A host-specific function is required for ligation of a wide variety of ribozyme-processed RNAs

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Hepatitis δ virus (HDV) replicates its circular RNA genome via a rolling circle mechanism. During this process, cis-acting ribozymes cleave adjacent upstream sequences and thereby resolve replication intermediates to unit-length RNA. The subsequent ligation of these 5′OH and 2′,3′-cyclic phosphate termini to form circular RNA is an essential step in the life cycle of the virus. Here we present evidence for the involvement of a host activity in the ligation of HDV RNA. We used both HDV and hammerhead ribozymes to generate a panel of HDV and non-HDV RNA substrates that bear 5′ hydroxyl and 2′,3′- cyclic phosphate termini. We found that ligation of these substrates occurred in host cells, but not *in vitro* or in *Escherichia coli*. The host-specific ligation activity was capable of joining RNA in both bimolecular and intramolecular reactions and functioned in a sequence-independent manner. We conclude that mammalian cells contain a default pathway that efficiently circularizes ribozyme processed RNAs. This pathway could be exploited in the delivery of stable antisense and decoy RNA to the nucleus.

Hepatitis δ is a small circular RNA virus that exists as a satellite of hepatitis B virus (1). Its genome consists of 1,679 nucleotides, of which ≈70% are involved in base pairing interactions, giving it a rodlike structure (2, 3). Hepatitis δ virus (HDV) is thought to replicate via a rolling circle mechanism (4) in which its circular genome serves as template in an RNA-dependent transcription reaction. During rolling circle replication, antigenomic multimers are produced. These multimers are resolved to unit-length monomers by the HDV antigenomic ribozyme, a cis-cleaving, catalytic RNA segment that forms a double-pseudoknot structure and is present once per unit length (5, 6). In the multimer, two cleavage events release a unit-length linear monomer bearing 5′hydroxyl and 2′,3′cyclic phosphate termini. Intramolecular ligation of these termini produces the circular antigenome, which serves as a template for genome production via an analogous round of rolling circle replication, which involves cleavage by the HDV genomic ribozyme.

After ligation, the cleavage site is reconstituted, and, at this stage, the ribozyme must be attenuated to prevent cleavage of the circular product. Ribozyme attenuation is promoted by sequences that reside opposite to the ribozyme in the circular rodlike structure. Through base pairing interactions, the ribozyme is prevented from adopting its active conformation (7). In the multimer (during replication), a host activity is proposed to promote transient disruption of this attenuation, allowing cleavage, release, and subsequent ligation of unit-length substrates. In support of this model, cleavage of circular HDV RNA by the resident ribozyme is promoted under conditions that prevent the attenuator–ribozyme interaction (7).

The HDV genomic and antigenomic ribozymes constitute a distinct structural class of catalytic RNA. Unlike hammerhead and hairpin ribozymes, in which base pairing interactions flanking both sides of the cleavage site are crucial for catalysis, the HDV ribozyme pairs only with sequences that reside immediately 3′ to the cleavage site (8). This lack of a requirement for 5′ pairing has allowed the HDV ribozyme to be used to generate defined 3′ ends after its fusion to heterologous RNA (9).

To date, our understanding of the ligation of HDV RNA remains limited. In one set of *in vitro* experiments, it was concluded that HDV RNA could self-ligate in an uncatalyzed reaction. Sharmeen *et al.* (10) proposed that, after cleavage of the multimer, the resulting monomer assumed the rodlike structure. In this conformation, the termini of the monomer would be brought into apposition, allowing sequences opposite the ligation junction to serve as a splint. Self-ligation could then occur in an uncatalyzed reaction involving an attack of the 2′,3′-cyclic phosphate by the proximal 5′ hydroxyl. To test this hypothesis, two precleaved RNAs were assayed for ligation after their hybridization to a third, partially complementary splint RNA. Although some ligation was observed, it was inefficient and occurred with very slow kinetics, under nonphysiological conditions (2 days at 4°C, in 1 M ethylenediamine). Ligation was not observed in the absence of the splint sequences. Isolation and reverse transcription of this *in vitro* ligated RNA suggested that ≈50% of the ligated bonds were 2′ to 5′ junctions. The relevance of these *in vitro* findings was brought into question by more recent studies of HDV RNA ligation *in vivo*. In these experiments, HDV RNAs lacking splint sequences were ligated at wild-type efficiencies (>90%) (7).

In another study of *in vitro* ligated RNAs, Wu and Lai (11) concluded that HDV genomic RNA was capable of undergoing reversible cleavage and ligation in a ribozyme-mediated manner that appeared to be regulated by the concentration of free Mg^{2+} ions. In their model, complete removal of Mg^{2+} ions by the addition of a molar excess of EDTA inhibited cleavage and promoted ligation of cleaved HDV RNA fragments.

In contrast to Wu and Lai, Rosenstein and Been (12) found that, although chelation of Mg^{2+} ions did inhibit the cleavage reaction, religation of the cleaved RNA fragments could not be detected. Instead, it was found that, in the presence of Mg^{2+}, ribozyme cleavage was actually enhanced by the denaturants as used by Wu and Lai in their loading buffers. This effect was abolished when molar excess of EDTA was included in the buffer. Therefore, it was suggested that Wu and Lai were interpreting a measurable lack of cleavage (in their EDTA containing buffers) as evidence for religation. In a later paper (13), Wu and Lai agreed with this alternate interpretation.

Although the results mentioned above provide some insight into the cleavage and ligation activities of HDV RNA in protein-free environments, our knowledge of these reactions as they occur *in vivo* remains limited. To date, it is not known whether the HDV ribozyme participates in the ligation of HDV RNA *in vivo*. Here we sought to further our understanding of the circularization of HDV by evaluating the ability of a panel of ribozyme-processed RNA substrates to be ligated inside cells. We found that the HDV ribozyme is not required for RNA

Abbreviations: HDV, hepatitis δ virus; RT, reverse transcription.

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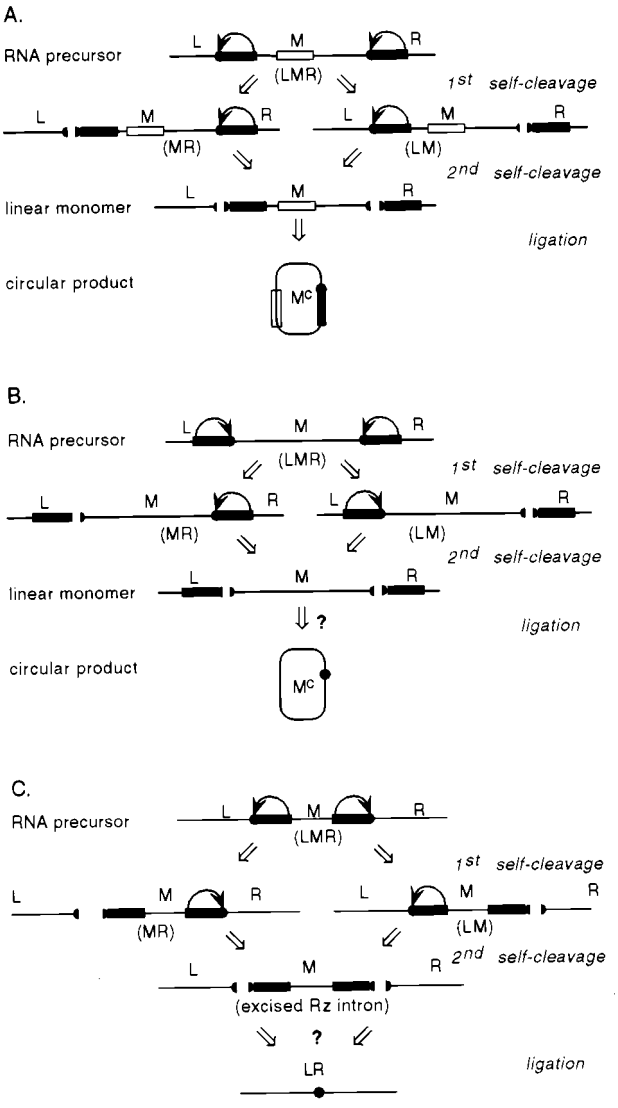

Fig. 1. Processing pathways of precursor RNAs. Ribozymes are depicted as rectangles adjacent to their respective cleavage sites (circles). For HDV genomic ribozymes, recognition and cleavage of the sequence U/GGCCGGC occurs between the U and G residues. The downstream-cleaving hammerhead ribozyme cleaves after the sequence GUC in its substrate domain. L, M, and R correspond to the leftward, middle, and rightward cleavage fragments, respectively, generated by ribozyme cleavage. (*A*) Two wild-type HDV ribozymes. The open rectangle depicts attenuator sequences. M^C represents circular (ligated) RNA. (*B*) Mutant (downstream-cleaving) and wild-type ribozymes. (*C*) Ribozyme-based intron.

ligation and present evidence for the involvement of a host-specific activity.

Materials and Methods

Plasmids. The RNA expression vector pDL625 was described previously (7) and expresses, under the control of a T7 RNA polymerase promoter and terminator, a truncated HDV precursor in which the two HDV genomic ribozymes are in their wild-type configuration and are flanked by natural sequences. pDL669 is a derivative of pDL625 in which the first ribozyme was converted to a downstream-cleaving mutant and the M region (see Fig. 1 *A* and *B*) was replaced with different HDV sequences, also able to fold into the rodlike structure. To generate pDL669, the LM-containing region of pDL625 was replaced between the *Sph*I and *Eae*I sites with the following HDV sequence (numbering as in gb_vi:hpdgen): 692–770 (genomic ribozyme catalytic region), 664–695 (ribozyme cleavage site), 5′ CCTGCAG 3′, 961–1,109 (left half of the rodlike structure from *Sal*I-*Sma*I), 482–685 (right half of the rodlike structure from *Sma*I-EaeI). pCR9 was designed to express a circular Kan^r RNA and was derived from pDL669. In pCR9, the HDV sequences of pDL669 between the two ribozyme cleavage sites were replaced with the following Kan^r sequences (numbering as in gb_ba1:TN5): 2,178–2,363 (3′ region of Kan^r gene and stop codon), 5′ ACTCTAGAG 3′, 1,516–2,177 (ribosome binding site and 5′ region of Kan^r gene). pKW23 is identical to pCR9 except that the 11-nt clamp sequence, 5′ CTCCGCCACAC 3′, was inserted immediately upstream of the catalytic region of the first HDV genomic ribozyme. This clamp sequence is complimentary to a 10-nt sequence in the Kan^r region 11 nucleotides downstream of the first ribozyme cleavage site. pCR41 was designed to express a circular fragment of the Tet^r gene and is identical to pKW23 except that the LM region of the latter was replaced with a downstream-cleaving hammerhead ribozyme followed by a 348-nt region, the Tet^r gene from pBR322. To generate pCR41, synthetic oligonucleotides oli226 and oli227 were used as PCR primers to amplify the Tet^r gene; the resulting product was cleaved with *Sac*I and *Eag*I and then was joined to the *Sac*I and EaeI sites of pKW23. The sequences of oli226 and oli227 are as follows: oli226 5′ ATAGGGAGCTCCGGTCTGATGAGTCCGTGAGGACGAAACAGGCTTGCGCCTGTCACCGGAGCTTCGAATTCCGACAGCATCGC 3′, oli227 5′CATGCCGGCCGTTTTTTGCCACCATACCCAC 3′.

pCR36 was designed to express a fragment of the Cat^r gene interrupted by a synthetic intron composed of a wild-type and a downstream-cleaving HDV genomic ribozyme (Fig. 1*C*). The backbone of this vector is identical to that of pKW23; however, the LMR region of pKW23 was excised with Ecl136II and *Acc*I and was replaced with the following: gb_ba1:trn9cat nucleotides 358–467, HDV nucleotides 687–785 (complete wild-type genomic ribozyme), 5′ CTCGCTCACCGTCACG 3′, which includes a 10-nt clamp complimentary to Cat^r sequences 10 nucleotides downstream of the second cleavage site, HDV nucleotides 694–770 (genomic ribozyme catalytic domain), HDV nucleotides 663–692 (genomic ribozyme substrate domain), Cat^r nucleotides 475–546. pTW102 expresses the 1,165-nt HDV deletion mutant, G-ΔApa (14), from a T7 RNA expression vector. It was constructed by using pDL539 (14) and pDL625 and was kindly provided by Ting-Ting Wu and John Taylor (Fox Chase Cancer Center, Philadelphia).

***In Vitro* Transcription.** Isotopically labeled RNAs were generated by *in vitro* transcription (1 hr) of cDNA using T7 RNA Polymerase (Promega) in the presence of [α-^{32}P]UTP (NEN). In these reactions, transcription buffer (Promega) was supplemented with 500 μM each ATP, CTP, and GTP and 5 mM DTT. Each 20-μl reaction contained 0.5 μg of linearized template DNA.

Transfection and RNA Preparation. The T7 RNA Polymerase expression vector, pKW87, was cotransfected with RNA expression vectors as calcium phosphate precipitants onto monolayers of Huh7 cells grown in a mixture of DMEM and Ham's F-12 media (50:50 vol/vol). The medium was supplemented with 10% FBS (heat-inactivated). Transfection efficiencies were determined by including a secreted alkaline phosphatase expression vector in each transfection as described (7). Total RNA was harvested from cells 3 days posttransfection by using the RNeasy RNA Isolation Kit (Qiagen, Chatsworth, CA) and was normalized according to transfection efficiencies (14) by elution in 96% formamide. All RNA preparations were visualized by ethidium bromide staining before Northern blot analysis.

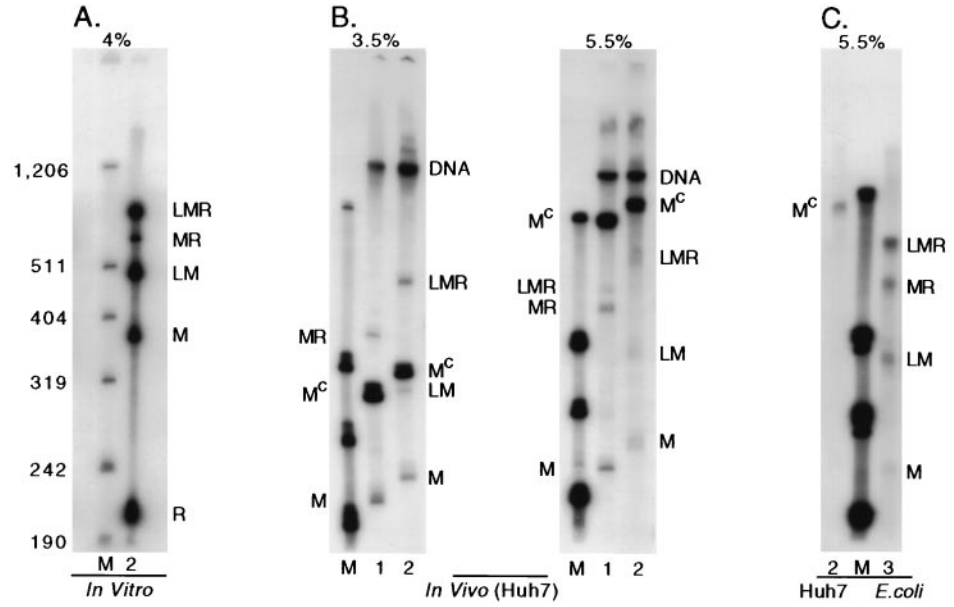

Fig. 2. Processing of HDV RNAs containing wild-type and mutant HDV ribozymes. (*A*) *In vitro* transcription. (*B*) Northern blot analysis of RNA harvested from transfected Huh7 cells. (*C*) Northern blot analysis of RNA generated from plasmid pDL669 harvested from Huh7 cells (lane 2) and from *E. coli* cells (lane 3). M, marker. Lane 1, HDV RNA generated from plasmid pDL625 (wild-type) and processed as in Fig. 1*A*. Product sizes (in nucleotides): LMR, 651; MR, 597; LM, 402; M, 348; R, 249. Lanes 2 and 3, HDV RNA generated from plasmid pDL669 (mutant) and processed as in Fig. 1*B*. Product sizes (in nucleotides): LMR, 756; MR, 638; LM, 484; M, 369. Percentages are percent polyacrylamide. The end-labeled DNA marker (M) is plasmid pDL616 linearized with *Msp*I (7). Fragment lengths of the DNA marker are notated in *A*.

RNA expression in *Escherichia coli* was carried out essentially as described (7), except that the cells were lysed with lysozyme and RNA was harvested with the RNeasy RNA Isolation Kit (Qiagen).

RNA Analysis. Northern blot analysis of total RNA harvested from transfected cells was performed essentially as described (7). In brief, 5 μl of each RNA sample was run through bis-acrylamide (1:19) gels containing 9 M urea, was electrophoretically transferred to nylon membranes (Zeta-Probe, Bio-Rad), and was UV cross-linked (Fisher catalog no. FB-UVXL-1000). Each blot was cross-linked two times at the "optimal cross-link" setting. [α-^{32}P] labeled probes were used for RNA detection. For detection of HDV RNA, plasmid pDL538 linearized with *Acc*I was transcribed *in vitro* by using T7 RNAP in the presence of [α-^{32}P] UTP. Kan^R and Tet^R RNAs were similarly detected by using antisense Kan^R and Tet^R probes, respectively. Relative signal strengths of all blots were quantitated with a digital phosphorimager (STORM 860, Molecular Dynamics).

Reverse Transcription (RT)–PCR and Sequencing of Ligated RNAs. The Access RT-PCR System (Promega catalog no. 1250) was used to prepare cDNA from ligated RNAs according to the manufacturer's protocol. cDNAs were reamplified with *Taq* polymerase and were cloned by using the TOPO TA Cloning Kit (Invitrogen catalog no. K4650-01) following the manufacturers protocol. Individual clones were then sequenced across the ligation junctions by using appropriate primers.

Results

An HDV RNA Lacking Ribozyme Sequences Is Efficiently Ligated *in Vivo*. During replication of HDV RNA, multimeric intermediates are resolved to unit-length linear RNAs, which are subsequently ligated to form a circular RNA. These monomeric RNAs are released from the multimer after two cleavages by cis-acting HDV ribozymes present once per unit length (see Fig. 1*A*). Because the ribozyme cleavage site resides upstream of the catalytic domain, each monomer will contain one copy of the ribozyme. Therefore, it is possible that ligation of the 5′ and 3′ ends to form the circular RNA is mediated by the ribozyme in the linear substrate by reversal of the cleavage mechanism. If this were correct, then removal of the ribozyme from the substrate should abolish ligation. To test whether an HDV RNA lacking ribozyme sequences is a substrate for ligation, we designed a cDNA construct which, when transcribed and cleaved, would produce a substrate RNA lacking ribozyme sequences and bearing the appropriate 5′ and 3′ ends (Fig. 1*B*).

A mutant genomic HDV ribozyme (pDL669) was made by removing the cleavage site from the 5′ end of the ribozyme and placing it at the 3′ end. In this way, we created an HDV ribozyme that is predicted to cleave downstream of the catalytic domain. We placed this downstream-cleaving ribozyme immediately upstream of HDV RNA, which was then followed by a wild-type upstream-cleaving ribozyme. In this construct, cleavage of the mutant and wild-type ribozymes is predicted to release a linear RNA containing 5′hydroxyl and 2′,3′-phosphate cyclic termini but lacking a resident ribozyme (see Fig. 1*B*).

We first transcribed this construct *in vitro* to test whether it would indeed generate an RNA capable of undergoing two cleavages by the mutant and wild-type ribozymes. After T7 RNA polymerase-directed transcription in the presence of [α-^{32}P]UTP, the RNA was analyzed on a denaturing polyacrylamide gel. Linear RNA corresponding in size to the doubly cleaved species was generated (Fig. 2*A*), demonstrating that the mutant downstream-cleaving ribozyme is functional and, in the context of a wild-type upstream-cleaving ribozyme, is capable of generating a linear (doubly cleaved) "M" species lacking a resident ribozyme. Furthermore, no ligation of M to "M^C" was observed *in vitro*.

We then tested whether the mutant ribozyme construct would function in a host cell in conjunction with a wild-type HDV genomic ribozyme to generate a doubly cleaved RNA lacking resident ribozyme sequences. Fig. 2*B* shows the results of a Northern blot of total RNA harvested from Huh7 cells cotrans-

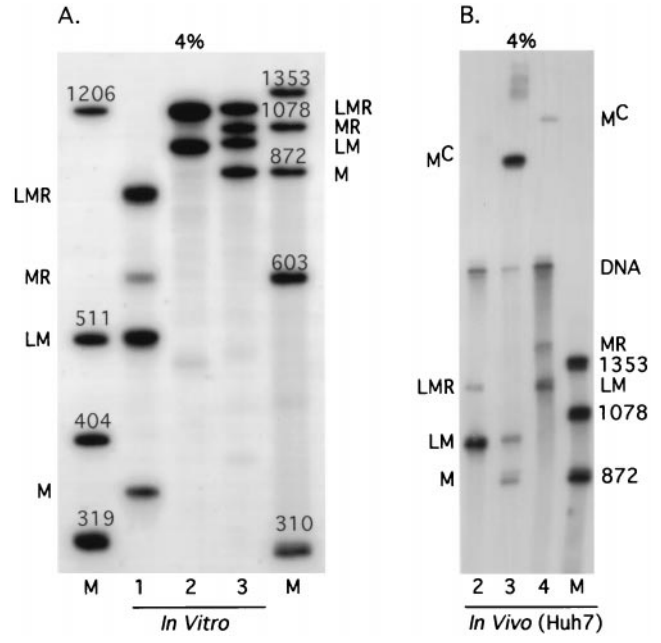

Fig. 3. Processing of Kan^R RNA containing wild-type and mutant HDV ribozymes. (*A*) *In vitro* transcription. M on the left is plasmid pDL616 linearized with *Msp*I. Marker on the right is ΦX174 linearized with *Hae*III. (*B*) Northern blot analysis of RNA harvested from transfected Huh7 cells. Lane 1, HDV RNA generated from plasmid pDL669 and processed as in Fig. 1*B*. Lane 2, Kan^R RNA generated from plasmid pCR9 and processed as in Fig. 1*B*. Product sizes (in nucleotides): LMR, 1,217; MR, 1.112; LM, 968; M, 863. Lane 3, Kan^R RNA generated from plasmid pKW23 and processed as in Fig. 1*B*. Product sizes (in nucleotides): LMR, 1,228; MR, 1,112; LM, 979; M, 863. The mutant downstream-cleaving ribozyme in lane 3 contains clamp sequences that are missing from the corresponding ribozyme in 2. Lane 4, HDV RNA generated from plasmid pTW102 and processed as in Fig. 1*A*. Product sizes (in nucleotides): LMR, 1,468; MR, 1,414; LM, 1,219; M, 1,165. Percentages are percent polyacrylamide.

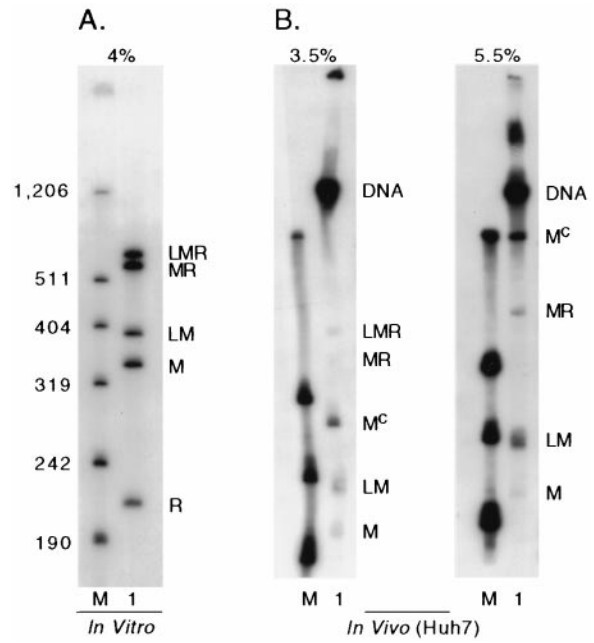

Fig. 4. Processing of Tet^R RNA containing hammerhead and HDV genomic ribozymes. (*A*) *In vitro* transcription. (*B*) Northern blot analysis of RNA harvested from transfected Huh7 cells. M, marker. Lane 1, Tet^R RNA generated from plasmid pCR41 and processed as in Fig. 1*B*. Product sizes (nucleotides): LMR, 648; MR, 597; LM, 399; M, 348; R, 249. Percentages are percent polyacrylamide. DNA marker (M) sizes are defined in *A*.

fected with our mutant (pDL669) or a control (pDL625) construct along with a vector that expresses T7 RNA polymerase. pDL625 contains two wild-type HDV ribozymes and was previously shown to generate a doubly cleaved RNA (M), which is then efficiently ligated inside host cells (7). Because the RNA species generated by pDL625 is of similar size to that predicted for our mutant construct, it is useful as both a size marker for identification of circular RNA and as a comparison for ligation efficiency. To detect circular RNA, two polyacrylamide gels of differing percentage are run (under denaturing conditions). This assay is diagnostic for circular RNA because the migration of this species will be differentially retarded (relative to linear species) as a function of the polyacrylamide percentage.

When transcribed *in vivo*, both constructs give rise to defined RNA species (of predicted sizes) that result from cleavage of one or both ribozymes, demonstrating that the mutant ribozyme was capable of functioning *in vivo*. The mutant ribozyme construct (pDL669) also functioned when transcribed in *E. coli*, generating the predicted linear RNA species (Fig. 2*C*, lane 3). However, ligation (M→M^C) of the doubly cleaved M species was only observed in host cells and not in *E. coli* (compare lanes 2 and 3 of Fig. 2*C*). This ligation occurred at an efficiency of 91% [M^C/(M + M^C)], equal to that of our control construct, pDL669 (91%). These results demonstrate that a doubly cleaved M species lacking a ribozyme is a substrate for ligation in host cells, but not *in vitro* or in *E. coli*. Furthermore, the mutant construct lacked attenuator sequences, suggesting that ribozyme attenuation is needed only for the stabilization of circular RNAs that contain a ribozyme.

***In Vivo* Ligation of Heterologous RNA.** To this point, we have established that an HDV RNA that lacks a ribozyme but bears 5′hydroxyl and 2′,3′-cyclic phosphate termini is a substrate for ligation in host cells but not *in vitro* or *E. coli*. We next wanted to know whether the observed ligation was a property of the HDV RNA or whether a heterologous RNA bearing the appropriate termini could also serve as a substrate. To this end, we replaced the HDV sequences in our mutant ribozyme construct with DNA from the neomycin phosphotransferase II gene from Tn5 (Kan^R) and tested the ability of the corresponding Kan^R RNA to be processed. The sequence naturally recognized by the HDV genomic ribozyme is U/GGCCGGC (where "/" denotes the site of cleavage). This sequence naturally occurs in the Kan^R open-reading frame and was fused to downstream-cleaving and upstream-cleaving HDV genomic ribozymes (refer to *Materials and Methods*). After cleavage by both ribozymes, a linear Kan^R transcript is released in which the open-reading frame is interrupted by ribozyme cleavage. If this M species were to be ligated, the Kan^R open-reading frame would be restored upon circularization. As before, we first tested our Kan^R construct *in vitro*. Here we found that, although doubly cleaved Kan^R RNA could be generated, the cleavage efficiency of the mutant downstream-cleaving ribozyme was considerably impaired (Fig. 3*A*, lane2; "LM" denotes that species of RNA in which the wild-type ribozyme has cleaved but the mutant has not). Analysis of the Kan^R construct using M-fold, an algorithm that predicts RNA secondary structure, revealed a possible interaction between the Kan^R and substrate portions of our precursor RNA. If correct, such an interaction would potentially disrupt proper folding of the HDV ribozyme and prevent efficient cleavage. In an effort to promote proper folding of the mutant ribozyme, we engineered a clamp sequence 5′ to the ribozyme sequence that is complementary to a sequence immediately 3′ of the substrate domain. As evidenced by the corresponding increase in the amount of M generated, this modification enhanced the cleavage

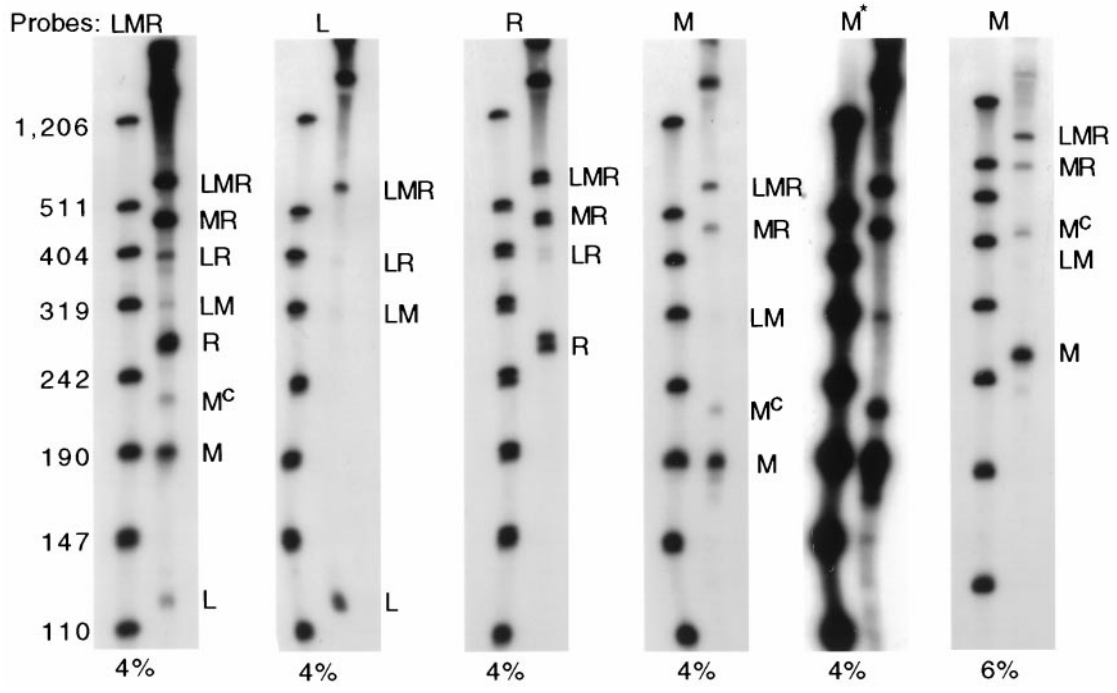

Fig. 5. Processing of Cat^R RNA containing an HDV ribozyme-based intron. Northern blot analysis of RNA harvested from Huh7 cells transfected with plasmid pCR36. Plasmid pCR36 generates Cat^R RNA, which is processed as in Fig. 1*C*. Product sizes (in nucleotides): LMR, 604; MR, 489; LR, 389; LM, 330; R, 274; M, 215; L, 115. Fragment-specific (L, M, R, etc.) ^{32}P-labeled probes were used individually on independent blots of the RNA sample. M* is a darker exposure of M.

efficiency of the mutant ribozyme to near wild-type levels (Fig. 3*A*, compare lanes 2 and 3). However, as with doubly cleaved HDV RNA, no ligation of the linear M species was observed *in vitro* (data not shown). Expression of this RNA in *E. coli* did not confer any measurable resistance to kanamycin, consistent with a lack of ligation in that cell (data not shown).

To study processing of the Kan^R construct in mammalian cells, we transfected it into host cells that express T7 RNA polymerase and analyzed total RNA for the presence of doubly cleaved Kan^R RNA. Fig. 3*B* shows that not only was doubly cleaved (M) Kan^R RNA produced within host cells, but that it was also efficiently ligated to form circular RNA (Fig. 3*B*, lane 3). This result demonstrated that host cells facilitate the ligation of non-HDV RNA lacking a resident ribozyme, and bearing 5′hydroxyl and 2′,3′ -cyclic phosphate termini. In support of this result, the size of RT-PCR products generated from RNA using Kan^R specific primers confirmed that M had been converted to M^C. The resulting cDNA was cloned, and seven independent isolates were sequenced across the predicted ligation junction. Analysis of these sequences revealed that the junction was correct to the nucleotide in each case. We have also observed host-specific ligation of a Cat^R RNA substrate similarly generated by ribozyme cleavage (data not shown).

Cleavage and Ligation of Tet^R RNA Using a Hammerhead Ribozyme. Circularization of HDV and non-HDV RNAs that lack resident ribozyme sequences argues against ribozyme participation in the ligation reaction. However, because the ribozyme recognition sequence GGCCGGC remains at the 5′ end of these doubly cleaved RNAs, ribozyme participation *in trans* cannot be formally ruled out. To rule out participation of the ribozyme *in trans*, the doubly cleaved RNA would have to lack such sequences that could promote reassociation of the ribozyme once it has been cleaved away from the RNA. To achieve this condition, we created a downstream-cleaving variant of the hammerhead ribozyme to generate the 5′ end of doubly cleaved Tet^R RNA. Here, a doubly cleaved portion of Tet^R RNA is generated by the action of the downstream-cleaving hammerhead ribozyme and an upstream-cleaving HDV ribozyme. As shown in Fig. 4, this RNA was properly processed *in vitro* (*A*) and *in vivo* (*B*). However, ligation of the doubly cleaved species to form circular RNA was only observed *in vivo*. Ligation occurred at an efficiency of 87%, comparable to that seen for wild-type HDV ligation (91%). Because the hammerhead ribozyme requires base pairing on either side of its cleavage site(15), and the doubly cleaved Tet^R RNA only provides one-half of this requirement, participation of the hammerhead ribozyme in this ligation event is unlikely. Similarly, the HDV ribozyme is not predicted to associate with this RNA *in trans* because it bears no sequence complementarity to this M. Ligation of Tet^R RNA was, therefore, ribozyme-independent (beyond generation of the termini). Coupled with the lack of ligation *in vitro* and in *E. coli*, our results to this point suggest that ligation of doubly cleaved RNA is catalyzed by a host activity.

Bimolecular Ligation of Cat^R RNA. Our observation that *in vivo* ligation of ribozyme-cleaved RNA was ribozyme- and sequence-independent led us to focus our attention on the termini of the doubly cleaved substrates, all of which contained 5′hydroxyl and 2′,3′- cyclic phosphate termini. Such termini are also generated (and ligated) during splicing of certain eukaryotic tRNAs (16). During this process, two tRNA half-molecules are joined in a bimolecular ligation reaction to form a mature tRNA. We sought to test whether ribozyme-generated half-molecules could be similarly joined *in vivo*. To this end, we created a ribozyme-based intron that is designed to auto-excise from within Cat^R RNA and generate two half-molecules of Cat^R RNA: a 5′ half bearing a 2′,3′- cyclic phosphate and a 3′ half with a 5′ hydroxyl (Fig. 1*C*). The ribozymes in this construct were active *in vitro* (data not shown) and *in vivo* (Fig. 5). Furthermore, the half-molecules were ligated when produced *in vivo* but not *in vitro*. Bimolecular ligation of the ribozyme-generated half-molecules of Cat^R RNA

occurred at an efficiency of 8%. This lower efficiency is not surprising for two reasons. Unlike with tRNA ligation, where the two half-molecules are held together by base pairing interactions, the two Cat^R half-molecules share little or no complementarity. In addition, unimolecular ligation (i.e., circularization) is, in general, a more efficient process than bimolecular ligation.

The ligation of L and R fragments was also confirmed by RT-PCR. Direct sequencing of six independent cDNA clones of this ligated RNA across the ligation junction revealed that all six contained the predicted junction.

Discussion

We have presented findings that contribute to our understanding of RNA ligation in eukaryotes. Although we fail to observe ligation of RNA substrates bearing 2′, 3′-cyclic phosphate and 5′ hydroxyl termini *in vitro* or *E. coli*, in mammalian cells, the same substrates are ligated at efficiencies >90%. Thus, a host-specific factor(s) is likely involved in the catalysis of the ligation reaction. In addition to HDV, this activity joined heterologous RNAs bearing 5′ hydroxyl and 2′,3′-cyclic phosphate termini and was capable of ligating these termini intramolecularly or intermolecularly.

To date, two RNA ligase activities have been biochemically identified in mammalian cell extracts (17, 18). Both are competent for the ligation step of tRNA splicing, an essential eukaryotic process (19) that removes introns from certain precursor tRNAs. During the reaction, an intron-containing precursor tRNA is cleaved by an endonuclease that removes the intron sequence, leaving behind two tRNA half-molecules. The 5′ half-molecule bears a 2′,3′-cyclic phosphate terminus, and the 3′ half-molecule bears a 5′ hydroxyl terminus. In a series of processing steps, the two half-molecules are ultimately ligated by an RNA ligase (16, 20).

The two mammalian RNA ligase activities are mechanistically different. The first activity uses the phosphate from the 2′,3′-cyclic phosphodiester bond to form the 3′-5′ phosphodiester linkage at the ligation junction (21, 22). Although it has been shown to ligate polyribonucleotides *in vitro*, very little is known about this activity *in vivo* and, for instance, whether it is able to join non-tRNA substrates. The second mammalian RNA ligase activity incorporates an exogenous phosphate (obtained from a nucleotide triphosphate) at the junction and creates a 2′ phosphomonoester, 3′–5′ phosphodiester linkage intermediate. A separate 2′ phosphatase activity later removes the 2′ phosphate moiety.

The second mammalian ligase activity is functionally analogous to that possessed by the only RNA ligase described in yeast. The yeast tRNA ligase can ligate substrates other than tRNAs and has been shown to ligate HAC1 mRNA, a messenger RNA involved in regulation of the unfolded protein response (23, 24).

The second mammalian ligase is also functionally analogous to an RNA ligase partially purified from wheat germ extract (25). This activity can ligate and circularize viroid RNAs (4). Like HDV, viroids are small circular RNAs that are thought to adopt an unbranched rodlike structure and replicate via a rolling circle mechanism. Because it remains possible that one or both mammalian tRNA ligase activities are able to ligate non-tRNA substrates, each remains a candidate for participation in HDV RNA ligation. Perhaps HDV has subverted the substrate promiscuity of one or both activities to replicate its genome.

The observation that heterologous ribozyme-generated RNAs are efficiently ligated to circular forms may have implications in the development of antisense technologies. The increased stability of ligated circular RNA would provide a greater half-life for RNAs directed against cellular targets, increasing the probability of their effectiveness (26). Furthermore, there is some evidence that antisense RNA can have a greater inhibitory effect when delivered to the nucleus (27). In general, RNAs whose 3′ end is specified by ribozyme cleavage rather than polyadenylation are retained in the nucleus (28). Hence, by flanking an antisense or decoy RNA with downstream and upstream-cleaving ribozymes, one may be able to achieve not only greater stability, but also preferred localization of the therapeutic RNA.

When two HDV ribozymes were used to generate the substrate, ligation of the termini formed a U/G junction whereas a U/A junction was formed with the hammerhead construct. A C/G junction is naturally formed upon ligation of HDV antigenomic RNA. Thus, these findings suggest that ligation of cellular RNAs bearing 5′ hydroxyl and 2′,3′-cyclic phosphate termini occurs with little or no sequence requirements with respect to both internal and terminal nucleotides. Identification and further characterization of this RNA ligase activity(s) should aid in our understanding of RNA processing and maturation pathways that operate within mammalian cells.

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