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Evolution

Circular RNAs: Relics of precellular evolution?

(self-processing RNAs/viroids/satellite RNAs/introns/ribozymes)

T. O. DIENER

Center for Agricultural Biotechnology and Department of Botany, University of Maryland, College Park, MD 20742; and Agricultural Research Service, U.S. Department of Agriculture, Beltsville, MD 20705

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ABSTRACT **The demonstration of enzymatic capabilities of certain RNAs, in addition to their well-known template properties, has led to the recognition that RNAs are the only biological macromolecules that can function both as genotype and phenotype, hence raising the possibility of Darwinian selection and precellular evolution at the RNA level in the absence of DNA or protein. Recent models of such precellular RNA systems are patterned after the properties of intron-derived ribozymes. On the basis of a phylogenetic analysis and known properties of certain small plant pathogenic RNAs (viroids and viroid-like satellite RNAs), I suggest that these plant RNAs are more plausible candidates than introns as "living fossils" of a precellular RNA world. Their small size and circularity would have enhanced probability of their survival in error-prone, primitive self-replicating RNA systems and assured complete replication without the need for initiation or termination signals. All of these RNAs possess efficient mechanisms for the precise cleavage of monomers from oligomeric replication intermediates. Some (most viroids) require a host factor, but others (viroid-like satellite RNAs and one viroid) function as self-cleaving RNA enzymes far smaller and simpler than those derived from introns. The question is raised whether introns could have evolved from viroids or viroid-like satellite RNAs rather than vice versa, as has been widely speculated.**

With the discovery that certain RNAs possess catalytic properties (1, 2), earlier suggestions that RNA preceded DNA as the carrier of genetic information during evolution have gained considerable credence. As discussed by Joyce (3), it is unlikely, however, that RNA represents the most archaic genetic system, but whatever the chemical identity of pre-RNA systems may have been, it appears plausible that RNA preceded DNA as the genetic material. Several circumstantial lines of evidence supporting this contention have repeatedly been stated (reviewed in ref. 3). Most compelling is the recognition that RNA is the only known macromolecule that can function both as genotype and phenotype—thus permitting Darwinian evolution to occur at the molecular level in the absence of DNA or functional proteins.

Most recent models for self-replicating, precellular RNA systems assume the existence of primitive RNA enzymes (ribozymes) with properties that are derived from known self-splicing mechanisms of certain introns (4, 5), notably the intron of the *Tetrahymena thermophila* rRNA gene (5). Indeed, Doudna and Szostak (6) have shown recently that a modified *Tetrahymena* ribozyme can splice together multiple oligonucleotides aligned on a template strand to yield a fully complementary product strand—thus demonstrating the feasibility of template-directed, RNA-catalyzed RNA replication.

As presently known, some of the prerequisites of this reaction are, however, difficult to envisage in a primitive RNA world. The *Tetrahymena* ribozyme is a relatively large molecule (300+ nucleotides) (6) with a complex secondary and tertiary structure essential for activity (5). How could such a complex ribozyme have evolved extracellularly (7), and how could replication have been initiated correctly at the exact beginning of the template and have proceeded precisely to its end?

Introns, however, are not the only catalytic RNAs known. Additional examples are the RNA component of RNase P (2) and certain small plant pathogenic RNAs (viroids and viroid-like satellite RNAs). In this paper I propose that the latter are more likely candidates than introns as "living fossils" of a precellular RNA world and show that their characteristic properties may reflect features necessary to overcome obstacles in the self-replication of primitive RNAs. In order to view either introns or plant pathogenic RNAs as remnants of a precellular RNA world one must assume, of course, that their ancestors were free-living molecules and that they have survived by acquiring an intracellular mode of existence sometime after the evolution of cellular organisms.

Viroids and Viroid-like RNAs

Viroids are small (246–375 nucleotides), single-stranded, covalently closed circular RNAs that, in contrast to viruses, are not encapsidated (reviewed in ref. 8). Like viruses, viroids replicate autonomously in susceptible plant cells despite their small size and severely limited amount of genetic information (9). Viroid replication occurs by means of RNA intermediates of opposite polarity and does not involve DNA. In sharp contrast to viral RNAs, viroids do not function as mRNAs; hence viroids must be replicated by preexisting host enzymes. The presence of oligomeric viroid forms (usually of opposite polarity) in infected plants suggests that replication occurs by a rolling-circle type mechanism (10).

Viroid-like satellite RNAs (one group of plant satellite RNAs; below referred to simply as satellite RNAs) are similar in many respects to viroids but are encapsidated and can replicate only in cells that are infected with specific helper viruses (11). They are circular single-stranded RNAs of about 350 nucleotides that are found, together with linear single-stranded viral RNAs of about 4500 nucleotides, in the isometric particles of several plant viruses of the sobemovirus group (their helper viruses). Little, if any, sequence similarity exists between these satellite RNAs and the RNA(s) of their helper viruses (11). These satellite RNAs, like viroids, lack mRNA activity and possess highly base-paired rod-like structures (12).

The encapsidated forms of another group of plant satellite RNAs, associated with nepoviruses [among others, the satellite RNA of tobacco ringspot virus (STobRSV RNA)], are

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Abbreviations: ASBVd, avocado sunblotch viroid; ASSVd, apple scar skin viroid; CCR, central conserved region; PSTVd, potato spindle tuber viroid; STobRSV, satellite of tobacco ringspot virus.

linear (13), but circular molecules can be isolated from infected tissue (14).

Because linear repetitive sequence dimers, trimers, and higher-order multimers occur in infected tissue (or in helper virus capsids), satellite RNAs, like viroids, appear to be replicated by a rolling-circle type process. Implicit in rolling-circle replication is a precise mechanism by which viroid or satellite RNA monomers are excised from oligomeric replication intermediates and ligated to form the circular, monomeric progeny RNAs. Conceptually, this process is analogous to the processing (cleavage-ligation) reactions by which introns are spliced out of precursor RNAs and exons joined to form functional RNAs.

Cleavage Processes

Experiments to elucidate the mechanisms of oligomeric viroid and satellite RNA processing have clearly demonstrated that several disparate cleavage processes operate. Whereas most satellite RNAs (15, 16) and one viroid [the avocado sunblotch viroid (ASBVd)] (17) are self-cleaving, other viroids require a factor present in cell nuclei (18). Structural requirements also are disparate. Whereas most self-cleaving plant pathogenic RNAs contain a highly conserved series of short nucleotide sequences and can assume, by base-pairing, a characteristic secondary structure called a "hammerhead" (Fig. 1 and Fig. 2 *Left*), viroids (except ASBVd) cannot assume such a structure (20).

Ability to assume the hammerhead structure appears to be a prerequisite for the self-cleavage of most plant satellite RNAs, but multimers of the minus sequence of the STobRSV RNA self-cleave but cannot form a hammerhead structure (16). With this RNA, the exact structure required for cleavage has not been determined, but two regions have been identified in which insertions inactivate the self-cleaving reaction; these are separated by a region in which insertions have no effect (21).

With viroids (except ASBVd), the mechanism of cleavage-ligation is less well known. However, infectivity studies of viroid-specific cDNAs and RNA transcripts therefrom have given clues as to a possible cleavage-ligation site. Such studies have shown that whereas cDNAs containing a monomeric viroid sequence are not infectious, constructs containing tandem repeats of the viroid sequence are highly infectious (reviewed in ref. 22). Further analysis has shown that (as judged by infectivity) far less than a complete dimer of the viroid sequence is sufficient for cleavage to take place, provided that the sequence duplication occurs in the central portion of the upper strand of the viroid molecule [in its conventional representation (23)]. Because in representatives of the potato spindle tuber viroid (PSTVd) group this region is highly conserved, it has become known as the "central conserved region" (CCR) (24) (Fig. 2 *Center*). Recently, viroids have come to light [the apple scar skin viroid (ASSVd) group (25–27)] whose central region differs from that of the PSTVd group but is nevertheless highly conserved within the group. With all viroids (except ASBVd), the CCR is flanked by inverted repeats of about nine nucleotides (hairpin I) (28) (Fig. 2 *Center*). ASBVd differs from all other viroids also by containing only a small portion of the CCR of the PSTVd viroids (24).

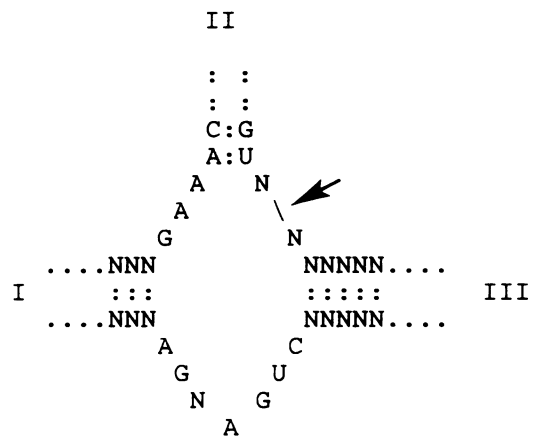

FIG. 1. Consensus sequence of the hammerhead self-cleavage cassette (19). I, II, and III, hairpin loops that vary in size; N, arbitrary nucleotide complementary to N in opposite strand of stem; arrow, cleavage site.

On the basis of these findings, a model for a putative cleavage-ligation site has been proposed (22). The model has identified a thermodynamically highly stable palindrome that viroid constructs with a duplication of the upper CCR and the flanking hairpin I sequences (as well as oligomeric viroids) can assume and posits that formation of this structure is required for cleavage-ligation to occur (22) (Fig. 2 *Right*). Recent results generally are compatible with this model (47) but also point to the existence of less favored secondary processing sites in different regions of viroids (29).

The existence, among these small plant pathogenic RNAs, of two disparate cleavage processes, one requiring a host factor and the other being self sufficient, is reminiscent of splicing mechanisms operative among different groups of introns, in which the splicing of mRNA precursors requires an external factor, whereas that of many group I and II precursor RNAs is RNA catalyzed. The RNA-catalyzed reaction has been proposed to represent the ancestral splicing process and the mechanism responsible for the splicing of mRNA precursors to be derived from it (4). Similarly, the self-cleavage mechanism operative with present-day satellite RNAs and ASBVd probably is the ancestral reaction and the mechanism operative with all other known viroids was derived from it. With introns (4), as well as with viroids, a major step in the transition would be the appearance of trans-acting factors that execute the excision.

Viroid and Satellite RNA Evolution

Results of a phylogenetic analysis of viroids and satellite RNAs (based on their computer-aligned nucleotide sequences) are consistent with the concept that these RNAs have a common origin and that ASBVd represents a connecting link between satellite RNAs and viroids evolved therefrom (unpublished data). In this view, viroids evolved from satellite RNAs while still free-living molecules and both acquired a dependence on their host (viroids) or helper virus (satellite RNAs) only after becoming intracellular pathogens.

Alignment of the upper portion of the viroid CCR discloses sequence similarities between the self-cleaving ASBVd and the other viroids. In particular, those sequences of ASBVd that form the upper left-hand portion of the hammerhead structure appear to be present in the right-hand portions of the putative cleavage sites of PSTVd and ASSVd group viroids, whereas their left-hand portions bear no resemblance to the ASBVd sequence (Fig. 2 *Center*). Hence, stem loop II plus stem loop III of the hammerhead have been converted to a more stable (and more helical) potential conformation. It is known that viroids (except ASBVd) cannot form the hammerhead structure (20) and, as shown in Fig. 2, oligomers of ASBVd cannot form the palindrome typical of all other known viroids. It is possible, therefore, that those sequences that are involved in forming the putative cleavage structure of most present-day viroids have evolved from sequences originally involved in forming an autocatalyzing cleavage structure, such as is still present in ASBVd and most satellite RNAs.

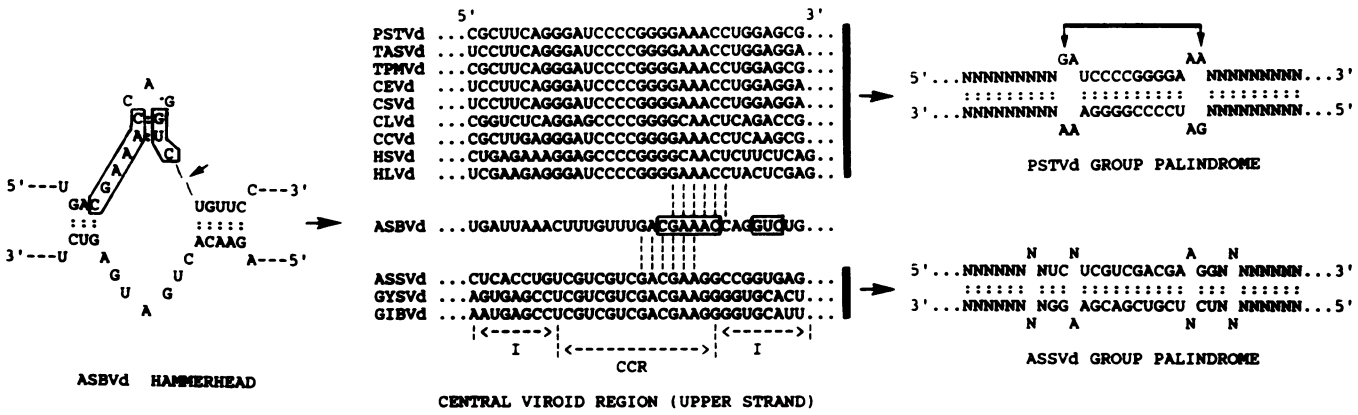

FIG. 2. Nucleotide sequences of the upper central regions of some viroids and their possible evolutionary relationships (see text). (*Left*) Hammerhead structure of the plus strand of the ASBVd (20). (*Center*) Comparison of the aligned central regions of the upper strands of viroids. (*Right*) The palindromic sequence, considered as a putative cleavage-ligation site, that oligomeric forms of all known viroids, except ASBVd, can assume. PSTVd, potato spindle tuber viroid; TASVd, tomato apical stunt viroid; TPMVd, tomato planta macho viroid; CEVd, citrus exocortis viroid; CSVd, chrysanthemum stunt viroid; CLVd, columnea latent viroid; CCVd, coconut cadang-cadang viroid; HSVd, hop stunt viroid; HLVd, hop latent viroid; GYSVd, grapevine yellow speckle viroid; GIBVd, grapevine IB viroid. Boxed sequences in ASBVd are upper portions of hammerhead consensus sequences (see Fig. 1). Small arrows, cleavage site in ASBVd and range of putative cleavage sites in PSTVd group viroids; large arrows, hypothetical change from self-cleaving hammerhead structure to sequences capable to form stable palindrome. I, hairpin I; vertical lines, sequence identities between ASBVd and other viroids; colons, base-pairing.

Minimal Ribozymes

Only relatively small portions of satellite RNA sequences are required as signals for self-splicing. Thus, deletion of RNA sequences from both the 3′ and 5′ termini of one satellite RNA (that of the lucerne transient streak virus) has shown that a 51-nucleotide remnant of the original RNA, containing little more than the sequences comprising the hammerhead, is capable of rapid and complete self-cleavage (16), and a synthetic 19-nucleotide RNA fragment can cause rapid, highly specific cleavage of a 24-nucleotide RNA fragment under physiological conditions (19). Because the 19-mer can participate in many cleavage reactions, it fulfills all criteria for a genuine ribozyme. The nucleotide sequences of the two fragments were constructed such that together they can form the hammerhead structure and hence conform to the model proposed for the self-cleaving domain of satellite RNAs (16). Similarly, the two mutationally sensitive regions of the minus strand of STobRSV have been produced as two separate oligonucleotides whose minimal sizes were determined to be 10 and 46 nucleotides for the substrate and ribozyme, respectively (30). It is evident, therefore, that ribozymes far simpler than those involved in intron splicing, but with similar capabilities, exist. This opens the possibility that they, rather than intron-derived ribozymes, may have been involved in precellular RNA self-replication.

Genomic Tags and Circular Genomes

Despite the now demonstrated feasibility of template-specific, RNA-catalyzed RNA synthesis by intron-derived ribozymes, their possible involvement in precellular RNA synthesis is still somewhat problematic. As has been pointed out (3, 31), one of the most serious problems is the need to identify a specific site for initiation in order to assure end-to-end copying of a linear template. Without a specific initiation site, replication could initiate anywhere on the template and complete replication would be unlikely. It has been suggested that this problem may have been solved in the RNA world by evolution of a 3′-terminal "genomic-tag" structure that directed the reaction to commence at a specific site (31).

I propose that viroids and satellite RNAs have solved the problem of complete replication in a different and far simpler manner. The covalently closed circular structure of most of these RNAs and their rolling-circle type synthesis assure complete replication provided that transcription continues at least once around the circle. Thus, no need exists for a genomic tag. What is required, however, is a mechanism for the precise cleavage of monomers out of the oligomeric replication intermediates and an efficient means by which monomers are ligated into circular progeny RNAs. With viroids and satellite RNAs, both of these requirements are fulfilled.

Although with most present-day viroids these functions have been usurped by protein enzymes (presumably by one of several host RNA polymerases plus a host RNA ligase), RNA-catalyzed cleavage-ligation is still evident with ASBVd and satellite RNAs. These self-cleavage reactions generate 5′-OH and 2′,3′-cyclic phosphodiester termini, as is the case with ordinary base- or acid-catalyzed hydrolysis of RNA, and the only cofactor required is a structure-stabilizing ion, such as magnesium (32). Hence, these reactions may represent very primitive RNA processes; they are clearly distinct from those involved in intron splicing, which are far more complex (33). The 2′,3′-cyclic phosphodiester termini generated during self-cleavage are suitable substrates for RNA ligase (15); however, spontaneous self-ligation resulting in covalently closed circular molecules has been demonstrated with the self-cleaved minus strand of STobRSV RNA in the absence of protein (32).

In principle, a ribozyme patterned after the hammerhead structure is capable of performing all of the steps involved in Sharp's hypothetical RNA self-replication scheme (4). As compared with intron models, the concept of precellular RNA replication based on viroid and/or satellite RNA precursors requires fewer assumptions and appears more plausible. Above all, nonenzymatic synthesis (34) of a simple 19-nucleotide ribozyme is far easier to accept than that of a sophisticated 300+-nucleotide RNA, as is necessary with the intron model.

"Polyploidy" and Circular Genomes

There are still other cogent reasons to consider viroids and satellite RNAs as relics of precellular self-replicating RNA systems. Eigen (35) has pointed out that the fidelity of replication in such systems sets a limit on the length of a

master sequence that can be maintained against its distribution of error copies. Also, because of the high stability of G·C base pairs, (G+C)-rich sequences would have been highly favored (36). Both with respect to their base composition and size, viroids and satellite RNAs fulfill these requirements. They are characterized by high G+C ratios and they represent the smallest replicating molecules known.

Yet, replication and faithful maintenance of 300- to 400-base molecules nonenzymatically or by primitive ribozyme systems may appear implausible (37). Here again, the characteristics of viroids, and particularly their circularity, may help resolve the problem.

Initially, the length of oligonucleotides synthesized in these primitive systems must have been very limited. Because circularization would assure complete replication, it would be advantageous for such oligonucleotides to be ligated into small circles. There is, however, another compelling advantage to a circular genome. The high error rate of prebiotic replication systems (estimated as 10^{-1}–10^{-2} misincorporations per base per generation) (34) would almost certainly favor systems with multiple copies of their genomes (37). One effective method to achieve such polyploidy would consist in rolling-circle transcription of a circular template, resulting in tandem repeats of the genetic information.

Structural Periodicity

It is probable that with improved fidelity of the copying mechanism the need for multiple copies would diminish but that, at the same time, the sequence required for cleavage would become more stringently defined. Hence, incorporation errors (point mutations) within the recognition site would abolish cleavage at that site and molecules twice the size of the previous ones would be synthesized.

Conceivably, this is one of the mechanisms by which larger genomes could have evolved. If so, remnants of structural periodicity might be detectable in present-day genomes of great antiquity. Interestingly, this is indeed the case with viroids. Juhasz *et al.* (38) have shown that all viroids (except hop stunt viroid) exhibit structural periodicities characterized by repeat units of 12-, 60-, or 80-nucleotide residues, depending on the viroid species (Fig. 3). Random sequences generated by using the same nucleotide composition as that of some representative viroids, small nuclear RNAs, or satellite RNAs do not exhibit structural periodicities (38).

With at least two viroids, each repeat unit contains either an exact portion of the hammerhead self-splicing cassette—namely, the sequence GAAAC—or the consensus motif GRRAY (Fig. 3). It is possible, therefore, that these sequences are remaining portions of now defunct self-cleavage sites. Why most viroids, but not satellite RNAs, display periodicity is not clear but, in view of the presumed long evolutionary history, it is not surprising.

Introns, Viroids, and Transposons

Based on the presence in viroids and satellite RNAs of sequences resembling conserved elements in group I introns whose presence is essential for splicing, much speculation has centered around the concept that viroids represent "escaped introns" (39–41). Results with self-cleaving satellite RNA fragments (16, 42) show, however, that these sequence elements cannot be of functional significance in the self-cleavage process of satellite RNAs: fragments from which all intron-like elements have been removed self-splice efficiently.

Why then are these intron-like sequences maintained in viroids and satellite RNAs? Conceivably, sequence similarities between viroids and introns could be coincidental. Indeed, probability calculations have shown that the presence of the conserved intron-like elements in one satellite RNA is only slightly more frequent than would be expected in random nucleotide sequences of identical base composition (significance at the 0.05 level) (43). Alternatively, they could fulfill another, presently unknown, function in today's viroids and satellite RNAs and may have become involved in intron splicing at a later stage in evolution. In the latter case it would be tempting to speculate that viroids and viroid-like RNAs are phylogenetically older than introns. The fact that no viroid is known to code for protein, whereas many introns do, is consistent with this contention. Although the evolutionary origin of introns is unknown, one hypothesis states that they arose by insertion of transposable elements into preexisting genes (39, 44). Recent findings indeed support the view that group I and group II introns have evolved from (or into) mobile elements (45). Because sequence similarities exist between viroids and transposable elements (46), it is conceivable that introns may be "captured" viroids, rather than viroids "escaped" introns.

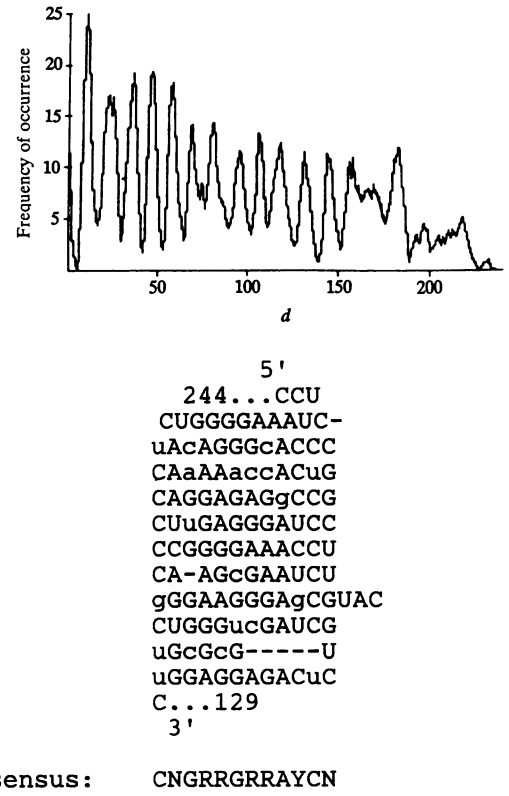

FIG. 3. (*Upper*) Structural periodicity of the small coconut cadang-cadang viroid nucleotide sequence. Frequency function, $f_m(d) = \Sigma_{i=d}^{d+\frac{k}{l}-1} f(i)/l$, where $m = 5$ = number of matches; d = distance between k-tuples; $k = 6$ = the number of nucleotides in each subsequence in terms of purines and pyrimidines; and $l = 4$ = number of averagings. (*Lower*) Periodicity of a portion of the 246-nucleotide sequence of circular coconut cadang-cadang viroid (nucleotides 244–246, 1–129) and deduced consensus sequence; R, purine; Y, pyrimidine. [Reproduced with permission from ref. 38 (copyright Elsevier).]

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