REVIEW

RNA-Dependent RNA Polymerases, Viruses, and RNA Silencing

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Most viruses have RNA genomes that are replicated and transcribed into messenger RNA by viral RNA–dependent RNA polymerases (RdRps), usually in concert with other viral and host factors. Many, if not most, eukaryotes also encode putative RdRps that have been implicated in sequence-specific, RNA-triggered gene silencing. Although the viral and cellular RdRps have no sequence homology, they share functional similarities such as copying messenger RNA templates and intercellular spread of the amplified sequences. Better understanding of viral and host RdRps will improve our ability to control viruses and to use RNA silencing and viruses as tools for research, biotechnology, and medicine.

The primary pathways of genetic information flow in cells, codified as the "central dogma" of early molecular biology, are the replication of DNA from DNA, transcription of RNA from DNA, and translation of proteins from RNA. A pathway outside of this scheme that is attracting growing attention is the copying of RNA from RNA, mediated by enzymes called RNA-dependent RNA polymerases (RdRps). RdRps must have played a vital role early in evolution, when RNA was the primary genetic material. These enzymes also have crucial functions in contemporary biology. RdRps are encoded by a wide variety of RNA viruses for genome replication, mRNA synthesis, RNA recombination, and other processes. Many, if not most, eukaryotes also encode demonstrated or putative RdRps, whose roles are still being uncovered. Recent experiments with the nematode *Caenorhabditis elegans* and the fruit fly *Drosophila* add to evidence that cell RdRps play important roles in RNA silencing (*1*–*3*), a mechanism in which double-stranded RNA (dsRNA) triggers sequence-specific gene repression in animals and plants. Here, I review the roles of viral and host RdRps in viral pathogenesis, host defense, and RNA silencing.

RNA Virus RdRps and Accessory Factors

Most known viruses store and replicate their genomes as RNA, with no natural DNA forms. A few examples of medically important RNA viruses are Ebola virus, which causes lethal hemorrhagic fevers; influenza virus, which in a single 1918–1919 pandemic killed tens of millions of people; and hepatitis C virus, which is carried by over 150 million people and can cause severe liver damage and cancer. RNA viruses are divided into three major classes differentiated by whether the infectious virion particles contain the genome as dsRNA, positive-strand (messenger-sense) RNA, or negative-strand RNA.

To replicate their genomic RNA, all known RNA viruses encode an RdRp. These RdRps share multiple sequence motifs that are conserved across all three major RNA virus classes. Crystal structures for RdRps of positive-strand RNA and dsRNA viruses show structural similarity not only to each other, but also to DNA-dependent RNA and DNA polymerases and reverse transcriptase (*4*–*10*). All of these polymerases share a structure similar to that of a right hand, with palm, thumb, and fingers domains. The palm domain structure is particularly conserved and contains four sequence motifs preserved in all RNA and DNA polymerases.

In natural infections, viral RdRps act in combination with other viral and host factors (*11*) involved in selecting template RNAs and initiation sites for RNA synthesis, initiating or maintaining elongation of RNA synthesis, differentiating genomic RNA replication from mRNA transcription, modifying product RNAs with 5′ caps or 3′ polyadenylate, and other functions. Thus, viral RdRps function in specific complexes that target the polymerase to appropriate templates and coordinate the various steps of RNA synthesis while protecting the viral RNAs from competing processes such as translation and degradation. In the case of negative-strand RNA viruses (Fig. 1A), only viral RNAs coated with nucleocapsid protein, a viral single-stranded RNA (ssRNA) binding protein, can be used as templates for RdRp (*12*). For dsRNA viruses (Fig. 1B), RNA templates and RdRp are packaged together in a core or shell of viral proteins within which RNA synthesis occurs (*13*, *14*). Positive-strand RNA virus replication occurs in membrane-bound, multiprotein complexes. For some, if not many, positive-strand RNA viruses, these complexes parallel dsRNA virus and retrovirus cores, with viral accessory proteins sequestering viral RNA replication templates with RdRp and additional viral functions such as RNA capping factors and putative RNA helicases (*15*). For all three virus classes, the mass of "accessory" proteins in such complexes usually exceeds that of the RdRp subunits or domains by a large margin.

RdRps are crucial to virus survival not only through replication but also as engines of genome variability and evolution. High error rates (typically on the order of 10^{-4}) in viral RdRp copying ensure wide variability in RNA virus populations, allowing rapid virus evolution under selective pressures imposed by the host immune response, drug treatments, and so on. (*16*). Strand switching during RdRp copying is also a mechanism for RNA recombination, allowing RNA viruses to repair deleterious mutations, rearrange genes, and acquire new

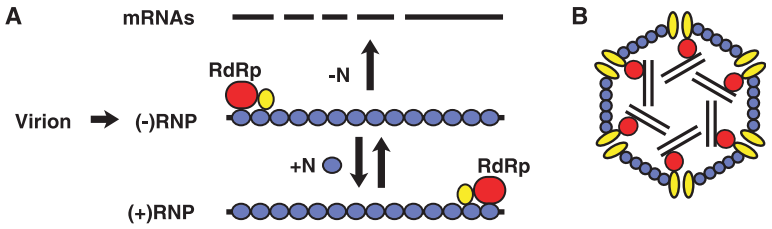

Fig. 1. Viral RNA replication complexes. (**A**) Negative-strand virus RNA synthesis. The negative-strand ribonucleoprotein (RNP) complex delivered by the virion includes RdRp (red), phosphoprotein cofactor (yellow), and the viral RNA genome (black line) covered with nucleocapsid protein N (blue). At the start of infection, in the absence of free N, partial mRNA transcripts are made. After translation, N availability directs production of full-length, N-coated positive-sense RNA, which is copied to make more genomic negative-strand RNPs. (**B**) Highly simplified cross section of a transcriptionally active dsRNA virus (reovirus) core, showing core shell proteins (blue). RNA synthesis complexes at each of 12 fivefold axes include RdRp (red), dsRNA template, and capping factors (yellow) that serve as an exit pore for nascent RNAs.

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genes from other viruses or their hosts (*17*). Genome comparisons imply that such RNA recombination has been the major force in RNA virus evolution.

Cellular RdRps

Although RdRps are most widely known as factors encoded by RNA viruses, RdRp activities have been reported in uninfected cells for more than 30 years (*18*). At least two types of cellular RdRp activities exist. First, in certain contexts, cellular DNA-dependent RNA polymerases can function as RdRps. For example, plant viroids—pathogenic, ~250- to 400-nucleotide (nt) circular RNAs that encode no proteins—are replicated in the nucleus by DNA-dependent RNA polymerase II or in chloroplasts by chloroplast RNA polymerase. Similarly, replication and transcription of the 1.7-kb circular RNA genome of human hepatitis delta virus involve cellular RNA polymerase II (*19*, *20*).

A second type of cellular RdRp appears distinct from DNA-dependent RNA polymerases. The best-characterized of these RdRps is from tomato (*21*). This enzyme can produce RNA products at least 100 nt long by extending primers on RNA templates or, for at least some RNAs, by initiating RNA synthesis without a primer opposite the 3′ end. These RdRp activities cofractionate with a 127-kD protein that lacks homology to proteins of known function, including virus-encoded RdRps, reverse transcriptases, and DNA-dependent RNA and DNA polymerases. The tomato RdRp might thus represent a structurally and functionally distinct class of polymerase, like some RNA primases (*22*). Alternatively, the 127-kD protein might be an accessory factor associated with an as-yet-unidentified polymerase. However, because the latter seems unlikely owing to the RdRp purification results and genetic results outlined below, the 127-kD protein is referred to here as the tomato RdRp. Homologs of the tomato *RDRP* gene encoding this 127-kD protein have been found not only in *Arabidopsis* and other plants, but also in *C. elegans*, *Neurospora crassa*, and the fission yeast *Schizosaccharomyces pombe*. Outside of plants, however, no direct evidence has been provided for RdRp activity of the relevant gene products.

RNA Silencing

RNA silencing (also called posttranscriptional gene silencing) refers to a group of interrelated, sequence-specific, RNA-targeted gene-silencing mechanisms common to animals, plants, and some fungi (*23*, *24*). In RNA silencing, endogenously synthesized or exogenously applied dsRNA is processed by a ribonuclease III–like nuclease, Dicer, into 21- to 25-nt dsRNAs with 2- to 3-nt 3′ overhangs. These dsRNA fragments, called short interfering RNAs (siRNAs), associate with an ~250- to 500-kD nuclease complex called RISC (RNA-induced silencing complex) and target this complex to complementary mRNAs. RISC then cleaves the targeted mRNAs opposite the complementary siRNA, which may render the mRNA susceptible to other RNA degradation pathways.

RNA silencing appears to have multiple natural functions, including the control of viral infection and transposon movement (*25*, *26*). In plants, RNA viruses induce and can be strongly inhibited by RNA silencing (*27*). The result is a race of viral replication and spread against the induction and spread (see below) of antiviral RNA silencing. The importance of this race to the success or failure of infection is shown by the fact that many plant viruses encode genes that suppress different steps in RNA silencing (*25*, *26*, *28*). Similarly, recent data show that an insect RNA virus induces RNA silencing in *Drosophila* cells and encodes an RNA silencing inhibitor (*29*). Because RNA silencing occurs in mammalian cells (*30*, *31*), mammalian viruses also may be affected by RNA silencing.

At the DNA level, in addition to inhibiting transposon movement, RNA silencing is linked to transcriptional silencing of chromosomal genes (*32*, *33*). Moreover, Dicer and pathways related to RNA silencing are required to process numerous endogenous precursor RNAs into ~22-nt microRNAs (miRNAs) that regulate developmental events and likely other processes (*34*).

RdRps and RNA Silencing

Diverse lines of evidence suggested that some aspects of RNA silencing might involve cellular RdRps. In early experiments on RNA silencing associated with sense transgenes, copying of target mRNAs into complementary RNA by cell RdRps was proposed to explain the sequence specificity of silencing (*35*). Possible RdRp amplification of inducing RNA signals was suggested by the surprising potency, spread, and persistence of RNA silencing responses. RNA silencing can be induced by injecting a few molecules of dsRNA per cell, spreads systemically in animals and plants, and persists from treated parents to untreated progeny (*24–26*).

Homologs of the tomato *RDRP* gene are required for RNA silencing in *Neurospora*, *C. elegans*, and *Arabidopsis* (*36–39*). *C. elegans*, for example, encodes four homologs: *rrf-1*, *rrf-2*, *rrf-3*, and *ego-1*. *rrf-1* deletion blocks RNA silencing of genes expressed in somatic but not germ line cells (*2*), whereas mutation of *ego-1*, which is selectively expressed in germ line, blocks RNA silencing of germ line–expressed genes (*37*). *ego-1* mutants also interfere with multiple aspects of germ line development, including meiosis, whereas *Neurospora* meiosis is blocked by mutants of *sad-1*, which encodes a putative RdRp required for meiotic silencing by unpaired DNA (*40*). Thus, some putative RdRps involved in silencing have essential roles related to development.

Consistent with the above genetic findings, recent biochemical results imply that RNA-dependent RNA synthesis is important for at least some manifestations of RNA silencing. Experiments with *Drosophila* cell extracts suggest that siRNAs may act as primers for RdRp synthesis on complementary RNA targets (*1*). Purified siRNAs were incorporated into dsRNA in a reaction requiring a complementary RNA template. The resulting dsRNA was processed into new siRNAs, potentially amplifying the RNA silencing response. ssRNA or dsRNA, but not DNA, directed siRNA incorporation into dsRNA, implying that siRNA might prime dsRNA synthesis on target mRNA, amplify the inducing dsRNA target, or both (Fig. 2A). The spectrum of dsRNA produced in some experiments suggested possible ligation of RNA products extended from multiple siRNA primers. Because no homolog of the tomato *RDRP* gene has been detected in the *Drosophila* or human genome, these results suggest the possibility of distinct or divergent RdRps.

Experiments with *C. elegans* have provided independent support for RdRp action in RNA silencing in vivo (*2*). Worms fed dsRNA corresponding to a defined target region in an mRNA produced siRNAs complementary to not only that region, but also to flanking sequences closer to the 5′ end of the same mRNA. This polarity is consistent with processing of the inducer dsRNA to siRNAs that prime RNA synthesis on the target mRNA to create new dsRNA that is then processed to yield the secondary siRNAs. The efficacy of these secondary siRNAs in RNA silencing was shown by "transitive" silencing. In animals expressing mRNAs with the structure A and AB (Fig. 2B), a dsRNA trigger complementary to region B silenced both mRNAs. As predicted for silencing through induction of secondary siRNAs, B dsRNA silenced mRNA A only if mRNA AB was expressed simultaneously, and BA mRNA was unable to generate such an effect. Mutation of the *C. elegans* RdRp homolog *rrf-1* blocked secondary siRNA production and transitive silencing, consistent with their inferred dependence on siRNA-primed RNA synthesis (*2*).

Transitive RNA silencing also has been observed in plants. RNA silencing can be induced in plants by promoterless DNA fragments corresponding to part of a transcribed gene. These trigger DNA fragments have been proposed to interact with the transcribed gene to produce aberrant RNAs whose altered termination or processing mark them

for RdRp copying into dsRNA. In plants expressing full-length green fluorescent protein (GFP) mRNA, GFP DNA fragments transitively silence RNA virus derivatives bearing nonoverlapping GFP sequences either 5′ or 3′ to the trigger (*41*). The bidirectionality of this transitivity may reflect copying of aberrant RNAs by the primer-independent activity of plant RdRps (*21*).

Additional evidence for RdRp action emerged from experiments showing that short antisense RNAs (asRNAs) injected into *C. elegans* can induce RNA silencing (*42*). Although siRNAs are only effective in a narrow size range around 20 to 23 nt, asRNAs of 22 to 40 nt were equally effective, implying that the asRNAs did not act directly as siRNAs. Such asRNA silencing also required the Dicer enzyme that degrades dsRNA into siRNA and, after injection into the gonad, asRNA silenced germ line–expressed genes but not genes expressed solely in the soma. Thus, asRNA only induced silencing in the immediate presence of a complementary mRNA target, consistent with asRNA-primed RdRp synthesis of dsRNA subsequently processed by Dicer into siRNAs (Fig. 2A).

Issues in RdRp Action

Like viral RdRps, cellular RdRps may function as part of a larger complex of factors that contribute to template and primer selection, initiation, elongation, or the fate of RNA products. siRNAs, which appear to act as RdRp primers, associate with one or more protein complexes like RISC that unwind siRNA, anneal it to target mRNA, and cleave the target (*43*). RdRp interaction with such complexes could facilitate initiation and help to regulate the balance of synthetic versus degradative pathways in RNA silencing. In or out of such complexes, RdRps may associate with some of the multiple helicases implicated in RNA silencing (*24*). Like the many viral helicase homologs involved in RNA replication, such helicases might facilitate RdRp initiation or elongation by unwinding dsRNA templates or removing secondary structure in ssRNA templates.

Because host RdRps act on mRNA targets, RNA synthesis and translation could be mutually antagonistic. Ribosomes and RdRp move in opposite directions, and their action on a single template is incompatible (*44*). Viruses have evolved specific pathways by which viral and host proteins recognize viral mRNA templates, inhibit their translation, and recruit them as RdRp templates (*15*, *44*, *45*). To copy mRNAs effectively, host RdRps may require similar processes. Translational modulation might involve members of the Argonaute family of proteins, eIF2C homologs that are required for RNA silencing and are found in the RISC complex (*46*).

The extent to which RdRp action contributes to RNA silencing may depend on the specific organism or tissue, the specific induction pathway, or even the specific target. Thus, although RdRp-dependent secondary siRNAs and transitive RNA silencing were seen in *C. elegans* (*2*), transfection of cultured human embryonic kidney 293T cells with a synthetic siRNA was found to suppress expression of target gene *TSG101* with no apparent transitivity (*47*). In the presence of the silenced, endogenous *TSG101* gene, mutation of the siRNA target region alone was sufficient to allow a coexpressed *TSG101* mRNA to escape silencing.

Viruses, RNA Silencing, and RdRps

Consistent with the antiviral effects of RNA silencing and the involvement of RdRp in at least some RNA silencing, mutation of the *Arabidopsis* RdRp homolog *SGS2/SDE1* was found to increase the accumulation of cucumber mosaic virus and a potato virus X replicon (*38*, *39*). This same *Arabidopsis* mutation did not affect the accumulation of several other positive-strand RNA viruses, possibly because these viruses already efficiently inhibit RNA silencing in wild-type plants. Also consistent with a role in virus control, expression of RdRp genes in many plants is greatly stimulated by virus infection. This long-recognized stimulation led to early consideration and subsequent rejection of the possibility that host RdRps might be responsible for viral RNA replication (*48*, *49*). In tobacco, a virus-inducible host RdRp gene with clear antiviral effects is also induced by salicylic acid, which stimulates generalized antiviral and antimicrobial defense responses (*50*).

Because RNA viruses replicate their genomes through complementary RNA strands, it has been widely suggested that viral dsRNA replication intermediates are primarily responsible for viral induction of RNA silencing. Nevertheless, in keeping with viral capacity for rapid adaptation, varied data imply that RNA viruses have evolved mechanisms to minimize accessibility of their replicative intermediates to host defenses such as RNA silencing and other dsRNA-stimulated pathways, including the interferon and 2′,5′-oligoadenylate/ribonuclease L pathways of animals (*51*).

For negative-strand RNA viruses, nucleocapsid protein (Fig. 1A) blocks annealing of negative- and positive-strand RNA replication templates to form dsRNA. Moreover, synthetic siRNAs against respiratory syncytial virus, a negative-strand RNA virus, induce degradation of viral mRNA but not nucleocapsid-coated genomic RNA in cultured human cells (*52*). Thus, nucleocapsid protein appears to block siRNA-targeted recognition and degradation. For dsRNA viruses, synthesis and retention of dsRNA templates in the viral core has long been viewed as a mechanism to protect against dsRNA-induced host defenses. Similarly, for at least

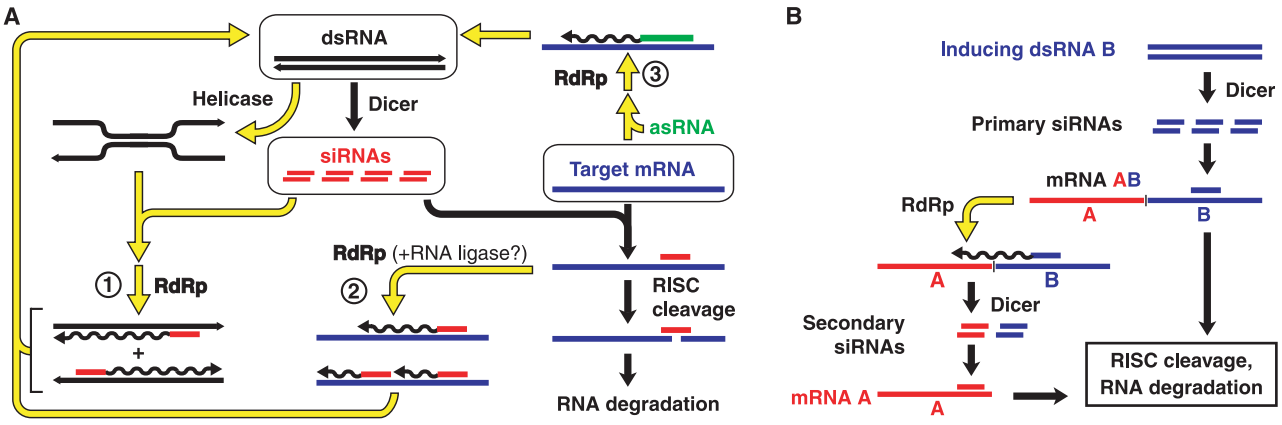

Fig. 2. (**A**) Degradative and synthetic pathways linking dsRNA, siRNAs, and target mRNA in RNA silencing. Black arrows denote classical RNA silencing degradative pathways. Yellow arrows denote RdRp-dependent synthetic pathways leading to generation or amplification of dsRNA. RdRp may act on siRNA-primed dsRNA (1), siRNA-primed mRNA (2), or asRNA-primed mRNA (3) to generate or amplify some or all of the inducing dsRNA sequences. (**B**) Transitive RNA silencing. Through the combined action of siRNA primers and RdRp on intermediary mRNA AB, inducing dsRNA B mediates degradation not only of mRNA AB, but also of mRNA A, which lacks inducing sequence B.

some positive-strand RNA viruses, positive- and negative-sense RNA replication templates in membrane-associated viral RNA replication complexes are strongly protected from degradative pathways in vivo and from outside enzymes including ribonucleases in vitro (*15*). This appears to be noteworthy because viral induction of RNA silencing has been studied primarily with positive-strand RNA viruses.

How then do RNA viruses induce RNA silencing and other dsRNA-dependent host defense pathways (*27*, *51*, *53*)? Virus shielding of dsRNA may greatly reduce or delay an otherwise massive RNA silencing response, but may not be perfect. Given potential amplification by host RdRp, eventual turnover of a very small fraction of replication complexes may be sufficient to trigger RNA silencing. Alternatively, high levels of viral mRNAs, which for many viruses reach ribosomal RNA levels, may make viruses sensitive to normally inefficient mechanisms for inducing RNA silencing. Such inefficient triggering mechanisms could include low-level Dicer action on structured regions of viral mRNAs, alternate pathways to initiate host RdRp copying, or other triggers. Such mechanisms would be consistent with previously observed threshold effects for RNA silencing by sense-RNA inducers and indications that RNA virus mRNAs can induce dsRNA-stimulated defense responses in animal cells (*25*, *26*, *33*, *51*).

Conclusions

The results outlined above pose many intriguing questions. For example, how are the potentially complex interactions of RNA synthesis and degradation in RNA silencing regulated (Fig. 2)? What is the relative importance of RdRp action in RNA silencing for different organisms, tissues, induction pathways, and RNA targets? Do cellular RdRps contribute to processes beyond RNA silencing, such as other dsRNA-stimulated antiviral responses (*51*) or regulatory RNA synthesis (*34*)? How do RNA silencing responses spread between cells, and is this spread functionally similar to virus spread?

Answers to many of these questions should emerge soon. Progress in RNA silencing has accelerated steadily as a result of growing interest in its natural roles and applications, and availability of model systems with synergistic genetic and biochemical strengths. Similarly, advances in RNA virus replication are being driven by needs for improved virus control and the ability of viruses to illuminate key cellular pathways. Ongoing progress in both arenas promises to reveal remarkable new facets of basic biology and increasingly powerful and flexible ways to manipulate gene expression at the RNA level.

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