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VIEWPOINT

RNA Silencing: The Genome's Immune System

Ronald H. A. Plasterk

Genomes are databases sensitive to invasion by viruses. In recent years, a defense mechanism has been discovered, which turns out to be conserved among eukaryotes. The system can be compared to the immune system in several ways: It has specificity against foreign elements and the ability to amplify and raise a massive response against an invading nucleic acid. The latter property is beginning to be understood at the molecular level.

All genomes of complex organisms are potential targets of invasion by viruses and transposable elements. Forty-five percent of the human genome consists of remnants of previous transposon/virus invasions and elements that are still active to date: 21% long interspersed nuclear elements, 13% short interspersed nuclear elements, 8% retroviruses, and 3% DNA-transposons, as compared with less than 2% that encodes (nontransposon) proteins. A priori, one would expect that organisms need to fight off such invasions to prevent the genome from being completely taken over by molecular invaders. The two problems with which the organism is faced in protecting the integrity of the genome are similar to those faced by the vertebrate immune system: (i) how to recognize self from nonself, and (ii) how to amplify an initial response in a specific fashion.

The vertebrate immune system fights off invaders using a two-step strategy: a large repertoire of antibody-encoding genes is generated from a limited set of gene segments by combinatorial gene rearrangements, and this repertoire is stored in a distributed fashion over large numbers of cells. After infection, clonal selection and expansion of a few of these cells results in an immune response specifically directed to the immunogen. The vertebrate immune system has solved the specificity problem by initially generating a more or less random repertoire, which, during a phase of early development, is limited by a filtering process, called tolerance induction: cells raised against self antigens are excluded from the mature immune system.

How does the genome recognize invaders and raise an overwhelming and specific "immune response" against them? One strategy to suppress transposons may be the selective methylation of transposon sequences in the genome (*1*), although it has also been argued that this phenomenon is a secondary effect of suppression (*2*). This will not be discussed further, but see a recent review for more information (*3*). In recent years, an RNA-based silencing mechanism has emerged that is ancient, conserved among species from different kingdoms (fungi, animals, and plants), and very likely acts as the "immune system" of the genome. This system was initially independently discovered and studied in different organisms before it was recognized that the underlying mechanisms are at some level identical. Posttranscriptional gene silencing (PTGS) and co-suppression in plants (*4*, *5*), as well as RNA-mediated virus resistance in plants (*6*), RNA interference in animals [first discovered in *Caenorhabditis elegans* (*7*)], and silencing in fungi ["quelling" in Neurospora (*8*)] and algae (*9*) are all based on the same core mechanism. This conclusion is based on the discovery of common mechanistic elements [such as the small interfering RNAs (siRNAs) (*10*)] and of homology between genes required for this mechanism in plants, animals, and fungi and algae.

The precise mechanism of this group of phenomena, now referred to as RNA silencing, is being rapidly unraveled. The aspect that I specifically address here is the equivalent in RNA silencing of "clonal selection," which allows the vertebrate immune system to raise a massive immune response (*11–14*).

The Function of RNA Silencing

Neither nematodes nor flies normally encounter highly concentrated double-stranded RNA (dsRNA) of identical sequence to one of their endogenous genes. Nevertheless, genetic analysis indicates that the number of genes required for gene silencing triggered by exogenous dsRNA is probably larger than 10 (*15–18*). What is the natural function of this elaborate pathway?

The clearest picture is seen in plants, where PTGS and virus-induced gene silencing are recognized as mechanisms that protect against frequently occurring viral infections (*6*, *19*). An advantage of this defense system is that the defensive signal can spread, such that inoculation in one area of a leaf can confer immunity on surrounding cells. A study in this issue shows that an animal virus also encodes a suppressor of RNA interference (RNAi), supporting the notion that RNAi may have an antiviral function in animals as well (*20*). In nematodes, loss of function of genes required for RNAi results in the activation of multiple transposable elements in the germline (*15*), indicating that they function to repress the spreading of transposons within the genome of subsequent generations of worms.

Protection against viruses and transposons may be the natural function of the core of the RNAi pathway, but it does not explain all aspects of what is now considered to be RNAi. One of the most striking features of RNAi in *C. elegans* is the systemic effect. Injection of naked dsRNA into one region of the animal may affect gene expression elsewhere, and dsRNA present in the lumen of the gut as part of the food is apparently taken up and affects gene expression in progeny that arises in the gonads (*21*). In plants, grafting experiments have shown immunity traveling over 30 cm of stem tissue (*22*); this

Hubrecht Laboratory, Centre for Biomedical Genetics, Uppsalalaan 8, 3584 CT Utrecht, Netherlands. E-mail: Plasterk@niob.knaw.nl

ability may add to the protective effect in case of repeated infections by a virus. This systemic effect is not seen in all systems (e.g., is not seen in *Drosophila*). In the case of *C. elegans*, it may be entirely coincidental that the RNA-silencing effect can be triggered by dsRNA in the food. *C. elegans* can take precursors for nucleic acids from its food. RNAi induced by feeding (*21*, *23*) may take advantage of two distinct pathways, one whose natural function is to import nucleic acids to be used as precursors for replication and transcription, and the other that functions as the virus/transposon shield.

Self and Nonself

Given the link of the genome's "immune system" to RNAi, which is triggered by dsRNA, one may ask how transposons or viruses induce dsRNA that corresponds to their own sequence. In *C. elegans*, at least three explanations have some plausibility. First, once an element has inserted multiple copies into random locations in the genome, read-through transcription from flanking promoters may generate RNA from both strands, forming dsRNA. The chance of this occurring would increase with the number of insertions, and this would provide a mechanism that senses copy number in combination with random integration, a sensor of a transposon spreading in the genome. Second, transposons known to be regulated by RNAi genes in *C. elegans* have terminal inverted repeats. Read-through transcription of a single copy could result in snap-back dsRNA corresponding to these termini. We have indeed observed such dsRNA corresponding to transposon termini in *C. elegans* (*24*). Third and finally, there may be some other sensor of the foreign nature of transposons. It is conceivable that all "good" genes share structural motifs in their mRNAs, possibly even in the interaction between the 5′ and 3′ termini, and proteins factors bound to them. mRNAs that lack such features might be turned into dsRNA by a specialized machinery. Several *C. elegans* mutants that are defective in transposon silencing are not defective in RNAi after administration of dsRNA, possibly revealing the hypothetical step that turns foreign mRNAs into dsRNA.

Plant mutants that are defective in transgene silencing are found not to be defective in virus-induced silencing (*18*). They contain a mutation in an RNA-directed RNA polymerase (RdRP), and its likely role is to convert the single-stranded RNA (ssRNA) of the "foreign" transgene into dsRNA. Thus, for viruses the nonself feature could simply be dsRNA, whereas for transgenes the nonself feature would be something that is recognized by an RdRP that converts ssRNA into dsRNA.

Amplification

Small amounts of dsRNA are able to silence a vast excess of target mRNA in *C. elegans* (*7*). There are at least three mechanistic explanations for this observation: (i) The Dicer enzyme cuts long dsRNA molecules into short "primary" siRNAs (Fig. 1). Because each siRNA can potentially target a homologous mRNA, this provides a level of amplification that, depending on the length of the dsRNA, could easily measure 10- to 20-fold. (ii) A catalytic mechanism, in which siRNAs are used multiple times, can provide further amplification. (iii) Short RNAs may be able to serve as primers on target mRNA and subsequent generation of "secondary siRNAs" (target-directed amplification) and thus initiate an RNA-directed RNA polymerization reaction (see Fig. 1).

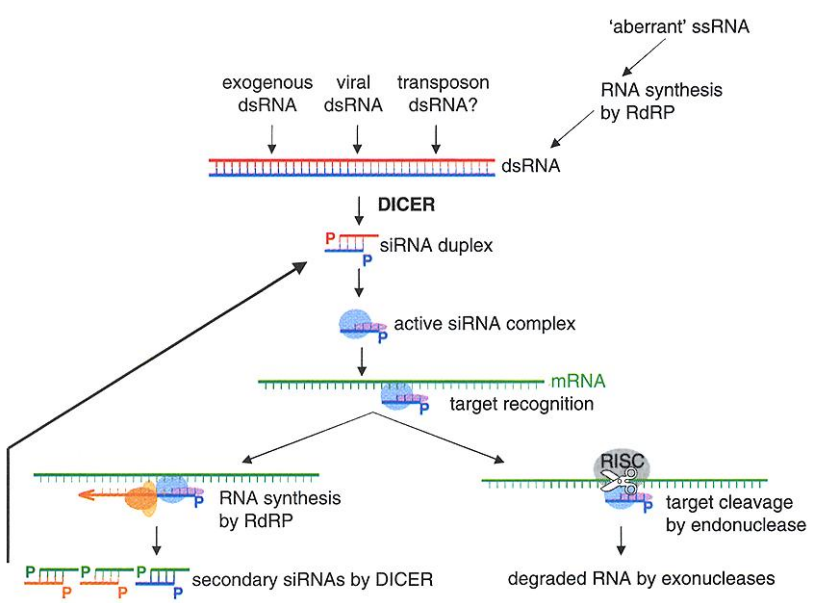

Fig. 1. A model for the molecular steps in RNA silencing.

Target-Dependent Amplification

In the first step of this reaction, the mRNA is recognized by primary siRNAs. The hypothetical sequence of events is as follows: dsRNA is cut into short siRNAs, presumably these are converted from dsRNA into ssRNA, and then two things can happen. These siRNAs (presumably bound to proteins) are by themselves unstable, and are degraded, unless they recognize homologous target mRNA, present in the cell, and base pair to it. The evidence for this in *C. elegans* is threefold: (i) RNAi directed against a marker gene [green fluorescent protein (*GFP*)] does not result in detectable siRNAs in vivo unless the *GFP* gene is expressed in the target tissue (*25*). (ii) Only the antisense strand of siRNAs is seen in vivo, not the sense strand (*26*, *27*). (iii) Many mutants defective in RNAi show no detectable steady-state levels of siRNAs in vivo, whereas activity of the Dicer enzyme could easily be demonstrated in vitro with crude cellular extracts (*11*). Apparently these mutants are able to make siRNAs at wild-type levels, but fail to stabilize them, presumably because they never reach the stage where siRNAs base pair to their target.

This stabilization provides a quick specificity filter. If dsRNAs are generated for whatever reason, but if there are no mRNAs that could potentially be silenced by them, then the reaction dies out immediately because the siRNAs are not stabilized. If, on the other hand, there is target RNA for these siRNAs, then the reaction continues.

Then, in the second step, after the antisense siRNA has base paired to the target mRNA, target-directed amplification can occur. In worms and plants, RNAi induced by dsRNA corresponding to a region in the middle of a gene results in the synthesis of siRNAs regions immediately flanking the target site (*11*, *12*). In worms [but not in plants and fly extracts (*28*)] this effect shows polarity (only 5′ secondary siRNAs are seen), and there is a clear influence of distance, as this so-called "transitive effect" does not extend further than a few hundred base pairs. Note that it is likely that many of the siRNAs derived from the region covered by the initial dsRNA [referred to as "primary siRNA" (*11*)] are probably also secondary and result from short elongation reactions within this region. A second indicator of transitive RNAi is provided by the demonstration that for RNAi directed against (transgenic) gene fusions, the effect can enter a 5′ domain from a 3′ domain and thus affect an unlinked nonfused gene that corresponds to the 5′ domain (*11*). Finally one can trigger efficient RNAi by injection of short antisense RNAs, provid-

ed that these find a corresponding target mRNA that they can immediately hop onto (*26*).

The polymerase required for this amplification is probably different in different tissues. In the germline of *C. elegans*, the *ego-1* gene has been implicated in RNAi; it has sequence homology to a factor previously isolated from the tomato as an RdRP (*29*). In somatic *C. elegans* cells, another RdRP homolog has been implicated: *rrf-1*. Mutation of the *rrf-1* gene results in loss of RNAi and in significant decrease of siRNAs. Inactivation of another RdRP homolog has the opposite effect, of enhancing RNAi [*rrf-3* (*11*)]. The *rrf-3* gene product may be less active and may compete with RRF-1 in the relevant complex. In *Dictyostelium*, three RdRP homologs have been described. Loss of one of them, *rrpA*, resulted in loss of RNAi and of detectable siRNAs (*13*).

The *Arabidopsis thaliana* RdRP homolog SDE1/SGS2 is also required for transitive RNAi (*12*). A significant difference between transitive RNAi in *C. elegans* and plants (*Nicotiana bethamiana* and *Arabidopsis*) is that, in plants, the transitive effect can occur in the 3′ as well as the 5′ direction, and as a consequence, secondary siRNAs are found both 5′ and 3′ of the targeted region. In plants, siRNAs may direct an RdRP to an mRNA, triggering unprimed RdRP activity of the complete RNA molecule. Alternatively, the initial reaction may show polarity, but frequent template jumps may occur.

The combination of siRNA stabilization and transitive RNAi results in a "chain reaction," in which multiple cycles of replication can occur, followed by Dicing, new priming, and a new round of amplification (Fig. 1).

Conclusion

We are beginning to dissect an ancient mechanism that protects the most sensitive part of a species: its genetic code. Like the vertebrate immune system, the machinery recognizes molecular parasites, raises an initial response, and stabilizes and amplifies this response. Given the conservation of parts of the RNAi-silencing machinery [see reviews (*30*, *31*)], this genome defense mechanism should be widespread, although details may differ. It is thus also possible that RNAi silencing refers to a family of mechanisms that are quite different in context and detail. This will almost certainly be the case for more specific aspects of the biology: for example systemic RNAi in *C. elegans* (*21*), spreading of silencing in plants (*22*), and suppression of silencing induced by several plant viruses (*32*).

Just as knowledge of immunology has laid the foundation for (experimental) immune therapy, a thorough understanding of the genome's immune system has great potential for applications in directed gene silencing, in experimental biology, and possibly also in disease therapy.

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VIEWPOINT

Ancient Pathways Programmed by Small RNAs

Phillip D. Zamore

Double-stranded RNA can now be used in a wide variety of eukaryotes to suppress the expression of virtually any gene, allowing the rapid analysis of that gene's function, a technique known as RNA interference. But how cells use the information in double-stranded RNA to suppress gene expression and why they contain the machinery to do so remain the subjects of intense scrutiny. Current evidence suggests that RNA interference and other "RNA silencing" phenomena reflect an elaborate cellular apparatus that eliminates abundant but defective messenger RNAs and defends against molecular parasites such as transposons and viruses.

Virtually any gene can now be disrupted in cultured human cells, flies, worms, and a growing list of other organisms in just a week or two (*1*, *2*) using new tools based on the cellular phenomenon of "RNA silencing" (Fig. 1). These new tools likely will soon be extended to whole mammals (*3*–*5*) and may one day form the basis of a new class of drugs to treat human disease. Knowing only the DNA sequence of a gene, molecular biologists can design potent, sequence-specific inhibitors—a form of double-stranded RNA—that block expression of just that gene. Using such inhibitors, we can now ask for each of the tens of thousands of human messenger RNAs (mRNAs) the central question of genetics: what does this gene do?

White Flowers and Silenced Worms

New tools for evaluating gene function (Fig. 1) sprang from the discovery that disparate and bizarre examples of RNA silencing are all man-

Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Lazare Research Building, Room 825, 364 Plantation Street, Worcester, MA 01605, USA. E-mail: phillip.zamore@umassmed.edu