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Posttranscriptional control of HBV gene expression

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1. ABSTRACT

Acute and chronic hepatitis B virus (HBV) infection is the cause of about 1 million deaths each year worldwide. Although a vaccine for HBV is available, new HBV infections are appearing at alarming rates and maternal-fetal transmission is a major cause of viral spread. Thus, new and effective antiviral strategies are necessary for the 300 million chronically HBV-infected individuals to reduce their morbidity and mortality. Precise processing of viral RNAs is essential for the hepatitis viral life cycle, and, to our knowledge, the processing, nuclear export, and stabilization/degradation of viral RNAs are exclusively mediated by host factors. Thus, identification of host factors required for viral RNA metabolism and subsequent molecular analysis of the interactions between viral RNAs and corresponding host factors might represent novel avenues for the development of new antiviral strategies. In this review, we summarize the current knowledge about the posttranscriptional control of HBV RNA metabolism.

2. INTRODUCTION

According to the World Health Organization (WHO) approximately 2 billion humans have been infected by the hepatitis B virus (HBV) and worldwide, approximately 350 million people are chronic carriers (63). The chronicity rate dramatically varies with the age of the person when the infection occurs - perinatal infection results in 95% to a chronic infection, whereas the infection of children (age 1 to 5 years) leads in about 30% to chronicity, and only 5 % of infected adults developing a chronic infection (21, 82). Chronic HBV infection can manifest in a severely pathological manner, developing into hepatic cirrhosis or hepatocellular carcinoma in later years, causing about 0.5 to 1.2 million deaths annually (63). The current treatment of chronically infected patients with antiviral drugs like nucleoside analogues (e.g. lamivudine) and interferon alpha-2b are only effective in about 20-30% of patients (21, 82). Therefore, the only hope for chronic carriers would be a cost-effective and robust antiviral

therapy, which is currently unavailable. The health and societal costs of chronic HBV infections are enormous, underscoring the urgent need for the development of novel and efficacious antiviral strategies.

HBV is a noncytopathic, hepatotropic virus with a 3.2-kb, circular, partial-double stranded DNA genome, which serves as template for transcription of the pregenomic/precore mRNAs and three subgenomic RNAs (for review (10, 98, 100)). Although no direct evidence is reported in the literature, we assume that a Cap at the 5' end modifies the viral RNAs. This view is supported by a recent study showing that encapsidation of the pregenomic RNA (pgRNA) is Cap dependent (59). Although there are several promoters, all viral RNAs terminate at a common polyadenylation signal (poly (A)). Terminal redundancy of the pg/preC RNAs, but not of the subgenomic RNAs, raises an interesting question regarding why the cellular RNA polymerase II uses the poly (A) signal only when encountering the poly (A) signaling the second time, effectively ignoring the signal at its first encounter. In contrast to the transcription of the pg/preC RNAs during transcription of the subgenomic RNAs, the poly (A) signal is used at the first encounter. Several reasons may explain why the RNA polymerase II bypasses the poly (A) signal after its first encounter: the HBV poly (A) signal (UAUAAA) differs from the consensus poly (A) signal (AAUAAA); the poly (A) signal is in close proximity to the core promoter; and elements upstream of the core promoter have been identified to stimulate poly (A) signal usage if the RNA polymerase II reaches the poly (A) signal the second time (9, 93, 94).

HBV replicates through the reverse transcription of its pgRNA and is, with respect to retroviruses, referred to as a pararetrovirus. HBV contains four overlapping open reading frames encoding the hepatitis B e-antigen (HBeAg); the core protein; the reverse transcriptase-polymerase (P) protein; and the large, middle, and small surface protein (preS1, preS2, S); and the X protein. The HBV genome displays a very high coding capacity in which each nucleotide (nt) codes for at least one viral protein. In addition to its function as a template for reverse transcription into genomic minus-strand DNA, the bicistronic pgRNA serves as mRNA for translation of the core and P proteins. The precore mRNA (preC RNA) initiates about 30 nucleotides (nts) upstream of the pgRNA and encodes the HBeAg. Interestingly, only the pgRNA is encapsidated but not the slightly longer preC RNA (60, 124). It appears that only the pgRNA is encapsidated due to the formation of a protein complex at the encapsidation signal consisting of viral proteins encoded by the pgRNA, which blocks scanning of the 40S ribosome subunit (78). The dual function of the pgRNA as a pregenome and an mRNA for the core and P protein suggests that accurate processing and stability of viral pgRNAs is of paramount importance for the hepadnaviral life cycle. It is noteworthy that the small difference in length between the precore and pregenomic RNA (~30 nts) makes it difficult to determine whether posttranscriptional events might affect only one or the other RNA species. Subgenomic 2.4-kb and 2.1-kb mRNAs (preS1 and preS2/S RNAs) encode three viral surface proteins, whereas the small 0.7-kb RNA encodes the X protein. For a recent review on hepatitis B virus gene expression please refer to (100).

Processing, maturation, nuclear export, and decay of viral RNAs are essential for viral replication and largely depend on the formation of ribonucleoprotein particle (RNP). Those RNPs are formed between the viral RNA and host factors. We assume that most, if not all, of the RNA processing steps are mediated by host factors. Those factors are essential for viral replication and spread because they guarantee, for example, the proper processing of the pgRNA, which carries the complete viral genomic information to the next virus generation. It is attractive to speculate that the identification of a specific interaction between a host factor and the viral RNA might be of tremendous therapeutic value. Hence, identification of host factors interacting with viral *cis*-acting RNA elements is required to understand HBV gene expression. In this review, we summarize the current knowledge about viral RNA metabolism. Further characterization of viral RNA metabolism should provide essential information for the development of novel antiviral strategies based on the disturbance of accurate viral RNA processing.

3. SPLICING OF HBV RNA

During its life cycle, HBV utilizes cellular machineries for processing its RNAs. In mammalian cells, it is well established that an RNA polymerase II transcript is co-transcriptionally processed. In fact, capping, splicing, and polyadenylation of an mRNA occurs co-transcriptionally and is in part mediated by the C-terminal domain of the RNA polymerase II, which recruits essential RNA processing factors in a phosphorylation-dependent manner (for review (72, 89, 134)). Because the covalently closed circular viral DNA (cccDNA) is transcribed by the cellular RNA polymerase II, we might consider that the viral RNA is also co-transcriptionally processed. In fact, polypyrimidine tract binding protein associated splicing factor (PSF) stimulates HBV RNA splicing (see below, (47)) and is associated with the C-terminal domain of RNA polymerase II (18). However, we are not aware of studies that directly address the co-transcriptional processing of HBV RNA.

Presently, it is not known whether viral proteins are involved in the processing of viral RNAs. The viral core protein is the only virus-encoded protein with unspecific DNA and RNA-binding activity (42, 87), which interacts with the pgRNA during encapsidation. In addition, nuclear localization of the HBV core protein has been documented in various systems (11, 12, 34, 68, 77, 80, 127, 128). A recent analysis of HBV-infected patients revealed that the HBV core protein is preferentially located in the nucleus during the immune-tolerance phase compared to patients with more severe symptoms (102). Importantly, Sheen *et al.* demonstrate a correlation between nuclear localization of the core protein and occurrence of viral RNA splicing (102). Interestingly, the core protein of the related duck hepatitis B virus (DHBV) was found in the nucleus, in close proximity to splicing speckles (68).

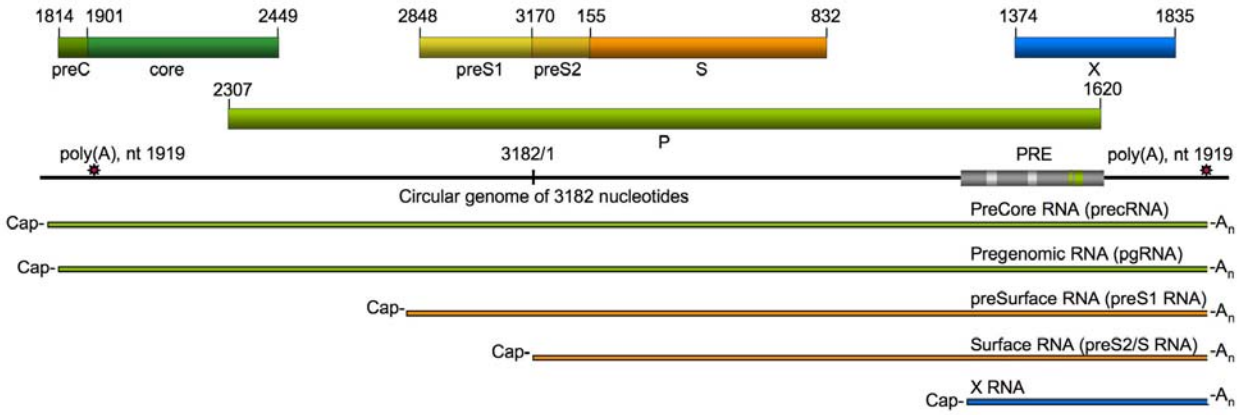

Figure 1. HBV gene expression. The circular HBV genome, 3,182 nts in length, is the template for five viral transcripts: the preCore (preC) RNA, the pregenomic (pg) RNA, the preS1 RNA, the preS2/S RNA and the X RNA. The RNAs are encoding 7 viral proteins: the precore, the core, the polymerase (P), the large (preS1), the middle (preS2), the small surface (S) and the X protein. The posttranscriptional regulatory element is indicated as grey box (for details see Figure 3) and the poly (A) signals are indicated as star. HBV nt position according to the Galibert nomenclature (20).

Furthermore, expression of a mutant of the core protein correlated with a decrease in spliced viral RNA levels, indeed indicating that the core protein might be functional in viral RNA processing (64). In addition, the splicing regulatory (SR)-domain protein kinases 1 and 2 (SRPK1 and 2) that regulate the activity of SR proteins (119) have been shown to phosphorylate the HBV core protein (14). In contrast, it has been shown that SRPK1 and 2 suppress HBV replication by inhibiting the packaging of the viral pgRNA without phosphorylation of the core protein (132). However, whether those kinases affect HBV RNA splicing was not addressed. Although many studies implicate a role of the core protein in viral RNA maturation, a direct function of the core protein in viral RNA metabolism has not been documented to date, indicating that most—if not all—of the viral RNA processing steps are mediated by host factors.

For the viral life cycle, it is crucial that all viral RNAs escape the cellular splicing machinery and are exported by a splicing-independent mechanism. However, it is well established that HBV mRNAs are spliced, and substantial effort has been undertaken to understand the role of splicing in the HBV life cycle and to learn about its regulation (8, 91, 111, 113-115, 126). Today, 13 splice variants of the pgRNA (37, 52, 91, 106, 115) and two splice variants of the preS2/S mRNA are known (40). In addition, spliced RNAs have been described for the duck and woodchuck hepatitis virus (38, 81, 83).

In mammalian cells, most of the cellular mRNAs contain short exons and much longer intronic sequences, in contrast to HBV, in which the exons are longer than the introns. To create a functional mRNA, the introns must be removed and the exons must be ligated precisely. The removal of the intron is mediated by the giant multiprotein/RNA complex referred to as a spliceosome, which assembles in a stepwise manner at the splice sites (for review see (2, 41, 122, 123)). The basic sequence elements required for splicing to occur are 5' splice sites (5'ss) and 3' splice sites (3'ss) and the branch site located within the intron. The utilization of the 3'ss is influenced by the polypyrimidine tract located at the 3'end of the intron close to the 3'ss. Consensus sequences are established for 5'ss and 3'ss in mammalian cells and computer programs are available to predict splice sites. Importantly, the utilization of a splice site does not only depend on its sequence since splicing regulatory elements might regulate its utilization (73). The activity of the splicing enhancer and the splicing silencer are regulated by *trans*-acting factors. SR proteins are known to stimulate splicing via binding to a splicing enhancer whereas hnRNP proteins are well known to suppress splicing by binding to splicing silencer elements. The splicing enhancer and silencer are eventually located in close proximity and the balance of active enhancers and silencers might regulate the frequency of splice-site utilization.

Although splicing is not required for the production of functional viral proteins, splicing of viral RNAs is well documented, which raises questions about why the virus retained splice sites and how the majority of viral RNAs escape the splicing machinery. Most studies focus on the characterization of the major HBV splice variant, referred to as SP1 (splice product 1), which is created by utilization of the 5'ss at position 2,447 and 3'ss at position 489 (Figure 2). Approximately 30% of the pg/preC RNA is spliced to SP1 in cell culture experiments (126). In contrast, analysis of patient samples revealed a broad variation of pg/preC RNA splicing, ranging from no splicing to extensive and genotype-dependent splicing (61, 106, 111, 112, 126). That encapsidation of spliced pgRNAs results in the production of non-functional viral DNA genomes in patient samples is well documented (37, 75, 91, 106, 115) and mutagenesis of the splice sites and subsequent transient transfection experiments revealed that SP1 and the potential protein encoded by spliced RNAs are not required for viral replication (111, 126). However,

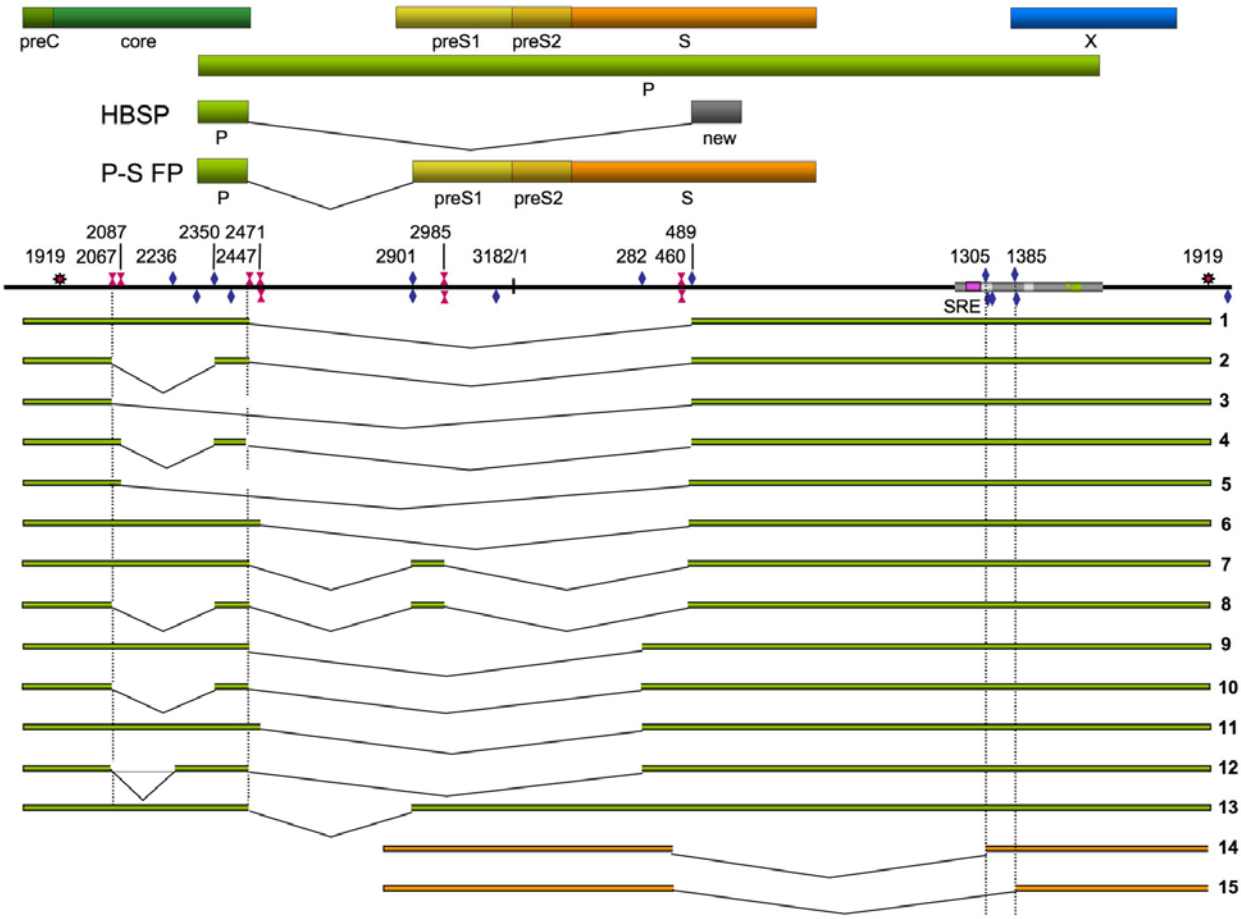

Figure 2. Summary of known HBV splice variants. HBV RNA is intensively spliced as summarized. Documented splice sites (ss) are indicated above the black line and predicted splice sites are indicated below the black line. Red symbols = 5′ss (splice donor), blue symbol = 3' ss (splice acceptor). The two known fusion proteins are illustrated below the viral open reading frames. HBSP = HBV spliced generated protein (107); P-S FP = Polymerase-surface fusion protein (52). The PRE is indicated as a grey box (for details see Figure 3) and the splicing regulatory element 1 (SRE) is shown as pink box within the PRE (grey box). Usage of different combinations of 5'ss and 3'ss led to the generation of 13 known splice variants (lanes 1–13, (37, 52, 91, 106, 115)) of the pg/preC RNA and 2 splice variants of the preS2/S mRNAs (lane 14 and 15, (40)). The poly (A) signal is indicated as star. HBV nt position according to the Galibert nomenclature (20).

documentation of spliced mRNAs in cell cultures and more importantly, during natural infection, suggested a certain function for those spliced RNAs and encoded proteins. Strikingly, proteins translated from spliced viral RNAs have been reported (Figure 2, (52, 107)). The function of the polymerase-surface fusion protein is suggested to function as a structural protein, and investigators speculate whether it plays a role in viral entry (52). The second known fusion protein, referred to as hepatitis B splice-generated protein (HBSP), consists of a part of the polymerase open reading frame and a new open reading frame. Antibodies against HBSP are found in patient sera (107), and an HBSP-specific T-cell response has been reported (71), which has been shown to be expressed in liver tissue of HBV-infected patients. It is suggested that HBSP plays a role in viral infection and/or in viral persistence (91, 107, 108). The detection of spliced viral RNAs and even proteins encoded by some spliced RNAs underscores the potential importance of viral RNA splicing during HBV infection.

As mentioned earlier and shown below, SR proteins and hnRNP proteins regulate splicing of cellular mRNAs. Some of the SR proteins and hnRNP proteins are regulated by phosphorylation and their subcellular localizations (97, 110), indicating that during the onset of HBV infection, activity of such factors might differ from cells of chronically infected patients and cell cultures in which most of the reported work was performed. Moreover, in Huh7 cells viral replication is suppressed by SR protein kinase 1 and 2 (SRPK 1 and 2) (132), and SRPK activity may be rendered during the onset of viral infection, modulating also viral RNA splicing. In addition the viral core protein might contribute to regulation of HBV splicing as suggested by a study showing that splicing is upregulated during the early phase of chronic infection and correlates with nuclear localization of the viral core protein (102).

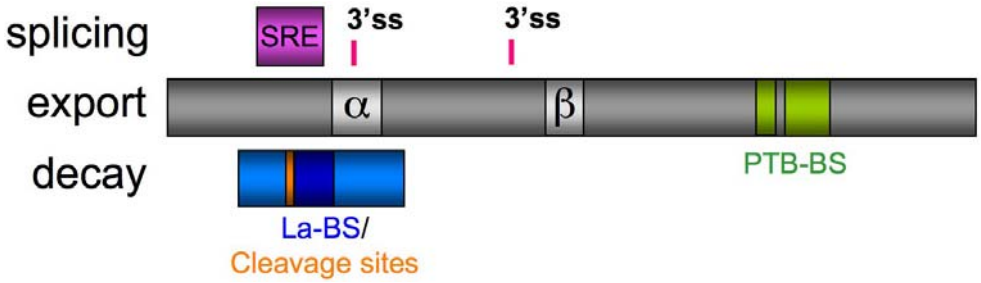

Figure 3. The multifunctional HBV posttranscriptional regulatory element (PRE). The PRE is composed of three functional domains regulating splicing, nuclear export, and the decay of HBV RNAs. Splicing of viral RNAs depends on the splicing regulatory element 1 determined in the 5´end of the PRE (SRE = Splicing regulatory element 1 (position 1,252–1,288). Two 3'ss (red lanes, nts 1,305 and 1,385, see Figure 2) are indicated. The PRE mediates the nuclear export of subgenomic RNAs. The PRE-alpha domain (nts 1,292–1,321), the PRE-beta domain (nts 1,410–1,434) and the binding sites for PTB (nts 1,527–1,536 and nts 1,542–1,569) are required for export capacity of the PRE. In addition, the decay of the viral RNA (see also Figure 4) can be initiated by an element called RNA.B (light blue box, nts 1,242–1,331), which contains the proposed minimal La binding site (La-BS, dark blue box, nts 1,270–1,331) and the two endoribonucleolytic cleavage sites (orange lanes, nts 1,268 and 1,270). HBV nt position according to the Galibert nomenclature (20).

In this context, it is important to note that splicing of the related DHBV is well documented and known to be important during the viral life cycle (81). DHBV uses the cellular splicing machinery to create a viral RNA, which encodes the large surface proteins (L-RNA). Although the L-RNA is also raised by transcription, a subset of the L-RNA pool is created by splicing, which is required for DHBV infection (81). Furthermore, Loeb *et al.* have shown that the usage of splice sites and their regulation depend on a secondary structure of the viral pgRNA which brings the 5'ss and the 3'ss in close proximity (66). Their elegant study points to another level of splicing regulation, namely the accessibility of splice sites. Some splice sites might be buried in secondary structures and those structures must be altered first to allow splice-site utilization. As shown below, a similar mechanism might regulate splice-site utilization in HBV. Clearly, additional studies are required to firmly establish that viral RNA splicing is not just a side effect tolerated by the virus but has an important function in the viral life cycle. However, we should not ignore the fact that the high conservation of splice sites and the modulation of splicing during the course of infection, as well as the presence of unique proteins encoded by spliced viral RNAs, are strongly suggestive of a functional role of splicing in the HBV life cycle.

The different degrees of splicing reported in cell culture experiments, patient samples and different genotypes of the viral genome (61, 106, 111-114, 126) suggests that HBV RNA splicing is balanced and not a constitutive process. Furthermore, if we compare known and actually used splice sites with the predicted splice sites, we find that some of the used splice sites are not necessarily predicted and that some predicted splices sites are not used (Figure 2). This indicates again that HBV splicing is likely to be regulated by *cis*-acting elements and *trans*-acting factors, not only leading to the activation of cryptic splice sites but also to silencing of strong splice sites.

3.1. Regulation of HBV RNA splicing

3.1.1. *Cis*-acting elements

During a search for *cis*-acting regulatory elements within the HBV genome, the posttranscriptional regulatory element (PRE) was identified (53, 56, 57). One of the first studies mapped the PRE to nts 1,200–1,684 (53) and the minimal boarders were later mapped to nts 1,217–1,582 comprising the minimal PRE (15, 104). The PRE is located in all viral RNAs (Figure 1). As discussed in more detail (see below §4.0), the primary function of the PRE is thought to be the mediation of nuclear export of unspliced preS/S RNAs (53, 56, 57). However, prediction of potential 5´ss and 3´ss within the preS/S subgenomic RNAs suggested that the PRE might also be required to suppress preS/S RNA splicing (53).

During our studies to identify PRE-interacting host factors and the functional characterization of PRE subelements, it was established that deletion of the PRE abolished viral RNA splicing. The major splice product (SP1) was hardly detectable in the absence of the PRE suggesting that the PRE stimulates viral RNA splicing (47). The PRE is located ~760 nts downstream of the 3'ss (nt 489) and ~1,985 nts downstream of the 5'ss (nt 2,447), which are utilized to generate SP1. Therefore, the PRE regulates splicing over a long distance, a known feature of splicing enhancer elements (28, 116). Using the default settings of the EseFinder software 2.0 (http://rulai.cshl.edu/tools/ESE2/; (6, 105)) many binding sites for the splicing regulatory factors SC35, SRp55, SRp45, and SF2/ASF were predicted within in the PRE. Interestingly, under more stringent settings, high-scoring binding sites for SR protein were found at the 5'end of the PRE between nts 1,250–1,350. Deletion of this element indeed significantly reduced splicing of viral RNA (47). Further analysis of the region led to the identification of a minimal element (nts 1,252–1,288), which was identified as a splicing enhancer and termed Splicing Regulatory Element 1 (SRE1, (47)). Although HBV RNA splicing was diminished after SRE1 deletion, deletion of the whole PRE led to a much stronger decrease in viral RNA splicing,

indicating that the PRE might contain additional splicing enhancer elements and/or a splicing silencer. The position of the SRE1 is of special interest because several other *cis*-acting elements are located in close proximity (Figure 3). The SRE1 is overlapping (Figure 3) with endoribonucleolytic cleavage sites (45), the proposed binding site for the La protein (46), composing an HBV RNA stabilizing element, (17) and is in close proximity to the PRE-alpha domain (15, 104). The PRE-alpha domain is essential for full PRE activity (15, 104). The overlapping elements functional in HBV RNA splicing, nuclear RNA export, and viral RNA stabilization raises the pressing questions of whether *trans*-acting host factors—proposed to interact with those elements—act in a synergistic or competitive manner. To address this question, we first obtained data showing that La overexpression diminishes HBV splicing (unpublished data, GS and TH), suggesting that binding of La protein to the La binding site might interfere with the function of the SRE1 (Figure 3). Much more work is required to sort out these puzzling findings and it is imperative that we identify host factors interacting with the SRE1 and the PRE -alpha domain modulating PRE function.

The recent finding that preS2/S RNAs are spliced led to the interesting link between the position of the utilized 3'ss which is located within the PRE-alpha domain. The NMR-based structural analysis (Schwalbe M *et al.*, in press NAR) revealed that the accessibility of the splice site is likely dependent on structural changes, potentially mediated by *trans*-acting factor (s). That splicing is regulated by structural determinants of the splice sites is well known (5, 49) and is documented for the splicing of the related DHBV (66). The initial correlations hint at another level of regulation. Structural constraints might be important features of the PRE, which are probably modulated by *trans*-acting factors.

Another potential *cis*-acting regulatory element is located between a potential 5'ss at position 460 and the frequently utilized 3'ss at position 489. Deletion of 30 nts located between both splice sites dramatically reduces the expression of the preS/S RNA (135). The authors speculate about a posttranscriptional, PRE-dependent mechanism, in which PRE-interacting proteins might interfere with components of the splicing machinery, abolishing utilization of the 5'ss (nt 460) and 3'ss (nt 489) (135). Such a model would implicate an increase in unspliced preS/S RNAs in the presence of the PRE and a decrease in preS/S RNAs in the absence of the PRE, as initially observed (53, 56, 57). The finding that the PRE contains a splicing enhancer might support this hypothesis. Furthermore, recently it has been reported that a natural occurring HBV mutation was detected in a viral subpopulation from a patient who had a reactivation of a latent HBV infection characterized by clearance of circulating surface antigen (HBsAg) (40). Strikingly, a single point mutation in the 5'ss at position 460 was identified as a cause for a strong posttranscriptional effect on preS2/S mRNA and HBsAg expression (40). Again, it might be reasonable to postulate that the PRE represses the utilization of splice sites upstream of the PRE, suggesting that RNA-RNA interactions bridging long distances and bringing the PRE in close proximity to splice sites and/or by protein-protein-interactions regulates the utilization of splice sites in a PRE-dependent mechanism. This view would indicate that the PRE, although containing a splicing enhancer, represses HBV RNA splicing.

Furthermore, analysis of complex HBV variants, isolated from immune-suppressed renal transplant recipient chronic HBV carriers, revealed an accumulation of variants defective in HBV RNA splicing (75). Sequence analysis of HBV variant genomes revealed that major splice sites and the SRE1 were not mutated. A follow-up study led to the identification of single point mutations responsible for a strong reduction in HBV RNA splicing (unpublished data, SM, TH, and HM).

Collectively, in the last few years, a significant amount of data suggested a complex regulatory mechanism underlying HBV RNA splicing. Future identification of host factors, additional *cis*-acting elements, single nts, RNA-RNA interactions, and protein-protein interactions are critical determinants for understanding the regulation of HBV RNA splicing, which then might indicate a role of HBV splicing during HBV infection.

3.1.2. Trans-acting factors

We must assume that the activity of the SRE1 and additional splicing-regulatory elements are regulated by *trans*-acting factors. We do not know yet whether splicing regulatory proteins SF2/ASF, SC35, SRp55, and SRp45, which are predicted to bind to the SRE1, indeed interact and regulate SRE1. However, the splicing factor PTB-associated splicing factor (PSF) (85)) was found to stimulate viral RNA splicing (47). Interestingly, PSF-mediated stimulation of splicing was even stronger if the PRE element was deleted, underscoring the important function of the PRE in repressing HBV RNA splicing, as postulated earlier. The splicing silencing capacity is at least able to reduce the potential of PSF to stimulate HBV RNA splicing. Besides other functions (for review see (101)), PSF is a splicing factor that specifically interacts with the polypyrimidine tract close to the 3`ss (85) and probably replaces $U2AF^{65}$ during the second catalytic step of splicing (27). Because overexpression of PSF results in a stimulation of viral RNA splicing, it would be of interest to determine whether PSF interacts with the polypyrimidine tract preceding the 3'ss at nt position 489, utilized for the major splice. Furthermore, it will be interesting to study whether PSF stimulates the utilization of additional splice sites or only the 3'ss at position 489. Those studies would define whether PSF activates specific splice sites or whether PSF generally enhances HBV splicing. Because PSF-dependent splicing stimulation was even higher in the absence of the PRE, we assume that the PRE inhibits PSF functionality on splice sites. Because PSF could only stimulate splicing when its RNA recognition motif was functional (47), it is likely that PSF interacts directly with the viral RNA.

Another splicing regulatory protein, polypyrimidine tract-binding protein (PTB), (22, 24) was

reported to interact with the 3'-end of the PRE and its function in HBV RNA export was implicated ((65, 130); see below). However, it is well established that PTB can inhibit splicing (103, 109). Because PTB interacts with the 3'-end of the PRE, we might consider that the PRE harbors two splicing regulatory elements: the enhancer SRE1 and a silencer, comprised of the PTB binding sites. Although the interaction between PSF and PTB is not firmly established, possibly PSF and PTB functionally interact and balance the utilization of viral splice sites. After deletion of the PRE, the generation of the major splice SP1 was significantly reduced, indicating that utilization of 5'ss (position 2,447) and of 3'ss (position 489) depended mainly on a positive regulatory function of the PRE. In contrast, as depicted earlier, utilization of the 5'ss (position 460) is assumed to be repressed by the PRE and prevents preS2/S RNA splicing.

However, SR proteins stimulate a splicing enhancer and hnRNP proteins activate the splicing silencer, so it is very likely that the splicing stimulatory potential of the PRE responds to the cellular context and different cellular conditions. It is well established that splicing regulatory factors are regulated by posttranslational modifications as well as their cellular distribution (97, 110). Thus, it would be interesting to know to what extent —if at all—HBV RNA splicing is modulated during viral infection. Therefore, the cellular changes in splicing regulatory factors paralleling viral infections might pose critical constraints, as underscored by the reported downregulation of the splicing factor PSF during HBV infection of primary human hepatocytes (95), the regulation of PSF by SR kinases (51), and the increase in splicing during early stages of chronic infection (102).

4. NUCLEAR EXPORT OF VIRAL RNA

The nuclear export of mature mRNAs is an energy-dependent mechanism mediated by export adapters interacting with the mRNA and export-receptors interacting with the RNA-adapter complex (cargo), thereby facilitating the export of the cargo through the nuclear pore complex (26, 69). During mRNA splicing, the protein complexes deposited are likely to be crucial for nuclear export. In contrast, intron-less mRNAs containing export elements are recognized by export-adapters. HBV RNAs are unspliced RNAs and are exported by a splicing-independent mechanism. Accordingly, HBV RNAs are likely to contain an RNA export element allowing the nuclear export of unspliced viral RNAs, similar to the of HIV Rev response element (RRE; (70)) and the Mason-Pfizer monkey virus constitutive transport element (CTE; (3); for review (13, 39, 96)). The HBV RNA export element, PRE, was discovered by two groups (53, 56, 57), and initial experiments suggested that the genetic element, which is located in all viral RNAs, is required for the expression of viral surface proteins. It was concluded that the PRE mediates the nuclear export of unspliced RNAs and that in the absence of the PRE, viral preS/S RNAs are degraded in the nucleus. (53, 56, 57). Interestingly, the unspliced pg/preC RNA is obviously exported by another element; deletion of the PRE only affected the expression of the preS/S mRNAs. The export element for the pg/preC RNA is still elusive.

The minimal PRE is located between nt position 1,217–1,582 and is about 365 nts long (15, 104). Detailed analysis revealed that the PRE consists of two functional domains: PRE-alpha and PRE-beta1 (15, 104). Both domains are required for full functionality, indicating that the PRE requires extended structural features and/or that the PRE recruits transacting host factors at separate regions. The PRE subdomains are highly conserved in different viral genotypes, underscoring the functional importance of those elements (104). Furthermore, structural predictions of the PRE indicate that the PRE-alpha and PRE-beta1 domains are likely to fold into stem-loop structures (86, 104) as confirmed for the PRE-alpha domain by NMR studies (Schwalbe M *et al.*, in press NAR). Studies to identify transacting factors that regulate PRE function led to the discovery of two host proteins, referred to as PRE interacting proteins 1 and 2 (PIP-1, -2), which specifically interact with the PRE (58). PIP-1 was subsequently identified as glyceraldehyde-3-phosphate dehydrogenase (GAPDH; (129)). However, the functional meaning of the GAPDH-PRE interaction is still elusive. PIP-2 was recently identified as PTB (130), which binds specifically to two sites at the 3' end of the PRE (Figure 3). Mutagenesis of the PTB binding sites revealed that those sites are indeed the binding sites, but more importantly, it indicated that PRE-mediated export of a reporter RNA was reduced when the binding sites were mutated (130). Further characterization of the PTB protein revealed its nuclear export sequence and its ability to shuttle between the nucleus and cytoplasm. Hence, PTB might be a candidate factor required for the PRE-dependent export of unspliced viral RNAs. However, a recent study suggests that PTB is not involved in the nuclear export of mRNAs (62). As described in more detail below, the La protein interacts with the 5' end of the PRE (Figure 3). Recently the nucleoplasmatic shuttling of the La protein has been demonstrated (19). However, no data are available to suggest that La is involved in the export of RNAs. In contrast, recent data argue for a La-dependent nuclear retardation of mRNAs (4). Taken together, three proteins are known to interact with the PRE GAPDH, PTB, and the La protein; however, more work is required to firmly establish a function for those proteins in the PRE-mediated nuclear export of unspliced preS/S RNAs.

The PRE can functionally substitute for the HIV-1 rev responsible element (RRE) and it was assumed that both viruses use the same export pathway for unspliced viral RNAs (92). The HIV rev protein binds to the RRE and mediates the export of unspliced viral RNAs by interacting with the export receptor CRM1. The CRM1-dependent pathway can be inhibited by the small molecule leptomycin B. However, PRE functionality was not inhibited by leptomycin B or by overexpression of deltaCAN, which competes with nucleoporins for binding to CRM1 (84, 88, 131) indicating that the PRE uses a different pathway. Further analysis revealed that the export pathway used by the PRE is also different from the tRNA export pathway, since overexpression of the vesicular stomatitis virus

matrix protein M protein blocks tRNA export (48) but not PRE-dependent export (131). In addition, the PRE obviously uses a different pathway than the CTE element, because the CTE-dependent export is inhibited by a mutant of the Ran-binding protein 1 (131, 133). Those studies indicate that the PRE uses a unique export pathway, which remains to be established.

In comparison to the HBV PRE (PRE) the related woodchuck hepatitis virus PRE (WPRE) is even more potent at facilitating gene expression of heterologous transgenes by a posttranscriptional mechanism. The functional difference between PRE and WPRE probably depends on an additional sequence element referred to as a WPRE-gamma domain, which is absent in the PRE (16). In addition, the WPRE contains PRE subelements referred to as PRE-alpha and PRE-beta domains, which are required for full activity (15, 104). Interestingly, WPRE-dependent stimulation of gene expression could be inhibited partially by leptomycin B or overexpression of deltaCAN, indicating that the WPRE utilizes other and/or additional mechanisms than the PRE to boost gene expression (88). It was suggested that the WPRE enhances gene expression at different posttranscriptional levels such as 3′ end processing, splicing, and export and that all three subelements are required for full activity (88, 99, 136). Similar conclusions were made for the PRE (29, 55, 67). Hence, comparison of the properties of PRE and WPRE reveals significant functional, structural, and mechanical similarities but also indicates distinct differences.

In summary, although 14 years have passed since the discovery of the PRE, it is unclear how the PRE mediates the expression of HBV preS/S RNAs and why the export of the pg/preC RNA is mediated by a different mechanism. Accumulating data suggest that the PRE is a splicing regulatory element, so we postulate that the function of the PRE is indeed the export of unspliced RNA, but that the PRE recruits splicing factors mediating the export. In light of this hypothesis, it is interesting that SR proteins, splicing regulatory factors interacting with splicing enhancer, are actually known to mediate nuclear export of unspliced RNA (54).

The HBV PRE, and more frequently the related WPRE, is used to enhance transgene expression in vectors developed for gene therapy approaches (76, 99, 120, 136). We do not know yet whether the WPRE also contains a splicing regulatory element, but because of the sequence similarities and the functional similarities between PRE and WPRE, we assume that the WPRE can activate and silence splice sites. Therefore, we must consider that the WPRE might lead to unintended splicing of the transgene of interest. The known cell-type-dependent functionality of the WPRE (121) strongly indicates that the WPRE function is regulated by cellular factors and it is easy to imagine that under certain cellular conditions the expression of the transgene is negatively affected by the WPRE.

5. MODULATION OF HBV mRNA STABILITY

Other than splicing viral RNAs and exporting mature RNA to the cytoplasm, the decay rate of viral RNAs is a critical determinant of viral gene expression. A recent review of mRNA decay reflects the complexity of the mechanism regulating mRNA stability (23). Similar to splicing and nuclear export of HBV RNAs, the decay of viral RNAs is most likely regulated exclusively by host factors. To understand the mechanism, we must identify viral *cis*-acting elements and *trans*-acting host factors. One of the most intensively described *cis*-acting elements involved in mRNA destabilization are AU-rich regions located within the 3' UTR (1), which are characterized by the presence of AUUUA pentamers embedded in AU-rich sequences. Analysis of the viral genome revealed the presence of four AUUUA elements; however, they were not surrounded by AU-rich sequences. Accordingly, mutagenesis of the AUUUA motifs revealed that the half-life of HBV pgRNA was not affected, and that the levels of subgenomic and spliced SP1 RNA were not changed (unpublished data, TH), indicating that they are not critical for HBV RNA expression.

Studies in HBV transgenic mice reveal a potent non-cytotoxic, posttranscriptional mechanism inducing viral RNA degradation. This mechanism was induced by the injection of cytokines IL-2 and TNF-alpha or after adoptive transfer of HBsAg-specific cytotoxic T lymphocytes (CTL) (25, 30-33, 35, 36, 117). Attempts to reveal the underlying posttranscriptional mechanism led to the discovery that the mouse RNA-binding protein La binds (for review on the La protein see (74, 125)) specifically to the viral RNA (46). The binding site was mapped to a 91-nt long stretch overlapping the 5' end of the PRE (Figure 3) and the two RNA-recognition motifs of La have been shown to be required for HBV RNA binding *in vitro* (50). The association of human La with HBV RNA was later documented by co-immunoprecipitation using extracts from HepG2 2.15 cells (17). Importantly, comparison of nuclear extracts prepared form the liver of HBsAg-specific CTL-treated or control-treated HBV transgenic mice and measurement of HBV RNA levels firmly established a transient and tight correlation between CTL-induced degradation of viral RNA and La protein fragmentation (44, 46). Strikingly, injection of neutralizing antibodies against TNF-alpha and IFN-gamma prior to the injection of HBsAg-specific CTLs not only abolished HBV RNA decay but also prevented La fragmentation. The concomitant fragmentation of La and HBV RNA degradation indicates a functional connection in which the La protein stabilizes HBV RNA. Accordingly, mutagenesis of the La-binding site located within HBV RNA induced a decrease in the HBV RNA half-life by about two hours as determined in cell cultures (17). The notion that the La protein stabilizes HBV RNA was further supported by the characterization of endoribonuclease activity, which cleaved HBV RNA in close proximity to the purported La protein binding site (Figure 4; (45)). Strikingly, HBV RNA cleavage was more pronounced if nuclear extracts prepared from HBsAg-specific CTL-injected mice were analyzed compared to extracts from control mice (45), suggesting a stimulation of the endoribonuclease after injection of HBsAg-specific CTLs. The endoribonuclease has been characterized (45) but is not yet identified; however, initial experiments are supportive of a model in which the functional interaction

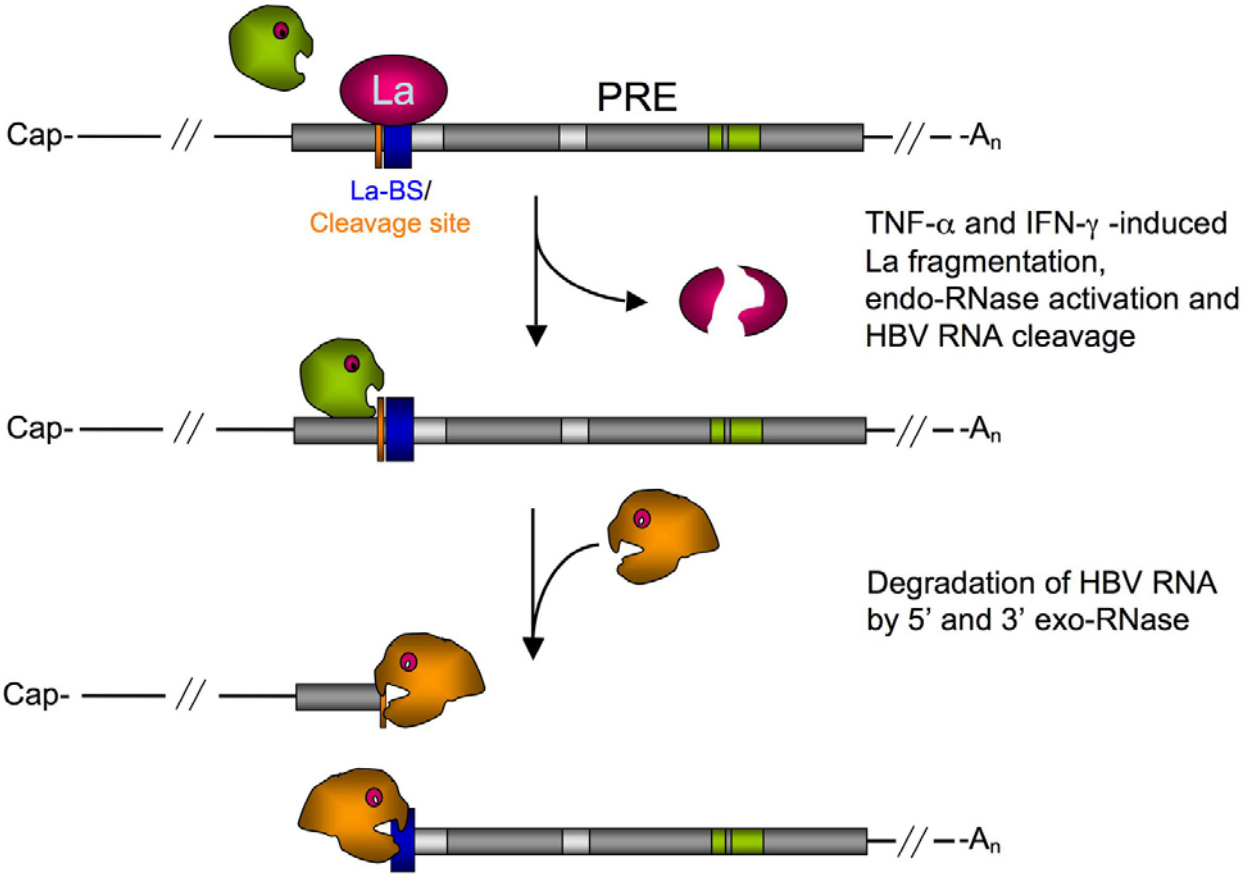

Figure 4. Model for the cytokine induced degradation of HBV RNA. The La protein (red) binds to the minimal binding site (dark blue box nts 1270-1331) located at the 5' end of the PRE (grey box, for details see Figure 3) and protects the endoribonucleolytic cleavage sites (orange lane, nts 1,268 and 1,270). After activation of HBsAG-specific CTLs cytokines TNF-alpha and IFN-gamma are induced. Induction of TNF-alpha and IFN-gamma induces the fragmentation of the La protein and the stimulation of an endoribonuclease (green). Fragmented La is unable to protect the cleavage sites and cleavage occurs. The unprotected 5' and 3'end of the viral RNA are rapidly degraded by 3'→5' and 5'→3' exoribonucleases (orange).

between La and HBV RNA controls HBV RNA stability. Collectively, those studies suggest a model of CTL-induced degradation of HBV RNA as depicted in Figure 4. The model is strongly supported by experiments that show a decrease in viral RNA levels and viral antigen expression in HepG2 2.15 cells after efficient siRNA-mediated knock-down of human La (79).

More recent data revealed that transcriptional as well as posttranscriptional processes affect HBV RNA expression. After adaptive transfer of HBsAg-specific CTLs or during persistent lymphocytic choriomeningitis virus (LCMV) infection, a posttranscriptional mechanism induces degradation of HBV RNA, whereas after acute murine cytomegalovirus (MCMV) infection or polyI/C injection, viral RNA decreases due to inhibition of viral transcription (118). Interestingly, La fragmentation was not only observed after CTL injection and LSMV infection but also after MSMV infection (44). It is unclear whether MCMV infection induces two concomitant mechanisms—the transcriptional shutoff and activation of a posttranscriptional mechanism—as depicted by La fragmentation. With respect to an efficient antiviral mechanism, induction of both mechanisms might be more potent.

A cytokine-induced posttranscriptional mechanism has been also described in cell culture models (7, 43, 90). After IFN-gamma treatment of HBV-DNA transfected HuH7 cells a remarkable downregulation of HBV RNA was observed (90). HBV deletion mutants were used to map the IFN-gamma response element between nt position 1 and 1,306. Interestingly, this region overlaps with the La binding site (position 1,270–1,295) and the HBV RNA endoribonucleolytic cleavage sites (position 1,267/68 and 1,269/1270).

Taken together, expression of viral gene products is not only determined by its rate of transcription or its rate of protein turnover but also by the rate of mRNA decay. Importantly, the stabilization of the viral RNA is likely mediated by host factors interacting with *cis*-acting elements in the viral RNA. The La protein is probably only an example and we assume that additional host factors are involved in the regulation of HBV decay. The existence of auxiliary

factors modulating the La-HBV RNA interaction has been postulated (17). The well-documented existence of a potent posttranscriptional mechanism leading to the degradation of the viral RNA is of significant therapeutic meaning.

6. REMARKS AND PERSPECTIVES

Although little is known about the posttranscriptional control of HBV gene expression, in recent years, studies suggested potential mechanisms regulating the maturation and stabilization of HBV RNAs. However, many questions remain to be answered in order to understand the mechanism underlying the regulation of viral RNA splicing, to define the nuclear export of viral RNA, and to understand how viral RNA is stabilized. The characterization of critical interactions between host factors and highly conserved viral *cis*-acting elements—at the molecular level—might reveal novel therapeutic strategies to target viral RNA maturation and stability.

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