Viroids and Hepatitis Delta Virus

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Abstract

There is a subviral world, whose most prominent representatives are viroids. Despite being solely composed by a circular, highly structured RNA of ~250 to 400 nucleotides without protein-coding ability (all viruses code for one or more proteins), viroids can infect and incite specific diseases in higher plants. The RNA of human hepatitis delta virus (HDV), the smallest genome of an animal virus, displays striking similarities with viroids: It is circular, folds into a rodlike secondary structure, and replicates through a rolling-circle mechanism catalyzed by host enzymes and *cis*-acting ribozymes. However, HDV RNA is larger (~1700 nucleotides), encodes a protein in its antigenomic polarity (the ð antigen), and depends for transmission on hepatitis B virus. The presence of ribozymes in some viroids and in HDV RNA, along with their structural simplicity, makes them candidates for being molecular fossils of the RNA world that presumably preceded our extant world based on DNA and proteins.

Keywords

- viroids
- hepatitis delta virus
- subviral pathogens
- rolling-circle replication
- ribozymes

The end of the 19th century witnessed a scientific revolution that ultimately led to the emergence of a new scientific discipline, virology, as a consequence of the discovery of *Tobacco mosaic virus* (TMV).[1] Later, numerous viruses were identified infecting animal (including human) cells, as well as fungi, bacteria, and even mycoplasmas, but virology, like genetics, was born linked to the plant world. The paradigm regarding viruses as the lowest step of the biologic scale remained firm and unchallenged along the following 65 to 70 years, being implicitly assumed that all acellular pathogens were viruses, even if occasionally the viral particles (the virions) could not be identified (presumably for their lability, low concentration or any other technical argument). However, this long stasis interval was interrupted by the discovery of the causal agent of potato spindle tuber (PST), a disease of presumed viral etiology that turned out to be incited by a new class of much simpler pathogens: the viroid. *Potato spindle tuber viroid* (PSTVd),[2–4] thus became the first described subviral pathogen endowed with autonomous replication. Soon after, by applying the methodology developed for PSTVd to other plant diseases of uncertain etiology, quite a few new viroids were discovered.[5,6] The picture that has come to light along the years is that viroids, despite their name, differ in structure, function, and evolutionary origin from viruses. Briefly, to facilitate understanding of this article, viroids: (1) are circular RNAs of only 250 to 400 nucleotides (nt), a size significantly smaller than the smallest viral genomes; (2) lack protein-coding capacity, whereas all viruses encode in their genomes at least one and frequently several proteins; and (3) cause symptoms similar to those induced by viruses in plants economically relevant, including herbaceous (potato, tomato, and some ornamentals) and woody species (citrus, grapevine, avocado, coconut palm, and some temperate fruit trees).[6–8] Many of these diseases are distributed worldwide. Are viroids restricted to the plant kingdom? Strictly speaking, yes, but there is a connection with *hepatitis delta virus* (HDV).

The Scale of Genomic Sizes

Viroids are the lower step of the biologic scale, at least in terms of genomic complexity (►**Fig. 1**). This is not the proper place to discuss whether viruses (and viroids) should be considered living microorganisms, a question that has been heatedly debated. They certainly do not have a metabolism of their own and consequently need to parasitize that of the cells they infect, but viruses (and viroids) share the most characteristic property of living beings: In an appropriate environment they are able to generate copies of themselves, in other words, they are endowed with autonomous replication (and evolution). It is in this framework where viroids represent the

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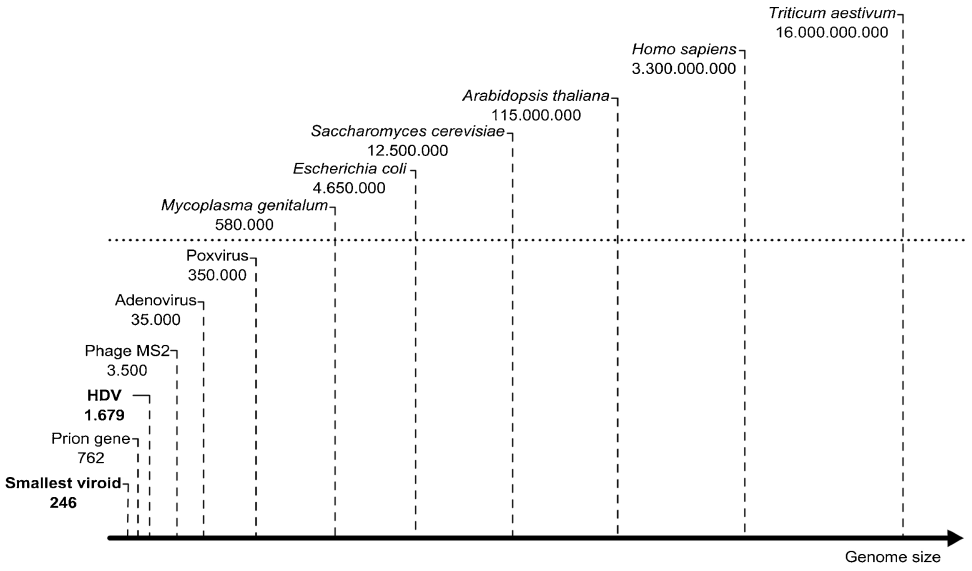

Figure 1 The scale of genomic sizes. Values are approximate and expressed in DNA base pairs, except for phage MS2, hepatitis D virus, and viroid genomes that are expressed in RNA bases. The genome size of wheat is an estimate. The horizontal line separates cellular organisms from viral and subviral agents.

frontier of life (246–401 nt) an aspect that should attract the attention of anybody interested in biology. Leaving aside prions, which are "infectious" proteins, the step in the biologic scale of genomic sizes immediately above viroids is occupied by the HDV genome (►**Fig. 1**), constituted by a circular RNA with a size only 4 to 7 times higher than that of viroids (see below). Thus, HDV has the smallest genome reported for an animal virus. Also worthy of note is that, like viroid RNAs, HDV RNA is circular, an uncommon structural feature with deep consequences in their replication (see below).

Viroid Discovery and Characterization

The working assumption that the causal agent of PST disease was a conventional virus could not be confirmed because initial attempts addressed to sediment by ultracentrifugation the hypothetical virions failed: the infectious principle remained in the supernatant. Moreover, following centrifugation in a sucrose gradient (that fractionates virions and particulate cell components essentially according to their size) this infectious principle migrated as an entity significantly smaller than conventional virions and what it was even more intriguing, the migration was not affected by a pretreatment with phenol (a chemical for removing proteins), thus suggesting that the infectious principle was a naked nucleic acid instead of a nucleoprotein as in typical viruses. Subsequent attempts applying polyacrylamide gel electrophoresis (PAGE; a technique appropriate for resolving complex nucleic acid preparations into their components) showed that the infectious principle was a tiny nucleic acid. More specifically, a small RNA (as revealed by its sensitivity to RNase but not to DNase), with circular structure (because of its resistance to exonucleases, which catalyze degradation of nucleic acids starting from their termini). All these studies were performed using tomato (belonging to the same botanical family as potato) because it was a more adequate experimental host: It can be easily grown in the greenhouse and expresses characteristic symptoms in a relatively short interval (2 weeks). The possibility that the causal agent of PST disease could be a small satellite RNA functionally dependent on a helper virus, was discarded given that the corresponding virions could not be identified in infected tissues. Moreover, RNase fingerprint analysis showed for this agent a structural complexity consistent with a single RNA molecule, dismissing the alternative of a population of similarly sized RNAs with different sequences altogether accounting for a conventional virus genome. Therefore, it had to be admitted that an autonomously replicating small circular RNA (a viroid) was the infectious principle of PST disease.[4] All this aforementioned evidence, however, was based on infectivity bioassays and compelling support for the novel concept of viroid had to await identification of the predicted small RNA by two physical approaches: (1) PAGE revealed that the infectivity of nucleic acid preparations from PSTVd-infected tomato was strictly associated with a ultraviolet- (UV-) absorbing small RNA absent in mock-inoculated preparations (►**Fig. 2**),[3] and (2) electron microscopy of purified PSTVd RNA provided direct observation and estimation of its small size (►**Fig. 3**).[9]

Sequencing of PSTVd by direct methods on the RNA itself (without cDNA cloning in vivo or in vitro because techniques like amplification by reverse transcription-polymerase chain reaction had not yet become available), stands as a landmark in biology:[10] PSTVd is the first eukaryotic pathogen for which the complete primary structure was determined, opening the current genomics era. Closer examination of the PSTVd sequence showed that it was to a good extent self-complementary, leading to a rodlike secondary structure and explaining why under the electron microscope PSTVd RNA displayed the same width as the phage double-stranded

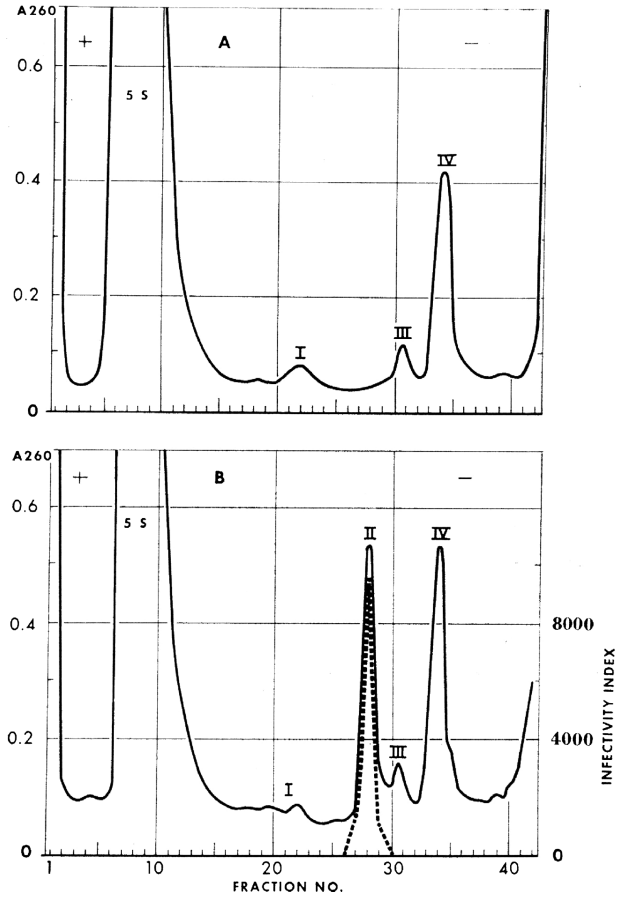

Figure 2 Absorbance profiles at 260 nm (continuous lines) of RNA preparations from healthy (A) and potato spindle tuber viroid- (PSTVd-) infected (B) tomato leaves following electrophoresis in 20% polyacrylamide gels. The broken line in (B) represents the infectivity distribution resulting from cutting the cylindrical gels into fine slices and inoculating the eluted RNAs into blocks of tomato plants; the infectivity index (right) is expressed in arbitrary units. 5S refers to 5S ribosomal RNA (~120 nt); I, III, and IV to unidentified minor components of tomato RNA, and II to PSTVd RNA. Electrophoretic migration is from right to left. Notice that the infectivity is strictly associated to the differential ultraviolet peak II only detected in the preparation from PSTVd-infected tissue. (Reproduced with permission from Diener, 1972).

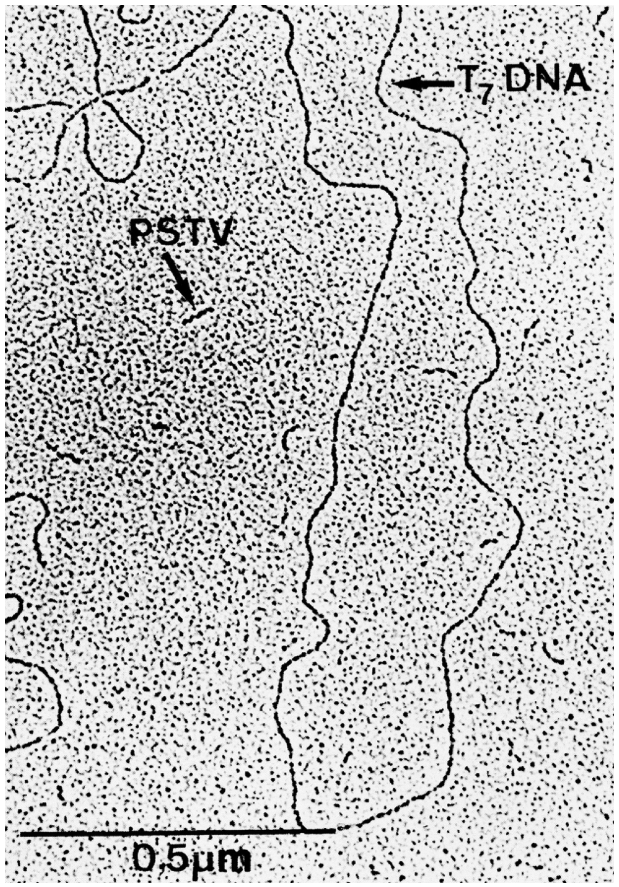

Figure 3 Electronic micrograph of purified potato spindle tuber viroid RNA mixed with purified DNA from the coliphage T7. Notice the size differences as well as the rodlike structure of the viroid RNA that displays a width similar to that of the double-stranded DNA. (Reproduced with permission from Diener TO, Viroids and Viroid Diseases. New York: Wiley;1979).

DNA used as internal control (►**Fig. 3**). Subsequent molecular characterization of other viroids (up to eight were already known by the beginning of the 1980s) confirmed the preservation of this rodlike folding and of a central conserved region (CCR) in most of them,[11] but not in *avocado sunblotch viroid* (ASBVd),[12] which thus appeared as the founding member of a second group of viroids. Initial reservations that ASBVd could rather be a viroid-like satellite RNA dissipated following discovery and characterization of *peach latent mosaic viroid* (PLMVd)[13] and subsequently of another two[6] with the same striking feature of ASBVd:[14] Their strands of both polarities self-cleave through hammerhead ribozymes, in other words, they are catalytic RNAs (as also is HDV RNA, see below), a property with profound connotations in the replication and evolutionary origin of these agents. The two groups of viroids, represented PSTVd and ASBVd, eventually resulted in the creation of two taxonomic families, *Pospiviroidae* and *Avsunviroidae*,[6] respectively, a classification that is independently supported by the different subcellular site of replication and accumulation (see below). Moreover, some members of the second family adopt clearly branched secondary structures, as illustrated by PLMVd.[13]

Discovery of HDV: Striking Similarities of Its Genomic RNA with Viroids

Immunofluorescence detection of a new antigen-antibody system (∂/anti-∂) associated with *hepatitis B virus* (HBV) in liver cell nuclei and in serum of patients with hepatitis B surface antigen (HBsAg)-positive chronic liver disease,[15] was soon followed by transmission experiments in chimpanzees indicating the association of ∂Ag with a transmissible pathogenic agent, the ∂ agent.[16] Moreover, ∂Ag in serum comigrated in gradient centrifugation with a discrete subpopulation of HBsAg particles, as well as with a small nucleic acid (specifically an RNA because it was sensitive to RNase but not to DNase) with an estimated molecular weight of only 5×10^5 (~14700 nt); if this RNA represented the genome of the ∂ agent, it would be smaller than those of the known RNA viruses, but larger than the viroids of higher

plants.[17] Here appeared a first connection between HDV and viroid RNAs.

Further studies confirmed that the genomic HDV RNA was indeed a single-stranded species of ~1.7-kilobases (kb).[18] Most importantly, not only the genomic HDV RNA isolated from infected chimpanzees or woodchucks (another experimental host), but also its antigenomic counterpart had a circular structure, a feature not reported so far for the genome of any animal virus, but characteristic of viroids.[19] Furthermore, these authors presciently proposed that once inside the infected cell HDV RNA, like viroids, could replicate without the help of a second virus, that is, HBV might simply provide the envelope proteins for transmission of the HDV genome from cell to cell and from animal to animal.[19] They also advanced that, if HDV replication occurred by a rolling-circle mechanism similar to that proposed for viroids known at that time,[20] then two types of rolling-circle should operate (see below).

The covalently closed circular structure of HDV RNA was confirmed by electron microscopy, and cloning and sequencing revealed a genome of 1678 or 1679 nt with an open reading frame in the antigenomic strand that would encode two possible isoforms of an antigen specifically recognized by antisera from patients with chronic hepatitis ∂ viral infection.[21] Moreover, HDV RNA, the sequence of which was independently corroborated by other groups (including a virus isolate directly obtained from a patient with acute ∂ hepatitis to exclude that serial passages in chimpanzees had altered the properties of human HDV),[22,23] could be folded on itself as a rodlike secondary structure with 61 to 70% of the bases paired.[21,23] This conformation was remarkably similar to that predicted for PSTVd (see above), thus extending the similarities between these two infectious agents. However, unlike PSTVd, HDV RNA was encapsidated (resembling in this respect plant satellite viruses and plant satellite RNAs) and encoded in its antigenomic polarity a protein (the ∂Ag), demanding for this purpose synthesis of an antigenomic mRNA of ~900 nt with the 5′-cap structure and 3′-polyA tail characteristic of this RNA class.[24] Editing by adenosine deaminase results in the generation of the large isoform (L-∂Ag), which is 19-aminoacid longer than the short isoform (S-∂Ag) of 195 aminoacids[25] and has a different functional role (see below).

Where and How Viroids and HDV Replicate?

The observation that the most abundant circular form, considered by convention as having the (+) polarity, is accompanied in tissues infected by PSTVd and other members of the family *Pospiviroidae* by lower amounts of oligomeric viroid RNAs of the complementary (−) polarity, led to the proposal that the latter resulted from reiterative transcription of the former through a rolling-circle mechanism[20] with only RNA intermediates.[26] Furthermore, fractionation of subcellular components by differential centrifugation revealed that both PSTVd strands accumulate in the nucleus,[27,28] a finding later corroborated by *in situ* hybridization combined with confocal laser scanning and transmission electron microscopy.[29,30] Intriguingly, similar studies with ASBVd and other members of the family *Avsunviroidae* (see for a review, ref. [6]) showed that (+) and (−) strands accumulated preferentially in the chloroplast. Given that nuclei and chloroplasts have different evolutionary origin and composition, deep distinctions in replication were anticipated for both viroid families. Regarding HDV, early Northern blot and *in situ* hybridizations with strand-specific probes located both the genomic and antigenomic RNAs in the nucleus of cultured woodchuck hepatocytes,[31] with subsequent experiments confirming this finding except that, after synthesis, the genomic RNA is preferentially exported from the nucleus to the cytoplasm.[32] Therefore, HDV RNA appeared more closely related to PSTVd than to ASBVd. Moreover, replication of HDV genome in cultured woodchuck hepatocytes did not seem to require coinfection with a hepadnavirus,[31] reinforcing the idea that HDV, like viroids, was dependent for replication exclusively on factors provided by the host cell.

A detailed comparative account of how viroids and HDV replicate through a rolling-circle mechanism has been recently published;[33] therefore, here we will just summarize the most salient features, focusing first on the templates and then on the enzymes (and ribozymes). Two alternative pathways of the mechanism, involving one or two rolling circles (the viroid literature refers to these pathways as asymmetric and symmetric, respectively), have been proposed. In the asymmetric pathway, the incoming monomeric circular (+) RNA is reiteratively transcribed into oligomeric (−) strands, which next serve as templates for synthesis of oligomeric (+) strands. In the symmetric pathway, the oligomeric (−) strands are first cleaved and ligated into the monomeric circular (−) RNA, which is the template for producing the oligomeric (+) strands. In both cases, the oligomeric (+) strands are cleaved and ligated to generate the final product of the replication cycle, the monomeric circular (+) RNA (►**Fig. 4**). Accordingly, the characteristic RNA of the symmetric pathway is the monomeric circular (−) form. Failure to detect this molecular species in tomato infected by PSTVd indicates that this and other members of the family *Pospiviroidae* replicate by the asymmetric pathway,[20,34] whereas its presence in avocado infected by ASBVd strongly supports that this and other members of the family *Avsunviroidae* follow the symmetric pathway.[35] Extending this line of thinking, detection in experimental hosts of the antigenomic HDV circular RNA, equivalent to the monomeric circular (−) form of viroids, led to the proposal that HDV replicates through the symmetric pathway with two rolling circles.[19]

The rolling-circle mechanism demands three catalytic activities that at least for viroids were initially presumed of host origin considering their lack of protein-coding ability: an RNA polymerase to produce the oligomeric strands, an RNase for cleavage to their unit-length linear counterparts, and an RNA ligase to generate the circular monomers. Characterization of these catalytic activities resulted in unexpected findings, prominent among which is that replication of viroid and HDV RNAs is mediated by DNA-dependent RNA polymerases forced to transcribe RNA templates (how this may happen is an unsolved, and intriguing, issue). More specifically,

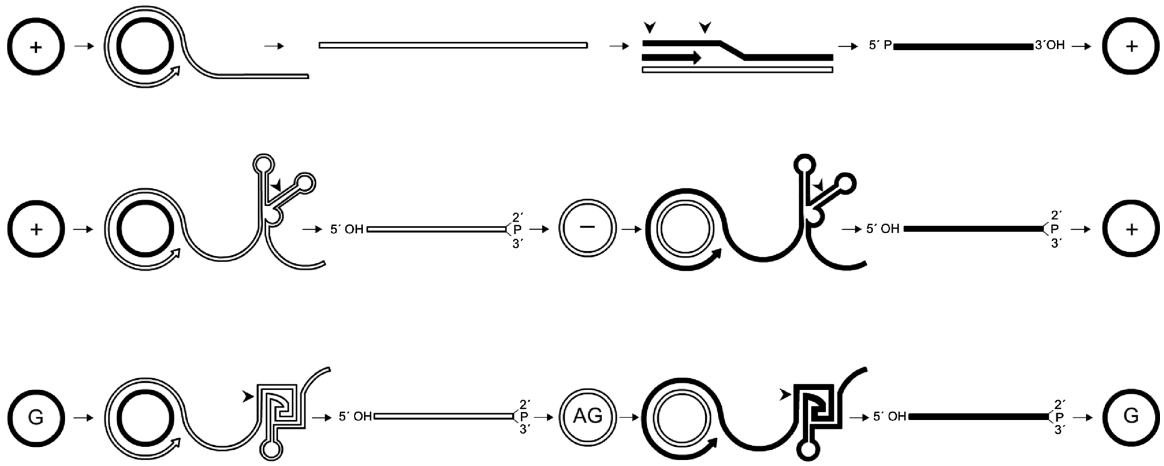

Figure 4 Alternative pathways of the rolling-circle mechanism proposed for replication of viroids and hepatitis D virus (HDV). Members of the family *Pospiviroidae* replicate in the nucleus through the asymmetric pathway with only one rolling-circle (upper panel), whereas members of the family *Avsunviroidae* and HDV replicate through the symmetric pathway with two rolling-circles in the chloroplast and nucleus (middle and lower panels, respectively). Solid and open lines refer to the plus (+) or genomic (G) polarity, and to the minus (−) and antigenomic (AG) polarity, respectively. Notice that cleavage (indicated with arrowheads) is catalyzed by host enzymes (HE), or alternatively by hammerhead or ð ribozymes, and results in linear monomeric RNAs with characteristic termini that are then circularized by host RNA ligases or autocatalytically.

synthesis of (+) and (−) strands of nuclear viroids (family *Pospiviroidae*) is prevented by the low concentrations of the mycotoxin α-amanitin characteristically inhibiting the nucleoplasmic RNA polymerase II (pol II), and both strands are immunoprecipitated from preparations of infected tissue by an antibody specific for the major subunit of this enzyme (see for a review, ref. [6]). Involvement of pol II in replication of HDV RNA is also well documented;[36] some studies have additionally implicated pol I and III,[37,38] although a recent reexamination of this issue with a proteomic-RNA interference search for host factors participating in HDV RNA replication has recovered subunits of pol II (but neither of pol I nor of pol III).[39] Furthermore, S-∂Ag binds pol II directly and stimulates transcription,[40] whereas viral RNA accumulation is negatively affected by L-∂Ag[36–38]; the activity of both isoforms can additionally be regulated by different posttranscriptional modifications.[41] In the family *Avsunviroidae,* the available evidence indicates that elongation of RNA strands is catalyzed by a nuclear-encoded RNA polymerase (NEP) that is translocated into plastids (mostly chloroplasts), rather than by a plastid-encoded polymerase (PEP). Evidence sustaining this view is based on the effects of the bacterial toxin tagetin, which inhibits PEP but not NEP,[42] and on detection of PLMVd replicating in peach leaves expressing a PLMVd-induced albinism wherein PEP-dependent transcription is essentially absent.[43]

A related issue is whether initiation of RNA strands is site-specific or not because reiterative transcription of the circular templates of viroid and HDV RNAs, followed by processing, ensures production of complete copies regardless of the initiation site. However, the available evidence indicates that initiation of viroid strands starts at precise sites located within specific structural domains.[44–46] The situation is less clear in this respect for HDV. On the one hand, identification of genomic and antigenomic HDV RNAs of 18–25 nt having defined 5′ termini with a cap structure (the chemical signature added cotranscriptionally to typical pol II transcripts), suggests that these small RNAs could prime site-specific initiation of HDV strands.[47] On the other hand, synthesis of HDV strands could be site-independent and primed by nascent RNAs transcribed from the host DNA, which associated with the polymerase could detach, jump on a HDV RNA, and resume transcription.[36] Template switching during HDV replication seems a realistic assumption because cotransfected less-than-unit HDV RNAs can generate the complete replicating form[48]; template switching, moreover, might be facilitated by the low processivity (inability to transcribe long nucleotide stretches) of host RNA polymerases when acting on RNA rather than on their typical DNA templates.

Characterization of the catalytic activity involved in the second replication step also provided surprises. Cleavage of the oligomeric (+) RNAs in the family *Pospiviroidae* appears determined by a double-stranded structure in which some specific phosphodiester bonds become particularly susceptible to an RNase III-like enzyme.[49] In the family *Avsunviroidae,* however, this reaction is not mediated by host-encoded enzymes, but by ribozymes embedded in both polarity strands.[50] Contrary to the long-standing biochemical dogma ("all enzymes are proteins"), some RNAs do express catalytic activity (and are termed ribozymes).[51,52] In ASBVd and members of its family, the ribozymes are of the hammerhead class, the smallest and most deeply studied. These findings opened the possibility, later confirmed, that ribozymes could also participate in replication of HDV RNA.[53] HDV ribozymes are of another class and endowed with very peculiar properties.[54] Summarizing, replication of HDV RNA occurs in the nucleus (like in the family *Pospiviroidae*), but through a symmetric double-rolling-circle mechanism with ribozyme-mediated self-cleavage of both strands (like in the family *Avsunviroidae*). Self-cleavage in vivo most likely occurs

cotranscriptionally,[55,56] and it is facilitated (but not catalyzed) by proteins termed RNA chaperones of host origin in viroids[57] and of viral origin (the ∂Ag) in HDV.[58]

The final ligation step of the replication cycle is poorly understood. Because the RNase III-like enzyme proposed to mediate cleavage of the oligomeric (+) strands in the family *Pospiviroidae* generates 5′-P and 3′-OH termini,[49] the existence of a plant RNA ligase activity able to catalyze their joining must be postulated; this activity has been recently identified (Nohales, Flores, and Daròs, unpublished data). In contrast, the hammerhead-mediated self-cleavage characteristic of the family *Avsunviroidae* results in 5′-OH and 2′,3′ cyclic phosphodiester termini. Because such termini are specifically joined by tRNA ligases, epitomized in plants by the wheat germ tRNA RNA ligase,[59] an enzyme of this class appears the best candidate for catalyzing circularization, particularly considering its predominant localization in chloroplasts and proplastids.[60] Other alternatives, however, cannot be excluded.[22] Lastly, although initial data pointed out that the 5′-OH and 2′,3′ cyclic phosphodiester termini produced by HDV ribozymes can self-ligate resulting in a mixture of 2′,5′- and 3,-5-phosphodiester bonds,[61] other data indicate that circularization is catalyzed by a host-specific enzyme.[62] This issue deserves further examination.

Viroid and HDV RNAS: Evasion of Host Defenses and Disease Induction

As indicated above, most viroids and HDV RNAs adopt typical rodlike secondary structures promoted by their circularity and extensive autocomplementarity.[9,10,19,23] These compact conformations must not have evolved by chance and likely result from selection pressures that include resistance against endoribonucleases (the majority of which have preference for single-stranded RNAs) and exoribonucleases (inactive on circular RNAs), and the need to provide small RNA motifs stabilized by non-Watson–Crick pairs and tertiary interactions[63,64] crucial for replication or movement.[65] In the specific case of HDV RNAs, their fold-back structure resembling an imperfect double-stranded RNA (dsRNA) probably emerged from a compromise between (1) eluding the interferon-induced antiviral response triggered in animals by the dsRNA-dependent protein kinase, with which HDV RNA interacts in a paradoxical manner[66] and (2) facilitating editing in the antigenomic RNA (eventually producing L-∂Ag) catalyzed by adenosine deaminase that has a preference for dsRNA domains.[25,67]

Given that viroids are non-protein-coding RNAs, they must incite disease as a consequence of the direct interaction of their genomic RNA (or derivatives thereof, see below) with host components. Initially, research focused on interactions between the most abundant viroid circular form and host proteins required for various purposes, for instance, replication. The finding that natural or artificial nucleotide changes mapping at specific short RNA motifs modulate symptom expression in both viroid families, was interpreted assuming that these motifs (the so-called pathogenic determinants) served as binding sites for recruiting host proteins, the sequestration of which from their normal roles would ultimately result in disease induction. Using different experimental approaches, a quite diverse suite of host proteins—including histones, phloem lectins, and protein kinases that mediate hormonal signaling cascades—were identified interacting with PSTVd, as well as some chloroplastic proteins interacting with ASBVd.[6,68] In addition, side-by-side comparisons of protein and transcript patterns from viroid-infected and mock-inoculated plants uncovered that PSTVd infection deeply modifies expression of many tomato genes involved in diverse pathways that comprise defense and stress,[69] cell growth,[70] and hormone biosynthesis.[71] However, most of these alterations may be late effects and, consequently, nonspecific.

Discovery of RNA silencing has provided new insights on how viroids may induce disease, changing the focus to their interactions with host nucleic acids. RNA silencing is a defense mechanism initially evolved in most eukaryotes for containing the invasion of foreign viruses and the undue proliferation of endogenous transposons. This mechanism is mediated by small interfering RNAs (siRNAs) produced by a family of class-III RNases (dicer or dicer-like [DCL], in plants) acting on the genomic viral RNA, and particularly on the dsRNA replicative intermediates; the resulting primary siRNAs, together with secondary siRNAs generated by an amplification circuit, are transferred to proteins of the family *Argonaute* (AGO; at the core of the RNA-induced silencing complex [RISC]) and guide them to specifically promote postranscriptional degradation of viral RNAs, or transcriptional inactivation of transposon DNAs. RNA silencing also regulates development through pathways having components in common with the defensive pathway, but mostly mediated by endogenous small RNAs termed microRNAs (miRNAs). RNA silencing appears particularly relevant in plants, with four DCL and 10 AGO paralogous genes in *Arabidopsis thaliana,* but only one and four counterparts, respectively, in human cells.[72] The finding that infections by members of both viroid families triggers the accumulation of viroid-derived small RNAs (vd-sRNAs) structurally similar to the siRNAs,[73–78] strongly supports that viroid RNAs are targeted by one or more host DCLs. Furthermore, there is circumstantial evidence indicating that vd-sRNAs may load RISC and downregulate viroid titer,[79] as well as prevent invasion of meristems, the niche for stem cells.[80] Alternatively, vd-sRNAs might mimic miRNAs and guide RISC against complementary regions of some host messenger RNA (mRNA), targeting it for degradation and promoting disease induction.[73,81]

The antiviral role of RNA silencing first reported in plants has been later extended to insects and nematodes;[82,83] however, its role in mammals, wherein virus infections are contained by an adaptive immune system, is less apparent. Consistent with this view, essentially no HDV-derived sRNAs were detected during HDV replication in hepatocytes of human, woodchuck, and mouse origin, or after incubation *in vitro* of unit-length HDV RNAs with human recombinant dicer.[84] Therefore, it is unlikely that RNA silencing may play a major role in disease induction by HDV, thus suggesting the involvement of alternative mechanisms.

Emergence and Evolution of Viroid and HDV RNAs: Moving Back to the Origin of Life

A paradigm generally accepted in evolution is that "the simplest is the oldest." In line with this view, viroids are postulated to have a very old origin (independent from that of viruses) that goes back to the RNA world assumed to have preceded our current world based on DNA and proteins. The need for such an RNA world is easy to understand considering that proteins can express genetic information (i.e., catalyzing chemical reactions) but they cannot store it, whereas DNA behaves the other way around. Given that the very different chemical composition of DNA and proteins excludes their simultaneous emergence in evolution, another macromolecule with the ability to concurrently store and express the genetic information appears the most plausible alternative. This macromolecule is RNA: It is the genetic material of many viruses, including HDV, and it may directly express a function (as illustrated by the ribozymes catalyzing self-cleavage during replication of HDV and members of the family *Avsunviroidae*). Other features of viroids (including their lack of protein-coding ability), support the tenet that they may be "living fossils" or "molecular relics" of the RNA world that, following the emergence of cellular organisms, would have adopted an intracellular mode of survival either in the nucleus (the typical eukaryotic organelle) or in the chloroplast (acquired by symbiosis with cyanobacteria).[85] Subsequent mutation events—a chloroplastic viroid has the highest mutation rate reported for any biologic entity[86]—and recombination episodes generated by template switching during replication of coinfecting viroids would have facilitated their evolution.

What are the ideas about HDV in this respect? For a full discussion of this topic, we refer the readers to a recent review.[87] We will only highlight here that dissection of the antigenomic HDV RNA has disclosed that it is composed of a catalytic viroidlike domain, containing the ribozymes and forming one of the terminal regions of the rodlike secondary structure, fused to a protein-coding domain. Such observation suggests the exciting scenario that this HDV RNA may have evolved from the capture by a self-replicating viroidlike RNA of the mRNA coding for a protein like the ∂Ag.[88] Having this protein as a positive influence on the replication of the resulting fusion product would have ensured its survival. Recent bioinformatics analyses, supported by biochemical evidence, have yielded a new surprising twist: hammerhead- and HDV-like self-cleaving ribozymes are ubiquitously present and expressed along the tree of life. The available data suggest key regulatory roles for these catalytic motifs (including mRNA biogenesis in humans) and that they have become integrated in the corresponding DNA genomes by retrotransposition.[89–91] In this same context, recent data obtained with a combined experimental/computational approach that enriches for circular RNAs and allows their unbiased profiling in whole genomes, have revealed the presence of circular non-protein-coding RNAs in *Archaea*, wherein they are relatively abundant and might have yet unidentified biologic roles.[92]

Concluding Remarks and Future Perspectives

Returning to the beginning of this review, research in plants led to the emergence of genetics and virology. Within the latter discipline, the unexpected discovery of viroids (restricted to plants so far) set the stage for rapid acceptance of the uncommon structure of the human pathogen HDV, and for predicting (rightly) some of its functional features: replication by a rolling-circle mechanism mediated by a template-redirected RNA polymerase(s) and by ribozymes embedded in the viral RNAs.[93] Viroids are unique in having developed the ability to parasitize the transcription machinery of their hosts (as indicated above, how they achieve this goal remains an intriguing conundrum), whereas viruses mostly parasitize their host translation machinery. HDV displays a dual behavior: It depends on both a preexisting host-encoded RNA polymerase (like viroids) and on a viral-encoded protein (like viruses). Work on viroids (and HDV) has also opened an unexpected window to figure out the nature of the initial replicons that populated our planet before the advent of cellular life. Moreover, viroid and HDV ribozymes have found wide applications in molecular biology and biotechnology. The final message of this article is that keeping an open mind, as opposed to a too narrow focus, is a wise strategy for a fruitful research: Inspiration can come from bordering and even apparently distant areas.

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Abbreviations

AGO	argonaute
ASBVd	avocado sunblotch viroid
CCR	central conserved region
cDNA	complementary DNA
∂Ag	delta antigen
DCL	dicerlike
DNase	deoxyribonuclease
dsRNA	double-stranded RNA
HBV	hepatitis B virus
HDV	hepatitis delta virus
L-∂Ag	large isoform delta antigen
miRNA	microRNA
mRNA	messenger RNA
NEP	nuclear-encoded RNA polymerase
PAGE	polyacrylamide gel electrophoresis
PEP	plastid-encoded RNA polymerase
PLMVd	peach latent mosaic viroid
pol	polymerase
PST	potato spindle tuber
PSTVd	potato spindle tuber viroid

RNase	ribonuclease
RISC	RNA-induced silencing complex
S-∂Ag	short isoform delta antigen
siRNA	small interfering RNA
TMV	tobacco mosaic virus
tRNA	transfer RNA
vd-sRNA	viroid-derived small RNA

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