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One single nucleotide difference alters the differential expression of spliced RNAs between HBV genotypes A and D

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ABSTRACT

Hepatitis B virus (HBV) is generally classified into eight genotypes (A to H) based on genomic sequence divergence. The sequence variation among the different HBV genotypes suggests that the spliced RNAs should be different from genotype to genotype. However, the *cis*-acting element involved in the modulation of the distinct expression profiles of spliced HBV RNAs remains unidentified. Moreover, the biological role of splicing in the life cycle of HBV is not yet understood. In this study, spliced RNAs generated from genotypes A and D were carefully characterized in transfected HepG2 cells. The species and frequency of the spliced RNAs were dramatically different in the two genotypes. Of note, a population of multiply spliced RNAs with intron 2067–2350 excision was identified in HBV genotype A-transfected HepG2 cells, but not in genotype D transfected HepG2 cells. Further, we found a single nucleotide difference (2335) located within the polypyrimidine tract of the splice acceptor site 2350 between the two genotypes, and a single base substitution at 2335 was able to convert the splicing pattern of genotype D (or genotype A) to that of genotype A (or genotype D). These findings suggest that different unique splice sites may be preferentially used in different HBV genotypes resulting in distinct populations of spliced RNAs. The possible significance of the distinct spliced RNAs generated from the different HBV genotypes in HBV infection is discussed.

1. Introduction

Hepatitis B virus (HBV) is a hepatotropic, noncytopathic, enveloped virus that causes acute and chronic hepatitis. Even after the development of a vaccine, chronic hepatitis B (CHB) remains one of the most serious human viral infectious diseases worldwide. More than 350 million people in the world suffer from chronic HBV infection, many of whom subsequently develop more severe liver diseases including cirrhosis and hepatocellular carcinoma (HCC) (Chiaramonte et al., 1999; Kao and Chen, 2002). To date, HBV genomes have been classified into eight well-defined genotypes (genotypes A to H), and two tentative newly-identified genotypes (genotype I, a novel inter-genotypic recombinant; and genotype J) based on sequence divergence of greater than 8% over the entire viral genome, or greater than 4% in the viral S region. In addition, more than 30 related subgenotypes have been identified so far (Shi et al., 2012). These genotypes display distinct geographic and ethnic distributions (Cao, 2009; Lin and Kao, 2011; Norder et al., 2004; Shi et al., 2012). Different HBV genotypes also show varying clinical relevance and response to antiviral treatment during chronic viral infection (Ding et al., 2001; Hou et al., 2007; Kao et al., 2000a; Orito et al., 2001; Yang et al., 2008; Zollner et al., 2004). The mechanisms involved in the differing pathogenic properties of the HBV genotypes are unknown.

Major HBV transcripts include a 3.5 kb preC mRNA that encodes HBeAg, a 3.5 kb pregenomic RNA (pgRNA) that encodes core and

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polymerase proteins, 2.4 kb and 2.1 kb subgenomic RNAs that encode surface proteins, and a 0.9 kb mRNA that encodes the HBx protein. The transcription of these unspliced viral RNAs is initiated from different transcription start sites but is terminated at a unique polyadenylation site located within the HBV genome (Seeger and Mason, 2000). In addition to these unspliced transcripts, several spliced viral RNAs have been identified in various systems including HBV-transfected hepatoma cells (Choo et al., 1991; Su et al., 1989a; Wu et al., 1991), HBV-transgenic mice (Choo et al., 1991), HBV-infected liver tissues (Chen et al., 1989; Su et al., 1989b; Wu et al., 1991), the sera of CHB patients (Gunther et al., 1997), and the defective HBV particles in hepatocytes (Abraham et al., 2008; Sommer et al., 2000; Terre et al., 1991). Several studies have demonstrated the significance of HBV spliced RNAs and their relevance in clinical events. For example, a duck hepatitis B virus (DHBV)-produced spliced RNA, named spliced L RNA, was identified as an essential factor for virus replication (Obert et al., 1996). The expression of defective HBV particles, which contain a truncated viral genome generated by a singly spliced 2.2 kb RNA (SP1), was closely related to the persistence of viral infection and the severity of liver disease (Rosmorduc et al., 1995; Soussan et al., 2008). And two novel fusion proteins encoded by HBV spliced RNAs (HBSP and P-S fusion protein) were also identified as being associated with viral structure, viral replication, cell apoptosis, and severity of liver disease (Huang et al., 2000; Park et al., 2008; Soussan et al., 2000, 2003). These studies indicate that HBV spliced RNAs are functional molecules that possibly evolved to regulate the viral life cycle and host-virus interactions during viral infection.

The sequence variations found among the different HBV genotypes suggest that the types and proportions of their spliced RNAs should vary from genotype to genotype (Gunther et al., 1997; Sommer et al., 2000). However, a systematic comparative analysis of heterogeneous spliced RNAs generated from different genotypes has not yet been performed. Moreover, the underlying *cis*-acting element involved in the generation of genotype-specific spliced RNAs has not been clarified. Here, we carried out a systematic study of types and proportions of spliced viral RNAs generated from genotype A and genotype D HBV. A comparative analysis of the expression patterns of the spliced RNAs showed that a significantly higher proportion of multiply spliced viral RNAs was generated in genotype A-transfected cells, leading to the identification of a subset of genotype-specific multiply spliced RNAs with intron 2067–2350 excision. Sequence alignment revealed a single nucleotide difference located in the polypyrimidine tract region of the 2350 acceptor site of the two genotypes. A single base substitution at this critical nucleotide significantly altered the splicing pattern of the HBV genome. In summary, we identified a critical *cis*-acting element that modulates the excision of intron 2067–2350 and is, at least in part, involved in the regulation of the heterogeneity of spliced HBV RNAs. The knowledge obtained from this study provides insights into the regulatory mechanism of diverse spliced viral RNAs generated by different HBV genotypes.

2. Materials and methods

2.1. Ethics statement

This study was approved by the Institutional Review Board, Chang Gung Memorial Hospital (IRB NO. 97-1583B). Written informed consent was obtained from all patients providing liver biopsy samples. Eleven consecutive chronic hepatitis B patients (genotypes A and D) who received liver biopsies were included in this study.

2.2. Cell culture and transfection

HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum. For transfection, HepG2 cells were grown to 60% confluence and transfected with plasmids comprising the HBV genome of genotype A (subgenotype: A2) (Yuh et al., 1992) or genotype D (subgenotype: D3) (Chou et al., 2007) using the jetPEI reagent (Polyplus transfection), according to the manufacturer's instructions.

2.3. Reverse transcription-polymerase chain reaction

Total RNA was extracted using the TRIzol reagent (Invitrogen, California, USA) according to the manufacturer's protocol. To eliminate genomic DNA contamination in RNA preparation, the total RNA was dissolved in diethylpyrocarbonate (DEPC)-treated water and incubated with RNase-free DNase I (DNase I, Amplification Grade) for 15 min at 25 °C. Then, EDTA was added and the DNase I-treated RNA sample was heated at 65 °C for 10 min to stop the reaction. cDNA templates were obtained by reverse transcription of total RNA using oligo(dT) and SuperScriptII Reverse Transcriptase (Invitrogen, California, USA), and then amplified by PCR using the following primer pairs: For the detection of intron excision(s) in the 5′ half of the 3.5 kb viral transcript the forward primer (1814–1837) was 5′-ATGCAACTTTTTCACCTCTGCCTG-3′ and reverse primer (630–655) was 5′-GAGGCCCACTCCCATAGGAATTTTC-3′; for the detection of intron excision(s) in the 3′ half of 3.5 kb viral transcript, the forward primer (630–655) was 5′-CGGAAAATTCCTATGGGAGTGGGCCTC-3′ and reverse primer (1799–1821) was 5′-GTTGCATGGTGCTGGTGCGCAGA-3′. The same thermo-cycling parameters were used for both sets of PCR analyses: 94 °C for 10 min; 5 cycles of 94 °C for 30 s, 50 °C for 30 s, and 72 °C for 90 s; 30 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 90 s; and 72 °C for 10 min. We confirmed that the amplification of all RT-PCR products had not reached the plateau at this cycle number.

2.4. Analyses of HBV spliced RNAs

To determine the relative amounts of individual HBV spliced RNAs, a previously established RT-PCR-based method was used with some modification (Gunther et al., 1997). In brief, cDNA was generated by reverse transcription of total RNA extracted from cells. The cDNA derived from HBV RNA was further amplified by PCR using a pair of HBV specific primers as mentioned above. The RT-PCR products from different HBV spliced RNAs were then separated in a 2% NuSieve 3:1 agarose gel (Lonza, USA) and the signal of each RT-PCR product, representing the amount of respective HBV spliced RNA, was quantified individually by densitometric analysis. For structural analysis of these spliced RNAs, the RT-PCR products were recovered from the gel and cloned into the pGEM-T vector for further sequence analysis. The resultant sequences were used to determine the junctions of spliced RNAs by comparison with the HBV genome of the respective genotype. The structures of the spliced RNAs, including the deleted regions and the splice donor/acceptor sites, were subsequently characterized. Nucleotide positions at the exon boundary were shown by numbering starting from the EcoRI site of the different HBV genotypes. The usage frequency of an individual splice donor (or acceptor) site was estimated according to a previously described method with modification (Gunther et al., 1997), and the usage frequency of a specific splice site was estimated from the sum of the detection frequency of the site in all spliced RNAs analyzed.