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Reversible Cleavage and Ligation of Hepatitis Delta Virus RNA

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A 148-nucleotide subfragment of hepatitis delta virus RNA was shown to undergo cleavage and ligation reversibly. The direction of the reaction is determined by the presence or absence of Mg^{2+} ions, with the presence of Mg^{2+} favoring the cleavage reaction. Ligation requires specific conformation of the RNA molecules involved and occurs only between two cleaved RNA fragments that are still held together by hydrogen bonds. The ligation reaction occurs rapidly on removal of Mg^{2+} by EDTA. This represents a new class of RNA enzymes.

HEPATITIS DELTA VIRUS (HDV) IS a defective virus often associated with fulminant hepatitis in humans (*1–3*). HDV infection is endemic in many parts of the world and is prevalent among intravenous drug users in the United States. The virus contains a single-stranded circular RNA genome of 1.7 kilobases (*4–6*) and has properties similar to those of plant viroid RNA. The mechanism of HDV RNA replication is still not clear. It has been suggested that HDV RNA replicates by a rolling circle mechanism similar to that of viroid RNA (*7*), since RNA intermediates that are larger than genome length have been detected in the hepatocytes of HDV-infected chimpanzees (*8, 9*). The demonstration that the antigenomic RNA of HDV can cleave itself (*10*) is consistent with a rolling circle mechanism of replication. Our laboratory has recently shown that a 133-nucleotide subfragment and several other larger fragments of HDV genomic RNA could be cleaved in the presence of Mg^{2+} (*11*). The cleavage reaction can occur immediately after in vitro transcription of these fragments. The efficiency of RNA cleavage varies with the length of the RNA fragment, suggesting the importance of secondary structure of RNA in self-cleavage. The cleavage reaction occurs very rapidly so that cleavage is complete within seconds after addition of Mg^{2+} at 37°C. We now report an equally rapid reverse reaction with a 148-nucleotide subfragment of HDV genomic RNA. We found that upon removal of Mg^{2+} by addition of EDTA, the two cleaved fragments can be religated covalently. This is the first ribozyme whose cleaving and ligating activities are mediated by a divalent cation. This religation is dependent on the conformation of the RNA fragments involved.

The pT7 plasmid (pHN54) containing the HDV sequence from nucleotide 654 through 801 (*6*) and a T7 RNA polymerase promoter (*11*) was linearized with restriction enzyme Eco RI and used for the synthesis of RNA in vitro by T7 RNA polymerase according to established protocols (*11*). The RNA transcript was heat-denatured and analyzed by polyacrylamide gel electrophoresis (PAGE) under 7*M* urea (lane 1 of Fig. 1A). In agreement with our earlier demonstration that this RNA transcript has cleavage activity (*11*), more than 97% of the transcripts appeared as two small fragments (labeled 3′ and 5′ in Fig. 1), and only a trace amount of RNA has the size of expected primary transcript (1°). Our earlier studies showed that the smaller of the two fragments is the 5′ end cleavage product (*11*). After the transcription products were incubated with 60 m*M* EDTA at 37°C for 10 minutes, almost 25% of the total RNA synthesized appeared as an RNA identical in size to that of the primary transcript (lane 2 of Fig. 1A). Because PAGE was performed under denaturing conditions, this result indicates that at least some of the RNA fragments religated covalently after the addition of EDTA. To rule out the possible participation of various transcription components in the religation reaction, such as DNA template and RNA polymerase, we isolated the religated RNA from the denaturing gel and incubated it in the cleavage buffer (40 m*M* tris-HCl, *p*H 8.1, and 12 m*M* $MgCl_2$) at 37°C for 30 minutes. Almost all of the RNA was cleaved into two smaller fragments (lane 1 of Fig. 1B). When these cleavage products were incubated in 60 m*M* EDTA at 37°C for 10 minutes, 10% to 15% of the cleavage products were converted to have the size of primary transcript (lane 2 of Fig. 1B). This result established that the cleaved products could be religated without extraneous proteins. However, the extent of ligation under this condition was not as high as that achieved in the original transcription buffer. The reason for this difference is not clear. To demonstrate that the RNA fragments were religated correctly, we isolated this religated RNA from the gel (lane 2 of Fig. 1B) and incubated it in the same cleavage buffer as described above. The religated RNA could efficiently cleave itself into two fragments of correct size and ligate itself upon addition of 60 m*M* EDTA (Fig. 1C). This result indicates that the RNA religated properly and kept the correct conformation necessary for cleavage and religation (see below).

These findings suggest that this RNA can cleave itself in the presence of Mg^{2+} and ligate itself when the Mg^{2+} is removed by EDTA. To establish that the effect of EDTA on RNA ligation was through the removal of Mg^{2+}, we performed the cleavage-ligation experiments under different concentrations of Mg^{2+} or EDTA. RNA ligation occurred only at molar concentrations of EDTA that exceeded the molar concentrations of Mg^{2+} used (Table 1). This result indicates that the direction of RNA cleavage or ligation was primarily determined by Mg^{2+} concentration. The ligation reaction occurred rapidly upon the addition of 60 m*M* EDTA, with a half-time ($t_{1/2}$) of less than 1 minute at 37°C.

We then examined the possibility that RNA ligation could occur in trans—that is, the possibility that the cleavage products of different RNA species could ligate to each

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other. We transcribed two RNA species of different length [one, pHN54, contains nucleotides 654 to 801 of HDV RNA, and the other, pHN7-3, contains nucleotides 654 to 820 of HDV RNA and an additional 85 nucleotides of foreign sequences (*11*)]. For each plasmid, one reaction was performed in the presence of ^{32}P-labeled uridine triphosphate ([α-^{32}P]UTP), and the other was performed without labeling. As expected, the ^{32}P-labeled transcripts of both plasmids were cleaved into two smaller fragments (lanes 1 and 3 of Fig. 2), although the efficiency of cleavage of pHN7-3 RNA was lower than that of pHN54 RNA. Both RNAs also religated upon addition of 60 m*M* EDTA after transcription and subsequent cleavage (lanes 2 and 4 of Fig. 2). We then mixed ^{32}P-labeled transcription products from one of the plasmids with unlabeled transcription products from the other plasmid and added 60 m*M* EDTA to the mixtures to initiate ligation. If trans-ligation occurred, two ^{32}P-labeled religated RNA species of different length would be detected. However, in both reactions, only one species of labeled ligated RNA was observed (lanes 8 and 10 of Fig. 2). In a control experiment, both transcription products were ^{32}P-labeled, mixed, and then incubated with EDTA; two religated RNAs were detected (lane 6 of Fig. 2). We have also mixed a tenfold excess of unlabeled transcription mixture from one of the plasmids with the other ^{32}P-labeled RNA. Only one religated RNA species was detected after the addition of EDTA (*12*). Therefore, we concluded that trans-ligation did not occur under these conditions. This result suggests that ligation could take place only between the two cleaved RNA fragments that were still associated in a complex after cleavage. This complex presumably would gradually dissociate into separate RNA fragments, which no longer have the correct conformation necessary for ligation. This interpretation is consistent with the finding that the two RNA cleavage products isolated from the denaturing or nondenaturing gels could not undergo ligation (*12*).

To prove this ligation mechanism, we performed in vitro transcription of pHN54 at 37°C under the typical conditions described above. After transcription, the reaction mixture was gradually heated to various temperatures. At each designated temperature a portion of the reaction mixture was removed; 60 m*M* EDTA was added to the removed portion, which was then incubated for an additional 10 minutes at 37°C. The reaction products were heat-denatured and analyzed by PAGE under 7*M* urea. The religated RNA could be detected only in samples that had been incubated at temperatures below 60°C (Fig. 3A). There was a dramatic decrease of the ligation efficiency between 50°C and 60°C.

We then performed a similar experiment except that no EDTA was added to the transcription products. Instead, portions of the transcription-cleavage products were removed at different temperatures and analyzed directly by PAGE under nondenaturing conditions. In addition to the two cleav-

Table 1. The effect of EDTA concentration on religation. The ^{32}P-labeled primary transcript of pHN54 was eluted from the gel (Fig. 1A) and incubated in cleavage buffer consisting of 40 m*M* tris-HCl, *p*H 8.1, and varying concentrations of Mg^{2+} as indicated. After incubation at 37°C for 30 minutes, various amounts of EDTA as indicated were added to the reaction mixtures. After further incubation for 10 minutes, the products were analyzed by PAGE under 7*M* urea. The total amounts of religated RNA were determined by scintillation counting and calculated as the percentage of total RNA transcripts. The background counts for the uncleaved RNA were subtracted.

EDTA (m*M*)	Religation (%) when Mg^{2+} in cleavage reaction is		
	7.2 m*M*	4.8 m*M*	2.4 m*M*
0	0	0	0
1.5	0	0	0
3.0	0	0	0
6.0	0	14	10
12.0	13	13	11
24.0	15	15	13
60.0	16	14	16

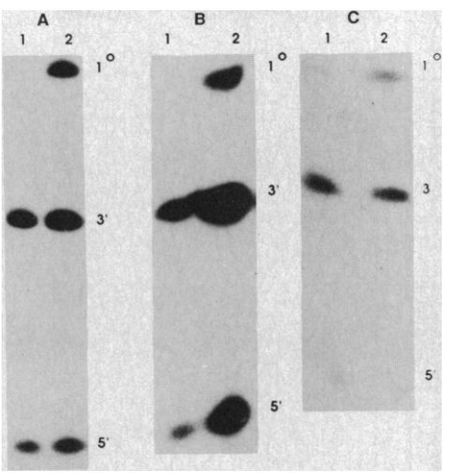


Fig. 1 (left). PAGE of RNA products of cleavage and ligation. The pHN54 plasmid DNA (0.5 μg) was linearized with restriction enzyme Eco RI and then incubated at 37°C for 1 hour in 30 μl of transcription buffer [40 m*M* tris-HCl, *p*H 8.1, 12 m*M* $MgCl_2$, 1 m*M* spermidine, 5 m*M* dithiothreitol, and bovine serum albumin (0.1 mg/ml)] containing 0.9 m*M* each of NTP (Sigma), including 0.1 mCi of [α-^{32}P]UTP, 30 units of ribonuclease inhibitor RNasin (Promega), and 15 units of T7 RNA polymerase (Amersham or Boehringer Mannheim). (**A**) After transcription, 10 μl of ^{32}P-labeled transcription mixture was mixed with 10 μl of 7*M* urea and heat-denatured at 100°C for 2 minutes before electrophoresis on 6% polyacrylamide gels containing 7*M* urea (lane 1). The rest of the transcription mixture was adjusted to a final concentration of 60 m*M* EDTA and incubated for an additional 10 minutes. The latter sample was then adjusted to 7*M* urea, heat-denatured, and analyzed as in lane 1 (lane 2). After electrophoresis, the gel was autoradiographed. (**B**) The largest RNA band in lane 2 of (A) was eluted from the gel by crushing and soaking in 0.3*M* sodium acetate and 20 m*M* tris-HCl, *p*H 8.0, at 4°C overnight. The eluted RNA was precipitated with ethanol and then redissolved in 30 μl of cleavage buffer (40 m*M* tris-HCl, *p*H 8.1, and 12 m*M* $MgCl_2$) and incubated at 37°C for 30 minutes. After incubation, one-tenth of the sample was directly resuspended in 7*M* urea and the rest was adjusted to 60 m*M* EDTA and incubated for an additional 10 minutes before being resuspended in 7*M* urea. Both samples were heat-denatured and analyzed by PAGE under 7*M* urea as in (A). (**C**) Similar to (B) except that the original RNA sample was derived from the 1° RNA in lane 2 of (B), and the same amounts of radioactivity were applied to both lanes. The symbol 1° represents the primary transcript or the religated product; 3′ and 5′ are the two cleavage products of the primary transcript.

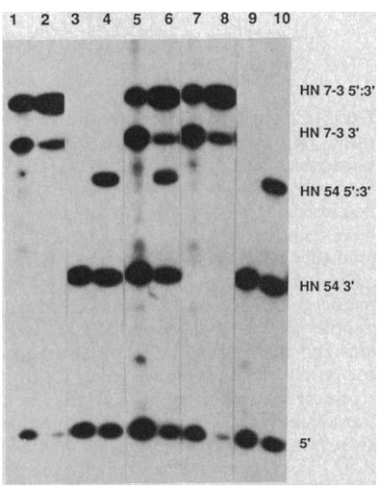
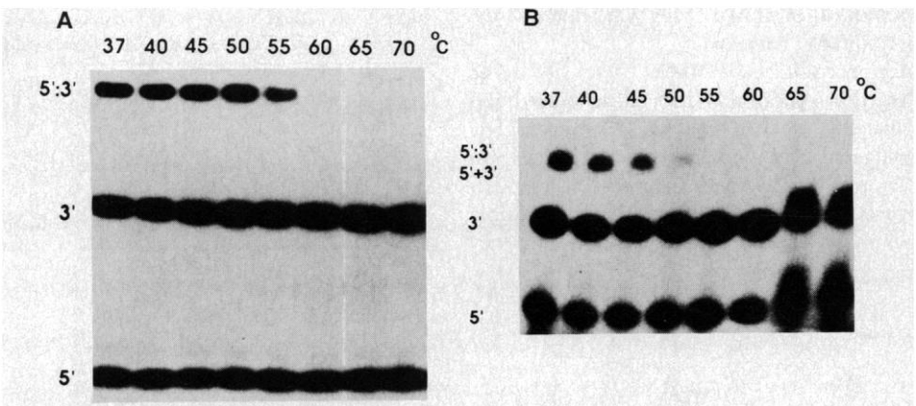


Fig. 2 (right). Demonstration of lack of trans-ligation. All of the transcription reactions were performed as in Fig. 1 in the presence or absence of [α-^{32}P]UTP. The transcription products were analyzed by PAGE under 7*M* urea. (Lane 1) ^{32}P-Labeled transcription products of pHN7-3; (lane 2) same as lane 1 but incubated with 60 m*M* EDTA at 37°C for 10 minutes after transcription; (lane 3) ^{32}P-labeled transcription products of pHN54; (lane 4) same as lane 3 but with 60 m*M* EDTA; (lane 5) mixture of ^{32}P-labeled transcription products of pHN7-3 and pHN54; (lane 6) same as lane 5 but with 60 m*M* EDTA; (lane 7) mixture of ^{32}P-labeled pHN7-3 transcription products and unlabeled pHN54 transcription products; (lane 8) same as lane 7 but with 60 m*M* EDTA; (lane 9) mixture of ^{32}P-labeled pHN54 transcription products and unlabeled pHN7-3 transcription products; (lane 10) same as lane 9 but with 60 m*M* EDTA.

age products, an RNA species similar in size to that of the primary transcript was detected in samples that had been incubated at temperatures lower than 60°C (Fig. 3B). The largest RNA species was probably formed by cleaved RNA products that were still associated by hydrogen bonds in a complex form, as evidenced by the finding that this RNA was not detectable after RNA was denatured (see Fig. 1). The amounts of RNA present in this complex form after cleavage and the amounts of RNA religated after addition of EDTA at different temperatures were determined by measuring the radioactivity of each RNA band. The amounts of RNA that religated paralleled closely the amounts of RNA present in the complex form at various temperatures (Fig. 3C). This result suggests that a fraction (approximately 25% at 37°C) of cleaved RNA fragments existed as a complex form after cleavage, and it is the RNA fragments in this complex that could undergo religation. This complex was disrupted at 60°C.

This study demonstrated that HDV RNA has autocleaving and autoligating activities, that are reversible. The balance between cleavage and ligation is apparently regulated by the concentration of Mg^{2+}. Cleavage could occur at a Mg^{2+} concentration as low as 500 μM (*11*), but the complete removal of Mg^{2+} resulted in religation. This reversible cleavage and ligation is a new property among the ribozymes. For instance, the satellite RNA of tobacco ringspot virus undergoes cleavage and ligation under the same Mg^{2+}-containing buffer (*13*). The self-cleaving ribozyme of *Tetrahymena* pre-ribosomal RNA also undergoes cleavage and polymerase reactions in a similar buffer that contains Mg^{2+} (*14, 15*). The direction of reaction in these cases is apparently determined by the concentrations of substrates rather than Mg^{2+} concentrations. Furthermore, the minimum Mg^{2+} concentration required for HDV RNA cleavage is lower than that for other ribozymes, and the rates of both its cleavage and ligation are faster than those of other ribozymes (*11*). Thus, HDV RNA represents a new class of ribozyme. Both cleavage and ligation activities are apparently required for the rolling-circle replication of HDV RNA in vivo (*7*). However, it is not clear whether the large RNA intermediates of HDV are cleaved and ligated by the same mechanism as the small RNA subfragments we observed.

It is interesting that only the RNA fragments in a complex, which is probably formed by hydrogen bonds disruptable by heating at 60°C, could undergo ligation. This finding places some constraints on the ability of RNA to ligate. However, if HDV RNA replicates by a rolling circle mechanism, the intermediate of the HDV RNA replication could be multimeric RNA. The cleavage of this RNA intermediate could be a monomeric RNA that contains the two ligatable ends in the same molecule. As a result, the two ends could be held in proximity by the secondary structure of the RNA molecule. Therefore, theoretically, the RNA cleavage products could undergo ligation under proper conditions. The products of such ligation reaction would be a circular RNA equivalent to the RNA genome of HDV. The optimum conformation required for cleavage and ligation may not be the most stable form of RNA. Conceivably, the secondary structure of the RNA in infected cells may be maintained by the binding of hepatitis delta antigen, which has an RNA-binding activity (*16*). It will be interesting to study the role of the delta antigen in HDV RNA cleavage and ligation.



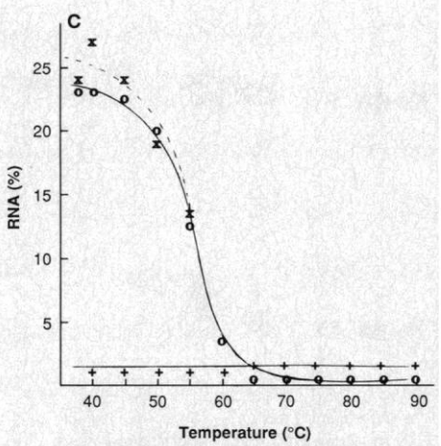


Fig. 3. Cleavage and ligation at different temperatures. (**A**) The Eco RI–linearized pHN54 was transcribed by T7 RNA polymerase in the presence of [α-^{32}P]UTP under the same conditions as described in Fig. 1. The transcription products were then incubated in a heat block, which was gradually heated (from 37°C to 95°C). At designated temperatures, a portion of sample was removed and 60 m*M* EDTA was added. These samples were heat-denatured at 100°C for 2 minutes and then separated by electrophoresis on a 6% polyacrylamide gel containing 7*M* urea; 5′:3′ represents the religated RNA. (**B**) The pHN54 transcription products were treated the same way as in (A), except that no EDTA was added and RNA was not heat denatured. The samples collected at different temperatures were mixed with 5% glycerol and then applied to a 6% polyacrylamide gel without urea. Electrophoresis was conducted at 4°C in 50 m*M* tris-acetate, *p*H 7. The symbols 5′:3′ and 5′+3′ represent the uncleaved primary transcript and the cleaved products in the complex form, respectively. (**C**) The amounts of (o) religated products, (x) cleavage products in the complex form, and (+) uncleaved primary transcripts were converted to the percentages of total RNA in each sample and are plotted as a function of temperature. Data of religated products and uncleaved primary transcripts in the complex form were obtained from the results of (A) and (B), respectively. The radioactivity of each RNA band was determined by scintillation counting and normalized to the relative concentration according to the number of moles of U residues in each RNA. The residual amounts of uncleaved primary transcripts, which were determined from the same samples as in (B) but run on a denaturing gel containing 7*M* urea (*12*), were subtracted from both the amounts of religated products and the amounts of cleavage products in the complex form.

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