RNA Silencing in Drosophila

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Received March 6, 2003; accepted March 14, 2003

RNA silencing is a highly conserved mechanism found in both the plant and animal kingdoms that is thought to protect the genome from disruption by transposons and viral integration events. Double stranded RNAs (dsRNA) produced by transposons or replicating virus result in the production of short 21–25 nucleotide double stranded RNA molecules containing a hydroxyl group on a two base-pair 3' overhang and a 5' phosphate residue. These siRNAs are the hallmark of RNA silencing, also known as post transcriptional gene silencing (PTGS) in plants, quelling in Neurospora, and RNA interference in C. elegans, Drosophila, and Dictyostelium. Remarkably these siRNAs direct the degradation of the complementary target RNA through a complicated mechanism that is just now being understood. From our studies on RNA interference in Drosophila we propose a model in which the siRNAs interact through complementarity with the target RNA and are extended by a cellular RNA-dependent RNA polymerase (RdRP) to form a critical length of dsRNA that is subsequently degraded by RNase III-related enzymes. Here, we discuss this model and the data produced in Drosophila to support it and, in turn, this model is compared to the proposed scheme for RNA silencing in mammalian systems.

Key words: RNAi, RdRp, siRNAs, 3'-hydroxyl, RNA ligase

I. Introduction

RNA silencing is the response by different organisms to double stranded RNA, a signature of transposon mobilization and viral attack. The double stranded RNA is specifically and rapidly processed into shorter pieces and this leads to the subsequent degradation of any complementary single stranded RNA in the cytoplasm. One of the proposed functions of RNA silencing is to protect the genome from transposable elements and viral integration events by acting like an immune system for the genome [17]. However, the story is more complex, for components of the RNA silencing mechanism also play a role in normal development by controlling gene function in a chromatin context [24] or regulating protein expression levels by modulating mRNA translation. Recently RNA silencing has attracted a great deal of attention as an inverse genetic tool in numerous developmental systems with potential application in gene-therapy for human diseases [20 and references therein].

Everything started more than ten years ago. Researchers working on plant pigmentation were trying to increase the coloration in different types of flowers. They injected a transgene for chalcone synthase, an enzyme involved in the synthesis of the purple pigment in the petunias. Surprisingly, they found that most of the flowers were lighter in color and some of them were even completely white. The phenomenon was called post-transcriptional gene silencing (PTGS) since the gene was transcribed but the mRNA was rapidly degraded inducing the silencing of the endogenous gene [15]. During the same years, identical observations were noted in the Neurospora crassa fungi [4]. It only became clear ten years later that the critical molecule in the process was the double stranded RNA. Fire and Mello, attempting to investigate the function of the unc gene in C. elegans, were using the antisense technique. They observed identical results with the injection of either antisense or sense strand RNA, the latter used as a control. The most potent effect was observed when both sense and antisense RNAs were co-injected. From this they surmised that both preparations of sense and anti-sense RNA were contaminated with the complementary strand and, therefore, small amounts of dsRNA could direct the degradation of a much greater amount of complementary RNA [7].

Biochemical and genetic studies quickly gave suffi-
cient information to propose a model for RNA silencing. Again, the first input was from the plant field. Hamilton and Baulcombe found small sized pieces of 21–25 nt dsRNA in plants that correlated with PTGS [8]. These 21–25 dsRNA, named ‘short interfering RNAs’ (siRNAs), were then found in Drosophila and other systems [27]. They were derived from the processing of large dsRNA. The enzyme responsible for the processing activity was cloned one year later and named Dicer [3]. Genetic studies in the meantime revealed different proteins involved in RNA silencing: RNA helicase, RNA-dependent-RNA-polymerase, 5'-3' exonuclease, and argonaute proteins [10]. Therefore a complex of proteins and RNA, called the RNA-induced silencing complex or RISC, has been proposed to degrade the target mRNA [9]. Different questions related to the nature of the RISC complex have still not been answered, such as, what are the proteins involved in the complex, what leads to the formation of the complex and, most importantly, how does the complex degrade the target mRNA.

RNA silencing is a remarkable process in that substoichiometric amounts of dsRNA can completely degrade the complementary target mRNA. suggesting there is an amplification step in the silencing process. RNA silencing depends strictly upon the presence of the corresponding short interfering RNAs (siRNA). The double-stranded 21–25 nt siRNAs contain a 2 nt overhanging at the 3’ hydroxyl end and a 5’ phosphate group [6]. The siRNAs exclusively recognize and target the degradation of the complementary mRNA and they are able to amplify the silencing effect through a mechanism involving RdRp [16]. Although it is unequivocal that the siRNAs are the essential nucleotide component in RNA silencing, it still under discussion whether or not they degrade the complementary mRNA “sic et simpliciter” through a “guide” mechanism that targets a catalytic nuclease complex, or, if they serve as primers that are extended by a cellular RNA-dependent RNA polymerase (RdRp) to generate a length of dsRNA sufficient to be degraded by RNase III-type activity to produce a second generation of siRNAs. Evidence for the latter idea has been clearly demonstrated in C. elegans with transitive RNAi and RNAi induced by short 40–50 bp antisense RNAs, both of which are dependent upon RdRp [1, 21, 22]. Genetic screens in several lower eukaryotic systems have already identified, RdRp, as one of the essential genes for RNA silencing. The question that has to be addressed is how do siRNAs effectively ensure the full degradation of the complementary mRNA?

We present data, using a Drosophila in vitro system, that supports a critical role for RdRp activity and the function of siRNAs in RNA silencing. Similarities and differences in the RNA silencing process between Drosophila and mammalian systems will be discussed.

II. Materials and Methods

mRNA target and siRNA trigger

Synthesis of capped and poly-adenylated S65T GFP and Pp-Luc messenger RNA were performed as previously described [25]. GFP siRNA trigger (GGGTTCATTTAG-CAGACCA, from residue 549 to residue 567 of GFP) was purchased from Dharmacon Research, Inc.

Silencing assay in vitro

The silencing assay in vitro was performed as previously described [25]. Briefly, capped and poly-adenylated S65T GFP and Pp-Luc mRNA transcribed in vitro with α-32P UTP label were incubated in presence of cold S65T GFP dsRNA, Drosophila embryo extract, nucleotides and the ATP regenerating system done by creatine phosphate and creatine phospho-kinase. Different time point were collected and treated with proteinase K. The reaction was run on 1.5% formaldehyde agarose gel. The gel was blotted onto Nytran-plus (Millipore), dried and exposed to Kodak AR film.

Block of 3’ end of siRNA with PGP

Block of 3’ end of siRNA with PGP and dephosphorilation with phosphatase was done as previously described [25].

Silencing assay in vivo

SL2 cell line co-transfected with cDNA encoding GFP and Pp-Luc were also transfected with the GFP siRNA. Cells were immunostained for GFP and β-gal and analyzed by using a BioRad confocal microscope.

RNA ligase activity assay

The ligase assay was done using Drosophila embryo extract adding either tRNA or GFP siRNA in the presence of 5'-P32 pGP (Amersham). Different time point were collected. The time point, 2 hour, was digested with RNase A. The material was run on 6% acrilammide urea-gel. The gel was fixed in 10% methanol and 10% acetic acid, dried and exposed to a autoradiographic film.

III. Results

To determine if the siRNAs were critical to RNA silencing and how they participated in the degradation of the complementary mRNA target, we established a protocol to isolate the siRNAs in a “native” state. 32P UTP uniformly labeled GFP dsRNA was incubated in Drosophila embryo extract prepared from 2 hour embryos in order to produce GFP siRNAs. The extract was then digested with micrococcal nuclease (Mn) to remove nucleic acids not protected by proteins and to degrade any GFP dsRNA not processed into siRNAs (Fig. 1A, compare lane: control to lane: +Mn). The resulting material was also resistant to RNase One treatment (Fig. 1B), showing that the siRNAs derived by the processing of large dsRNA continue to be double stranded molecules.

We tested the activity of the siRNAs derived from Mn digestion (Mn-siRNAs) in silencing reactions performed in Drosophila embryo extracts (Fig. 2). In this assay, 32P UTP uniformly labeled mRNA target and control mRNAs are in-
cubated in *Drosophila* embryo extract with dsRNA trigger and monitored for the specific disappearance of the target mRNA. We routinely use full length GFP and *Pp*-Luc as the target and control mRNAs, respectively, and full length GFP dsRNA as the trigger. Unexpectedly, when we used the GFP Mn-siRNAs to trigger silencing, they did not degrade the complementary RNA (Fig. 2). However, we realized that Mn digestion of RNA leaves a phosphate moiety on the 3’ hydroxyl group of the siRNAs, unlike the natural siRNAs that have a free 3’ hydroxyl group. Treatment of the Mn siRNA preparation with the calf intestinal phosphatase (CIP) to remove the 3’ phosphate restored RNA silencing (Fig. 2). Therefore, active siRNAs require a hydroxyl group at the 3’ end.

**Fig. 1.** Double stranded siRNAs are produced with micrococcal nuclease (Mn) treatment. A: control, processing of GFP dsRNA; +Mn, addition of Mn to control; +Mn+dsRNA, addition of dsRNA to +Mn. Addition of dsRNA to +Mn competes for dsRNA processing. Note the reduction of labeled siRNAs. B: siRNA are resistant to RNaseI.

**Potential role of the siRNA 3’ hydroxyl group in priming activity for a cellular RNA dependent RNA polymerase (RdRp)**

Since RdRP has been shown to be a critical protein in RNA silencing in other lower eukaryotes, we tested the ability of the siRNAs to act as primers that could be incorporated into the complementary target RNA. The 32P UTP labeled siRNAs were incubated in a silencing reaction to see if they could be incorporated into dsRNA corresponding only into the complementary mRNA strand (Fig. 3). This was the case and the product of the reaction was resistant to both RNase One and RNase T1, two enzymes that digest single strand but not double stranded RNA (data not shown). The product of this reaction was isolated from a 4% acrylamide sequencing gel, annealed and processed again in the presence of
Fig. 2

Fig. 3
Drosophila embryo extract. Identical to the double stranded RNA transcribed in vitro, the siRNA labeled dsRNA was also processed to produce a second generation of siRNAs (Fig. 4). As shown in a time course, the siRNAs were incorporated into dsRNA product (Fig. 5A) and were then rapidly processed into a second generation of siRNAs (Fig. 5B).

To analyze whether or not multiple siRNAs were incorporated into the complementary RNA, we cut the product band from a 4% acrylamide sequencing gel, annealed and digested it with RNase T1 to generate a type of RNA fingerprint since RNase T1 cleaves only after G residues. Only the complementary strand of the labeled siRNAs was incorporated all along the template strand (Fig. 6). This result suggested an RNA ligase activity might also present in the reaction to join the adjacent partially extended siRNAs on the template strand. We checked this in the Drosophila embryo extract and we found that both siRNAs and tRNA were labeled with 5'-32P-pGp, suggesting ligase activity was indeed in the extract. Furthermore, the label was resistant to phosphatase (CIP) (data not shown), as expected for

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**Fig. 4.** Full length siRNA labeled GFP dsRNA is processed in new siRNAs. The product of incorporation of siRNAs into sense RNA (shown at the left) is processed into new siRNA as well as dsRNA transcribed in vitro (shown at the right). MW, molecular weight; 1, dsRNA synthesized in vitro; 2, primer labeled RNA.

**Fig. 2.** SiRNAs require the 3' OH group to function in RNA silencing. The treatment with calf intestinal Phosphatase (CIP) restores the silencing activity of Mn-siRNAs.

**Fig. 3.** Mn-siRNAs are specifically incorporated into the complementary mRNA. 32P UTP labeled siRNAs (indicated at the top of the gel) were incubated in the presence of the cold target (shown at the bottom of the gel).
an internal phosphate, but was completely digested with RNase A (Fig. 7).

However, a single siRNA generally drives a single cut on the target mRNA since a minimal extension to 39 bp is all that is required for Dicer cleavage (Fig. 8A). To investigate if the cut requires the hydroxyl group at the 3’ end, we ligated pGp to the 3’ hydroxyl with T4 RNA ligase. The presence of a 3’ pGp blocks the clipping activity produced by the siRNA (Fig. 8). Most importantly, when the siRNAs blocked with pGp were treated with CIP they re-acquired the silencing and clipping activity, consistent with the requirement for a 3’ hydroxyl for siRNA function. This is shown in vitro with the cleavage of target mRNA (Fig. 8A) and in vivo with the silencing of GFP expression in the SL2 cell line (Fig. 8B). A single siRNA can also drive the production of full-length dsRNA and consequently of other siRNAs in a region 5’ to the siRNA position. This has been observed in extension assays and in RNase protection assays using as a trigger an siRNA in the 3’ portion of the GFP target RNA and an RNA probe almost 250 nt 5’ to the siRNA position (Fig. 9).

Therefore, we propose a model in which the siRNAs are extended up to a sufficient length to guarantee the formation of new dsRNA, primarily through the action of a cellular RdRp activity, and possibly an RNA ligase. These new dsRNAs are then degraded by RNase III related enzyme(s), such as Dicer, to generate a population of secondary siRNAs that are capable of repeating the process.

IV. Discussion

An important function of RNA silencing is to protect the genome from any kind of endogenous or exogenous disruption by viral elements or transposons that could
RNA silencing in Drosophila potentially damage the genome. RNA silencing appears to be the primary mechanism operating in lower eukaryotes to perform this important protective function. The mechanism is conserved in both the plant and animal kingdoms, including the vertebrates. The main difference between lower and higher eukaryotes is the differential tolerance to the presence of double-stranded RNA within the cell. It is known that Drosophila can tolerate mRNA-length double-stranded RNAs with no difficulty. This likely reflects the fact that Drosophila do not appear to have the equivalent of the PKR gene, the kinase activated by dsRNA greater than 40 bp in length that triggers the interferon pathway and induces apoptosis in vertebrate cells. In fact, when mouse PKR is expressed in Drosophila cells, the introduction of dsRNA induces apoptosis, similar to mammalian cells (unpublished observations). This suggests that the initial trigger in the pathway is absent—possibly the PKR-related proteins—but the downstream components are still present in the fly.

Although there is no current genomic evidence for a cellular RdRp in Drosophila, our demonstration that siRNAs are incorporated into dsRNA that is subsequently processed by RNAse III activity to generate a second generation of siRNAs fits very well with the genetic and biochemical data derived from experiments in C. elegans [1, 21, 22]. It is highly unlikely that Drosophila developed an RNA silencing mechanism that is vastly different from the highly conserved one seen in most of the lower eukaryotes. We are currently in the process of purifying the RdRp activity we have identified in Drosophila embryo extracts. It should be noted that the Drosophila genome is not fully annotated or com-

Fig. 6. SiRNAs are incorporated all along the complementary template. Primer labeled reaction is cut from gel, digested with RNase T1 and run on acrilammide gel.
plete so the RdRp equivalent to the enzymes identified in *C. elegans*, Neurospora, and Dictyostelium will likely be identified in the near future.

At this point, it is important to note which aspects of RNA silencing are common to both lower eukaryotes and mammals. In all the organisms that show RNA silencing there is a Dicer-related RNase III activity that has been shown, both genetically and biochemically, to be essential for the process to occur. RNA silencing can be mediated by synthetic siRNAs in mammals and lower eukaryotes, but only the lower eukaryotes can tolerate the presence of dsRNA greater that 40 bp, since this is the minimal length of dsRNA that can trigger PKR and apoptosis in mammalian cells [2]. The key step in the initiation of RNA silencing is the generation of the siRNAs. Dicer is the only known RNase III related activity in humans that can carry out this process. Consistent with our siRNA primer model, which suggests Dicer clips the extended dsRNA primed by the siRNA, Dicer has also been shown to be required for the function of synthetic siRNAs in mammalian cells [5], and we have found similar results in *Drosophila* SL2 cells (in preparation, CL and BMP). Contrary to the data for *Drosophila* Dicer [13], recombinant human Dicer does not require ATP for dsRNA processing to produce siRNAs [18, 28]. According to the RISC model, Dicer is not required once the trigger dsRNA is processed into siRNAs, so this discrepancy clearly indicates there is a great deal we need to understand about the mechanism of RNA silencing. Although several genes have been identified as important for RNA silencing in lower eukaryotes: RdRP, Dicer, Argonaute, and helicase

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**Fig. 7.** Both synthetic siRNA and tRNA bind 32P UTP labeled pGp in the presence of embryo extract. This binding was completely digested with RNase A.
Fig. 8. The presence of a 3' pGp blocks the clipping activity produced by the siRNA in vitro (A) and the silencing activity induced in vivo (B). siRNA* interacts with GFP from 610 to 631, while siRNA from 549 to 567 nt.
among them, the corresponding genes are only now being identified as components of RNA silencing in mammalian cells.

A great deal of effort has been put forward to utilize RNA silencing in human cells as a technology to delineate gene function and as a new approach for the treatment of viral infections, cancer, and some genetic disorders [19]. Application of the process in mammalian cells is more complicated than in lower eukaryotes due to the mammalian cell’s intolerance to large dsRNA molecules. Thus the application of RNA silencing to mammalian cells requires that one use only siRNAs even though the efficacy of a particular siRNA is highly dependent on the target position. So far the best target position is not predictable, nor is it conserved in the same gene in different cell backgrounds. Furthermore, unlike the case in lower eukaryotes, the block of the 3’ hydroxyl group on the siRNA as well as single base pair mismatches within the siRNA are well tolerated in mammals. By contrast to the lower eukaryotes, the most effective siRNA silencing in mammalian cells is only observed when equimolar or greater levels of siRNA are used in targeting. Very few molecules of dsRNA can target several hundred molecules of mRNA in C. elegans, consistent with the idea that an amplification step involving RdRp is part of the process in lower eukaryotes. If siRNAs can work catalytically in a RISC complex as proposed, they should be more efficient in targeting on a molar basis in mammalian cells. The exact nature of the RISC complex has yet to be determined and only very preliminary data suggests that a single mammalian let7 RISC complex in mouse cells can target more than a few mRNAs [11], far less than the molar efficiency of dsRNA in C. elegans. Proof of the catalytic nature of the RISC complex will have to await its biochemical purification.

Different strategies have been developed to create siRNAs (Table 1). The best reagents are chemically synthesized and are easy to use, but cost is a concern. Conversely, it is possible to synthesize siRNAs in vitro with cDNA oligos containing the minimal promoters for the bacterial polymerases, such as T7, T3 and SP6. An additional

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**Fig. 9.** SiRNA extends the 5’ end of the complementary target. Scheme of the experiment is shown at the left. The $^{32}$P UTP probe that covers the 3’ end 200 nt of GFP is used in RNase protection assay.

**Table 1. Scheme for the synthesis of siRNAs to knock down genes in mammalian cells**

<table>
<thead>
<tr>
<th>Step</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Chemically synthesized</td>
</tr>
<tr>
<td>2.</td>
<td>Synthesis in vitro with cDNA oligo (kit, T7 promoter)</td>
</tr>
<tr>
<td>3.</td>
<td>DNA vectors to create short hairpin RNA (Pol III promoter expression)</td>
</tr>
<tr>
<td>4.</td>
<td>Synthesis in vitro by processing of full length dsRNA + size-separation</td>
</tr>
<tr>
<td>5.</td>
<td>Synthesis in vitro with E. Coli RNase III or Dicer</td>
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</table>
problem arises because one cannot predict the efficacy of a given siRNA since there are no current rules for siRNA design. The potency of each siRNA must be determined empirically. A way to get around this problem is to create a design. The potency of each siRNA must be determined given siRNA since there are no current rules for siRNA problem arises because one cannot predict the efficacy of a silencing purification of these components to try and reconstruct RNA silencing as well as key aspects of development. A greater components in different combinations to affect RNA crucial effectors for RNA silencing, but the exact way in 'knock down' phenomenon.

In conclusion, we now know that the siRNAs are the crucial effectors for RNA silencing, but the exact way in which these short dsRNA exert a silencing effect on target RNA is only beginning to be understood in both lower eukaryotes and mammals. Genetics has identified several RNA is only beginning to be understood in both lower orders of magnitude [12]. Notwithstanding all the difficul-

V. References


pol III promoter. The difficulties with this approach are two fold: first, one must know the position of the most efficient siRNA; and second, the expression level from the different pol III promoters is cell background specific over several orders of magnitude [12]. Not withstanding all the difficul-
ties, the siRNA approach does work in mammalian cells, but at present we do not understand the mechanism behind the ‘knock down’ phenomenon.


