technically speaking

RNAi: RNA Interference

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Abstract

RNA interference (RNAi) is a rapidly emerging and powerful technique used to investigate gene function by degrading a specific mRNA target in a cell or organism and thus knocking out or knocking down the level of the encoded protein. This specific mRNA degradation is mediated by complementary double-stranded RNA. This article reviews RNAi and its relationship to other RNA-mediated mechanisms generally referred to as RNA silencing. This review also addresses the involvement of short interfering RNAs (siRNAs), microRNAs (miRNAs), small temporal RNAs (stRNAs), and short hairpin RNAs (shRNAs) in various RNA silencing mechanisms.

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(Editor's Note: *Technically Speaking* appears in this issue of *Promega Notes* in the form of an article so that we can more thoroughly discuss this exciting new technology.)

History

RNA interference (RNAi) is a phenomenon in which double-stranded RNA (dsRNA) specifically suppresses expression of a target protein by stimulating the specific degradation of the complementary target mRNA (see references 1–8 for reviews on RNA silencing in general, including RNAi). It burst onto the scene at the RNA Society meeting in May 2001, but the first hints of the phenomenon appeared in 1990, when Rich Jorgensen was attempting to engineer petunias with more intense purple color by introducing exogenous transgenes, which unexpectedly showed variegated pigmentation (9). The introduced DNA sequences somehow affected expression of the endogenous loci. The phenomenon was given the name "co-suppression". At the same time, plant laboratories were finding that plants responded to infection by RNA viruses by specifically targeting the viral RNA for degradation (10–13). These phenomena observed in plants have since been termed "post-transcriptional gene silencing" (PTGS) or "viral-induced gene silencing" (VIGS) (14,15).

In 1995 Guo and Kemphues found that sense RNA was as effective as antisense RNA for suppressing gene expression in *C. elegans* (16). This work was extended in the seminal RNAi paper by Fire *et al.*, in which they demonstrated that double-stranded RNA (dsRNA) was tenfold more potent at reducing gene expression in *C. elegans* as compared to sense or antisense RNAs alone (17).

Since its report in *C. elegans* (17), RNAi has been reported to occur in a variety of organisms, including zebrafish (18), planaria (19), hydra (20), fungi (21), *Drosophila* (22), and mammalian mouse embryo systems (23,24). The response to dsRNA in fungi like *Neurospora crassa* has been termed "quelling", which refers to the silencing of an endogenous gene by introduction of a transgenic copy of that gene (4,15,21).

These phenomena have been collectively termed RNA silencing and appear to use a common set of proteins and short RNAs. These processes are mechanistically similar, though not identical.

Mechanism

In most mammalian systems, the introduction of longer dsRNAs (> 30bp) induces a potent antiviral response that results in activation of a dsRNA-activated protein kinase, PKR, which phosphorylates EIF-2α, inducing in turn a generalized inhibition of translation (25). In addition, dsRNA activates the 2′–5′ oligoadenylate polymerase/RNase L system and represses IkB, which can induce cell death via apoptosis. Therefore, it was welcome news when Tuschl and coworkers and Fire and colleagues showed that chemically synthesized short-interfering RNAs (siRNAs) could induce specific gene silencing in a wide range of mammalian cell lines without causing apoptosis (26,27). This was also observed in vitro in *Drosophila* embryo extracts (28–31).

Mechanistically, RNAi involves a multistep process (Figure 1). The dsRNA is recognized by an RNase III family member (e.g., Dicer in *Drosophila*) and is cleaved into siRNAs of 21–23 nucleotides (28,32,33). In the next step, the siRNAs are incorporated into an RNAi targeting complex known as RISC (RNA-induced silencing complex), which destroys mRNAs that are homologous to the integral siRNA (32,33). The target mRNA is cleaved in the center of the region complementary to the siRNA (28), with the net result being a rapid degradation of the target mRNA and a decrease in protein expression. The most potent siRNA duplexes are 21 nucleotides long, comprising a 19bp sequence with a 2-uridine 3′ overhang at each end (28).

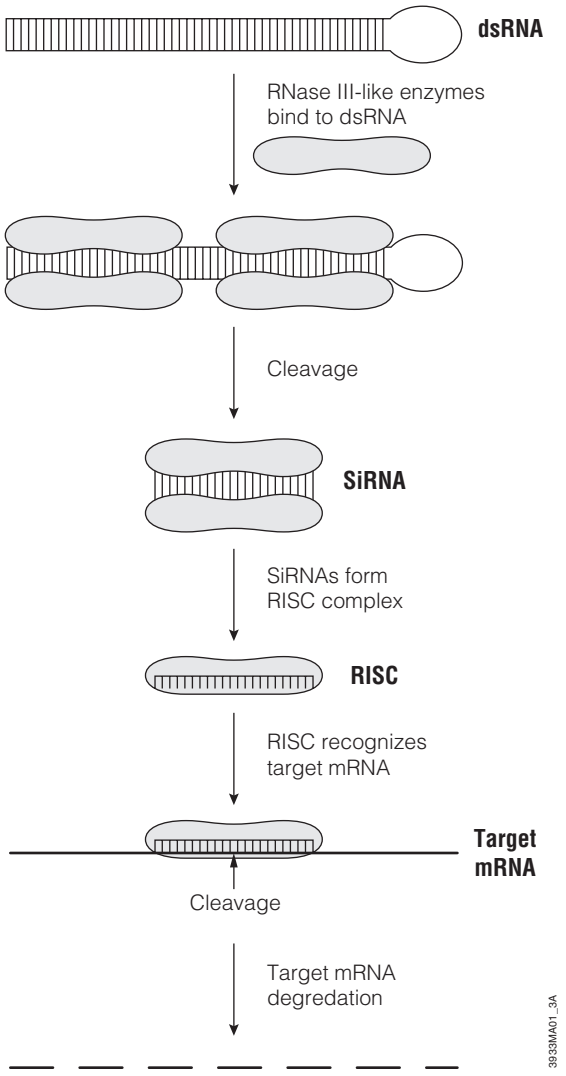

Figure 1. Proposed mechanism of RNAi. The dsRNA-processing proteins, which belong to a family of RNase III, bind to the dsRNA. The RNase III cleaves the dsRNA into siRNA. The siRNA are proposed to form multicomponent nuclease complexes (RISC). The target mRNA recognized by RISC is cleaved in the center of the region complementary to the siRNA, and the target mRNA is quickly degraded.

The Human homolog of Dicer has been cloned, expressed and extensively characterized and was found to colocalize with calreticulin in the endoplasmic reticulum (34,35). Dicer is a large multidomain enzyme including a putative DexH/DEAH RNA helicase/ATPase domain, a PAZ signature, two neighboring RNase III-like domains, and a dsRNA-binding domain (RBD). The cleavage of dsRNA by Dicer does not require ATP, but ATP may be required for product release (34). RNA interference in human cells was shown to be restricted to the cytoplasm or to those mRNAs undergoing nuclear export (36).

Notable differences between RNAi in various eukaryotic organisms include the heritable and systemic nature of silencing in *C. elegans* compared to the apparently autonomous and nonheritable silencing observed in *Drosophila* and mammals (7). Similar systemic silencing has been observed in plants, and a recently identified phenomenon termed "transitive RNAi" may provide insight into the differences between *C. elegans* and higher eukaryotes.

Transitive RNA refers to the movement of the RNAi silencing signal along a particular gene. For example, in *C. elegns* targeting the 3′ portion of an mRNA results in production of siRNAs homogous to the targeted region and suppression of that transcript. In addition, siRNAs complementary to regions of the mRNA upstream of the area initially targeted by the silencing trigger also appear to accumulate. If these siRNAs are complementary to other mRNAs, those mRNAs are targeted for silencing as well. Thus, silencing can travel in a 3′ to 5′ direction on a specific mRNA target and can also proceed to other mRNA targets that share regions of sequence homology to the initial target.

The transitive RNA phenomenon appears to require an RNA-dependent RNA polymerase (RdRP) and has led to a model in which siRNAs might prime synthesis of additional dsRNA (and siRNAs) by RdRPs (37). However, RdRPs do not appear to be necessary for RNAi in flies or mammals (38,39).

Synthesis and Use of dsRNA

The synthesis of either longer double-stranded RNAs (~200–1,000bp) or shorter siRNAs (21–23bp) has been accomplished using in vitro transcription with T7 RNA polymerase (17,40,41). siRNAs can also be chemically synthesized, and there are a number of commercial suppliers for such siRNAs, with Dharmacon Research, Inc., being the most widely utilized. Yu *et al.* (41) demonstrated comparable efficacy between in vitro synthesized siRNA and chemically synthesized siRNA. In addition, siRNAs may be generated from longer dsRNAs by in vitro digestion with purified *E. coli* Exonuclease III (42,43). Enzymatically processing long dsRNAs to siRNAs allows for the generation of a great variety of siRNAs capable of interacting with multiple sites on the target mRNA, increasing the likelihood of a decrease in protein levels and a detectable biological response.

More recently, the use of specially designed in vivo expression vectors is obviating the synthesis and introduction of dsRNA/siRNA into cells or organisms. The use of RNA polymerase III promoters, which possess a well-defined transcription start signal and a termination signal consisting of five consecutive thymidine residues, in which the cleavage of the nascent RNA transcript occurs after the second uridine (44), has allowed for the in vivo synthesis of siRNAs with optimal structure. The HI (45,46) and U6 (41,47) promoters have been used for this purpose. In addition, the use of viral

or retroviral vectors has shown successful for in vivo synthesis of siRNAs in cells or tissues (48,49). The in vivo expression of siRNAs generally takes the form of short hairpin RNAs (shRNAs), in which ~19 nucleotides of perfectly matched base pairing is connected by various spacer regions and ends in a 2-nucleotide 3´-overhang (41,45–49). McManus *et al.* (47) demonstrated that even slight modifications in hairpin structure affect silencing activity, so design of the full transcript is critical.

The introduction of dsRNA/siRNA to initiate RNAi may be accomplished using a number of techniques. In the case of *C. elegans*, dsRNA may be injected (17,50), soaked (51), or fed (52–54). *Drosophila* cells in culture are simply exposed to the dsRNA in the culture medium (55), while the introduction of siRNA into mammalian cells requires the use of a transfection reagent or method (26,27).

RNA Silencing and Other Small RNAs

Although hundreds of small, single-stranded RNAs, termed micro-RNAs (miRNAs), have been identified, only two have documented functions, the *lin-4* and *let-7* miRNAs, which are temporally expressed in *C. elegans* and are therefore called small temporal RNAs (stRNAs) (56). *Let-7* is present in most, if not all, bilaterally symmetric animals, including *Drosophila* and humans (57). The small temporal RNAs induce developmental progression by negatively regulating the expression of proteins encoded by mRNAs whose 3' untranslated regions contain sites complementary to the stRNAs (56). This process has been called "translational repression" and does not require perfect complementarity to the hybridized mRNA (58). It has been shown that stRNAs are processed from stable larger (~70 nucleotide) stem-loop precursors by Dicer and its homologs, suggesting a common processing machinery for generating siRNAs and stRNAs (59–61). Synthetic hairpin RNAs that mimic siRNAs and miRNAs can effectively target a gene for silencing through mRNA degradation (47). It has been suggested that RISC is a flexible platform upon which different regulatory RNA species may be superimposed, resulting in different RNA silencing mechanisms (7). However, the outcome always involves the decrease in the amount of target proteins.

RNAi and the Genome

Recent work has demonstrated that the protein components and short RNAs that mediate RNAi are also necessary for essential chromosomal functions, linking RNAi and maintenance of genomic integrity (reviewed in references 5–8). RNA-based silencing mechanisms may act as an ancient "immune system" for the genome to protect itself against viruses and transposable elements and to eliminate defective mRNAs. The RNAi machinery appears to induce genomic methylation in plants and alter chromatin structure in *Drosophila*, *C. elegans* and fungi (7).

Applications and Future Directions

RNAi has been used in mammalian cells to identify gene products essential for cell growth (62). In addition, Irie *et al.* demonstrated the sub-type and species-specific knockdown of various PKC isoforms in both human and rat cells and detected UV-irradiated siRNA-transfected cells using a thymine dimer antibody (63).

RNAi has been used to specifically target degradation of an oncogene product, which resulted in the subsequent death by apoptosis of leukemia cells transformed by this protein (64). The use of RNAi to specifically target and prevent viral infections by either HIV-1 (65) or HCV (66) in cell culture (65) or intact animals (66) has been demonstrated as well. Poliovirus infection was shown to be attenuated with siRNAs targeted to either a capsid-protein mRNA or the viral polymerase gene (67). Studies by Xia *et al.* explored the use of viral-mediated delivery methods in vitro and in vivo to eventually target polyglutamine diseases (48). These observations open the field for further studies toward novel gene therapy approaches for anti-cancer or anti-viral treatments using RNAi and siRNAs or shRNAs (68).

Conclusions

RNA interference and other complex RNA silencing phenomena that are regulated by short RNA molecules, either endogenously expressed or exogenously introduced, have begun to revolutionize biology today in organisms from single-cell protozoa to mammals. It will permit loss-of-function genetic screens and rapid tests for genetic interactions in mammalian cells, which up to this point have been quite difficult to perform quickly (7).

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