Alternative splicing of viral transcripts: the dark side of HBV

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ABSTRACT

Regulation of alternative splicing is one of the most efficient mechanisms to enlarge the proteomic diversity in eukaryotic organisms. Many viruses hijack the splicing machinery following infection to accomplish their replication cycle. Regarding the HBV, numerous reports have described alternative splicing events of the long viral transcript (pregenomic RNA), which also acts as a template for viral genome replication. Alternative splicing of HBV pregenomic RNAs allows the synthesis of at least 20 spliced variants. In addition, almost all these spliced forms give rise to defective particles, detected in the blood of infected patients. HBV-spliced RNAs have long been unconsidered, probably due to their uneasy detection in comparison to unspliced forms as well as for their dispensable role during viral replication. However, recent data highlighted the relevance of these HBV-spliced variants through (1) the trans-regulation of the alternative splicing of viral transcripts along the course of liver disease; (2) the ability to generate defective particle formation, putative biomarker of the liver disease progression; (3) modulation of viral replication; and (4) their intrinsic propensity to encode for novel viral proteins involved in liver pathogenesis and immune response. Altogether, tricky regulation of HBV alternative splicing may contribute to modulate multiple viral and cellular processes all along the course of HBV-related liver disease.

INTRODUCTION

In eukaryotes, alternative splicing is a trivial event. The overall function of alternative splicing is to increase the diversity of mRNAs by coordinating the generation of isoforms from one single gene. In humans, more than 90% of genes are alternatively spliced by several mechanisms such as exon skipping or intron retention, leading to the exclusion or inclusion of a specific RNA sequence.[1] This gives rise to mRNA populations that increase by around fourfold to fivefold the diversity of proteins, essential for development, differentiation and organ functions.[2–4] Furthermore, mis-splicing can trigger human diseases including genetic disorders and cancers.[5] The splicing process is coordinated by the spliceosome, a large ribonucleoprotein complex.[6] Alternative splicing is regulated by cis-acting domains, located in the intronic or exonic regions (silencers and enhancers), and by transacting splicing factors (repressors and activators).[2 3 7 8]

Like in the eukaryotic transcriptome, alternative splicing is important for the completion of the replication cycle of multiple virus families, through the expression of proteins contributing to the productive infection.[9 10] Considering the HIV-1, an unspliced genomic transcript is responsible for the generation of Gag and Gag-Pol precursor structural proteins. HIV-1 primary transcripts undergo extensive and complex alternative splicing for the expression of the regulatory viral proteins. Indeed, most HIV-1 strains use four different splice donor (SD) and eight different acceptor sites which produce more than 40 different spliced mRNA species for Rev, Vpu, Vpr, Vif and Tat expression.[11] Likewise HIV, the human papilloma DNA virus (HPV) also requires constitutive and alternative splicing to generate the 20 different mRNAs, encoding proteins that are essential to complete its life cycle.[12] This is driven both by cis-acting regulatory sequences present on viral RNA as well as by trans-acting splicing factors such as proteins belonging to the SR (Serine-Arginine) or hnRNPs (heterogeneous nuclear ribonucleoproteins) families. In contrast to HIV-1 or HPV, alternative splicing regulation of the HBV transcripts seemed not critical for viral replication.

HBV is a hepatotropic DNA virus member of the hepadnavirus family.[13] Worldwide, more than 250 million people are living with a chronic HBV infection.[14] This chronic infection is associated with hepatic lesions of variable severity, ranging from a practically normal liver to severe lesions of active chronic hepatitis that can progress to cirrhosis.[13] HBV chronic infection may also be associated with the development of hepatocellular carcinoma (HCC).[15] HBV is not directly cytopathic for hepatocytes, the onset of HCC is mainly linked to the development of chronic diseases on a cirrhotic liver.[15 16] There is also evidence for a direct role of HBV in HCC development, through integration of the viral DNA into the host genome as well as via viral protein expression.[15] There is no complete cure for the majority of chronically infected patients, although current lifelong oral treatments lead to effective viral suppression.[17]

The viral genome of HBV consists of a partially double-stranded, circular molecule of 3.2 kb. There are 10 genotypes of HBV (A–J), presenting DNA sequence divergence of about 8%. HBV genotypes are differentially distributed worldwide and this geographical repartition might be associated with different outcomes in disease progression and responses to treatment.[13 16] The circulating HBV particle comprises an outer lipid viral envelope and an icosahedral nucleocapsid that contains a relaxed circular DNA associated with viral

polymerase.[13 16] Internalisation of the HBV particle is driven by the sodium taurocholate cotransporting polypeptide receptor, a transmembrane transporter mainly expressed on hepatocytes.[18] After entry into the hepatocyte, the partially double-stranded, relaxed, circular DNA is repaired in the nucleus, to form the covalently closed circular DNA (cccDNA), matrix of the viral transcription.[13 14 16 19] Four unspliced transcripts of 3.5, 2.4, 2.1 and 0.8 kb are synthesised from the HBV genome and encode for three surface proteins (large PreS1/S2/S, middle PreS2/S, small S), core protein (forming viral capsid), HBe protein (translated from the 3.5 kb preC RNA, marker of viral replication), DNA polymerase protein and HBx transactivator protein (involved in viral replication and carcinogenesis).[15 16] Among the viral transcripts, the 3.5 kb pregenomic mRNA (pgRNA) is packaged through its ε-stem-loop structure with the viral polymerase into the nucleocapsid, where its reverse transcription occurs.[13] Then, the nucleocapsid is enveloped and secreted or recycled from the cytoplasm back into the nucleus, leading to cccDNA amplification.[13 14 16]

In addition to unspliced HBV mRNAs, accumulation of evidence has demonstrated that the pgRNA and, more recently, the PreS2/S surface mRNA may undergo single or multiple splicing events. The existence of alternative spliced viral RNAs has also been reported in other members of the *Hepadnaviridae* such as the woodchuck and duck hepatitis viruses.[20 21] Although the existence of HBV-spliced RNAs has been known for decades, their importance in viral biology is not clearly understood. This review will update the knowledge on the mechanism by which HBV-spliced viral RNAs are generated and their biological impact on the course of HBV infection and during liver disease.

Alternative splicing of HBV RNAs and formation of defective HBV particles

The first identification of a spliced HBV RNA processed from the viral pgRNA was made in 1989 in the liver of infected patients.[22] Two spliced pgRNA isoforms, resulting from single splicing (sp1) or double splicing (sp10) excision, were detected by northern blot.[22–24] The preservation of the ε-stem-loop structure at the 5′-terminal region of spliced isoforms argues for their probable packaging and reverse transcription ability. This was confirmed by the detection of HBV-defective DNA generated from sp1 or sp10 RNAs in circulating particles.[25–33] These circulating viral particles are defective considering their failure to self-replicate in the absence of trans-complementation with a wild-type virus.

Nowadays, 20 HBV-spliced variants (sp1 to sp20) derived from the pgRNA and expressed at different amounts are reported, suggesting a sharp regulation of splicing machinery (figure 1). Among them, seven spliced variants were detected, mainly by real-time (RT)qPCR, in infected liver of patients with either chronic active infection or HCC (figure 1).[22 34–36] Twelve splicing isoforms were indirectly observed as circulating defective particles in blood samples as well as in supernatants of HBV transfected human hepatoma cells.[26 27 30 31 33 36–42] At last, the isoform derived from sp18 was identified in HBV DNA-transfected cell (figure 1).[43 44] Recently, several new putative spliced isoforms were reported by RNA-seq analysis in transfected Huh7 cells or liver biopsy samples.[35] However, these new spliced isoforms need further confirmation using direct identification methods.

Computational analysis identified numerous splice sites throughout the HBV pgRNA. Only some of these predicted splice sites have been attributed a role in the synthesis of the 20 HBV alternative splicing variants.[45] This observation highlighted that usage of splice sites also depends on a dynamic secondary structure of the HBV pgRNA.[46–48] The splice donor (SD) and splice acceptor (SA) sites involved in the generation of the 20 variants are located at positions 2067, 2087, 2447, 2471 and 2985 for SD or at positions 2236, 2350, 2902, 3169, 282 and 489 for SA (HBV genotype D nucleotides numbering). Combinations between donor/acceptor sites generating single and multiple splicing variants are illustrated in figure 1. The sp1 isoform is the main splicing variant (2447SD/489SA), accounting for up to 30% of total pgRNA and generated up to 70% of whole HBV DNA circulating particles.[25 29–33 35 38 40 43 49–52] Regarding the other spliced HBV RNA variants and related defective particles, their amount was less frequently studied and quantified and may vary from 0% to 15% of total circulating particles.[30 31 43 52] Altogether, the SD 2447 and SA 489 sites are frequently recognised, contributing to the synthesis of 32% and 43% of HBV RNA-spliced variants, respectively (figure 2A).

Besides pgRNA, it should be noticed that alternative splicing regulation of the subgenomic RNA encoding HBV envelope proteins was also reported (figure 1). Alternative splicing of the PreS/S mRNA relies on the splicing donor and acceptor sites at positions 458 and 1305 (sp21) or 1385 (sp22), respectively.[53 54]

Detection of HBV RNA splicing and related defective particles

As previously mentioned, the first identification of an alternative splicing regulation of HBV pgRNA was performed in 1989 by northern blot analysis.[22–24 55] Two years later, Terre *et al*, reported the detection of HBV-defective particles containing DNA derived from spliced RNA by PCR in the blood of infected patients.[33 41] Then, amplification of the full-length HBV genome by PCR highlighted the occurrence of various defective particles generated from pgRNA-spliced forms.[26] Development of (RT)qPCR approaches using specific primers of spliced RNAs and related defective genomes has largely contributed to better characterise the amount of circulating defective particles in vitro as well as in vivo.[32 38] Thereby, clinical studies established a correlation between circulating defective HBV particles from sp1RNA and their wild-type viral counterpart.[25 32 38 39 49] Yet, some variations of the proportion of circulating defective particles revealed a modulation of HBV alternative splicing during liver disease progression.[39 49 50] However, the complete pattern of HBV-spliced isoforms and related defective particles remain hardly assessable by (RT)qPCR. Only semiquantitative universal approaches such as Rapid Amplification of cDNA Ends (RACE PCR), or next-generation sequencing (NGS) after amplification of every single viral genome, allowed to define this pattern.[35 56] Second-generation NGS, which relies on short read sequences, may show limitations in characterising the splice junctions. Nevertheless, such approach has consolidated the diversity of HBV-spliced isoforms expression.[35] In contrast, recently developed third-generation NGS, whose method achieves full-length sequencing in one single read of HBV genomes (both wild-type and splice-derived), would provide the proportion of each viral form in biological samples.[57–59]

Cis-regulation and trans-regulation of HBV RNA splicing

Numerous studies have reported that the isoforms and amount of HBV RNA-spliced variants as well as related defective particles may differ between patients.[25 26 29 32 38–40 49–51] Regulation of pgRNA splicing is contingent on either cis-regulation, which depends on viral genetic variability, or trans-regulation, through the regulation of the spliceosome machinery activity.[26 31 52 60]

Regarding HBV cis-acting sequences, splicing sites (SD and SA) were described in HBV consensus sequences of genotypes A to

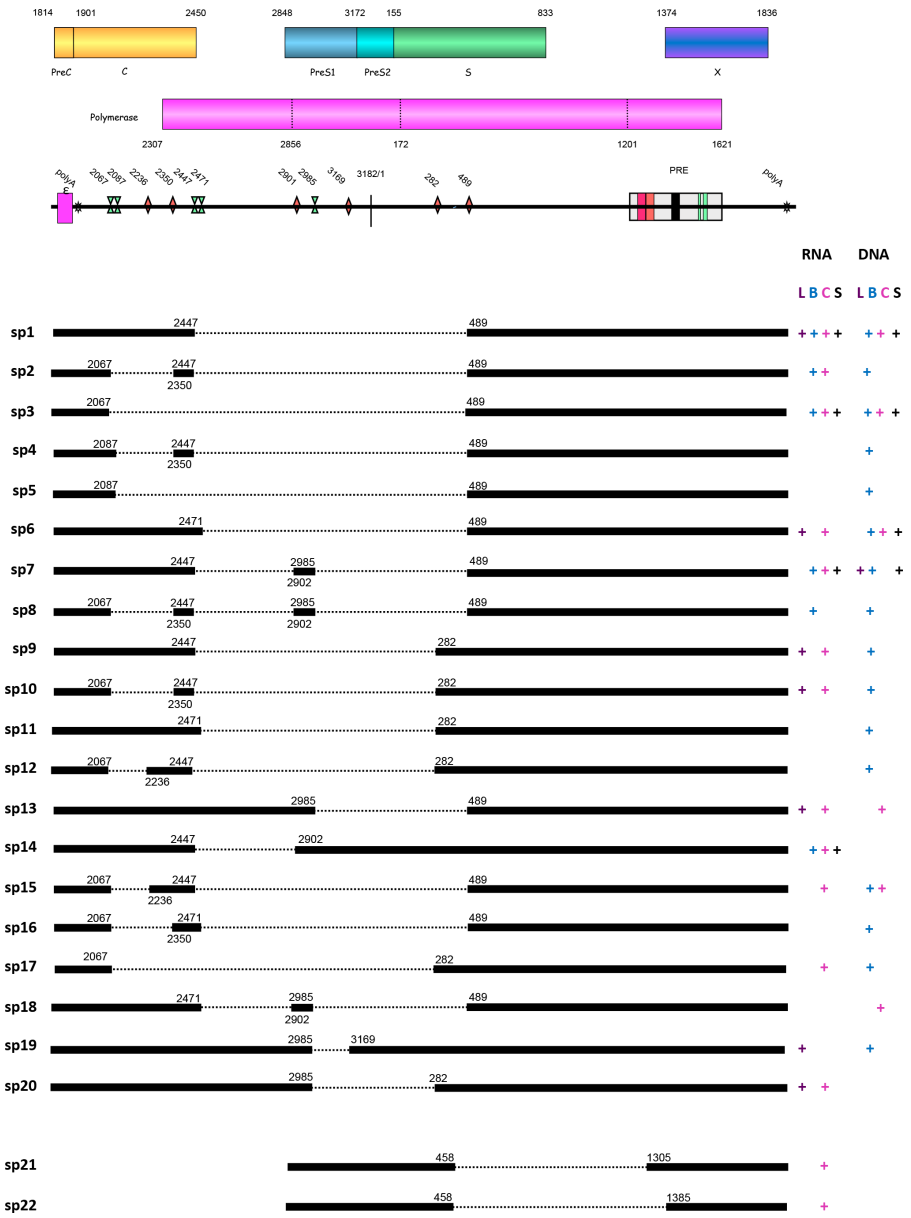

Figure 1 Splicing variants generated from alternative splicing regulation of HBV RNAs. Upper panel HBV pregenomic RNA sequence (genotype D numbering from nucleotide 1–3181)which includes the open reading frames (boxes) of PreC-C (yellow), PreS1/PreS2/S (green), HBx (blue) and polymerase (pink, dotted lines delimitate terminal protein, spacer, reverse transcriptase and RNAseH subdomains) genes. Splice donor (square) and acceptor (diamond) sites are indicated all along the sequence of the pgRNA. Middle panel 20 spliced pgRNA isoforms can be generated by alternative splicing of HBV pgRNA. Due to the lack of a consensus nomenclature in the naming of the different spliced isoforms, we propose the following labels: sp1–sp20. Detection of each splicing-generated isoform in biological samples (L, B, C, S) either under RNA (spliced transcripts) or DNA (defective particles) forms was indicated in the right size. Lower panel alternative splicing regulation of the subgenomic HBV envelope RNA leads to the sp21 and sp22 variants. *Bibliographic references associated*: sp1RNA[22–24 26 31–35 39 40 43 44 49 50 52 55 68 90 118 119]; sp2RNA[25 26 44 113]; sp3RNA[25 26 31 43 44 113 119]; sp4RNA[25 26 35]; sp5RNA[25 26 35]; sp6RNA[25 26 31 33 35 41 43 44]; sp7RNA[25 26 35 36 44 85 94 116]; sp8RNA[26 113]; sp9RNA[26 35 44]; sp10RNA[25 26 34 44 52 88]; sp11RNA[26 35]; sp12RNA[25 35]; sp13RNA[35 43 55 119]; sp14RNA[33 35 41 94 116]; sp15RNA[25 31 44]; sp16RNA[25]; sp17RNA[25 44]; sp18RNA[35 43 44]; sp19RNA[28]; sp20RNA[35]; sp21RNA[53 54]; sp22RNA.[53 54] L B C S: liver, blood, cell and supernatant of cells.

D but their intra-genotypic conservation remains poorly studied. Furthermore, several reports suggested a relationship between HBV genotype and the regulation of alternative splicing through an unknown mechanism.[25 31 44 60] Thus, to precise the influence of HBV genetic variability on alternative splicing regulation, we compared the sequence of the 11 SD and SA sites of the pgRNA across HBV genotype A, B, C, D and E from 5962 sequences extracted from the HBV databank.[61] As expected, the eukaryotic consensus sequences of splice sites (GT for SD and AG for SA) were preferentially identified in HBV (table 1).

Conservation of consensus splice site sequences was higher than 95% across all genotypes, except for SD 2067 and 2087 (table 1). The main sequence of the donor site 2067 (GC), detected in over than 99% of the genotypes, might be also

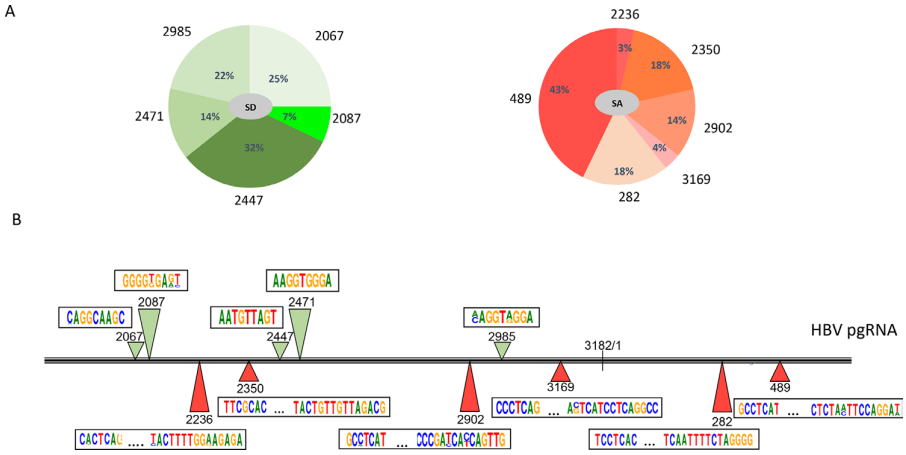

Figure 2 Splice sites commitment in pgRNA: frequency of use and nucleotide sequences environment in genotypes A, B, C, D and E. (A) Frequency of use of the donor (left panel) and acceptor (right panel) splice sites in the synthesis of the different spRNAs. (B) Genetic variability of HBV sequence surrounding splice sites in 5962 whole HBV sequences extracted from HBVdb.[57] The nucleotide variability is illustrated between −3 and +6 for donor sites and −12 and +3 for acceptor sites. The predictive splice branch point sequence of pgRNA alternative splicing, located between −40 and −200 nucleotides, is indicated downstream of each acceptor site.[59] These data were generated using the weblogo V.3 software. pgRNA, pregenomic RNA.

recognised by the spliceosome machinery.[62 63] Regarding the site 2087, a consensus GT sequence was predominantly observed in the genotypes B and C, while a GG sequence was mainly found in the genotypes A, D and E. Although the GG sequence could also be considered as a SD site,[4 62 63] splicing forms generated from the SD 2087 (sp4 and sp5) were only detected in genotype B and C.[25 26 64] Altogether, the robust level of conservation of the splice sites highlights a critical role of alternative splicing during HBV infection. Additional nucleotide sequence elements located close to the splice sites may also contribute to the splicing regulation. Like splice sites, these surrounding regions may be affected by the viral genetic variability. Alignment of these HBV nucleotide sequences, including the splicing branch point, also revealed a remarkable conservation across genotypes (figure 2B). One exception was detected in the genotype D, where SD 2350 does not seem recognised in splicing regulation (ie, no synthesis of sp10RNA), probably due to a nucleotide substitution (G2335A) in an adjacent regulatory sequence.[44] The clinical relevance of this apparent genotype-specific splicing regulation of HBV needs further investigation.

Reports have demonstrated, by analogy with the Rev response element of HIV-1, the existence of a post-transcriptional regulatory element (PRE) located at the 3′ end of HBV transcripts. The PRE sequence (nucleotides 1151–1684 in genotype D) was

Table 1 Genetic variability of splice donor and acceptor sites used in HBV pgRNA for genotypes A, B, C, D and E

		Genotype (sequence number)				
Splice sites Genotype D numbering		A (n=850)	B (n=1718)	C (n=2079)	D (n=1028)	E (n=287)
Splice donor	2067	GC 99.9%	GC 99.7%	GC 99.5%	GC 99.9%	GC 100%
	2087	GG / GT 92.4 %/0.2%	GT / GG 89 %/6%	GT / GG 93.6 %/0%	GG / GT 94.3 %/0.2%	GG / GT 87.8 %/0%
	2447	GT 100%	GT 99.7%	GT 99.8%	GT 99.5%	GT 99.7%
	2471	GT 99.6%	GT 100%	GT 99.9%	GT 99.6%	GT 100%
	2985	GT 99.8%	GT 99.7%	GT 99.9%	GT 99.7%	GT 100%
Splice acceptor	2236	AG 99.6%	AG 98.5%	AG 98.8%	AG 99.0%	AG 99.3%
	2350	AG 100%	AG 100%	AG 99.9%	AG 99.9%	AG 100%
	2902	AG 98.6%	AG 99.8%	AG 99.7%	AG 99.7%	AG 100%
	3169	AG 99.3%	AG 95.1%	AG 99.5%	AG 96.9%	AG 99.0%
	282	AG 99.8%	AG 99.9%	AG 99.7%	AG 99.3%	AG 100%
	489	AG 99.9%	AG 99.9%	AG 99.9%	AG 99.4%	AG 99.0%

first attributed a role in the nuclear export of unspliced viral subgenomic RNAs through CRIM1-independent pathways.[65–67] However, the PRE is a multifunctional highly structured cis-element with silencer and enhancer regions controlling viral splicing in a cell type-dependent manner.[46 47 68 69] Indeed, by using gene reporter approaches, some regions with positive or negative regulation of the splicing of the pgRNA have been described within the PRE.[47 70]

A second regulatory region, located in the intronic fragment of the pgRNA (nucleotides 2951–3163 in genotype D), was identified as a long intronic splicing silencer (ISS_L). This sequence which covers silencer and enhancers sites, suppresses the alternative splicing of sp1RNA.[46] Notably, like for the PRE, modification of the ISS_L sequence altered splicing efficiency but also affected the nuclear export of HBV transcripts (and in a lesser extent their stability), highlighting a close relationship between both mechanisms.[46 47 68 70]

A third region regulating the splicing of the pgRNA encompasses the HBV enhancer II and the basal core promoter (EN-II/BCP). These latter sequences have pivotal roles in HBV replication and possibly in viral pathogenesis.[71 72] In a previous report, it was found that LUC7 like 3 (LUC7L3), an RNA splicing factor belonging to the SR family, is a negative regulator of the HBV replication process, through the downregulation of the EN-II/BCP activity.[73] Furthermore, overexpression of LUC7L3 increased the ratio of spliced over unspliced pgRNA.[73] Thus, it could be hypothesised that negative regulation of the ENII/BCP activity is associated with the promotion of the pgRNA alternative splicing. In line with this hypothesis, it was recently demonstrated, *in vitro*, that another splicing factor, PUF60, suppresses the splicing of the pgRNA and promotes its degradation by enhancing the activity of the HBV core promoter.[74]

As reported, the alternate splicing activity of these regions also depends on numerous trans-acting splicing factors. In order to identify the mechanisms of HBV alternative splicing trans-regulation, nuclear proteins interacting with the HBV pgRNA were explored by RNA pull-down assay.[50] Among the 389 proteins interacting with HBV pgRNA, 15% were directly related to the splicing machinery.[50] These factors act as splicing inhibitors or activators and in addition, contribute to the nuclear export of unspliced HBV RNAs, as it was recently reported for the SRSF10.[75] Moreover, it was demonstrated that the amount of spliced HBV RNAs was higher in cells derived from human HCC than in non-hepatic cancer cells, suggesting tissue specificity of HBV splicing related to the expression of trans-acting factors.[60] A modulation of expression of trans-regulating splicing factors was also reported during liver disease progression towards HCC.[76–78] In the case of HBV, the proportion of defective particles containing spliced RNA-derived genomes increases with the course of liver disease.[21 32 38 39 49 51] Thus, we used an appropriate transgenic mouse model expressing the whole HBV genome (unconcerned by a cis-regulation impact) to explore the trans-regulation of HBV alternative splicing events according to liver damage. We took advantage of this model to demonstrate that the modulation of some trans-regulatory splicing proteins such as SF1, PSF, SRSF1 and La was related to liver injuries and subsequently increased the level of HBV-spliced sp1RNA.[50] This upregulation of HBV alternative splicing contributed to liver disease and may participate to the viral persistence.[50]

Besides RNA-interacting splicing factors, other host proteins, known to interact in trans with DNA, may also participate in HBV alternative splicing regulation. For instance, histone deacetylase 5, which removes acetyl groups from histone and non-histone proteins, has been shown to enhance splicing of HBV RNAs.[79] By contrast, the trans-active response DNA-binding protein has been speculated to downregulate the splicing of pgRNA by activation of the core promoter of HBV DNA as well as in a post-transcriptional level during nuclear export.[80]

In summary, HBV alternative splicing events depend on multifactorial mechanisms. Despite the involvement of cis-regulation and its modulation according to the viral genetic variability, trans-acting splicing factors play a major role in HBV alternative splicing regulation during liver disease.

HBV RNA-spliced variants in viral life cycle

In contrast to other viruses, generation of HBV RNAs from alternative splicing seems dispensable for viral replication.[23] However, the multiplicity of spliced transcript forms raised the question of their impact on the viral life cycle. Despite similarities between the outcome of unspliced and spliced pgRNAs (packaging, reverse transcription and secretion as Dane wild-type or defective particles, respectively), several studies have emphasised differences in the behaviour of both RNA isoforms (figure 3). Indeed in nucleocapsids, HBV pgRNA is reverse transcribed and generates double-strand DNA either under linear (dslDNA) or relaxed circular (rcDNA) forms. rcDNA viral particles were largely involved in the cccDNA formation either after repooling from the cytoplasm towards the nucleus or after de novo infection.[13] By contrast, dslDNA cannot contribute to the formation of cccDNA. Nevertheless, this dslDNA is the main integrative form into host genome.[13 19]

In contrast, spliced RNAs are probably unable to generate defective cccDNA. Indeed, it was shown that encapsidated HBV-spliced RNAs mainly supported the synthesis of minus-strand DNA and duplex linear DNA but not relaxed circular defective DNA (rcdDNA).[43] This is probably associated with the excision of a cis-acting sequence, required for the synthesis of relaxed circular HBV DNA, after splicing (figure 3).[37] Subsequently, the absence of rcdDNA impaired the possibility to generate defective cccDNA. Additionally, taking into account that duplex linear DNA is the main HBV integrated form into the host genome, defective DNA genomes derived from alternative splicing of pgRNAs should be easily detected in the chromosomal sequence of infected hepatocytes. Yet, HBV-defective sequences have never been reported into the human genome to date.[81] Taken together, the failure of HBV nucleocapsids containing spliced RNA to produce on one hand rcdDNA, and to generate on the other hand integrative defective DNA forms may support a deficient mechanism of nuclear readdressing. In line with this hypothesis, an accumulation of nucleocapsids containing genome derived from spliced RNAs might be observed in the cytoplasm of infected cells.[43] In addition, it was established that the proportion of intracellular spliced RNAs was significantly higher than related defective particles released in the blood or cell supernatant.[25 31 39 43] Such higher ratio of spliced RNAs in the intracellular compartment could be explained by a lower efficiency of the reverse transcription process than for unspliced pgRNA. However, these results could also be attributed to a defect of viral maturation or secretion of HBV-defective particles.

Another question raised by the alternative splicing regulation of HBV is that viral proteins encoded by spliced RNAs may contribute to modulate the replication of HBV. The HBV core is a structural protein of 21 kDa that self-assembles to form the viral nucleocapsid, supporting the reverse transcription step of viral replication.[82 83] Spliced RNAs, and particularly the sp1RNA, contain an open reading frame encoding a precore/core protein deleted of its last C-terminal amino-acid (ΔC183).

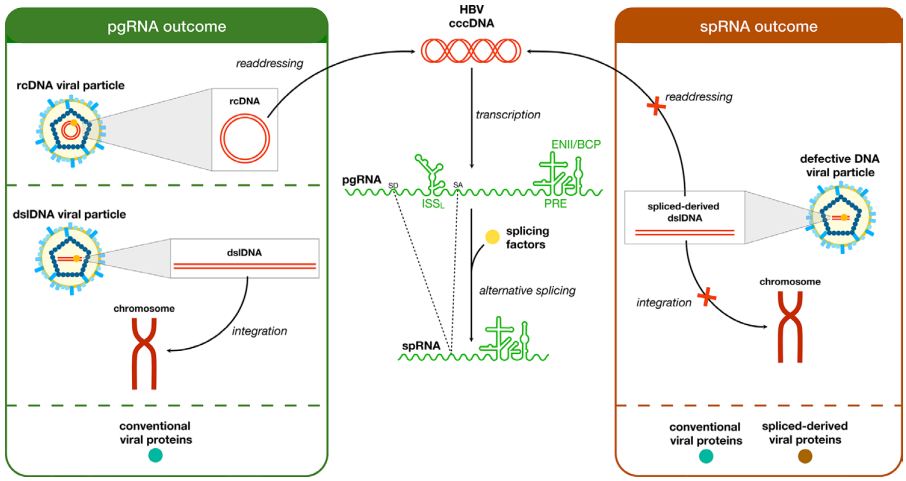

Figure 3 Outcome of pregenomic and spliced HBV RNAs during viral cycle.The outcome of pgRNA and spRNA was illustrated in left and right panel, respectively. Unspliced and spliced viral RNAs may be packaged, reverse transcribed and secreted as wild-type or defective DNA particles, respectively. Viral DNA genome in wild-type particles contains partial dslDNA or rcDNA forms. Focus on rcDNA showed its implication in the cccDNA formation after either readdressing from the cytoplasm towards the nucleus or de novo infection. Double–strand linear DNA is the main integrative form into host genome. Translation of pg and subgenomic RNAs led to the conventional HBc, HBe, HBx, Pol and envelops proteins expression (blue dot). Alternative splicing of pgRNA depends on cis-regulation elements (pre, ISS_L and EnhII/BCP) and trans-regulatory factors including SF1, PSF, SRSF1 and La proteins (yellow dot). Spliced RNA generates dsl-defective DNA, which does not contribute to the cccDNA formation or host integration form. Spliced RNAs may be translated in splice-derived truncated or original viral proteins (brown dot) termed as HBc ΔC183, RT′-RH/HpZ/P′, HBSP, PS, HBDSP, spPS1. dslDNA, double-strand DNA under linear form; pgRNA, pregenomic RNA; rcDNA, relaxed circular DNA.

On one side, the truncated precore protein interferes with the assembly of the nucleocapsid, which may inhibit HBV replication.[82] Intriguingly, this report suggests that HBV preC RNA (encoding precore protein) undergoes similar alternative splicing regulations than pgRNA. On the other side, the truncated core protein remains either fully replication competent[82] or produces nucleocapsids containing preferentially spliced RNA isoforms.[84] It was also reported that a viral protein, generated by the doubly spliced sp7RNA, enhances the replicative competency of full-length HBV genome. This is probably through activation of HBV regulatory elements including viral promoters, Enhancer I and core upstream regulatory sequences.[36 85] Cotransfection studies have reported that the spliced RNA variants (sp1, sp8, sp10 and sp16) modulated HBV replication.[36 42 86] Mechanisms implicated in this modulation remain to be characterised, although in vitro studies have already shown that sp10 RNA reduces the transcription level of pgRNA.[87 88] Finally, it was also suggested that an increased level of HBx protein, which may be translated from all HBV RNA-spliced variants, might contribute to the viral replication.[36] Altogether, these data support that spliced RNA may interfere with HBV replication.

Viral interference was largely reported in the field of virology but not for HBV, to date.[89] Viral interference is an asynchronous cycle event where one virus inhibits the replication of other forms of virus, contributing to viral persistence.[89] For HBV, we could hypothesise that defective particles generated from spliced RNA may interfere with wild-type virus particles. However, viral quantification of both wild-type and defective particles, in clinical samples, did not support an interfering influence on replication. Indeed, defective particles derived from sp1RNA were almost detected in highly replicative patients and despite variations of their proportion (from undetectable to more than 70% of the whole circulating viruses), their mean ratio remained consistent and weak (3% to 4%) across all clinical studies.[21 25 26 29–33 38 39 49–51]

Collectively, these data suggest a specific outcome of spliced RNAs compared with pgRNA and a putative role on HBV life cycle. Nevertheless, to date, these reports did not support a significant interference mechanism of the spliced RNAs (and the related defective particles) on viral replication.

Alternative splicing of HBV and liver pathogenesis

Whereas spliced RNAs do not seem essential to viral replication, accumulated evidence suggests a role during liver pathogenesis. Therefore, two questions may arise: (1) can HBV-spliced RNAs be useful biomarkers for the clinical management of HBV liver disease? (2) can HBV-spliced RNAs encode unconventional viral proteins which play a role in liver injury?

HBV-spliced variants as biomarkers of liver disease?

The frequent detection of spliced forms in HBV infected patients (as well as in woodchuck or duck hepatitis samples) presumed for their impact on the course of liver disease. Clinical studies reinforced this hypothesis by positively correlating the proportion of circulating defective particles, mainly generated from sp1RNA, with the severity of liver inflammation and fibrosis.[32 50 51 90] In line with this result, a longitudinal retrospective study revealed an increased proportion of sp1-derived defective particles prior to the development of HCC.[49] Additionally, the production of sp1RNA-derived defective particles is altered in presence of HBV mutation associated with the lamivudine-drug resistance.[38] Altogether, these clinical studies stressed on the relationship between spliced RNAs and liver disease progression, although all these findings were almost obtained from quantification of sp1RNA and its related defective particles.

Up to date, only a few studies have focused on the whole pattern of spliced RNA isoforms and their associated defective particles. As previously reported in studies on sp1RNA,[39 49] a deep sequencing analysis highlighted a fluctuation of circulating

defective viruses derived from multiple spliced isoforms along the course of infection.[91] Furthermore, the level but not the pattern of total circulating defective HBV DNA negatively correlated with the response to interferon-α therapy.[25] Finally, the expression profile of RNA-spliced variants differed in HCC samples from patients infected with two different HBV genotypes.[35] However, the viral diversity, related to the whole HBV-spliced RNAs and related defective particles, needs to be better investigated.

Unfortunately, these circulating defective DNA forms will not be helpful as biomarkers for the clinical management of HBV infection, considering their sensitivity to nucleos(t)ide analogues therapy.

HBV-spliced variants as a source of pathogenic unconventional viral proteins?

It is tempting to speculate that enrichment of spliced transcripts, which initiate defective particles, might contribute to liver pathogenesis through their ability to encode new viral proteins. Numerous potential truncated or original proteins could derive from HBV-spliced RNAs. However, only few studies, reported below, focused on this feature. Besides being the main spliced viral transcript, sp1RNA encodes for original and truncated splicing-derived proteins detected in vitro and in vivo.[40 92] Additionally, three other splicing-generated proteins have been reported, mainly in vitro, namely: (1) the 43 kDa polymerase-surface (PS) protein which derives from a spliced pgRNA lacking intron 2447/2902 (sp14RNA), (2) the hepatitis B doubly spliced protein (HBDSP) which derives from a spliced pgRNA lacking intron 2447/2902 and 2985/489 (sp7RNA) and (3) the splicing PreS1 deletion protein (spPS1) which is translated from a spliced RNA lacking intron 2985/3169 (sp19RNA).[87 93–95]

Viral protein generated from sp1RNA

The sp1RNA encodes for the eighth HBV protein named HBSP.[40] The HBSP sequence shares its N-terminal amino acids with the N-terminal part of the viral polymerase (47 aa), while, due to the splicing event, its C-terminal moiety (64 aa) consists of an original sequence. In vivo, HBSP has been detected in the liver of patients with chronic HBV infection and anti-HBSP antibodies have been detected in 30%–50% of sera of HBV chronic carriers.[40 51] Detection of these antibodies is significantly associated with severe liver fibrosis.[51] In addition, the relationship between HBSP and the progression towards advanced stages of the liver disease were also suggested by the ability of HBSP to activate T-cell responses in HBV-infected patients[96]; this without a major contribution to the hepatic inflammatory process.[97] However, our group reported on an attenuation of liver damage in HBSP transgenic mice following the induction of local inflammation and fibrosis through the stimulation of the TNF-α-regulated signalling pathways.[50 90] The protective effect of HBSP on the liver resulted from a downregulated expression of C–C motif chemokine ligand 2 (CCL2) by hepatocytes and a subsequent decreased recruitment of inflammatory monocyte/macrophages.[50] Furthermore, such reduced expression of CCL2 in the liver of HBV chronic carriers was associated with upregulation of HBV pgRNA splicing.[50] These findings argue for mechanisms whereby a viral protein hacks some signalling pathways involved in innate immunity and limits the extent of liver inflammation and fibrosis, which may contribute to viral persistence. In agreement with a role of HBSP to modulate liver disease, a recent report demonstrated that HBSP suppresses Fas-mediated hepatocyte apoptosis by enhancing the activity of PI3K/Akt signalling.[98]

In the opposite way, in vitro studies have shown that HBSP induces several hallmarks of cell apoptosis through a putative BH3 homology domain in its N-terminal region.[99 100] It was proposed that HBSP, by interacting with the fibrinogen γ chain or with microsomal epoxide hydrolase, may interfere with cell homeostasis and may participate to hepatic metabolic perturbation during HBV infection.[101 102] In addition, it was shown that HBSP can interact with the cathepsin B and eventually contribute to cell migration and invasion during cancer.[103] Nevertheless, these investigations were most of the time performed in a situation where HBSP was expressed outside of the whole HBV genome context. The study of HBSP function in the HBV context remains complex considering the liver disease-dependence of its expression through HBV alternative splicing regulation, particularly during liver carcinogenesis.

Additionally, sp1RNA may also encode for a truncated polymerase protein of 42,3 kDa, initiated from the start codon $A^{2446}T^{2447}G^{489}$ generated by the splicing junction and named either RT′-RH or HpZ/P′.[25 92] Expression of this truncated protein is depending on the splicing factor SRSF2 and impaired HBV replication through an interaction either with the RNA helicase SUPV3L1 or with proteins involved in chromatin and histone functions.[92]

Finally, in vitro expression of sp1RNA also leads to the intracellular accumulation of ‘conventional’ HBV core proteins, which are able to inhibit interferon-mediated induction of the antiviral protein MxA.[29 86]

Viral protein generated from sp14RNA

The PreS1 domain of the 43 kDa PS protein, which is encoded by the sp14RNA has only been detected in vitro. It was suggested that this fusion protein might be a substitute for the HBV large surface protein during viral maturation.[87 94] In cotransfection experiments, this protein, which is localised in the perinuclear region, inhibited HBV replication through a mechanism that remains to be characterised. Its biological implication in HBV pathogenesis was evoked through inhibition of HBs secretion (mediated by ER retention) which contributes to viral immune escape.[87]

Viral protein generated from sp7RNA

The doubly spliced variant lacking ‘introns’ 2447/2902 and 2985/489, initially isolated from HCC tissue sample, encodes for HBDSP.[36 85] The HBDSP protein was predicted to begin at the start codon of the polymerase and to share its 47 N-ter amino acids with the latter as a consequence of the first splicing. The C-ter domain of HBDSP consists of 27 amino acids shared with the PreS1 protein as a result of the second splicing.[85] The chimeric 74 amino acids HBDSP was detected in hepatoma cell lines transfected with the sequence of a defective HBV genome resulting from the 2.2 kb doubly spliced transcript. This protein shows weak transactivating properties and pleiotropic effects mediated through the activator protein-1-binding and CCAAT/enhancer-binding sites.[85] However, precise mechanisms associated with the regulatory functions of HBDSP and its biological implication in HBV pathogenesis needs further investigation.

Viral protein generated from sp19RNA

Deletions in the PreS region have recurrently been associated with the development of liver diseases and particularly HCC.[93 104–109] Intriguingly, spliced RNAs lacking intron 2985/3169 generates an in-frame deletion in the 3′ end of the PreS1 region.[28 95] This variant encodes for the splicing PreS1

deletion protein (183-nucleotide deletion in the C-ter) named spPS1. In vitro, immunofluorescence staining showed that spPS1 protein accumulated within the endoplasmic reticulum (ER).[95] This observation probably results from abnormal transmembrane topologies of the spPS1 protein in the ER. It was hypothesised that the truncated protein may induce oxidative stress, through enhanced production of reactive oxygen species, contributing to inflammation.[95] However, understanding the role of the ER and oxidative stresses in HBV spPS1-related liver disease will require further investigation. Clinical study concluded that sp19 detection, in combination with BCP and Pre-C mutations, was associated with the development of liver cirrhosis.[28]

CONCLUDING REMARKS

The role of alternative splicing in HBV infection and pathogenesis is slowly but steadily getting recognised. Accumulated data clearly demonstrate that HBV splicing is a common event during HBV chronic infection and liver diseases. Regarding virus replication, regulation of alternative splicing of HBV transcripts contributes to the diversity of circulating viral particles in the blood of infected patients. Furthermore, recent studies report on the existence of HBV particles containing RNA, including spliced RNA, in sera of chronically infected patients with HBV. The role of these unspliced or spliced RNA particles remains to be clarified although current study suggests their failure to generate a productive HBV infection.[110] Already, their titre in the bloodstream may show a predictive value of response to antiviral therapy.[56 111–116] In contrast to numerous viruses, splicing of viral RNAs does not seem essential for the life cycle of HBV. However, the faculty of these HBV-spliced transcripts to encode original proteins that are able to interfere with viral replication and the outcome of the nucleocapsids highlight the requirement of further studies to better define the biological meaning of these features.

In human cancers, dysregulation of alternative RNA splicing contributes to disease progression by modulating RNA isoforms encoding for proteins involved in cell proliferative pathways.[117] Furthermore, the nature of the liver microenvironment can also influence which isoforms are expressed in a given cell type. A better understanding of HBV alternative splicing accurate control (ratio spliced over unspliced isoforms) will enlarge our knowledge on direct role of splicing in HBV liver pathogenesis. In this context, is the regulation of HBV alternative splicing a hallmark of changes in the liver microenvironment or does it contribute to enhance or control liver disease development? The described correlation between an increased proportion of splicing variants in the blood and the severity of liver fibrosis or HCC argues for a significant role of the HBV splicing variants in liver pathogenesis. In this line, the microenvironment, modified by liver injury, tends to promote the sp1RNA synthesis and subsequently the expression of the HBSP protein. In turn, HBSP interferes with the hepatocyte production of chemokines, which may contribute to viral persistence by favouring immune escape.

To date, tricky regulation of HBV alternative splicing may impact on many processes all along the course of HBV liver disease. Further studies are necessary to better decipher its contribution to viral persistence and liver pathogenesis.

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REFERENCES

1 Black DL. Mechanisms of alternative pre-messenger RNA splicing. *Annu Rev Biochem* 2003;72:291–336.
2 Hallegger M, Llorian M, Smith CWJ. Alternative splicing: global insights. *Febs J* 2010;277:856–66.
3 Irimia M, Blencowe BJ. Alternative splicing: decoding an expansive regulatory layer. *Curr Opin Cell Biol* 2012;24:323–32.
4 Kornblihtt AR, Schor IE, Alló M, *et al*. Alternative splicing: a pivotal step between eukaryotic transcription and translation. *Nat Rev Mol Cell Biol* 2013;14:153–65.
5 Scotti MM, Swanson MS. RNA mis-splicing in disease. *Nat Rev Genet* 2016;17:19–32.
6 Han SP, Tang YH, Smith R. Functional diversity of the hnRNPs: past, present and perspectives. *Biochem J* 2010;430:379–92.
7 Kelemen O, Convertini P, Zhang Z, *et al*. Function of alternative splicing. *Gene* 2013;514:1–30.
8 Pan Q, Shai O, Lee LJ, *et al*. Deep surveying of alternative splicing complexity in the human transcriptome by high-throughput sequencing. *Nat Genet* 2008;40:1413–5.
9 Hernandez-Lopez HR, Graham SV. Alternative splicing in human tumour viruses: a therapeutic target? *Biochem J* 2012;445:145–56.
10 Meyer F. Viral interactions with components of the splicing machinery. *Prog Mol Biol Transl Sci* 2016;142:241–68.
11 Karn J, Stoltzfus CM. Transcriptional and posttranscriptional regulation of HIV-1 gene expression. *Cold Spring Harb Perspect Med* 2012;2:a006916.
12 Graham SV, Faizo AAA. Control of human papillomavirus gene expression by alternative splicing. *Virus Res* 2017;231:83–95.
13 Seeger C, Mason WS. Molecular biology of hepatitis B virus infection. *Virology* 2015;479-480:672–86.
14 Seto W-K, Lo Y-R, Pawlotsky J-M, *et al*. Chronic hepatitis B virus infection. *Lancet* 2018;392:2313–24.
15 Brechot C, Kremsdorf D, Soussan P, *et al*. Hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC): molecular mechanisms and novel paradigms. *Pathol Biol* 2010;58:278–87.
16 Yuen M-F, Chen D-S, Dusheiko GM, *et al*. Hepatitis B virus infection. *Nat Rev Dis Primers* 2018;4:18035.
17 Zeisel MB, Lucifora J, Mason WS, *et al*. Towards an HBV cure: state-of-the-art and unresolved questions--report of the ANRS workshop on HBV cure. *Gut* 2015;64:1314–26.
18 Yan H, Zhong G, Xu G, *et al*. Sodium taurocholate cotransporting polypeptide is a functional receptor for human hepatitis B and D virus. *Elife* 2012;1:e00049.
19 Seeger C, Mason WS. Hepatitis B virus biology. *Microbiol Mol Biol Rev* 2000;64:51–68.
20 Hantz O, Baginski I, Fourel I, *et al*. Viral spliced RNA are produced, encapsidated and reverse transcribed during in vivo woodchuck hepatitis virus infection. *Virology* 1992;190:193–200.
21 Obert S, Zachmann-Brand B, Deindl E, *et al*. A splice hepadnavirus RNA that is essential for virus replication. *Embo J* 1996;15:2565–74.
22 Su TS, Lui WY, Lin LH, *et al*. Analysis of hepatitis B virus transcripts in infected human livers. *Hepatology* 1989;9:180–5.
23 Su TS, Lai CJ, Huang JL, *et al*. Hepatitis B virus transcript produced by RNA splicing. *J Virol* 1989;63:4011–8.
24 Suzuki T, Masui N, Kajino K, *et al*. Detection and mapping of spliced RNA from a human hepatoma cell line transfected with the hepatitis B virus genome. *Proc Natl Acad Sci U S A* 1989;86:8422–6.
25 Chen J, Wu M, Wang F, *et al*. Hepatitis B virus spliced variants are associated with an impaired response to interferon therapy. *Sci Rep* 2015;5:16459.
26 Günther S, Sommer G, Iwanska A, *et al*. Heterogeneity and common features of defective hepatitis B virus genomes derived from spliced pregenomic RNA. *Virology* 1997;238:363–71.
27 Günther S, Sommer G, Plikat U, *et al*. Naturally occurring hepatitis B virus genomes bearing the hallmarks of retroviral G-->A hypermutation. *Virology* 1997;235:104–8.

28 Lin Y-M, Chen B-F. A putative hepatitis B virus splice variant associated with chronic hepatitis and liver cirrhosis. *Virology* 2017;510:224–33.
29 Rosmorduc O, Petit MA, Pol S, *et al*. In vivo and in vitro expression of defective hepatitis B virus particles generated by spliced hepatitis B virus RNA. *Hepatology* 1995;22:10–19.
30 Sommer G, Günther S, Sterneck M, *et al*. A new class of defective hepatitis B virus genomes with an internal poly(dA) sequence. *Virology* 1997;239:402–12.
31 Sommer G, van Bömmel F, Will H. Genotype-specific synthesis and secretion of spliced hepatitis B virus genomes in hepatoma cells. *Virology* 2000;271:371–81.
32 Soussan P, Pol J, Garreau F, *et al*. Expression of defective hepatitis B virus particles derived from singly spliced RNA is related to liver disease. *J Infect Dis* 2008;198:218–25.
33 Terré S, Petit MA, Bréchot C. Defective hepatitis B virus particles are generated by packaging and reverse transcription of spliced viral RNAs in vivo. *J Virol* 1991;65:5539–43.
34 Chen PJ, Chen CR, Sung JL, *et al*. Identification of a doubly spliced viral transcript joining the separated domains for putative protease and reverse transcriptase of hepatitis B virus. *J Virol* 1989;63:4165–71.
35 Lim CS, Sozzi V, Littlejohn M, *et al*. Quantitative analysis of the splice variants expressed by the major hepatitis B virus genotypes. *Microb Genom* 2021;7.
36 Ma Z-M, Lin X, Wang Y-X, *et al*. A double-spliced defective hepatitis B virus genome derived from hepatocellular carcinoma tissue enhanced replication of full-length virus. *J Med Virol* 2009;81:230–7.
37 Lewellyn EB, Loeb DD. Base pairing between cis -acting sequences contributes to template switching during plus-strand DNA synthesis in human hepatitis B virus. *J Virol* 2007;81:6207–15.
38 Preiss S, Littlejohn M, Angus P, *et al*. Defective hepatitis B virus DNA is not associated with disease status but is reduced by polymerase mutations associated with drug resistance. *Hepatology* 2008;48:741–9.
39 Redelsperger F, Lekbaby B, Mandouri Y, *et al*. Production of hepatitis B defective particles is dependent on liver status. *Virology* 2012;431:21–8.
40 Soussan P, Garreau F, Zylberberg H, *et al*. In vivo expression of a new hepatitis B virus protein encoded by a spliced RNA. *J Clin Invest* 2000;105:55–60.
41 Terré S, Rosmorduc O, Kremsdorf D, *et al*. Reverse transcription and packaging of hepatitis B virus (HBV)—RNA generates in vivo defective serum HBV particles. *J Hepatol* 1991;13:S42–8.
42 Yuan TT-T, Lin M-H, Chen D-S, *et al*. A defective interference-like phenomenon of human hepatitis B virus in chronic carriers. *J Virol* 1998;72:578–84.
43 Abraham TM, Lewellyn EB, Haines KM, *et al*. Characterization of the contribution of spliced RNAs of hepatitis B virus to DNA synthesis in transfected cultures of Huh7 and HepG2 cells. *Virology* 2008;379:30–7.
44 Huang C-C, Kuo T-M, Yeh C-T, *et al*. One single nucleotide difference alters the differential expression of spliced RNAs between HBV genotypes A and D. *Virus Res* 2013;174:18–26.
45 Sommer G, Heise T. Posttranscriptional control of HBV gene expression. *Front Biosci* 2008;:5533–47.
46 Chowdhury JB, Roy D, Ghosh S. Identification of a unique splicing regulatory cluster in hepatitis B virus pregenomic RNA. *FEBS Lett* 2011;585:3348–53.
47 Huang C, Xie M-H, Liu W, *et al*. A structured RNA in hepatitis B virus post-transcriptional regulatory element represses alternative splicing in a sequence-independent and position-dependent manner. *Febs J* 2011;278:1533–46.
48 Loeb DD, Mack AA, Tian R. A secondary structure that contains the 5′ and 3′ splice sites suppresses splicing of duck hepatitis B virus pregenomic RNA. *J Virol* 2002;76:10195–202.
49 Bayliss J, Lim L, Thompson AJV, *et al*. Hepatitis B virus splicing is enhanced prior to development of hepatocellular carcinoma. *J Hepatol* 2013;59:1022–8.
50 Duriez M, Mandouri Y, Lekbaby B, *et al*. Alternative splicing of hepatitis B virus: a novel virus/host interaction altering liver immunity. *J Hepatol* 2017;67:687–99.
51 Soussan P, Tuveri R, Nalpas B, *et al*. The expression of hepatitis B spliced protein (HBSP) encoded by a spliced hepatitis B virus RNA is associated with viral replication and liver fibrosis. *J Hepatol* 2003;38:343–8.
52 Wu HL, Chen PJ, Tu SJ, *et al*. Characterization and genetic analysis of alternatively spliced transcripts of hepatitis B virus in infected human liver tissues and transfected HepG2 cells. *J Virol* 1991;65:1680–6.
53 Candotti D, Lin CK, Belkhiri D, *et al*. Occult hepatitis B infection in blood donors from South East Asia: molecular characterisation and potential mechanisms of occurrence. *Gut* 2012;61:1744–53.
54 Hass M, Hannoun C, Kalinina T, *et al*. Functional analysis of hepatitis B virus reactivating in hepatitis B surface antigen-negative individuals. *Hepatology* 2005;42:93–103.
55 Suzuki T, Kajino K, Masui N, *et al*. Alternative splicing of hepatitis B virus RNAs in HepG2 cells transfected with the viral DNA. *Virology* 1990;179:881–5.
56 Stadelmayer B, Diederichs A, Chapus F, *et al*. Full-length 5′RACE identifies all major HBV transcripts in HBV-infected hepatocytes and patient serum. *J Hepatol* 2020;73:40–51.
57 McNaughton AL, D'Arienzo V, Ansari MA, *et al*. Insights from deep sequencing of the HBV Genome-Unique, tiny, and misunderstood. *Gastroenterology* 2019;156:384–99.
58 McNaughton AL, Roberts HE, Bonsall D, *et al*. Illumina and nanopore methods for whole genome sequencing of hepatitis B virus (HBV). *Sci Rep* 2019;9:7081.
59 Sauvage V, Boizeau L, Candotti D, *et al*. Early MinION™ nanopore single-molecule sequencing technology enables the characterization of hepatitis B virus genetic complexity in clinical samples. *PLoS One* 2018;13:e0194366.
60 Ito N, Nakashima K, Sun S, *et al*. Cell type diversity in hepatitis B virus RNA splicing and its regulation. *Front Microbiol* 2019;10:207.
61 Hayer J, Jadeau F, Deléage G, *et al*. HBVdb: a knowledge database for hepatitis B virus. *Nucleic Acids Res* 2013;41:D566–70.
62 Burset M, Seledtsov IA, Solovyev VV. Analysis of canonical and non-canonical splice sites in mammalian genomes. *Nucleic Acids Res* 2000;28:4364–75.
63 Desmet F-O, Hamroun D, Lalande M, *et al*. Human splicing finder: an online bioinformatics tool to predict splicing signals. *Nucleic Acids Res* 2009;37:e67.
64 Lee GH, Wasser S, Lim SG. Hepatitis B pregenomic RNA splicing--the products, the regulatory mechanisms and its biological significance. *Virus Res* 2008;136:1–7.
65 Donello JE, Beeche AA, Smith GJ, *et al*. The hepatitis B virus posttranscriptional regulatory element is composed of two subelements. *J Virol* 1996;70:4345–51.
66 Huang J, Liang TJ. A novel hepatitis B virus (HBV) genetic element with Rev response element-like properties that is essential for expression of HBV gene products. *Mol Cell Biol* 1993;13:7476–86.
67 Zang WQ, Yen TS. Distinct export pathway utilized by the hepatitis B virus posttranscriptional regulatory element. *Virology* 1999;259:299–304.
68 Heise T, Sommer G, Reumann K, *et al*. The hepatitis B virus pre contains a splicing regulatory element. *Nucleic Acids Res* 2006;34:353–63.
69 Visootsat A, Payungporn S, T-Thienprasert NP. A conserved RNA structural element within the hepatitis B virus post-transcriptional regulatory element enhance nuclear export of intronless transcripts and repress the splicing mechanism. *Mol Biol Rep* 2015;42:1603–14.
70 Roy D, Bhanja Chowdhury J, Ghosh S. Polypyrimidine tract binding protein (PTB) associates with intronic and exonic domains to squelch nuclear export of unspliced RNA. *FEBS Lett* 2013;587:3802–7.
71 Moolla N, Kew M, Arbuthnot P. Regulatory elements of hepatitis B virus transcription. *J Viral Hepat* 2002;9:323–31.
72 Quarleri J. Core promoter: a critical region where the hepatitis B virus makes decisions. *World J Gastroenterol* 2014;20:425–35.
73 Li Y, Ito M, Sun S, *et al*. LUC7L3/CROP inhibits replication of hepatitis B virus via suppressing enhancer II/basal core promoter activity. *Sci Rep* 2016;6:36741.
74 Sun S, Nakashima K, Ito M, *et al*. Involvement of Puf60 in transcriptional and post-transcriptional regulation of hepatitis B virus pregenomic RNA expression. *Sci Rep* 2017;7:12874.
75 Chabrolles H, Auclair H, Vegna S, *et al*. Hepatitis B virus core protein nuclear interactome identifies SRSF10 as a host RNA-binding protein restricting HBV RNA production. *PLoS Pathog* 2020;16:e1008593.
76 Tremblay M-P, Armero VES, Allaire A, *et al*. Global profiling of alternative RNA splicing events provides insights into molecular differences between various types of hepatocellular carcinoma. *BMC Genomics* 2016;17:683.
77 Wang H, Lekbaby B, Fares N, *et al*. Alteration of splicing factors' expression during liver disease progression: impact on hepatocellular carcinoma outcome. *Hepatol Int* 2019;13:454–67.
78 Webster NJG. Alternative RNA splicing in the pathogenesis of liver disease. *Front Endocrinol* 2017;8:133.
79 Taha TY, Anirudhan V, Limothai U, *et al*. Modulation of hepatitis B virus pregenomic RNA stability and splicing by histone deacetylase 5 enhances viral biosynthesis. *PLoS Pathog* 2020;16:e1008802.
80 Makokha GN, Abe-Chayama H, Chowdhury S, *et al*. Regulation of the hepatitis B virus replication and gene expression by the multi-functional protein TARDBP. *Sci Rep* 2019;9:8462.
81 Yang L, Ye S, Zhao X, *et al*. Molecular characterization of HBV DNA integration in patients with hepatitis and hepatocellular carcinoma. *J Cancer* 2018;9:3225–35.
82 Wang Y-L, Liou G-G, Lin C-H, *et al*. The inhibitory effect of the hepatitis B virus singly-spliced RNA-encoded p21.5 protein on HBV nucleocapsid formation. *PLoS One* 2015;10:e0119625.
83 Zheng C-L, Fu Y-M, Xu Z-X, *et al*. Hepatitis B virus core protein dimer-dimer interface is critical for viral replication. *Mol Med Rep* 2019;19:262–70.
84 Köck J, Nassal M, Deres K, *et al*. Hepatitis B virus nucleocapsids formed by carboxy-terminally mutated core proteins contain spliced viral genomes but lack full-size DNA. *J Virol* 2004;78:13812–8.
85 Chen W-N, Chen J-Y, Lin W-S, *et al*. Hepatitis B doubly spliced protein, generated by a 2.2 KB doubly spliced hepatitis B virus RNA, is a pleiotropic activator protein mediating its effects via activator protein-1- and CCAAT/enhancer-binding protein-binding sites. *J Gen Virol* 2010;91:2592–600.
86 Rosmorduc O, Sirma H, Soussan P, *et al*. Inhibition of interferon-inducible MxA protein expression by hepatitis B virus capsid protein. *J Gen Virol* 1999;80:1253–62.
87 Park G-S, Kim H-Y, Shin H-S, *et al*. Modulation of hepatitis B virus replication by expression of polymerase-surface fusion protein through splicing: implications for viral persistence. *Virus Res* 2008;136:166–74.

88 Tsai K-N, Chong C-L, Chou Y-C, *et al*. Doubly spliced RNA of hepatitis B virus suppresses viral transcription via TATA-binding protein and induces stress granule assembly. *J Virol* 2015;89:11406–19.
89 Manzoni TB, López CB, Defective LCB. Defective (interfering) viral genomes re-explored: impact on antiviral immunity and virus persistence. *Future Virol* 2018;13:493–503.
90 Pol JG, Lekbaby B, Redelsperger F, *et al*. Alternative splicing-regulated protein of hepatitis B virus hacks the TNF-α-stimulated signaling pathways and limits the extent of liver inflammation. *FASEB j*. 2015;29:1879–89.
91 Betz-Stablein BD, Töpfer A, Littlejohn M, *et al*. Single-Molecule sequencing reveals complex genome variation of hepatitis B virus during 15 years of chronic infection following liver transplantation. *J Virol* 2016;90:7171–83.
92 Yuan S, Liao G, Zhang M, *et al*. Translatomic profiling reveals novel self-restricting virus-host interactions during HBV infection. *J Hepatol* 2021;75:74–85.
93 Chen B-F. Hepatitis B virus pre-s/s variants in liver diseases. *WJG* 2018;24:1507–20.
94 Huang HL, Jeng KS, Hu CP, *et al*. Identification and characterization of a structural protein of hepatitis B virus: a polymerase and surface fusion protein encoded by a spliced RNA. *Virology* 2000;275:398–410.
95 Lin C-M, Wang G-M, Jow G-M, *et al*. Functional analysis of hepatitis B virus pre-s deletion variants associated with hepatocellular carcinoma. *J Biomed Sci* 2012;19:17.
96 Mancini-Bourgine M, Bayard F, Soussan P, *et al*. Hepatitis B virus splice-generated protein induces T-cell responses in HLA-transgenic mice and hepatitis B virus-infected patients. *J Virol* 2007;81:4963–72.
97 Bayard F, Godon O, Nalpas B, *et al*. T-Cell responses to hepatitis B splice-generated protein of hepatitis B virus and inflammatory cytokines/chemokines in chronic hepatitis B patients. ANRS study: Hb EP 02 HBSP-FIBRO. *J Viral Hepat* 2012;19:872–80.
98 Wu S-X, Chen W-N, Jing Z-T, *et al*. Hepatitis B spliced protein (HBSP) suppresses Fas-mediated hepatocyte apoptosis via activation of PI3K/Akt signaling. *J Virol* 2018;92. doi:10.1128/JVI.01273-18. [Epub ahead of print: 01 12 2018].
99 Lu YW, Ren YD, Bai J, *et al*. The spliced variant of hepatitis B virus protein, HBSP, interacts with Bcl-2/Bcl-xL in vitro and induces apoptosis in HepG2 cells. *IUBMB Life* 2008;60:700–2.
100 Lu YW, Tan TL, Chan V, *et al*. The HBSP gene is expressed during HBV replication, and its coded BH3-containing spliced viral protein induces apoptosis in HepG2 cells. *Biochem Biophys Res Commun* 2006;351:64–70.
101 Chen J-Y, Chen W-N, Liu L-L, *et al*. Hepatitis B spliced protein (HBSP) generated by a spliced hepatitis B virus RNA participates in abnormality of fibrin formation and functions by binding to fibrinogen γ chain. *J Med Virol* 2010;82:2019–26.
102 Chen J-Y, Chen W-N, Jiao B-Y, *et al*. Hepatitis B spliced protein (HBSP) promotes the carcinogenic effects of benzo [alpha] pyrene by interacting with microsomal epoxide hydrolase and enhancing its hydrolysis activity. *BMC Cancer* 2014;14:282.
103 Chen W-N, Chen J-Y, Jiao B-Y, *et al*. Interaction of the hepatitis B spliced protein with cathepsin B promotes hepatoma cell migration and invasion. *J Virol* 2012;86:13533–41.
104 Chen B-F, Liu C-J, Jow G-M, *et al*. High prevalence and mapping of pre-s deletion in hepatitis B virus carriers with progressive liver diseases. *Gastroenterology* 2006;130:1153–68.
105 Yeung P, Wong DK-H, Lai C-L, *et al*. Association of hepatitis B virus pre-s deletions with the development of hepatocellular carcinoma in chronic hepatitis B. *J Infect Dis* 2011;203:646–54.
106 Pollicino T, Cacciola I, Saffioti F, *et al*. Hepatitis B virus PreS/S gene variants: pathobiology and clinical implications. *J Hepatol* 2014;61:408–17.
107 Melegari M, Scaglioni PP, Wands JR. The small envelope protein is required for secretion of a naturally occurring hepatitis B virus mutant with pre-S1 deleted. *J Virol* 1997;71:5449–54.
108 Gerner P, Schäfer HMC, Prange R, *et al*. Functional analysis of a rare HBV deletion mutant in chronically infected children. *Pediatr Res* 2003;53:891–7.
109 Gerner PR, Friedt M, Oettinger R, *et al*. The hepatitis B virus seroconversion to anti-HBe is frequently associated with HBV genotype changes and selection of preS2-defective particles in chronically infected children. *Virology* 1998;245:163–72.
110 Shen S, Xie Z, Cai D, *et al*. Biogenesis and molecular characteristics of serum hepatitis B virus RNA. *PLoS Pathog* 2020;16:e1008945.
111 Hatakeyama T, Noguchi C, Hiraga N, *et al*. Serum HBV RNA is a predictor of early emergence of the YMDD mutant in patients treated with lamivudine. *Hepatology* 2007;45:1179–86.
112 Jansen L, Kootstra NA, van Dort KA, *et al*. Hepatitis B Virus pregenomic rna is present in virions in plasma and is associated with a response to pegylated interferon alfa-2a and nucleos(t)ide analogues. *J Infect Dis* 2016;213:224–32.
113 Lam AM, Ren S, Espiritu C, *et al*. Hepatitis B virus capsid assembly modulators, but not nucleoside analogs, inhibit the production of extracellular pregenomic RNA and spliced RNA variants. *Antimicrob Agents Chemother* 2017;61.
114 Rokuhara A, Matsumoto A, Tanaka E, *et al*. Hepatitis B virus RNA is measurable in serum and can be a new marker for monitoring lamivudine therapy. *J Gastroenterol* 2006;41:785–90.
115 Wang J, Shen T, Huang X, *et al*. Serum hepatitis B virus RNA is encapsidated pregenome RNA that may be associated with persistence of viral infection and rebound. *J Hepatol* 2016;65:700–10.
116 Wang J, Sheng Q, Ding Y, *et al*. HBV RNA virion-like particles produced under nucleos(t)ide analogues treatment are mainly replication-deficient. *J Hepatol* 2018;68:847–9.
117 Ward AJ, Cooper TA. The pathobiology of splicing. *J Pathol* 2010;220:152–63.
118 Cox LE, Arslan O, Allain J-P. Characterization of hepatitis B virus in Turkish blood donors, and the prevalence of the SP1 splice variant. *J Med Virol* 2011;83:1321–5.
119 Köck J, Rösler C, Zhang J, *et al*. Human hepatitis B virus production in avian cells is characterized by enhanced RNA splicing and the presence of capsids containing shortened genomes. *PLoS One* 2012;7:e37248.