Mimicry of the hepatitis delta virus replication cycle mediated by synthetic circular oligodeoxynucleotides

Amy M Diegelman and Eric T Kool

Background: Hepatitis delta virus (HDV) is a circular single-stranded RNA pathogen whose monomeric form results from self-processing. Although studies have examined minimal HDV ribozyme activities, the mechanism for forming the circular virus remains unclear, and the *trans* catalytic properties of self-processed forms of HDV ribozymes have not been studied. In addition, HDV ribozymes have not previously been engineered to cleave a non-HDV sequence.

Results: Long repeating RNAs have been produced from *in vitro* rolling-circle transcription of synthetic circular oligodeoxynucleotides encoding catalytically active subsets of the entire antigenomic RNA virus. Like full-length HDV, these multimeric RNAs undergo self-processing to monomer length; importantly, cyclization is found to occur efficiently, but only in the presence of the circular template. Linear and circular monomer ribozymes and engineered variants are shown to be active in cleaving HDV and HIV RNA targets in *trans*, despite having self-binding domains.

Conclusions: Mimicry of the rolling-circle replication pathway for HDV replication has led to a new proposal for cyclization of HDV RNA. Under these conditions, cyclization is mediated by the complementary circular template. In addition, it has been shown that self-processed HDV ribozymes can be catalytically active in *trans* despite the presence of antisense sequences built into their structure.

Address: Department of Chemistry, University of Rochester, Rochester, NY 14627, USA.

Correspondence: Eric T Kool
E-mail: etk@etk.chem.rochester.edu

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Introduction

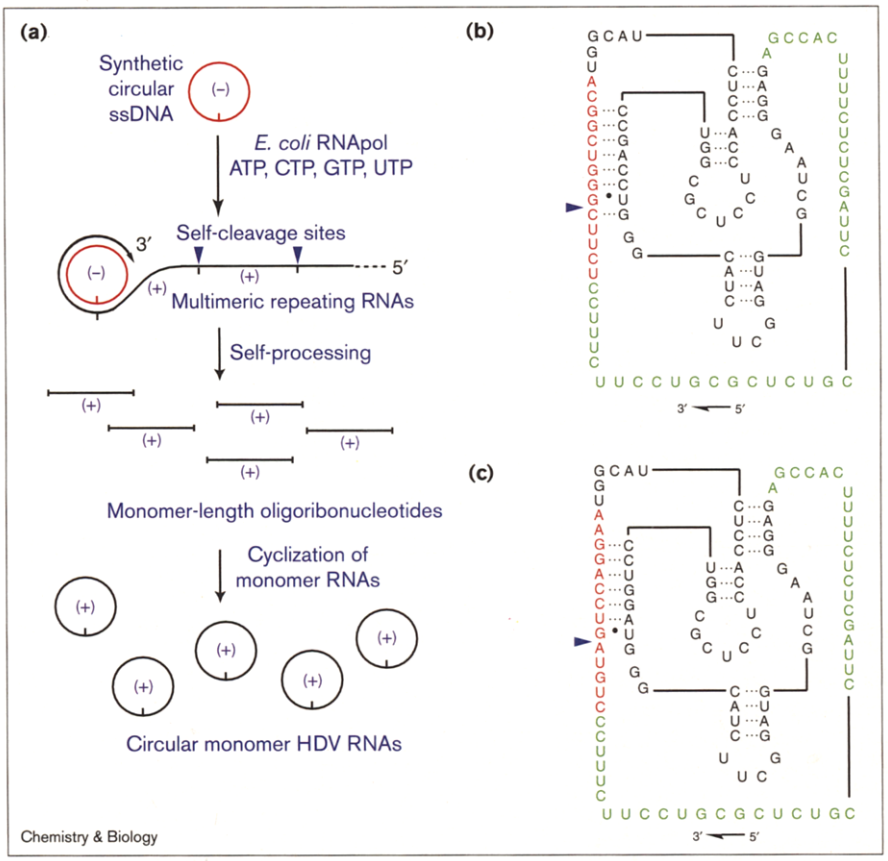
Hepatitis delta virus (HDV), a satellite of the hepatitis B virus, consists of a 1678 nucleotide (nt) closed, circular single-stranded RNA [1–4] and is the only pathogenic RNA known in the animal kingdom [5]. It has been proposed that replication of this RNA is viroid-like, proceeding by a rolling-circle mechanism (reviewed in [5,6]), possibly assisted by RNA polymerase II [7,8]. The self-cleaving ability of genomic and antigenomic HDV RNAs is well characterized [9–16] and is an essential feature of the rolling-circle replication pathway.

In addition to the studies of native HDV RNAs, genomic [14,17–24] and antigenomic [25,26] forms of the ribozyme motif have been engineered for *trans* cleavage of hepatitis-delta-derived RNAs by removing nonessential sequences, which frees the substrate-binding domain for binding a target in intermolecular fashion. It has been postulated that the limited sequence requirements in the substrate-binding domain of HDV allow for the design of a ribozyme that can cleave a non-HDV RNA sequence in *trans* [27]. To our knowledge this has not yet been done, although a recent report describes altering an HDV ribozyme to cleave other sites in HDV RNA [28].

Also crucial to a rolling-circle replication mechanism is the ability of HDV linear monomer RNAs to be ligated to circular form. The ligation ability of HDV ribozymes has been explored [29–32] and it has been shown that the equilibrium for cleavage versus ligation lies far on the side of cleavage in the presence of magnesium [31]. Despite this, HDV RNA monomers exist primarily in circular form when isolated [33,34], a fact that is not well understood at present. For plant-infecting viroids and satellites, cyclization mechanisms may be varied. For example, monomer RNAs derived from the minus satellite RNA of the tobacco ringspot virus (–)sTRSV, may cyclize spontaneously from self-ligation by reversing the cleavage reaction, which is possible due to the facile ligation of hairpin ribozyme motifs [35,36]. Other viroids that utilize hammerhead [37], or nonribozyme motifs for self-processing [38], probably rely instead on template-dependent ligation for closure to circular form, which could also be the case for HDV [30].

We recently reported on the synthesis of self-processed hammerhead [39] and hairpin [40] ribozymes by rolling-circle transcription (RCT) of circular single-stranded DNA oligonucleotides (Figure 1). This method of RNA synthesis allows for efficient production of ribozymes *in vitro*. Moreover, the ribozymes created by RCT closely resemble those in natural viroids and virusoids, in that they contain ribozyme-processed 5′ and 3′ ends, and their *trans*-cleaving domains are potentially occupied by self-complementary sequences. In addition, like those pathogenic

Figure 1



Transcription and sequences of HDV RNAs. **(a)** Scheme for rolling-circle transcription (RCT) of circular DNAs encoding delta ribozymes followed by self-processing and ligation. Similar steps are also involved in the replication of naturally occurring viroids and virusoids [5]. **(b)** HDV109 and **(c)** POL109 RNA. Red sequences correspond to designed RNA sequences for *trans* cleavage, green sequences correspond to nucleotides added to join 5′ and 3′ ends to create a circular species. Arrows represent sites of cleavage (self-processing) in multimeric RNAs. Secondary structures are shown here in analogy to conserved antigenomic pseudoknot structure [52].

RNAs, the smaller ribozymes produced by RCT are generated from complementary circular templates, and the presence of these complementary sequences can lead to chemistry not available for smaller ribozymes produced from linear monomeric template DNAs or produced synthetically. RCT, therefore, allows the study of ribozyme properties in a context more closely resembling that which occurs naturally, and could lead to new insights into these RNA motifs. Despite the significant differences between catalytic RNAs generated by self-processing, HDV RNA catalytic activities have rarely been studied with RNAs in which both ends are self-processed.

We now describe studies on the properties of self-processed RNAs derived from antigenomic hepatitis delta RNA. Biomimetic RCT coupled with self-cleavage is found to result primarily in monomer-length HDV-derived RNAs. Interestingly, circular monomers are the predominant products, and they are formed only in the presence of the circular DNA template. All three steps of HDV replication are, therefore, successfully mimicked *in vitro*. Significantly, the linear and circular RNA products are found to be catalytically active in *trans*, despite having potentially inhibitory self-complementary sequences. The results have possible implications in the biological activity and mechanism of circularization of natural HDV RNAs, as well as in the possible use of HDV-derived ribozymes in RNA-targeted therapies.

Results

Two circular single-stranded DNAs were constructed to explore the properties of self-processed HDV RNAs (Figure 1) by mimicking the replication pathway. We constructed the circular sequences with the plan of testing both T7 and *Escherichia coli* RNA polymerases for transcriptional activity, because previous work with circular single-stranded (ss)DNAs has shown varied transcription activity from sequence to sequence [39,40]. The sequences of the circular DNAs (HDV109 and POL109) were derived from antigenomic HDV RNA, and encoded antigenomic RNAs including the ribozyme motif, as well as its self-cleavage site (Figure 1). The minimal active ribozyme includes approximately 75 nt of sequence; we included another ~40 nt of adjacent HDV sequence (green nt in Figure 1) to act as a bridge, which was presumably long enough to link the domains without altering the structure necessary for activity. The first circular DNA, HDV109, has its complete sequence derived directly from antigenomic HDV. The second circular DNA, POL109, has 13 nucleotides altered from HDV109, making the

transcribed RNA complementary to a site in HIV-1 *pol* RNA [40]. The circular DNAs were constructed using T4 DNA ligase to join two approximately half-length sequences [39]; short 20-0mer oligodeoxynucleotides were used as splints to aid in joining the circle precursors.

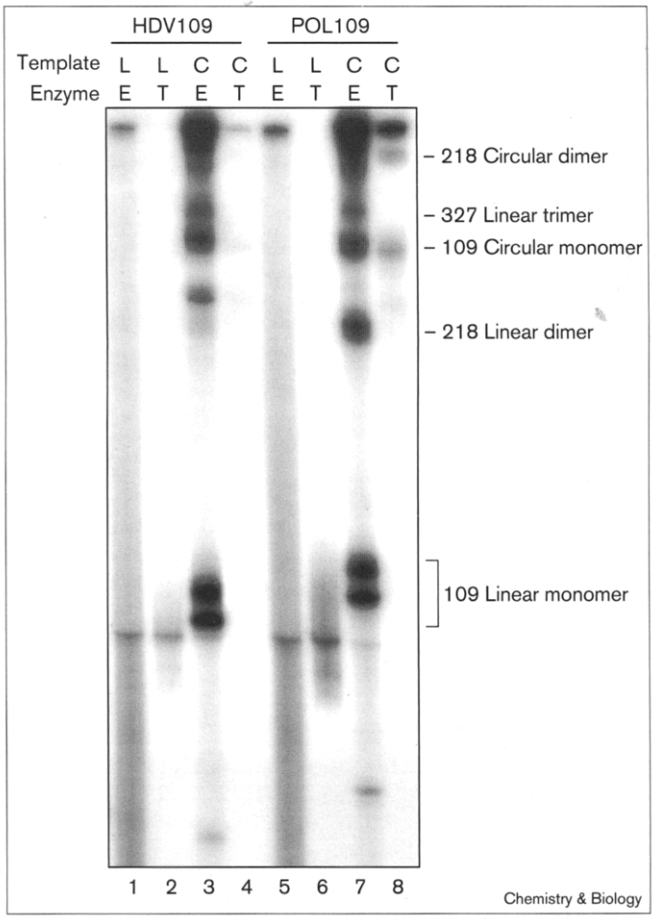
Initial tests of transcription were carried out with both 109-mer, circles as well as with the full-length linear precursors to these circles (Figure 2). The results show that both delta-derived DNA circles were transcribed with greater efficiency by *E. coli* RNA polymerase than by the T7 polymerase. The products after 10 h appear to be long RNAs not resolved by the gel, as well as two distinct bands that size markers indicate fall into the apparent 100–120 nt size range. By contrast, the linear precursor DNAs were weakly transcribed by both enzymes, and did not produce these distinct RNAs.

Both circular DNAs yielded similar chief products, which were shown to be circular and linear 109-nt monomer RNAs. These bands were isolated, and the faster migrating linear monomers were characterized both by comparison to size markers and by sequencing with RNase T1 (data not shown). In each case, the sequence was that expected for RCT of the 109mer circular DNAs followed by self-cleavage. Circular and linear monomer RNAs, produced in approximately equal amounts, were among the major products from RCT (Figure 2). The circular monomers (identified as such by partial alkaline hydrolysis) traveled on the 10% denaturing gel considerably more slowly than the linear variants; the circular RNA traveled 0.35 and 0.4 the rate of the linear RNA for HDV109 and POL109, respectively. The finding of circular products as the major products was surprising, based on previous reports showing that HDV cleavage–ligation equilibrium lies far to the side of cleavage in the presence of Mg^{2+} [31].

To investigate the reactivity of the isolated self-processed HDV ribozymes, we incubated linear and circular monomer RNAs from both HDV109 and POL109 for 24 h in 12 mM Mg^{2+}-containing buffer and analyzed products by denaturing polyacrylamide gel electrophoresis (PAGE). We hypothesized that the presence of the circular DNA template might affect the products of the self-processing. For that reason, incubation was carried out both in the presence and absence of 1 μM linear and circular DNA templates. The results are shown in Figure 3. Additionally, incubation was also carried out with unpurified ribozyme RNAs produced from the DNA circles to investigate the influence of *trans*-cleaving RNAs on this reactivity.

When isolated linear monomer RNAs were incubated in the presence of complementary circular DNA, we found that the product is a circular monomer RNA (Figure 3, lane 4) that is identical in mobility to the original circular RNA produced in the transcription reaction (lanes 1,8).

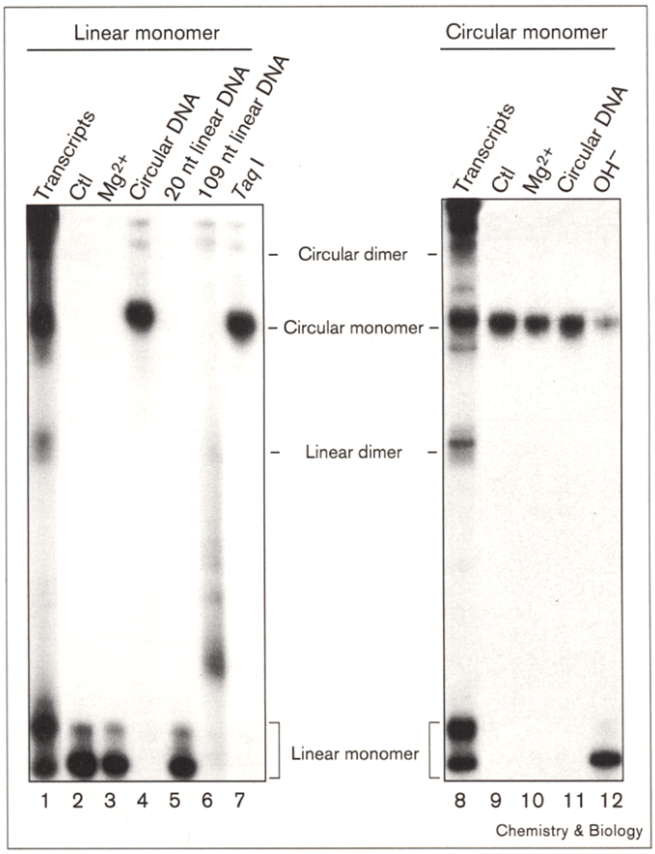
Figure 2



Transcription of linear and circular ssDNAs encoding hepatitis delta antigenomic ribozymes showing appearance of long RNAs, as well as shorter, specific bands. Topology of template DNA is denoted by L (linear) and C (circular); enzyme is denoted by E (*E. coli* RNA polymerase) and T (T7 RNA polymerase). RNAs are denoted by length and topology. Both bands marked linear monomer appear to be different conformations of the same RNA.

The cyclization proceeds to more than 95% for both HDV109 and POL109 (data not shown for POL109). Incubation in alkaline buffer (lane 12) opened the circular RNA to create a species which migrates the same as the 109mer linear monomers, a result that is characteristic for circular nucleic acids. The circular products were also digested with *Taq*I, RQ1 DNase and RNase H. The finding that the mobility of this band remains unchanged after *Taq*I digestion (lane 7) supports its identification as a circular RNA and not an RNA/DNA catenane, as does the gel mobility relative to other circular and linear RNAs. Interestingly, incubation of the linear monomer RNAs with the full-length precursor of the circular DNA gave no observable monomeric circular product, and a small amount of circular dimer (lane 6). A shorter 20-mer DNA complementary to the junction gave no apparent ligation (lane 5).

Figure 3



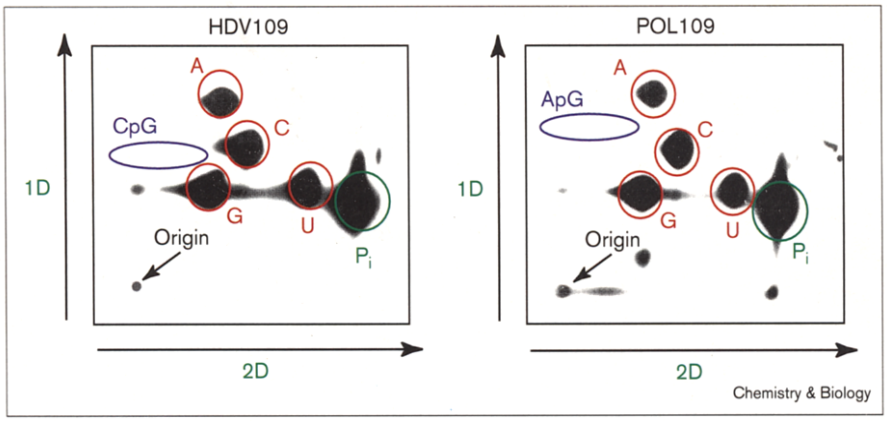
Incubation of linear monomer and circular monomer RNAs from HDV109 with Mg^{2+} and with and without templating DNAs of various sizes. Note cyclization of linear monomer RNA only in the presence of circular DNA template (lane 4) and the lack of self-cleavage activity of circular monomer RNA (lane 10). Ctl is the control reaction, which contained the isolated RNA dissolved in loading buffer.

The observation of ligation only with the circular DNA complement suggested a templated reaction of 2′,3′-cyclic phosphate with 5′-OH on the opposite end of the RNA, rather than reversal of the self-cleavage mechanism. Incubation of the circular RNA monomers isolated after RCT in the same Mg^{2+} buffer adds support to this hypothesis. In the absence or presence of the DNA circle (lanes 9–11) or RNAs able to cleave the circle *in trans* (data not shown), the cyclic RNA is stable, producing no linear monomer or dimer. This lack of self-cleavage ability in the circular monomer would be consistent with an uncleavable 2′–5′ junction at the ribozyme cleavage site, or with an inactively folded conformation.

To test for the presence of a 2′–5′ junction, we digested the circular monomers completely with RNase T2. Two-dimensional thin-layer chromatography (TLC) was used to examine the products, which were expected to be 3′-monophosphate nucleotides and 2′–5′ linked dinucleotides [37]. The results showed the presence of the expected mononucleotides but not the expected dinucleotides, which were expected to co-elute with authentic guanylyl(2′–5′)adenosine for POL109 and guanylyl(2′–5′)-cytidine for HDV109 (Figure 4). This result is consistent with the absence of a 2′–5′ junction in the circular monomer RNAs of both sequences (HDV109 and POL109), and suggests the possibility of an inactively folded conformation.

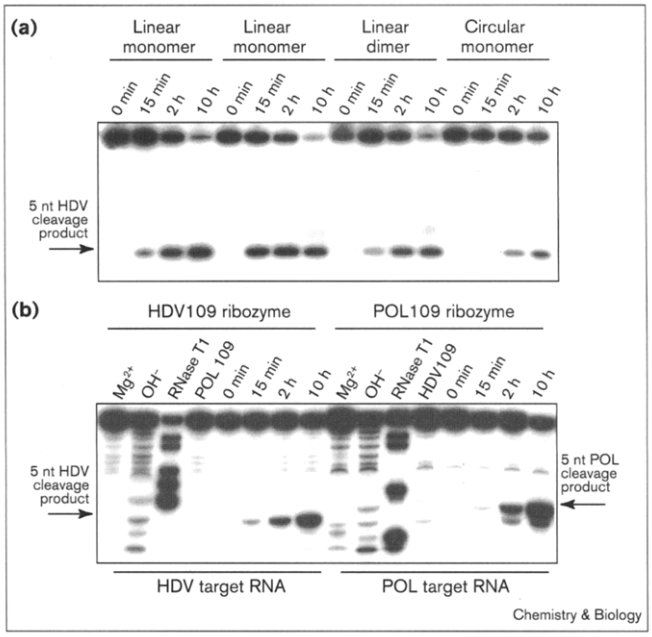
To test for *trans*-cleaving activity by the monomer-length RNAs, we incubated the chief products from both circular DNA templates with shorter synthetic 14mer RNAs in buffers containing between 0.5 and 12 mM Mg^{2+}. The 14mers corresponded to the complementary target sequences from HDV antigenomic RNA and from HIV-1 *pol*. The results are shown in Figure 5. The data show that the linear monomer RNAs can, in fact, cleave the expected RNA targets, and more surprisingly, the circular RNAs can also cleave the target RNAs in *trans* (Figure 5a). Cleavage was found to be relatively efficient at magnesium concentrations as low as 1 mM (data not shown). In addition, the two linear monomer ribozyme RNAs (HDV109 and POL109) cleave their targets specifically, as

Figure 4



Two-dimensional TLC of RNase-T2-digested circular RNAs from HDV109 and POL109. The four 3′-monophosphates (which co-elute with authentic samples) are observed, but not the proposed dinucleotide, which would co-elute with authentic guanylyl(2′-5′)adenosine (ApG) for POL109 and guanylyl(2′-5′)cytidine (CpG) for HDV109.

Figure 5



Cleavage of **(a)** 14-nt target RNAs (HDV and POL) in a 12 mM magnesium buffer by discrete RNAs or **(b)** a mixture of ribozyme transcripts derived from HDV109 and POL109.

indicated by the lack of cross-cleaving activity for the two different targets (Figure 5b). Comparison with previously described self-processed hairpin ribozymes at Mg^{2+} levels of 12 mM found similar cleavage activity [41].

Discussion

Mimicking HDV replication

The results show that the three chief steps of HDV replication (RCT, self-processing and cyclization) can be recapitulated *in vitro* using a much smaller synthetic construct and a bacterial RNA polymerase. Our circular DNAs contain only 6.5% of the entire viral sequence, suggesting that much of the viral RNA serves purposes beyond the simple steps described here. Indeed, viroid RNAs replicate successfully in plants with complete sequences of less than 300 nt in size. Of course, the RNAs in the present system do not replicate, chiefly because the circular products do not encode a ribozyme that would be active after the next round of transcription. Probably the most surprising result from the present experiments is that cyclization occurs quite readily, and that the circular template mediates this reaction. Possible mechanisms for this are discussed below.

Transcription of promoterless ssDNAs

We have established that HDV ribozymes can be produced by transcription of small synthetic circular DNAs, using both *E. coli* and T7 RNA polymerases. Previous studies have shown significant variations in a given polymerase's ability to transcribe various circular ssDNA sequences [39,40]. It is important to note that none of these circles contains a canonical promoter for either polymerase. In a potentially related finding, a single-stranded promoter for *E. coli* RNA polymerase was recently identified [42], although its sequence is different from those in this study. The present finding brings to three the number of small ribozyme motifs that can be produced using the rolling-circle approach. Studies are underway to identify any promoter-like sequence and/or structural motifs that allow for efficient initiation with different polymerases. We propose that it might be possible to use such small circular DNAs as vectors to deliver biologically active RNAs (such as ribozymes) into human cells. It remains to be seen, however, whether eukaryotic RNA polymerases can transcribe such structures.

Mechanisms of cyclization

The mechanism for cyclization of these self-processed HDV RNAs is of particular interest. It is noteworthy that a similar cyclization of self-processed RNA has been observed for a hammerhead ribozyme in the presence of a complementary circular template [43], suggesting that, whatever the mechanism, it might be operative for multiple classes of catalytic RNAs. In the present HDV system, our data establish that linear monomer RNAs are only converted to circular monomer RNAs in the presence of a complementary circular DNA template, which suggests a simple template-dependent ligation of the 5′-OH and 2′,3′-cyclic phosphate. The resulting circular species does not appear to be a RNA/DNA catenane, however, and appears to be resistant to cleavage at the ligation junction. One possibility is that the proposed template-assisted ligation produces a 2′–5′ junction. This junction is known to prevent hammerhead-ribozyme cleavage [37], and in the HDV system would probably inhibit self-cleavage back to linear form, accounting for the stability of the circular monomer RNAs. In fact, it is possible that some of the junction might be transiently formed as a normal 3′–5′ junction, but such a junction would be expected to be self-cleaved again. This process could eventually accumulate the majority of the RNA population as a circular species with the noncleavable junction.

Contrary to this hypothesis, however, our enzymatic digestion experiments were unable to find evidence for a dinucleotide containing such a 2′–5′ junction. An alternative hypothesis to explain this apparent lack of *cis*-cleavage activity in the circular monomers is that the template-directed ligation yields circular RNA in a ribozyme-inactive conformation, where the intramolecularly cleavable RNA does not occupy the cleavage active site. Interestingly, these circular RNAs are evidently able to cleave another RNA in *trans*, which is consistent with this hypothesis. It is also noteworthy that cyclized full-length HDV and subfragments have also been observed not to self-cleave in other studies, also possibly because of unproductive secondary structure [32]. Studies probing the secondary and tertiary structure of

these circular monomers in comparison to the linear species will be necessary to explore these hypotheses further.

The short (20 nt) DNA template for ligation does not appear to promote any ligation of linear monomer RNAs, and incubation with the full-length (109 nt) linear DNA results in only a small change in product distribution (a weak band that is possibly circular dimer). The linear and circular DNA templates used here for ligation are of the same size and sequence. Additionally, the disruption of continuity in the linear sequence occurs at a position ~50 nt distal from the region complementary to the RNA ligation site, which should allow for the possibility of efficient templating. Why then does only the circular form allow ligation? We surmise that the two may be different in secondary structure. For example, the covalently closed circular form might be conformationally restricted in a way that favors a structure active in ligation, whereas the linear form might fold to block the region necessary for binding the RNA.

A number of additional questions remain in this cyclization mechanism. Although we have shown that a circular DNA can act as a template in ligating the linear monomer RNAs, it remains to be seen whether a circular RNA (more directly cogent to HDV replication) would also serve as a template. A linear RNA template has been used to ligate two linear complementary HDV RNA fragments [30], and the suggestion was made that partially complementary sequences within the same viroid could template closure of linear HDV. Evidence for a similar cyclization pathway has been recently described for hammerhead-containing viroids [37]. A recent report suggests that a 377-mer HDV catalytic segment does not ligate intramolecularly *in vitro*, however [29]. Here we propose a second mechanism, in which the complementary circular genomic HDV might template the closure of the antigenomic RNA and *vice versa*. In the present case, a circular, as opposed to linear, complement would allow for more efficient ligation; however, ligation on a circular template is expected to yield a catenated DNA/RNA product if at least one full turn of duplex is formed. In this work we see no evidence for a catenated product, either by gel mobility or by endonuclease treatments. We hypothesize, therefore, that although some duplex must form between circular DNA template and the linear monomer RNA during ligation, the product linking number is zero either because the duplex is short, or because the right-handed turn is compensated for elsewhere in the complex.

Irrespective of the mechanism by which circular monomers are formed, the results clearly show that, although self-processed HDV ribozymes are not efficient at ligation by reversing the cleavage mechanism, they can be efficiently ligated in the presence of a complementary circular template. This has potentially important implications in the replication cycle of the HDV, because the most common form of the virus is circular [33,34]. Although the present RNAs are only a subset of the entire virus, their design and method of transcription mimic important features of full-length antigenomic HDV. They are produced by a rolling-circle mechanism followed by self-processing, and the self-cleavage produces linear RNAs that are subsequently converted to circular form.

Trans-cleavage by self-processed HDV RNAs

The data also establish that linear and circular HDV ribozymes derived from self-processing can have significant RNA-cleavage activity in *trans*, despite the presence of self-complementary sequences. This is, to our knowledge, the first report of such *trans* cleavage. A particularly attractive feature of the self-binding domains in self-processed ribozymes is their ability to enhance sequence specificity of *trans* cleavage (T. Omichi and E.T.K., unpublished observations). Been and coworkers [44] have reported circular HDV constructs that cleave HDV sequences in *trans*, but these did not contain such self-complementary domains. The HDV ribozyme motif is different from hammerhead and hairpin motifs in that the RNA target is not cleaved between two separately bound domains. In addition, the helix length required for activity is longer for the HDV motif. For the sequences used here there is the possibility of an 8-bp helix that arises from self-binding. This helix is predicted to have a free energy of –9.3 kcal/mol and a T_m of 62.4°C for POL109 and free energy of –11.9 kcal/mol and a T_m of 70.8°C for HDV109 [45]. It would not have been surprising, therefore, if the *trans* cleavage were found to be inactivated by this substantial antisense complementarity. Because it is not, we surmise that, if this helix is indeed present (it must have been transiently present to allow for self-processing), the binding of an RNA target in *trans* must await the dissociation of this structure. Further study will be needed to delineate the actual secondary structure of these monomer ribozymes as well as their catalytic efficiency relative to ribozymes lacking such self-complementarity. In any case, the results showing that the sequence can be engineered for cleavage of non-HDV targets suggest that self-processed antigenomic RNAs might be useful in biomedical applications. The only other report of HDV *trans* cleavage of an altered substrate [28] demonstrated the ability of nonself-processed antigenomic HDV ribozymes to cleave HDV RNA in its delta-antigen coding region.

Finally, the finding of *trans*-cleaving activity for these self-processed ribozymes could have implications in the activities of the whole HDV virus. It seems quite possible that linear or circular forms of HDV could possess the ability to cleave other RNA targets in *trans*. Such RNA targets may include multimeric HDV replication intermediates, as well as RNAs from the host. The remaining noncatalytic ~1300 nt (in the antigenomic strand) code for a nuclear, RNA-binding phosphoprotein, hepatitis delta antigen (HDAg), which has been found to bind both genomic and

antigenomic HDV RNAs specifically [46] and which has sequence homology to a protein in humans [47]. Although HDAg is not required for cleavage and ligation of HDV RNAs, its presence does enhance this activity and is apparently required for replication ([48–50]; reviewed in [8,51]). It is notable that HDV has been proposed to have arisen from a more primitive viroid-like RNA by capture of such a host-derived translatable RNA [47]. The first step of this capture might well have been cleavage of a homologous host sequence in *trans*, as we have observed for a small subset of HDV RNA.

Significance

Hepatitis delta virus (HDV), a human pathogen comprised entirely of RNA, is proposed to replicate by a rolling-circle mechanism, yet the details surrounding this process are unclear. Our results show that a very small subset of the virus successfully mimics several of the steps of this replication. The biomimetic pathway utilized here is important: like the full-length HDV, the present small ribozyme domains are produced by a rolling-circle mechanism followed by self-cleavage, and circular RNAs are found as chief products. We have, therefore, successfully mimicked the three main steps of HDV replication *in vitro* using a synthetic circular oligonucleotide (only a small fraction of the entire virus) as the starting point. The finding that cyclization of the monomer RNAs occurs readily, and only in the presence of the circular template, is significant because it suggests that HDV itself may also be cyclized this way. We have also demonstrated that self-processed HDV ribozymes can have significant RNA-cleaving activity in *trans*, despite the presence of self-complementary sequences that might have inhibited substrate binding. This suggests that linear or circular forms of full-length HDV may possess the ability to cleave other RNA targets in *trans*, conceivably including RNAs from the host. Finally, the results suggest that self-processed antigenomic HDV ribozymes might be useful in biomedical applications, and that synthetic circular single-stranded DNA templates are useful vectors for generating them.

Materials and methods

Synthesis of circular DNAs HDV109 and POL109

The preparation of ssDNA circles was carried out by a double-ligation scheme using T4 DNA ligase and short synthetic DNA splints as previously described [40]. The splint oligomers were 20 nt long, complementary to 10 nt on each of the ends to be ligated. Sequences were as follows:

HDV109: 5′-pGAC CCG AAG AGG AAA GAA GGA CGC GAG ACG GAA TCG AGA GAA AAG TGG CTC T-3′ and 5′-pCCC TTA GCC ATC CGA AGA TGC CCA GGT CGG ACC GCG AGG AGG TGG AGA TGC CAT GCC-3′. Splint #1: 5′-TCT TCG GGT CGG CAT GGC AT-3′. Splint #2: 5′-TGG CTA AGG GAG AGC CAC TT-3′.

POL109: 5′-pTGG ACT ACA GGG AAA GAA GGA CGC GAG ACG GAA TCG AGA GAA AAG TGG CTC T-3′ and 5′-pCCC TTA GCC ATC CGA AGA TGC CCA TCC AGG ACC GCG AGG AGG TGG AGA TGC CAT TCC-3′. Splint #1: 5′-CTG TAG TCC AGG AAT GGC AT-3′. Splint #2: 5′-TGG CTA AGG GAG AGC CAC TT-3′.

Transcription reactions

Conditions for an internally labeled RCT reaction were: 1 μM circle or precircle, 3 units *E. coli* RNA polymerase holoenzyme (Boehringer Mannheim) or 25 units T7 RNA polymerase (New England Biolabs), 0.5 mM ATP, CTP, UTP, 60 μM GTP, 0.30 μCi of α-[^{32}P] GTP in a pH 8.1 (25 mM Tris–HCl) buffer containing 20 mM NaCl, 12 mM $MgCl_2$, 0.4 mM spermine–HCl, 100 μg/ml acetylated bovine serum albumin, 10 mM dithiothreitol (DTT), and 12.5 units/ml RNase inhibitor (Promega), in a total reaction volume of 15 μl. Reactions were incubated at 37°C for 10 h and analyzed by 10% denaturing PAGE at 4°C. Unlabeled RNAs were prepared using 12 mM $MgCl_2$ and *E. coli* RNAP with all four rNTPs at 0.5 mM, and isolated by ethanol precipitation after 10% denaturing PAGE. Half of each unlabeled reaction was desalted by Chroma-spin size-exclusion column chromatography (Clontech) to remove $MgCl_2$ giving the ribozyme RNA solution. Sequencing of linear monomer RNAs was as previously described [40].

Cleavage in trans *of 14-nt HDV and POL RNAs*

The POL RNA target oligonucleotide (5′-CUGUAGUCCAGGAA-3′, corresponding to positions 3605–3618 of HIV-1 *pol* in strain HXB2) and HDV RNA target oligonucleotide (5′-UCUUCGGGUCGGCA-3′, corresponding to positions 900–913 of antigenomic HDV RNA) were prepared on an Applied Biosystems 392 synthesizer using the standard RNA cycle. The RNA was 5′-end labeled and purified by preparative 20% polyacrylamide denaturing gel electrophoresis. Ribozyme RNAs for study were either the ribozyme RNA solution (see above) or isolated RNA bands as described above. Cleavage reaction conditions were as follows: 5′-end-labeled target RNA and ribozyme in a pH 7.5 (40 mM Tris–HCl) buffer containing 0.5–12 mM $MgCl_2$, 2 mM spermine and 12.5 units/ml RNase inhibitor, reacted at 37°C for 5 min–10 h as indicated. Gel analysis was carried out by 20% denaturing PAGE.

Magnesium incubation of isolated RNAs

Radiolabeled linear monomer and circular monomer RNAs were equilibrated in a pH 7.5 (40 mM Tris–HCl) buffer containing 12 mM $MgCl_2$, 2 mM spermine HCl, 12.5 units/ml RNase inhibitor (Promega) with or without 1 μM appropriate templating DNA (as indicated) or an unquantified amount of RNA from a RCT reaction. Incubation was for 24 h. Post-magnesium incubation digestion was performed using either RQ1 DNase (1 unit, 3 h, 37°C), *Taq*I endonuclease (5 units, 3 h, 37°C), or RNase H (3.4 units, 30 min, 37°C) in the above buffer. Alkaline hydrolysis conditions were as previously described [40], with 5 min reaction time. Control reactions contained the isolated RNA band dissolved in loading buffer. Gel analysis was carried out with 10% denaturing PAGE at 4°C.

Two-dimensional thin-layer chromatography

Circular monomers from HDV109 and POL109 were digested with 10 units RNase T2 (GIBCO BRL) in a 10 mM sodium acetate, pH 4.5 buffer at 37°C for 24 h [37]. The reaction was stopped on ice and 5 × 1 μl aliquots were spotted on cellulose TLC plates (10 × 10 cm with fluorescent indicator; EM-Science) along with ~10 μg samples of the four 3′-monophosphates, adenosine, cytosine, uridine (Sigma) and guanosine (ICN Biomedicals) and the two proposed 2′,5′-linked dinucleotides: guanylyl(2′-5′)adenosine for POL109 and guanylyl(2′-5′)cytidine for HDV109 (Sigma). The first dimension was run in 50 ml of 5:3 (v/v) isobutryic acid:0.5 M ammonium hydroxide. The solvent front was run to the end of the plates, which were dried overnight prior to running the second dimension after rotating the plate 90° and eluting with 50 ml of 70:15:15 (v/v/v) isopropanol/concentrated HCl/water. The solvent front was again run to the edge of the plate and the plates were dried overnight. Standards were observed by UV shadowing, and radioactive RNase T2 digests by autoradiography and phosphorimaging.

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