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RNA Recombination of Hepatitis Delta Virus in Natural Mixed-Genotype Infection and Transfected Cultured Cells

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Most RNA viruses encode their own RNA polymerases for genome replication, and increasing numbers of them appear to be capable of undergoing RNA recombination. Here, we provide the first report of intergenotypic recombination in hepatitis delta virus (HDV), the only animal RNA virus that requires host RNA polymerase(s) for viral replication. In vivo, we analyzed RNA species derived from the serum of a patient with mixed genotype I and genotype IIb HDV infection by using multiple restriction fragment length polymorphism (RFLP) assays and sequence analysis of cloned reverse transcription (RT)-PCR products. Six HDV recombinants were isolated from 101 tested clones, and HDV recombination in this patient was further confirmed by RT-PCR with genotype-specific primer pairs. Analysis of the recombination junctions suggested that the HDV genome rearrangement occurred through faithful homologous recombination. We then used an RNA cotransfection cell culture system to investigate HDV RNA recombination in vitro. We found that HDV recombinants could indeed be detected in the transfected cells; some of these possessed recombination junctions identical to those identified in vivo. Furthermore, using a PCR-independent RNase protection assay, we were able to readily identify the recombined HDV RNA species in cultured cells. Taken together, our results demonstrate that HDV RNA recombination occurs in both natural HDV infections and cultured cells, thereby presenting a novel mechanism for HDV evolution.

Hepatitis delta virus (HDV) is a subviral agent of the hepatitis B virus that requires hepatitis B surface antigen for its assembly and transmission (48, 49). HDV is unique among animal viruses in that it resembles some plant pathogens, such as viroids, in having a circular RNA with an unbranched rod-like structure (25, 59), and a replication strategy that utilizes host RNA polymerases (Pols) (37, 41). Upon entering a cell, genomic HDV RNA is transcribed into complementary subgenomic mRNA for the delta antigen (HDAg) (15) and multimeric antigenomic RNAs (31, 57). These then undergo self-cleavage and ligation to generate unit-length circular RNA (27, 46, 53, 63). Similarly, the circular antigenomic RNA serves as a template for production of additional genomic RNA.

In both virions and cells, the HDV RNA forms a ribonucleoprotein complex with HDAg (9, 35). There are two forms of HDAg, small and large, which play distinct biological roles. The small HDAg (195 amino acids) is required for HDV RNA replication (8, 29). The large HDAg is produced through specific RNA editing by the small form of the cellular enzyme ADAR1 (20, 44, 62), which converts the UAG stop codon for the small HDAg into a UGG tryptophan codon (36). This process leads to translation of an additional 19 amino acids and creates the large HDAg (214 amino acids), which in turns inhibits genome replication and is required for packaging (6, 8).

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Viral hepatitis due to HDV infection has been found worldwide, and the complete nucleotide (nt) sequences of HDV genomes have been reported from many locations (5, 10, 11, 16, 28, 32, 38, 51, 59). Comparison of these by Casey et al. (5) indicated that there are at least three HDV genotypes: I, II, and III. HDV genotype III has been isolated only in northern South America (5) and is associated with severe acute hepatitis, whereas the milder HDV genotype II is found primarily in Asia (64). In contrast, HDV isolates of genotype I have been reported in every part of the world, and the pathogenesis of genotype I infections varies from fulminant hepatitis to asymptomatic chronic liver disease (10, 11, 16, 32, 38, 51, 59). Phylogenetic analysis of additional HDV sequences further suggested that HDV genotype IIb (isolated from Taiwan and the Miyako Islands, Okinawa, Japan) and genotype IIb-M (isolated from the Miyako Islands) form genetic subgroups (50, 60, 61, 65). It is worth noting that most of the above-mentioned sequences were isolated from non-African countries. Recently, characterization of 22 African sequences revealed that 15 of them form three new lineages and that the other 7 are scattered within genotype I (45). These analyses also suggested that genotype IIb sequences form a distinct clade instead of being a subtype of genotype II. Therefore, recent work has indicated that the various HDV genotypes fall into at least seven clades (45). Interestingly, HDV genotype I is the only genotype detected in some locations, including Europe and North America (5, 61), whereas multiple genotypes have been detected in Africa (45) and in Asian locations, including Japan, Russia, and Taiwan (16, 17, 33, 60, 61, 65). Furthermore, mixed infections of genotypes I and II or II and IIb have been reported in Taiwan (66). However, the roles of these mixed HDV

TABLE 1. List of primers used for PCR amplification of the HDV genome

Primer type	Primer name	Sequence (5′-3′)	Location (nt)
Consensus	18	ATGCCATGCCGACCCGAAGAGGAA	889–912
	55′	GGAGAGACGGGATCACCGAAGAAGGAAGGC	1310–1281
Genotype specific	I5′-1	TGGAAACCCGCTTTATTCAC	940–959
	I3′-1	CAAGAGGAAGCAGCTATCGG	1271–1252
	I3′-2	CAAGAGGAAGCAGCTATCGT	1271–1252
	IIb5′-1	TGGAACCCTCGATCCTTTAT	940–959
	IIb3′-1	CAAGAAGAAGCAGCTCTCCT	1271–1252

infections in viral biology and evolution remain to be elucidated.

It has been reported that specific RNA editing as well as random mutations occurring during RNA replication lead to sequence heterogeneity in the HDV genome (5, 12, 16, 32, 36, 42, 55, 59). Genetic recombination is an important process for eliminating detrimental mutations and ensuring greater genetic diversity. Recombination occurs in mixed infections of some closely related RNA viruses that encode their own RNA polymerases (30) and in viroids that are dependent on the cellular enzymatic activities for replication (1, 4, 14, 23, 24, 43, 47, 52, 54). However, although phylogenetic analysis suggests that genetic recombination can occur both between and within HDV genotypes (67), HDV recombinants have never been isolated from HDV mixed-genotype infections (66) either in nature or in the laboratory. In this report, we demonstrate that intergenotypic recombination of HDV RNA occurs both in a patient with a mixed genotype I and IIb infection and also in a RNA cotransfection cultured cell system.

MATERIALS AND METHODS

RNA extraction and RT-PCR. HDV-related sequences were characterized from serum sample HDM2-18, which was derived from a 39-year-old chronic hepatitis patient (patient HDM-2) who was positive for serum hepatitis B surface antigen and antibodies to HDV. Total RNA was extracted from the serum using the TRI REAGENT LS for liquid samples (Molecular Research Center, Inc.) as described previously (60). In vitro-transcribed HDV RNAs from pG4B-D1I containing a full-length genotype I cDNA clone obtained from the woodchuck-adapted HDV genome of an Italian patient (28, 60), pG4B-D1IIb (containing a full-length cDNA of the HDV genome derived from the Taiwan-IIb-1 clone of genotype IIb HDV) (60), and pG4B-D1II-1 (containing a full-length cDNA of the HDV genome derived from the Taiwan-3 clone of genotype II) (34) were synthesized as described previously (34, 60) and used as controls for the subsequent genotyping and recombination analyses. Reverse transcription (RT)-PCR amplification was performed using a Titan One Tube RT-PCR system (Boehringer Mannheim). The reaction conditions were as reported elsewhere (60), except that a longer elongation time (2 min) and fewer cycles (25 to 30 cycles) were employed to suppress in vitro recombination between related PCR template sequences, as suggested in a previous report (21). The sequences of the primers used are summarized in Table 1. The nucleotide numbering system used in this report is in accordance with that of Wang et al. (60). Primers 18 and 55′ were consensus primers used to amplify sequences of all HDV genotypes. The resulting fragment 1855′ represents a region (nt 889 to 1310) that is generally used for the classification of HDV genotypes (5). A second region (nt 940 to 1271) was produced using genotype-specific primer pairs (I5′-1–IIb3′-1 and IIb5′-1–I3′-2) for specific amplification of 5′-I–IIb-3′ and 5′-IIb–I-3′ recombinants, respectively. Total RNA was also extracted from cultured cells cotransfected with genotype I and IIb RNAs and then subjected to RT-PCR using primer pair 18-55′ for HDV genotyping analysis, as well as genotype-specific primer pairs I5′-1–IIb3′-1 and IIb5′-1–I3′-1 for the examination of recombination junctions.

HDV genotyping and analysis of HDV RNA recombinants. XhoI restriction fragment length polymorphism (RFLP) analysis, previously shown to effectively differentiate between HDV genotypes within PCR fragment 1855′ (nt 889 to 1310) (64), was used to screen mixed infections of HDV. Genotypes I, IIb, II, and III yielded different XhoI-digested PCR products, so mixed HDV infections could be defined by RFLP patterns showing combinations of the variously sized bands. PCR products showing an XhoI RFLP pattern with multiple bands were then cloned into a T vector (TOPO TA cloning vector; Invitrogen). Plasmids were extracted from 101 colonies and subjected to double digestion with EcoRI (to release the HDV cDNA inserts) and XhoI (to differentiate the HDV genotypes). To further investigate the sequence heterogeneity of the HDV-related sequences, clones were also subjected to multiple RFLP assays, including double digestion of clones with EcoRI plus SacII and EcoRI plus SphI. Finally, 10 clones each of genotype I and IIb candidates and clones with unique RFLP patterns (clones 8, 23, 59, 60, 83, and 85) were subjected to sequence analysis using an ABI 377 DNA sequencing system (Perkin-Elmer/Applied Biosystems). For analysis of genotype-specific amplicons (nt 940 to 1271), the PCR products were cloned into the TOPO TA cloning vector (Invitrogen), and 10 clones of each recombination type were subjected to a XhoI RFLP assay and sequence analysis.

RNA transfection and posttransfection analysis of HDV RNA recombinants. COS7-SmT1 cells stably expressing small HDAg from an integrated genotype I small-HDAg-encoding plasmid under the control of the simian virus 40 late promoter (34) were selected and cultured as described previously (60), with the addition of 100 μg of gentamicin/ml. One day before transfection, COS7-SmT1 cells (5×10^5) were seeded in six-well (35-mm-diameter) plates. Cells were transfected with HDV RNA monomers for initiation of HDV genome replication, as reported previously (13). Transfections were carried out with RNA mixtures containing 1 μg each of in vitro-transcribed 1.7-kb genotype I (28, 60) and genotype IIb (60) RNAs by using 2 μl of SuperFect transfection reagent (QIAGEN), according to the manufacturer's instructions. The RNAs were transcribed in vitro from linearized plasmids pG4B-D1I and pG4B-D1IIb as previously described (60). After in vitro transcription, the template DNA was removed with RNase-free DNase I (Promega), and the RNA transcripts were purified by using an RNeasy mini kit (QIAGEN). Six days posttransfection, RNA was extracted and subjected to RT-PCR and XhoI RFLP genotyping. In addition, HDV RNA recombinants were amplified by RT-PCR using genotype-specific primers as described above, inserted into the TOPO TA vector, and sequenced.

HDV RNA recombinants with recombination junctions mapping to nt 1159 to 1207 were further confirmed by an RPA III RNase protection assay, according to the manufacturer's instructions (Ambion). Briefly, a [^{32}P]UTP-labeled probe was transcribed with T7 RNA polymerase from BamHI-linearized TOPO-R6 plasmids (containing a 5′-I–IIb-3′ recombinant with a crossover region located between nt 1159 and 1207), to produce HDV RNA in the antigenomic orientation. The HDV insert in the TOPO-R6 plasmid was also subcloned into another T vector (YT&A; Yeastern Biotech). The resulting plasmid, YT&A-R6, was digested by BamHI and transcribed with T7 RNA polymerase to produce a genomic RNA containing a 5′-I–IIb-3′ recombinant with the crossover region located between nt 1159 and 1207. Three genomic HDV RNAs transcribed in vitro from linearized YT&A-R6, pG4B-D1I, and pG4B-D1IIb (60) were also hybridized with the probe and served as controls for RNase protection. Approximately 10^6 cpm of labeled probe was hybridized with 10 μg of total RNA extracted from transfected cells. After hybridization, the sample was digested by RNase A and T1, precipitated, resuspended in gel loading buffer, and subjected to gel electrophoresis in a 5% acrylamide–8 M urea gel. The protected bands were finally visualized by autoradiography.

Nucleotide sequence accession numbers. The sequences discussed in this work can be found in the GenBank database under accession numbers M21012, AF209859, AY452981, AY526577, AY546081, and AY546082.

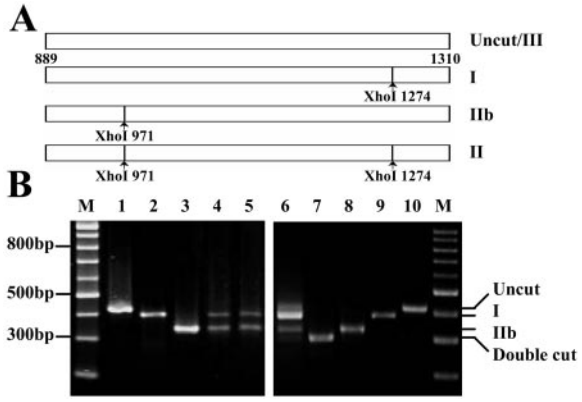

FIG. 1. XhoI RFLP analysis for detection of different HDV genotypes in a mixed infection. (A) XhoI restriction sites in the amplified sequences of different genotypes. The PCR products of genotypes I and IIb each have a single XhoI site, at nt 1274 and 971, respectively. Genotype II PCR products contain both of these XhoI sites, whereas genotype III contains no XhoI site in this region. (B) The XhoI-cleaved PCR products of HDV genomes were electrophoresed on a 3% agarose gel and stained with ethidium bromide. Lanes: M, 100-bp-ladder molecular size markers; 1 and 10, undigested PCR products; 2 and 9, digested genotype I PCR products; 3 and 8, digested genotype IIb PCR products; 4, digested PCR products of mixed in vitro-transcribed genotype I and IIb RNAs; 5, digested PCR products of mixed total cellular RNAs extracted from cells transfected separately with genotype I and IIb RNAs; 6, a complex RFLP pattern obtained from the HDV patient; 7, digested genotype II PCR products.

RESULTS

Discovery of a complex XhoI RFLP pattern in an HDV PCR product from patient serum. Analysis of HDV isolates from around the world has revealed multiple phylogenetically distinct genotypes (5, 16, 17, 33, 45, 60, 61, 65), and researchers have extensively studied the genetic heterogeneity of HDV sequences in various single-genotype infections and transfections, of both animals and cultured cells (5, 12, 16, 26, 32, 36, 42, 55, 59). Although mixed-genotype infections of HDV have been reported (66), there has been little previous work investigating the genetic diversity in mixed-genotype HDV infection. To address this issue, we examined the HDV-related RNA species present in mixed-genotype HDV infections. HDV genotyping based on the XhoI RFLP pattern of reverse-transcribed PCR products covering nt 889 to 1310 was used to screen HDV samples as previously described (64). Within the amplified fragment, genotypes I and IIb each have a single XhoI site, at nt 1274 and 971, respectively (Fig. 1A), genotype II contains both of these XhoI sites, and genotype III does not contain any XhoI site. Consequently, after XhoI digestion of the PCR products, the larger bands of genotypes I, IIb, and II were 387 bp (Fig. 1B, lanes 2 and 9), 340 bp (lanes 3 and 8), and 304 bp (lane 7), respectively, in size. Mixed infections of HDV were thereby defined by RFLP patterns showing combinations of the above-described bands.

To confirm that this RFLP assay was able to detect various viral genotypes in the same patient, and to rule out the possibility that the detected bands were artifacts generated during RT-PCR, equal amounts of in vitro-transcribed HDV genotype I and IIb RNAs were mixed together for a positive control analysis. As expected, only two bands corresponding to the sizes of the input HDV RNAs were observed (Fig. 1B, lane 4). Similar results were obtained when a mixture of genotype I and genotype IIb RNAs extracted from separately transfected cells was subjected to RT-PCR and XhoI RFLP analysis (lane 5). It has been reported that strand mixing during PCR could result in formation of heteroduplex products containing a restriction site on one strand but not the other, thus leading to digestion resistance (21). Under the reaction conditions used in this study, the XhoI-undigested band was extremely faint or undetectable (Fig. 1B, lanes 4 and 5), suggesting that this was not a problem in our experiments. Artificial recombination is known to occur between related sequences during PCR, leading to the generation of different RFLP patterns, but this may be suppressed by using PCR programs with increased elongation times and a minimal number of cycles (21). Using these PCR conditions, we performed RT-PCR on the premixed RNAs and inserted the resulting fragment into a T vector with two EcoRI cutting sites flanking the insertion site. Plasmid DNAs extracted from 100 colonies were then subjected to double digestion with EcoRI (leading to the release of HDV cDNA inserts) and XhoI (allowing differentiation of the HDV genotypes). As expected, no recombinants were detected under these conditions.

In marked contrast, when RNA extracted from the serum of a mixed-infection HDV patient was analyzed in this way, we observed complex RFLP patterns, including four bands (Fig. 1B, lane 6). The electrophoretic mobilities of three of these bands matched genotypes I (lane 9), IIb (lane 8), and II (lane 7), with the fourth band corresponding to the size of the uncleaved PCR product (lane 10). Although genotype III has no XhoI cutting site within the amplified region, it has been found only in northern South America and is associated with severe acute hepatitis (5). Thus, it is unlikely that this patient would bear genotype III. However, it is theoretically possible that this patient's serum contained unidentified novel HDV sequences that were not recognized by our PCR-RFLP strategy. Overall, our finding of a variety of HDV-related RNA species in the same patient suggests that genetic diversity of clinical HDV is much more complicated than previously thought.

Identification of HDV recombinants in a mixed-genotype HDV infection. To further investigate the HDV RNA species present in this case, we next cloned the PCR products into T vectors, extracted plasmid DNA from 101 colonies, and subjected the samples to EcoRI-XhoI double digestion. HDV genotypes I and IIb were identified in 70 and 25 clones, respectively, representing a ratio of 3:1. This ratio was consistent with the relative intensities of the bands corresponding to HDV genotypes I and IIb in the RT-PCR–XhoI RFLP assay (Fig. 1B, lane 6). As summarized in Table 2, clones 8, 23, and 85 showed the genotype II XhoI RFLP pattern (containing two XhoI sites), and clones 59, 60, and 83 were unable to be cleaved by XhoI. According to a previous report, SacII sites were present in the PCR products of most genotype I and II Taiwan isolates (64). By sequence analysis of the published data, we found that a SphI (nt 1091) site was highly conserved in genotype IIb Taiwan clones, while such a conserved site was not observed in genotype II Taiwan clones and was present only in some genotype I sequences. Furthermore, SacII and SphI restriction sites were not found in the PCR products of genotype III HDV. Therefore, the genome organization of 10

TABLE 2. Summary of multiple RFLP patterns observed in clones carrying HDV genomes obtained from patient serum

Restriction site (nt)	Cleavage in[a]:								
	Genotype I clones	Genotype IIb clones	Clone no.						Genotype II T3 clones
			8	23	85	59	60	83	
XhoI (971)	−	+	+	+	+	−	−	−	+
XhoI (1274)	+	−	+	+	+	−	−	−	+
SacII (1160)	−	+	−	+	−	−	+	+	+
SphI (1091)	+	+	+	−	+	+	+	+	−

[a] Cleavage was present (+) or absent (−) in the indicated clone(s).

clones of each genotype I and IIb candidates, three clones of a potential genotype II candidate, and three XhoI-undigested plasmids were further evaluated by SacII and SphI RFLP analysis.

As summarized in Table 2, all of the tested genotype I clones were sensitive to SphI and resistant to SacII, while all tested genotype IIb clones were sensitive to both enzymes. Surprisingly, the SacII- and SphI-RFLP patterns for one potential genotype II clone (clone 23), but not the other two (clones 8 and 85), were identical to the RFLP patterns of a control genotype II T3 clone (33, 34). The SacII and SphI RFLP patterns for the three XhoI-undigested clones (clones 59, 60, and 83) were not identical to those expected for the published genotype III clones and not consistent with one another. Therefore, a significant degree of sequence heterogeneity was present in the populations with or without two XhoI sites.

To evaluate genomic diversity, all clones analyzed by SacII and SphI RFLPs were then subjected to sequence analysis. For the analyzed clones of genotypes I and IIb, the genotyping results obtained by multiple RFLP assays were in complete agreement with those from sequencing analysis. Two representative sequences for each genotype are shown in Fig. 2A and B. Interestingly, the sequencing data for the six clones with or without two XhoI sites revealed that they were recombinants between parental genotype I and IIb sequences (Fig. 2C), instead of being genotype II, genotype III, or new variants. Based on the assumption that only one recombination event occurred between two XhoI sites, the 5′-I–IIb-3′ recombinant should not contain a XhoI site (Fig. 2D). Similarly, sequences containing two XhoI sites could be detected in 5′-IIb–I-3′ recombinants (Fig. 2D). Therefore, the genome organization obtained from the XhoI RFLP assays was consistent with that from our sequence analysis, except that the latter identified the recombination junctions (Fig. 2C and Table 3). Four crossover regions were identified: nt 1071 to 1085, 1087 to 1102, 1134 to 1148, and 1186 to 1207. The last two could be detected in the reciprocal recombinants, 5′-I–IIb-3′ and 5′-IIb–I-3′. Mixed infections of HDV genotypes I and II and of genotypes II and IIb have been reported previously (66), but this is the first report of a mixed infection of genotypes I and IIb in a human patient. Furthermore, the identification of recombinants in this patient indicates for the first time that HDV RNA recombination occurs during natural mixed-genotype infection.

Detection of HDV recombinants using genotype-specific RT-PCR primers. As shown above, we observed RT-PCR products corresponding to recombinant RNAs from mixed-genotype infection of HDV. However, large-scale RFLP and sequencing of cloned PCR products is time-consuming and labor-intensive. Therefore, we next studied HDV RNA by using genotype-specific RT-PCR primer pairs to specifically amplify 5′-I–IIb-3′ and 5′-IIb–I-3′ recombinants (covering nt 940 to 1271). This method had the benefit that the genotype-specific primers were able to suppress potential artificial recombination during PCR, as described previously (19, 56). cDNAs were produced from primers specific to either genotype I or genotype IIb; thus, the use of the recombinant-specific primers in the subsequent PCRs should amplify only the recombinant sequences. When in vitro-transcribed genotype I and IIb RNAs were mixed and then subjected to RT-PCR using the recombinant-specific primer pairs, no signals were observed (Fig. 3, lanes 1 and 2), suggesting that recombination did not occur during RT-PCR amplification. However, bands corresponding to the potential 5′-I–IIb-3′ (lane 3) and 5′-IIb–I-3′ (lane 6) recombinants were observed in samples amplified from the patient's serum. As expected, 5′-I–IIb-3′ recombinants were resistant to XhoI digestion (lane 4), while 5′-IIb–I-3′ recombinants were sensitive to XhoI digestion (lane 5).

To investigate the recombination junctions, PCR products were cloned into T vectors, and 10 clones each of the 5′-I–IIb-3′ and 5′-IIb–I-3′ recombinants were subjected to sequencing analysis. As summarized in Table 3 and Fig. 4, the crossover regions were mapped to nt 1087 to 1102, 1110 to 1125, 1134 to 1148, 1172 to 1184, 1186 to 1207, and 1211 to 1222. Three of these (nt 1087 to 1102, 1134 to 1148, and 1186 to 1207) were also detected in recombinants when the consensus primers were used during PCR. Detection of the same recombination junctions by two independent analyses further proved that the observed HDV RNA recombination occurred in the patient rather than being a random event. Sequence analysis of the recombinants clearly illustrated that the observed HDV recombination was likely a homologous process, since all crossovers occurred at homologous regions between the two parental HDV RNAs, and no insertions, deletions, or mismatches were observed (Fig. 4). No consensus sequence was identified around the HDV recombination junctions (Fig. 4).

Although the two XhoI sites span nt 971 to 1274, the observed HDV recombination sites were clustered from nt 1071 to 1222 (Fig. 4). However, this is explained by the fact that nt 971 to 1070 overlaps the highly divergent C terminus of the large HDAgs, which would not favor homologous recombination. The observation that two pairs of crossover regions (nt 1071 to 1085 and 1087 to 1102, and nt 1172 to 1184 and 1186 to 1207) were separated from each other by only a single nucleotide further implicates the nonrandom distribution of crossover regions for HDV recombination.

HDV RNA recombination in cultured cells. We recently showed that a cloned HDV genotype IIb (Taiwan-IIb-1) could replicate in cultured cells (60). To mimic the natural mixed HDV infection, we cotransfected in vitro-transcribed genomic genotype I (28, 60) and IIb (60) RNAs into COS7 cells stably expressing small HDAg. Six days after transfection, RNA was extracted from the cells and subjected to RT-PCR with consensus primer pair 18–55′. Similar to our results with the natural infection, multiple bands were observed after XhoI digestion of the PCR products (Fig. 5A, lane 4). Similarly, multiple bands were detected after XhoI RFLP analysis of PCR products from cells cotransfected with antigenomic genotype I and

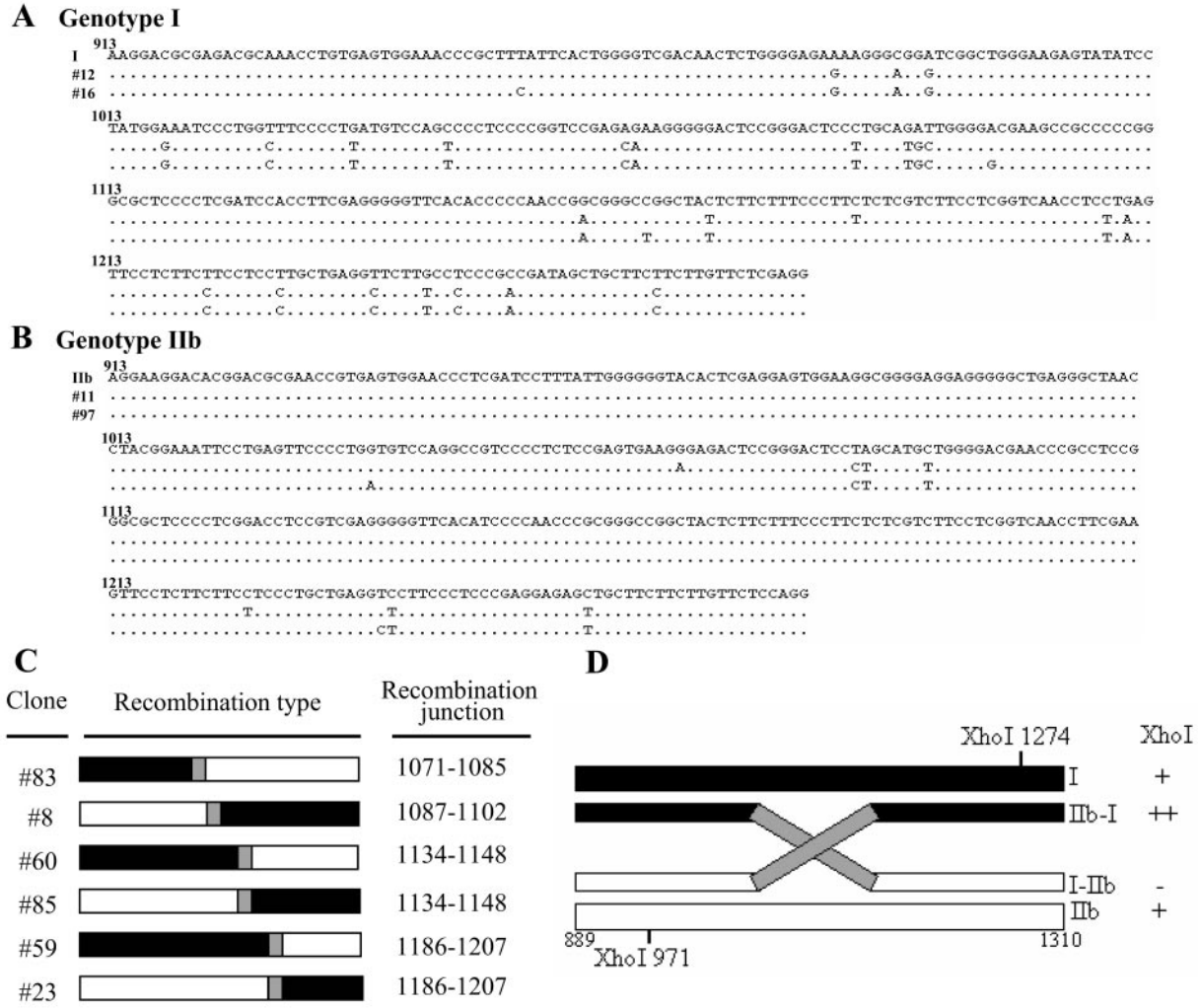

FIG. 2. HDV-related sequences detected in the serum of a patient with mixed genotype I and IIb infection. (A and B) Alignment of nucleotide sequences (nt 913 to 1280) from representative HDV isolates of genotypes I (A) and IIb (B), and sequences identified in the mixed infection of HDV. Dots indicate conserved nucleotides. Sources of the representative isolates of different genotypes are as follows: I, HDV genotype I isolated from Italy (GenBank accession number M21012) (28); IIb, genotype IIb from the Taiwan-IIb-1 clone (GenBank accession number AF209859) (60). The sequences obtained from two clones each of HDV genotypes I (clones 12 and 16) and IIb (clones 11 and 97) from the mixed infection are shown. (C) Schematic demonstration of HDV recombinants identified by sequence analysis. The recombination junctions are shaded in gray and summarized on the right. The genotype I and IIb sequences are indicated by closed and open bars, respectively. (D) Restriction maps of the potential clones carrying recombinant HDV genomes. The XhoI RFLP profiles and the predicted genome organization of recombinants 5′-I–IIb-3′ and 5′-IIb–I-3′ are summarized on the right.

IIb RNAs (data not shown). The sizes of the two major bands (lane 4) were undistinguishable from those of parental genotypes I and IIb (lanes 2 and 3), while the sizes of the two faint bands (lane 4) were consistent with those of uncleaved (lane 1) and double-cleaved bands. These data imply that HDV RNA recombination also occurred in cultured cells.

To further investigate the molecular nature of the HDV recombinants in cultured cells, genotype-specific primers were used for RT-PCR, and the recombination junctions of the cloned PCR products were analyzed. The crossover regions of 10 clones each of the 5′-I–IIb-3′ (designated TOPO-R1~R10) and 5′-IIb–I-3′ (designated TOPO-R11~R20) recombinants were analyzed (Fig. 5B). The results indicated five crossover regions, mapping to nt 1050 to 1054, 1110 to 1125, 1134 to 1148, 1159 to 1207, and 1212 to 1229. Again, all crossovers

TABLE 3. Categories of recombinant genomes and localization of recombination junctions

Localization of recombination junctions (nt)	No. of recombinant genomes identified by PCR using:			
	Consensus primers		Genotype-specific primers	
	I-IIb	IIb-I	I-IIb	IIb-I
1071–1085	1	0	0	0
1087–1102	0	1	2	0
1110–1125	0	0	1	1
1134–1148	1	1	1	2
1172–1184	0	0	1	1
1186–1207	1	1	3	4
1211–1222	0	0	2	2

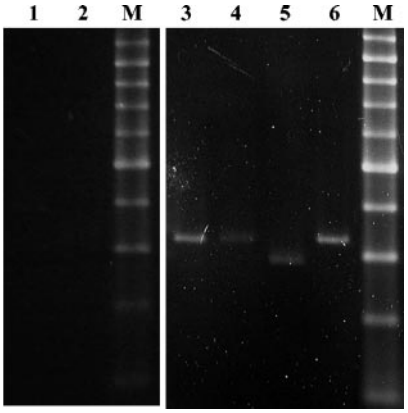

FIG. 3. Detection of HDV recombinants by use of genotype-specific primers. cDNAs generated with genotype I- or IIb-specific primers served as the templates for subsequent PCRs with recombinant-specific primer pairs. The PCR products were cleaved with XhoI, separated on 3% agarose gels, and stained with ethidium bromide. The observed 5′-IIb–I-3′ recombinant contains one XhoI site (nt 971), whereas the 5′-I–IIb-3′ recombinant lacks a XhoI site. Lanes: M, 100-bp-ladder molecular size markers (the dominant band is 500 bp in size); 1 and 2, undigested PCR products of mixture of in vitro-transcribed genotype I and IIb RNA by use of primer pairs I5′-1–IIb3′-1 and IIb5′-1–I3′-1, respectively; 3 and 6, undigested PCR products obtained from the HDV patient with primer pairs I5′-1–IIb3′-1 and IIb5′-1–I3′-2, respectively; 4 and 5, digested PCR products obtained from the HDV patient with primer pairs I5′-1–IIb3′-1 and IIb5′-1–I3′-2, respectively.

occurred at homologous regions shared between the two parental HDV RNAs (Fig. 5B). Although the transfected sequences were different from those identified in the mixed-genotype infection (Fig. 2A and 2B), four crossover regions (nt 1110 to 1125, 1134 to 1148, 1159 to 1207, and 1212 to 1229)

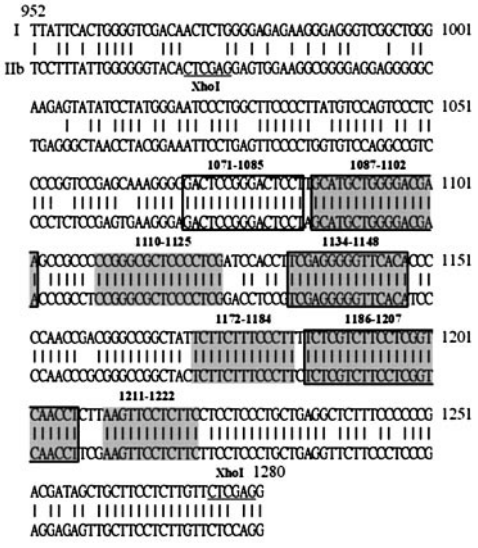

FIG. 4. Primary structure of the crossover regions of intergenotypic HDV recombinants identified in a natural mixed-genotype HDV infection. Genomic sequences (nt 952 to 1280) of HDV genotypes I and IIb are given. Short lines depict the homologous bases between two genotypes. The crossover regions identified using genotype-specific primers are indicated by gray shading, while those obtained using consensus primers are indicated by rectangles.

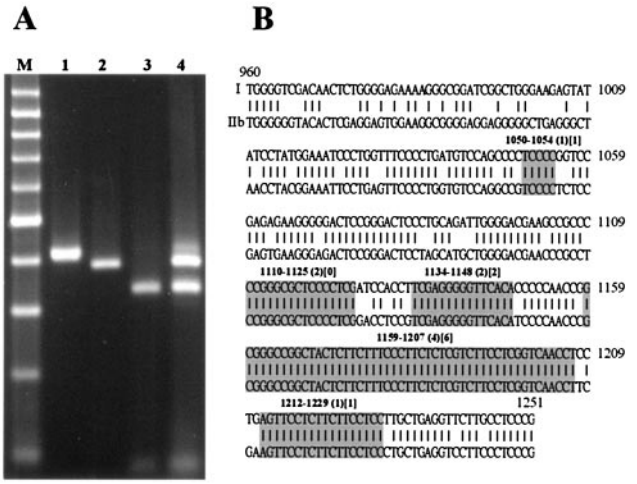

FIG. 5. HDV recombination in transfected cultured cells. (A) XhoI RFLP analysis of PCR products amplified from total RNA extracted from cotransfected cultured cells by use of consensus primers. Lanes: M, 100-bp-ladder molecular size markers (the dominant band is 500 bp in size); 1, undigested PCR products; 2, digested genotype I PCR products; 3, digested genotype IIb PCR products; 4, a complex RFLP pattern obtained from transfected cultured cells. (B) Primary structure of the crossover regions of intergenotypic HDV recombinants identified in transfected cultured cells by use of genotype-specific primers. The nucleotide sequences of the genomic segments (nt 960 to 1251) of HDV genotypes I (Italian clone) and IIb (Taiwan-IIb-1 clone) are given. Short lines depict the homologous bases between the two genotypes. The crossover regions identified are indicated by gray shading. Numbers given in parentheses and square brackets indicate the number of 5′-I–IIb-3′ and 5′-IIb–I-3′ recombinant clones, respectively.

detected in the cotransfected cultured cells were identical to or overlapped with those observed in the patient's serum (Fig. 4 and 5B). Therefore, this work shows the establishment of a cotransfected cell culture system that could serve as an experimental system for future study of HDV RNA recombination.

Detection of HDV recombinants using RNase protection assay. As shown in Fig. 5B, the recombination junction of 10 of 20 tested clones from the cotransfected cultured cells mapped to nt 1159 to 1207. This finding prompted us to directly detect the HDV RNA recombinants by an RNase protection assay using a 5′-I–IIb-3′ probe (in vitro transcribed from the TOPO-R6 plasmid) with the recombination junction located at nt 1159 to 1207. This method avoided the use of PCR amplification, thus guarding against the possibility that the detected recombinants were artifacts generated during RT-PCR. To avoid detection of HDAg mRNA, cells were cotransfected with antigenomic genotype I and IIb RNAs and the extracted RNA was hybridized with an antigenomic RNase protection probe specific to the HDV genomic RNA, which should be present only after HDV replication. As demonstrated in Fig. 6, a TOPO-R6 plasmid containing a recombinant HDV sequence (recombination junction nt 1159 to 1207) was transcribed in vitro to produce a 446-base RNA probe. After hybridization and RNase treatment, the protected bands for genotypes I and IIb were 267 and 113 bases, respectively. In contrast, genomic 5′-I-(1159 to 1207)–IIb-3′ recombinant sequences yielded a 332-base protected band. The probe alone was sensitive to RNase digestion (data not shown), whereas it was resistant to RNase treatment after hybridization with an in vitro-tran-

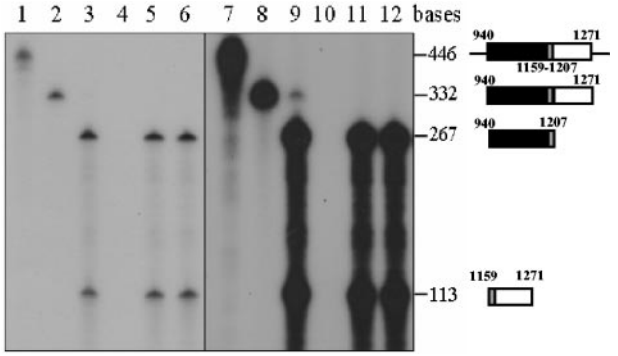

FIG. 6. Detection of HDV RNA recombinants with an RNase protection assay. Probes specific to the HDV RNA recombinant with the crossover region located at nt 1159 to 1207 were synthesized in vitro, hybridized with various RNA samples, and subjected to RNase digestion for detection of the HDV RNA recombinants. Lanes 7 to 12 represent the results of a longer exposure of the data shown in lanes 1 to 6. Lanes: 1 and 7, no-target, no-RNase control lanes corresponding to the full-length probe; 2 and 8, probe hybridized with a complementary in vitro-transcribed HDV RNA; 3 and 9, probe hybridized to RNA samples extracted from cotransfected cultured cells; 4 and 10, probe hybridized to RNA samples extracted from untransfected cells; 5 and 11, probe hybridized to mixtures of in vitro-transcribed genotype I and IIb RNAs; 6 and 12, probe hybridized to mixtures of total RNA extracted from cells independently transfected with genotype I and IIb HDV RNAs. The probe and protected bands are schematically depicted on the right. The genotype I, genotype IIb, and vector sequences are indicated by a closed box, open box, and thin line, respectively.

scribed complementary recombinant sequence (Fig. 6, lane 2). When RNA samples extracted from cotransfected cells were subjected to the RNase protection assay, we observed two dominant bands corresponding to genotypes I and IIb (lane 3). The gel was then subjected to extended film exposure (lanes 7 to 12) to detect the less common HDV RNA recombinants. As expected, a 332-base band identical to that of the protected HDV RNA recombinant of interest (lane 9) appeared following the longer exposure. Furthermore, when a mixture of in vitro-transcribed genotype I and IIb HDV RNAs was subjected to this RNase protection assay, only two bands with the expected sizes (267 and 113 bp) were observed (lanes 5 and 11). Similarly, when genotype I and IIb RNAs obtained from cells separately transfected with genotype I and IIb RNAs were mixed with the probe and subjected to the RNase protection assay, no recombinant bands were observed (lanes 6 and 12). No band was found when RNA extracted from untransfected cells was subjected to the RNase protection assay (lanes 4 and 10). This finding confirmed the recombination junction in an RT-PCR-independent fashion, showing that the result was not a PCR-based artifact. Taken together, these data suggest that HDV RNA recombination did occur at the junction mapped to nt 1159 to 1207 in cultured cells expressing two replicating HDV clones. The identification of recombinant HDV RNAs by an RNase protection assay provided direct evidence for HDV RNA recombination in cultured cells.

DISCUSSION

Although phylogenetic analysis has suggested that genetic recombination can occur in HDV (67), HDV RNA recombinants have not been previously identified from natural mixed-genotype infections (66). Here, we provide the first identification of HDV RNA recombinants in natural mixed-genotype infections and cotransfected cultured cells.

In this study, PCR methods were used to investigate HDV RNA recombination. Several lines of evidence showed that the HDV recombination observed here was not the result of artifacts caused by reannealing and extension of incompletely synthesized strands during PCR. First, control RT-PCR amplifications using template mixtures of two RNAs (either produced from in vitro transcription or extracted from single-transfection cells) showed no evidence of recombination. In contrast, HDV recombinants were readily detectable by RT-PCR from infected or cotransfected samples by using either consensus primers or genotype-specific primers. Second, instead of being randomly distributed, the crossover regions identified in this study all mapped to homologous regions between two RNA species. Furthermore, identical recombination junctions were observed in reciprocal 5′-I–IIb-3′ and 5′-IIb–I-3′ recombinants. Third, some crossover regions detected in the cultured cells were identical to or overlapped those observed in the patient's serum, even though the transfected sequences differed from those identified in the mixed-genotype infection. This nonrandom HDV RNA recombination suggests that the crossovers were guided by a mechanism that was more specific than premature termination and reinitiation during RT-PCR amplification. And, last, HDV RNA recombination at junctions identified from RT-PCR amplification could be detected in transfected cultured cells by an RNase protection assay (Fig. 6), which avoided PCR amplification. Taken together, these observations and controls clearly illustrate that true HDV RNA recombination was identified in this study.

HDV mixed infections of genotypes II and IIb and genotypes I and II have been reported, but RNA recombinants have not been identified in the individuals with these infections (67). It has been reported that the frequency of homologous recombination between more distantly related viruses is significantly lower (22, 39, 40, 58). However, the sequence homologies between genotypes I and II and genotypes II and IIb are 76 and 78%, respectively, and that between genotypes I and IIb is 75%. Therefore, sequence homology alone does not appear to explain the absence of HDV recombination in the previously reported mixed infections with genotypes I and II and genotypes II and IIb (66). Based on the fact that the minor strain of HDV always constituted less than 10% of the total colony population in the previous report (66), it is likely that the replication levels of each individual HDV strain in the mixed infections played an important role in determining the recombination frequency. In addition, genotype II HDV was detected in all of the mixed-genotype HDV infections in the previous report (66); recently, the replication level of a Taiwanese genotype II clone was shown to be 100-fold lower than that of a genotype I prototype (34). These observations support a hypothesis in which the HDV replication level is an important factor in dictating the HDV recombination rate. Thus, we propose that HDV RNA recombination is performed via a replication-dependent mechanism.

The template switching mechanism is a well-accepted model of recombination for RNA viruses encoding their own RNA polymerases. The idea that HDV RNA recombination is a replication-dependent event suggests that HDV recombina-

tion might also occur via a template switch mechanism. Recently, Pol II was implicated in intramolecular template switching, which is a process that can occur with precision or that can involve deletions and even nontemplated insertions (7). We propose that host polymerases might also have the ability to undergo precise intermolecular template switching during HDV replication to generate recombinants. In the conventional template switch mechanism, the polymerase pauses during RNA recombination, perhaps in response to RNA secondary structures around the recombination site, and then jumps from the original donor template to the acceptor template (18, 30). During HDV RNA replication, polymerase locally unwinds the duplex region of the HDV rod-like structure; the HDV genome is GC-rich, making it likely to form local secondary structures, such as hairpins, where the polymerase might pause and initiate a crossover. About 70% of the HDV genome is base paired to form the rod-like structure; however, only 53% of the bases are paired between nt 1182 to 1196 and the side opposite (nt 405 to 412) on the predicted HDV rod-like genome, perhaps suggesting that there is another mechanism causing the polymerase complex to dissociate from the template during HDV replication. The role of RNA secondary structures in facilitating HDV RNA recombination remains to be investigated.

HDV is the only animal virus that utilizes host polymerases to replicate the viral genome. From our data, we suggest that host RNA polymerases might have the ability to undergo precise intermolecular template switching during HDV replication to generate recombinants. According to this hypothesis, RNA recombination might also occur between cellular and viral RNAs. It has been proposed that HDV might have evolved from a viroid-like RNA via capture of a cellular transcript (2, 3). Indeed, a cellular homolog of HDAg has been claimed (3). Therefore, the original incorporation of the HDAg coding sequence and its complementary sequence (to maintain the rod-like structure) into the HDV viroid-like domain might have occurred through RNA recombination.

In this study, we found that in the region analyzed, the recombination frequency between genotypes I and IIb in a natural HDV infection was 6% (the recombination frequency was determined by dividing the yield of recombinants by the sum of the yields of the parental viruses). Considering the typically high reversion rate and the occurrence of intragenotypic recombination, this value might underestimate the HDV recombination frequency. Also, if two recombination events occurred in the analyzed region, the RFLP pattern might be undistinguishable from that of the parental RNAs. In addition, the HDV recombinants observed here were isolated from patient serum rather than from infected liver, thus representing the virion RNA that has been packaged and released into the bloodstream. The recombinant population detected in the serum could thereby represent a selective growth advantage in the infected liver cells and/or greater resistance against the various selective pressures in the host. Thus, the data reported here may not completely reflect the actual recombination frequency and mechanism. We are currently using the RNA cotransfection cell culture system established in this report to investigate recombination across the entire HDV genome. Characterization of the HDV recombinants present in the cells instead of in the released viral particles should provide more insights into the mechanism of HDV recombination.

HDV has been reported to acquire highly modified genomes via both incorporation errors during RNA replication and RNA editing (5, 12, 16, 32, 36, 42, 55, 59). Our results further suggest that RNA recombination occurs during HDV replication and thereby represents another mechanism for HDV genome heterogeneity. The fact that crossover sites could be detected in the HDAg-coding region suggests that recombination might yield viral pathogens with new biological properties. Therefore, such genetic heterogeneity will make the prevention and control of viral hepatitis even more difficult. Understanding the mechanism and the results of genetic heterogeneity of HDV will allow us to further comprehend the biological roles of HDV in human liver diseases.

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