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ROLE OF THE RIBOZYME OF HEPATITIS DELTA VIRUS ON THE TRANSCRIPTION AFTER POLYADENYLATION

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Summary: We have previously demonstrated that the ribozyme located 34 nucleotides downstream of the polyadenylation site on the antigenomic RNA of the hepatitis delta virus can stabilize the downstream transcript after polyadenylation. Here, we have reports on further investigations of the molecular mechanism of this stabilization effect and the potential role of the small and large delta antigens. We found that the downstream transcripts after polyadenylation were stabilized by the ribozyme independently of either the small or large delta antigen. The stabilization effect was abolished as the ribozyme activity was eliminated by mutations on either the enzyme domain or target site of the ribozyme. These findings suggested that it was the ribozyme activity rather than the RNA structure or the delta antigens that contributed to the stabilization effect.

Hepatitis delta virus is a satellite virus of the hepatitis B virus (1). Its genome is a single-stranded RNA molecule about 1700 bases in size and replicates in the nuclei of infected cells via the synthesis of another complementary RNA, the antigenome. Both the genome and antigenome have closed-circular conformation and can fold intramolecularly to form an unbranched rod-like structure with about 70% of the bases paired (2, 3, 4, 5). The viral genome replication probably proceeds via a "double-rolling circle model"(6,7). On both the genome and the antigenome, there is an unique catalytic sequence, so called "ribozyme", which was proposed to process the on-going RNA into unit-length in size and to become a closed-circular conformation via the reverse reaction of ribozyme function (8, 9, 10, 11, 12). HDV encodes one viral protein, the delta antigen (HDAg), on its antigenome. The two forms of HDAg were found in both HDV infected patients and the experimental tissue culture system. The small HDAg is 195 amino acids in length and the large HDAg is 214 amino acids long with an extra 19 amino acids in its carboxyl terminal. The small HDAg is essential for HDV RNA replication (13), while the large HDAg inhibits HDV RNA replication (14, 15) but is required for virion assembly (16, 17).

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Abbreviations: HDV, hepatitis delta virus; HDAg, hepatitis delta antigen; PCR, polymerase chain reaction.

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The mRNA for HDAg is 800 bases long with a 3' polyadenylated tail and is located in the cytoplasm (18). Since both the unit-length antigenomic RNA and the mRNA are synthesized by using the genomic RNA as the template, it is interesting to investigate whether distinct mechanisms direct the synthesis of either the mRNA or the unit-length antigenome or if both of them are generated from the same transcription. Recently, we have reported that the synthesis of either the mRNA or the unit-length antigenome was regulated by selective usage of the polyadenylation signal on the antigenome (19, 20). We found that polyadenylation was suppressed by the delta antigen only as the nascent transcript was long enough to fold to form the rod-like structure as the transcription passed through the polyadenylation signal. Based upon this, we proposed that during synthesis of HDV antigenomic RNA, the transcript is initially polyadenylated to become mRNA as the transcription goes through the polyadenylation signal at the first time. When the transcription goes farther and passes through the polyadenylation signal in the subsequent cycles, the polyadenylation is suppressed to allow the transcript to become an unit-length antigenome (20). However, it is already known that during the synthesis of most mammalian mRNA, transcription is terminated right after polyadenylation, and the downstream transcripts are rapidly degraded (21, 22, 23). Thus, proceeding the rolling-circle model of HDV genome replication, the mRNA needs to have a novel mechanism to stabilize the downstream transcripts and then prevent the transcription from being terminated. One possibility is that the ribozyme located 3' close to the polyadenylation site on the antigenomic RNA may be involved in this regulation, since the ribozyme cleavage site is only 34 bases 3' of the polyadenylation site. We, thus, hypothesize that the 3'-juxtapositioned ribozyme might endow the downstream transcripts after polyadenylation with extra-stability.

We have previously demonstrated that the preservation of the 3' juxtapositioned ribozyme to the polyadenylation site on the HDV antigenomic RNA leads to stabilization of the downstream transcripts after the mRNA was polyadenylated (19). This finding supports our hypothesis that after the mRNA is polyadenylated, transcription can go farther to generate one or more copies of the unit-length antigenomic RNA. In this report, we further investigated the molecular mechanism of this stabilization effect and the potential role of both the small and the large HDAgs in this stabilization effect.

Materials and Methods

Plasmid constructions. The various HDV cDNA fragments were inserted in the mammalian vector, pSVL (Pharmacia), so that the HDV RNA would be generated from the SV40 late promoter as described before(20). Plasmids, pSVL(sAg) and pSVL(lAg) contained the HDV cDNA fragment from *BglII* to *XbaI*. This fragment comprised not only the open-reading-frame for either the small or large HDAg respectively, but also the HDV ribozyme (Figure 1). Plasmid pSVL(mAg) contained the same *BglII* to *XbaI* fragment with the exception of a two-base deletion at position 1569 and 1570, so that no HDAg would be produced after introduction into mammalian cells (20). The insert of pSVL(Sc) was the HDV cDNA fragment from *XhoI* to *SalI*, so that part of the open-reading frame for the HDAg and the whole ribozyme domain were deleted (Figure 1). To construct the mutant pSVL(R) which contained a three-base mutation on the ribozyme targeting site (position 898 to 900), the three-step PCR-based site-directed

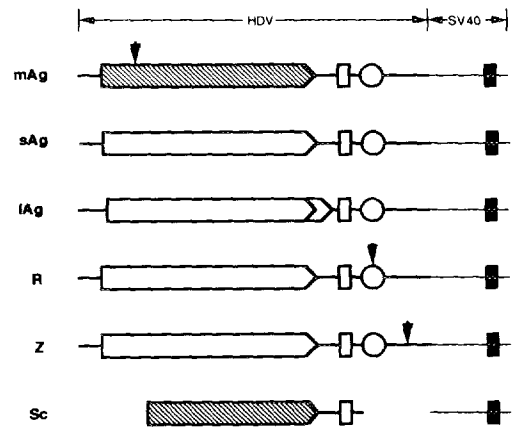

Figure 1. Diagram of the region of HDV cDNA inserted into the eukaryotic expression vector pSVL (Pharmacia). All the HDV cDNA inserts contain the region from *BglII* to *XbaI*, except the insert of pSVL(Sc) which is from position 1508 to 894, in accordance with the numbering system of Kuo et al. (4). The open arrow represents the open-reading frame of the delta antigen, while the striped ones the open-reading frame containing mutation. The open rectangle and the open circle represent the HDV polyadenylation site and the HDV ribozyme targeting site. The enzymatic domain of the ribozyme is located 3' of the ribozyme cleavage site. The flanking SV40 sequences are indicated by the thin line and the SV40 polyadenylation site is illustrated by the stippled rectangle. The arrowheads indicate the regions containing mutation.

mutagenesis was applied. The first and the second PCR were done with the primer pairs of A and B, and C and D, respectively. The nucleotide sequences for primer A is 5'-GGAGAGGCAGGATCACCGAGCAAG-3'; for primer B, 5'-CCGAAATGAAG AGGAAAGAAGGAC-3'; for primer C, 5'-CTCTTCATTCGGCATGGCATCTCCA-3'; for primer D, 5'-GCTGGCGCCGGCTGGGCAACATTC-3'. The underlined nucleosides indicate the mutated sequences. Primer B and primer C were in opposite polarity but both contained the three-base mutations at the same corresponding positions on HDV sequences. The products of first and the second PCR were denatured, annealed and then elongated for 5 cycles followed by the third PCR, which used the primer pair of A and D. The third PCR product was used to replace the fragment from *SalI* to *XBaI* on pSVL(sAg) to generate pSVL(R). Plasmid pSVL(Z) was constructed by deletion of three bases (869 to 871) at *RsrII* site of pSVL(sAg). This was done by trimming the S1 exonuclease at the *RsrII* cleavage site. Both the mutation sequences on pSVL(R) and pSVL(Z) were confirmed by dideoxynucleotide sequencing and the ribozyme activities were examined by an *in vitro* assay as mentioned below.

Transfections. The DEAE-dextran mediated transfection described by Cullen (24) was employed. Three million Cos7 cells were seeded on a 100-mm plate 24 hours before the transfection. Five micrograms of plasmid DNA were used for each plate. Culture medium (DMEM with 10% of FCS) was replaced every day till the day of harvest.

RNA extraction and poly(A) selection. Total cellular RNA was harvested on day 2 after transfection by direct lysis of cells in 4 M Guanidine isothiocyanate followed by equilibrium centrifugation in a CsCl gradient as previously described (24). Polyadenylated RNA was further isolated twice with oligo(dT)-cellulose (19).

Northern blot analysis. Three micrograms of each polyadenylated RNA sample were glyoxalated prior to electrophoresis in a 1.5% agarose gel. After transfer to a nylon membrane and deglyoxalation, the RNA was hybridized with a [α-^{32}P]UTP-labeled HDV strand-specific riboprobe made by the T7 *in vitro* transcription system (Promega)(19), and then analyzed by autoradiography.

Immunoblot analysis. Cells were lysed in Laemmli's buffer (25), boiled for 10 minutes and then electrophoresed in 12% SDS-polyacrylamide gel. After transfer to a nitrocellulose filter, the total cellular protein was reacted with diluted serum of an HDV infected patient, followed by treatment with alkaline-phosphatase conjugated anti-human IgG for colorimetric reaction, as described in the manufacturer's instruction (Promega).

***In vitro* synthesis of HDV RNA and *in vitro* examination of ribozyme activity.** HDV cDNA fragments were inserted into plasmid pGEM 4Z (Promega), so that specific HDV RNA species could be generated with the bacteriophage T7 or SP6 RNA polymerase in an *in vitro* system (20). To examine the ribozyme activity, the [α-^{32}P]UTP-labeled HDV RNA was subjected to three cycles of heating to 70°C for 10 minutes and cooling on ice for 5 minutes in the buffer containing 40 mM Tris.HCl pH 7.5 and 6 mM $MgCl_2$. The final products were analyzed on a 6% polyacrylamide sequencing gel and autoradiography.

Results and Discussion

It has previously been established that the small HDAg is essential for HDV RNA replication, while the large HDAg interferes with HDV RNA replication. Therefore, it is interesting to know whether either form of HDAg is implicated in the stabilization effect of the ribozyme on the downstream transcripts after the mRNA for HDAg has been polyadenylated. The experiment strategy is summarized in Figure 2A. After transfection, the polyadenylated RNA was extracted and examined by Northern blot analysis. The antigenomic RNA, which was generated from the SV40 late promoter, contained both the HDV ribozyme domain located 34 nucleotides 3' of the HDV poly(A) site and the SV40 polyadenylation signal. As a result, after the mRNA was polyadenylated at the HDV poly(A) site (RNA species **a**), the downstream transcripts were cleaved at their 5'-end by the ribozyme and then polyadenylated at the farther downstream SV40 poly(A) site (RNA species **b**). This would allow the downstream transcripts to be assayed easily with Northern blot analysis according to the size difference between two RNA species, as shown in Figure 2B. The mRNA species were expected to be about 1.7 kb in length (RNA species **a**), while the downstream transcripts were predicted to be about 0.5 kb long (RNA species **b**). The plasmid pSVL(Sc) contained the deletion on the ribozyme domain and additional deletion on the 5' side of the open-reading frame for the HDAg (Figure 1). Therefore, the mRNA and the downstream transcripts generated from pSVL(Sc) would be smaller (1.0 kb and 0.38 kb respectively). In Figure 2B. the upper bands (shown by **a**) were the mRNA species and the lower bands (shown by **b**) were the downstream transcripts after the mRNA was polyadenylated at the HDV poly(A) site. In the absence of the ribozyme domain (Figure 2B, lane 6), the downstream transcripts could not be detected (RNA species **b** of lane 6). This was consistent with the general phenomenon that during the synthesis of most mammalian mRNAs, the downstream transcripts after polyadenylation are rapidly degraded and transcription is terminated. However, as the ribozyme domain was preserved on the HDV antigenomic RNAs, the downstream transcripts were readily detected (RNA species **b** of lane 1, Figure 2B). These findings suggested that presence of the ribozyme on the post-polyadenylated downstream transcripts led to stabilization of the downstream transcripts (19). In addition, when either the small

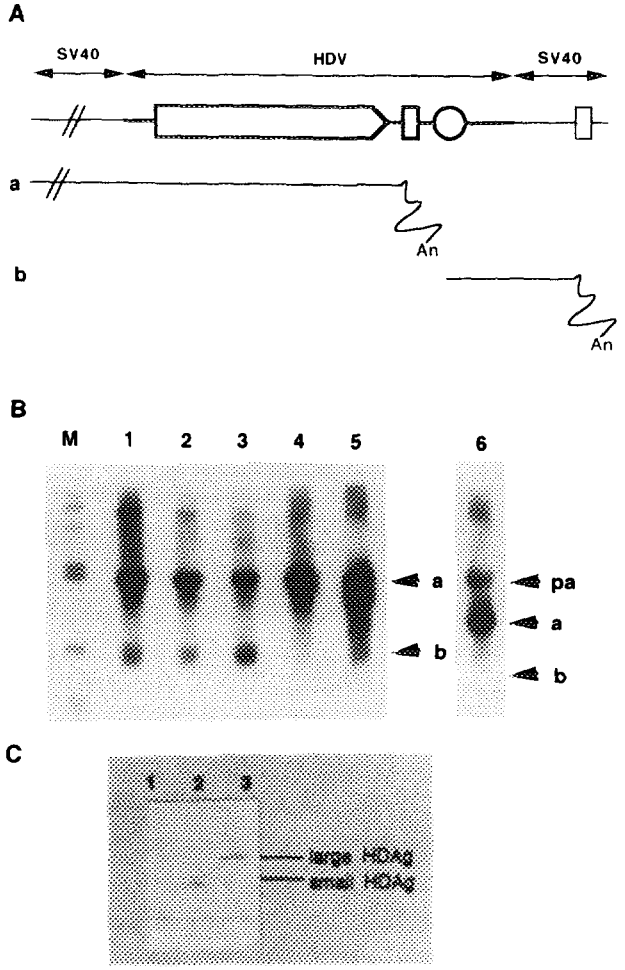

Figure 2. The effects of the ribozyme and the delta antigen on the downstream transcripts after polyadenylation. (A) Schematic diagram of the transcription of HDV antigenomic RNA. The flanking SV40 sequences are indicated by the thin line. On the HDV sequences, the open arrow indicates the open-reading frame for the delta antigen; the rectangle, the HDV poly(A) addition site; the circle, the ribozyme cleavage site. The rectangle on the 3' flanking SV40 sequences represents the SV40 polyadenylation site. The mRNA polyadenylated at the HDV poly(A) site is about 1.7 kb in length (**a**), while the downstream transcript after the mRNA was polyadenylated at the HDV poly(A) site and cleaved by the HDV ribozyme is about 500 bases in size (**b**). (B) Northern blot analysis. The mRNA polyadenylated at HDV poly(A) addition site is indicated by **a**, while the downstream RNA species after the mRNA was polyadenylated is indicated by **b**. Lanes 1 to 6 are the results of the cells transfected with pSVL(mAg), pSVL(sAg), pSVL(lAg), pSVL(R), pSVL(Z) and pSVL(Sc), respectively. Of notice, in lane 6, the size of transcripts of **a** and **b** are 1.0 kb and 0.38 kb respectively, due to extra-deletion at both the 5' HDV sequences and the HDV ribozyme domain in pSVL(Sc), as demonstrated in Figure 1. RNA species **pa** refers to the precusor of RNA species **a** that still contains the SV40 intron (19). Lane M is the *HindIII* fragments of the bacteriophage lambda DNA. (C) Immunoblot for detection of the small and large delta antigen. Lane 1, cells were transfected with pSVL(mAg); lane 2, pSVL(sAg); lane 3, pSVL(lAg).

HDAg or the large HDAg was supplied at the same time (Figure 2C), the downstream transcripts were also detectable (lanes 2 and 3 of Figure 2B) and there was no significant difference in their amount (Figure 2B, lane 1, 2 and 3). These results indicated that the

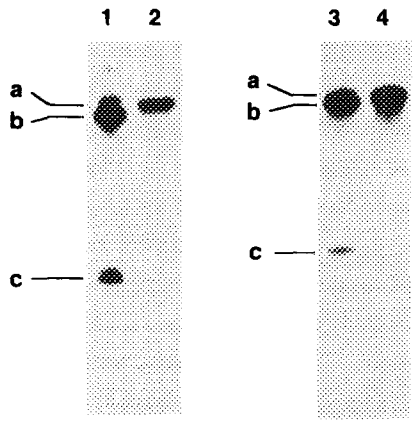

Figure 3. In *vitro* examination of the ribozyme activity. [α-^{32}P]UTP-labeled HDV antigenomic RNA fragments were synthesized *in vitro* with bacteriophage T7 RNA polymerase followed by three cycles of heating to 50°C for 10 minutes and cooling on ice. The products were then separated on a 6% polyacrylamide sequencing gel followed by autoradiography. Lane 2 and lane 1 are the RNA samples derived either from pSVL(R) or from the corresponding wide type sequences, respectively; lane 4 and lane 3 are those from pSVL(Z) or from the corresponding wide type sequences, respectively. RNA species **a** represents the intact RNA; species **b** and species **c** represent the 3' and the 5' fragments cleaved by the ribozyme, respectively. They are 652 bases, 590 bases and 62 bases in length, respectively, for those in lanes 1 and 2; and 796 bases, 700 bases and 96 bases long, respectively, for those in lanes 3 and 4.

ribozyme, which is located only 34 bases 3' of the HDV poly(A) site, exerted the stabilization on the downstream transcripts after the mRNA was polyadenylated and that the stabilization effect was independent of the presence of either the small or large HDAg.

The RNA structure required for the ribozyme activity has been elucidated (26, 27). Such a novel, complicated structure might allow the downstream transcripts after polyadenylation to be more resistant to cellular RNase degradation. Alternatively, the ribozyme activity *per se* is essential for the stabilization. To distinguish these two possibilities, two mutant clones, pSVL(R) and pSVL(Z), were created to abolish the ribozyme activity with relative conservation of the RNA structure. To examine the ribozyme activity of the RNA generated from these mutants, their HDV cDNA inserts were moved into pGEM 4Z (Promega). Therefore, the HDV RNAs could be synthesized *in vitro* with bacteriophage T7 RNA polymerase and their ribozyme activities were examined by an *in vitro* assay. As shown in Figure 3, the RNAs generated with the mutant clones derived from pSVL(R) and pSVL(Z) were shown in lane 2 and lane 4, respectively. The wide type RNAs generated from the corresponding regions were shown in lane 1 and lane 3, respectively. The top bands (**a**) represent the full-length RNA species, while the middle (**b**) and the bottom (**c**) ones are the 3' side and the 5' side of the ribozyme-cleavage fragments, respectively. Clearly, more than 90% of the wide type RNA species were cleaved by the ribozyme (lanes 1 and 3), while none of the either mutant RNA species was cleaved (lanes 2 and 4). These results confirmed that the mutations on both pSVL(R) and pSVL(Z) efficiently eliminated the ribozyme activity at the corresponding RNA level. They were used to transfect Cos7 cells and the results of Northern blot analysis were shown in lanes 4

and 5 of Figure 2B, respectively. The lower bands (RNA species **b**, representing the downstream transcripts after the mRNAs were polyadenylated, as mentioned above) were undetectable in lanes 4 and 5, as compared to those with wide-type ribozyme in lanes 1, 2 and 3. It was worthy to note that both the RNA species generated from pSVL(R) and pSVL(Z) contained three-base changes to eliminate the ribozyme activity with minor disturbance of the different stem-loop structures of the ribozyme. Therefore, these results indicated that it was the ribozyme activity rather than the complicated RNA structure which endowed the post-polyadenylated downstream transcripts with extra-stability.

How does ribozyme activity contribute to the stabilization of the downstream transcripts after polyadenylation? The main difference between the downstream transcripts generated from either the polyadenylation or the polyadenylation followed by the ribozyme cleavage is the structure of the 5'-ends. The 5'-end of the former is a phosphorylated group (the usual result from cleavage by most nucleases), while the 5'-end of the latter is a hydroxyl group (28, 29). Therefore, it is reasonable to speculate that the stabilization is directly related to this unusual 5'-hydroxyl group. By using a cell-lysate assay with the [α-^{32}P]UTP-labeled RNA, we found that the RNAs with 5'-phosphorylated end were more susceptible to degradation than those with 5'-hydroxyl end (data not shown). The half life of the former was estimated to be 4 minutes, while that of the latter was 8 minutes. Since both of them were degraded rapidly in the cell-lysate assay, it is not clear whether this difference is significant *in vivo* . Perhaps, cellular RNA-binding proteins (30), the cellular transcription machinery, or even the cellular polyadenylation machinery (31) are involved in this stabilization effect. Further investigation is currently in progress.

In summary, HDV utilized a novel mechanism to overcome the instability of the downstream transcripts and the transcription termination after polyadenylation during the synthesis of the antigenomic RNA. The juxtaposition of the ribozyme 3' to the HDV poly(A) addition site endowed the downstream transcripts after polyadenylation with unusual stability to allow the transcription to generate one or more copies of the unit-length antigenomic RNAs. This stabilization effect was contributed by the ribozyme activity and was independent of either HDAg.

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