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# Role of the Hepatitis B virus proteins in pro- and anti-apoptotic processes

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## TABLE OF CONTENTS



## 1. ABSTRACT

The Hepatitis B virus (HBV) can induces severe liver diseases as chronic hepatitis and hepatocellular carcinoma. Actually, apoptosis can play an important role in the progress of these diseases. As apoptosis goes through various extrinsic or intrinsic pathways, with activation of caspases and the possible involvement of mitochondria, HBV proteins can interfere with the various apoptosis processes. So far, four HBV proteins were reported to have such effect: the Large envelope protein, a truncated form of the Middle envelope protein, the HBx protein and HBSP, a protein generated from a spliced mRNA. In addition, our recent results suggest that indirectly the precore protein could have a function in apoptosis. This review focuses on the putative roles of HBV proteins as pro- or anti-apoptotic factors and the relationship which could exist with the HBV life cycle.

## 2. THE HEPATITIS B VIRUS

The Hepatitis B virus (HBV) is a virus which specifically infects the liver. It causes a wide spectrum of liver diseases ranging from an asymptomatic carrier state to chronic hepatitis with progression to hepatocellular carcinoma. A major factor is the chronic infection which can influence apoptosis (1).

It belongs to the hepadnavirus family which included several animal viruses. Among them, the Woodchuck Hepatitis Virus (WHV), the Ground Squirrel Hepatitis Virus (GSHV) and the Duck Hepatitis B Virus (DHBV) which infect respectively the woodchuck, the ground squirrel and the Peking duck have been also extensively studied.

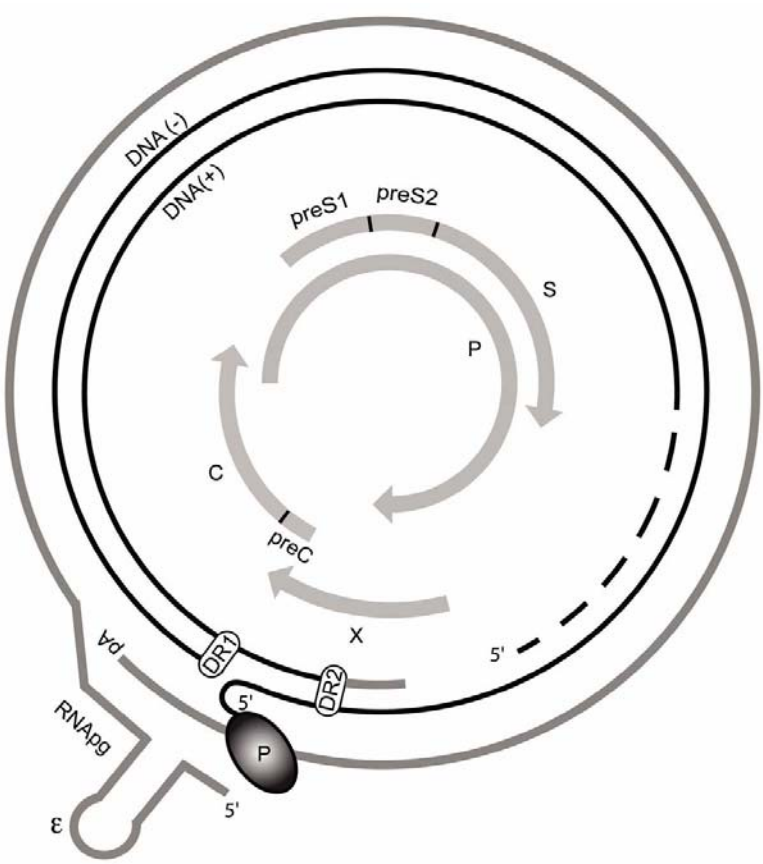


**Figure 1.** The HBV genome. The HBV genome is a partially double-stranded circular DNA. The complete chain (negative strand) consists of 3182 nucleotides while the size of the incomplete (positive strand) varies from 1700 to 2800 nucleotides, from one virion to another. The circularity of the genome is achieved by a 200 nucleotides overlap of its 5'ends. In addition, two repeated sequences of 11 nucleotides (DR1 and DR 2) are present on the complete strand. Another unusual design of the HBV genome is the covalent attachment at the 5' extremities of the DNA strand of the viral Polymerase (negative strand) or of a short RNA sequence (positive strand).

**2.1. Virion structure, genome and genetic organization**

Electron microscopy studies have revealed three features of HBV. First, the virion is a 42-47 nm particle, consisting of a 22-25 nm nucleocapsid (core) and an outer envelope of about 7 nm (2). Second, during the acute phase of the illness, up to $10^9$/ ml virions are present in the sera of the patient. Third, in addition to the virions, other particles (up to $10^{13}$/ ml) consisting of empty envelopes are also detected (3).

The HBV genome consists of a partially double-stranded circular DNA molecule. The full length strand contains 3182 nucleotides while the size of the short one varies from a virion to another, usually between 1700 to 2800 nucleotides (Figure 1). Its genetic organization is known since its sequence was determined (4). Four open reading frames (ORF) were present on the complete DNA strand, encoding respectively the reverse transcriptase/DNA polymerase (P ORF), the envelope proteins (S ORF), the core and precore protein (C ORF) and the HBx antigen (X ORF). The P ORF covers about 80% of the genome and consequently overlaps entirely the S ORF and partially the C and X ORFs (Figure 1). Identical ORFs were present in the WHV and GSHV genomes while the X ORF seems absent from the DHBV genome.

**2.2. Viral proteins**

Seven proteins are encoded by the HBV genome: the 3 envelope proteins referred as L (Large), M (Middle) and S (Small) proteins, the replicative protein P (which stands for Polymerase protein), the core protein (also named HBc Antigen or HBcAg), the precore protein which is the precursor of the HBe Antigen (HBeAg) and the X protein (also named as HBxAg).

**2.2.1. The envelope proteins**

Three proteins are the viral components of the HBV envelope: the S protein is the major one (70% of the envelope proteins), the M (10 %) and the L (20%) being the minor ones. The 3 proteins are encoded by the S ORF, using three in-frame start codons. The L, M and S proteins are respectively the translation product of the entire ORF (S gene + the pre-S1 and pre-S2 sequence), the S gene and the pre-S2 sequence and the S gene alone. In other words, the L, M and S proteins share the same C-terminal region but the M protein lacks the N-terminal 119 aminoacids (aa) of

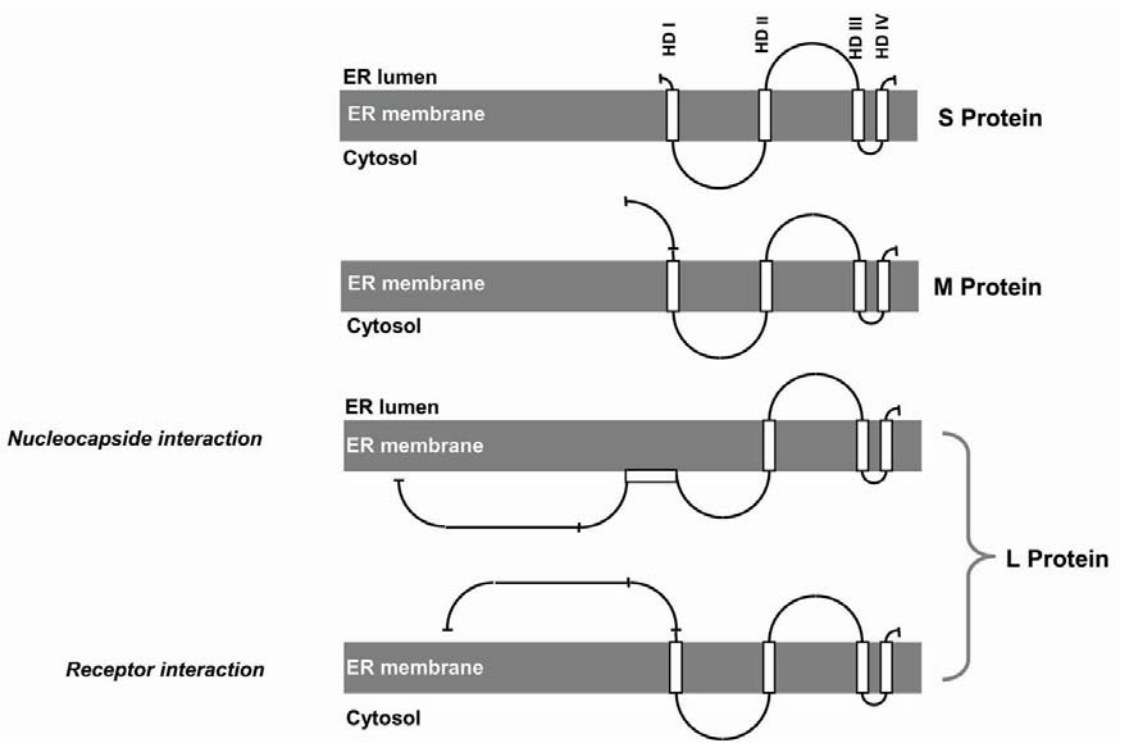


**Figure 2.** Topologies of the envelope proteins. The topology of each envelope protein S, M or L is shown in relation with the endoplasmic reticulum membrane. The N-terminal extremities of the proteins are on the left. The four transmembrane domains are indicated by a white rectangle and indicated as HD (hydrophobic domain) I, II, III and IV. For the M protein, the small horizontal line fixes the boundary between the preS2 and the S gene encoded domains. For the L protein, the two topological forms are shown. The small lines fix the domains encoded by the preS1, preS2 and S. Adapted from an original schema of Camille Sureau (Reference number) with his permission.

the L protein while the S protein lacks the first 55 aa of the M protein. They all bring the "a" determinant responsible of the HBs antigenicity. The three proteins are synthesized at the endoplasmic reticulum (ER) membrane and have a complex trans-membrane topology (5). The S and M proteins contain 4 hydrophobic domains (HD I, II, III or IV) which are inserted in the membrane of the ER. The situation is different for the L protein which displays a dual-membrane topology as the N-terminal pre-S (pre-S1 plus pre-S2) is either at the surface of the particles or facing the inner side of the virion (Figure 2).

The S protein is translated from a 2.1 kb mRNA species (Figure 3) as a membrane protein. This protein can be N-glycosylated at the asparagin residue 146, leading to a 27 kDa protein. However, about half of the molecules produced remain unglycosylated with a molecular mass of 24 kDa.

The M protein is translated from the longest 2.1 kb mRNA. It has the same topology than the S protein with its N-terminal domain exposed at the virion surface. The M protein possesses two N-glycosylation sites, the Asn 146 and a second one encoded by the pre-S2 sequence. At the opposite of the Asn 146 site, the second site is always recognized by the N-glycosylation machinery and thus the M protein is either a mono or a di-glycosylated form, with respective apparent Mr of 33 and 36 kDa.

Synthesized from the 2.4 kb mRNA, the L protein is the most complex surface protein of HBV. As mentioned above, the protein has two different topologies: its N-terminal region is either exposed at the surface or oriented to the interior of the virion. This particular feature allows the L protein to interact with the hepatocyte receptor at the entry step and with the nucleocapsid at the assembly step (Figure 2). As the S protein, the L protein is synthesized as an unglycosylated (43 kDa) or a glycosylated protein (46 kDa).

**2.2.2. The DNA polymerase-reverse transcriptase (P protein)**

HBV replicates *via* reverse transcription of a 3.5 kb RNA intermediate, referred as the pregenomic RNA (pgRNA). The replicative enzyme is the larger HBV protein (90 kDa). It contains three domains and has 4 enzymatic activities. The N-terminal domain is responsible of the priming activity which allows the initiation of the reverse transcription. Between the N-terminal domain and the third one, the spacer domain most likely gives flexibility to the molecule. The third domain brings the reverse transcriptase, the DNA polymerase and the RNAse H activities (see below). The enzyme is encoded by the P

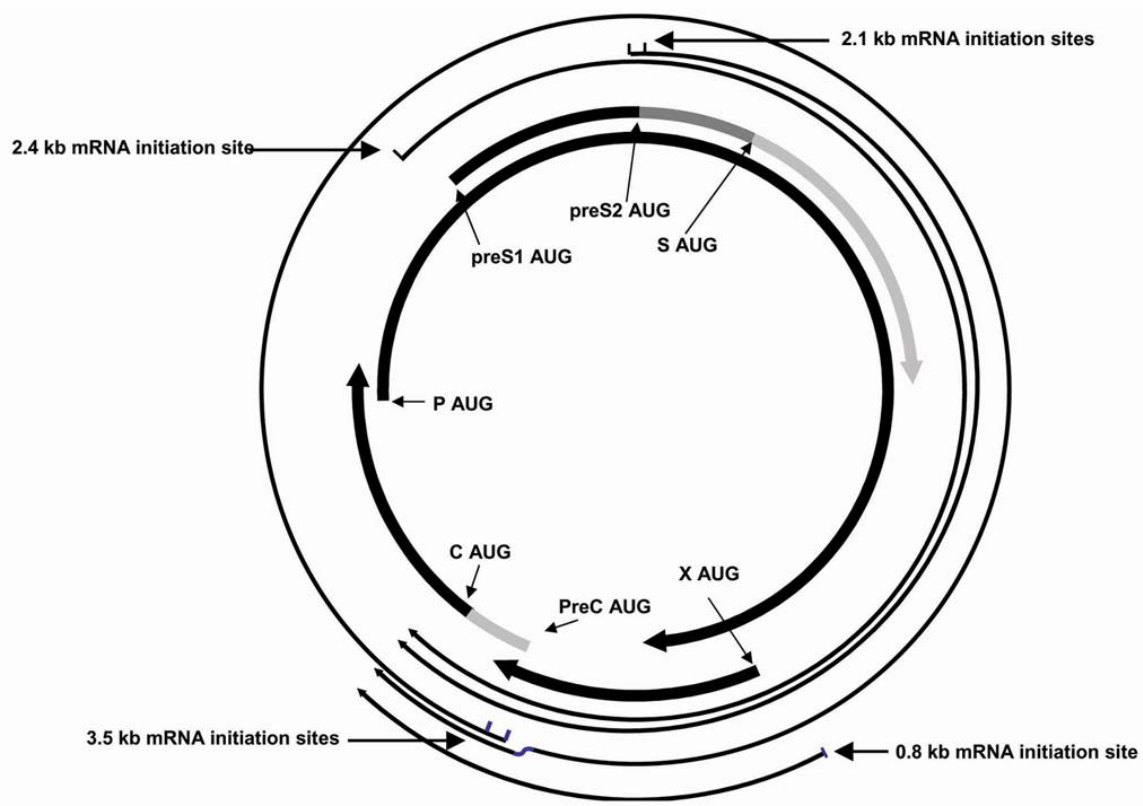


**Figure 3.** HBV mRNA. The wide lines represent the ORF P, S, C and X while mRNAs are represented as thin lines. Initiation transcription sites are indicated by short and thin lines. Adapted from an original schema of Camille Sureau with his permission.

ORF and is translated from the pgRNA - which also serves as template for the translation of the core protein - by a leaky scanning mechanism (6).

**2.2.3. The core protein**

The 21 kDa core protein is a phosphoprotein of 183 aa. The C-terminal part (34 aa) presents similarities with DNA binding proteins and is responsible for the strong interaction between the core protein and the DNA genome. The N-terminal domain is involved in the encapsidation of the viral genome and in its replication. As the P protein, the core protein is translated from the pgRNA.

**2.2.4. The HBx protein**

This protein was named thirty years ago HBx as no precise function could be predicted from its amino acids sequence. The HBx protein is encoded by the X ORF and translated from a 0.8 kb mRNA. This 154 aa protein (17 kDa) is conserved among hepadnaviruses which infect mammals but have no counterparts in the infected hosts. HBx is mainly localized in the cytoplasm with a mitochondrial fraction (7). However, in infected woodchuck hepatocytes a small amount of WHBx (the WHV counterpart of HBx) has been detected in the nuclear matrix (8). Accordingly, HBx contains a transactivation domain and a Nuclear Export Signal (NES) motif (9,10). Whereas numerous experimental data were reported but the precise functions of HBx in the viral life cycle and the natural course of HBV infection are still not clear. It was reported to have several cellular partners which could be potential targets of HBx. *In vitro,* HBx has been shown to be a potent transactivator of cellular and viral genes, an activity in agreement with its nuclear location. Other studies suggested that HBx is involved directly or indirectly in the viral infection and/or multiplication, in agreement with its cytoplasmic localisation. The possible role of HBx in apoptosis will be envisaged later in this review. Whatever the actual functions of HBx are, different results suggest that the protein could be involved in hepatocarcinoma development (11).

**2.2.5. The precore protein**

The precore protein is encoded by the precore sequence plus the core gene (Figure 1) and translated from a specific 3.5 kb mRNA (Figure 3). The primary translation product, a 25 kDa protein, undergoes two proteolytic modifications to yield HBeAg which is present in the serum of infected patients. However, about 15% of the precore molecules go back to the cytosol. The precore protein is dispensable for HBV infection but its precise function remains to be determined. So far, there is a consensus about a role of the precore protein in HBV persistence, based on two hypotheses which are not exclusive. The first one involved the cytoplasmic fraction of the precore protein which would diminish the amount of mature capsids (12) while the second implies that HBeAg would induce an immunological tolerance *in utero* (13).

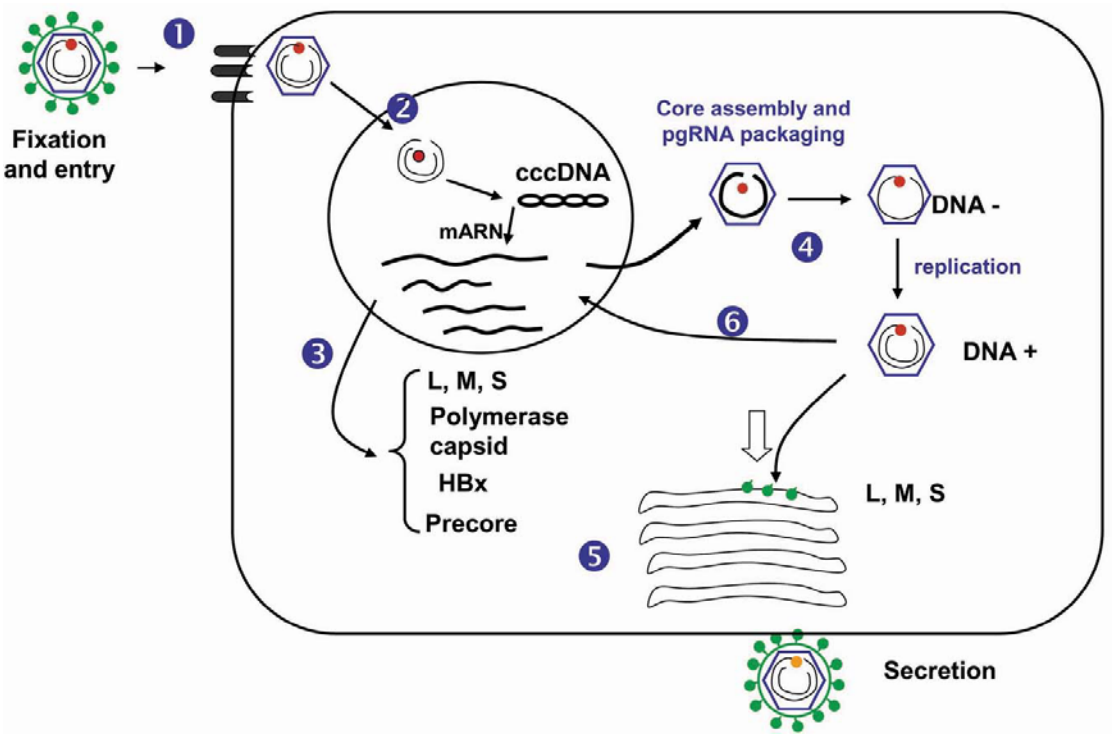


**Figure 4.** HBV multiplication cycle. An interaction between the HBV L protein and a unidentified receptor allows the HVB virion to attach to the hepatocytes (step 1). Nucleocapsids reach the nucleus before to dissociate in the nuclear basket and release their genomes (step 2). Viral genome is transformed in a covalently circular closed DNA (cccDNA) which serves as template for the cellular RNA polymerase. Viral mRNAs are then transcribed (step 3). The pregenomic RNA (pgRNA) is then encapsidated and retro-transcribed. The negative strand is then replicated (step 4). The nucleocapsids are then enveloped in the endoplasmic reticulum and the intraluminal virons exocyted in the blood (step 5). However, some nucleocapsids go back to the nucleus to amplify the cccDNA stock (step 6).

**2.3. Viral cycle**

A schematic view of the HBV life cycle is shown in Figure 4.

**2.3.1. Attachment of the viral particles to the receptor, internalisation and early steps of the cycle**

The details of the first steps of the HBV life cycle are still under discussion. So far, the nature of the actual receptor of HBV is still unknown (for review (14)) but it was demonstrated that the first 25 aa of the viral L protein are involved in the binding of virion to the receptor. After its binding, it is likely that HBV enters in the cell as DHBV does, via a receptor-mediated endocytosis. The release of the nucleocapsids is most likely pH-independent but once again, the actual mechanism remains to be determined.

The cytoplasmic nucleocapsids are then transported to the nucleus, depending of the microtubules network (15). After reaching the nuclear membrane the nucleocapsids interact with the cellular importins and go through the nuclear pore. Kann and co-workers has shown that the nucleocapsids dissociate in the nuclear basket to allow the entry of the genome in the nucleoplasm (15).

**2.3.2. Formation of the covalently closed circular DNA and mRNA synthesis**

The unusual design of the HBV genome does not allow any transcription process and consequently it must be modified as soon as it has entered in the nucleus. After removal of both the oligoribonucleotide and the DNA polymerase covalently linked to 5' extremities of the two strands, the DNA is converted by cellular enzymes into the covalently closed circular DNA (ccc DNA) which is supercoiled. Then the ccc DNA is transcribed by the RNA polymerase II from the four promoters present on the negative strand: the preC/C, the preS1, the preS2/S and the X promoter.

Different mRNAs have been identified which are capped and polyadenylated and for most of them unspliced. Among the mRNAs, two are about 3.5 kb in size: the pgRNA and the preC mRNA. Both are synthesized under the control of the PreC/C promoter but differ by their 5' extremity (Figure 3). The shorter one is the pgRNA which has a double function in the viral cycle as it serves as template for the viral reverse transcriptase and as mRNA for the translation of the core and the DNA polymerase/reverse transcriptase. The transcription of the preC mRNA begins upstream of the preC ATG, allowing the synthesis of the precore protein.

Another family of unspliced RNA allows the translation of the three envelope proteins. The most important species (2.1 kb) is synthesized under the control of the preS2/S promoter. Actually, the species is a mix of

two mRNA which slightly differ by their 5' extremities and serve as messengers for the translation of the M and S proteins (Figure 3). The minor species (2.4 kb) is under the control of preS1 promoter and allow the translation of the L protein. The last unspliced mRNA is the shorter (0.8 kb) mRNA. It is synthesized under the control of the X promoter and allows the synthesis of HBx.

In addition to the unspliced mRNAs, different spliced mRNAs have been identified in HBV-infected liver (16). Two of them could be encapsidated and reverse transcribed leading to the presence of defective particles in the sera of chronically infected patients. Interestingly, one of this spliced mRNA encodes a new HBV protein named Hepatitis B Spliced Protein (HBSP) which could have an important role in apoptosis (see below).

**2.3.3. Capsid formation, pregenomic RNA encapsidation and genome replication**

The capsid assembly takes place in the nucleus in a genome independent manner. The final structure exhibit two distinct icosahedral geometries composed of either 180 or 240 or subunits, with have similar stabilities (17).Whatever the case, the capsid is not totally closed and channels allow the entry of nucleotides and other components required for genome replication. Interactions between the pgRNA and both the P and the core protein are necessary for the encapsidation of the genome (18).

The replication process is complex and partially similar to that described for the retroviruses. It takes place in the capsids and can be schematized in four main steps. The first step is the initiation of the negative strand synthesis, through a covalent link between the P protein and a guanosine, followed by the addition of three residues, close to the 5' extremity (19). As replication progress in the 5' to 3' direction, a jump of the primer in front of a complementary sequence located at the 3' end of the pgRNA is required. The second step is the full synthesis of the negative strand with a concomitant degradation of the RNA template by the RNAseH activity of the P protein. However, the last 18 ribonucleotides are not degraded and serve as primer for the synthesis of the positive strand, with the DNA negative strand as template (third step). Finally, the synthesis of the positive strand is arrested before completion, leading to the characteristic DNA genome described above.

**2.3.4. Virion assembly and virion exocytose**

The envelope acquisition takes place in the ER through interactions between the L protein and the nucleocapsid. Then, the intraluminal virions are secreted in the blood stream via the exocytic pathway. However, all the nucleocapsids are not enveloped and interestingly these nucleocapsids go back to the nucleus. Thus, the pgRNA serves as template for the formation of new ccc DNA which is present at a 20 to 50 copies per nucleus. How is regulated the balance between enveloped and non-enveloped nucleocapsids remains to be determined. It seems that, at the beginning of the infection, most of the nucleocapsids are directed to the nucleus when the L protein is not translated in large quantities. It is worth noting that subviral particles containing only envelope proteins are secreted into the blood in large excess over the virions.

## 3. ROLE OF THE VIRAL PROTEINS IN LIVER APOPTOSIS

The liver has special features which is necessary to recall rapidly before envisaging the role of the HBV viral proteins in apoptosis. Among them is the possibility of hepatocytes proliferation after partial hepatectomy, a complex mechanism which involves several intricate pathways. However, in a normal liver, hepatocytes normally divide extremely infrequently but most likely hepatocyte's apoptosis could induce a proliferate state in the adjacent cells (20). As viral DNA insertion in the cellular genome occurs during liver-cell proliferation, apoptosis can indirectly favour the persistence of the viral genome in the cell and thus appears as a fundamental component of chronic hepatitis.

### 3.1. HBx

As explained above, HBx is a multifunctional regulator protein. Thus, before to envisage its possible role in apoptosis, it is worth to evoke briefly its other functions. First, HBx has been described as a powerful transactivator involved in the regulation of HBV genes expression. In addition, HBx was also shown to transactivate the expression of cellular genes like IL6 (21), NOS (22), FasL (23), NF-AT (24). However, these reports were obtained *in vitro* and thus it cannot be excluded that the transactivatory function of HBx is more specific during the course of a natural infection.

Later, HBx has been involved in viral replication. The first indication came from experiments performed with the Woodchuck hepatitis B virus (WHV). Sitterlin and co-workers have shown that a complex made of WHBx and UVDDB, a DNA repair factor, is essential for viral replication (25). More recently, Xu *et al.* reported that HBx was essential for viral replication in transgenic mice expressing the HBV genome (26). It was also observed that the interaction between HBx and UVDDB causes the translocation of HBx into the nucleus, an event correlated with an increase of HBx-mediated transactivation (27). Thus, it is possible that the transactivatory function of HBx and its role in viral replication are strongly related.

Finally, a role of HBx in oncogenesis has been evoked. The first results supporting this hypothesis were obtained with transgenic mice in which HBx induces hepatocarcinoma (28,29). More recently a systematic analysis of human liver cancers has revealed that in these tumors frequent integrations of HBV genomes exist. Interestingly these genomes have often mutations in the X gene, in particular 3'-deletions. These findings suggest that HBV integration may play an important role in the HCC development, notably when the HBx gene is 3' deleted during this integration process (30).

A role of HBx in apoptosis has been often reported. Once again it is worth to underline that the studies of HBV regulation mechanisms remain incomplete due to a

lack of an efficient HBV infection system. Thus, the data reported in the literature were derived from *in vitro* or *in cellulo* experiments or from experiments performed with an animal model infected with another hepadnavirus - close but not quite identical - to HBV. Taken together, these elements are informative on the mechanism of action of the virus, but we must remain cautious about their interpretation.

Apoptosis goes through various extrinsic (TNF) or intrinsic pathways (Bcl2), with usually activation of caspases and the possible involvement - or not - of mitochondria. Thus, it is not surprising that a pleiotropic protein as HBx was reported to have a role in these various apoptosis processes. However, the data are sometimes contradictories as some of them lead to the conclusion that HBx was pro-apoptotic while other lead to the opposite conclusion. Clearly, this antagonist role of HBx on apoptosis depends on the type of system used in the pertinent study (permissive cells or not, primary or transformed cells). However, these discrepancies could reflect that the role of HBx in apoptosis processes could varies during the viral life cycle. For example, HBx acts on cell cycle checkpoints and has a role in carcinogenesis, elements which are closely associated with the apoptosis.

On the other hand, we must keep in mind that apoptosis protects the cell from the deleterious effect of viral replication (31).

**3.1.1. HBx is a pro-apoptotic factor**

The pro-apototic effect of HBx was either direct or indirect. In the latter case the transactivatory function of HBx was often involved as the activation of the caspase 3 (32). However, other indirect effects were reported in experiments performed with hepatoma derived cell (HepG2) as non hepatic cell (HeLa cells) (33). These indirect effects are related to the extrinsic stimulation pathways of apoptosis. For example, when apoptosis is induced into hepatocytes by serum deprivation, a concomitant expression of HBx accentuates the apoptotic effect by activation of the p38MAPK and Jun kinase pathways (34). Similarly, HBx could sensitize hepatocytes to apoptosis induced by various apoptotic stimuli (TRAIL, TNF alpha) (35,36) through the activation of c-Myc and the MAP kinases pathways (36). Furthermore apoptosis induced during chronic HBV infection would involve the FAS pathways (37-39).

Once again, another indirect pro-apototic effect of HBx could be related to its capacity to transactivate the expression of some cellular genes. It was shown that transfection of HepG2 hepatoblastoma cells with either HBV genome or HBV X gene resulted in induction of TNF alpha expression (40). Interestingly TNF alpha could induce the expression of NOS2 (41). Keeping in mind that HBx was able to increase *in vitro* the transcription of NOS2 (22), HBx seems to be able by two different pathways to modulate positively the NO increase in hepatocytes. At the end of the process, the release of ROS leads to apoptosis (42).

HBx is also able to induce apoptosis through an interaction with mitochondria. Indeed, it was shown that overexpression of HBx induces an alteration of mitochondria (43) and a fall of the mitochondrial membrane potential (44). The alteration of mitochondia was explained by an interaction of HBx with a matrix protein Hsp60 that facilitates HBx induced apoptosis (45,46) while the decrease in the membrane potential was due to the interaction of HBx with VDAC, a external membrane protein (44). In addition, the alteration of the mitochondrial membrane significantly increases the concentration of cytosolic $Ca^{2+}$, leading to the activation of the caspase 3 and consequently can be interpreted as an indirect pro-apoptotic effect of HBx. Similar mechanisms have been reported for other viruses that are able to establish a persistent infection (33). However, conflicting results have been reported about the role of HBx on caspase 3 mediated- apoptosis (see below).

As mentioned before, there is a correlation between HCC development and integration of viral genome including a mutated form of HBx. Finally, analysis of HBx tumour-derived mutants from HBV chronic carriers has revealed that these mutants had a decrease of their pro-apoptotic potential (47). This pro-apoptotic pathway would involve (48). The authors propose that this event could cause a selective advantage to the development of HCC.

**3.1.2. HBx is an anti-apoptotic factor**

One of the first HBx partner characterized was the pro-apoptotic protein P53 (49). HBx/ P53 interaction impedes the binding of P53 to DNA *in vitro* (50) or confines P53 in the cytoplasm that consequently cannot interact anymore with its physiological partners or bind to DNA. This phenomenon would prevent apoptosis mediated by P53 (51)

The anti-apoptotic role of HBx could be more indirect. For example, the transactivation of the NFkapaκ-B genes family by HBx is associated in HepG2 cells to death's resistance through the anti-FAS pathway (52,53). This resistance may be due to the activation of SAPK / JNK pathways in which HBx would be in relation to the protein14-3-3 (21). The stimulation by HBx of PI3K activity in hepatocytes will induce a protecting effect from apoptosis (54) and would help cell survival via a PI3K/Akt/Bad pathway.

Finally, HBx could also act as an anti-apoptotic factor through an interaction with different caspases. A data reported ten years ago showed that overexpression of HBx leaded to a strong interaction with caspase 3 which consequently inhibit the caspase activity in rat fibroblast and human hepatoma (55). As indicated above, this result is in discrepancy with the more recent papers but these differences could report antagonist effects which would take place at different stages of the infection. More recent observations suggest that indirectly HBx could favour the inhibition of caspase 9. Indeed, HBx was shown to up regulate the survivin protein, an IAP (Inhibitor of Apoptosis Protein) which is overexpressed in many cancers (56). The surviving protein interacts with a cellular protein

named HBXIP (which stands for hepatitis B X-interacting protein), and the complex binds to the pro-caspase 9. Thus, the surviving/HBXIP complex precludes the formation of the apoptosome Apaf1/caspase 9. As HBx can bind through its association with HBXIP, it can indirectly suppress caspase activation in a survivin-dependent manner. Thus, in this case, HBX modulates apoptosis pathways through interactions with HBXIP/survivin complexes and acts as an anti-apoptotic factor (57).

### 3.2. Envelope proteins

A relationship between the envelope proteins and apoptosis was clearly established for the L protein and for a truncated form of the M protein found in liver tumours but there is no report of a possible effect of the S protein on the apoptotic process although these proteins share a significant part of their primary sequence. Concerning the L protein and the truncated M protein both have been shown to be transcriptional activators (58-60), a property related to their particular topology (see above).

#### 3.2.1. The L protein

The first indication that the L protein could have a pathogenic effect came from expression studies of the protein in transgenic mice. It was shown that hepatocellular damages in transgenic mice expressing the L protein were predominantly due to apoptosis (61). Later on, it was observed that in these conditions, the L protein aggregates in a pre-Golgi compartment within the hepatocytes leading to cell death (37). The same year, Xu *et al.* showed that accumulation of the L protein in the ER-Golgi intermediate compartment of hepatoma cells induces the stress of the ER (62). More recently, the same group demonstrated that expression of the L protein in hepatoma cells induced apoptosis (63) in agreement with the results obtained with transgenic mice.

#### 3.2.2. The M protein

In most than 80% of the HBV induced liver tumours, viral sequences were found in the cellular genome, without any specificity. These viral sequences could notably encode truncated forms of the Middle envelope protein, known as MHBst, which are found in at least 1/3 of the hepatocellular carcinomas (64). Actually, existence of two types of MHBst proteins has been reported, with two different intracellular localizations (60). Both of them are devoid of their C-terminal domain and consequently, the shorter one, truncated at aa 63 ($MHBst^{63}$) is soluble while the longer one ($MHBst^{76}$) is still inserted in the ER membrane. However, $MHBst^{76}$ does not present the same topology that the wild type M protein as its N-terminal domain is localised in the cytoplasm. In addition to its function as a transcriptional activator, MHBst confers a pro-apoptotic potential to infected liver cells (unpublished result cited in (65)). Very recently Liang *et al.* (66) showed that the truncated MHB protein renders the hepatocytes susceptible to TRAIL-induced apoptosis. In this paper, the authors demonstrated that this process involves the activation of the ERK2 and the degradation of procaspases-3 and 9. Interestingly, the same group has previously reported that HBx also sensitizes hepatocytes to TRAIL-induced apoptosis, through another mechanism (35). Taken together, these results give new insights in HBV infection-induced apoptosis.

### 3.3. HBV spliced generated protein (HBSP)

Data in the literature have shown that HBV pgRNA, the matrix for viral replication, may undergo simple or multiple splicing (16). These RNA are differentiated by the site of splicing. The major spliced HBV mRNA lacking intron 2447/489, may account for up to 30% of total HBV pgRNA as revealed in transfected cells and in the liver of chronically infected patients. However, the role of these spliced RNA in the natural history of viral infection has not yet been established although they might be encapsidated and retro-transcripted and gave rise to the secretion of defective particles (67,68). Recent report demonstrated that *in vivo*, at least one of these defective particle forms is as expected, associated with viral multiplication and more interestingly, related to the severe liver disease of HBV chronic carriers. Moreover, a new HBV protein, referred as HBV spliced protein (HBSP), is encoded by the major spliced RNA and related-defective particle (69). This protein is translated from the AUG of polymerase and because of splicing, is endowed in its C-terminal moiety with a sequence, which differs from that of other viral proteins (Figure 5). *In vivo*, HBSP protein has been detected in liver biopsy specimens from patients with active chronic hepatitis. High levels of HBSP could be detected in liver biopsy tissue from HBV chronic hepatitis infected patients in contrast to the barely detectable level of HBx protein. In addition, antibodies directed against this protein have been found in serum of approximately 50% of HBV infected patients with chronic hepatitis (69,70).

Although the mechanisms of liver injury are usually described as being due to the host immune response, evidence is now in favour of the direct impact of the expression of certain HBV proteins on liver disease. In this context, HBSP ectopic expression, after transient transfection, induced several hallmarks of cell apoptosis without associated cell-cycle block in Chang liver cells (69). The role of mitochondria-dependent apoptotic pathway, governed by Bcl-2 family of proteins, has been implicated during the development of HBV related liver disease. Recently, a BH3 homology domain was identified in the N-terminal part of HBSP protein. This BH3 region, also present in the HBV polymerase sequence, may interact as a pro-apoptotic motif with members of the Bcl-2 family proteins and therefore, might play a role in HBSP-related apoptosis. Evidences of the BH3 domain influence on HBSP-related apoptosis were suggested either by directed mutagenesis or by the relationship between HBSP genetic variability regarding to HBV genotype and the intensity of cellular apoptosis, in HepG2 cells (71,72). Finally, although the precise role of HBSP protein expression remains to be clarified in the context of HBV liver diseases, *in vitro* data are in favour of an impact on cell viability.

### 3.4. Precore protein

The precore protein has never been reported to be a pro- or an anti-apoptotic factor. However, our recent results suggested that it could play an indirect role in apoptosis. Indeed, seven years ago, a cellular partner of the

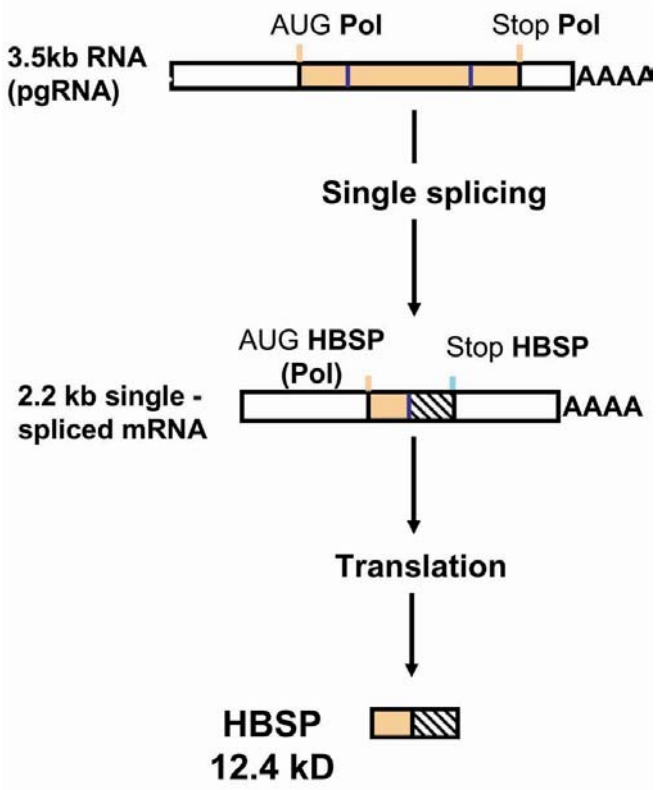


**Figure 5.** Schematic representation of singly spliced HBV RNA and HBSP translation product, pgRNA referred to the pregenomic RNA and Pol to the P gene.

HBV precore protein has been described (73), which was then identified as the p32/gC1qR protein (74). This cellular protein (also named HABP1) belongs to a particular class of proteins, which are both multifunctional and multicompartmental (75). Numerous data, obtained by different methods on a variety of cells or tissues, suggest that p32/gC1qR is located primary in the mitochondria and could be involved in the apoptotic process (76). This hypothesis was supported by recent studies which reported a proapoptotic effect of p32/gC1qR (77-80). We assume that the interaction between the HBV precore protein and p32/gC1qR could negatively modulate the pro-apoptotic effect of p32/gC1qR and we are currently investigating this hypothesis with Vincent Rincheval (LGBC, Universite de Versailles St-Quentin ).

As gC1qR was also reported to modulate the splicing factor SF2 (75,81), it is possible that gC1qR could favour the major spliced RNA leading to the synthesis of HBSP (see above). As preliminary results support this hypothesis, we have undertaken experiments to determine if the precore protein through its association with gC1qR can modulate HBSP expression and consequently apoptosis.

### 3.5. Other viral proteins

A potential role of the reverse transcriptase or the core protein in liver apoptosis has never been reported. The only data published so far indicate that, at the opposite of HBx, the precore, the core and the reverse transcriptase proteins do not activate the NF-kappaB–, SRE-, and AP-1–Associated Signals (82).

## 4. CONCLUDING REMARKS

Since several years, a close relationship between HBV infection and hepatocellular carcinogenis has been established (for review (83)). However, pathogenesis of HBV-induced tumours is still incompletely understood but apoptosis is most likely a key event in this process. From the data of the literature, 4 HBV proteins can interfere with the apoptosis machinery, directly or most of the time indirectly. It could be consider surprising that HBV utilize at least 4 proteins for the modulation of apoptosis, however we would like to underline that one of them (MHBst) is only detected in liver tumours and another one HBSP in defective particles found in the sera of chronically infected patients. In addition, these viral proteins do not seem to interfere in the same way with the cellular apoptosis machinery. Finally, two others important points must be recall: *i)* the liver has special features as the possibility of hepatocytes proliferation after partial hepatectomy, a complex mechanism which involves several intricate pathways, *ii)* progression to a hepatocellular carcinoma is a long process which takes usually more than thirty years.

Taking all these points in account, the implication of 4 HBV proteins could reflect that they play a role either

at the beginning of HBV infection, for the establishment of a chronic infection, during the chronic infection and finally during tumour liver progression. A hypothetical model could be the following. At the beginning of infection, the L protein and HBx would have a pro-apoptotic function in order to induce a proliferate state in the adjacent cells which favours new infections. For the establishment of a persistent infection, the anti-apoptotic functions of HBx and precore will be required. During chronic infection, as HBx is quite undetectable, HBSP would assure a pro-apoptotic function to maintain a low level of infection but this function would be regulated by precore. Finally, at the hepatocellular carcinoma state, MHBst would play a pro-apoptotic role in some of the tumor cells for a r unexplained reason.

Even if these hypotheses are highly speculative, HBV seems to be an excellent model to unravel the link between apoptosis and a viral pathogen.

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**Abbreviations:** AP-1: activating protein 1; ccc DNA: covalently closed circular DNA; DHBV: duck hepatitis B virus; ER: endoplasmic reticulum; ERK2: extracellular signal regulated kinase 2; GSHV: ground squirrel hepatitis virus; HBcAg: HBc Antigen; HBeAg: HBe Antigen; HBSP: HBV spliced generated protein; HBV: hepatitis B virus; HBx: HBV protein x; HBXIP: hepatitis B X-interacting protein; HCC: hepatocellular carcinoma; HSP60: heat shock protein 60; IAP: inhibitor of apoptosis protein; IL6: interleukin 6; MAP: mitogen-activated protein; MHBst: truncated middle envelope protein; NF kappa B: necrosis factor kappa B; NF-AT: nuclear factor of activated T-cells; NO: nitric oxide; NOS: nitric oxide synthase; ORF: open reading frame; pgRNA: pregenomic RNA; ROS: reactive oxygen species; SAPK / JNK: stress-activated protein kinase/c-Jun NH2-terminal kinase; SRE: serum response element; TNF: tumor necrosis factor; TRAIL: TNF-related Apoptosis-inducing ligand; UVDDB: ultraviolet light-damaged DNA binding protein; VDAC: voltage-dependent anion channel; WHV: woodchuck hepatitis virus

**Key Words:** Human Hepatitis B virus, apoptosis, HBV Proteins, Review

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