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Identification of a unique splicing regulatory cluster in hepatitis B virus pregenomic RNA

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ABSTRACT

HBV particles with genome derived from spliced mRNAs accumulate in patients with virus-derived severe liver necrosis and fibrosis. We investigated the role of an intronic element (intronic splicing silencer-long, ISS_L) on splicing of HBV minigene transcripts. Removal of the entire ISS_L showed two-fold increase in splicing while shorter deletions within ISS_L indicated isolated clusters of activator and repressor domains. Activator domains stimulated splicing in presence of PRE, a long HBV 3′ exon and even when present in a heterologous context. Mutations in the repressor domain unexpectedly augmented repression. The role of this intronic splicing regulatory element could be important for HBV pathogenesis.

1. Introduction

Hepatitis B virus (HBV), a member of the hepadnaviridae family, is a 42 nm particle with an enveloped 28 nm nucleocapsid. Transcription on the HBV genome by host RNA polymerase II produces 3′-coterminal pregenomic/precore RNA (pgRNA, 3.5 kb) and three subgenomic RNAs (2.4, 2.1 and 0.8 kb) [1]. Individual promoters and related enhancer elements that are regulated by ubiquitous or liver enriched transcription factors, drive transcription of these viral RNAs [2].

Splicing of viral transcripts in hepadnaviridiae family has diverse effects in viral life cycle. Replication of duck hepatitis B virus (DHBV) in infected primary duck hepatocytes and ducks requires a spliced mRNA [3]. Even though no direct role of splicing could be established for HBV replication [4], the load of defective HBV particles with shortened genome increased with liver necroinflammation and fibrosis [5]. In cultured hepatocytes, about 50% DNA within intracellular capsids is derived from spliced RNAs [6]. In patient sera and HBV infected cultured hepatocytes, eleven smaller than full-length genomes are generated from spliced pgRNA [7,8]. Of these spliced RNAs, two products (SP1 and SP2) predominate. The SP1 product is obtained by removal of intron with 5′ donor and 3′ acceptor sites positioned at 2447nt and 489nt, respectively, but generation of SP2 requires the donor at 2067nt and the same acceptor site. The search for *cis*- and *trans*-acting factors regulating pgRNA splicing has identified both activator and repressor regions in Post-transcriptional Regulatory Element (PRE) present in the 3′ exon of pgRNA [9]. Recently a double-hairpin structure in PRE, when placed in a heterologous intron, silenced splicing in a structure and position-dependent fashion [10]. However effect of HBV intronic elements in splicing of a homologous transcript has never been reported.

At least two proteins encoded by spliced HBV RNAs are found in patient sera and cultured hepatocytes. An endoglycosylase H (endo-H) sensitive 43 kDa polymerase-surface fusion protein translated from a HBV spliced RNA was reported in cells expressing HBV P protein. HBSP, encoded by a 2.2 kb singly spliced mRNA generated by the same splice sites used for generating SP1 species [11], bound to the fibrinogen γ-chain and inhibited fibrin formation [12]. Taken together the role of splicing in HBV infection remains a very crucial unanswered question.

To gain insight of how intronic splicing signals dictate splicing in HBV, we investigated the role of an intronic element (intronic splicing silencer-long, ISS_L) with a pronounced secondary structure in homologous minigene context. The minigene transcript is very similar to the HBSP pre-mRNA and thus should recapitulate the events leading to its processing. Our data reveal that the ISS_L is actually composed of interspersed repressor and activator domains. The activator domains could stimulate splicing even when

Abbreviations: HBV, hepatitis B Virus; pgRNA, pregenomic RNA; ISS_L, intronic splicing silencer-long; PRE, post transcriptional regulatory element; HBSP, hepatitis B splice-generated protein; RT-PCR, reverse transcriptase-polymerase chain reaction

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the 3′ exon harbored PRE or the almost entire length of HBV 3′ exon. Moreover these activator domains were also functional in a heterologous pre-mRNA. A separate repressor motif within the intron was also identified. To our knowledge this is the first report of *cis*-acting intronic splicing regulators from HBV RNA.

2. Materials and methods

2.1. Cell lines and oligonucleotides

HuH7 cell line was maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum. The primers used in cloning and PCR are depicted in Supplementary Table 1.

2.2. Preparation of minigene constructs

Different regions of the HBV genome were amplified from a plasmid containing the entire HBV sequence (NCBI No. AY945307) and cloned in pcDNA 3.1(+) expression vector (Invitrogen) under CMV-IE promoter. CPS, the basal construct contained sequences spanning 2231–3182 bp followed by 20–621 bp of HBV genome to include the nucleotide positions 2447 and 489 as splice donor and acceptor sites with a 1204nt intron separating them. CPS and CPSISS$_L$ΔISR1 backgrounds were used for PCR with mutagenic primers to generate ISS$_L^M$ mutants. PRE (1151–1684nt) and 3′ exonic region of pgRNA (622–1800nt) were inserted downstream of CPS by EcoRI and KpnI sites to construct CPSPRE and CPSpg minigenes respectively. For reporter assays using pCI-neo mammalian expression vector (Promega), respective primer pairs were annealed (Supplementary Table I), and inserted into the intron present in the vector backbone. All the constructs were confirmed by DNA sequencing.

2.3. Transfection

Transient transfections were performed in 6-well plates by $CaCl_2$ using standard protocols. Each well typically received a mixture of 2 μg of test plasmid, 1 μg of pEGFP-C1 (Clontech), and 2 μg of pUC19. Total RNA was isolated 48 hours post-transfection using TRI reagent (Sigma) following manufacturer's instructions.

2.4. Semiquantitative determination of transcripts

Reverse transcription of isolated total RNA was done with random hexamers. Hp8 and Hp2 primer pair was used for amplification of cDNA from precursor RNA and Hp1 and Hp2 pair was used for amplification of cDNA from spliced RNA. Serial 10-fold dilutions of target RNA was simultaneously assayed alongside the cellular RNAs to ascertain that the amplification of the products was on the exponential phase. Each RNA sample was normalized with respect to GFP and GAPDH transcripts. All the tubes were simultaneously PCR amplified with [α-^{32}P]dCTP and analyzed in 6% nondenaturing polyacrylamide gel. To compare the band intensities across multiple gels, all the gels under analysis were exposed together to a phosphorimager screen and analyzed by ImageQuant TL software. Splicing efficiency was calculated as [pixel value of spliced/pixel values of (spliced + unspliced)] for each sample.

Splicing in pCI-neo background was assayed with pCIF and pCIR primer pair that amplifies both spliced (161 bp) and unspliced (293 bp for pCI-neo transcript and 313 bp for pCI-neo + 20nt insert transcript) species.

2.5. Real-time RT-PCR

After reverse transcription, cDNA was amplified in StepOnePlus™ real-time PCR System (Applied Biosystems) using Maxima™ SYBR Green/ROX qPCR Master Mix(2×) (Fermentas) and specific primers. HpRT1 and HpRT2 primer pair was used for amplification of cDNA from spliced species and HpRT11 and HpRT2 pair was used for amplification of total minigene cDNA. For standard curves of C_t vs. log[RNA in ng], two step RT-PCR was performed on known amounts of in vitro transcribed target RNA. The relative quantities of spliced or total RNA in each sample under investigation were obtained from the respective standard curves. Data shown here represents mean of at least three independent experiments with error bar representing ±standard deviation. Student's *t*-test was performed with GraphPad Prism 5 software.

2.6. RNA stability assay

Twenty-four hours post transfection, cells were treated with 1 μg/ml Actinomycin D and kept at 37 °C for indicated time and total RNA was extracted. The isolated RNA was analyzed by phosphorimager quantitation as described above. RNA levels were normalized against GAPDH mRNA.

3. Results and discussion

3.1. The HBV intron contains a splicing silencer

To establish an in vitro system that recapitulates the splicing regulation of HBV pre-mRNA, we designed minigenes under the control of CMV-IE promoter and transfected them into HuH7 human hepatocarcinoma cell line. The CMV-IE promoter has been shown to enhance the accumulation of spliced transcripts for retroviral genes when compared to their endogenous promoters and also for HBV where the combination of both CMV-IE promoter and HuH7 cells as transfection hosts were noted to produce more splice variant RNA [13]. We constructed a basal minigene (CPS, as it includes regions from these three HBV genes) to recapitulate splicing of HBV major spliced product SP1 and the HBSP mRNA (Fig. 1). Both semiquantitative radioactive RT-PCR and real-time RT-PCR analyses were carried out to elucidate the level of splicing of the transcripts. We explored the secondary structure of the intron of the minigene with MFOLD (http://www.bioinfo.rpi.edu/applications/mfold) using default settings to give us idea about the presence of structured regulatory motifs [14]. We concentrated on an element spanning 2951–3163nt that was conserved in all the predicted structural conformations of HBV intron in different free

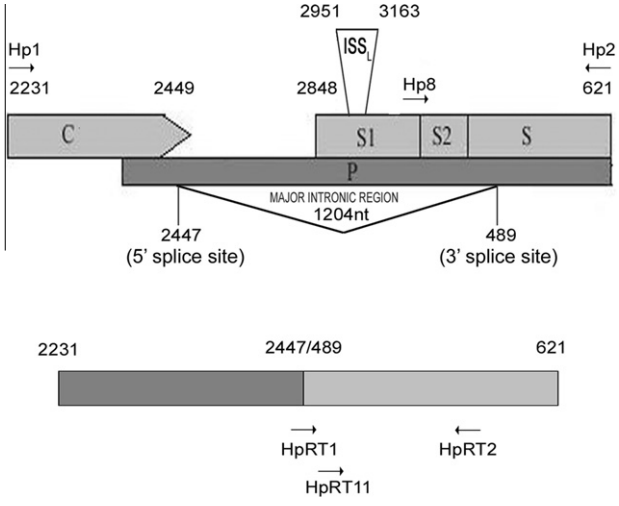

Fig. 1. Construction of the basal minigene, CPS. The 5′ and 3′-splice sites in pgRNA and their positions in HBV genes are indicated. ISS$_L$ was deleted from CPS (CPSΔISS$_L$), when indicated (upper panel). Arrows show location of the primers used for amplification of cDNA. Primers for real-time PCR for identification of spliced and total minigene transcripts are shown in lower panel.