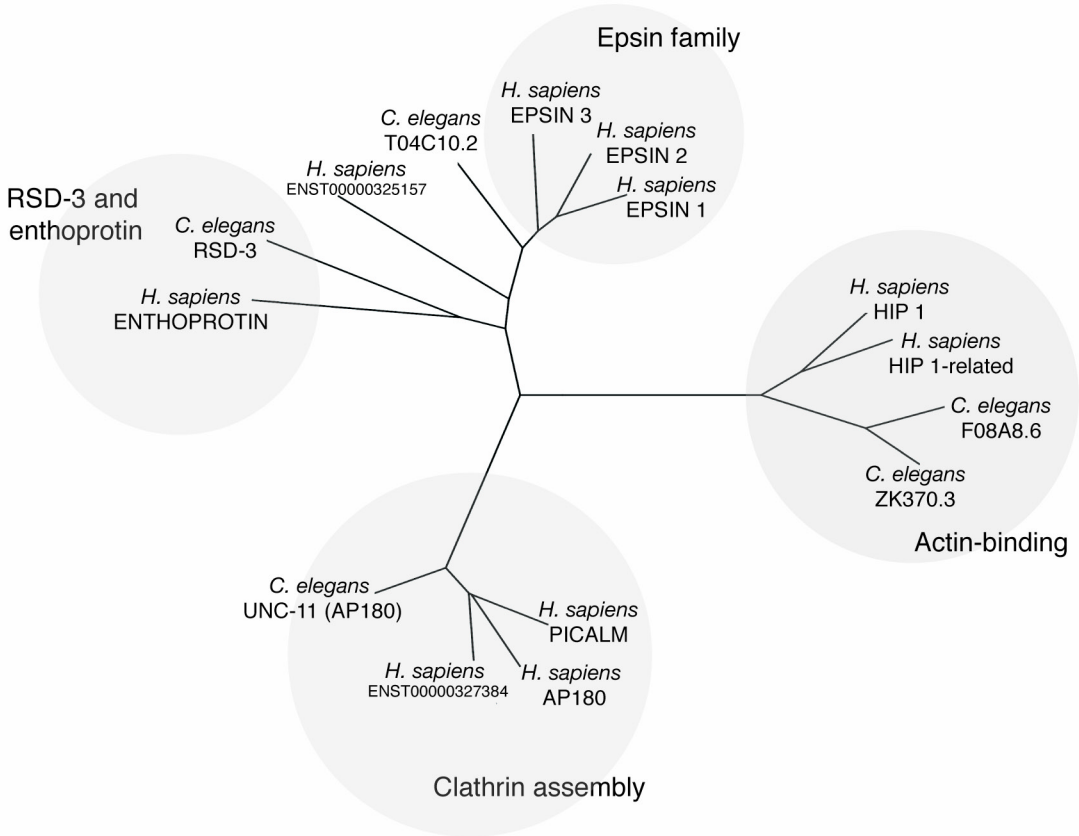
C.



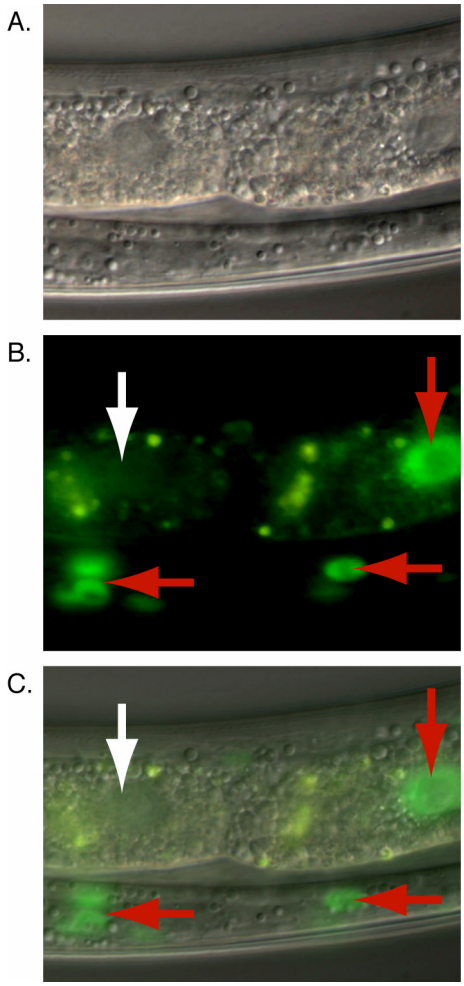


Figure 4. Hairpin Induced RNAi Does Not Spread
(A) Nomarski image of middle body region. (B) Fluorecent image of the same region. (C) Merged image.
Systemic RNAi does not occur when dsRNA is expressed endogenously from a hairpin construct. *pkIs1582* animals show widespread expression of GFP (Simmer et al., 2002a). The expression in intestinal cells can be extinguished by expressing dsRNA for GFP from a hairpin GFP construct under control of the *elt-2* promoter, but loss of expression is only seen in cells containing the hairpin (white arrow). Neighbouring cells without the hairpin continue to express GFP (red arrows), indicating that dsRNA against GFP is unable to spread from cell to cell.

Experimental procedures

***C. elegans* culture**
Animals were cultured according to standard protocols (Hope, 1999). The Bristol strain N2 was used as the standard wild-type strain. Strains and alleles used were: MT464[*unc-5*(*e53*)IV;*dpy-11*(*e224*)V;*lon-2*(*e678*)X], MT465[*dpy-5*(*361*)I;*bli-2*(*e768*)II;*unc-32*(*e189*)III], DR102[*dpy-5*(*e61*);*unc-29*(*e403*)]I, SP1478[*unc-29*(*e193*);*dpy-24*(*s71*)]I, CB4856 (Hawaiian polymorphic strain), CB4857 (polymorphic strain from CA), DR101[*dpy-5*(*e61*);*unc-55*(*e1170*)]I, EJ275[*unc-29*(*e1072*)/*dxDf1*]I, DH1033 (Grant and Hirsh, 1999), AZ212 (Praitis et al., 2001), DH1201[*rme-1*(*b1045*)], DH1206[*rme-8*(*b1023*)], CX51[*dyn-1*(*ky51*)], VC205[*cav-1*(*ok270*)], BA1090[*cav-2*(*hc191*)], NM1568[*ehs-1*(*ok146*)].

Mutagenesis
We mutagenised approximately 3000 hermaphrodites from the Bristol N2 strain with 50 mM ethyl methanesulfonate (EMS) for four hours. Mutagenised worms were then cultured on OP50 food for 6 days at 20°C. After treatment with sodium hypochlorite to kill hatched worms, the eggs were plated on a bacterial lawn expressing *par-1* dsRNA (Fraser et al., 2000). We also searched for spontaneous mutants using approximately 3 million *mut-6* animals (in which Tc1 transposons are activated in the germline (Mori et al., 1988)). In both cases, after several generations of growth, surviving mutants were singled, allowed to produce progeny and 12 of these F1 worms were again transferred to plates containing bacteria expressing *par-1* dsRNA. One from the twelve was kept as a mutant resistant to dsRNA feeding. A number of offspring from each mutant line were then injected with 100ng/µl *pos-1* dsRNA and scored for subsequent production of dead eggs. Mutants resistant to RNAi by feeding but sensitive to RNAi by injection were designated *rsd*.
Complementation testing, genetic mapping and transgenic manipulations were carried out according to standard protocols (Hope, 1999). The *rsd-3* and *rsd-6* mutant animals could be rescued following transgenesis with the corresponding cosmid (*rsd-6*) or cosmid fragment (*rsd-3*). Rescue of *rsd-8* animals has been previously demonstrated by Winston et al (Winston et al., 2002).

Yeast two-hybrid screening
Full-length or partial fragments of *rsd* genes were obtained by standard PCR on a *C. elegans* cDNA library and ligated into pPC97. Screening was carried out using standard protocols (Clontech) against a cDNA library from mixed-stage *C. elegans* extracts. Positive clones were tested for Gal4-dependent transcription of β-galactosidase by 'lifting' onto Hybond-N membrane (Amersham), lysing in liquid nitrogen and incubating overnight at 30°C on filter paper soaked in Z-buffer (60mM Na2HPO4, 60mM NaH2PO4, 10mM KCl, 1mM MgSO4, 50mM β-mercaptoethanol, 0.025% X-gal). Clones testing positive on both selective plates and by β-galactosidase assay were sequenced, subcloned from the bait vector and serial truncation constructs made in order to map the minimal interface required for interaction.

Bioinformatics
Protein alignments were generated using the Multalin program (http://prodes.toulouse.inra.fr/multalin/multalin.html). Evolutionary trees were generated using the Mega2.1 program (Kumar et al., 2001) on sequence data derived from the ENSEMBL database (www.ensembl.org) and the Wormbase database (www.wormbase.org).

Hairpin expression of dsRNA
A fragment of the GFP cDNA was cloned into the pJM67 vector behind the *elt-2* promoter in two inverted orientations separated by a short non-complementary loop and then injected into *pkIs1582* animals (that show widespread GFP expression (Simmer et al., 2002a)). Following injection, the GFP hairpin construct forms an extrachromosomal array that is carried in a mosaic fashion. Any individual transgenic worm will therefore have some cells that carry the array and some that do not, allowing one to monitor RNAi effects spreading from hairpin positive cells to neighbouring cells that lack the hairpin. These experiments were also repeated using the *ncl-1* marker (data not shown) by injecting the GFP hairpin together with a *ncl-1* rescuing marker plasmid into *ncl-1/pkIs1582* animals. *ncl-1* animals have enlarged nucleoli, a phenotype that is rescued cell-autonomously by the *ncl-1* plasmid. In the case of injections with the *ncl-1* rescuing marker into *ncl-1* worms, cells containing the array will have a normal nucleolar morphology whilst cells without the array have enlarged nucleoli, allowing one to distinguish cells carrying the array from cells without it. In both cases, silencing effects were never seen to spread from cells harbouring the hairpin to neighbouring cells without the transgene.

Acknowledgements
Several strains used in this work were kindly provided by the Caenorhabditis Genetics Centre and by Johnny Fares. We would like to thank Eugene Berezhikov for assistance with bioinformatics, Rene Ketting for help with the mut-6 transposon screen and other members of the Plasterk lab for critical reading of the manuscript. R.C.M is supported by a Human Frontier Science Program fellowship. F.S. is supported by the Netherlands Organisation for Scientific Research (Grant CW97045).

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Summary

Viruses exploit their host by using many compounds of the host and cause damage by replication and spreading through the organism. Transposons are DNA elements that can move and multiply themselves within their host genome. As a result, they can cause damaging mutations. This thesis is about a defense mechanism against viruses and transposons, which is called RNA interference (RNAi). This mechanism is conserved in many organisms including plants, fungi, mouse, human and the fruit fly *Drosophila*.

Much effort is used to unravel the mechanism underlying RNAi. Double stranded RNA (dsRNA) is the initiator of the process. First, the dsRNA is cleaved into small pieces (siRNAs). Next, these effecter molecules bind to RNAs. These RNAs are subsequently cleaved and degraded. Viruses and transposons can no longer replicate or spread due to the degradation of their RNAs.

RNAi can also be applied to target specific RNAs. This is done to study gene functions. By analysis of the effects of RNAi mediated destruction of an mRNA, which results in loss of the protein, information on the function of a gene can be obtained. It is also possible to use RNAi to improve food products or in disease treatment. In order to use RNAi in the different applications it is necessary to know more about the mechanism.

During my research I have identified components involved in RNAi. I have used the model organism *Caenorhabditis elegans*. This a small worm, which has been studied for many years by investigators of different specialities. Therefore, many details about this organism are known and numerous research methods are developed.

Chapter 1 of this thesis gives an overview of what is currently known about the mechanism of RNAi.

Chapter 2 is about an amplification step in the RNAi mechanism. RRF-1 is an enzyme implicated in this process. We propose that this enzyme produces new dsRNA using the RNA that has to be broken down as a template. This results in more effecter molecules that help to finish the RNAi process.

Chapter 3 concerns RRF-3, a family member of RRF-1. RRF-3 seems to inhibit RNAi; removal of RRF-3 results in an increase in the efficiency of RNAi. This can be useful when RNAi is applied.

We used the worms without RRF-3 to generate new data on the genes of *C. elegans*. This is described in chapter 4. Each mRNA was targeted using RNAi and the effects on the worms were determined. New information on approximately 400 genes was obtained.

Another aspect of RNAi is the spreading of the process throughout the worm. The dsRNA that triggers RNAi (or a modified form) is able to spread; this results in mRNA breakdown in distant tissues. Chapter 5 discusses several components that seem to be involved in the spreading of RNAi in *C. elegans*.

Samenvatting

Virusen gebruiken voor hun vermeerdering de bouwstenen van hun gastheer en beschadigen door hun vermeerdering en verspreiding het organisme. Transposons zijn stukjes DNA die zich kunnen verplaatsen en vermeerderen binnnen het genoom van een organisme. Hierdoor kunnen ze in het genoom van hun gastheer schadelijke veranderingen veroorzaken. Dit proefschrift gaat over een afweerproces, RNA interferentie (RNAi), dat tegen virusssen en transposons werkt. Het bestaat in veel organismen, bijvoorbeeld in planten, schimmels, muizen, mensen en het fruitvliegje *Drosophila*.
Er wordt veel onderzoek gedaan naar hoe RNAi precies werkt. Dubbelstrengs RNA (dsRNA) blijkt de initiator voor het process. Eerst wordt het dsRNA geknipt in kleine stukjes (siRNAs). Vervolgens kunnen deze effector moleculen binden op RNAs. Die RNAs worden uiteindelijk kapot geknipt en verder afgebroken. De RNAs kunnen dus virus RNAs of transposon RNAs zijn. Door de afbraak kunnen virusen en transposons niet meer repliceren en verspreiden.
RNAi kan ook geexploiteerd worden om doelgericht bepaalde RNAs af te breken. Dit wordt bijvoorbeeld gedaan in onderzoek naar de functies van genen. Door gericht een mRNA af te breken, waardoor er minder eiwit ontstaat, en te kijken naar de gevolgen wordt er informatie verkregen over de functie van een gen. Ook kan RNAi worden gebruikt voor het verbeteren van voedingsmiddelen of in ziektebestrijding. Om RNAi goed te gebruiken moeten we echter nog veel meer weten van hoe het mechanisme precies werkt.
Tijdens mijn onderzoek heb ik componenten van het RNAi mechanisme geidentificeerd. Om dit te doen heb ik gebruik gemaakt van het modelorganisme *Caenorhabditis elegans*. Dit is een wormpje dat al vele jaren wordt bestudeerd door onderzoekers van verschillende vakgebieden. Hierdoor is er veel over bekend en zijn er allerlei onderzoeksmethoden ontwikkeld.
Een uitgebreide uitleg over wat er tot nu toe bekend is over het mechanisme van RNAi staat in hoofdstuk 1.
Hoofdstuk 2 gaat over een stap in het RNAi mechanisme die zorgt voor vermeerdering van het dsRNA. Een enzym, RRF-1, betrokken bij dit proces wordt ook beschreven. We denken dat dit enzym het RNA dat moet worden afgebroken gebruikt om meer dsRNA te maken. Daardoor komen meer effector molekulen om RNAi af te ronden.
Hoofdstuk 3 gaat over een eiwit, RRF-3, uit dezelfde familie als RRF-1. RRF-3 lijkt RNAi tegen te werken; als je RRF-3 weghaalt gaat RNAi efficienter. Dit kan handig zijn als je RNAi wilt gebruiken.
Met de wormen waarin RRF-3 is weggehaald hebben we een grootschalig experiment gedaan om meer gegevens te krijgen over de genen van *C. elegans*. Dit is beschreven in hoofdstuk 4. Stuk voor stuk werden alle mRNAs met behulp van RNAi afgebroken en werd er gekeken naar de effecten op de worm. Dit gaf nieuwe informatie over ongeveer 400 genen.
Een ander aspect van RNAi is de verspreiding van het proces door de hele worm. Het dsRNA dat RNAi initieert (of een bewerkte vorm van het dsRNA) kan worden verspreid, zodat mRNA in bepaalde verder gelegen weefsels wordt afgebroken. Hoofdstuk 5 gaat over onderdelen die betrokken lijken bij het verspreiden van RNAi door *C. elegans*.

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Curriculum Vitae

Femke Simmer werd geboren op 13 augustus 1976 te Amsterdam. In 1994 behaalde zij het atheneum diploma aan de Openbare Scholengemeenschap Nieuwer Amstel te Amstelveen. In hetzelfde jaar begon zij met de studie Medische Biologie aan de Vrije Universiteit van Amsterdam. Zij deed stage bij de vakgroep Medische Chemie aan de Vrije Universiteit van Amsterdam, onder begeleiding van dr. I.M. van Die en prof.dr. D.H. van den Eijnden, en bij de afdeling Moleculaire Genetica van het Nederlands Kanker Instituut te Amsterdam, onder begeleiding van dr. H.C. Korswagen en prof.dr. R.H.A. Plasterk. In 1999 behaalde zij het doctoraal examen Medische Biologie. Vanaf april 1999 was zij werkzaam als onderzoeker in opleiding bij de afdeling Moleculaire Biologie van het Nederlands Kanker Instituut te Amsterdam, onder begeleiding van prof.dr. R.H.A. Plasterk. In maart 2000 verhuisde de onderzoeksgroep van prof.dr. R.H.A. Plasterk naar het Hubrecht Laboratorium te Utrecht. Daar werd het onderzoek vervolgd. De resultaten van dit onderzoek staan beschreven in dit proefschrift getiteld “RNA Interference in *Caenorhabditis elegans* – Mechanism and Application” .

Femke Simmer was born on August 13th in Amsterdam. She attended the Openbare Scholengemeenschap Nieuwer Amstel in Amstelveen and graduated in 1994. She then studied Biomedical Sciences at the Vrije Universiteit of Amsterdam. During her undergraduate work, she did a research project at the department of Medical Chemistry of the Vrije Universiteit of Amsterdam, under the supervission of Dr. I.M. van Die and Prof. D.H. van den Eijnden, and a second research project at the Netherlands Cancer Institute in Amsterdam, under the supervission of Dr. H.C. Korswagen and Prof. R.H.A. Plasterk. She graduated in 1999. From April 1999 she worked as a PhD student at the Netherlands Cancer Institute in Amsterdam, under the supervission of Prof. R.H.A. Plasterk. In March 2000 the research group of Prof. R.H.A. Plasterk moved to the Hubrecht Laboratory in Utrecht. There the research was continued. The results of this research are described in this thesis called “RNA Interference in *Caenorhabditis elegans* – Mechanism and Application”.