Document downloaded from:

http://hdl.handle.net/10251/84540

This paper must be cited as:

Flores Pedauye, R.; Grubb, CD.; Elleuch, A.; Nohales Zafra, MA.; Delgado, S.; Gago Zachert, SP. (2011). Rolling-circle replication of viroids, viroid-like satellite RNAs and hepatitis delta virus: variations on a theme. RNA Biology. 8(2):200-206. doi:10.4161/rna.8.2.14238.

The final publication is available at

http://doi.org/10.4161/rna.8.2.14238

Copyright Taylor & Francis

Additional Information

Rolling-circle replication of viroids, viroid-like satellite RNAs and hepatitis delta virus: variations on a theme

Ricardo Flores*, Douglas Grubb, Amine Elleuch, María-Ángeles Nohales, Sonia Delgado and Selma Gago

Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC); Campus Universidad Politécnica; Avenida de los Naranjos; Valencia, Spain

Abstract

Viroids and viroid-like satellite RNAs from plants, and the human hepatitis delta virus (HDV) RNA share some properties that include small size, circularity and replication through a rolling-circle mechanism. Replication occurs in different cell compartments (nucleus, chloroplast and membrane-associated cytoplasmatic vesicles) and has three steps: RNA polymerization, cleavage and ligation. The first step generates oligomeric RNAs that result from the reiterative transcription of the circular templates of one or both polarities, and is catalyzed by either the RNA-dependent RNA polymerase of the helper virus on which viroid-like satellite RNAs are functionally dependent, or by host DNA-dependent RNA polymerases that, remarkably, viroids and HDV redirect to transcribe RNA templates. Cleavage is mediated by host enzymes in certain viroids and viroid-like satellite RNAs, while in others and in HDV is mediated by *cis*-acting ribozymes of three classes. Ligation appears to be catalyzed mainly by host enzymes. Replication most likely also involves many other non-catalytic proteins of host origin and, in HDV, the single virus-encoded protein.

Running title: Replication of viroids, viroid-like satellite RNAs and hepatitis delta virus

Key words: small infectious RNAs, small catalytic RNAs, ribozymes, protein-RNA interactions, RNA binding proteins

*Correspondence to: Ricardo Flores; Instituto de Biología Molecular y Celular de Plantas (UPV-CSIC); Campus Universidad Politécnica; Avenida de los Naranjos; 46022 Valencia, Spain; Tel.: +34.963877861; Fax: 34.963877859; Email: rflores@ibmcp.upv.es

Douglas Grubb's present address: Department of Molecular Signal Processing; Leibniz-Institut für Pflanzenbiochemie; Weinberg 3, 06120 Halle, Germany

Amine Elleuch's present address: Laboratoire des Biotechnologies Végétales Appliquées à l'Amélioration des Cultures; Faculté des Sciences de Sfax BP 1171; 3000 Sfax, Tunisia

Introduction: the subviral RNA world

Discovery of satellite viruses[1] and, particularly, of satellite RNAs[2], viroids[3] and human hepatitis delta virus (HDV)[4], overturned the more than 50-year-old paradigm regarding viruses as the lowest step of the biological scale, and revealed the existence of a subviral world populated by a quite diverse spectrum of small RNA replicons. Plant satellite viruses and satellite RNAs depend for their replication and transmission on co-infection of the host cell by a helper virus; while the former encode a structural protein encapsidating their genome, the latter are encapsidated by the coat protein of the helper virus. On the other hand, HDV RNA encodes a non-structural protein that promotes its accumulation —and possibly its autonomous replication— in infected cells, but depends for transmission on hepatitis virus B. Finally, viroids are non-protein-coding and autonomously-replicating RNAs that infect plants without a concurrent helper virus. There are reviews dealing with different aspects of the infectious cycle of these singular RNA replicons[5-11]. Here, we will focus on the replication of: i) viroids and a special class of satellite RNAs, the so-called viroid-like satellite RNAs, as representatives of the plant kingdom, and ii) HDV as the single representative of the animal kingdom. All replicate through an RNA-based rolling-circle mechanism involving circular templates and longer-than-unit intermediates, which are cleaved by host enzymes —or *cis*-acting ribozymes embedded in their strands— and subsequently ligated[12]; however, comparative analysis reveals significant variations on this theme.

Structural similarities (and differences)

Early recognition of the similarities existing between viroids, viroid-like satellite RNAs and HDV RNA[12] led to the classification of these replicons within "the brotherhood of the small RNA circular pathogens", thus highlighting their shared properties: small size, circular structure, and pathogenicity. Leaving apart pathogenicity, which lies outside the scope of the present review, this common denominator is punctuated by some differences. Regarding size, the genomes of viroids and viroid-like satellite RNAs (between 220 and 400 nt)[5,13] are too small to encode a protein of minimal complexity, whilst HDV RNA is significantly larger (~1700 nt)[14], sufficient for encoding in its antigenomic polarity a protein, the delta antigen (∂Ag). However, a closer examination of HDV RNA has led to the proposal that it comprises a ~350-nt viroid-like domain, wherein the ribozymes critical for replication reside[15], fused to another domain containing the region coding for the ∂Ag; implicit in this proposal is the recombinant origin of HDV RNA (see below).

The circular structure of viroid, viroid-like satellite and HDV RNAs, in addition to affording protection against exonucleases, ensures full-genome replication without recurring to initiation or termination tags —a mechanism potentially relevant for primitive RNA self-replicating systems (see below)— and may provide specific signals for encapsidation. Indeed, the circular form is the predominant RNA encapsidated in viroid-like satellite RNAs of sobemoviruses, for which the term virusoid was coined, while the linear form is predominantly encapsidated in viroid-like satellite RNAs of nepo- and luteoviruses[5,16]. Besides, only the HDV genomic RNA is assembled into viral particles[17], requiring resolution of the problem posed by circular RNAs, which cannot be translated through a conventional ribosome scanning pathway that demands a 5'-cap structure and a 3'-polyA tail, by producing an antigenomic mRNA of ~900-nt with these characteristics[18].

Finally, sequence similarities between viroids and viroid-like satellite RNAs allow their subdivision into specific taxonomic classes: viroids are clustered into families *Pospiviroidae* (type species *Potato spindle tuber viroid,* PSTVd) and *Avsunviroidae* (type species *Avocado sunblotch viroid,* ASBVd), and viroid-like satellite RNAs into groups that parallel those of their helper RNA viruses (despite lacking sequence similarities with them)[19]. Other functional properties support this classification scheme (see below). However, the higher-order structural similarities are even more appealing. Due to their extensive self-complementarity, viroid, viroid-like satellite and HDV RNAs adopt a characteristic rod-like or quasi-rod-like secondary structure[20-22]. Exceptions to this rule are the clearly branched secondary structure of certain viroids[23]. These compact but imperfect secondary structures must have evolved from a compromise and most likely: i) confer resistance against endonucleases like those involved in RNA silencing, a process that seems particularly active in plants[8,24], ii) avoid the animal-specific defensive response triggered by the dsRNA-dependent protein kinase, iii) contain small RNA motifs that are essential for replication or movement, as revealed by a recent mutational analysis of PSTVd[25], and iv) facilitate editing by an adenosine deaminase characteristically operating on dsRNA substrates, a process occurring during replication of HDV RNA leading to synthesis of the large isoform of the delta antigen (L-∂Ag), which is 19-aminoacid longer than its short counterpart (S-∂Ag)[26]. Moreover, physical, biochemical and reverse genetic approaches have unveiled elements of tertiary structure that endow these RNAs with additional stability and functional abilities[11,27-29].

Subcellular replication sites

Because viroids and HDV RNA rely on pre-existing host RNA polymerases for their replication, this process must take place in subcellular compartments harboring such enzymes. Early studies detected the most abundant PSTVd circular RNA —arbitrarily regarded as having (+) polarity— and its oligomeric (-) strands in nuclear fractions, strongly implicating a nuclear RNA polymerase in replication[8,12]. Employing finer tools, these results were confirmed for PSTVd and extended to other members of the family *Pospiviroidae*[30,31], which are usually referred to as nuclear viroids. Remarkably, similar experiments showed the preferential accumulation in plastids of (+) and (-) strands of ASBVd and other members of the family *Avsunviroidae,* which are usually referred to as chloroplastic viroids and assumed to be replicated by a chloroplastic RNA polymerase[8,32,33]. These data also supported a rolling-circle mechanism in which the oligomeric (+) and (-) strands were considered replication intermediates arising from reiterative transcription of circular templates of one or both polarities[12].

In parallel with the situation observed for PSTVd and related viroids, initial studies found the genomic and antigenomic HDV RNAs in the nucleus[34], and subsequent experiments substantiated this result, although the genomic RNA has been also observed in the cytoplasm[35]. Nuclear import of HDV RNA is mediated by the S-∂Ag, which has a nuclear localization signal and an RNA binding motif[36,37]. It is plausible that host proteins may play a similar role in translocating viroid RNAs to the nucleus and, particularly, to the chloroplast. Incidentally, members of the family *Avsunviroidae* are among the very few RNAs able to cross the chloroplastic membrane.

On the other hand, given their dependence on RNA polymerases encoded (at least one of the subunits) by their helper RNA viruses, which replicate in the cytoplasm and more specifically in membranous vesicles connected with the cytoplasm[38], replication of viroid-like satellite RNAs presumably also occurs within these vesicles. However, in the absence of definitive experimental evidence, the possibility that the helper virus may contribute protein(s) redirecting a host RNA polymerase to catalyze transcription of viroid-like satellite RNAs must be also entertained[5].

Polymerization of RNA strands

In striking contrast with viroid-like satellite RNAs, viroid and HDV RNAs are replicated by DNA-dependent RNA polymerases redirected to accept RNA templates. How this change of template specificity occurs in nuclear (and chloroplastic) RNA polymerases is intriguing, as also is the question of why evolutionary processes selected these enzymes rather than

RNA-dependent RNA polymerases, which are particularly well-represented in plants[39] and have been also described in mammals[40]. Perhaps viroid and HDV RNAs need additional nuclear (and chloroplastic) factors for their replication, whereas viroid-like satellite RNAs have adapted themselves to the subcellular microenvironment in which their helper viruses replicate.

Setting aside some initial discrepancies, there is solid biochemical and immunological evidence supporting the involvement or RNA polymerase II (Pol II) in replication of nuclear viroids: synthesis of their (+) and (-) strands is inhibited *in vitro* and *in vivo* by low concentrations of α-amanitin, and both polarity strands are pulled down from preparations of infected tissue by an antibody against the major subunit of Pol II[8]. Notably, there is also a general consensus involving Pol II in replication of HDV but, while some studies implicate this enzyme in the nucleoplasmic synthesis of genomic and antigenomic strands (in addition to the subgenomic mRNA of antigenomic sense encoding ∂Ag), others indicate that elongation of antigenomic strands is catalyzed by Pol I in the nucleolus; to complicate even more the situation, new data have additionally implicated Pol III. Evidence supporting these partially contradictory views, as well as the experimental approaches on which they are based, can be found in the original publications and in recent reviews[41-43]. Crystallographic analysis has shown that an RNA template-product duplex occupies in Pol II the same site as the DNA-RNA hybrid during transcription, although processivity *in vitro,* but not necessarily *in vivo,* is limited[44]. Also pertinent to this context are the findings of a combined proteomic-RNAi screen for host factors involved in HDV RNA replication in which, in addition to other proteins, subunits of Pol II (but neither of Pol I nor of Pol III) were retrieved (see below). Moreover, S-∂Ag is essential for accumulation (and perhaps replication) of HDV RNA[45], and can be regulated by different post-transcriptional modifications as also can be L-∂Ag[46], which has a dominant negative effect on viral RNA accumulation[41-42].

RNA elongation in the family *Avsunviroidae* appears to be catalyzed by a nuclear-encoded RNA polymerase (NEP) that is translocated into plastids, rather than by a plastid-encoded polymerase (PEP). Data supporting this notion include the effects of tagetitoxin, which inhibits PEP but not NEP, on *in vitro* synthesis of host and viroid RNAs by chloroplastic preparations from ASBVd-infected tissue[47], together with active PLMVd replication in peach leaves showing a PLMVd-induced albinism wherein PEP-dependent transcription is essentially abolished[48]. Other data, however, have proposed the involvement of PEP in PLMVd replication, although they have been obtained with a heterologous *in vitro* system (replacing PEP by the RNA polymerase of *Escherichia coli)*[49].

Initiation of RNA strands: site- or non-site-specific? Unprimed or primed?

In theory, reiterative transcription of circular templates —like those of viroid, viroid-like satellite and HDV RNAs— and subsequent transcript cleavage (see below), lead to the same products irrespective of whether initiation is site-specific or not. This latter question has been addressed in the family *Avsunviroidae* taking advantage of the observation that, in chloroplasts, primary RNA transcripts contain a characteristic 5′-triphosphorylated group that can be mapped by biochemical approaches (namely, *in vitro* capping followed by either RNase protection assays or RNA ligase-mediated rapid amplification of cDNA ends). Using this strategy, initiation of (+) and (-) strands in ASBVd has been mapped at the right terminal A+U-rich loops of their proposed quasi-rod-like secondary structures[50], and in PLMVd at short double-stranded RNA motifs that also contain the self-cleavage sites[51]. Therefore, two structurally distinct elements, hairpin-loops and double-stranded RNA motifs, serve the same purpose, with their adjacent regions being also possibly involved in recruiting both NEP and auxiliary transcription factors.

The picture is less complete in the family *Pospiviroidae,* wherein initiation of PSTVd (-) strands has been mapped to a specific position in the left terminal loop[52]. Yet, this result has been obtained with a potato nuclear extract supplemented with the PSTVd monomeric (+) circular RNA, and assumes that the initiation complex reconstituted *in vitro* reproduces that existing *in vivo.* Moreover, the transcription initiation site for (+) strands remains unidentified.

HDV is a particularly appealing case because, besides the genomic and antigenomic circular forms and their oligomeric linear replicative intermediates, the subgenomic ∂Ag-mRNA of antigenomic polarity (with the characteristic 5′-cap and 3′-polyA tail) is also generated. These termini imply that Pol II, irrespective of whether it is transcribing DNA or RNA templates, has the potential to act in concert with the corresponding 5′-capping and 3′-processing cellular machinery. Does Pol II also recruit the 5′-capping machinery when catalyzing the synthesis of the genomic and antigenomic strands? Interestingly, capped small RNAs (18-25 nt) have been recently detected and associated with HDV replication: one of antigenomic polarity has the same 5′-terminus as the ∂Ag-mRNA and interacts with ∂Ag and Pol II, while the 5′-terminus of the other maps at a structurally analogous region (the so-called pode) of the genomic RNA[53]. These capped small RNAs may well reflect the HDV transcription initiation sites, because typical Pol II transcripts are capped co-transcriptionally (incidentally, this rationale could be extended to members of the family *Pospiviroidae,* the

transcription of which is also catalyzed by Pol II). In the proposed replication model[53], annealed capped small RNAs would prime initiation of replication, given that primed HDV RNA-directed transcription has been observed *in vitro*[44]. Alternatively, synthesis of HDV RNA strands could be non-site-specific and primed by nascent host RNAs[41]. The possibility that the complex formed by a host RNA polymerase and a host RNA being transcribed from a DNA template could detach, jump and resume transcription on a HDV RNA, has experimental support because template switching appears particularly feasible in HDV replication. Actually, co-transfected less-than-unit HDV RNAs can reconstitute the full-length replicating form[54], a finding that must be explained by template switching or by invoking an unknown, homology-based RNA *trans*-splicing mechanism. Template switching in this case may be stimulated by the low processivity of host RNA polymerases transcribing RNA templates instead of their physiological DNA counterparts. A final related issue, still controversial, is whether synthesis of the subgenomic ∂Ag-mRNA is linked to the HDV replication, or the processes are independent and reside in distinct complexes[42]. This complication does not exist in viroids and viroid-like satellite RNAs, which do not encode any protein in their genomes and, therefore, there is no possible interference between replication and transcription.

Cleavage or self-cleavage of concatemeric RNA intermediates

Study of the processing of the oligomeric RNA intermediates resulting from the rolling-circle mechanism into their unit-length counterparts has led to unanticipated findings: it may occur in strands of one or both polarities (resulting in two variants of the rolling-circle mechanism), and be catalyzed by host-encoded enzymes or by RNA-embedded ribozymes.

Because only the oligomeric (-) strands, and not their monomeric circular derivative, have been identified in PSTVd-infected tomato, this and other members of the family *Pospiviroidae* presumably replicate via an asymmetric pathway with a single rolling-circle[55,56] (Fig. 1). The available evidence indicates that cleavage of oligomeric (+) RNAs is determined by a specific RNA conformation and, although several alternatives have been offered[57], the most recent data support a double-stranded structure that makes certain phosphodiester bonds particularly vulnerable to an RNase III-like enzyme typically producing 5′-P and 3′-OH termini[58,59]. Moreover, while PSTVd (-) strands accumulate in the nucleoplasm, (+) strands are found in the nucleolus as well as in nucleoplasm; these results suggest that synthesis of viroid RNAs of both polarities is catalyzed by Pol II in the nucleoplasm, but only (+) strands

are subsequently transferred to and processed in the nucleolus, where processing of the rRNA and tRNA precursors also takes place[31].

On the other hand, because the monomeric circular (-) RNA has been detected in ASBVd-infected avocado, replication of this viroid and other members of its family is proposed to occur by a symmetric pathway with two rolling-circles[32] (Fig. 1). Furthermore, cleavage of the oligomeric (+) and (-) RNAs to their unit-length forms is autolytic and mediated by *cis*-acting hammerhead ribozymes, which were first reported in ASBVd and a viroid-like satellite RNA[60,61] (Fig. 1). In contrast with the hammerhead structures of the other chloroplastic viroids, those of ASBVd are thermodynamically unstable and self-cleavage most likely occurs through double-hammerhead structures transiently formed during replication by the oligomeric intermediates[62]. *In vivo,* self-cleavage presumably takes place during transcription[63], is catalyzed by the central conserved core of the hammerhead structure, and is facilitated by interactions between loops that flank this core[64-66] and with host proteins acting as RNA chaperones[67].

Replication of HDV RNA is somewhat a hybrid sharing features of the two pathways operating in viroids. As in the family *Pospiviroidae,* it takes place in the nucleus (see above) but, like in the family *Avsunviroidae,* through a symmetric double-rolling-circle mechanism with self-cleavage catalyzed by ribozymes of a unique class[68]; these ribozymes also operate *in vivo* co-transcriptionally[69] and are stimulated by a specific RNA chaperone: the ∂Ag[70].

Viroid-like satellite RNAs represent another hybrid situation: i) three satellite RNAs from sobemoviruses replicate through an asymmetric rolling-circle pathway with hammerheads only in the (+) strand, ii) the other two satellite RNAs from sobemoviruses and the single one associated with a luteovirus replicate using a symmetric rolling-circle pathway with hammerheads in both polarity strands, and iii) the three satellite RNAs from nepoviruses also use a symmetric rolling-circle replication pathway, but with hammerheads in the (+) strand and another class of ribozyme, dubbed hairpin, in the (-) strand[71-74]. Moreover, in the luteovirus satellite RNA, adoption of an active single-hammerhead structure in the unit-length RNA is prevented by a pseudoknot while, in oligomeric RNAs, a double-hammerhead structure lacking the pseudoknot can be formed and mediate their efficient self-cleavage[75]. Similar elements of tertiary structure (pseudoknots) have been reported in HDV ribozymes[76].

Circularization of unit-length linear RNAs

This third replication step is the least-well studied. A corollary of the recent proposal that an RNase III-like enzyme mediates *in vivo* cleavage of the oligomeric (+) strands in the family *Pospiviroidae* (see above), is that plants must have an RNA ligase able to catalyze ligation of the resulting 5′-P and 3′-OH termini, which after cleavage are brought in physical proximity through a conformational switch seemingly promoted by an element of tertiary structure (the loop E in PSTVd and closely-related viroids)[58]. Preliminary experimental evidence in support of the existence of such an RNA ligase activity has been obtained recently (Nohales, Flores and Daròs, unpublished data).

In the family *Avsunviroidae,* the monomeric RNA resulting from the hammerhead-assisted self-cleavage contains 5′-OH and 2′,3′ cyclic phosphodiester termini, which are those characteristically demanded by the wheat germ tRNA ligase[77]. This enzyme is a good candidate for mediating circularization because, together with the 2′-phosphotransferase (which catalyzes splitting of the 2′-phosphomonoester group generated by the tRNA ligase), are predominantly targeted to chloroplasts and proplastids[78]. As in the previous case, a conformational switch is also needed to bring into close proximity and proper orientation the two termini resulting from self-cleavage; recent mutational analysis of a chloroplastic viroid suggests that the ligation site is located in an internal loop, within a quasi-double stranded motif, of the most stable secondary structure predicted for the viroid RNA[79]. Alternatively, circularization of chloroplastic viroids might be an RNA-mediated mechanism: i) catalyzed by the hammerhead ribozyme and producing a 3′,5′-phosphodiester linkage, thus resembling the mode of action of the hairpin ribozyme (see below), or ii) autocatalyzed (self-ligation) and producing an atypical 2′,5′-phosphodiester bond[80]. A critical assessment of these options has been presented elsewhere[57].

Directly related with the situation described for the family *Avsunviroidae,* ligation *in vivo* of the two termini arising from hammerhead-mediated self-cleavage of the (+) strands of one viroid-like satellite RNA results in a 2′-phosphomonoester, 3′,5′-phosphodiester group; this is the expected finger print of a tRNA ligase, if it is assumed that the 2′-phosphotransferase is blocked by, for instance, encapsidation of the circular RNA by the coat protein of its helper virus[81]. The 2′-phosphomonoester, 3′,5′-phosphodiester group may also promote *in vitro* jumping of the reverse transcriptase, thus explaining the deletions of residues flanking the ligation site observed in cDNA clones of some viroid-like satellite RNA[82] and chloroplastic viroids[83]. Implicit in the involvement of tRNA ligase in circularization of viroid-like satellite RNAs is that a fraction of this enzyme must co-localize in the same subcellular compartment where replication of their helper viruses take place (see above). However, the hairpin

ribozyme, which in the three satellite RNAs of nepoviruses mediates self-cleavage of (-) strands, also seems to catalyze their ligation producing a 3′,5′ phosphodiester bond[71,72]; it must be noted in this context that the RNA ligase activity of the hairpin ribozyme is significantly higher than that of the hammerhead ribozyme.

Finally, the HDV ribozymes, like their hammerhead and hairpin counterparts, generate 5′-OH and 2′,3′ cyclic phosphodiester termini. Early results suggested that they can self-ligate producing a mixture of 2′,5′- and 3,-5-phosphodiester bonds, but more recent data support the view that circularization is catalyzed by a host-specific enzyme[84]. Further characterization of this enzyme has not been achieved, although tRNA ligase appears a likely candidate.

Role of other host proteins

Due to their minimal genomic size and lack of protein-coding capacity, replication of viroids most likely needs the participation of many host proteins that complement and modulate the enzymes (and ribozymes) directly involved in catalyzing the three steps of the rolling-circle mechanism: RNA polymerization, cleavage and ligation. The search for these host factors has been addressed with *in vitro* binding assays between viroid RNAs and host proteins extracted from infected tissues or expressed from cDNA libraries, or with *in vitro* analysis of subcellular fractions. These approaches have met with partial success and have left unanswered whether the observed interactions also occur *in vivo* and their possible functional role (see for a discussion ref. 67), although at least in one case a host protein with a major role in PSTVd infection has been identified[85]. To focus on viroid-protein interactions relevant *in vivo,* ASBVd-infected avocado leaves were directly irradiated with ultraviolet light: mass spectrometry (MS) of the most abundant ASBVd RNA-host protein adducts formed *in vivo* identified two closely-related chloroplast RNA-binding proteins of a family whose members are involved in stabilization, maturation and editing of chloroplast transcripts[67]. One of these two proteins acts as an RNA chaperone and facilitates *in vitro,* and presumably *in vivo,* the hammerhead-mediated self-cleavage of oligomeric ASBVd RNAs. Attempts to extend the same methodology to PSTVd-infected tissues have been unsuccessful so far, at least in part because PSTVd accumulates *in vivo* to levels significantly lower than ASBVd (Nohales, Flores and Daròs, unpublished data).

The interactions of host proteins with HDV components (RNAs or ∂Ag isoforms), and the approaches used, have been very recently reviewed[43]. Given the intrinsic experimental limitations of some of these approaches, and the lack of confirmatory work on the

physiological relevance of a good number of the interactions detected, a combined proteomic-RNAi screen for host proteins interacting with S-∂Ag has been performed[86]. To this end, a stable cell line expressing a Flag-tagged S-∂Ag, which supports in *trans* replication of an HDV variant with an early nonsense mutation in S-∂Ag, was created and subsequently used to immunoprecipitate and identify by MS more than 100 polypeptides. Interestingly, they include: i) nine of the 12 subunits of Pol II, highlighting again the key role of this polymerase in HDV replication, and ii) the putative RNA helicase MOV 10 reported to interact with an Argonaute complex and, like its Arabidopsis homologue SDE3, involved in RNA amplification. Moreover, RNAi-knockdown of a fraction of these polypeptides, including MOV 10 and Argonaute 4 (an specific effector of RNA silencing), affected negatively HDV replication[53,86], thus validating the reliability and potential of this approach, and establishing a link between HDV replication and RNA silencing. Viroid replication, and even pathogenesis, might also be connected to RNA silencing[57].

Additional effects of replication and concluding thoughts

Replication of viroid, viroid-like satellite and HDV RNAs, being the first step of their infectious cycle, has a strong influence on the properties of the resulting progeny. This is because replication is mechanistically linked to mutation rate and recombination, which together with subsequent selection, shape the final population structure. Remarkably, the mutation rate of a chloroplastic viroid is the highest reported for any biological entity[87], possibly due to the low fidelity of a single-subunit and proofreading-deficient NEP redirected to transcribe RNA templates. Nuclear viroids and HDV, being transcribed by Pol II that structurally and functionally is more complex than NEP, may not follow the same trend (whether Pol II retains proofreading activity when transcribing an RNA template has not been explored). Template switching also highlights the impact that replication can have on evolution because, in the three classes of small RNA replicons here compared, there are examples of mosaic sequences most likely emerging from recombination events between pre-existing RNAs; actually, the HDV genome itself has been proposed to result from the fusion of a catalytic viroid-like RNA and a protein-coding RNA[88]. In addition, early findings of certain newt transcripts with hammerheads[89], and of a carnation viroid-like RNA with hammerheads that also exists as a DNA form fused to DNA sequences of a pararetrovirus (leading to consideration of this peculiar RNA/DNA system as a retroviroid-like element[90,91]), have been extended recently: HDV- and hammerhead-like ribozymes are widely distributed throughout the biological scale, with their location in the host genomes

strongly suggesting a regulatory role and the involvement of retrotransposition in their genesis[92,93].

The frequent presence of ribozymes in viroid, viroid-like satellite and HDV RNAs, strongly supports the view that they represent "living fossils" of an ancestral "RNA world"[94]. Following the emergence of proteins and DNA, a common ancestor of Pol II and single-subunit RNA polymerases —predicted to catalyze replication of early RNA genomes[95,96]— presumably evolved to transcribe DNA templates. However, the initial ability to transcribe RNA templates may have been retained in present-day RNA polymerases, with the involvement of Pol II and NEP in the transcription of viroid and HDV RNAs representing the tip of an iceberg mostly composed by cellular RNAs. If so, the study of these minimal RNA replicons will have opened a new window into the metabolism of their host cells, as has recurrently happened with the study of some viruses.

Throughout this article we have highlighted the remarkable structural and functional similarities existing between three classes of small circular RNA endowed with the ability to replicate in plant or animal cells. We also refer readers to a recent review[97]; if results advanced therein by one of the coauthors are fully confirmed, the analogies between viroids and HDV RNA would extend to unanticipated levels.

Acknowledgements

This work was supported by the Ministerio de Ciencia e Innovación of Spain (grant BFU2008-03154/BMC) and by the Generalidad Valenciana (ACOMP/2010/278) to R.F, by the National Science Foundation of the USA (grant IRFP-0602042) to D.G, and by the Agencia Española de Cooperación Internacional (A/022313/08) to A.E. Due to space limitations we have been unable to refer to the original work of many authors and, instead, we have recurred to reviews. We apologize for any inconvenience this may have caused.

References

1. Kassanis B. Properties and behaviour of a virus depending for its multiplication on another. J Gen Microbiol 1962; 27:477-88.
2. Schneider IR. Satellite-like particles of tobacco ringspot virus that resemble tobacco ringspot virus. Science 1969; 166:1627-9.
3. Diener TO. Potato spindle tuber virus. IV. A replicating, low molecular weight RNA. Virology 1971; 45:411-28.
4. Rizzeto M, Canese MG, Arico J, Crivelli O, Bonino F, Trepo CG, Verme G. Immunofluorescence detection of a new antigen-antibody system associated to the hepatitis B virus in the liver and in the serum of HBsAg carriers. Gut 1977; 18:997-1003.
5. Symons RH, Randles JW. Encapsidated circular viroid-like satellite RNAs (virusoids) of plants. Curr Top Microbiol Immunol 1999; 239:81-105.
6. Taylor JM. Replication of human hepatitis δ virus: influence of studies on subviral plant pathogens. Adv Virus Res 1999; 54:45-60.
7. Diener TO. Discovering viroids—a personal perspective. Nat Rev Microbiol 2003; 1:75-80.
8. Flores R, Hernández C, Martínez de Alba E, Daròs JA, Di Serio F. Viroids and viroid-host interactions. Annu Rev Phytopathol 2005; 43:117-39.
9. Lai MM. RNA replication without RNA-dependent RNA polymerase: surprise from hepatitis delta virus. J Virol 2005; 79:7951-58.
10. Tsagris EM, Martínez de Alba AE, Gozmanova M, Kalantidis K. Viroids. Cell Microbiol 2008; 10:2168-79.
11. Ding B. The biology of viroid-host interactions. Annu Rev Phytopathol 2009; 47:105-31.
12. Branch AD, Levine BJ, Robertson HD. The brotherhood of the small RNA circular pathogens. Semin Virol 1990; 1:143-52.
13. Gross HJ, Domdey H, Lossow C, Jank P, Raba M, Alberty H, Sänger H. Nucleotide sequence and secondary structure of potato spindle tuber viroid. Nature 1978; 273: 203-8.
14. Makino S, Chang MF, Shieh CK, Kamahora T, Vannier DM, Govindarajan S, Lai MMC. Molecular cloning and sequencing of a human hepatitis delta virus RNA. Nature 1987; 329:343-6.
15. Kuo, MY, Sharmeen L, Dinter-Gottlieb G, Taylor J. Characterization of self-cleaving RNA sequences on the genome and antigenome of human hepatitis delta virus. J Virol 1988; 62:4439-44.

16. Bruening G, Passmore BK, van Tol H, Buzayan JM, Feldstein PA. Replication of a plant virus satellite RNA: evidence favors transcription of circular templates of both polarities. Mol Plant-Microbe Interact 1991; 4:219-25.
17. Sureau C. The role of HBV envelope proteins in the HDV replication cycle. Curr Top Microbiol Immunol 2006; 307:113-31.
18. Gudima S, Wu SY, Chiang CM, Moraleda G, Taylor J. Origin of the hepatitis δ virus mRNA. J Virol 2000; 74:7204-10.
19. Elena SF, Dopazo J, De la Peña M, Flores R, Diener TO, Moya A. Phylogenetic analysis of viroid and viroid-like satellite RNAs from plants: a reassessment. J Mol Evol 2001; 53:155-9.
20. Sogo JM, Koller T, Diener TO. Potato spindle tuber viroid X. Visualization and size determination by electron microscopy. Virology 1973; 55:70-80.
21. Chen PJ, Kalpana G, Goldberg J, Mason W, Werner B, Gerin J, Taylor J. Structure and replication of the genome of the hepatitis delta-virus. Proc Natl Acad Sci USA 1986; 83:8774-8.
22. Kos A, Dijkema R, Arnberg AC, van der Meide PH, Schellekens H. The hepatitis delta (δ) virus possesses a circular RNA. Nature 1986; 323:558-60.
23. Hernández C, Flores R. Plus and minus RNAs of peach latent mosaic viroid self-cleave in vitro via hammerhead structures. Proc Natl Acad Sci USA 1992; 89:3711-5.
24. Wang MB, Bian XY, Wu LM, Liu LX, Smith NA, Isenegger D, Wu RM, Masuta C, Vance VB, Watson JM, Rezaian A, Dennis ES, Waterhouse PM. On the role of RNA silencing in the pathogenicity and evolution of viroids and viral satellites. Proc Natl Acad Sci USA 2004; 101:3275-80.
25. Zhong X, Archual AJ, Amin AA, Ding B. A genomic map of viroid RNA motifs critical for replication and systemic trafficking. Plant Cell 2008; 20:35-47.
26. Casey JL, Gerin JL. Hepatitis δ virus RNA editing: specific modification of adenosine in the antigenomic RNA. J Virol 1995; 69:7593-600.
27. Branch AD, Benenfeld BJ, Baroudy BM, Wells FV, Gerin JL, Robertson HD. An ultraviolet-sensitive RNA structural element in a viroid-like domain of the hepatitis delta virus. Science 1989; 243:649-52.
28. Bussière F, Ouellet J, Côté F, Lévesque D, Perreault JP. Mapping in solution shows the peach latent mosaic viroid to possess a new pseudoknot in a complex, branched secondary structure. J Virol 2000; 74:2647-54.

29. Gago S, De la Peña M, Flores R. A kissing-loop interaction in a hammerhead viroid RNA critical for its in vitro folding and in vivo viability. RNA 2005; 11, 1073-83.
30. Bonfiglioli RG, Webb DR, Symons RH. Tissue and intra-cellular distribution of coconut cadang cadang viroid and citrus exocortis viroid determined by in situ hybridization and confocal laser scanning and transmission electron microscopy. Plant J 1996; 9:457-65.
31. Qi Y, Ding B. Differential subnuclear localization of RNA strands of opposite polarity derived from an autonomously replicating viroid. Plant Cell 2003; 15:2566-77.
32. Daròs JA, Marcos JF, Hernández C, Flores R. Replication of avocado sunblotch viroid: evidence for a symmetric pathway with two rolling circles and hammerhead ribozyme processing. Proc Natl Acad Sci USA 1994; 91:12813-7.
33. Navarro JA, Daròs JA, Flores R. Complexes containing both polarity strands of avocado sunblotch viroid: Identification in chloroplasts and characterization. Virology 1999; 253:77-85.
34. Taylor J, Mason W, Summers J, Goldberg J, Aldrich C, Coates L, Gerin J, Gowans E. Replication of human hepatitis delta virus in primary cultures of woodchuck hepatocytes. J Virol 1987; 61:2891-5.
35. Macnaughton TB, Lai MM. Genomic but not antigenomic hepatitis delta virus RNA is preferentially exported from the nucleus immediately after synthesis and processing. J Virol 2002; 76:3928-35.
36. Chou HC, Hsieh TY, Sheu GT, Lai MM. Hepatitis delta antigen mediates the nuclear import of hepatitis delta virus RNA. J Virol 1998; 72:3684-90.
37. Tavanez JP, Cunha C, Silva MCA, David E, Monjardino J, Carmo-Fonseca M. Hepatitis delta virus ribonucleoproteins shuttle between the nucleus and the cytoplasm. RNA 2002; 8:637-46.
38. Kopek BG, Perkins G, Miller DJ, Ellisman MH, Ahlquist P. Three-dimensional analysis of a viral RNA replication complex reveals a virus-induced mini-organelle. PLoS Biol 2007; 5:2022-34 (e220).
39. Wassenegger M, Krczal G. Nomenclature and functions of RNA-directed RNA polymerases. Trends Plant Sci 2006; 11:142-51.
40. Maida Y, Yasukawa M, Furuuchi M, Lassmann T, Possemato R, Okamoto N, Kasim V, Hayashizaki Y, Hahn WC, Masutomi K. An RNA-dependent RNA polymerase formed by TERT and the RMRP RNA. Nature 2009; 461:230-5.

41. Taylor JM. Replication of hepatitis delta virus RNA genome. Adv Virus Res 2009; 74:103-21.
42. Tseng C-H, Lai MM. Hepatitis delta virus RNA replication. Viruses 2009; 1:818-31.
43. Greco-Stewart V, Pelchat M. Interaction of host cellular proteins with components of the hepatitis delta virus. Viruses 2010; 2:189-212.
44. Lehmann E, Brueckner F, Cramer P. Molecular basis of RNA-dependent RNA polymerase II. Nature 2007; 450:445-9.
45. Yamaguchi Y, Filipovska J, Yano K, Furuya A, Inukai N, Narita T, Wada T, Sugimoto S, Konarska MM, Handa H. Stimulation of RNA polymerase II elongation by hepatitis delta antigen. Science 2001; 293:124-7.
46. Huang WH, Chen CW, Wu HL, Chen PJ. Post-translational modification of delta antigen of hepatitis D virus. Curr Top Microbiol Immunol 2006; 307:91-112.
47. Navarro JA, Vera A, Flores, R. A chloroplastic RNA polymerase resistant to tagetitoxin is involved in replication of avocado sunblotch viroid. Virology 2000; 268: 218-25.
48. Rodio ME, Delgado S, De Stradis A, Gómez MD, Flores R, Di Serio F. A viroid RNA with a specific structural motif inhibits chloroplast development. Plant Cell 2007; 19:3610-6.
49. Pelchat M, Grenier C, Perreault JP. Characterization of a viroid-derived RNA promoter for the DNA-dependent RNA polymerase from *Escherichia coli*. Biochemistry 2002; 41:6561-71.
50. Navarro JA, Flores R. Characterization of the initiation sites of both polarity strands of a viroid RNA reveals a motif conserved in sequence and structure. EMBO J 2000; 19: 2662-70.
51. Delgado S, Martínez de Alba E, Hernández C, Flores R. A short double-stranded RNA motif of peach latent mosaic viroid contains the initiation and the self-cleavage sites of both polarity strands. J Virol 2005; 79:12934-43.
52. Kolonko N, Bannach O, Aschermann K, Hu KH, Moors M, Schmitz M, Steger G, Riesner D. Transcription of potato spindle tuber viroid by RNA polymerase II starts in the left terminal loop. Virology 2006; 347:392-404.
53. Haussecker D, Cao D, Huang, Y, Parameswaran P, Fire AZ, Kay MA. Capped small RNAs and MOV10 in human hepatitis delta virus replication. Nat Struct Mol Biol 2008; 15:714-21.
54. Gudima SO, Chang J, Taylor JM. Reconstitution in cultured cells of replicating HDV RNA from pairs of less than full-length RNAs. RNA 2005; 11:90-8.

55. Branch AD, Benenfeld BJ, Robertson HD. Evidence for a single rolling circle in the replication of potato spindle tuber viroid. Proc Natl Acad Sci USA 1988; 85:9128-32.
56. Feldstein PA, Hu Y, Owens RA. Precisely full length, circularizable, complementary RNA: an infectious form of potato spindle tuber viroid. Proc Natl Acad Sci USA 1998; 95:6560-5.
57. Flores R, Gas ME, Molina-Serrano D, Nohales MA, Carbonell A, Gago S, De la Peña M, Daròs JA. Viroid replication: rolling-circles, enzymes and ribozymes. Viruses 2009; 1:317-34.
58. Gas ME, Hernández C, Flores R, Daròs JA. Processing of nuclear viroids in vivo: an interplay between RNA conformations. PLoS Pathog 2007; 3:1813-26 (e182).
59. Gas ME, Molina-Serrano D, Hernández C, Flores R, Daròs JA. Monomeric linear RNA of citrus exocortis viroid resulting from processing in vivo has 5′-phosphomonoester and 3′-hydroxyl termini: implications for the ribonuclease and RNA ligase involved in replication. J Virol 2008; 82:10321-5.
60. Hutchins C, Rathjen PD, Forster AC, Symons RH. Self-cleavage of plus and minus RNA transcripts of avocado sunblotch viroid. Nucleic Acids Res 1986; 14:3627-40.
61. Prody GA, Bakos JT, Buzayan JM, Schneider IR, Bruening, G. Autolytic processing of dimeric plant virus satellite RNA. Science 1986; 231:1577-80.
62. Forster AC, Davies C, Sheldon CC, Jeffries AC, Symons RH. Self-cleaving viroid and newt RNAs may only be active as dimers. Nature 1988; 334:265-7.
63. Carbonell A, De la Peña M, Flores R, Gago S. Effects of the trinucleotide preceding the self-cleavage site on eggplant latent viroid hammerheads: differences in co- and post-transcriptional self-cleavage may explain the lack of trinucleotide AUC in most natural hammerheads. Nucleic Acids Res 2006; 34:5613-22.
64. De la Peña M, Gago S, Flores R. Peripheral regions of natural hammerhead ribozymes greatly increase their self-cleavage activity. EMBO J 2003; 22:5561-70.
65. Khvorova A, Lescoute A, Westhof E, Jayasena SD. Sequence elements outside the hammerhead ribozyme catalytic core enable intracellular activity. Nat Struct Biol 2003; 10:708-12.
66. Martick M, Scott WG. Tertiary contacts distant from the active site prime a ribozyme for catalysis. Cell 2006; 126:309-20.
67. Daròs JA, Flores R. A chloroplast protein binds a viroid RNA in vivo and facilitates its hammerhead-mediated self-cleavage. EMBO J 2002; 21:749-59.

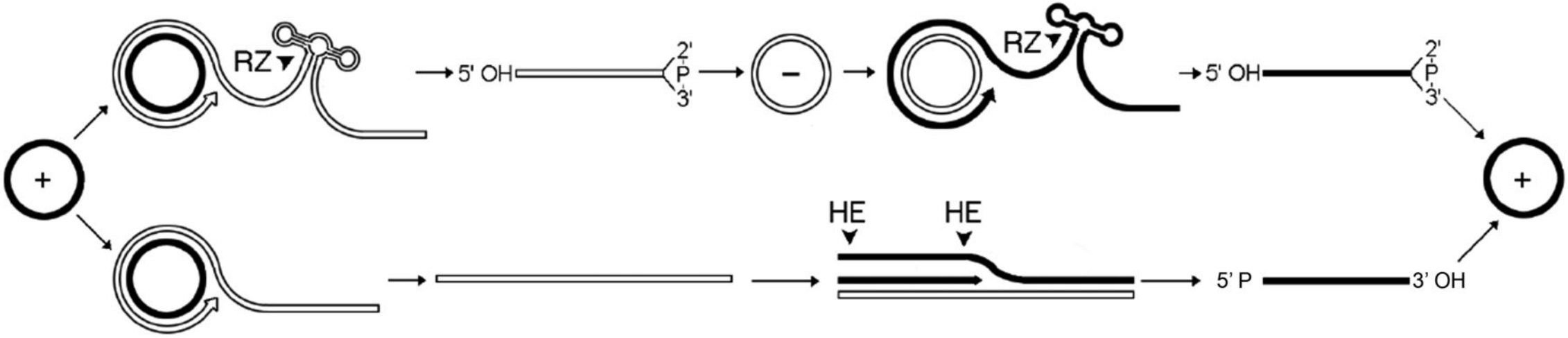
68. Ferre-D'Amare AR, Zhou K, Doudna JA. Crystal structure of a hepatitis delta virus ribozyme. Nature 1998; 394: 567-74.
69. Chadalavada DM, Cerrone-Szakal AL, Bevilacqua PC. Wild-type is the optimal sequence of the HDV ribozyme under cotranscriptional conditions. RNA 2006; 13:2189-201.
70. Lazinski DW, Taylor, JM. Intracellular cleavage and ligation of hepatitis delta virus genomic RNA: regulation of ribozyme activity by cis-acting sequences and host factors. J Virol 1995; 69:1190-200.
71. Buzayan JM, Gerlach WL, Bruening G. Nonenzymatic cleavage and ligation of RNAs complementary to a plant-virus satellite RNA. Nature 1986; 323:349-53.
72. Flores R, Hernández C, De la Peña, M, Vera A, Daròs JA. Hammerhead ribozyme structure and function in plant RNA replication. Meth Enzymol 2001; 341:540-52.
73. Rubino L, Di Serio F, Martelli G. Viroid-like satellite RNAs. In: Hadidi A, Flores R, Randles JW, Semancik JS, Eds. Viroids. Collingwood: CSIRO Publishing 2003; 76-84.
74. Rupert PB, Massey AP, Sigurdsson ST, Ferre-D'Amare AR. Transition state stabilization by a catalytic RNA. Science 2002; 298:1421-4.
75. Song SI, Silver SL, Aulik MA, Rasochova L, Mohan BR, Miller WA. Satellite cereal yellow dwarf virus-RPV (satRPV) RNA requires a double hammerhead structure for self-cleavage and an alternative structure for replication. J Mol Biol 1999; 293:781-93.
76. Perrotta AT, Been MD. A pseudoknot-like structure required for efficient self-cleavage of hepatitis delta-virus RNA. Nature 1991; 350:434-6.
77. Englert M, Beier H. Plant tRNA ligases are multifunctional enzymes that have diverged in sequence and substrate specificity from RNA ligases of other phylogenetic origins. Nucleic Acids Res 2005; 33:388-99.
78. Englert M, Latz A, Becker D, Gimple O, Beier H, Akama K. Plant pre-tRNA splicing enzymes are targeted to multiple cellular compartments. Biochimie 2007; 89:1351-65.
79. Martínez, F, Marqués J, Salvador ML, Daròs JA. Mutational analysis of eggplant latent viroid RNA processing in *Chlamydomonas reinhardtii* chloroplast. J Gen Virol 2009; 90:3057-65.
80. Côté F, Lévesque D, Perreault JP. Natural 2′,5′-phosphodiester bonds found at the ligation sites of peach latent mosaic viroid. J Virol 2001; 75:19-25.
81. Kibertis PA, Haseloff J, Zimmern D. 2′ phosphomonoester, 3′-5′ phosphodiester bond at a unique site in a circular viral RNA. EMBO J 1985; 4, 817-27.

82. Sheldon CC, Symons RH. Is hammerhead self-cleavage involved in the replication of a virusoid in vivo? Virology 1993; 194:463-74.
83. Ambrós S, Hernández C, Flores R. Rapid generation of genetic heterogeneity in progenies from individual cDNA clones of peach latent mosaic viroid in its natural host. J Gen Virol 1999; 80:2239-52.
84. Reid CE, Lazinski DW. A host-specific function is required for ligation of a wide variety of ribozyme-processed RNAs. Proc Natl Acad Sci USA 2000; 97:424-9.
85. Kalantidis K, Denti MA, Tzortzakaki S, Marinou E, Tabler M, Tsagris M. Virp1 is a host protein with a major role in potato spindle tuber viroid infection in Nicotiana plants. J. Virol 2007; 81:12872-80.
86. Cao D, Hausseckner D, Huangt Y, Kay MA. Combined proteomic-RNAi screen for host factors involved in human hepatitis delta virus replication. RNA 2009; 15:1971-9.
87. Gago S, Elena SF, Flores R, Sanjuán R. Extremely high variability of a hammerhead viroid. Science 2009; 323:1308.
88. Brazas R, Ganem D. ∂-interacting protein A and the origin of hepatitis ∂ antigen. Science 1997; 276:824-5.
89. Epstein LM and Gall JG. Self-cleaving transcripts of satellite DNA from the newt. Cell 1987; 48:535-43.
90. Hernández C, Daròs JA, Elena SF, Moya A, Flores R. The strands of both polarities of a small circular RNA from carnation self-cleave in vitro through alternative double- and single-hammerhead structures. Nucleic Acids Res 1992; 20:6323-9.
91. Daròs JA, Flores R. Identification of a retroviroid-like element from plants. Proc Natl Acad Sci USA 1995; 92:6856-60.
92. Webb C-HT, Riccitelli NJ, Ruminski DJ, Luptak A. Widespread occurrence of self-cleaving ribozymes. Science 2009; 326:953.
93. De la Peña M, Garcia-Robles I. Ubiquitous presence of the hammerhead ribozyme motif along the tree of life. RNA 2010; 16:1943-50.
94. Diener TO. Circular RNAs: relics of precellular evolution? Proc Natl Acad Sci USA 1989; 86:9370-4.
95. Poole AM, Logan DT. Modern mRNA proofreading and repair: clues that the last universal common ancestor possessed and RNA genome? Mol Biol Evol 2005; 22: 1444-55.

96. Salgado PS, Koivunen MRL, Makeyev EV, Bamford DH, Stuart DI, Grimes JM. The structure of an RNAi polymerase links RNA silencing and transcription. PLoS Biol 2006; 4:2274-81 (e434).
97. Taylor J, Pelchat M. Origin of hepatitis delta virus. Fut Microbiol 2010; 5:393-402.

LEGENDS TO FIGURES

Fig. 1. Mechanism proposed for replication of viroids, viroid-like satellite RNAs and HDV. The asymmetric pathway with one rolling-circle (upper) is used by viroids of the family *Pospiviroidae* and by some viroid-like satellite RNAs, while the symmetric pathway with two rolling-circles (lower) is used by the other viroid-like satellite RNAs, viroids of the family *Avsunviroidae* and HDV. Solid and open lines refer to plus (+) and minus (-) polarities, respectively, and processing sites are marked by arrowheads. Cleavage is alternatively mediated by ribozymes (RZ) or host enzymes (HE), generating linear monomeric RNAs with characteristic termini that are subsequently ligated by host enzymes or autocatalytically. [Reproduced with modifications from Viroids (Hadidi A, Flores R, Randles JW, Semancik JS, eds) 2003; CSIRO Publishing, Collingwood, Australia].



View publication stats