Virus Research 195 (2015) 79–85

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Reduced genetic distance and high replication levels increase the RNA recombination rate of *hepatitis delta virus*

Chia-Chi Lin[a], Zhi-Wei Yang[a], Shan-Bei Iang[a], Mei Chao[a,b,*]

[a] Division of Mcrobiology, Graduate Institue of Biomedical Sciences, College of Medicine, Chang Gung University, Tao-yang 333, Taiwan
[b] Department of Microbiology and Immunology, College of Medicine, Chang Gung University, Tao-yang 333, Taiwan

ARTICLE INFO

Article history:
Received 27 June 2014
Received in revised form 13 August 2014
Accepted 19 August 2014
Available online 27 August 2014

Keywords:
RNA recombination
Hepatitis delta virus
Template-switching
Viral evolution

ABSTRACT

Hepatitis delta virus (HDV) replication is carried out by host RNA polymerases. Since homologous inter-genotypic RNA recombination is known to occur in HDV, possibly *via* a replication-dependent process, we hypothesized that the degree of sequence homology and the replication level should be related to the recombination frequency in cells co-expressing two HDV sequences. To confirm this, we separately co-transfected cells with three different pairs of HDV genomic RNAs and analyzed the obtained recombinants by RT-PCR followed by restriction fragment length polymorphism and sequencing analyses. The sequence divergence between the clones ranged from 24% to less than 0.1%, and the difference in replication levels was as high as 100-fold. As expected, significant differences were observed in the recombination frequencies, which ranged from 0.5% to 47.5%. Furthermore, varying the relative amounts of parental RNA altered the dominant recombinant species produced, suggesting that template switching occurs frequently during the synthesis of genomic HDV RNA. Taken together, these data suggest that during the host RNA polymerase-driven RNA recombination of HDV, both inter- and intra-genotypic recombination events are important in shaping the genetic diversity of HDV.

1. Introduction

Hepatitis delta virus (HDV) is a human pathogen that consists of a small (1.7-kb) negative-sense RNA genome that is encapsidated by the *hepatitis B virus* envelope proteins (Rizzetto et al., 1980; Sureau, 2006; Wang et al., 1986). HDV is unique among the animal viruses in that it has a rod-like circular RNA genome with autocatalytic cleavage activity and a replication strategy that redirects host DNA-dependent host RNA polymerases to transcribe RNA templates (Flores et al., 2009; Taylor, 2006). Similar to many of the RNA viruses that encode their own RNA-dependent RNA polymerases (Lai, 1992; Worobey and Holmes, 1999), however, HDV has the ability to undergo RNA recombination in infections and transfected cultured cells (Chao et al., 2006; Gudima et al., 2005; Wang and Chao, 2005; Wu et al., 1999). Template-switching recombination has been studied extensively, and is known to involve a RNA polymerase that jumps from the original donor template to the acceptor template, where it continues RNA synthesis (Lai, 1992; Worobey and Holmes, 1999). Therefore, it seems likely that HDV recombination may occur *via* a cellular RNA polymerase-driven template-switching mechanism that may be favored by the low processivity of the polymerase when transcribing an atypical template (Chao, 2007).

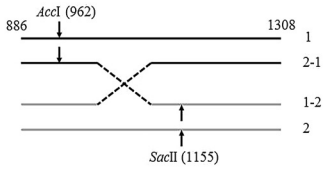
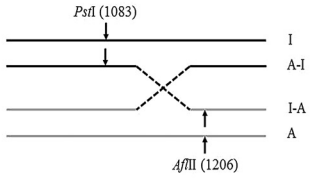
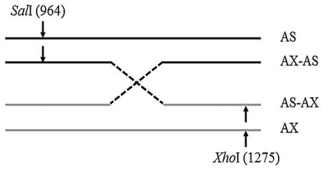
Based on phylogenetic analyses, HDV isolates from around the world may be divided into as many as eight genotypes (HDV-1 to HDV-8); the between-genotype divergence may be as high as 40% over the entire RNA genome, whereas the sequence heterogeneity among the isolates of the same genotype may be as high as 16% (Casey et al., 1993; Deny, 2006; Hughes et al., 2011; Imazeki et al., 1990; Shakil et al., 1997; Wu et al., 1998). Different HDV genotypes show different geographic distributions and associated disease patterns. For example, genotypes HDV-2 and HDV-4 are found exclusively in Asia; HDV-3 is found in northern South America; HDV-5–7 are observed among individuals of African origin; and HDV-8 has been identified in Africa and Brazil (Barros et al., 2011). HDV-3 is associated with severe acute hepatitis (Casey et al., 1993), whereas HDV-2 has been associated with less severe outcomes (Wu et al., 1995). In contrast, isolates of HDV-1 are geographically widespread and have been associated with a wide spectrum of diseases, from fulminant hepatitis to asymptomatic chronic disease (Rizzetto and Ciancio, 2012).

Abbreviations: HDV, *hepatitis delta virus*; HDAg, hepatitis delta antigen; RFLP, restriction fragment length polymorphism.

* Corresponding author at: Department of Microbiology and Immunology, College of Medicine, Chang Gung University, 259, Wen-Hwa 1st Road, Kwei-Shan, Tao-yang 333, Taiwan. Tel.: +886 3 2118800x5134; fax: +886 3 211 8956.
E-mail addresses: d9801203@stmail.cgu.edu.tw (C.-C. Lin), russelltools1201@gmail.com (Z.-W. Yang), isbisb@mail.cgu.edu.tw (S.-B. Iang), pa0728@mail.cgu.edu.tw (M. Chao).

http://dx.doi.org/10.1016/j.virusres.2014.08.011
0168-1702/

Table 1
Summary of RFLP patterns in various HDV RNA co-transfections.

Parental RNAs used	Restriction enzymes	Predicted HDV genome in the cells	RFLP profiles	No. of clones
Clone I of HDV-1 and HDV-2	*Acc*I + *Sac*II		346+76 422 193+153+76 269+153	195 0 1 4
HDV-1: clones I and A	*Pst*I + *Afl*II		225+197 422 197+123+102 320+102	14 5 8 23
Clone A mutants: AS , AX	*Sal*I + *Xho*I		344+78 422 311+78+33 389+33	8 10 9 13

Since HDV RNA recombination is homologous (Chao et al., 2006; Wang and Chao, 2005) and may occur *via* a replication-dependent process, we hypothesized that the frequency of HDV RNA recombination between two HDV sequences should be affected by their replication levels and sequence homology. Here, we observed efficient intra-genotypic HDV RNA recombination in a co-transfection system, and found that HDV RNA recombination occurs frequently during HDV genomic RNA synthesis. These results suggest that HDV RNA recombination is an important mechanism for the natural evolution of HDV, and provide important new insights into the process of HDV RNA recombination.

2. Materials and methods

2.1. Plasmids for in vitro transcription

We previously described the construction of cDNA clones for HDV using the transcription vector, pG4B (Promega) (Lin et al., 2003; Wang and Chao, 2003). The pG4B-D1I and pG4B-D1II-1 transcription vectors contained monomer cDNA inserts of an HDV-1 of Italian origin (hereinafter referred to as clone I) (Kuo et al., 1988) and a HDV-2 (Taiwan-3 isolate) sequence (Lin et al., 2003), respectively. To construct pG4B-D1A, which contained a monomeric cDNA insert of a HDV-1 sequence originally isolated from the United States (hereinafter referred to as clone A) (Makino et al., 1987), the 1.7-kb *Pst*I (nt 651) (the nucleotide numbering system used in this report is in accordance with that of Kuo et al., 1988) fragment was excised from a PECE plasmid containing a head-to-tail cDNA dimer of clone A (Lee et al., 1993), and cloned into the *Pst*I site of the pG4B multiple cloning site. The GenBank accession numbers of the genomic sequences for HDV-1 clones I and A and the HDV-2 clone used in this study are M21012, M28267, and U19598, respectively. The generated pG4B-D1A construct was applied as the PCR template for a single-step, single-primer mutagenic technique (Makarova et al., 2000) that introduced two point mutants, thereby destroying a *Sal*I site (nt 962) and an *Xho*I site (nt 1272); these mutants were designated pG4B-D1AS and pG4B-D1AX, respectively. The oligonucleotide primers used to generate pG4B-D1AS and pG4B-D1AX were I-AS (nt 946-984, 5′-CGCTTTATTCACTGGGGTTGACAACT CTGGGGAGAGAAG-3′) and I-AX (nt 1256-1295, 5′-GCTGCTT CCTCTTGTTCTCTAGGGCCTTCCTTCGTCGGTG-3′), respectively (the underlined nucleotides represent mutations). PCR was performed as previously reported (Makarova et al., 2000), and the PCR products were digested with *Dpn*I for 2 h at 37 °C, and then transformed into DH5α competent cells. The resulting mutants were confirmed by both restriction enzyme digestion and sequencing. To synthesize HDV genomic RNAs for the subsequent RNA transfection experiments, these constructs were linearized and subjected to *in vitro* transcription (Lin et al., 2003; Wang and Chao, 2003). More specifically, pG4B-D1A, pG4B-D1AS and pG4BZ-D1AX were cleaved with *Xba*I, while pG4B-D1II-1 was linearized with *Eco*RI. The genomic HDV RNAs were synthesized *in vitro* with T7 RNA polymerase (Promega). To produce the genomic HDV RNA monomers, the pG4B-D1I plasmids were cut with *Hind*III and transcribed with SP6 polymerase (Promega).

2.2. Cells, RNA transfection, and post-transfection analyses of HDV RNA recombinants

COS7-SmT1 cells (Wang and Chao, 2005), which express the small HDAg, were co-transfected with *in vitro*-transcribed HDV-1 and HDV-2 RNAs, clone I and A HDV-1 RNAs, or mutated AS and AX HDV-1 RNAs. RNA transfections were performed as previously described (Wang and Chao, 2005). Six days post-transfection, RNA was extracted and subjected to RT-PCR-restriction fragment length polymorphism (RFLP) analysis, as previously described for the characterization of HDV-related sequences in co-transfected cultured cells (Wang and Chao, 2005). Briefly, a region covering nt 886-1308 was amplified from both parental and recombinant HDV sequences using consensus primers 18 and 55′, and RFLP assays were used to distinguish among the HDV-related sequences. The restriction enzymes used for each co-transfected HDV pair and the predicted RFLP patterns of the HDV-related sequences in the co-transfected cells are summarized in Table 1. To further investigate the recombination frequency and distribution of crossover regions,