sulfide) came from analyses of their stable carbon (^{13}C) and nitrogen (^{15}N) isotope contents (*7*, *8*). The amount of these isotopes detected in dominant fauna (tube worms, mussels, and clams) did not reflect normal deep-sea carbon and nitrogen. Later studies demonstrated the presence of intracellular chemoautotrophic symbionts in these animals, confirming the nonphotosynthetic nutritional source of carbon and nitrogen. Mussels and clams had δ^3C values of about –30 per mil (‰), in the range that was expected for carbon that is derived from chemoautotrophic bacteria. However, the $\delta^{13}C$ value of *Riftia* was much higher (~ –15‰) and consequently more difficult to understand. A variety of explanations have been put forward to explain these isotope values, but none has proven completely satisfactory (*9*–*11*).

Markert *et al*. find high amounts of enzymes involved in the reductive tricarboxylic acid cycle in extracts of the *Riftia* symbiont and suggest that this is an important pathway of carbon fixation by the symbiont. In addition to the implications for more energy-efficient carbon fixation, this finding may help explain the anomalously high carbon isotope values that have puzzled researchers for decades.

Far less than 1% of the microbes present in nature have been successfully cultured in the laboratory. No chemoautotrophic symbiont has yet been cultured, and it is possible that many never will be. Not only is the milieu of a living host difficult to imitate in vitro, but in some cases, the exchange and integration of host and symbiont genes may have yielded a symbiont more analogous to an organelle than to a free-living microbe. In such instances, genomic and proteomic approaches provide valuable information on the symbiont's metabolic capabilities and evolutionary history. Quantitative proteomics has the additional value of allowing one to use protein expression levels as a metric for studying the importance of metabolic pathways used by these symbiotic microbes in situ.

Many questions remain about these enigmatic animals and their rather extreme life styles. *Riftia*'s trophosome, which is packed with billions of bacteria per gram of tissue, is intertwined with the animal's gonads. Considering the rarity of active hydrothermal vents on the sea floor, and the improbability of larvae finding a suitable home, it is likely that a high percentage of the nutritional input from the symbionts goes directly to reproduction. How is this accomplished and coordinated? Furthermore, transmission of the symbionts between generations is not direct because the larvae are aposymbiotic and newly settled tube worms must acquire their symbionts anew each generation from an apparently free-living pool. How is the metabolism of the free-living stage different from that of the symbiotic stage? Once contact with a host is made, how do the symbionts contribute to successful establishment of the symbiosis? Molecular approaches like that of Markert *et al*. may help answer such questions about life and relationships in this remote and inhospitable environment.

References

1. S. Markert *et al*., *Science* **315**, 247 (2007).
2. A. J. Arp, J. J. Childress, *Science* **213**, 342 (1981).
3. A. J. Arp, J. J. Childress, *Science* **219**, 295 (1983).
4. J. J. Childress, C. R. Fisher, *Oceanogr. Mar. Biol. Annu. Rev.* **30**, 337 (1992).
5. P. R. Girguis, J. J. Childress, *J. Exp. Biol.* **209**, 3516 (2006).
6. H. Akashi, T. Gojobori, *Proc. Natl. Acad. Sci. U.S.A.* **99**, 3695 (2000).
7. G. H. Rau, J. I. Hedges, *Science* **203**, 648 (1979).
8. G. H. Rau, *Can. J. Fish. Aquat. Sci.* **37**, 742 (1980).
9. C. R. Fisher, M. C. Kennicutt II, J. M. Brooks, *Science* **247**, 1094 (1990).
10. J. J. Robinson, J. L. Stein, C. M. Cavanaugh, *J. Bacteriol.* **180**, 1596 (1998).
11. K. M. Scott, *Environ. Microbiol.* **5**, 424 (2003).

10.1126/science.1137739

MOLECULAR BIOLOGY

Amplified Silencing

Small RNA molecules that silence gene expression are amplified by different mechanisms in nematodes and plants.

David C. Baulcombe

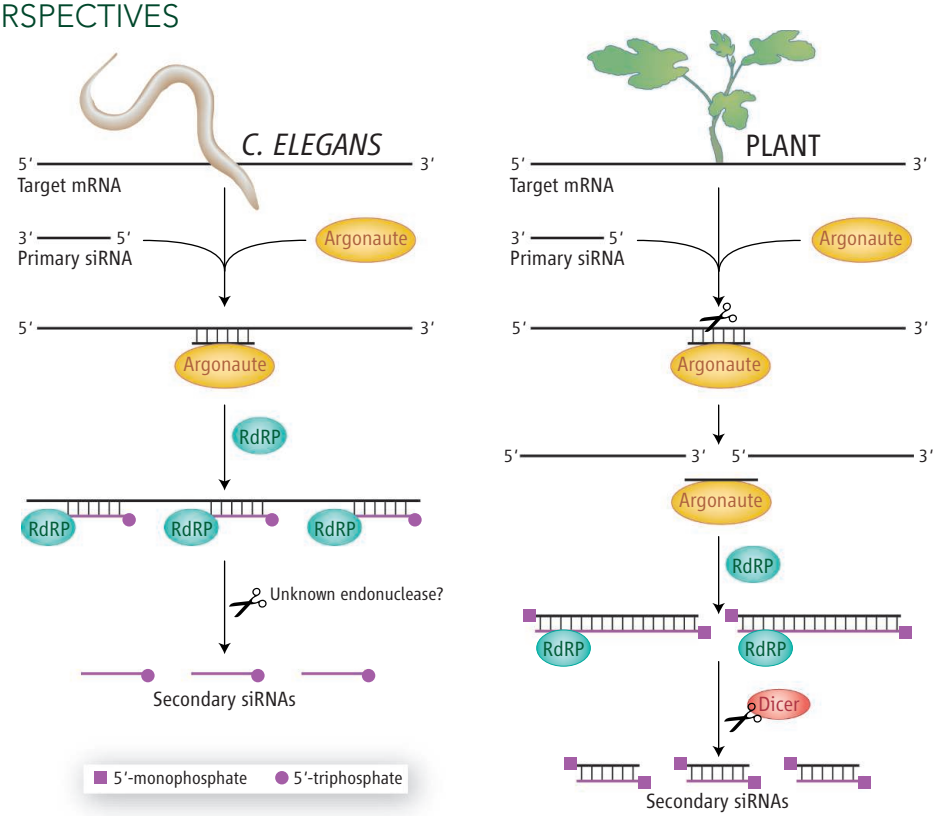
Ten years ago, we knew nothing about how double-stranded RNA blocks gene expression through the silencing of targeted RNA. We now have a good understanding of this process, and current interest is turning to variations on the basic mechanism. Recent studies involving plants and the nematode *Caenorhabditis elegans* continue this trend, including those reported in this issue by Pak and Fire on page 241 (*1*) and Sijen *et al.* on page 244 (*2*). Two other papers by Axtell *et al.* (*3*) and Ruby *et al.* (*4*) are also relevant. These studies deal with the amplification of silencing-related RNA and explain how strong, persistent silencing can be initiated with small amounts of "initiator" double-stranded RNA. The amplification process has implications for application of RNA interference to control gene expression in biotechnology and for understanding the effects of silencing RNAs on cell function and organism development.

Specifically, these new studies investigate how the target of silencing can spread (or transit) within a single strand of RNA. The initiator of transitivity is a double-stranded RNA that is first processed by Dicer, a ribonuclease III–like enzyme, into short interfering RNA (siRNA) or a related type of RNA referred to as microRNA (miRNA). These 21- to 25-nucleotide single-stranded RNAs are the primary silencing RNAs in the transitive process. A primary silencing RNA binds to a ribonuclease H–like protein of the Argonaute class. The resulting Argonaute ribonucleoprotein can target long RNA molecules by Watson-Crick base pairing. The targeted RNA then becomes a source of secondary siRNAs. Transitivity occurs when the secondary siRNAs correspond to regions adjacent to the target sites of the primary silencing RNA.

RNA-directed RNA polymerases (RdRPs) produce secondary siRNA, and the new results indicate that they catalyze two different mechanisms of silencing amplification. One mechanism is characterized by Axtell *et al*. (*3*), who investigated endogenous secondary siRNAs in plants. They show that efficient secondary siRNA production occurs if a single-stranded RNA has two target sites for the Argonaute ribonucleoprotein. Optimal secondary siRNA production occurs when the targeted RNA is cleaved by Argonaute. Cleaved RNA then recruits RdRP, which generates double-stranded RNA. Dicer then produces transitive secondary siRNAs (see the figure).

Another biogenesis mechanism of secondary siRNAs has, so far, only been described in *C. elegans*. The discovery of this distinct mechanism by Sijen *et al*., Pak and Fire, and Ruby *et al*. follows from the observation that a type of siRNA is underrepresented in

The author is in the Sainsbury Laboratory, John Innes Centre, Norwich NR4 7UH, UK. E-mail: david.baulcombe@sainsbury-laboratory.ac.uk



Secondary siRNA production in plants and animals. Secondary siRNAs are produced by RdRP-mediated transcription of RNA that has been targeted by a primary siRNA or miRNA. In *C. elegans* (**left**), an Argonaute protein associated with a primary siRNA targets a long single-stranded RNA and recruits an RdRP that synthesizes 22–23 nucleotide secondary siRNAs directly. In plants (**right**), the recruitment of RdRP is optimal when the long single-stranded RNA has two targets for primary siRNA or miRNA (only one is shown). The targeted RNA is then converted to long double-stranded RNA by the RdRP and secondary siRNAs are generated after cleavage by Dicer.

sequence databases. This scarceness is because these siRNAs have a 5′-triphosphate and are thus excluded by the standard methods for cloning and sequence analysis. These methods are normally specific for RNA with a 5′-monophosphate, the hallmark of Dicer cleavage.

In addition to their 5′-triphosphorylation, these siRNAs are distinct from the primary silencing RNAs in that they have a strand bias. They predominantly are antisense to the target of the primary silencing RNA. The secondary siRNAs also have the surprising characteristic that they are phased relative to each other (*2*, *4*): The first siRNA covers 22 nucleotides starting close to the target site of the primary siRNA, the second siRNA is then the adjacent 22 nucleotides, and so on (see the figure). One explaination for these features might be that the 5′-triphosphorylated secondary siRNAs are generated when RdRPs are recruited to a target of the primary silencing RNA. Short antisense RNAs are then synthesized de novo, and the presence of the 5′-triphosphate in the first incorporated nucleotide is diagnostic of secondary siRNAs made by this mechanism. Sijen *et al*. rule out primary siRNA as a primer in this mechanism because mismatches in its sequence relative to that of a target RNA are absent in the secondary siRNAs. To explain the rather precise size (22 or 23 nucleotides) of the secondary sRNAs, this model requires that the RdRP automatically terminates RNA synthesis at a defined site or that the transcription products be cleaved at their 3′ end by an unidentified endonuclease.

What is the natural role of these transitive secondary siRNAs? In plants, they target messenger RNAs (mRNAs) (*3*), and it is likely that they do the same in *C. elegans* because endogenous siRNAs with 5′-triphosphate correspond to the antisense of mRNA coding sequences (*1*). Moreover, Yigit *et al*. (*5*) describe how secondary siRNAs are bound to a specific class of Argonaute proteins and that they direct RNA cleavage. It is likely, therefore, that secondary siRNAs regulate gene expression in situations where amplification of silencing is important.

A clue to the type of situation in which secondary siRNA might be important comes from experimental RNA interference in *C. elegans* and transitive transgene silencing in plants. In both systems, transitivity and secondary siRNA production amplify silencing-related RNAs so that silencing persists in the absence of the initiator double-stranded RNA. In some instances associated with this persistence, there are epigenetic effects at the DNA or chromatin level (*6*, *7*). On the basis of these observations, and reasoning that experimental systems may illustrate elements of the natural mechanisms, it seems likely that the endogenous secondary siRNAs could mediate effects of silencing that persist in the absence of the initiator double-stranded RNA. Perhaps the amplified secondary siRNAs influence processes such as developmental timing in which the effects of a silencing trigger might persist after their initial induction. Consistent with this idea, secondary siRNAs in the plant *Arabidopsis thaliana* affect the timing of the developmental transition between adult and juvenile growth phases (*8*).

In addition to the biological implications of the amplification mechanisms, there are two technical issues. First, from a biotechnological perspective, it would be advantageous if the amplification mechanisms could be harnessed to enhance silencing in therapeutic or genomic applications. The absence of RdRP genes in the fly *Drosophila melanogaster* and in mammalian genomes indicates that this effect might not be possible in all organisms. However, there are recently described siRNA-like species in *Drosophila* (*9*) with the phased and strand-bias characteristics of secondary siRNAs in *C. elegans*. Perhaps there are other enzymes in mammals that can substitute for the RdRP proteins in an amplification process. The second technical point is a cautionary message about methods for high-throughput sequencing of siRNA populations. Secondary siRNAs with 5′-triphosphates are excluded from many of the methods associated with this technology, and amplified siRNAs would be missed. Fortunately, two of the *C. elegans* papers (*1*, *2*) describe methods for cloning and sequencing siRNA with 5′-triphosphate. We will now see to what extent the existing sequence databases will need to be revised to account for 5′-triphosphorylated siRNAs.

References

1. J. Pak, A. Fire, *Science* **315**, 241 (2007); published online 23 November 2006 (10.1126/science.1132839).
2. T. Sijen, F. A. Steiner, K. L. Thijssen, R. H. A. Plasterk, *Science* **315**, 244 (2007); published online 7 December 2006 (10.1126/science.1136699).
3. M. J. Axtell, C. Jan, R. Rajagopalan, D. P. Bartel, *Cell* **127**, 565 (2006).
4. J. G. Ruby *et al.*, *Cell* **127**, 1193 (2006).
5. E. Yigit *et al.*, *Cell* **127**, 747 (2006).
6. N. L. Vastenhouw *et al.*, *Nature* **442**, 882 (2006).
7. O. Voinnet, P. Vain, S. Angell, D. C. Baulcombe, *Cell* **95**, 177 (1998).
8. N. Fahlgren *et al.*, *Curr. Biol.* **16**, 939 (2006).
9. V. V. Vagin *et al.*, *Science* **313**, 320 (2006).

10.1126/science.1138030

Amplified Silencing

David C. Baulcombe

Science **315** (5809), 199-200.
DOI: 10.1126/science.1138030