RNA INTERFERENCE TECHNOLOGY AND ITS POTENTIAL USE IN FUNCTIONAL GENOMIC ANALYSIS

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ABSTRACT

RNA interference (RNAi) refers to the introduction of homologous double stranded RNA (dsRNA) to specifically target a gene's product, resulting in null or hypomorphic phenotypes. RNAi is highly gene specific,so it is much more effective than either sense or antisense RNA for producing gene interference activity. RNAi has recently emerged as a powerful reverse genetic tool to silence gene expression in the field of functional genomic analysis, including plants, *Caenorhabditis elegans*, and Drosophila for it allows researchers to study the effects of genomic loss of function in developing embryos without the complications of the gene knockout method.

Keywords: RNAi, functional genomic analysis

1. INTRODUCTION

1.1 Discovery of RNA Interference (RNAi)

Recently scientists working in different research fields observed a phenomenon they could not immediately understand. Plant biologists were attempting to boost the activity of the gene for chalcone synthase, an enzyme involved in the production of anthocyanin pigments, by introducing a powerful promotor sequence into their petunias. However, instead of a deep purple colour, many of the flowers grew variegated, or virgin white. The researchers concluded that the introduced chalcone synthase gene had somehow muted both itself and a normal petunia gene. Joergensen *et al.* termed this phenomenon of gene silencing "cosuppression" [1]. Cosuppression is the ability of some transgenes to silence both themselves and homologous chromosomal loci simultaneously. The initiator molecule for cosuppression is believed to be aberrant RNA, possibly dsRNA, and some components of the RNAi machinery are required for post-transcriptional silencing by co-suppression [2, 3, 4].

These findings were supported by another group studying plant RNA viruses. Baulcombe *et al.* [5] were expressing genes from the potato virus X in tobacco plants. The researchers hoped that viral proteins produced by the plants would stimulate its defence allowing the plants to resist subsequent attack by the virus itself. To their surprise the plants with the strongest resistance were those in which the introduced gene was silent. The researchers concluded that the introduced gene was co-suppressing both itself and the same gene in the virus.

Guo and Kemphues [6] working with *Caenorhabditis elegans* obtained strange results in their antisense experiments. The theory behind the antisense approach is to inject complementary RNA sequences into the target organism to block the targeted mRNA. The two sequences should then hybridize stopping the production of the encoded protein. To their surprise even the injected sense strand was active [6]. This was later explained as the sense strand used was contaminated with very small amounts of the corresponding antisense strand. In a classic antisense approach these small contaminations would have shown no effect at all.

The mystery was solved in 1998 by Fire *et al.* (1998), who showed that injection of dsRNA for specific genes into *C. elegans* caused a specific disappearance of the gene products from both the somatic cells and the F1 progeny. Injection of single stranded RNA for the genes had little effect. Use of dsRNA unrelated to the specific gene had no effect. They also showed that the effect was on the stability of the mRNA. The dsRNA appeared to cross cellular barriers and could work at a distance from the injection, and only a few molecules of dsRNA per cell were required, suggesting that a catalytic or amplification process was occurring. Only dsRNA sequences from exons had an effect; sequences from introns had no effect.

This phenomenon, termed RNA interference or RNAi, was soon shown to occur in a variety of organisms, including plants, zebra fish, Drosophila, mice, planaria, and even the protozoan, *Trypanosoma brucei*. The phenomenon was shown to be post-transcriptional by Montgomery *et al.* (1998).

1.2 Post-transcriptional gene silencing and RNA interference

As often happens in science, another phenomenon was being studied in another system that initially was thought to be unrelated but turned out to be identical to RNAi. In plants and fungi, a phenomenon known as post-transcriptional gene silencing (PTGS) had been intensively studied for the last 10 years. PTGS is a regulatory process in which the steady-state level of a specific messenger RNA (mRNA) is reduced by sequence-specific degradation of the transcribed, usually fully processed mRNA without an alteration in the rate of transcription of the target gene itself. If PTGS is explicitly mediated by dsRNA, the term RNA interference (RNAi) is preferred, but there may also be non-dsRNA sources, often termed aberrant RNAs, that may function as initiators of PTGS. Such aberrant RNAs may serve as templates for the putative RNA-dependent RNA polymerases (RdRPs) which have been identified in plants [7, 8, 9±11], fungi [12], and *C. elegans* [13] and which are believed to produce dsRNA in order to initiate and maintain silencing. This idea was derived from the pioneering biochemical analysis of RdRP purified from tomato leaves [14]. Tomato RdRP synthesizes short RNAs from single-stranded RNA or DNA oligonucleotide templates and it initiates transcription near the 3' end of an RNA template without the requirement for an oligonucleotide primer. It should, however, be noted, that RdRP activity has not yet been demonstrated for any other RdRP homologue.

Natural sources for aberrant RNAs or dsRNAs may be repetitive and mobile genetic elements such as transposons, or natural viruses. Integration of such elements nearby the promoters of endogenous genes is hypothesized to lead to unexpected antisense transcripts that at least partially anneal to the sense transcript to form dsRNA. Similarly, randomly integrated transgenes are responsible for activation of PTGS in plants. The probability of inducing PTGS by transgene integration is especially high if sense and antisense transcripts are expressed simultaneously [15], or if inverted repeat genes are introduced, in which the RNA transcript can fold back on itself to produce a dsRNA hairpin [16]. It is also debated whether tandem or dispersed copies of genes, which are subject to transcriptional silencing are capable of triggering post-transcriptional silencing [17].

Calculations of the amount of dsRNA injected into *C. elegans* or *Drosophila melanogaster* suggest that only a few molecules of dsRNA per cell are sufficient to mount an RNAi response [18, 19]. It may therefore be extremely difficult to detect aberrant RNAs or dsRNAs that trigger cosuppression and RNAi in an organism. The extraordinary sensitivity of the cell towards aberrant RNAs or dsRNA is also illustrated by the success of earlier experiments in *C. elegans* in which silencing was observed after injection of what was thought to be single-stranded sense or antisense RNAs [20]. It was only realized later that the effect was due to the small amount of dsRNA that generally contaminates RNA transcribed in vitro [21].

2. THE MECHANISM OF RNAi

So how does injection of dsRNA lead to gene silencing? Many research groups have diligently worked over the last few years to answer this important question. A key finding by Baulcombe and Hamilton provided the first clue. They identified RNAs of ~25 nucleotides in plants undergoing cosuppression that were absent in non-silenced plants. These RNAs were complementary to both the sense and antisense strands of the gene being silenced [22].

Further work in Drosophila — using embryo lysates and an in vitro system derived from S2 cells — shed more light on the subject [23, 24, 25]. In one notable series of experiments, Zamore and colleagues found that dsRNA added to Drosophila embryo lysates was processed to 21-23 nucleotide species. They also found that the homologous endogenous mRNA was cleaved only in the region corresponding to the introduced dsRNA and that cleavage occurred at 21-23 nucleotide intervals (25). Rapidly, the mechanism of RNAi was becoming clear.

3. MODELS OF THE RNAi MECHANISM

Both biochemical and genetic approaches have led to the current models of the RNAi mechanism. In these models (Figure 1), RNAi includes both initiation and effector steps. In the initiation step, input dsRNA (RNAs could be injected directly into the target tissue) is digested into 21-23 nucleotide small interfering RNAs (siRNAs), which have also been called "guide RNAs" [26, 27]. *C. elegans* and other nematodes can uptake dsRNA when soaked in dsRNA solution or fed plasmids that make dsRNA and consequently exhibit RNAi effects (Tabara *et al.*, 1998). This is the special characteristic of RNAi where dsRNAs can be transported across cell boundaries and be distributed to target tissues. Evidence indicates that siRNAs are produced when the enzyme Dicer, a member of the RNase III family of dsRNA-specific ribonucleases, processively cleaves dsRNA (introduced directly or via a transgene or virus) in an ATP-dependent, processive manner. Successive cleavage events degrade the RNA to 19-21 bp duplexes (siRNAs), each with 2-nucleotide 3' overhangs [27, 28].

In the effector step, the siRNA duplexes bind to a nuclease complex to form what is known as the RNA-induced silencing complex, or RISC. An ATP-depending unwinding of the siRNA duplex is required for activation of the RISC. The active RISC then targets the homologous transcript by base pairing interactions and cleaves the mRNA ~12 nucleotides from the 3' terminus of the siRNA [18, 27, 29]. Although the mechanism of cleavage is at this date unclear, research indicates that each RISC contains a single siRNA and an RNase that appears to be distinct from Dicer [27].

Due to the remarkable potency of RNAi in some organisms, an amplification step within the RNAi pathway has also been proposed. Amplification could occur by copying of the input dsRNAs, which would generate more siRNAs, or by replication of the siRNAs themselves. Alternatively or in addition, amplification could be effected by multiple turnover events of the RISC [18, 27].

4. RNAi AS A TOOL FOR FUNCTIONAL GENOMICS

Although the history and mechanism of RNAi and PTGS are fascinating, many researchers are most excited about RNAi's potential use as a functional genomics tool. Already RNAi has been used to ascertain the function of many genes in Drosophila, *C. elegans*, and several species of plants. With the knowledge that RNAi can be induced in mammalian cells by the transfection of siRNAs, many more researchers are beginning to use RNAi as a tool in human, mouse and other mammalian cell culture systems.

In early experiments with mammalian cells, the siRNAs were synthesized chemically (Ambion is one of several companies that offer custom siRNA synthesis). Recently, Ambion introduced a kit (the Silencer™ siRNA Construction Kit) to produce siRNAs by in vitro transcription, which is a less expensive alternative to chemical synthesis, particularly when multiple different siRNAs need to be synthesized. Once made, the siRNAs are introduced into cells via transient transfection. Due to differences in efficacy, most researchers will synthesize 3–4 siRNAs to a target gene and perform pilot experiments to determine the most effective one. Transient silencing of more than 90% has been observed with this type of approach [30-32, 33, 34].

So far, injection and transfection of dsRNA into cells and organisms have been the main method of delivery of siRNA. While the silencing effect lasts for several days and does appear to be transferred to daughter cells, it does eventually diminish. Recently, however, a number of groups have developed expression vectors to continually express siRNAs in transiently and stably transfected mammalian cells [35-41]. Some of these vectors have been engineered to express small hairpin RNAs (shRNAs), which get processed in vivo into siRNAs-like molecules capable of carrying out gene-specific silencing [35, 38, 39, 41]. The vectors contain the shRNA sequence between a polymerase III (pol III) promoter and a 4-5 thymidine transcription termination site. The transcript is terminated at position 2 of the termination site (pol III transcripts naturally lack poly(A) tails) and then folds into a stem-loop structure with 3' UU-overhangs. The ends of the shRNAs are processed in vivo, converting the shRNAs into ~21 nt siRNA-like molecules, which in turn initiate RNAi [35]. This latter finding correlates with recent experiments in *C. elegans*, Drosophila, plants and Trypanosomes, where RNAi has been induced by an RNA molecule that folds into a stem-loop structure [42].

Another siRNA expression vector developed by a different research group encodes the sense and antisense siRNA strands under control of separate pol III promoters [37]. The siRNA strands from this vector, like the shRNAs of the other vectors, have 5 thymidine termination signals. Silencing efficacy by both types of expression vectors was comparable to that induced by transiently transfecting siRNA.

The recent studies on RNAi have taken the research world by storm. The ability to quickly and easily create loss-of-function phenotypes has researchers rushing to learn as much as they can about RNAi and the characteristics of effective siRNAs. In the future, RNAi may even hold promise for development of gene-specific therapeutics. Much has been learned about this powerful technique, but additional information becomes available on an almost daily basis. It is not an understatement to say that the field of functional genomics is being revolutionized by RNAi.

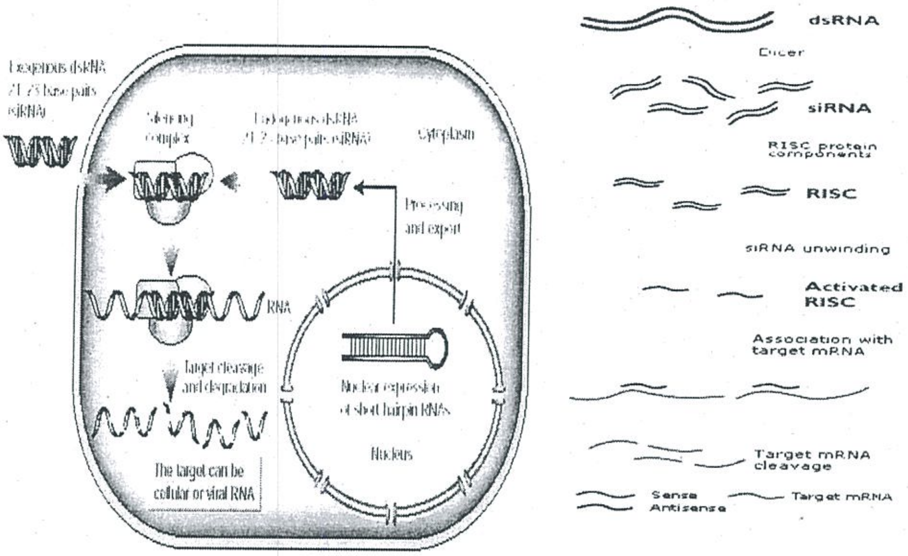

Figure 1. Models of the RNAi Mechanism

Dicer

- Enzyme belonging to the RNase III ribonuclease family
- Responsible for the processing of dsRNA into siRNAs
- Initiates RNAi

Small Interfering RNA (siRNA)

- 21-23 nucleotide dsRNA that mediate PTGS
- Produced in vivo by cleavage of dsRNA
- Amplification by an RNA-dependent RNA polymerase (RdRP) may occur
- Incorporated into the RISC guiding it to mRNA

RNA-Induced Silencing Complex (RISC)

- Nuclease complex composed of proteins and siRNA
- Targets and destroys endogenous mRNAs complementary to the siRNA

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