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Ribozyme-independent replication of a defective hepatitis D virus RNA derived from hepatitis B/D patients receiving antiviral therapy

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ABSTRACT

Novel hepatitis D virus (HDV) RNA mutants carrying large fragment deletions were identified in the serum samples of two hepatitis B/D patients receiving antiviral therapy. Sequence analysis revealed that the deleted regions encompassed both ribozyme domains. The mutant persisted in the serum samples for at least 2 and 10 months, respectively in the two patients, raising the question of whether such mutants could replicate in the absence of ribozyme domains. Thirty anti-HDV antibody-positive serum samples derived from 17 patients receiving antiviral therapy were submitted for RT-PCR detection of HDV RNA deletion mutants. Large fragment HDV RNA deletions were found in 4 patients. Of them, two had liver biopsy samples available. Northern blot analysis revealed high molecular weight HDV RNA replication intermediates, genomic and anti-genomic senses, in the liver tissues. Transfection of an in vitro transcribed HDV RNA deletion mutant (1.28 kb) into Huh7 and HepG2 cells also resulted in generation of high molecular weight HDV RNA species in the hepatoma cells (>6.5 kb) with secretion of a 6.5 kb HDV RNA species into the medium. In conclusion, we discovered novel large fragment deletion mutants of HDV RNA in hepatitis B/D patients receiving antiviral therapy. Such mutants did not contain ribozyme domains but could replicate in the liver cells to generate high molecular weight but not unit-length HDV RNA.

1. Introduction

Hepatitis D virus (HDV) is a defective RNA virus identified by Rizzetto in 1977 [1]. The virus contains a 1.7 kb negative stranded RNA genome which harbors an unbranched rod-like circular conformation, carrying ribozyme activities. HDV needs hepatitis B virus (HBV) surface antigen (HBsAg) to constitute its envelope during virion maturation. Its genome encodes two in-frame proteins, the small and large hepatitis D antigen (HDAg). The small HDAg enhances HDV replication whereas the large HDAg is required for virion assembly [2]. Replication of HDV RNA is carried out by cellular RNA polymerase via a rolling circle mechanism to generate linear concatemers and the ribozyme activities are required for cleavage and re-circularization of the unit-length HDV genome. Presently, approximately 5% of HBV carriers worldwide are co-infected with HDV [3]. Diagnosis of active HDV infection is made by the detection of HDV RNA in serum by reverse transcription (RT)-PCR [4].

Deletion mutants of HDV have been reported previously, but the deletion areas are usually very short and the ribozyme domains are always preserved. Here we reported the discovery of novel large fragment deletions in HDV RNA in two hepatitis B/D patients receiving antiviral therapy. In these mutants, the ribozyme domains were deleted. Subsequently, we provide northern blot evidence in the liver biopsy samples as well as in the mutant-transfected hepatoma cells to demonstrate that such mutants could replicate in the absence of ribozyme domains.

2. Methods

2.1. Patients

This study was conducted under approval of Institutional Review Board, Chang Gung Medical Center, Taiwan. Longitudinal

Abbreviations: HBV, hepatitis B virus; HDV, hepatitis D virus; HDAg, hepatitis D antigen; HBsAg, hepatitis B surface antigen; Anti-HDV, antibodies against HDAg; AST, aspartate aminotransferase; ALT, alanine aminotransferase; HBeAg, hepatitis B e antigen; Anti-HBe, antibodies against HBeAg; RT-PCR, reverse transcription-polymerase chain reaction.

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serum samples were obtained from two hepatitis B/D patients receiving antiviral therapy. Serum and liver biopsy samples of 17 chronic hepatitis D patients receiving antiviral therapy were retrieved from tissue bank of Chang Gung Medical Center.

2.2. HDV RNA assays

Serum anti-HDV antibodies were detected by anti-HDV test (Formosa Biomedical Technology Corp., Taiwan) and HDV RNA was detected by RT-PCR. Two sets of primers were used in this study (see Fig. 1A for locations of all primers). All nucleotide numbers were assigned according to a previously published Taiwanese genotype II HDV clone (GenBank accession number U19598). The first set included a primer located next to one of the ribozyme site, HDV-L, 5′-CATGGTCCCAGCCTCCTCGCTGGC-3′ (nt. 695–718; sense), and a primer located further downstream, HDV-R, 5′-GAAGGAAGGCCCTCGAGAACAAGA-3′ (nt. 1287–1264; antisense). The second set was designed to detect the ribozyme-deleted mutants. They were HDV-B1, 5′-ACCTCCAGAGGACCCCTTCAGC-GAA-3′ (nt. 304–328; sense), and HDV-B2, 5′-GAG-GGAGCTCCCCCGGCGAAGAG-3′ (nt. 1401–1379; antisense). In order to obtain the complete deletion clone, a third set of primers were used to amplify the remaining part of HDV genome. They were HDV-A1, 5′-GCGGGCCGGCTACTCTTCTTT C-3′ (nt. 1155–1176; sense), and HDV-A2, 5′-CTAGCCCCGTTGCTTTCTTTGCTTT-3′ (nt. 434–410; antisense). Sequence analysis was performed using an automatic DNA sequencer (CEQ 2000; Beckman Instruments Inc., Fullerton, CA).

2.3. Quantitative HBV-DNA assay

HBV-DNA was quantitatively assessed by use of the standardized real-time PCR test (COBAS TaqMan HBV test; Roche, Branchburg, NJ). In this assay, 1 IU/mL was equal to 5.82 copies/mL.

2.4. Cell culture and plasmid construction

HepG2 cells were maintained in minimal essential medium containing 10% fetal bovine serum. Huh7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. To obtain genomic and anti-genomic sense transcripts from a deletion mutant clone, a greater-than-unit-length of cDNA clone from nt. 304 (5′ end of the HDV-B1 primer) to nt. 434 (5′ end of the HDV-A2 primer) (totally 1279 nt. with 130 nt. overlapped sequence) was inserted into pGEM-T Easy vector (Promega corporation, Madison, WI). The insert was flanked by T7 and SP6 promoters on the vector and the plasmid was used for in vitro transcription after linearization. The anti-genomic and genomic specific probes were generated from this clone using single-sided PCR.

A plasmid, pCMV-HBV, was generated by inserting a copy of greater-than-unit-length HBV genome (3.37 kb; nt. 1820–1990; derived from pECE-C [5]) into a vector, pRc/CMV (Invitrogen,

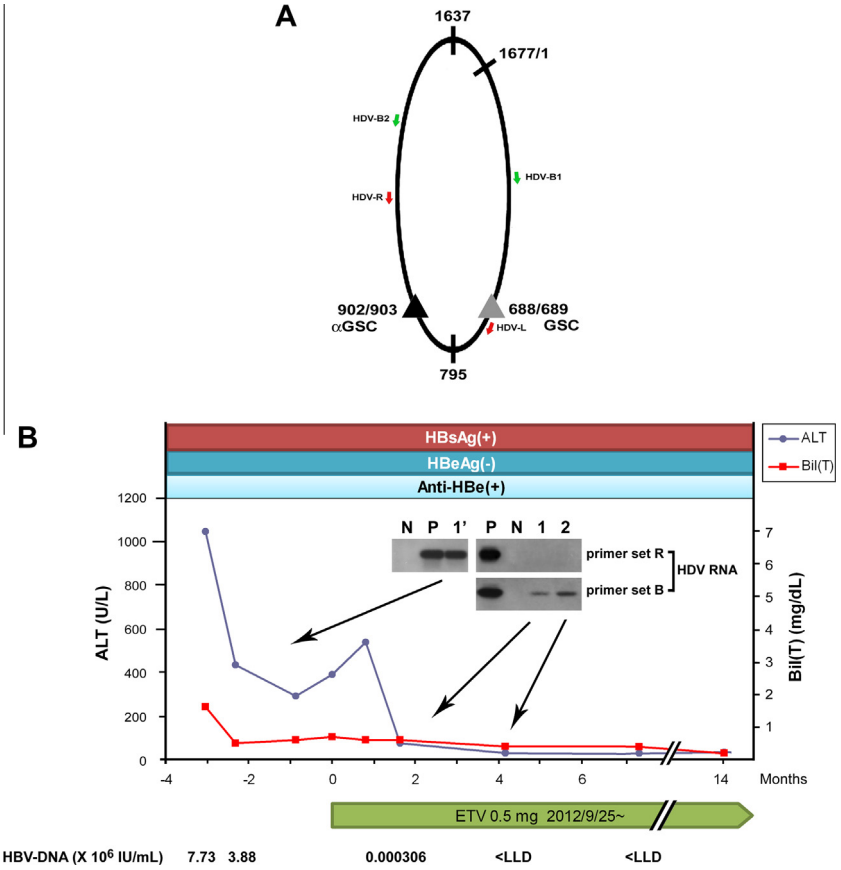

Fig. 1. Clinical and virological data of patient-1. (A) The positions of primers used for HDV RNA detection. Red arrows, primer set HDV-L and HDV-R; Green arrows, primer set HDV-B1 and HDV-B2; GSC and αGSC, self-cleavage sites for genomic (gray triangle) and antigenomic (black triangle) RNA. (B) Clinical course of Patient-1. ALT (gray blue circles) and bilirubin (red squares) levels were depicted along the clinical course. The starting point of entecavir (ETV) treatment (green bar) was given below the clinical course. HBV DNA levels were provided at the bottom. LLD, lower limit of detection. HDV RNA was detected using primer set R (HDV-L and R) and primer set B (HDV-B1 and B2) at different time-points. P, 20 pg of HDV RNA specific PCR product as positive hybridization control; N, 20 pg of cloning vector pCR2.1-TOPO (Invitrogen) as negative control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

San Diego, CA). This plasmid was stably transfected into HepG2 and Huh7 cells, respectively, to maintain HBV replication [5]. Huh7-HDV cells were the same as Huh7-D2T cells, which stably expressed HDV genome from an integrated plasmid containing cDNA dimer of a genotype I HDV cDNA (GenBank accession number, M21012) [6]. This cell line supported complete HDV RNA replication.

3. Results

3.1. Identification of a defective HDV RNA sequence in two patients with chronic hepatitis D

Serum samples from two chronic hepatitis B/D patients were studied in this report. Patient-1 was a 41-year-old male, who had chronic hepatitis B with severe activities. ALT level elevated up to 1080 U/L and bilirubin level reached 1.9 mg/dL. Serum HDV RNA was positive by RT-PCR (primer set: HDV-L and HDV-R) (Fig. 1B). HBV DNA was 7.73×10^6 IU/mL. Because of economical consideration, entecavir 0.5 mg/d but not peginterferon was given since September 2012. HBV DNA became undetectable 4 months after the start of treatment. Interestingly, HDV RNA was also undetectable by use of the primer set, HDV-L and R, 2 and 4 months after the start of treatment. To further confirm this result, we designed another set of primers, HDV-B1 and B2, to amplify a larger fragment of HDV RNA. Strikingly, a RT-PCR product, shorter than expected, was detected 2 months after the start of treatment. Sequence analysis was performed, which revealed a defective clone with deletion of nt. 524–1050, encompassing both the ribozyme domains. The same product (sequence verified) was detected again 4 months after the start of treatment, suggesting that the deletion mutant was persistent in this patient.

Patient-2 was a 48-year-old male, who had chronic hepatitis B/D for more than 10 years and developed liver cirrhosis. Esophageal varices bleeding occurred once 2 years before visiting our clinic. During the follow-ups, HBV DNA was undetectable while ALT elevated to 265 U/L. RT-PCR to detect HDV RNA using primer set HDV-B1 and 2 showed two products, one had the expected full length size while the other had a smaller size (Fig. 2). Pegylated interferon was given from April 2013 to February 2014. The shorter RT-PCR product could be detected 8 months after the start of treatment while the wild type disappeared. At the end of treatment, both forms of HDV RNA were undetectable. Sequence analysis of the shorter HDV RNA product showed a deletion of nt. 340–1247, again encompassing both the ribozyme sites.

3.2. Northern blot analysis for liver biopsy tissues obtained from chronic hepatitis D patients carrying the deletion mutants

To understand the prevalence of large fragment HDV RNA deletions, we retrospectively analysis HDV RNA by RT-PCR using primer set HDV-B1 and 2 using serum samples from 17 chronic hepatitis B/D patients receiving antiviral therapy. All patients were positive for anti-HDV antibody. Of the 17 patients, 10 were treated with entecavir, 3 with telbivudine, and 4 with lamivudine. Of the 10 patients treated with entecavir, 4 patients carried large fragment HDV RNA deletion mutants. Two of these 4 patients had a small piece of liver biopsy tissues stored in the −70 °C freezer in our Liver Research Center. Northern blot analysis was thus performed using probes specific to genomic and antigenomic sense HDV RNA (Fig. 3). It was found that positive hybridization signal appeared as a high molecular smear for both tissue samples using genomic sense specific probe (Fig. 3B, lanes 2 and 7). Besides the high molecular signal, a prominent species located at the 6.5 kb position was found in one of the sample using antigenomic sense specific probe (Fig. 3B, lane 2). In contrast, in Huh7-HDV cells, which were Huh7 cells stably transfected with a wild type HDV cDNA dimmer, only the 1.7 kb HDV RNA was seen (Fig. 3B, lane P).

3.3. Ribozyme-independent replication of a defective HDV RNA clone

To obtain a full length cDNA clone of the large fragment HDV RNA deletion mutant, HDV RNA derived from the patient whose sample was loaded on lane 2 in Fig. 3 was amplified by RT-PCR. Cloning and sequencing of the RT-PCR product indicated that a region spanning nt. 524–1051 was deleted. Again, the ribozyme domains of both orientations were missing in this mutant. Accordingly, a greater-than-unit-length of cDNA clone (1279 bp with 130 bp overlapped sequence, see Method) was constructed using the amplicons. In vitro transcription was performed to obtain both genomic-sense and anti-genomic sense defective HDV RNAs. These two species of RNAs were transfected into HepG2 and Huh7 cells, respectively, by electroporation. As shown in Fig. 4, positively hybridized smears were found from the loading well down to the

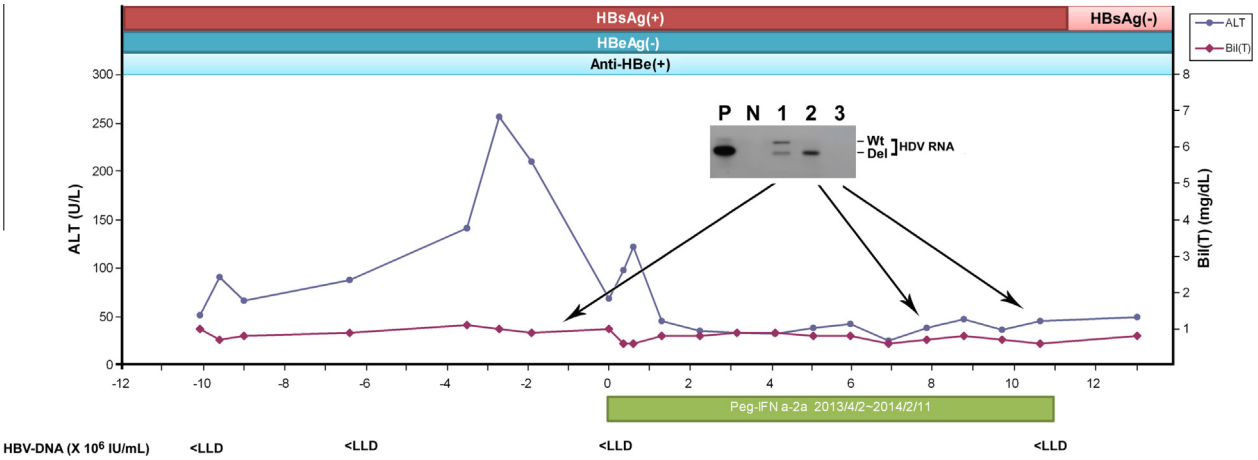

Fig. 2. Clinical and virological data of patient-2. ALT (gray blue circles) and bilirubin (red diamonds) levels were depicted along the clinical course. The time period of peginterferon (Peg-IFN) treatment (green bar) was given below the clinical course. HBV DNA levels were provided at the bottom. LLD, lower limit of detection. HDV RNA was detected using primer set B (HDV-B1 and B2) at different time-points. Wt, wild type; Del, deletion mutant; P, 20 pg of HDV RNA specific PCR product as positive hybridization control; N, 20 pg of cloning vector pCR2.1-TOPO (Invitrogen) as negative control. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

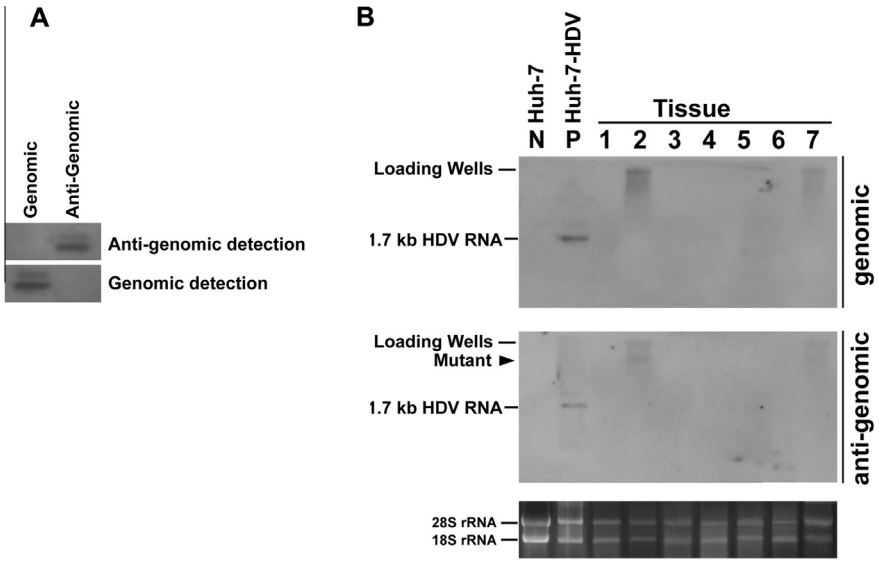

Fig. 3. Northern blot analysis to detect HDV RNA in the liver biopsy tissues of patients carrying defective HDV RNA mutants. (A) Specificity of the genomic and antigenomic senses detection probes. One pg of in vitro transcribed genomic sense (left) and antigenomic sense (right) HDV RNA was loaded onto the agarose gel respectively for northern analysis. Detection was made by hybridization with the antigenomic (upper) and genomic (lower) sense specific probes. (B) Detection of HDV RNA derived from liver biopsy samples. Liver biopsy samples obtained from 2 patients carrying defective HDV RNA mutants were included for Northern blot analysis (lanes 2 and 7). The other biopsy samples (lanes 1, 3–6) were obtained from patients with chronic HBV infection but negative for anti-HDV antibodies (as negative controls). Northern blot was performed using genomic (upper panel) and antigenomic (lower panel) sense specific probes. Cellular RNA from Huh7 and Huh7-HDV cells were used as negative and positive controls. 18S and 28S rRNA were used as loading controls. Solid triangular arrowhead, the position of a putative dominant species of HDV RNA mutant (6.5 kb according to the RNA molecular weight marker loaded in parallel).

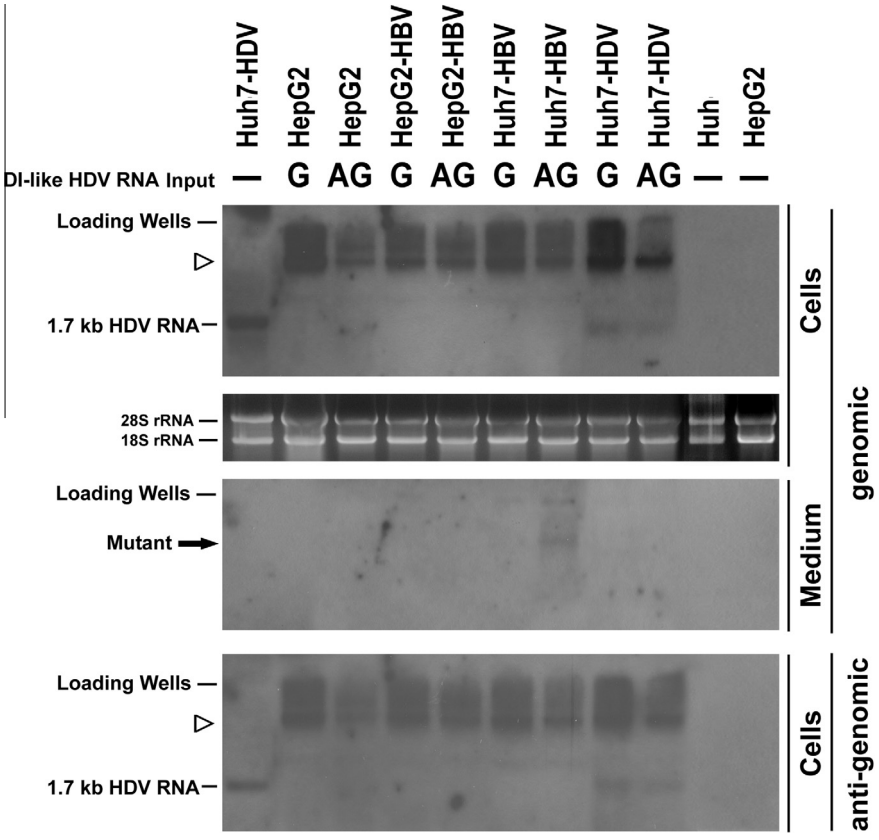

Fig. 4. Northern blot analysis of HDV RNA in defective HDV RNA transfected hepatoma cells. The greater-than-unit-length mutant HDV RNA transcripts (1.279 kb), genomic (G) or anti-genomic (AG) sense, were transfected into HepG2 or Huh-7 cells by electroporation. The culture medium was replaced (with fresh medium) 6 h after transfection. The medium and trypsinized/washed cells were harvested 24 h after transfection for RNA extraction and northern blot analysis. The genomic (upper two panels) and antigenomic (lowest panel) sense specific probes were used for detection. Empty triangle, the dominant species of putative high molecular weight replication intermediates of mutant HDV RNA. Arrow, the secreted high molecular weight HDV RNA mutant species (6.5 kb according to the RNA molecular marker loaded in parallel).

6.5 kb position with the 6.5 kb HDV RNA species as the most prominent species. The positive signals appeared in both the genomic-sense (G) transfected and anti-genomic-sense (AG) transfected cells. In Huh7-HDV cells, the amount of 1.7 kb wild type HDV RNA was significantly suppressed after transfection of the defective RNA, indicating an interference effect. In the medium, a positively hybridized HDV RNA species (6.5 kb) only appeared in Huh7-HBV cells.

4. Discussion

Pegylated interferon with or without combination of oral antiviral agent has been demonstrated to be effective in treating chronic hepatitis B/D infection [7,8]. Minor beneficial effect has also been shown if only oral antiviral agent was given [9,10]. Because of the absence of adverse effect, presence of minor beneficial effect and lower cost, patients with chronic hepatitis B/D infection are mostly treated with oral antiviral agents as the initial treatment as in Patient-1. Pegylated interferon was considered only when hepatitis activities still remained after complete suppression of HBV DNA as in Patient-2.

In this study, we discovered large fragment genomic deletion mutants of HDV. Previously, naturally occurring HDV mutants with deletions or insertions of a few nucleotides have been reported, but large fragment genomic deletions have never been observed [11]. A major concern of such findings was the possibility of PCR artifact. Several reasons argue against this view. Firstly, the authenticity of deletion could be confirmed by use of primers located in the deleted region as in Patient-1 (Fig. 1A and B). Such confirmation experiments were also done for Patient-2 and other patients (data not shown). Secondly, the deletion mutants in both cases were detected over a considerably long period of time (2–10 months), suggesting that the deletion mutants were likely stably persistent in the patients, which raised the question of whether it was capable of replication. Finally, northern blot experiments using liver biopsy samples revealed replication intermediates (genomic and anti-genomic senses) with a high molecular weight HDV RNA species (presumably derived from the mutant), instead of the 1.7 kb (wild type) HDV RNA.

By use of the cDNA clone carrying the deletion, defective HDV RNA was generated by in vitro transcription. Transfection of this 1.28 kb defective mutant (genomic and anti-genomic) into hepatoma cell lines also resulted in generation of high molecular weight HDV RNA replication intermediates. Interestingly, a major species of 6.5 kb was found. Previously, some HDV deletion mutants have been artificially generated for studies to understand the minimal length of sequence and important *cis*-elements required for HDV replication. In all studies, the ribozyme domains were believed to be essential and were absolutely required for HDV replication. Unexpected, the ribozyme domains in both orientations were absent from all of the deletion clones identified in this report.

The genome of HDV is circular and it is replicating via a double rolling circle mechanism. The fact that HDV ribozyme is absent from the genome of HDV deletion variants suggests that these HDV variants might employ a unique mechanism for replication. In some viroids, replication is achieved through an asymmetric replication pathway with only one rolling circle, where linear oligomeric antigenomic strands are formed and used as templates for synthesis of the genomic strands. The genomic strands are then processed by an RNase III-like enzyme (but not ribozyme), resulting in monomeric sequences [12]. In this model, the ribozyme sites may not be needed. In HDV, previous studies have indicated that in vitro transcribed HDV linear RNA is able to initiate HDV RNA replication in cultured cells, leading to the accumulation of HDV RNA circles [13,14]. It has also been proposed that host RNA polymerase can undergo intra-molecular template switch to produce HDV RNA circles [15]. Therefore, based on our northern blot and sequencing data, we hypothesize that during HDV replication, an intra-molecular template-switch occurs, resulting in deletion mutation. A small amount of the mutant could re-circularize through a putative host RNA ligase. Then, replication occurs through the rolling circle mechanism to generate high molecular weight concatemers. These concatemers serve as templates to generate the other sense of multiples and so on. In this model, no ribozyme activity is involved and therefore no unit-length HDV RNA is generated.

In Huh7-HBV cells, where complete HBV replication was maintained, the HDV mutant genome with suitable length was secreted (Fig. 4). Presumably, such processes required HBsAg. Because of such requirement, in patients with lower HBsAg levels, it is possible that multiple forms of replication competent, high molecular weight HDV RNA mutants could accumulate in hepatocytes. At this time, it is not clear why only transfection of the antigenomic-sense but not genomic-sense transcripts allows for mutant virion maturation and secretion in Huh7-HBV cells. We speculate that perhaps exogenous antigenomic-sense transcript can be directly used for mutant HDAg translation and thus facilitate the virion maturation. On the other hand, it is also unclear why only Huh7-HBV but not HepG2-HBV cells support virion maturation and secretion. It is likely that other Huh7 specific cellular factors are involved in this process.

In conclusion, novel large fragment deletion mutants were found in HDV infected patients receiving antiviral therapy. Northern blot analysis using the liver biopsy tissues as well as the mutant-transfected hepatoma cells revealed novel high molecular weight HDV RNA replication intermediates. Our data suggested a ribozyme-independent replication mechanism, where only mutant HDV RNA concatemers were generated but unit-length HDV RNA could not be produced.

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