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Genetics

Efficient trans cleavage and a common structural motif for the ribozymes of the human hepatitis δ agent

(RNA structure/RNA therapeutics)

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ABSTRACT **Cis-active ribozymes are potential therapeutic agents; however, to be used in this capacity, they must first be converted to trans-active ribozymes, a process facilitated by analysis of their structures. We present evidence that the genomic and antigenomic ribozymes of the human δ hepatitis agent share a structural ("axehead") motif that has conserved sequence elements and a stable hairpin. Guided by the features of the axehead, we divided each of the δ ribozymes into two subdomains, which we synthesized as separate RNA transcripts to give an enzyme and substrate for each ribozyme. Incubation of a substrate subdomain with its matching enzyme resulted in efficient and accurate trans cleavage. This work forms the basis for kinetic studies and for adapting the δ ribozymes for cleavage of selected target RNAs.**

The human δ hepatitis agent has a small covalently closed circular genomic RNA (1, 2). Like the RNA of other circular subviral pathogens, such as viroids and some plant viral satellite RNAs, δ RNA is thought to be copied into longer-than-unit-length complementary strands by an RNA-to-RNA rolling circle pathway (3, 4). *In vitro* studies have shown that certain forms of both genomic and antigenomic δ RNA are capable of self-cleavage (5–7), suggesting how δ precursor RNAs may be processed during their replication cycle.

Self-cleavage is a property of many viroid-like infectious RNAs (8). These cleavage reactions all produce 2′,3′-cyclic phosphate termini. "Hammerhead" structures were the first of the ribozyme motifs present in small infectious RNAs to be detected (9, 13) and defined (10). A second distinct structure, referred to as either a "hairpin" or a "paperclip," has also been described (11, 12, §). The δ ribozymes differ from each of these other two ribozyme subtypes. With the delineation of the consensus hammerhead model, experiments soon revealed that hammerhead structures, which are natural cis-acting ribozymes, can be converted into trans-acting enzymes (14). The hairpin/paperclip ribozyme motif has been similarly engineered to carry out trans cleavage (11, 12).

Work from a number of laboratories has succeeded in defining the approximate boundaries of the δ self-cleavage structures (6, 15, 16), and models for the genomic and antigenomic ribozymes have been proposed (6, 16, 17). However, few experiments have been carried out to determine how the δ self-cleavage structure might be separated into two subdomains: a "substrate" containing the site of cleavage and an "enzyme" composed of sequences needed to obtain efficient trans cleavage of the first RNA. This separation is a prerequisite for both kinetic studies of enzyme–substrate interactions of the δ ribozymes and for the development of RNA therapeutic agents based on these ribozymes.

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In this communication, we present a common structural motif for the genomic and antigenomic ribozymes of δ RNA. This motif incorporates information from partial nuclease digestion studies of the genomic RNA, emphasizes the strong primary sequence homologies of the δ genomic and antigenomic ribozymes, and has led directly to experiments revealing that both of the δ ribozymes can be divided into enzyme and substrate components capable of high-efficiency trans cleavage under physiological conditions *in vitro*. While further experiments are needed to determine the kinetic constants of the trans cleavage reactions, preliminary experiments demonstrate that readily detectable turnover takes place.

MATERIALS AND METHODS

RNA transcripts were synthesized in 1.5-ml reaction mixtures from synthetic DNA templates using α-^{32}P-labeled GTP according to Milligan *et al.* (18) and purified as before (19, 20). The genomic substrate was transcribed from the DNA sequence 3′-CGGACTACCGGCCGTACCAGGGTCGGAGG-AGCGACCGCGGCCGACCCGTTGTAAGGCTCCCC-TGG-5′; the genomic enzyme, from the DNA sequence 3′-CCAGGGGAGCCATTACCGCTTACCCG-5′; the antigenomic substrate, from the DNA sequence 3′-CGGAGAAGC-CCAGCCGTACCGTAGAGGTGGAGGAGCGCCAGGC-TGGACCCGTAGGCTTCCTCCTGCG-5′; and the antigenomic enzyme, from the DNA sequence 3′-CTGCA-GGTGAGCCTACCGATTCCCTCG-5′. The residue numbers of the δ genomic and antigenomic sequences encoded by these templates are given in the legends to Figs. 3 and 5, respectively. Trans cleavage was assayed by using conditions also provided in these figure legends. Cleavage sites were mapped by RNase T1 fingerprinting (20) and RNA secondary analysis (21).

RESULTS

The striking primary sequence homology between the δ genomic and antigenomic self-cleavage domains (compare *B* and *C* in Fig. 1) suggested that they share a common structure; however, in isolation, the sequence homology did not provide enough constraints to specify a single common motif. Direct structural information was needed to select among the possibilities. It seemed likely that at least some elements of the δ ribozyme would be present in conformational isomers of δ RNA transcripts, the majority of which fold into the rodlike secondary structure characteristic of viroid RNAs (1, 22). Formation of the cis-active δ self-cleavage structures is a tightly controlled event during the

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§Feldstein, P. A., Buzayan, J. M., van Tol, H. & Bruening, G., Abstracts of Meeting on RNA Processing, May 15–19, 1991, Cold Spring Harbor Lab., Cold Spring Harbor, NY, p. 298.

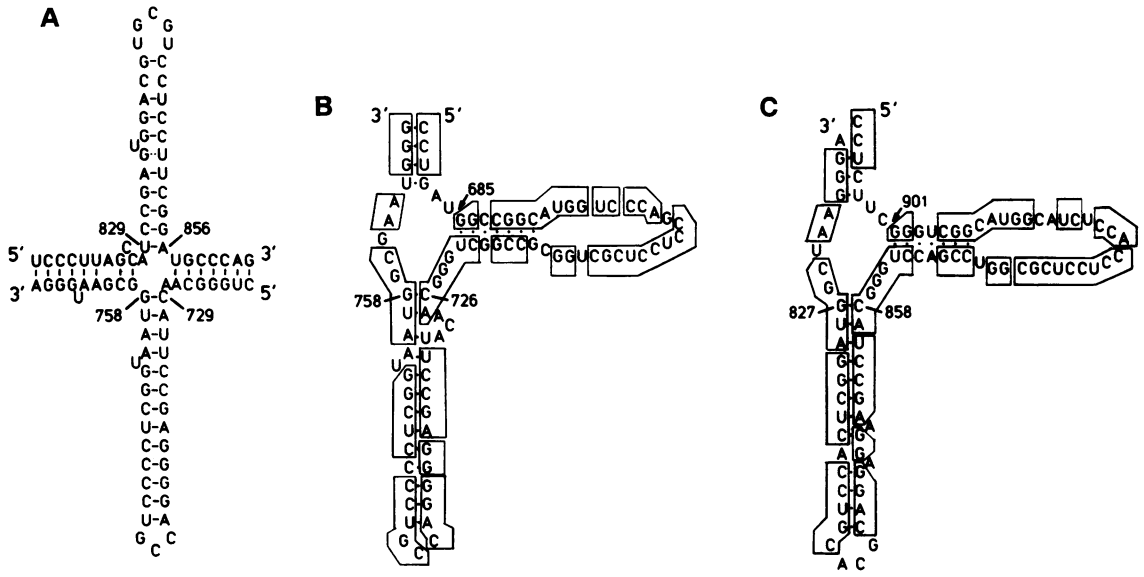

FIG. 1. Common structural axehead motif with conserved sequence elements and a stable hairpin for the δ genomic and antigenomic ribozymes. RNase T1-resistant fragments of δ RNA transcripts suggested the existence of the cruciform-containing conformational isomer shown in *A*. The stem–loop for the genomic ribozyme (*B*) is based on the lower stem–loop of the cruciform; the analogous stem–loop in the antigenomic ribozyme (*C*) is derived from the complement of the upper hairpin of the cruciform. Sequences and numbering are from Wang *et al.* (1). Regions of primary sequence homology between the genomic and antigenomic self-cleavage domains are indicated by boxes. Self-cleavage sites are marked by arrows. In the genomic ribozyme, the arrow identifies the phosphodiester bond between residues 685 and 686. The arrow in the antigenomic ribozyme is between residues 901 and 900.

replication cycle of the δ agent. *In vitro*, this process requires conformational switching if the RNA has been previously folded into the rodlike secondary structure (5, 8). We previously reported that certain portions of genomic δ RNA transcripts survive treatment with RNase T1 (1 mg/ml) at 37°C in a low salt buffer (23). We subsequently found that, although most of the RNase T1 partial digestion products are derived from molecules in the rodlike conformation, a stem–loop element (residues 729–758) was detected which is not compatible with the rodlike structure, but was compatible with the cruciform structure shown in Fig. 1*A* (A.D.B., H.D.R., and Bonnie J. Levine, unpublished results). The 5′ end of the stem–loop element is only about 45 bases away from the genomic self-cleavage site. In the cruciform conformation, this stem–loop structure lies opposite the template for a similar stem–loop structure whose 5′ end would be located about 45 residues away from the antigenomic self-cleavage site. We concluded that the cruciform structure—particularly residues 729–758, which are present in cis-active genomic ribozymes (6, 15–17)—could represent a transition state in which an active ribozyme structure was forming. The analogous cruciform structure could arise during the rearrangement of antigenomic RNAs in preparation for cis cleavage of these molecules.

Primary sequence homologies were combined with evidence of stable stem–loop elements (obtained through studies of nuclease-resistant regions in genomic transcripts) to give the models of the δ genomic and antigenomic self-cleavage structures shown in Fig. 1. In the resulting "axehead" motif, homologous sequence elements from genomic and antigenomic RNAs (boxed regions) are placed in the same relative positions in the structures, even at the expense of base pairs that could otherwise be drawn in one of them. This approach does not produce the maximum number of base pairs and is one reason why the structures we present differ from other models of the δ ribozymes. A prominent stem–loop appears in several previous models of the genomic self-cleavage structure; however, the one in our model is somewhat shorter than the analogous feature in the model of Wu *et al.* (6) and begins at a slightly different point in the RNA sequence than in models of Perrotta and Been (16) and Belinsky and Dinter-Gottlieb (17), to permit a consistent overall structure for the genomic and antigenomic ribozymes to be developed.

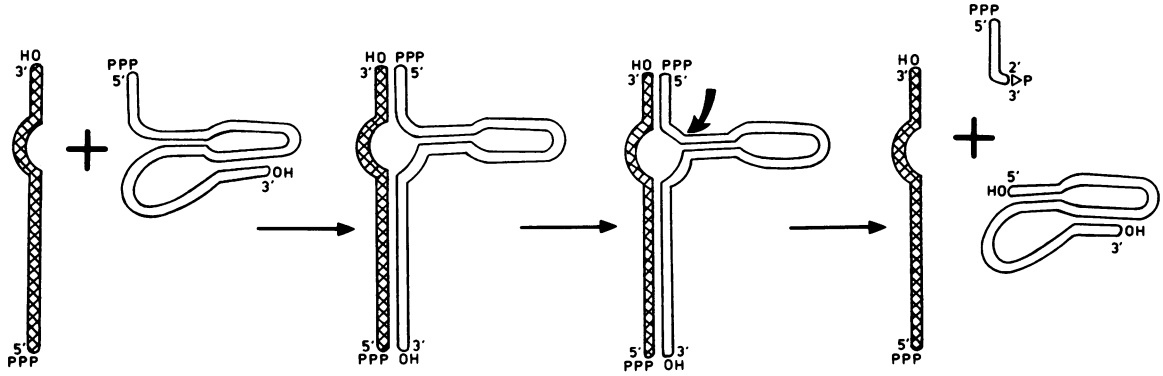

FIG. 2. Diagram of a δ trans cleavage reaction. A mixture of enzyme (crosshatched structures) and substrate (open structures) transcripts (with 5′ triphosphate and 3′ hydroxyl termini; first drawing) form an axehead structure composed of two RNA molecules (second drawing). Correct folding leads to cleavage (indicated by the arrow in the third drawing) and may be followed by release of the two products (fourth drawing). The sequences of the DNA templates for all transcripts are given in *Materials and Methods*.

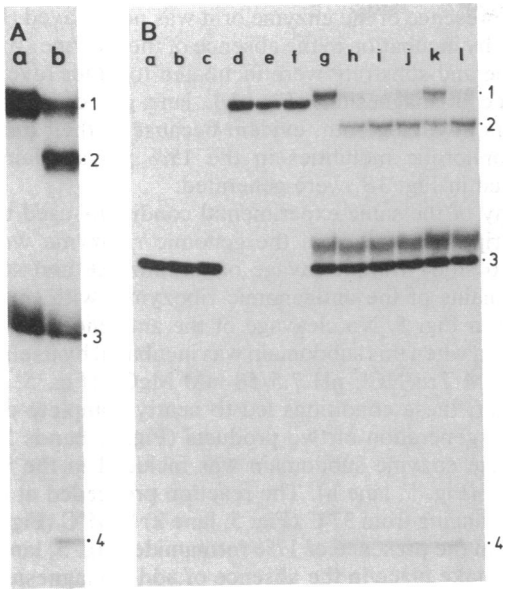

FIG. 3. Trans cleavage of a 65-base-long transcript with the sequence of δ genomic RNA. (*A*) A substrate oligonucleotide (band 1, residues 680–743) was mixed with a 26-base-long transcript (band 3, residues 744–767) and incubated for 60 min at 60°C in either EDTA (lane a) or $MgCl_2$ (lane b) prior to electrophoresis in a 10% polyacrylamide/7 M urea gel. Two cleavage products (bands 2 and 4) were generated by incubation in $MgCl_2$. (*B*) Properties of the δ trans cleavage reaction were analyzed further by carrying out a series of 60-min incubations followed by electrophoresis in a 15% polyacrylamide/7 M urea gel. The 26-base-long RNA was either incubated at 50°C in 50 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$ (lane a), incubated at 50°C in 20 mM Tris·HCl, pH 7.4/2 mM EDTA (lane b), or stored on ice (lane c). The 65-base-long substrate RNA was either stored on ice (lane d), treated at 50°C in 50 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$ (lane e), or treated at 50°C in 20 mM Tris·HCl, pH 7.4/2 mM EDTA (lane f). The substrate RNA and the 26-base-long RNA were mixed and either incubated in 20 mM Tris·HCl, pH 7.4/2 mM EDTA at 50°C (lane g), in 50 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$ at 50°C (lane h), in 50 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$ at 70°C (lane i), in 17% formamide/25 mM Tris·HCl, pH 7.5/50 mM $MgCl_2$ at 50°C (lane j), in 50 mM Tris·HCl, pH 7.5/50 mM $MgCl_2$ at 37°C (lane k), or in 50 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$ at 60°C (lane l). All samples were heated for 3 min at 90°C in a formamide/dye mixture containing 50 mM Tris·HCl, pH 7.5/50 mM EDTA prior to electrophoresis.

Whether the prominent stem–loop in the axehead motif is part of a transitional form or part of the actual cis-acting ribozyme, its extensive complementary base pairing suggested that, if a δ ribozyme were divided into two portions by opening it up in the terminal loop region, the two halves would subsequently associate and effect trans cleavage. The Milligan method (18) was used to produce pairs of *in vitro*-synthesized RNA transcripts with enzyme and substrate subdomains derived from each of the δ genomic and antigenomic ribozymes. The 5′ end of both enzyme subdomains contained sequences from the terminal loop region; the 5′ end of both substrate RNAs began upstream of the cleavage site and extended to the bottom of the terminal loop region. A diagram of a hypothetical trans cleavage reaction involving these transcripts appears in Fig. 2: a pair of enzyme and substrate subdomain transcripts are mixed and interact with each other, leading to cleavage of the substrate at the proper phosphodiester bond and dissociation of the products from the enzyme.

To test for trans cleavage experimentally, a 26-base-long transcript containing genomic residues 744–767 was mixed with a 65-base-long genomic substrate (containing residues 680–743) and incubated in Tris buffer with either EDTA or $MgCl_2$. In the presence of $MgCl_2$, the majority of the substrate RNA (Fig. 3*A*, band 1) was cleaved to produce two fragments of unequal length (Fig. 3*A*, bands 2 and 4). To identify the sequence content and the termini of the cleavage products, a preparative-scale reaction was carried out. RNA fingerprinting and secondary analysis were then used to characterize gel-purified RNAs.

Three spots in the RNase T1 fingerprint of the substrate RNA (Fig. 4*A*), pppGp, CCUGp, and AUGp, were of particular interest because they represent sequences from the 5′ end of this transcript and span the previously mapped cis cleavage site, the bond between residues uridine 685 and guanosine 686 (6, 15). Secondary analysis of RNA synthesized in the presence of α-^{32}P-labeled GTP confirmed the identity of these three species in the fingerprint of the substrate and revealed that two of them, pppGp and CCUGp, were present in the fingerprint of RNA from band 4 (Fig. 4*C*), indicating that band 4 RNA was derived from the 5′ end of the substrate. The oligonucleotide AUGp was absent from the fingerprint of the 5′ cleavage product and was replaced by a phosphatase-insensitive oligonucleotide that comigrated with AUp following treatment with pancreatic RNase A. These results identify the novel species in the fingerprint of the 5′ cleavage product as AU>p and show that accurate trans cleavage occurred.

Additional experiments were carried out to determine whether the trans cleavage of the genomic substrate could take place under a wide variety of conditions. The reaction proceeded at temperatures ranging from 37°C (Fig. 3*B*, lane k) to 70°C (Fig. 3*B*, lane i), in the presence of 17% formamide (Fig. 3*B*, lane j), and at $MgCl_2$ concentrations from 5 to 50 mM. Under identical experimental conditions (treatment at

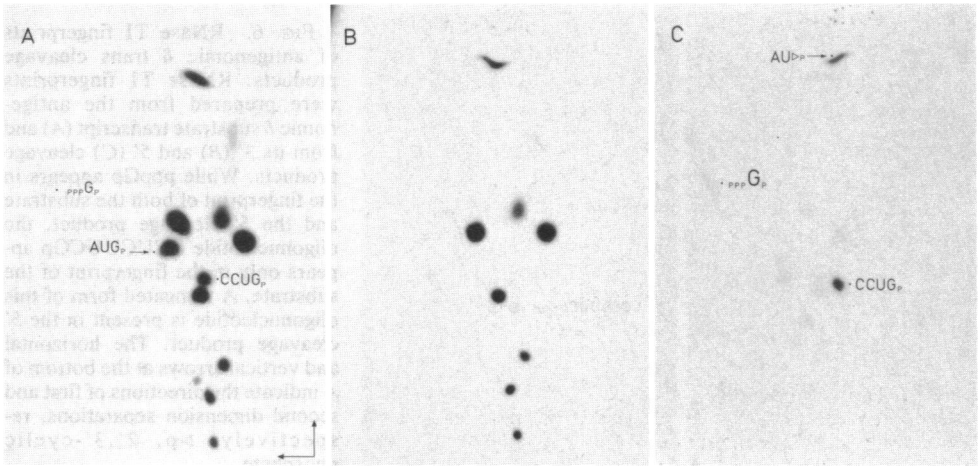

FIG. 4. Mapping of the δ genomic trans cleavage site by RNase T1 fingerprinting. After a preparative-scale cleavage reaction and elution of RNA bands from a polyacrylamide gel, RNase T1 fingerprints were prepared from the untreated 65-base-long substrate RNA (*A*) and from its relatively long 3′ cleavage product (*B*) and its short 5′ cleavage product (*C*). AUGp is only present in the fingerprint of the starting material, but its relative, an AU dinucleotide with a 2′,3′-cyclic phosphate (▷p) terminus, appears in the fingerprint of the 5′ cleavage product. The horizontal and vertical arrows at the bottom of *A* indicate the directions of first and second dimension separations, respectively.

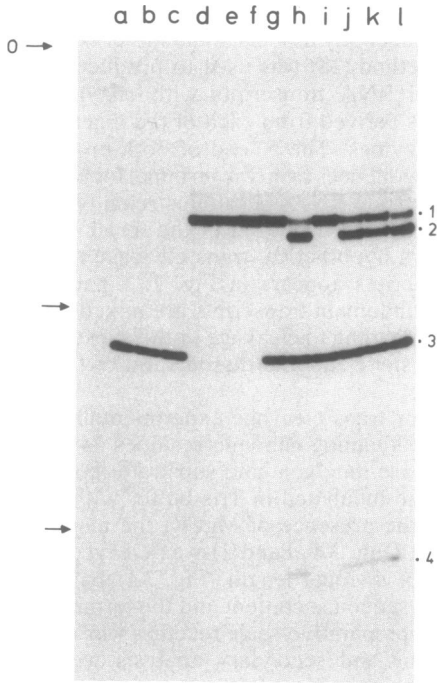

FIG. 5. Trans cleavage of a δ antigenomic RNA transcript. After a series of 60-min incubations, δ antigenomic RNA transcripts were fractionated by electrophoresis in a 15% polyacrylamide/7 M urea gel. A 27-base-long transcript (representing antigenomic residues 841–818) was either treated at 65°C in 50 mM Tris·HCl, pH 7.5/50 mM $MgCl_2$ (lane a), treated at 65°C in 20 mM Tris·HCl, pH 7.4/2 mM EDTA (lane b), or kept on ice (lane c). An antigenomic substrate RNA (residues 907–842) was either kept on ice (lane d), incubated at 65°C in 50 mM Tris·HCl, pH 7.5/50 mM $MgCl_2$ (lane e), or incubated at 65°C in 20 mM Tris·HCl, pH 7.4/2 mM EDTA (lane f). The antigenomic substrate RNA and the 27-base-long transcript were mixed with each other and then incubated in either 20 mM Tris·HCl, pH 7.4/2 mM EDTA at 65°C (lane g), in 50 mM Tris·HCl, pH 7.5/50 mM $MgCl_2$ at 65°C (lane h), in 50 mM Tris·HCl (pH 7.5) at 65°C (lane i), in 17% formamide/25 mM Tris·HCl, pH 7.5/50 mM $MgCl_2$ at 40°C (lane j), or in 50 mM Tris·HCl, pH 7.5/50 mM $MgCl_2$ at 37°C (lane k). A 40-min incubation was carried out in 50 mM Tris·HCl, pH 7.5/50 mM $MgCl_2$ at 50°C (lane l). All samples were heated for 3 min at 90°C in a formamide/dye mixture containing 50 mM Tris and 50 mM EDTA prior to electrophoresis. The arrows (from top to bottom) mark the origin of electrophoresis, the position of xylene cyanol blue, and the site of bromphenol blue. RNA band 1 denotes the antigenomic substrate; band 2, the 3′ cleavage product; band 3, the 27-base-long transcript; band 4, the 5′ cleavage product.

50°C in 50 mM Tris·HCl, pH 7.5/5 mM $MgCl_2$), the substrate was either cleaved efficiently (Fig. 3*B*, lane h) by incubation in the presence of the enzyme or it was not cleaved (Fig. 3*B*, lane e) by incubation in the absence of the enzyme. When the enzyme and substrate were incubated together [even in the absence of magnesium (Fig. 3*B*, lane g)], conformational isomers involving both, evident because of their distinctive electrophoretic mobilities in the 15% polyacrylamide gel depicted in Fig. 3*B*, were generated.

Many of the same experimental conditions used to study transcripts derived from the genomic ribozyme were also used to study trans cleavage of the enzyme and substrate subdomains of the antigenomic ribozyme, with the results shown in Fig. 5. No cleavage of the antigenomic substrate occurred when this subdomain was incubated by itself at 65°C in 50 mM Tris·HCl, pH 7.5/50 mM $MgCl_2$ (Fig. 5, lane e); however, these conditions led to nearly complete cleavage and the generation of two products (Fig. 5, bands 2 and 4) when the enzyme subdomain was included in the reaction mixture (Fig. 5, lane h). The reaction proceeded at temperatures ranging from 37°C (Fig. 5, lane k) to 65°C (Fig. 5, lane h) and in the presence of 17% formamide (Fig. 5, lane j), but did not take place in the absence of added magnesium (Fig. 5, lane i). Conformational isomers analogous to those produced during genomic reactions were not detected.

If trans cleavage of the substrate hydrolyzed the same phosphodiester bond broken during cis cleavage, (*i*) a small RNA (pppGCCUCUUC>p) would be released from the 5′ end of the substrate; (*ii*) the oligonucleotide CCUCUUC>p and pppGp would be present in the RNase T1 fingerprint of the 5′ cleavage product; and (*iii*) the oligonucleotide CCUCUUCGp would be missing from the fingerprints of both cleavage products but present in the fingerprint of the uncleaved substrate. RNA fingerprinting and secondary analysis of RNAs synthesized in the presence of α-^{32}P-labeled GTP were used to evaluate the accuracy of the antigenomic trans cleavage reaction. As shown in Fig. 6, one oligonucleotide (marked by the arrow in *A*) was present in the fingerprint of the substrate but was missing from the fingerprints of both cleavage products (Fig. 6 *B* and *C*). This oligonucleotide, the second slowest in the fingerprint, was sensitive to phosphatase and released Cp and Gp when treated with pancreatic RNase A, characteristics that indicate that it is CCUCUUCGp. The fingerprint of the smaller of the two cleavage products contained pppGp (Fig. 6*C*) and a second oligonucleotide identified as CCUCUUC>p because it (*i*) was phosphatase insensitive, (*ii*) released a novel product (distinct from pG) upon treatment with nuclease P1, and (*iii*) yielded Cp, but not Gp, upon treatment with pancreatic RNase A. These results show that trans cleavage occurred at the same site as cis cleavage.

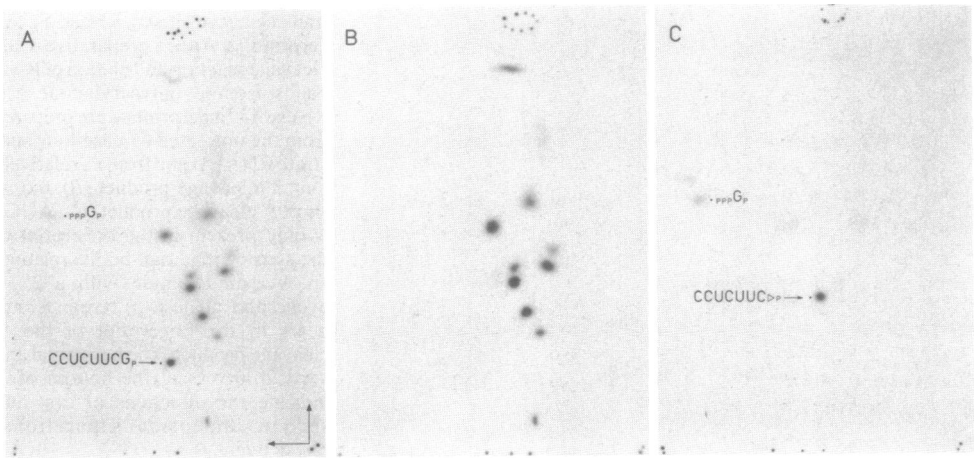

FIG. 6. RNase T1 fingerprints of antigenomic δ trans cleavage products. RNase T1 fingerprints were prepared from the antigenomic δ substrate transcript (*A*) and from its 3′ (*B*) and 5′ (*C*) cleavage products. While pppGp appears in the fingerprint of both the substrate and the 5′ cleavage product, the oligonucleotide CCUCUUCGp appears only in the fingerprint of the substrate. A truncated form of this oligonucleotide is present in the 5′ cleavage product. The horizontal and vertical arrows at the bottom of *A* indicate the directions of first and second dimension separations, respectively. ▷p, 2′,3′-cyclic phosphate.

DISCUSSION

Analysis of RNase T1-resistant fragments of δ genomic RNA and consideration of primary sequence homologies suggested that the self-cleavage domains of the genomic and antigenomic RNAs of the human δ hepatitis agent can be folded into a common structural (axehead) motif. Further experiments are needed to determine whether the prominent stem–loop in the axehead is present in active ribozymes or is part of a transitional form. In either case, it is likely that the stability of the hairpin could help to bring other parts of the self-cleavage domain into alignment. Thus, we opened the δ ribozymes in the loop of this hairpin, dividing each self-cleavage domain into two parts. Enzyme and substrate subdomains were synthesized as separate RNA transcripts, which were later combined to give efficient RNA processing reactions and the correct RNA termini. As might be expected if the axehead motif accurately depicts an important conformation of the δ ribozymes, we also found that the antigenomic enzyme cleaves the genomic substrate RNA with moderate efficiency (unpublished observations). Separation of the δ self-cleavage structure into two active subdomains will make it easier to identify elements of sequence or structure essential for catalytic activity and specificity and to delete portions found to be expendable.

The axehead motif differs in detail from models developed through studies of cis-acting δ ribozymes; however, a number of its broad features have appeared previously. In particular, some form of the major stem–loop structure was present in models of Wu *et al.* (6), Perrotta and Been (16), and Belinsky and Dinter-Gottlieb (17). While the site of trans cleavage is the same as that of cis cleavage, some of the distal sequence elements required under certain experimental conditions for cis cleavage (16, 17) are not present in the transcripts we studied. Thus, the axehead does not depict the recently described pseudoknots (16) because the genomic enzyme subdomain we used lacks two of the six bases needed to form the major pseudoknot helix. In addition, the interaction between segments 658–664 and 764–769 proposed by Belinsky and Dinter-Gottlieb (17) does not appear to be required for trans cleavage. Our results may suggest that certain structural features identified in past studies facilitate a folding process that produces active ribozymes, but these structural features may not themselves be needed for RNA processing reactions. Perrotta and Been (7) have shown that the first five or six residues present in our substrate transcripts are not required for cis cleavage, a result consistent with our preliminary studies of antigenomic transcripts containing only three bases on the 5′ side of the cleavage site. Further kinetic studies will be needed to determine how the efficiency of trans cleavage is affected by potential base pairing between the 5′ end of the substrate and the 3′ end of the enzyme. The potential for such a base-pairing interaction was enhanced in our trans reactions by the addition of residues not present in δ RNA to the 3′ end of the enzyme transcripts (see *Materials and Methods*).

Finally, while the axehead motif for δ ribozymes lacks the particular conserved primary sequence elements characteristic of the hammerhead self-cleavage domain, its simplicity and requirement for stem–loop structures suggest a relationship between the axehead and the hammerhead (10, 14). Since both hammerhead ribozymes and the δ ribozymes produce 2′,3′-cyclic phosphate termini, indicating that they have a common reaction mechanism, the lack of primary sequence homology between them may reflect the evolutionary distance separating viroid-like pathogens of plants and humans.

The ability of genomic and antigenomic δ enzyme subdomains to cleave substrate molecules suggests an approach that could be used to combat δ hepatitis: delivery of active RNA segments to human liver cells may prevent δ RNA replication. The therapeutic use of ribozymes derived from δ RNA may benefit from the natural ability of the cis-active δ ribozymes to function efficiently in the presence of nuclear proteins. In preliminary experiments we have found that addition of a nuclear extract (a kind gift of Umberto Pace, Cornell University Medical College) accelerates the rate of trans cleavage at 30°C. Furthermore, by analogy to the targeted cleavage of selected RNAs by hammerhead ribozymes (24), it should be possible to retarget the δ ribozymes so that they cleave specific RNA sequences in other viral and cellular RNAs.

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1. Wang, K. S., Choo, Q. L., Weiner, A. J., Ou, J. J., Najarian, R. C., Thayer, R. M., Mullenbach, G. T., Denniston, K. J., Gerin, J. L. & Houghton, M. (1986) *Nature (London)* **323,** 508–514.
2. Kos, A., Dijkema, R., Arnberg, A. C., van der Meide, P. H. & Schellekens, H. (1986) *Nature (London)* **323,** 558–560.
3. Branch, A. D. & Robertson, H. D. (1984) *Science* **223,** 450–454.
4. Chen, P. J., Kalpana, G., Goldberg, J., Mason, W., Werner, B., Gerin, J. & Taylor, J. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 8774–8779.
5. Sharmeen, L., Kuo, M. Y. P., Dinter-Gottlieb, G. & Taylor, J. (1988) *J. Virol.* **62,** 2674–2679.
6. Wu, H.-N., Lin, Y.-J., Lin, F.-P., Makino, S., Chang, M.-F. & Lai, M. M. C. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1831–1835.
7. Perrotta, A. T. & Been, M. D. (1990) *Nucleic Acids Res.* **18,** 6821–6827.
8. Branch, A. D., Levine, B. J. & Robertson, H. D. (1990) *Sem. Virol.* **1,** 143–152.
9. Prody, G. A., Bakos, J. T., Buzayan, J. M., Schneider, I. R. & Bruening, G. (1986) *Science* **231,** 1577–1580.
10. Forster, A. C. & Symons, R. H. (1987) *Cell* **49,** 211–220.
11. Feldstein, P. A., Buzayan, J. M., van Tol, H., DeBear, J., Gough, G. R., Gilham, P. T. & Bruening, G. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 2623–2627.
12. Hampel, A., Tritz, R., Hicks, M. & Cruz, P. (1990) *Nucleic Acids Res.* **18,** 299–304.
13. Hutchins, C. J., Rathjen, P. D., Forster, A. C. & Symons, R. H. (1986) *Nucleic Acids Res.* **14,** 3627–3640.
14. Uhlenbeck, O. C. (1987) *Nature (London)* **328,** 596–600.
15. Kuo, M. Y. P., Sharmeen, L., Dinter-Gottlieb, G. & Taylor, J. (1988) *J. Virol.* **62,** 4439–4444.
16. Perrotta, A. T. & Been, M. D. (1991) *Nature (London)* **350,** 434–436.
17. Belinsky, M. G. & Dinter-Gottlieb, G. (1991) *Nucleic Acids Res.* **19,** 559–564.
18. Milligan, J. F., Groebe, D. R., Witherell, G. W. & Uhlenbeck, O. C. (1987) *Nucleic Acids Res.* **15,** 8783–8798.
19. Branch, A. D., Benenfeld, B. J., Paul, C. P. & Robertson, H. D. (1990) *Methods Enzymol.* **180,** 418–442.
20. Branch, A. D., Benenfeld, B. J. & Robertson, H. D. (1990) *Methods Enzymol.* **180,** 130–154.
21. Barrell, B. J. (1971) in *Procedures in Nucleic Acids Research,* eds. Cantoni, G. L. & Davies, D. R. (Harper & Row, New York), Vol. 2, pp. 751–775.
22. Gross, H. J., Domdey, H., Lossow, C., Jank, P., Raba, M., Alberty, H. & Sänger, H. L. (1978) *Nature (London)* **273,** 203–208.
23. Branch, A. D., Benenfeld, B. J., Baroudy, B. M., Wells, F. V., Gerin, J. L. & Robertson, H. D. (1989) *Science* **243,** 649–652.
24. Haseloff, J. & Gerlach, W. L. (1988) *Nature (London)* **334,** 585–591.