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Characterization of Self-Cleaving RNA Sequences on the Genome and Antigenome of Human Hepatitis Delta Virus

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Recently we reported that in vitro RNA transcripts complementary to the genome of hepatitis delta virus (HDV) contain a unique site at which self-cleavage can occur. Subsequent studies showed that a similar self-cleavage site was present on in vitro RNA transcripts of genomic HDV RNA. The same self-cleavage reactions were also found to occur on HDV RNAs from the livers of infected chimpanzees. Using the in vitro RNA it was also possible to determine that the minimum length of contiguous sequence needed for self-cleavage of genomic RNA was 30 bases 5′ and 74 bases 3′ of the cleavage site. This sequence was not compatible with the "hammerhead" structure hypothesized to be important in the self-cleavage reactions of other RNAs.

Human hepatitis delta virus (HDV) can be considered as a naturally occurring satellite of human hepatitis B virus. The genome is a 1700-base circular single-stranded RNA that has the ability to fold on itself by base pairing (about 70%) to form an unbranched rod structure (9, 10, 16). Inside the infected cell it is possible to detect not only the circular genomic RNA but also its complement, the so-called antigenomic RNA (3). As reviewed elsewhere (J. Taylor, L. Sharmeen, M. Kuo, and G. Dinter-Gottlieb, UCLA Symp. Mol. Cell. Biol., in press), these and other properties of HDV suggest that the agent has strong similarities to certain pathogenic RNAs of plants: the viroids, virusoids, and satellite RNAs (8). HDV genome replication, like that of the plant agents, seems to be consistent with a rolling circle model (1). One aspect of such a model is that RNA transcription produces a linear multimeric RNA that is then "processed" to produce monomeric linear and circular RNAs. Such processing must include both cleavage and ligation events, and it should occur for both the genomic and antigenomic RNAs. In addition, others have shown in in vitro studies that the RNAs of certain of the plant agents can undergo such cleavage and ligation events in the complete absence of proteins (2, 7, 12).

Recently we reported that antigenomic HDV RNA sequences synthesized in vitro from cloned DNA are able to self-cleave at a unique location simply as a result of incubation in the presence of magnesium ions (14). These findings have now been extended to the complementary strand of HDV RNA, the genomic RNA. In addition we studied the self-cleaving ability of HDV RNAs isolated from the livers of infected animals. Finally, we determined the minimum amounts of contiguous in vitro HDV RNA sequence that are needed to achieve self-cleavage.

MATERIALS AND METHODS

HDV RNA synthesis, cleavage, and analysis. Most of the relevant procedures for HDV RNA synthesis, cleavage, and analysis have been described in a previous report (14). DNA clones of HDV sequences were inserted into the RNA transcription vector pGem4B (Promega Biotec, Madison, Wisc). RNA was then transcribed with either bacteriophage T7 or SP6 RNA polymerase. The self-cleavage of such in vitro RNA was achieved as follows. The RNA samples, in 0.1 mM EDTA, were heat denatured and then snap cooled on ice. After adjustment to 10 mM $MgCl_2$ and 50 mM Tris (pH 7.5), the samples were incubated for 1 h at 50°C. For primer extension we used synthetic DNA primers, either 35 or 15 bases long, that had been 5′ end labeled by using T4 polynucleotide kinase and [γ-^{32}P]ATP and then gel purified (14). This primer was hybridized to the in vitro RNA by incubation in 400 mM NaCl–10 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (pH 6.4). The hybrids were collected by precipitation with ethanol and then subjected to primer extension with reverse transcriptase (14, 17).

RNA from the livers of HDV-infected chimpanzees was extracted with guanidine isothiocyanate as previously described (3). To obtain self-cleavage and primer extension on this RNA, it was necessary to use a procedure more drastic than that used with the in vitro RNA. The RNA and the DNA primer, in 10 mM $MgCl_2$–100 mM Tris (pH 8.2), were placed in boiling water and then allowed to cool slowly to room temperature before the conditions were adjusted for primer extension with reverse transcriptase.

To compare the primer extensions on in vivo RNA relative to in vitro RNA, it was first necessary to quantitate the amount of relevant HDV template in the samples. This was done by applying the RNAs as slots to a nylon membrane, followed by hybridization using an end-labeled oligonucleotide.

RESULTS

Self-cleavage of RNA synthesized in vitro. In our previous study (14) we were able to locate the unique self-cleavage site on the antigenomic RNA of HDV, synthesized in vitro from cloned DNA, by use of a primer extension procedure (17). Briefly, we used a 35-base primer complementary to a site just downstream of the approximate site of self-cleavage. We elongated this end-labeled primer on a self-cleaved RNA template by using reverse transcriptase. This product was then subjected to gel analysis relative to an adjacent dideoxy sequencing ladder (13). Using the notation of Kuo et al. (9) for the 1,679-base genome, we showed that the self-cleavage site on the antigenomic RNA occurred between nucleotides 901 and 900, as read from the primer at nucleotides 801 to 835 (14).

We have since detected a similar unique cleavage site on

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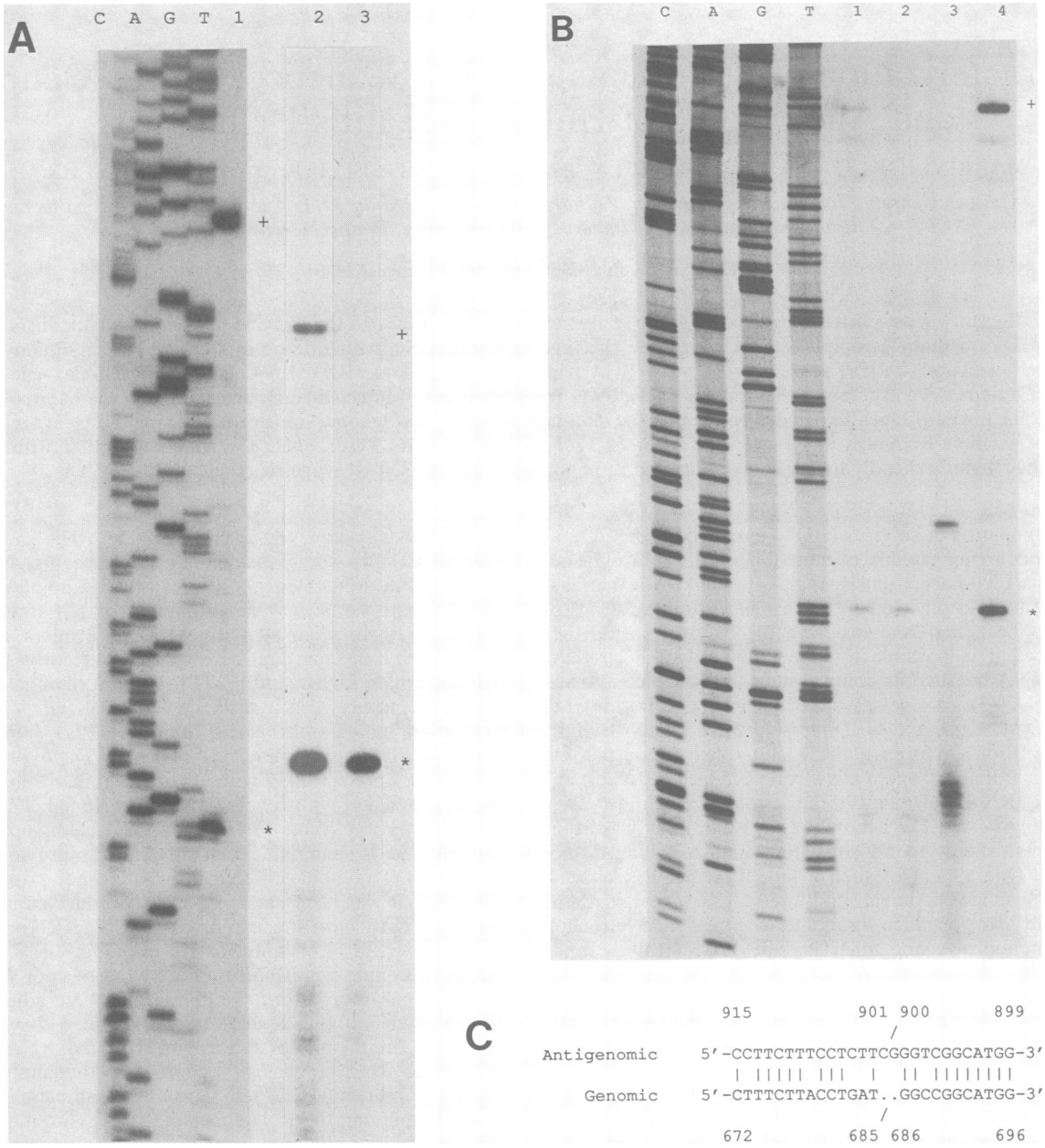


FIG. 1. Detection of self-cleavage sites by primer extension. The 5'-labeled DNA primers were hybridized to self-cleaved template RNAs and extended with reverse transcriptase as described previously (14, 17) and in the text. (A and B) Analysis of samples on DNA sequencing gels (13) relative to an adjacent dideoxy sequencing ladder (13) through a corresponding region. (A) Results with genomic RNAs: lanes 1 and 2, in vitro RNA; lane 3, in vivo RNA. Lanes 2 and 3 were obtained from a separate experiment. (B) Results with antigenomic RNAs: lanes 1 to 3, in vivo RNAs; lane 4, in vitro RNA. The in vivo RNA used in lane 3 was from the liver of a chimpanzee infected in The Netherlands with human HDV, whereas the RNAs in lanes 1 and 2 were from animals infected in the United States. The symbols in panels A and B are defined in the text. (C) Relationship between the cleavage sites on antigenomic and genomic HDV RNAs.

the genomic HDV RNA synthesized in vitro. As before, we used primer extension to exactly locate the cleavage site. Typical results are shown in Fig. 1A, lane 1. The labeled primer was from nucleotides 771 to 737, and the self-cleavage site (*) was located relative to the adjacent sequencing ladder as position 685/686. As before, the upper more slowly migrating band (+) corresponded to primer extension on uncleaved in vitro RNA.

The actual RNA sequences surrounding the cleavage sites on genomic and antigenomic RNAs are shown in Fig. 1C. It can be seen that there is a significant level of similarity between the two sequences (80%). This similarity derives from the fact that the two sites lie opposite each other on the base-paired rod, but occur on different strands. A similar correspondence between self-cleavage sites on genomic and antigenomic RNAs was also seen in the study of one plant agent RNA, lucerne transient streak virusoid (8), although there are other examples of plant agents that do not conform (8).

Self-cleavage of RNA synthesized in vivo. In the experiments described above we used HDV RNAs synthesized in vitro, using cloned HDV sequences as templates. After such

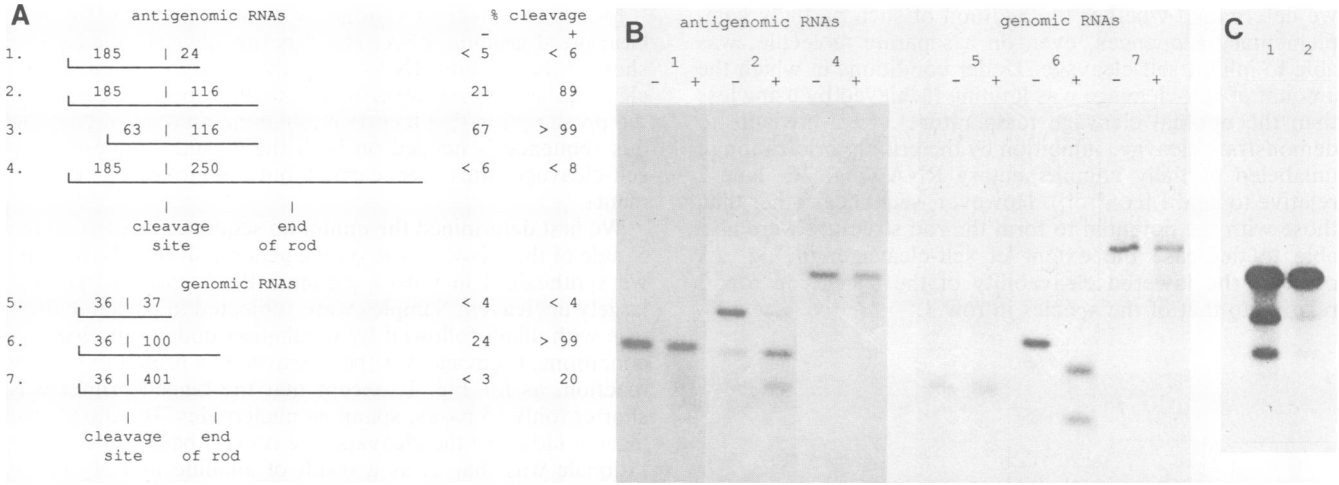


FIG. 2. Effects of adjacent sequences on ability of HDV RNAs to self-cleave. (A) Features of uniformly labeled in vitro RNAs that span the self-cleavage sites of either antigenomic (rows 1 to 4) or genomic (rows 5 to 7) RNA. The amount of HDV sequences adjacent to the cleavage sites are indicated. The numbers on the right indicate the extent to which these molecules self-cleaved, either during synthesis (−) or after an additional treatment under self-cleavage conditions (+). (B) Examples of agarose gel electrophoresis used to obtain the data shown in panel A. − and +, RNA transcripts either just after RNA synthesis or after the additional incubation under self-cleavage conditions, respectively. (C) Ability of the 301-base antigenomic RNA (panel A, row 2) to self-cleave. The uncleaved uniformly labeled RNA was gel purified after synthesis. It was then hybridized in the absence (lane 1) or presence (lane 2) of an excess of unlabeled 136-base antigenomic RNA from positions 785 (*Xba*I) to 650 (*Pst*I) on the other side of the rod structure. The two samples were collected by ethanol precipitation and subjected to mild self-cleavage conditions, followed by glyoxalation and agarose gel electrophoresis (14).

experiments it was possible to address the more complicated question of whether such self-cleavage also occurs on HDV RNAs synthesized in the livers of infected animals. We began with genomic HDV RNA since we have previously shown that in the liver tissue of an HDV-infected chimpanzee, genomic RNA is about 10-times more abundant than antigenomic HDV RNA (3).

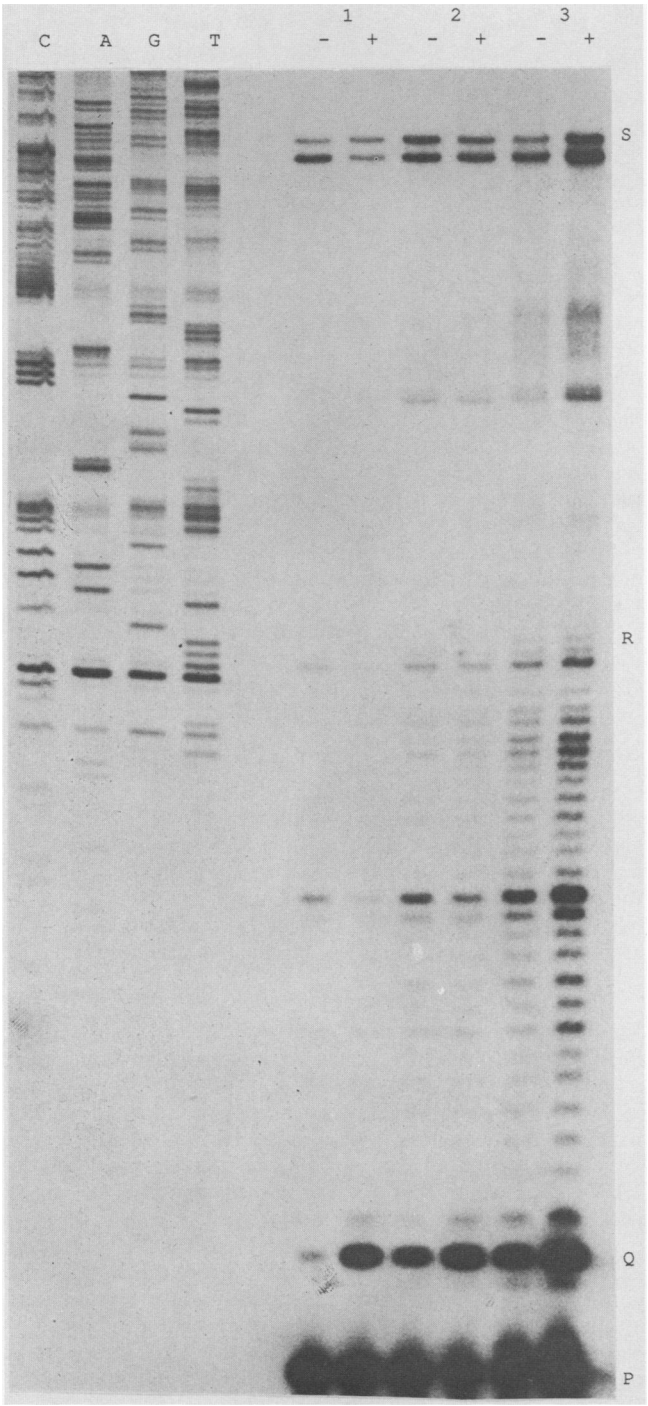
Primer extension was carried out with samples of such liver RNA both before and after incubation under self-cleavage conditions. In the latter instance we obtained significant amounts of a discrete product in the primer extension reaction. However, it was necessary to use the following more drastic conditions to detect self-cleavage. The RNA in 10 mM $MgCl_2$–100 mM Tris (pH 8.2) was placed in boiling water and then allowed to cool slowly. (These conditions were considered necessary to remove the folding of the RNA into the rod structure, which we subsequently show to be an inhibitor of self-cleavage.) The band obtained (Fig. 1A, lane 3) was identical to that obtained with in vitro RNA (lane 2). That is, self-cleavage occurred at the same site. RNA from the liver of a second chimpanzee gave the same result (data not shown). Relative to the signal strength obtained with an equivalent amount of in vitro-synthesized genomic RNA, we could deduce that 50 to 100% of the in vivo RNA was cleaved. In the absence of any self-cleavage treatment, the amount of in vivo RNA cleaved at this site was only 0.5 to 1% (data not shown).

We next attempted to demonstrate self-cleavage of the antigenomic RNA from an infected liver. The results of such an experiment, using the RNAs from three different animals, are shown in Fig. 1B, lanes 1 to 3, relative to the primer extension on in vitro RNA (lane 4). The first two RNAs (lanes 1 and 2) gave the same primer extension result as was obtained with the in vitro-synthesized RNA (lane 4). The third liver RNA gave a different band, 12 bases longer (lane 3). The second liver RNA actually contained both the in vitro band and the same novel band (lane 2). Our interpretation of the origin of the novel band was that the distance between the cleavage site and the 5′ end of the primer was 12 bases longer than that obtained with the other RNAs. We speculate that the difference was due to the presence in the infected livers of a variant form of the HDV genome; a precedent for this is the heterogeneity among clones detected by Wang et al. (16).

Effect of adjacent sequences on self-cleavage. On the basis of previous experiments (14) and those described above, we knew the sites on both the genomic and antigenomic HDV RNAs at which self-cleavage occurred. The following experiments were undertaken to determine the effects on self-cleavage of the sequences adjacent to the self-cleavage sites. Species of antigenomic and genomic RNAs were transcribed in vitro from cloned sequences (Fig. 2A). The amounts of HDV RNA sequence present both 5′ and 3′ of the self-cleavage sites are indicated in Fig. 2A. These RNAs were subjected to self-cleavage conditions and then assayed by glyoxalation and electrophoresis on 2.5% agarose gels. Typical results are shown in Fig. 2B. In some instances, some of the cleavage occurred during RNA synthesis itself (−), prior to the application of self-cleavage conditions (+). The amounts of cleavage were determined by densitometry (Fig. 2A).

The smallest species failed to cleave (Fig. 2A, rows 1 and 5). As will be explained later, such molecules lack sequences necessary for cleavage. Second, note that somewhat longer molecules were able to cleave with efficiencies exceeding 99% (rows 3 and 6). Finally, certain even longer molecules were actually less able to be cleaved (rows 2, 4, and 7). As indicated above, both the genomic and antigenomic RNAs of HDV have the ability to fold by intramolecular base pairing and form an unbranched rod structure. The ends of the rod mark the positions at which self-complementarity begins. As indicated in Fig. 2A, both the largest antigenomic and genomic RNAs (rows 4 and 7, respectively) extend beyond an end of the rod structure and thus have high degrees of self-complementarity. We suggest that the resulting base pairing may inhibit net self-cleavage. To test this hypothesis,

we determined whether the addition of such partially complementary sequences, even on a separate molecule, was able to inhibit self-cleavage. Under conditions in which the amount of self-cleavage was limiting (achieved by using less than the optimal cleavage temperature), we were able to demonstrate cleavage inhibition by the prior hybridization of unlabeled partially complementary RNA (Fig. 2C, lane 2 relative to lane 1 [control]). However, sequences other than those with the potential to form the rod structure were also able to decrease the extent of self-cleavage; in Fig. 2A contrast the lowered cleavability of the species in row 2 relative to that of the species in row 3.



Minimum extent of contiguous sequence needed for self-cleavage of genomic RNA. The experiments described above showed that certain RNAs, even though they contained the cleavage site, were nevertheless unable to self-cleave. On the presumption that a certain minimum amount of contiguous sequence is needed on both the 5′ and 3′ sides of the self-cleavage sites, we carried out the following experiments.

We first determined the minimum sequence needed on the 5′ side of the cleavage site on the genomic RNA. To do this, we synthesized in vitro a genomic RNA species that was largely uncleaved. Samples were subjected to partial hydrolysis with alkali followed by incubation under self-cleavage conditions. Cleavage was then assayed in a primer extension reaction, as for Fig. 1, except that the tagged primer was shorter (only 15 bases, spanning nucleotides 705 to 691) and located closer to the cleavage site (only 5 bases away). The rationale was that if, as a result of alkaline hydrolysis, an RNA species no longer had sufficient bases 5′ of the cleavage site, then it would not be cleaved and primer extension would read through the self-cleavage site to the alkali-hydrolyzed end. In contrast, molecules that still had sufficient bases to allow self-cleavage would be cleavable and when used in primer extension would read out (the five bases) to the cleavage site. The results of this approach are shown in Fig. 3. We can deduce that on this genomic RNA 30 bases were needed 5′ of the cleavage site.

A different approach was needed to determine the minimum amount of sequences needed 3′ to the self-cleavage site. The method we used was basically that used by Forster and Symons (5, 6) to address the identical question for the self-cleaving RNA of lucerne transient streak virusoid. Consider the genomic HDV RNA. We used [γ-^{32}P]GTP to label the 5′ end of genomic RNA, which was largely uncleaved during synthesis. This RNA was then submitted to various degrees of alkaline hydrolysis, followed by incubation under self-cleavage conditions. As shown in Fig. 4, 74 bases on the 3′ side of the cleavage site were needed in order to obtain self-cleavage.

In summary, these experiments indicated that self-cleavage of genomic RNA required no more than 30 bases 5′ and 74 bases 3′ of the cleavage site. These results were an improvement relative to those of earlier experiments, which indicated a need for at most 36 bases 5′ and 100 bases 3′ (Fig. 2A, row 6).

FIG. 3. Minimum sequences needed 5′ of the HDV genomic self-cleavage site. Primer extension studies were carried out as described in the legend to Fig. 1, with modifications as described in the text. Samples were analyzed on a DNA sequencing gel (13) relative to an adjacent dideoxy-sequencing ladder (13) through the corresponding region. Prior to primer extension, samples of unlabeled genomic RNA were submitted to limited alkaline hydrolysis for 0, 2, or 6 min (lanes 1, 2, and 3, respectively), and then subjected (+) or not subjected (−) to self-cleavage conditions. On the sequencing gel the following primer extension bands are indicated: P, 15-base primer; Q, primer extended 5 bases to the self-cleavage site; R, primer extended 29 bases beyond the cleavage site (the molecules were still unable to cleave); S, primer extension on molecules that were unaffected by both the partial alkaline hydrolysis and the self-cleavage conditions. The presence of not one but two bands at position S may have been due to 5′ heterogeneity in these transcripts or a major pause in reverse transcription. It should be noted that even in the reaction mixtures which did not undergo a deliberate self-cleavage treatment (−), the primer extension reaction itself, with incubation at 42°C in the presence of magnesium ions, is an almost optimal self-cleavage reaction (14).

Nature of the cleavage junction. In our initial study (14), we obtained preliminary evidence that the self-cleavage event on antigenomic HDV RNA synthesized in vitro produced a 5′ OH and a cyclic 2′,3′-monophosphate. To substantiate this, we carried out end labeling experiments with gel-purified self-cleavage products. Specifically, we synthesized the RNA species indicated in Fig. 2A, row 2. This RNA was subjected to self-cleavage, and the two fragments were purified on a polyacrylamide gel. These fragments were submitted to different end-labeling strategies to determine the nature of the cleavage junction (Fig. 5). The purified 3′ fragment was directly labeled with T4 kinase and [γ-^{32}P]ATP (lane 2). Thus, it must have had a 5′ OH group. The 5′ fragment was not labeled with T4 ligase and [α-^{32}P]pCp, even after treatment with calf intestinal phosphatase (lane 3). Thus, it did not have either a 3′ OH or a 3′ phosphate group. Actually in lane 3 (and also lanes 4 and 5) there was labeling of the 3′ fragment (which was present as a contaminant) and does have a 3′ OH. Our initial interpretation of these data was that the block at the 3′ terminus of the 5′ fragment was a cyclic 2′,3′-monophosphate. Others have shown two ways to split such a cyclic phosphate linkage to allow removal of the phosphate with calf intestinal phosphatase and release a 3′ OH which can then be labeled with pCp. One method is to first incubate the RNA in the presence of 10 mM $MgCl_2$ for 16 h at 37°C (4). The second method is to treat the RNA with 100 mN HCl for 4 h at 25°C (11). Both methods did allow end labeling of the 5′ fragment (Fig. 5, lanes 4 and 5, respectively), supporting the interpretation that self-cleavage of HDV RNA released a 5′ OH and a cyclic 2′,3′-monophosphate. Thus, HDV was no different in this respect from the self-cleaving RNAs of certain plant agents (2, 5–7) and the self-cleaving RNA of the newt satellite (4).

DISCUSSION

In the experiments discussed above we not only showed that there are sites of self-cleavage on both the antigenomic and genomic HDV RNAs but we determined the minimum contiguous sequence needed for self-cleavage. For the genomic RNA we found that there is a requirement for 30 bases on the 5′ side and 74 bases on the 3′ side. Similar experiments with the antigenomic RNA indicated the need for 17 bases 5′ and 69 bases 3′ (data not shown). Further experiments, similar to those of Uhlenbeck (15), are now necessary to determine whether additional nucleotides within such a molecule can be altered, by removal or replacements, without destroying the ability to self-cleave.

The type of self-cleavage ability described here has been extensively studied in other systems, especially with certain of the plant viroids, virusoids, and satellite RNAs (2, 5–8) and also for the satellite RNA of the newt (4). Keese and Symons (8) have examined such self-cleaving RNAs for conserved features in the RNA sequence and putative structure. Most of them fit what was originally described by Hutchins et al. (7) as a "hammerhead" structure. More recently, Uhlenbeck (15) has characterized this structure further. However, neither of the two HDV sequences fit this model. Similarly, the minus strand of the satellite of tobacco ringspot virus is able to self-cleave and yet it also lacks the hammerhead structure (2, 8). Thus, we think that self-cleavage ability depends upon features of nucleic acid sequence and structure that we are not yet able to recognize.

There are not only sequences that are essential for self-cleavage but there are sequences located away from the self-cleavage sites that can interfere with net cleavage. We

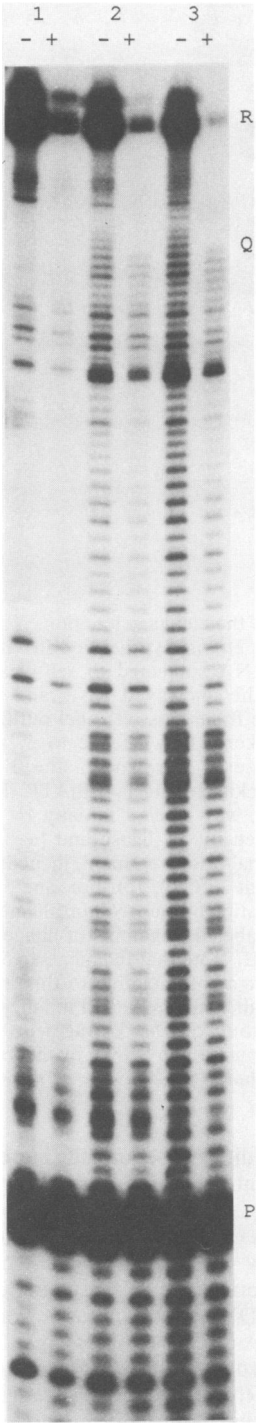


FIG. 4. Minimum sequences needed 3′ of the HDV genomic self-cleavage site. The genomic RNA was end-labeled during synthesis with [γ-^{32}P]GTP. Samples were submitted to limited hydrolysis with alkali, as for Fig. 3, for 0, 2, or 6 min (lanes 1 to 3, respectively). Prior to the final electrophoretic analysis these samples were further divided and subjected (+) or not subjected (−) to self-cleavage conditions. On the sequencing gel the following bands are indicated: P, self-cleaved RNA; Q, RNA fragments that extend 73 bases beyond the self-cleavage site but were nevertheless unable to cleave; R, full-length RNA molecules that were unaffected both by the partial alkaline hydrolysis and the self-cleavage conditions. It must be allowed that there was a consistent absolute loss of counts in the + lanes relative to the − lanes, presumably because of inefficient recovery of RNA subjected to the self-cleavage conditions.

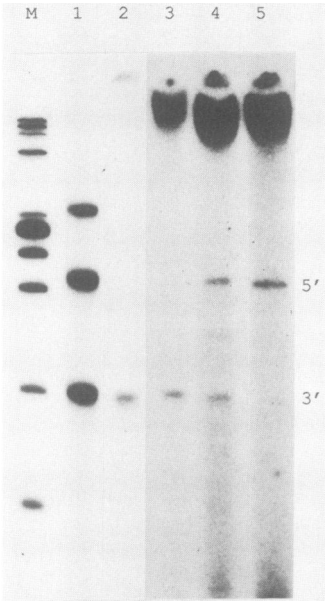


FIG. 5. Nature of the cleavage junction. A 301-base antigenomic RNA (Fig. 2A, row 2) was synthesized in vitro. The products of uniformly labeled RNA subjected to self-cleavage conditions are shown in lane 1. The uncleaved RNA and the 185-base 5′ and 116-base 3′ cleavage fragments were gel purified and then used as electrophoretic markers. For lanes 2 to 5, the RNAs were not labeled during synthesis. For lane 2, the 3′ fragment was then labeled by using T4 kinase and [γ-^{32}P]ATP. For lanes 3 to 5, the purified 5′ fragment was labeled initially by using calf intestinal phosphatase, followed by T4 ligase and [α-^{32}P] pCp. For lane 4, there was an additional prior treatment, incubation of the RNA in 10 mM $MgCl_2$ for 16 h at 37°C (2, 12). For lane 5, the additional prior treatment was incubation of the RNA in 100 mN HCl for 4 h at 25°C (11). Lane M, End-labeled fragments of phage ϕX replicative-form DNA cut with *Hae*III. Note that in lanes 3 to 5, there was also pCp labeling of the 3′ fragment. Relative to this, the labeling of the 5′ fragment is much higher in lane 5 than in lane 4. Our interpretation is that the cyclic group on the 5′ fragment was more efficiently opened by acid treatment (lane 5) than by incubation in the presence of magnesium ions (lane 4).

suggest that the additional sequences increase the chances of the RNA folding into a structure that is either incompatible with self-cleavage or which favors rapid religation. We showed that one example of such inhibitory sequences are those which allow the cleavage site to fold into the unbranched rod structure. Thus, we would expect that folding of the circular HDV RNAs into the rod structure would protect these RNAs from self-cleavage. However, another role for the rod structure may be to promote the ligation of the self-cleaved RNA. As we have described elsewhere (Taylor et al., in press), our working model for HDV genome replication requires that self-cleavage occur on nascent RNA transcripts, before they have had a chance to fold into structures that would be inhibitory. This would ultimately lead to the accumulation of linear unit-length RNA molecules. These molecules would in turn fold by means of the highly stable unbranched rod structure so as to bring into approximate apposition the two nucleotides that need to be ligated to produce circular RNA. Such a model is consistent with our observations that in the liver of an infected animal the majority of both the genomic and antigenomic RNAs are of unit length and, of these, about half are in a circular conformation (3). It is also consistent with recent experiments in which we have used HDV RNAs synthesized in vitro to obtain efficient self-ligation (L. Sharmeen et al., submitted for publication).

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