An Ultraviolet-Sensitive RNA Structural Element in a Viroid-Like Domain of the Hepatitis Delta Virus

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The RNA genome of the hepatitis delta virus (HDV) appears to be made up of two parts: a small domain with a high degree of sequence conservation and structural features likely to promote replication; plus a second, larger domain that is less conserved and encodes the delta antigen. This report focuses on one of the several sets of data that have led to the proposal of this model: the existence of a novel structural element in HDV genomic RNA. This structural element lies within the highly conserved domain of HDV RNA and may be related to the local tertiary structure previously mapped to the central conserved region of the plant viroid genome. Both elements occur in regions with no apparent coding capacity and are distinctively responsive to ultraviolet (UV) light. Transcripts containing partial and full-length genomic sequences of HDV readily undergo a UV-induced crosslinking reaction, which establishes a covalent bond between two noncontiguous segments. By locking two segments of the overall structure into place, this crosslink has permitted the unbranched, rodlike model of HDV RNA to be examined and confirmed in the portion of the RNA analyzed. The clustering of the novel tertiary structure and the recently discovered self-cleavage sites into a highly conserved, but apparently noncoding, portion of the genome defines a viroid-like domain in HDV RNA and raises questions about the possible events leading up to the association of free-living RNAs with messenger RNAs and other RNA molecules.

THE HEPATITIS DELTA VIRUS (HDV), previously referred to as the delta agent, is a mammalian pathogen bearing a strong similarity to the viroids and related satellite RNAs of plants. Like viroid genomes (*1, 2*), the genome of HDV is a covalently closed RNA circle (*3, 4*) and is smaller than the genome of any conventional virus. Furthermore, unlike defective interfering particles, HDV RNA has no significant sequence homology with its helper virus, hepatitis B virus (*5*), and thus more closely resembles many plant satellite RNAs (*6*). In addition, multimeric forms of both the genomic and antigenomic strands have been reported in tissues replicating HDV (*7–9*). As in the rolling circle replication pathway first proposed for the small infectious RNAs of plants (*10*), these multimeric HDV precursor RNAs are thought to be cleaved and ligated to yield progeny circles (*9*). Finally, a rodlike secondary structure has been drawn for HDV RNA (*3*), which is very much like the one that has emerged from detailed studies of plant viroid sequence and structure (*6, 11*). We report evidence placing an ultraviolet (UV)-sensitive site within transcripts of HDV RNA; these data provide experimental support for the proposed secondary structure of HDV RNA. A similar UV-sensitive site was mapped in potato spindle tuber viroid RNA (*12*) and is being used to define the structure of the viroid central conserved region. Our results with UV-induced crosslinking of HDV RNA constitute one feature of several that have helped us to identify a viroid-like domain in the genomic RNA of this pathogenic agent (*13*).

To obtain material for structural analysis, RNA of the genomic polarity was transcribed in vitro from a full-length cDNA clone of HDV RNA and several partial clones. The full-length clone begins at residue 1200 and terminates at residue 1199, according to the numbering scheme of the circular map of HDV genomic RNA (*3*); a second cDNA clone (1537 bases long) contains residues 1110 to 967; two smaller cDNA clones have the same 5′ terminal base, residue 482, but extend to different positions, residue 964 or 1109 (*14*).

For initial screening, transcripts of these cDNA clones were exposed to UV light for 90 s, under conditions previously used to crosslink UV-sensitive tertiary elements in viroids and eukaryotic 5*S* ribosomal RNA (rRNA) (*12, 15*). Both irradiated and control delta transcripts were digested at 37°C with ribonuclease T1 (RNase T1). The products were fractionated by gel electrophoresis to seek a UV-dependent band that might contain a photo-induced crosslink, which would signal the presence of a novel tertiary structure in the genomic strand of HDV RNA. A prominent band was present in UV-irradiated samples of both genome-length RNA and smaller partial transcripts (Fig. 1). This RNase T1 digestion method permits even large RNAs to be analyzed. Because their shorter length would allow two-dimensional gel electrophoresis to be used as a purification step for intact crosslinked forms, transcripts of the two partial

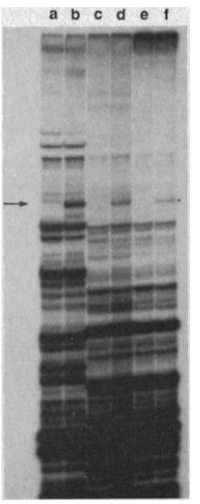

Fig. 1. Gel electrophoresis of an RNase T1–resistant product from UV-treated, genome-length transcripts of HDV. Control (lanes a, c, and e) and UV-treated (lanes b, d, and f) transcripts of partial (lanes a and b) or full-length (lanes c to f) HDV cDNA clones were digested with 2 μl of RNase T1 (1 mg/ml) in the presence of 10 μg of transfer RNA for 45 min at 37°C, and then the products were fractionated in a 20% ultrathin polyacrylamide gel containing 7*M* urea. An arrow identifies a partial digestion product present only in the UV-treated samples. Radioactive hieroglyphs were used to guide gel band excision.

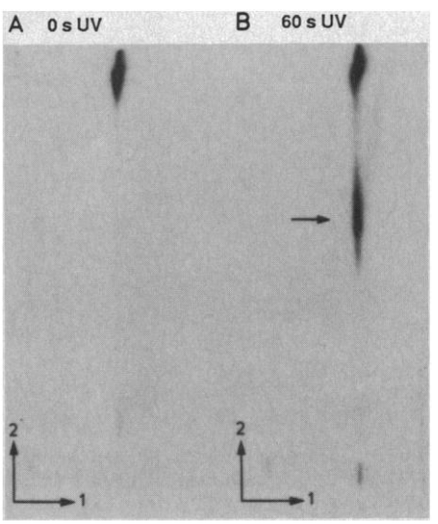

Fig. 2. Resolution of a crosslinked form of delta RNA by two-dimensional gel electrophoresis. Samples of genomic strand transcripts, prepared from a cDNA clone, which contained bases 482 to 1109 and had the same sequence as the variant reported by Wang *et al.* (*3*), were moved by electrophoresis into a 4% nondenaturing polyacrylamide gel (*32*). The gel was sliced into individual lanes, which were chilled on ice, and then either maintained as controls (**A**) or irradiated with UV light for 60 s (**B**), as described (*15*). Gel lanes were cast into individual 3.5% polyacrylamide gels containing 7*M* urea and the RNAs were fractionated by electrophoresis into the second gel in order to separate crosslinked (marked by arrow) from noncrosslinked forms. The pair of horizontal and vertical arrows indicate the directions of first and second dimension separation, respectively.

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clones mentioned above were used for further investigations.

In past studies, two-dimensional gel electrophoresis has been used to detect UV-induced crosslinks and to separate molecules containing an RNA-RNA crosslink from the remaining population. When this fractionation procedure is used, the UV irradiation is often carried out with the RNA immobilized in a polyacrylamide gel. In this configuration, overheating can be more easily avoided than when irradiation is carried out in a drop. To apply this technique to studies of HDV RNA, transcripts were first moved by electrophoresis into nondenaturing gels, and then individual gel lanes were exposed to UV light. After irradiation, the gel slices were cast into polyacrylamide gels containing urea, and samples were fractionated in the second dimension. A slowly migrating form of the delta transcript (Fig. 2, arrow) separated from the control molecules. This result indicates that the crosslink induced by UV treatment had altered the overall geometry of the RNA, connecting two noncontiguous segments of the genomic transcript and thus creating a circular component that conferred slow mobility in the second dimension urea gel.

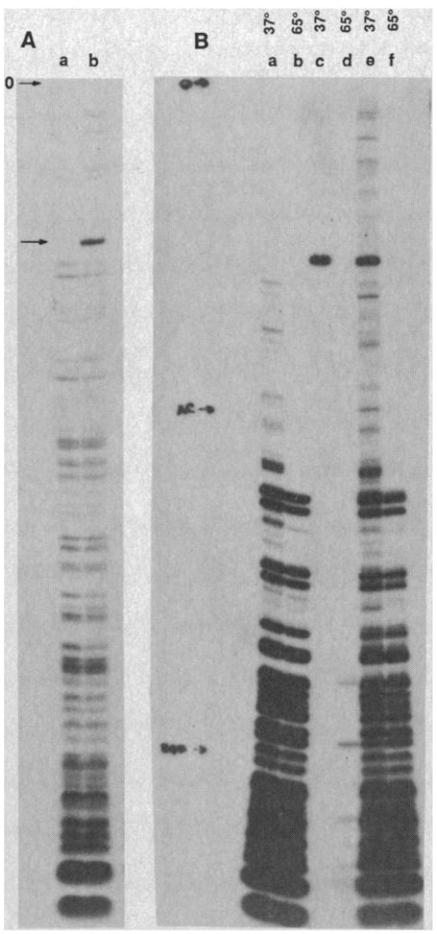

Fig. 3. Gel electrophoresis of an RNase T1–resistant species from crosslinked delta RNA. (**A**) A partial digestion product present only in samples of crosslinked delta RNA (arrow) was detected when samples of control (lane a) or crosslinked (lane b) transcripts (residues 482 to 1109) were digested for 45 min at 37°C under standard conditions [in a 2-μl volume of RNase T1 (1 mg/ml); 0.001*M* EDTA; 0.01*M* tris-HCl, *p*H 7.6; and 10 μg of tRNA] and then analyzed by electrophoresis in a 20% polyacrylamide gel containing 7*M* urea. (**B**) To seek conditions leading to complete digestion by RNase T1, control (lanes a and b) or crosslinked (lanes e and f) transcripts were digested under standard conditions at either 37°C for 45 min (lanes a and e) or 65°C for 1 hour (lanes b and f) before electrophoresis. A purified sample of the crosslink-specific RNase T1 partial digestion product was also digested at either 37°C (lane c) or 65°C (lane d). Ink marks (from top to bottom) identify the origin (O), xylene cyanol (XC), and bromphenol blue (BPB).

Crosslinked and control RNAs were eluted from two-dimensional gels, treated with RNase T1 at 37°C, and analyzed as in Fig. 1. An intense band specific to the digest of the crosslinked form of the transcript is marked by an arrow in Fig. 3A. This species was eluted and recovered in pure form (Fig. 3B, lane c). Further analysis revealed it to be a partial digestion product: the UV-sensitive site in delta RNA occurs in a highly structured region of the genome, in an area that resists ribonuclease digestion. After digestion at a higher temperature (65°C), the complete RNase T1 oligonucleotide constituents of the UV-specific band were released and could be seen after electrophoresis in polyacrylamide gels (Fig. 3B, lane d). This same oligonucleotide pattern was obtained when the UV-specific band from genome-length RNA was digested by RNase T1 at 65°C.

To permit nearest-neighbor analysis of their primary and secondary digestion products, we prepared separate delta transcripts from partial clones using each of the four α-^{32}P-labeled ribonucleoside triphosphates. Parallel crosslinking reactions were carried out on each of the four transcripts. The crosslink-specific RNase T1 partial digestion product was prepared from transcripts of each label and from transcripts containing mixed labels. After digestion with RNase T1 at 65°C or with pancreatic RNase A at 37°C, these (primary) digestion products were fractionated into two-dimensional fingerprints. The pattern obtained from a mixed label transcript digested by RNase T1 is shown in Fig. 4. With knowledge of the sequence of the DNA template for this in vitro transcript (*14*), the technique of RNA secondary analysis was used to identify the oligonucleotides.

Our data demonstrate that only two segments of the transcripts could give rise to the oligonucleotides present in the fingerprint of the crosslink-specific RNase T1–resistant region of HDV genomic sequence shown in Fig. 4. The released oligonucleotides define two noncontiguous segments of the delta genome. One segment contains residues 703 to 735, and the other, bases 856 to 876. The portion of the secondary structure map containing the RNase T1–resistant region is shown in the insert to Fig. 4. From this part of the map, it is evident that the RNase T1 partial digestion product contained a minimum of 54 bases, including 16 G residues. Since G residues specify cleavages by RNase T1 (*16*), the failure of this ribonuclease to cleave after such a large number of G residues implies that the crosslinking site lies within a region of tight structure that is locked into place by the UV-induced crosslink. The stability of various parts of delta RNA is revealed by the presence of partial digestion products in digests of control transcripts (see Fig. 3B,

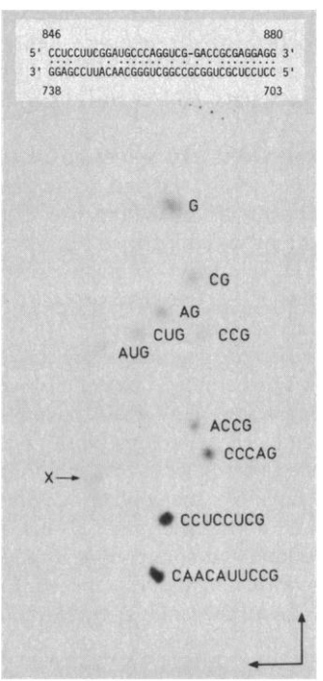

Fig. 4. Oligonucleotides released from the crosslink-specific RNase T1 partial digestion product. After elution from a 20% polyacrylamide gel, the crosslink-specific partial digestion product was incubated with RNase T1 (1 mg/ml) at 65°C for 1 hour, and then the oligonucleotides resulting from this treatment were fractionated into a two-dimensional fingerprint (*33*). Standard conditions for secondary analysis [with pancreatic RNase A, RNase U2, RNase T2, and nuclease P1 (*34*)] were followed and permitted the oligonucleotides to be identified. The fingerprint spot containing the covalent crosslink is marked with an X. The pair of horizontal and vertical arrows indicate the directions of first (gel electrophoresis) and second (homochromatography) dimension separations, respectively. The inset at the top of the fingerprint shows the secondary structure Wang *et al.* (*3*) proposed for this portion of the delta genomic RNA.

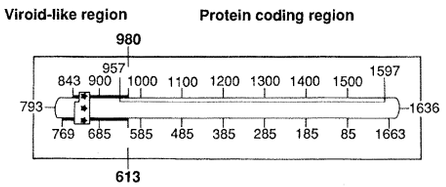

Fig. 5. Genomic map of the delta agent. The genomic RNA of the delta agent is drawn as a collapsed circle. A viroid-like domain at the left end of the molecule contains conserved segments (marked by heavy lines), the UV-sensitive element present in an RNase T1–resistant region (boxed and starred), and autocatalytic cleavage sites at residues 685 and 900. In the protein-coding region, the portion of the molecule specifying the delta antigen spans the distance between residues 957 and 1597 (designated by an open box); the actual mRNA for the antigen is composed of antigenomic sequences.

lane a). Although further analysis will be needed to identify the exact two residues that become covalently linked as a result of UV treatment, they appear to be in the central portion of the two segments shown in the inset to Fig. 4 (*17*).

It is likely that the two bases that become joined by the UV-induced crosslink lie within an element of local tertiary structure containing non–Watson-Crick bonds. The UV-sensitive sites in potato spindle tuber viroid and eukaryotic 5*S* rRNA occur in similar regions, which are devoid of conventional bonds and are flanked by helical regions (*12*). In these molecules, the unusual spatial orientation of two bases within the structural element causes them to become crosslinked by UV treatment. UV crosslinking experiments have provided essential information concerning the structure of several other types of RNA (*18–20*); delta RNA is part of the group that can now be studied by this powerful technique. Tertiary bonds in transfer RNA help to establish its three-dimensional structure (*21*). Similarly, non–Watson-Crick bonds may be important in other kinds of RNA. In particular, RNAs whose functions extend beyond the encoding of genetic information may require interactions that cannot be carried out solely by Watson-Crick base pairing.

Our study of a UV-sensitive site in transcripts of delta RNA provides support for the part of the secondary structure model proposed by Wang *et al.* (*3*), which predicts extensive base pairing between the two segments in which the crosslink is embedded. The oligonucleotide composition of the region surrounding the crosslinking site indicates that the two segments, which are widely spaced in the genomic RNA, are closely associated in the native molecule. This finding is compatible with the model in which delta RNA is depicted as a collapsed circle with left-hand and right-hand terminal loops formed around bases 793 and 1636, respectively (Fig. 5) (*3*). Although our study tested only a portion of this model, it suggests an approach that could be used to analyze additional regions. Several ribonuclease-resistant partial digestion products were released from samples that received no UV treatment. These species could provide starting material for further investigations. A variety of techniques will be needed for all parts of the viroid-like unbranched secondary structural model of delta RNA to be rigorously examined. Viroids are the prototypes of circular RNA molecules in which a series of short helical regions are interrupted by regions with no conventional base pairs. The structure of viroid RNA permits denaturation at temperatures comparable to those that denature other stable single-stranded RNAs but confers on the viroid denaturation process a cooperativity normally characteristic of double-stranded RNAs (*22*). These physical properties are thought to be important for viroids and may also be critical for delta RNA if it replicates through a similar pathway.

In a separate communication (*13*), we explain how the novel structural element described here and several other findings led to the two-domain model for HDV RNA. Briefly, the UV-sensitive site in delta RNA is in a conserved region of the genome. Although the four reported sequences of HDV (representing two strains) differ by about 10% overall, two stretches extending over 295 bases and containing the crosslinking site have but a single base change (*3, 23, 24*), defining a domain about the size of conventional viroids [which range from about 250 to 400 bases (*6*)]. The UV-sensitive site in potato spindle tuber viroid lies in the most highly conserved region of the plant viroid genome (*25*), in a portion of the molecule thought to be involved in replication. The proximity of the UV-sensitive element in HDV RNA to self-cleavage sites in the genomic (*26, 27*) and the antigenomic (*28*) strands, and the location of these components within the only extensive region of conserved delta sequence, suggested to us (*13*) that the delta agent is composed of two parts (Fig. 5). In this model, structures required for a number of replication functions cluster into a viroid-like domain, which makes up the left quarter of the genome. Of the remaining three-quarters, almost half is dedicated to encoding the delta antigen (*3, 23*), and much of the other half may serve to stabilize this coding region, in addition to specifying any functions of its own.

The possible events leading up to the association of a free-living RNA with an mRNA are discussed elsewhere (*13*). When HDV RNA is compared to other viroid-like RNAs, it is evident that there are two small infectious plant RNAs that also appear to be composites with a replication-competent domain linked to some added sequences. The additional sequences found in certain forms of cadang cadang viroid, for example, are duplications of sequences present in the unit-length genome (*29*). It may be that such a duplication could later be followed by genetic drift, with the evolution of a coding sequence. In the virulent RNA satellite of turnip crinkle virus, one domain bears strong similarity to a nonvirulent satellite RNA, whereas the other domain is 93% homologous to part of the helper virus (*30, 31*), suggesting that the virulent satellite originates from two different RNA molecules. The newly acquired sequences might become associated with the free-living RNA by a variety of mechanisms, including either a "jumping polymerase," as suggested for cadang cadang viroid (*25*), or by the ligation of separate RNA chains. Whatever the origin of the two domains of HDV, it is likely that their identification—and particularly that of the conserved, viroid-like domain—will help to plan strategies for the diagnosis and treatment of HDV infection. In addition, studies of the UV-induced crosslink reported here, combined with similar experiments carried out on other HDV strains, may reveal further insights into the origin and evolution of what appears to be a conjoined RNA molecule.

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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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