INTRODUCTION

Hepatitis delta virus (HDV) is a small defective RNA virus that is only found in patients that are also infected with hepatitis B virus (HBV) (Rizzetto et al., 1977, 1980), due either to co-infection of the two viruses or superinfection of a chronic HBV carrier. HDV induces a broad range of clinical manifestations in humans, ranging from asymptomatic cases to patients with fulminant hepatitis and hepatocellular carcinoma (Farci, 2003; Hughes et al., 2011). The HDV virion, which is 36 nm in diameter, has an outer envelope that contains HBV envelope proteins (Bonino et al., 1986; He et al., 1989). Inside the virion is a negative-stranded RNA of \( \sim 1.7 \) kb in size complexed with two forms (the large and small forms) of the only known virally encoded protein, hepatitis delta antigen (HDAg) (Weiner et al., 1988). The circular HDV genome and its complement antigenome are folded into an unbranched, rod-like structure with \( \sim 70 \% \) intramolecular base pairing (Chen et al., 1986; Kos et al., 1986) and they have ribozyme activity (Kuo et al., 1988b; Wu et al., 1989). HDAg lacks RNA polymerase activity, but it plays an important role in the life cycle of HDV; the small form of the HDAg is required for HDV RNA replication, whilst the large form is involved in virion assembly (Chang et al., 1991; Kuo et al., 1989). The N-terminal 195 aa of the two HDAgs are the same. For production of the longer form, RNA editing by cellular adenosine deaminase acting on RNA 1 (ADAR1) (Casey, 2012; Jayan & Casey, 2002; Wong & Lazinski, 2002) changes the UAG stop codon of the small HDAg to the UGG tryptophan codon, allowing translation to proceed by an additional 19–20 aa. The HDV genome is replicated via a double rolling-circle mechanism performed in the host cell nucleus by host polymerase (Chang et al., 2008; Moraleda & Taylor, 2001; Tseng & Lai, 2009). In this model, when the ribozyme domains appear during genomic and antigenomic HDV RNA synthesis, the nascent RNAs are cleaved and then ligated (possibly by host ligase activity) (Reid & Lazinski, 2000) to form circular RNA species that act as templates for the subsequent synthesis of HDV RNA.

Eight clades have been defined based primarily on nucleotide sequence relatedness and in some cases also on global distributions (Dény, 2006). Their genome sizes differ by \( \leq 30 \) nt,
but the sequence divergences of HDV clones within and between clades are up to 16 and 40 %, respectively. HDV-1 is prevalent worldwide, whereas HDV-2 and HDV-4, HDV-3, and HDV-5–HDV-8 are exclusively Asian, South American and African, respectively, in origin (Casey et al., 1993; Dény, 2006; Imazeki et al., 1990; Shakil et al., 1997; Wu et al., 1998). However, the geographical associations of the HDV clades can sometimes be blurred by population movements (Barros et al., 2011; Dény, 2006). Pathogenicity may vary among the HDV clades: HDV-1 has been associated with outbreaks of a severe form of fulminant hepatitis (Casey et al., 1993).

Similar to other RNA viruses that encode their own RNA-dependent RNA polymerases, the RNA genome of HDV is highly variable. In addition to the variants generated by host RNA polymerase not only performs RNA-directed RNA transcription, but also carries out template switching (Wu et al., 1999a). Mixed-genotype HDV infections are known to occur (Wu et al., 1999b). Homologous HDV RNA recombinants, possibly arising via host RNA polymerase-driven intermolecular template switching, have been reported to occur in Taiwanese patients co-infected with HDV-1 and HDV-4 (Wang & Chao, 2005), and in cultured cells co-transfected with two HDV RNAs of different or identical clades (Chao, 2007; Chao et al., 2006; Lin et al., 2015). The data indicated that reduced genetic distance and high replication levels increased the frequency of HDV RNA recombination (Lin et al., 2015). Recently, an HDV-1/HDV-2 recombinant has been reported in Vietnam using phylogenetic approaches (Sy et al., 2015).

Here, we established the first whole-genome recombination map using two cloned HDV-1 sequences replicated in cultured cells and provided evidence that chimeric HDAsgs with crossovers identified in this map were biologically functional. Finally, we propose a model that accounts for the HDV RNA recombination.

RESULTS

Establishment of a recombination map of HDV-1

To understand the biological significance of HDV recombination, we used a previously established experimental system (Lin et al., 2015; Wang & Chao, 2005) to construct a whole-genome recombination map of HDV. In this system, cultured cells were co-transfected with two HDV-1 RNA sequences, and reverse transcription (RT)-PCR products were cloned and sequenced. We had two replication-competent HDV-1 clones: clones I and A (Kuo et al., 1988a; Makino et al., 1987). The sequence homology between clones I and A of HDV-1 is 89 %. These two clones have been employed extensively (but independently) by different laboratories to study the life cycle of HDV (Kuo et al., 1988b, 1989; Wu et al., 1989). The region analysed previously for HDV RNA recombination was limited to nt 886–1308 (fragment A‘; Lin et al., 2015).

We employed the same total cellular RNA sample used to generate the recombination data for fragment A’, and sought to identify recombination events occurring across the whole HDV genome in cultured cells co-transfected with in vitro-transcribed clone I and clone A RNAs of HDV-1. Three additional partially overlapping fragments [B‘ (nt 1184–1679/1–257), C‘ (nt 190–746) and D‘ (nt 565–1015); Fig. 1a] were RT-PCR-amplified and subjected to RFLP assays. The utilized restriction enzymes and the predicted sizes of the parental and recombinant RNAs are summarized in Fig. 1(a). For example, the RT-PCR products obtained for fragment B‘ in clone I and clone A each have a single EcoRI site, at nt 1427 and 131, respectively. Following EcoRI digestion of the PCR products, the larger bands of clone I and clone A should be 510 and 627 bp, respectively. If one recombination event occurred between two EcoRI sites, the 5’-A-I-3’ and 5’-I-A-3’ recombinants would have bands consistent with the uncleaved (753 bp) and double-cleaved (384 bp) fragments, respectively. If RNA recombination occurred in co-transfected cells, we would see RFLP patterns reflecting combinations of the above-described bands. Indeed, complex RT-PCR/RFLP patterns with bands corresponding to parental and recombinant species were readily observed for fragments B‘, C‘ and D‘ using total cellular RNA extracted from co-transfected cells (Fig. 1b), suggesting that significant RNA recombination occurred. To further examine the molecular nature of the obtained HDV recombinants, the PCR products were cloned into T-vectors and DNA samples extracted from 50 colonies per fragment were subjected to sequencing. In our previous study, 14 recombinants with 16 crossovers were identified for fragment A’, including two recombinants with two crossovers each (nt 991–1016/1170–1182 and 1091–1156/1158–1168) (Lin et al., 2015). From the same RNA samples, we herein identified four, 16 and 16 recombinants with four, 20 and 20 crossovers for fragments B‘, C‘ and D‘, respectively (Table 1). For fragments A’, B‘, C‘ and D‘, the recombination frequencies (determined by dividing the yield of recombination events by the number of clones analysed) were 32, 8, 40 and 40 %, respectively (Table 1). The degrees of sequence homology between clones A and I for these four fragments were 92, 84, 85 and 96 %, respectively. Interestingly, although the degrees of homology were similar for fragments B‘ and C‘, the recombination frequency...
of fragment B' was fivefold lower than that of fragment C'.

In summary, from 200 clones obtained from four overlapping PCR products, we identified a total of 50 recombinants with 60 crossovers mapping to 22 different junction sites (Fig. 2). Seven, four, eight and three crossovers were mapped in fragments A', B', C' and D', respectively. Four recombinant clones with two intra-genotypic junctions each, mapping to nt 293–328/436–487, 389–413/436–487, 613–785/843–980 and 843–980/982–986, were identified. Two of these recombinants had three crossovers in the same molecule, mapping to nt 613–785/843–980/982–986. Together, these data indicate that there was a high frequency of intra-genotypic recombination in our experimental system. Sequence alignment of HDV-1 clones I and A indicated that all of the identified crossovers were located within regions that were homologous between the two parental sequences (Fig. S1, available in the online Supplementary Material). No consensus sequence was identified within or around the HDV recombination junctions.

Functionally active recombinant small HDAg

Nine of the 22 crossovers detected in the whole-genome recombination map fell within the ORF for the small HDAg (Fig. 2), which is required for replication of the HDV genome. Thus, we investigated the trans-activation activity of the recombinant small HDAg. Expression plasmids encoding mixtures of clone I and clone A sequences for the small form of HDAg were constructed and analysed for their ability to support HDV RNA replication. Small HDAg chimeras with arbitrary crossovers have been used to identify the region in small HDAg determining trans-activating efficiency between two HDV clones (Hsu et al., 2004). The results indicated that the N-terminal 56 residues of HDV-1 small HDAg determined the relative ability to trans-activate HDV replication. The recombination junction site used here was thereby nt 1491–1533, corresponding to aa 23–36 of small HDAg (Fig. 2). Plasmid pSVL-SmI22A expressed a small HDAg chimera comprising

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**Table 1. Summary of HDV recombinants obtained by sequencing of cloned PCR products**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Fragment</th>
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<tr>
<td></td>
<td>B'</td>
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<tr>
<td>Recombinant pattern</td>
<td>I-A</td>
</tr>
<tr>
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<td>0</td>
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<tr>
<td>I-1-A</td>
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<tr>
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<td>0</td>
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<tr>
<td>I-A-I-A</td>
<td>0</td>
</tr>
<tr>
<td>I-A-I-A</td>
<td>0</td>
</tr>
<tr>
<td>Sequence homology (%)</td>
<td>84</td>
</tr>
<tr>
<td>Recombination frequency (%)</td>
<td>8</td>
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*Data for fragment A' were adapted from our previous report (Lin et al., 2015).*
residues 1–22 from clone I and the rest from clone A, whilst pSVL-SmA22I expressed a small HDAg chimera having residues 1–22 from clone A and the rest from clone I. WT and chimeric HDAg-expressing constructs were co-transfected with plasmid pSVL-D2IM, which expressed HDV-1 clone I genomic RNA defective for HDAg (Kuo et al., 1989). Six days after transfection, total cellular RNA was harvested and HDV RNA replication was analysed by Northern blotting (Fig. 3a). The various small HDAgs were expressed at similar levels, as shown by Western blotting (Fig. 3b). As expected, cells transfected with HDV-defective HDV genotype I RNA construct alone showed no evidence of HDV RNA replication (Fig. 3a, lane 1), whilst HDV RNA replication was observed upon co-transfection of a WT HDAg expression plasmid (Fig. 3a, lanes 2 and 3). The level of RNA replication varied, with clone A HDAg showing 35 % less activity than clone I HDAg (Fig. 3a, lanes 2 and 3). Substitution of the N-terminal 22 aa of clone I HDAg with those from clone A (I22A) increased the activity by twofold compared with the WT HDAg of clone A (Fig. 3a, lanes 3 and 4). Conversely, expression of chimeric A22I HDAg decreased HDV replication to ~12 % that seen with WT clone I HDAg (Fig. 3a, lanes 2 and 5). These data clearly show that recombinant small HDAg could trans-activate HDV RNA replication, although with varied activity levels. Interestingly, small HDAg chimera I22A exhibited a trans-activation activity higher than those of WT small HDAgs I and A. This is consistent with the notion that RNA recombination might contribute to the generation of HDV strains with higher replication fitness, thereby playing important roles in the evolution and pathogenesis of HDV.

Serine-6 promotes the activity of the small HDAg in assisting HDV RNA replication

The data shown in Fig. 3 indicated that the N-terminal 22 aa of HDAg play an important role in regulating the trans-activation activity of the viral protein. The first 22 aa of the clone I and clone A HDAgs differ by four residues (Fig. 4a). The serine (S6) of clone I HDAg is a potential phosphorylation site for protein kinase C and an inhibitor of protein kinase C was previously shown to impair HDV RNA replication (Yeh et al., 1996). However, the role of S6 in the trans-activation activity of small HDAg has not been elucidated. We thereby constructed a series of point mutants at the sixth residue of WT and chimeric HDAgs, and investigated their trans-activation abilities. As shown in Fig. 4(b), when S6 of clone I HDAg was changed to R (I R) or A (I A), the trans-activation abilities of the mutants were reduced by 12- and 10-fold (Fig. 4b, lanes 3 and 4), respectively, compared with that of WT clone I HDAg (Fig. 4b, lane 2). In contrast, substitution of R6 to S6 in clone A HDAg (AS) substantially increased RNA replication (to 1.5-fold...
that of the WT clone A HDAg; Fig. 4b, lanes 5 and 6). Notably, the RNA replication level supported by A S was similar to that of I 22A, further confirming that the presence of a serine at the sixth residue plays a crucial role in the trans-activation ability of the small HDAg. Similar results were obtained when the mutants were constructed based on chimeric I22A and A 22I HDAgs: an S6 to R6 substitution of I 22A( I 22AR) decreased the trans-activation activity (Fig. 4b, lanes 7 and 8), whereas changing R6 to S6 in A 22I (A22IS) increased the HDV RNA replication (Fig. 4b, lanes 9 and 10). The various small HDAgs were expressed at similar levels, as shown by Western blotting (Fig. 4c).

Our results indicated that the genome-replication level of HDV is sensitive to the sixth amino acid within the N-terminal 22 aa of HDAg. Therefore, the recombination map established in this study provided a powerful tool for not only understanding HDV RNA recombination, but also elucidating the replication-related mechanisms.

**DISCUSSION**

In addition to the well-documented RNA editing at the amber/W site (Casey, 2012) and the random mis-incorporation of nucleotides during RNA synthesis (Imazeki et al., 1990; Lee et al., 1992), accumulating data indicate that homologous RNA recombination represents another mechanism responsible for generating genomic heterogeneity in HDV (Chao, 2007; Lin et al., 2015). Here, to the best of our knowledge, we established the first whole-genome recombination map. Our detailed examination of the distribution of the 22 crossovers summarized in Fig. 2 yielded several interesting observations. (1) Most of the identified crossovers appeared as pairs on opposite sides of the rod--like HDV RNA. Consequently, if template switching occurred twice on opposite sides of the same HDV RNA
molecule during a single round of replication (e.g. once at nt 63–89 and again at nt 1491–1533), the rod-like structure (which is important for replication) could be maintained and the resulting recombinant HDV RNA could be selected for subsequent replication. It is worth mentioning that the recombination event occurring at nt 1491–1533 could generate a small HDAg chimera with trans-activation activity (Fig. 3). The detection of recombination events favouring virus survival strongly suggests that HDV recombination is important to viral evolution. Furthermore, these data suggested that the recombination map established herein should be biologically relevant. (2) Of the 60 detected recombination events, 21 were found within the HDAg ORF, suggesting that the resulting chimeric HDAGs might be functional. Indeed, we showed that the first 22 aa of small HDAG are important for the trans-activation levels of small HDAG. (3) According to the rolling-circle replication strategy of HDV, a single template switch will produce a recombinant with two crossovers: the recombination site and the self-cleavage site (Chao, 2007). Our mapping of 18 recombination events to the ribozyme domains is consistent with a model in which HDV RNA recombination occurs via a replication-dependent process. Thus, a template-switching model for HDV RNA recombination involving viral ribozyme and host RNA polymerase was proposed (summarized in Fig. 5). As the HDV genome is circular and its replication occurs via a double rolling-circle mechanism, two (or an even number of) crossovers should be identified in HDV recombinants. If only one recombination event (or an odd number of events) occurs in the replicated HDV genome, the recombinant RNA will contain two junctions: one corresponding to the real recombination site and one representing the self-cleavage site (steps 1 and 2'). Conversely, if another recombination event occurs prior to the appearance of the self-cleavage site, both junctions between the two parental sequences will represent real recombination sites (steps 1 and 2).

As expected, the patterns observed herein for genetic recombination in HDV indicated that natural selection (functional HDAg and unbranched rod-like RNA genome) has acted to ensure that recombinants will be minimally deleterious (steps 3, 3' and 4). This template-switching model for HDV RNA recombination is similar to that proposed for RNA viruses encoding their own RNA-dependent RNA polymerases, except that viral ribozyme and host polymerases are involved in the generation of HDV RNA recombinants.

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**Fig. 5.** Schematic of a model for HDV RNA recombination. The individual steps are described in the text. Ovals drawn with black lines and dashed lines represent parental HDV sequences. Lines with arrowheads represent the newly synthesized nascent-strand RNA. Recombination junctions are depicted as closed circles. The self-cleavage site for genomic RNA is indicated with an asterisk.
Phosphorylation of small HDAg occurs on both serine and threonine residues (Mu et al., 1999). Three conserved serine residues of small HDAg, S2, S123 and S177, have been observed and their roles in HDV RNA replication have been reported previously (Mu et al., 2001; Yeh et al., 1996). The former two were potential protein kinase CKII recognition sites, and HDV RNA replication was profoundly suppressed in cultured cells by the addition of protein kinase CKII and protein kinase C inhibitors (Yeh et al., 1996). Furthermore, mutations at S2 and S177, but not S123, significantly impaired the activity of the small HDAg in assisting HDV RNA replication (Mu et al., 2001; Yeh et al., 1996). However, alanine replacement of S177 resulted in reduced phosphorylation of small HDAg, whilst the mutations at S2 and S123 had little effect. The fact that the S177 mutant still retained significant phosphorylation suggested that additional phosphorylated serine or threonine in small HDAg may be expected (Mu et al., 2001). The data presented here suggested the functional involvement of S6, which is a putative protein kinase C site in the HDAg, in HDV RNA replication. We thereby downloaded 231 full-length HDV genomic sequences from GenBank. Among them, 139 belonged to HDV-1, whilst HDV-2–HDV-8 were represented by 16, eight, 37, 15, six, five and five sequences, respectively. The majority of published HDV-1 and HDV-2 sequences had a serine residue at the sixth amino acid of HDAg. Specifically, 82, 94, 0, 27, 27, 0 and 20 % of HDV-1–HDV-8 sequences had a serine residue at the sixth amino acid of HDAg, respectively. The potential role of S6 phosphorylation in HDV RNA replication and pathogenesis remains to be investigated.

Similar to the identification of HDV RNA recombinant in natural mixed-genotype infections (Wang & Chao, 2005), the HDV recombinants reported here were obtained from RT-PCR amplification of RNA samples harvested from co-transfections. As shown in Fig. S2, we also mixed total RNA samples extracted from cells separately transfected with clone I or clone A RNAs, and subjected the mixtures to the same RT-PCR/RFLP analyses for fragments B and D. Only the bands corresponding to clone I and clone A were observed in these control reactions (Fig. S2). In contrast, HDV recombinants were readily detectable by RT-PCR from co-transfected samples (Fig. 1b). These data provided a line of evidence that the HDV recombination observed in cultured cells was not the result of artefacts during RT-PCR. As shown in Table 1, the degrees of sequence homology were similar for fragments B’ and C’, and the recombination frequency of fragment B’ was fivefold lower than that of fragment C’. The facts that the recombination junctions were not randomly distributed and that the crossovers were all mapped to the homologous regions between two RNA species (Fig. S2) also supported that the HDV recombination observed here was not the result of artefacts during RT-PCR. Furthermore, strain-specific primer pairs were used in a previous report studying HDV-1/HDV-4 recombination to successfully amplify recombinants from the RNA extracted from co-transfected cells, but not from mixed RNA samples extracted from cells separately transfected with two RNAs. Similar crossovers were detected by using consensus primers and strain-specific primer pairs (Chao et al., 2006; Wang & Chao, 2005). Importantly, an HDV-1/HDV-4 recombination junction, nt 1159–1207, identified from RT-PCR amplification could be detected in the co-transfected cells by an RNase protection assay, which avoided PCR amplification (Wang & Chao, 2005). The crossovers (nt 1158–1168, 1170–1182 and 1184–1206) identified here overlapped with that identified in HDV-1/HDV-4 (nt 1159–1207). Taken together, these data suggested that HDV RNA recombination detected by RT-PCR and sequencing was not an artefact, and was guided by a mechanism that was more specific than premature termination and reinitiation during RT-PCR.

Homologous recombination is generally thought to be rare in negative-sense RNA viruses, perhaps reflecting that the ribonucleoprotein complex never disassembles from the RNA (Conzelmann, 1998). However, accumulating reports indicated that RNA recombination occurs in negative-sense RNA viruses. For example, a recombinant lineage has also been identified for Ebola virus, which is a non-segmented negative-sense RNA virus (Wittmann et al., 1996). The data presented here suggested the functional involvement of S6, which is a putative protein kinase CKII recognition sites, and HDV RNA replication could be detected in the co-transfected cells by an RNase protection assay, which avoided PCR amplification (Wang & Chao, 2005). The crossovers (nt 1158–1168, 1170–1182 and 1184–1206) identified here overlapped with that identified in HDV-1/HDV-4 (nt 1159–1207). Taken together, these data suggested that HDV RNA recombination detected by RT-PCR and sequencing was not an artefact, and was guided by a mechanism that was more specific than premature termination and reinitiation during RT-PCR.

In vitro transcription from vectors containing the 1.7 kb SalI/SalI (nt 962) and PstI/PstI (nt 651) fragments of the clone I and clone A HDV-1 cDNAs, respectively, in a process that uses intramolecular template switching to generate a circular HDV RNA template for subsequent replication (Chang & Taylor, 2002). This mechanism has been shown to produce small deletions of HDV sequences and even insertions of non-template sequences. Therefore, template switching could occur imprecisely on linear HDV RNAs during the production of circular HDV RNAs. However, we observed correct sequences occurring at the site of the original discontinuity [SalI (nt 962) for clone I and PstI (nt 651) for clone A]. This may indicate that the circular HDV RNAs with authentic sequences from end to end have a survival advantage for subsequent replication. Furthermore, we did not identify any insertion, deletion or misincorporation at the recombination junctions (Fig. S1). Therefore, the HDV RNAs detected in the present work
represented RNAs that had undergone both intramolecular template switching (to generate HDV RNA circles; Chang & Taylor, 2002) and intermolecular template switching (to produce HDV recombinants). In a previous publication (Wang & Chao, 2005), we observed recombinant HDV sequences in the serum of a patient co-infected with HDV-1 and HDV-4. Thus, we believe that the recombination events observed in cells transfected with linear RNAs can mirror the events that occur during natural infections in which replication is initiated from circular HDV RNAs.

We conclude that HDV RNA recombination, which may be linked to the template switching of a host RNA polymerase acting on an atypical viral RNA template, represents a mechanism for generating genetic diversity of HDV. Our data also demonstrated that there are some similarities between RNA recombination events driven by host RNA polymerase and those driven by viral RNA-dependent RNA polymerase, specifically a replication-dependent template-switching mechanism. It is also worth mentioning that the investigation of the trans-activation abilities of the small HDAg chimeras further indicated that the genome-replication level of HDV was sensitive to the sixth amino acid within the N-terminal 22 aa of HDAg. Therefore, studies regarding HDV RNA recombination provide insights into not only the generation of genetic diversity of HDV, but also the elucidation of unknown molecular mechanisms for HDV replication. Future studies are thus warranted to elucidate the details of HDV RNA recombination, especially in laboratories that have access to multiple HDV clades. Our laboratory has access to HDV-1 and HDV-4 clones, and we are currently seeking to construct an inter-clade recombination map and examine the biological consequences of the resulting chimeric viral genomes and proteins. For example, although fragment B' had the lowest recombination frequency across the whole genome, we observed a pair of crossovers (nt 63–89 and 1491–1533) near the boundaries of a previously reported potential RNA promoter (nt 1541–60) (Greco-Stewart et al., 2006). The resulting recombinant is predicted to maintain a perfect rod-like structure, but has a heterogeneous RNA promoter. The possible effect of intra- and inter-clade promoter switching in HDV RNA replication should be investigated in the future. Furthermore, one important issue for the template-switching mechanism of HDV RNA recombination (Fig. 5) is the identification of RNA structures promoting template switching. We proposed that some intrinsic features in HDV rod-like RNA structure, such as an asymmetrical bulge, might play an important role in polymerase pausing and subsequent template switching. Experiments are ongoing to investigate the molecular mechanism of HDV RNA recombination.

**METHODS**

**RNA transfections.** To produce HDV RNAs for RNA transfection and subsequent analysis of HDV RNA recombination, we used transcription vectors pG4B-D11 (Wang & Chao, 2003) and pG4B-D1A (Lin et al., 2015), which contained monomeric cDNA inserts of clone I (HDV-1 of Italian origin; GenBank accession number M21012) (Kuo et al., 1988a) and clone A (HDV-1 of US origin; GenBank accession number M28267) (Makino et al., 1987), respectively. More specifically, pG4B-D1I and pG4B-D1A contained a unit-length 1.7 kb Sall/SalI (nt 962) and PstI/PstI (nt 651) fragment of clone I and clone A HDV-1 cDNAs, respectively. The nucleotide numbering system used here is in accordance with that of Kuo et al. (1988a, b). The genomic RNA monomers were transcribed in vitro from linearized plasmids pG4B-D1I and pG4B-D1A, as recommended by Promega. When pG4B-D1A was cut with XbaI and copied with T7 polymerase (Promega), genomic RNA was produced. The pG4B-D1I plasmids were cut with HindIII and transcribed with SP6 polymerase (Promega). HDV RNA recombination was investigated using the previously described experimental system (Lin et al., 2015; Wang & Chao, 2005). Briefly, RNA monomers of the HDV genome were in vitro-transcribed, the template DNA was removed and the RNA transcripts were purified as reported previously (Wang & Chao, 2005). SuperFect (Qiagen) was used to transfect in vitro-transcribed clone I and clone A HDV-1 RNA into COS7-SmT1 cells stably expressing small HDAg (Wang & Chao, 2005) to initiate replication of the HDV genome. As HDV-1 clone I replicated more efficiently than HDV-1 clone A, a transfection mixture containing clone I and clone A RNAs in a 1 : 3 ratio was used. Therefore, similar levels of these two parental HDV RNAs were observed once the replication was initiated in the transfected cells (Lin et al., 2015). At 6 days post-transfection, RNAs were extracted (Wang & Chao, 2005) and HDV RNA recombination was analysed.

**Analysis of HDV RNA recombinants.** HDV-related sequences present in the co-transfected cells at 6 days post-transfection were characterized by RT-PCR/RFLP analysis and sequencing of the cloned RT-PCR products. The primers used are summarized in Table S1 and were described previously (Wang & Chao, 2005). RT-PCR amplification was performed using the Titan One Tube RT-PCR System (Boehringer Mannheim) (Wang & Chao, 2005). RNA recombination in a region covering nt 886–1308 (fragment A') was previously investigated in cells co-transfected with clone I and clone A RNA (Lin et al., 2015). Here, we used the same RNA samples to identify the HDV RNA recombination occurring across the whole genome and to determine the recombination frequencies in co-transfected cells. Three additional overlapping fragments corresponding to nt 1184–257 (fragment B'), 190–746 (fragment C') and 565–1015 (fragment D') were PCR-amplified with consensus primer pairs F-B'/R-B', F-C'/R-C' and F-D'/R-D', respectively (Table S1). The sequences complementary to these primers did not vary between clone I and clone A of HDV-1. The utilized PCR conditions were intended to suppress in vitro recombination between related PCR template sequences (Judo et al., 1998; Wang & Chao, 2005). After amplification, RFLP analyses were used to differentiate the HDV-related sequences present in the co-transfected cells. The restriction enzymes used in the RFLP assays and the predicted RFLP profiles (only the sizes of the larger post-digestion bands are shown) are summarized in Fig. 1(a). The digested products were separated by 3 % agarose gel electrophoresis and stained with ethidium bromide. The PCR products were also cloned into a T-vector (TOPO TA cloning vector; Invitrogen). Plasmids extracted from 50 colonies for each fragment were subjected to sequencing analyses using an ABI377 DNA sequencing system (Perkin-Elmer/Applied Biosystems).

**HDAg-expressing plasmids.** To construct plasmids expressing WT small HDAs, the small HDAg ORFs in pSVL-D2I and pSVL-D2A (containing head-to-tail HDV cDNA dimer inserts of clone I and clone A, respectively) were PCR-amplified using primer pair 771/78 and the previously reported conditions (Wang & Chao, 2003). The PCR products were gel-purified and subcloned into a T-vector...
(pCR3.1; Promega) containing a human cytomegalovirus immediate-early promoter. The orientation and sequences of the resulting plasmids, which were designated pCR3.1-Sml and pCR3.1-SmA, were determined by restriction digestion and sequencing. To construct pCR3.1-SmI22A (expressing a chimeric HDag in which the N-terminal 22 aa were from clone I and the remainder of the sequence corresponded to clone A), overlapping HDV cDNA fragments corresponding to nt 889–1532 and 1491–1659 were PCR-amplified from pSVL-D2A and pSVL-D2I, respectively, using primer pairs 18 (Wang & Chao, 2005)/23 and 17/22, respectively. These overlapping PCR fragments were joined by overlapping extension (OE)-PCR (Horton et al., 1989) using primer pair 17/18. The resulting PCR product was gel-purified and cloned into pCR3.1, and the sequence and orientation of the resulting plasmid, designated pCR3.1-SmI22A, were confirmed by sequencing. Similarly, amplification of overlapping HDV cDNA fragments from pSVL-D2A and pSVL-D2I using primer pairs 18/23 and 17/22, respectively, followed by OE-PCR using primer pair 17/18, cloning and orientation/sequence confirmation yielded pCR3.1-SmA22I (expressing an HDag in which the N-terminal 22 aa corresponded to clone A whilst the remainder came from clone I). Plasmids expressing mutant small HDag carrying an amino acid substitution at residue 6 (a serine→arginine or serine→alanine mutation for the clone I sequence and an arginine→serine mutation for the clone A sequence) were derived from WT and chimeric HDag expression plasmids by a PCR-based site-directed mutagenesis method (Makarova et al., 2000). The oligonucleotides used for mutation were primers 43 (S6→R6), 44 (R6→S6) and 43’ (S6→A6). HDV cDNA fragments were PCR-amplified from pCR3.1-Sml using primer pairs 43/5 and 43’/5 and cloned into pCR3.1 to generate pCR3.1-SmI22A and pCR3.1-SmA22A, respectively. When the HDV cDNA was amplified from pCR3.1-SmI22A using primer pair 43/5, subsequent cloning yielded pCR3.1-SmI22A. Similarly, pCR3.1-SmA22A and pCR-SmA22I were generated when templates pCR3.1-SmA and pCR3.1-SmA22I were amplified with primer pair 43’/5. The PCR products were gel-purified and subcloned into pCR3.1. All mutant sequences were confirmed by sequencing.

DNA transfections and analyses of HDV replication. Plasmid pSVL-D2IM (Kuo et al., 1989) contained a head-to-tail HDV cDNA dimer insert and expressed an HDV-1 genomic RNA with a 2 bp deletion in the HDag ORF. It thus required the provision of a functional small HDag in trans to enable viral replication (Kuo et al., 1989). For DNA transfection experiments, COS7 monkey kidney cells were cultured and co-transfected with Lipofectamine (Invitrogen) (Wang & Chao, 2003) using equal amounts of pSVL-D2IM and various HDag-expressing constructs. At 6 days post-transfection, HDV RNA and HDag were extracted and analysed by Northern and Western blotting, respectively, as described previously (Wang & Chao, 2003). Experiments included duplicate transfections and were repeated at least twice, and representative results of Northern and Western blotting are shown.

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REFERENCES


Whole-genome analyses of HDV RNA recombination


