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Molecular cloning and expression of the hepatitis delta virus genotype IIb genome

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Abstract

Analysis of hepatitis delta virus (HDV) genome sequences has revealed multiple genotypes with different geographical distributions and associated disease patterns. To date, replication-competent cDNA clones of HDV genotypes I, II, and III have been reported. HDV genotypes I, II, and IIb have been found in Taiwan. Although full-length sequences of genotype IIb have been published, its replication competence in cultured cells has yet to be reported. In order to examine this, we obtained a full-length cDNA clone, Taiwan-IIb-1, from a Taiwanese HDV genotype IIb isolate. Comparison of the complete nucleic acid sequence of Taiwan-IIb-1 with previously published genotype IIb isolates indicated that Taiwan-IIb-1 shares 98% identity with another Taiwanese isolate and 92% identity with a Japanese isolate. Transfection of Taiwan-IIb-1 into COS7 cells resulted in accumulation of the HDV genome and appearance of delta antigens, showing that cloned HDV genotype IIb can replicate in cultured cells.

Keywords: Hepatitis delta virus; Genotype; Delta antigen; Viral replication

Hepatitis delta virus (HDV) is a subviral human pathogen composed of a 1.7-kb negative-sense circular RNA, the viral protein delta antigen (HDAg), and the hepatitis B surface antigens [1]. HDV RNA has the ability to form an unbranched rod-like structure [2] and to undergo self-cleavage and ligation [3–5]. HDV RNA replication is carried out by host RNA polymerase(s) via a double rolling circle mechanism [6–8]. The RNA species found in the HDV virion has been referred to as genomic RNA. When HDV enters cells, genomic RNA serves as template to synthesize mRNA for HDAg [9,10], and unit-length circular antigenomic RNA which serves as a template to produce circular genomic RNA.

HDV RNA forms a ribonucleoprotein complex with HDAg in both virions and infected cells [11,12]. There are two forms of HDAg, small (195 aa) and large (214 aa). A specific RNA editing event, which is performed by cellular double-stranded RNA adenosine deaminases [13], converts the UAG stop codon for the small HDAg to a UGG tryptophan codon [14,15]. Therefore, the large and small HDAgs are identical except for an additional 19 amino acids at the carboxyl terminus of the large HDAg. However, these two forms of HDAg have distinct functions; the small HDAg is required for RNA replication, while the large one suppresses genome replication and is required for packaging [16,17].

Viral hepatitis due to HDV infection is found worldwide, and the complete nucleotide sequence of HDV has been reported from many locations. Comparison and analysis of the published sequences indicate there are at least three HDV genotypes, referred to as I, II, and III [18], with different geographical distributions and associated disease patterns. HDV genotype IIb, which formed a monophyletic group and was most closely related to genotype II, was isolated in Taiwan and Japan [19,20]. Nucleotide sequence divergence among genotypes can be as high as 40%. HDV replication from cloned cDNA of genotypes I, II, and III has been established in cultured cells [21–23]. Several studies have investigated the role of HDV genotypic factors in the viral life cycle. There is genotype-specific complementation of HDV RNA replication by the small HDAg between genotypes I and III

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[21]. In addition, the secondary structures required for RNA editing differ between genotypes I and III [24]. It has also been reported that viral assembly and RNA editing for HDV genotype I are more efficient than those for genotype II [22]. However, a molecular study of HDV genotype IIb replication has not been reported. Here we report the successful construction of the first HDV genotype IIb genome expression plasmid and show replication competence of genotype IIb in cultured cells.

Materials and methods

Molecular cloning and sequence analysis. The isolate Taiwan-IIb-1 was obtained from a male patient with chronic active hepatitis. A serological profile indicated triple infection with hepatitis B, C, and D viruses. Furthermore, mixed infection of HDV genotypes I, II, and IIb was observed in this patient. He has a history of sexual contact with prostitutes, which is a common transmission route of HDV infections in Taiwan [25], and is at high risk of re-exposure to HDV [26]. TriReagent LS (Molecular Research Center) was used according to the manufacturer's instructions to isolate total RNA from the serum of this patient. RNA equivalent to 25 μl serum was reverse-transcribed and PCR-amplified using the Titan One Tube RT-PCR System, according to the manufacturer's instructions (Boehringer–Mannheim). The reaction mixture was incubated in a thermocycler equilibrated at 50 °C for 30 min, followed by 40 cycles of amplification, with each cycle consisting of 94 °C for 1 min, 50 °C for 1 min, 68 °C for 1 min, and then 10 min at 68 °C. For cloning the entire HDV genome, two overlapping fragments corresponding to positions 886–449 (fragment A) (numbering according to Kuo et al. [27]) and 260–1308 (fragment B) (Fig. 1A) were amplified by PCR (Fig. 1B) with primer pairs 18/56 and 30/55, respectively (Table 1). The amplification products were cloned into the T-vector, pGEM-T (Promega), to yield clones pGEM-T-1856 and pGEM-T-3055. *Xho*I restriction fragment length polymorphism was used to screen for HDV genotype IIb clones [20]. Three independent clones of each plasmid were then subjected to sequence analysis using an ABI377 DNA sequencing system (Perkin–Elmer/Applied Biosystems). The genome sequence is available in GenBank (Accession No. AF209859).

Plasmids for HDV genome expression. As shown in Fig. 2, the 0.83-kb *Sph*I fragment from pGEM-T-3055 was cloned into the calf intestinal alkaline phosphatase (CIP)-treated *Sph*I-linearized pGEM-T-1856 to yield pGEMT-1.1 × IIb. The unit-length 1.7-kb *Nhe*I–*Nhe*I(430) fragment of genotype IIb HDV cDNA from pGEMT-1.1 × IIb was cloned into the *Xba*I site of pSVL plasmid (Pharmacia), an eukaryotic expression vector containing the SV40 late promoter. The clone containing a head-to-tail dimer of the 1.7-kb *Nhe*I–*Nhe*I(430) fragment of HDV cDNA in a genomic orientation was obtained and referred to as pSVL-D2IIb. To construct the replicating HDV genotype I expression vector, the1.7-kb *Sal*I–*Sal*I(962) fragment of HDV cDNA was excised from pSVL-D3 [23] and inserted into pSVL pretreated with *Xho*I and CIP. The plasmid containing a head-to-tail dimer of the 1.7-kb *Sal*I–*Sal*I(962) fragment of HDV genotype I cDNA in a genomic orientation was obtained and referred to as pSVL-D2I.

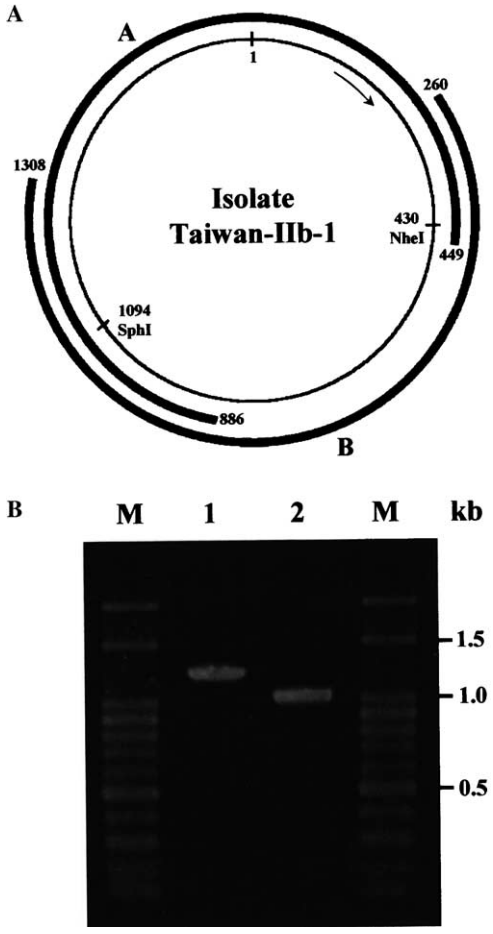

Fig. 1. (A) Schematic diagram of HDV Taiwan-IIb-1 cDNA clones (fragments A and B), and the relevant restriction enzyme sites. Nucleotide numbers (according to Kuo et al. [27]) are indicated at both ends of the clones. The circle shows the sequence of HDV. Arrow represents the orientation of HDV genomic RNA. (B) PCR-amplified cDNA products of HDV RNA. PCR products were separated on a 1.2% agarose gel and stained with ethidium bromide. Lanes M, 100-bp ladder DNA markers; lane 1, 1242-bp PCR product of fragment A; and lane 2, 1049-bp PCR product of fragment B.

Cell lines and DNA transfection. pSVL-D2IIb and pSVL-D2I were transfected into the COS7 monkey kidney cell line [28]. Cells (5×10^5 per well) were seeded in 6-well (35-mm diameter) plates and grown for 18 h to reach 60–70% confluence. Transfections were carried out with 4 μg DNA and 10 μl Lipofectamine (Invitrogen), according to the manufacturer's instructions. Transfection experiments were performed

Table 1
List of primers used for PCR amplification of HDV genome

Primer	Sequence (5′–3′)	Location
18	ATGCCATGCCGACCCGAAGAGGAAAG	886–911
56	CGGAACATCCACTCGCTAGC	449–430
30	ACCCACGGTCGGGTGATCCACCAGG	260–284
55	GGAGAGGCAGGATCACCGCCGAAGGAAGGC	1308–1279

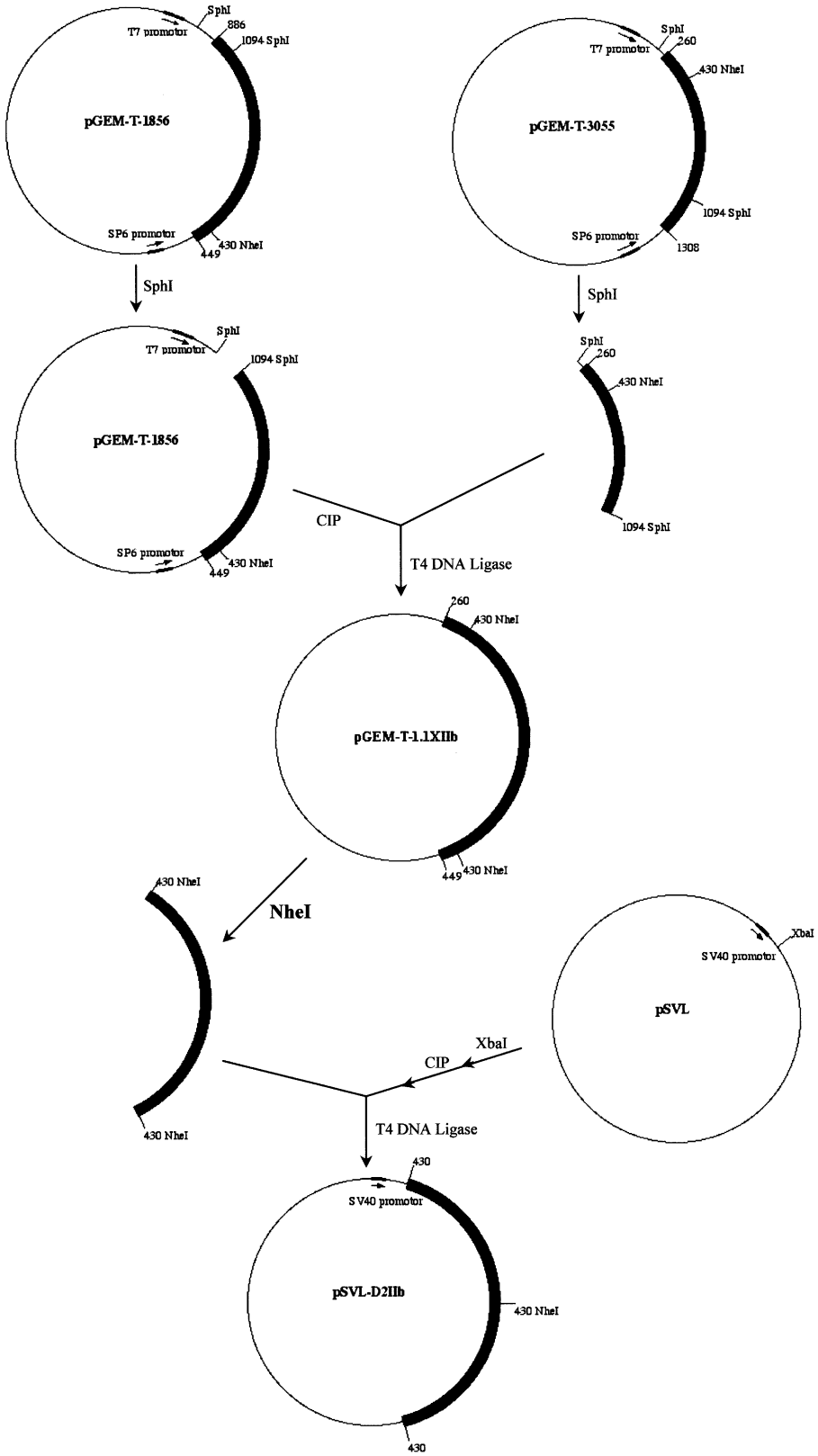

Fig. 2. Schematic diagram of the construction of the HDV genotype IIb genome expression vector. The thick line represents the HDV sequence and the thin line represents the vector sequence.

in duplicate and were repeated twice. The medium was changed every second day until the time of harvest. One third of the cells were subjected to Western blot analysis to monitor HDAg expression. RNA extracted from the other two thirds of the cells was subjected to Northern blot analysis to check the presence of HDV RNA.

RNA extraction and Northern blot analysis. HDV RNA was extracted from transfected cells with TriReagent (Molecular Research Center) following the instructions provided by the manufacturer. RNA was diluted in a buffer containing 320 mM Na_2HPO_4, 40 mM NaH_2PO_4, 8% formaldehyde, and 65.7% formamide, and boiled for 10 min. The heat-denatured RNA was electrophoresed on a 1.2% formaldehyde gel, then transferred, and UV cross-linked (Stratalinker; Stratagene) to Hybond-N^+ (Amersham Pharmacia Biotech.). The membrane was pre-hybridized at 58 °C for 3 h in buffer A [50% formamide, 0.6 M NaCl, 60 mM sodium citrate, 50 mM sodium phosphate, 50 mM Tris–HCl (pH 7.1), 0.1% sodium dodecyl sulfate (SDS), 0.002% Ficoll 400, 0.002% PVP, and 0.5 mg/ml sheared salmon sperm DNA] [29]. Hybridization was then carried out at 58 °C overnight in buffer A containing 10% dextran sulfate (w/v) and heat-denatured strand-specific ^{32}P-labeled RNA probe, which were monomeric genotype I RNA. The membrane was then washed twice at room temperature for 10 min each in a buffer consisting of 2× SSC (1× SSC is 150 mM NaCl and 15 mM sodium citrate) and 0.1% SDS, and then twice washed at 68 °C for 15 min each in a buffer consisting of 0.1× SSC and 0.1% SDS. The washed filter was then subjected to autoradiography. An additional hybridization using a probe specific for actin mRNA was performed using the same filter to monitor the amount of total RNA in each lane. Membranes were stripped of probe by boiling for 10 min in a buffer containing 0.1× SSC and 0.1% SDS.

In vitro transcription. The Promega in vitro transcription method was used. pG4B-D1IIb [containing 1.7-kb *Sal*I–*Sal*I(1355) Taiwan-IIb-1 isolate cDNA] and pG4B-D1I [containing 1.7-kb *Sal*I–*Sal*I(962) cDNA of a genotype I HDV isolate] [27] were cleaved with appropriate restriction enzymes and RNAs were synthesized with phage SP6 or T7 RNA polymerases in the presence (to make strand-specific radioactive RNA probes for Northern blot analysis) or absence (to serve as positive controls for Northern blot analysis) of [α-^{32}P]UTP (3000 Ci/mmol, 10 mCi/ml; Amersham Pharmacia Biotech). When pG4B-D1IIb and pG4B-D1T plasmids were cut with *Bam*HI and copied with T7 polymerase, they produced antigenomic HDV RNA which was used to detect genomic RNA. However, when they were linearized with *Hin*dIII and transcribed with SP6 polymerase, they yielded genomic RNAs, which were used to detect antigenomic RNA.

Determination of HDAg expression by Western blot analysis. Cells were solubilized as described previously [30] and proteins were separated by 12.5% SDS–PAGE. The protein samples were then electrotransferred to Immobilon-P membrane (Millipore) and HDAg was detected using a standard immunoblot procedure. Briefly, the membrane was blocked, incubated with rabbit anti-HDAg polyclonal antibody, washed, and then incubated with goat anti-rabbit IgG antibody conjugated with alkaline phosphatase (1:2000 dilution; Santa Cruz). After washing, HDAg was visualized by incubation with CDP-Star (Roche), a chemiluminescent substrate for alkaline phosphatase.

Results and discussion

The Taiwan-IIb-1 isolate was obtained from a patient with a mixed HDV genotype infection. Two partial but overlapping cDNA fragments covering the entire genome of HDV genotype IIb (Fig. 1) were cloned and

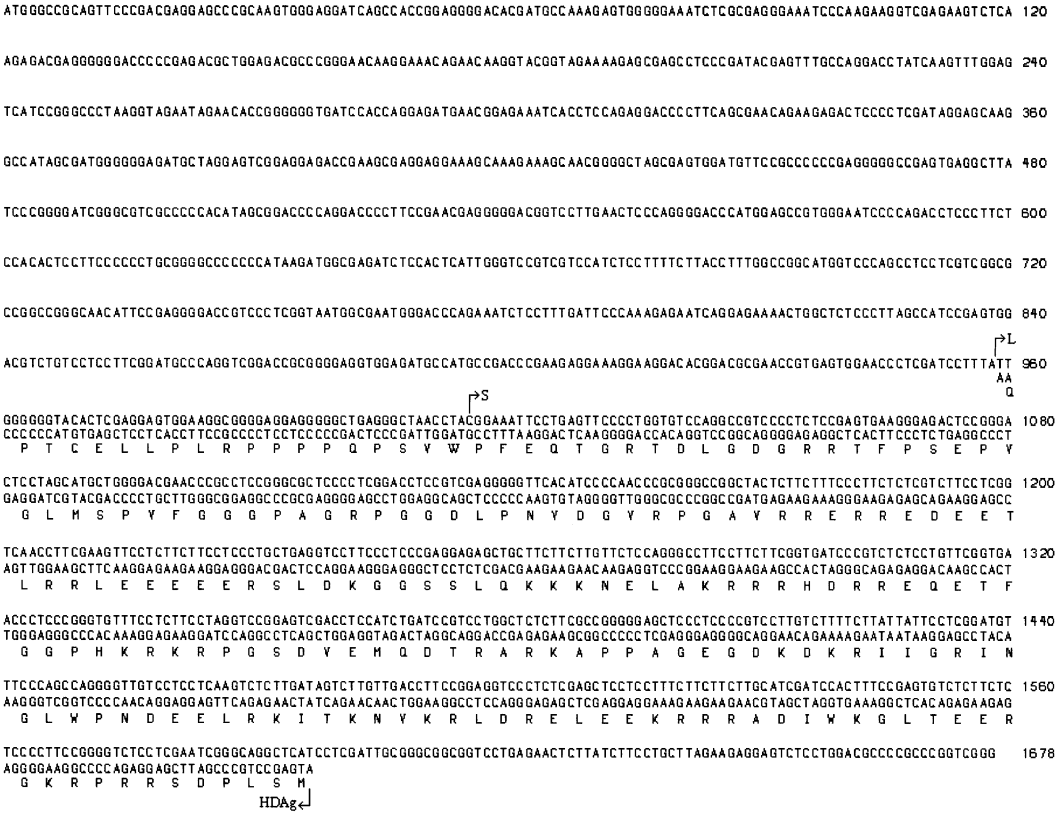

Fig. 3. The complete nucleotide sequence of Taiwan-IIb-1 HDV isolate. The translation of the open reading frames for the two forms of delta antigen in the antigenomic RNA strand is shown.

Table 2
Nucleotide and amino acid (aa) sequence identities among HDV isolates[a]

	Percentage identity of entire RNA genome/aa of small HDAg/aa of large HDAg							
	Taiwan-IIb-1	TWD60 (IIb)	2-05 (IIb)	T3 (II)	S-1 (II)	I (I)	T1 (I)	P-1 (III)
Taiwan-IIb-1		98.0/94.9/95.3	92.2/91.0/89.8	78.8/77.3/77.5	79.1/78.9/79.3	75.5/75.3/71.4	74.4/75.3/71.4	65.7/65.1/62.9
TWD60 (IIb)			91.3/87.7/86.9	78.4/76.3/76.5	78.2/77.3/77.9	75.5/74.2/70.4	75.0/74.7/70.9	66.2/64.1/62.0
2-05 (IIb)				79.6/75.9/75.7	80.5/75.9/75.7	76.8/75.4/73.0	75.9/73.3/71.1	69.6/61.2/58.3
T3 (II)					94.1/89.2/89.7	76.0/77.8/73.2	76.5/75.3/70.9	66.2/66.5/63.4
S-1 (II)						75.6/79.4/74.6	75.3/74.2/70.0	66.3/69.6/66.3
I (I)							88.0/88.2/88.8	64.4/69.1/65.3
T1 (I)								63.9/64.9/61.5
P-1 (III)								

[a] Genotypes of each isolate are given in parentheses. Sources of HDV isolates are as indicated: TWD60 (GenBank Accession No. AF018077) [20], T3 (U19598) [31], and T1 (M36590) [32] are isolates from Taiwan; 2-05 (AB015443) [19] and S-1 (X60193) [33] are isolates from Japan; I is obtained from the woodchuck-adapted HDV genome of an Italian patient (M21012) [27]; and P-1 is isolated from Peru (L22063) [18].

sequenced. The complete nucleotide sequence and the predicted amino acid sequence of HDAg for the Taiwan-IIb-1 isolate are shown in Fig. 3. The Taiwan-IIb-1 isolate is 1678 nt in length, similar to the size reported for other HDV isolates.

Comparison of the complete nucleic acid sequence of Taiwan-IIb-1 with published complete sequences of other HDV isolates indicates that Taiwan-IIb-1 is 75.5%, 78.8%, and 65.7% identical to HDV isolates of genotypes I (I) [27], II (T3) [31], and III (P-1) [18], respectively (Table 2). The predicted amino acid sequence of Taiwan-IIb-1 HDAg (small/large) shows 75.3/71.4%, 77.3/77.5%, and 65.1/62.9% identity to HDV isolates of genotypes I (I) [27], II (T3) [31], and III (P-1) [18], respectively (Table 2). Although the nucleotide sequence of Taiwan-IIb-1 is 98.0% identical to another genotype IIb isolate (TWD60) found in Taiwan [20], it is only 92.2% identical to an HDV genotype IIb (2-05) isolated from Japan [19]. HDV genotype IIb was first isolated from Taiwan, although with low frequency (only three out of 46 patients were singly infected with genotype IIb) [20]. In contrast, genotype IIb predominates in Okinawa, Japan, where the HDV infection is associated with low pathogenicity [19]. Furthermore, mixed genotype infections of HDV, which are frequently found in prostitutes, have been observed in Taiwan. Interestingly, five out of seven prostitutes examined had mixed infections of genotypes II and IIb [26]. Whether there are geographically based genome differences in HDV genotype IIb awaits sequence and functional analysis of more genotype IIb isolates from these areas.

Studies of HDV replication in cultured cells have been performed using clones of HDV genotype I [23]. Subsequently, functional differences between genotype I and III clones [21,24], and between genotype I and II clones [22], have also been determined. However, a replication clone for HDV genotype IIb has never been established. The fact that genotype IIb was frequently found in mixed HDV genotype infections in Taiwan

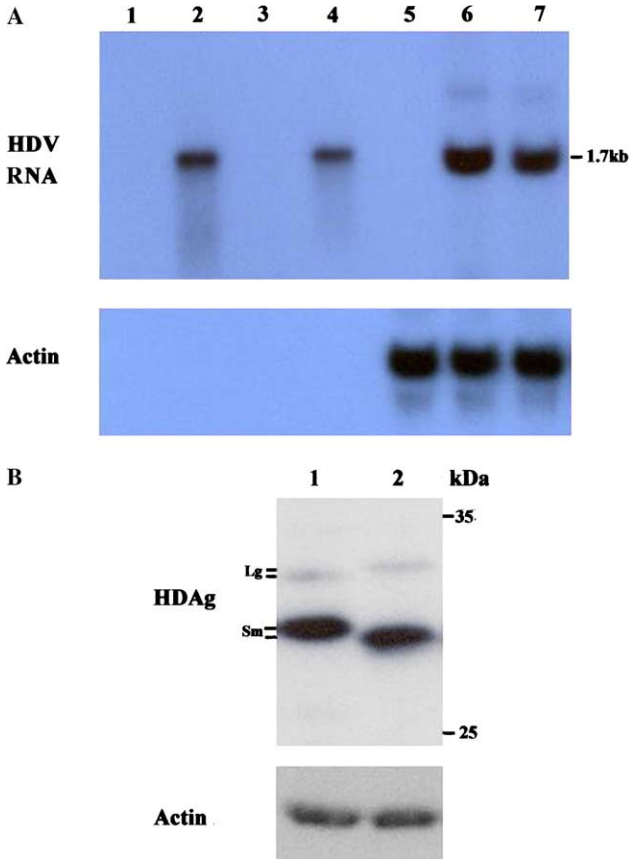

Fig. 4. HDV replication and HDAg expression in transfected COS7 cells. (A) Northern blot analysis to detect replicating HDV RNA following transfection. Lanes 1–4 contain in vitro-transcribed genomic genotype I, antigenomic genotype I, genomic genotype IIb, and antigenomic genotype IIb HDV RNAs, respectively. Lane 5 contains RNA extracted from untransfected COS7 cells; lanes 6 and 7 contain RNA samples extracted from COS7 cells transfected with pSVL-D2I and pSVL-D2IIb, respectively. The RNA integrity and loading equivalence were monitored by hybridization with an actin mRNA probe. (B) Western blotting to detect HDAgs from transfected cells. Lanes 1 and 2 contain samples of COS7 cells transfected with pSVL-D2I or pSVL-D2IIb, respectively. Lane 3 contains protein extracted from untransfected COS7 cells. Actin protein levels were determined to compare total protein levels in each lane.

prompted us to examine the replication competence of HDV genotype IIb. Two overlapping cDNA fragments covering the entire genome of Taiwan-IIb-1 isolate were used to construct a plasmid expressing HDV genome (Fig. 2). This plasmid was named pSVL-D2IIb, in which the SV40 late promoter directs synthesis of HDV genomic RNA containing two HDV self-cleavage/ligation domains, which in turn produces circular RNA for subsequent synthesis of antigenomic RNA. To compare the replication levels of genotypes I and IIb, a similar plasmid pSVL-D2I expressing the HDV genotype I genome (Italy) [27] was also constructed. Following transfection of pSVL-D2IIb and pSVL-D2I into COS7 cells, RNA was extracted from cells at six days post-transfection and HDV RNA was analyzed by Northern blotting using a probe specific for detecting HDV antigenomic RNA (Fig. 4). Note that the plasmid DNA would be expected to produce only genomic RNA dimers. The appearance of unit-length antigenomic RNA indicates that HDV replication occurred in transfected cultured cells. Since the sequence homology between genotypes I and IIb is only 75%, equal amounts of in vitro transcribed genotype I and IIb RNA monomers were also included (Fig. 4A, lanes 1–4) to serve as controls for quantitation. When in vitro-transcribed genomic RNAs were probed with a unit-length HDV genotype I genomic RNA probe, we observed no signal in such assays (Fig. 4A, lanes 1 and 3), indicating the strand-specificity of hybridization. Furthermore, homologous hybridization was favored over heterologous hybridization (2-fold difference) under the experimental conditions used (Fig. 4A, lanes 2 and 4). After quantitation, the replication level of genotype IIb (Taiwan-IIb-1 isolate) (lane 7) was found to be comparable with that of genotype I (Italy isolate) (lane 6). Furthermore, using an antigenomic RNA probe, we found that the levels of genomic RNA of genotypes I and IIb were similar (data not shown). These data strongly suggest that cDNA clone of genotype IIb HDV undergoes replication in cultured cells.

HDAg production is another indication of initiation of HDV replication. Although only the small HDAg was encoded in the transfected constructs, the large HDAg was produced via RNA editing that occurs during viral RNA replication. As expected, both forms of genotype IIb HDAg were detectable at 6 days after transfection. Interestingly, genotype IIb large HDAg (Fig. 4B, lane 2) migrated slower than genotype I large HDAg (lane 1). In contrast, genotype IIb small HDAg (Fig. 4B, lane 2) migrated faster than genotype I small HDAg (lane 1). It has been shown that both forms of genotype III HDAg migrate faster than their genotype I counterparts [21]. It has been proposed that the different migration patterns could be due to differences in post-translational modifications and/or structural variations [21], though the precise reasons remain unknown.

Although the sequence homology between genotype I and IIb isolates used in this report was only 75.5% at the nucleotide level, and 75.3%/71.4% (small/large HDAg) at the amino acid level, the replication abilities of these two isolates were comparable. The *cis* elements, such as the activities of RNA promoters and the efficiency of RNA editing, and the *trans* factors, such as the trans-activation ability of small HDAg and trans-inhibitory activity of large HDAg, may contribute to the different replication levels of HDV isolates. Therefore, unidentified functional domains, rather than overall genetic distance, accounted for the different levels of HDV RNA replication. The establishment of a replication-competent cDNA clone of HDV genotype IIb, together with the published clones of genotypes I, II, and III, provides an excellent experimental model to elucidate functional differences between HDV genotypes.

Acknowledgments

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