Hepatitis B Virus and Liver Disease

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To our mentor Professor Juei-Low Sung
Jia-Horng Kao
Ding-Shinn Chen
In memory of my parents Ping-Pei Chen and Shiu-Chin Tseng Chen
By Ding-Shinn Chen
To my parents Shi-Yang Kao and Wen-Shu Ho
By Jia-Horng Kao

Foreword

This compact monograph represents a welcomed update on hepatitis B virus (HBV) and the disease it causes. The 17 chapters review the full spectrum of issues regarding HBV—its structure, molecular virology, replicative life cycle, immune pathogenesis, modes of transmission, epidemiology, natural history, complications, prevention, and treatment. Special chapters deal with the important issues of maternal-infant transmission; the disease in children, in immunosuppressed individuals, and in hepatitis C virus-, hepatitis D virus-, and human immunodeficiency virus-coinfected individuals; carcinogenesis; fibrosis progression; noninvasive means of assessment; and the needs for future basic and translational research. The authors are internationally recognized experts from Asia, Australia, the United States, and Europe, reflecting the global distribution and burden of HBV. Importantly, this book goes far beyond what can be covered in standard textbooks of medicine, hepatology, infectious diseases, and even virology.

There are several ways to view this monograph: a big book for a small topic, or a small book for a big topic, or perhaps both. For one thing, the book has two topics—one is small and one big—the hepatitis B virus (small) and the disease that it causes (big).

HBV is small. With its circular, partially double-stranded genome of only 3200 bases, it is the smallest known human DNA virus. This number of bases equates to about ten base pairs per page, or hundreds of words for each base. If HBV had a single open reading frame, it would produce a single moderately sized protein only. HBV, however, produces seven different polypeptides (pre-S1, pre-S2, small HBsAg, HBV polymerase, HBcAg, HBeAg, and X), each with a different structure and distinct function. In addition, the 7 HBV polypeptides contain far more amino acids than could be encoded by 3200 bases. How does this small DNA virus accomplish this big task?

The answer is that the HBV genome is small but efficient. The four open reading frames of HBV (S, C, P, and X) partially overlap each over, but produce different proteins because they are translated in different reading frames. The gene regions also have no introns. By frameshifting and not using introns, the same nucleic acid sequences can produce two or three different amino acid sequences, and each base pair in the genome can be used twice if not three times (particularly in view of the gene regulatory regions). In addition, some of the gene regions have several start sites so that polypeptides of different lengths are produced. The S gene region

possesses three start signals which allow it to encode three forms of HBsAg differing in their length and tertiary structure as well as their functions. The C region has two potential start sites. One start signal encodes the nucleocapsid core antigen (HBcAg) which serves as a structural component of the virus. The second C region start site includes a pre-core region and, after further posttranslational editing, produces HBeAg, a secreted, small molecular weight protein that circulates in the serum. The P region overlaps with the C, S, and X regions and produces a large multifunctional polymerase (both DNA and RNA dependent) and a separate ribonuclease activity. Finally, the small X region produces a polypeptide which is retained intracellularly and probably acts as a transcription factor. Each of the seven HBV polypeptides is essential; deletion of any of them results in a marked decline or termination of replication.

Thus, HBV is small in size but versatile in function and complex in structure. It also has a unique replicative strategy – through an RNA intermediate. Currently, the reasons for the complexity of structure and replicative cycle remain only partially understood. Why does HBV produce such excessive amounts of HBsAg that circulate as incomplete, non-virion forms in microgram amounts during acute and chronic infection? What is the function of HBeAg that circulates in patients with HBV infection with high levels of viral replication and seems necessary to produce chronic infection but not necessarily to sustain it? How does HBV blunt or circumvent the host innate and adaptive immune response to its presence? With its compact structure and multistep replicative cycle, how and when did this virus arise during human evolution?

In contrast to the virus itself, the disease that HBV produces in humans is a very large topic. When HBV was first discovered in the late 1960s, chronic infection with hepatitis B was found to affect 5–10% of the earth's population and to be the major cause of cirrhosis and hepatocellular carcinoma worldwide. Virtually, every human population, even those in the most remote areas of the world, harbored evidence of HBV infection. In China and Southeast Asia, with the highest rates, more than 200 million persons were believed to be chronically infected. In these areas, HBV was the most frequent cause of chronic liver disease and cirrhosis. In these areas and worldwide, hepatocellular carcinoma, the most dreaded long-term consequence of chronic HBV infection, ranked among the most common causes of cancer death. At that time, there was no means of prevention or treatment of this disease. This is not changed: all as a result of the discovery of HBV and the rapid subsequent advances in diagnosis, prevention, and now treatment.

The discovery of HBV was a major milestone of twentieth-century medicine. Quite aptly, Baruch Blumberg, the discoverer of the Australia antigen, which was later found to be the surface antigen of HBV and named HBsAg, was awarded the 1976 Nobel Prize in Medicine. The global implications of this discovery were immense. Once the Australia antigen was linked to HBV, it was rapidly found to be a reliable diagnostic marker for infection leading to means of screening donor blood and elimination of posttransfusion hepatitis B. More importantly, HBsAg could be purified in high quantities from serum, inactivated by heat and chemical treatment and used as the first, effective vaccine against this disease. Recombinant vaccines

(the first in humans) then followed and are now sensibly priced and used worldwide. Therapies for HBV followed means of prevention, but have now become clearly integral to any attempt to eradicate this disease worldwide. Current oral nucleoside analogues are highly effective in suppressing HBV replication and induce clinically significant remissions in disease in almost all patients. The combined effects of vaccination and treatment have begun to have major effects on the global burden of this disease. Eradication of HBsAg and all evidence of HBV replication by therapy is still limited, but new insights and innovative approaches are now zeroing in on this next step in HBV control.

These considerations make this small, compact monograph a welcome addition to our understanding of this small, compact virus and the very important disease that is causes.

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Preface

Hepatitis B virus (HBV) was identified more than 50 years ago, and it was soon found that the infection is among the most frequent and important in humans. It causes a wide spectrum of liver diseases, spanning from fulminant hepatitis to cirrhosis and hepatocellular carcinoma. In the last couple of decades, the understanding of HBV infection, especially the management of chronic infection, has evolved drastically. The pathogenesis of this virus has become clearer after basic, clinical, and epidemiological studies. More constructively, the infection can now be prevented effectively, and the chronic infection can be suppressed efficiently, shedding light at the end of the tunnel toward the elimination of HBV infection.

However, the rapid progresses are still not well taken by many people in the medical profession. And thus, it is timely and necessary to have a monograph on this subject. We edited a book *Hepatitis B Virus and Liver Disease* which is published by Springer Science + Business Media Singapore Pte Ltd. We aimed to provide a comprehensive, state-of-the-art review of HBV infection and liver disease.

The book updated the results of basic and translational medicine including hepatitis B viral life cycle, immunopathogenesis of HBV-induced chronic liver disease, viral and host genetic factors affecting disease progression, molecular mechanism of HBV-induced hepatocarcinogenesis, and the clinical implications. The clinical aspects of chronic HBV infection were elucidated by experts in epidemiology, natural history, hepatitis B vaccination, coinfection with hepatitis C or D viruses and human immunodeficiency virus, and management of special populations like children, pregnant women, and those under immunosuppressive therapy. The implications of occult HBV infection were also discussed. Finally, the advances and perspectives in the development of novel treatments for the cure of HBV infection were included.

We hope this book can serve as a useful resource for students, health-care providers, and researchers who are interested in the management and study of patients with hepatitis B.

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Molecular Virology and Life Cycle

1

Darren J. Wong and Stephen A. Locarnini

Abstract

The hepatitis B virus is a prevalent human infection with no cure at present. It is a significant cause of global morbidity and mortality and has achieved its persistence in humans via its complex life cycle and ability to use its few protein products in a multifunctional manner to subvert and evade immune detection and clearance. These aspects of the virus are discussed in detail, as are the development of clinically important mutations in the viral genome that develop as a result of host immune selection, as well as those selected by the introduction of antiviral therapy or vaccination.

Keywords

Hepatitis B • Molecular Virology • Lifecycle • Antiviral Resistance

1 Classification

The hepatitis B virus (HBV) is the most well-known member of the virus family *Hepadnaviridae*. The species in this family are split amongst two genera—*Avihepadnavirus* and *Orthohepadnavirus*—with human HBV belonging to the latter. HBV has been further classified into ten genotypes, A to J, which are based

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on whole-genome nucleotide diversity of at least 8% (Norder et al. 1994; Stuyver et al. 2000; Kann and Gerlich 2005; Tatematsu et al. 2009; Olinger et al. 2008). There is distinct geographical variation amongst these genotypes (Table 1.1).

Table 1.1 Overview of the ten genotypes of HBV. Adapted from Locarnini et al. (2013). Note that genotype J has yet to be verified, as only one case has been isolated

Genotype	Subtype	Genome length (nt)	Frequency of mutation[a]		Global distribution
			PC	BCP	
A	A1	3221	Uncommon	Common	Africa, Asia
	A2				Northern Europe, North America
B	B1	3215	Common	Uncommon	Japan
	B2				Rest of Asia
	B3				Indonesia, China
	B4				Vietnam, Cambodia
C	C1	3215	Common	Common	Far East
	C2				Far East
	C3				Polynesia
	C4				Australian indigenous population
	C5				Philippines
D	D1	3182	Common	Common	Europe, Middle East, Egypt, India, Asia
	D2				Europe, Japan
	D3				Europe, Asia, South Africa, USA
	D4				Australia, Japan, Papua New Guinea
E		3212	NK	NK	Sub-Saharan Africa, UK, France
F	F1	3215	Uncommon	NK	Central and South America, Bolivia
	F2				Brazil, Venezuela, Nicaragua
	F3				Venezuela, Panama, Columbia
	F4				Argentina, Bolivia, France
G		3248	Very common	NK	USA, Germany, Japan, France
H		3215	NK	NK	USA, Japan, Nicaragua
I		3215	–	–	Vietnam, Cambodia
J		3182	–	–	Japan

Modified from Locarnini et al. (2013)

[a]PC, precore mutations such as G1896A; BCP, basal core promoter mutations such as A1762T, G1764A; very common (most isolates); common (up to 50% of isolates); uncommon (less than 10% of isolates); NK, not known

Several of the genotypes (A through D, and F) can be further subclassified into subgenotypes, which are defined by a nucleotide diversity of between 4 and 8%.

The relevance of genotype classification emerges in clinical practice, where there are notable differences in pathogenic potential, and response to immunomodulatory antiviral therapy (Funk et al. 2002; Kao 2002). Genotype D is more pathogenic than genotype A2 and less responsive to interferon therapy, whereas genotype C is more pathogenic than genotype B (Kao and Chen 2003; Chan et al. 2002a, b; Orito et al. 2001).

2 Structure of the Hepatitis B Virus

2.1 Viral and Subviral Particles

Three types of viral particles are present in the blood of persons infected with HBV. The whole virion is the classical Dane particle, which is spherical in shape with a diameter of 42 nm. Virions have two layers—an outer envelope, coated with hepatitis B surface antigen (HBsAg) proteins—and an inner nucleocapsid, composed of dimers of hepatitis B core antigen (HBcAg). HBcAg exists as two distinct populations that exhibit T3 or T4 symmetry, consisting of 180 or 240 core proteins, resulting in sizes of 32 nm or 36 nm, respectively (Crowther et al. 1994). The nucleocapsid encloses the HBV genome and intimately associated, endogenous DNA polymerase. Two subviral particles are also present in the sera, both of which are composed solely of HBsAg and are not infectious. One is a smaller spherical structure of 17–25 nm diameter, and the other is filamentous, being approximately 20 nm in diameter and of variable length. The function of these subviral particles is not clear but is probably immune decoys.

2.2 Genome Organization and Viral Proteins

The genome of HBV is a circular, partially double-stranded, relaxed circular (RC) DNA molecule (Lok et al. 1994). The two DNA strands are held in a circular configuration by a 226 base pair overlap between the 5′ ends that contain 11 nucleotide repeat sequences called DR1 and DR2 (Ganem and Schneider 2001). Genome length is between 3181 and 3221 bases, depending on the genotype. The minus DNA strand is not a closed circle and has a gap between its 3′ and 5′ ends, which is bridged by the plus DNA strand. The viral polymerase is covalently bound to the 5′ end of the minus strand. The 5′-end of the plus strand is formed by an 18-base oligoribonucleotide, which is capped in the same manner as typical messenger RNA (mRNA), (Will et al. 1987). The 3′ end of the plus strand is not at a fixed position, so there is single-stranded gap region of variable length, which can be from 20 to 80% of total genomic length, which can be filled in by the endogenous viral DNA polymerase. The minus strand encodes the complete viral genome and contains four overlapping open reading frames (ORF). The longest ORF encodes the viral

polymerase (Pol). The envelope ORF (Pre-S1, Pre-S2 and S) overlaps entirely with the Pol ORF, whilst the precore/core (Pre-C/C), and the X ORFs overlap partially with it. The ORFs overlap in a frameshifted manner, meaning that the virus strand is read approximately one and one-half times during transcription.

The transcriptional template of the virus is the covalently closed circular (ccc) DNA, which exists in the cell nucleus as a viral minichromosome. From this template, four mRNA transcripts are produced of varying kilobase (kb) length—the pregenomic (3.5 kb), the pre-S1 (2.4 kb), the pre-S2/S (2.1 kb) and the X (0.7 kb). Each transcript has its expression controlled by a different gene promoter, namely, the enhancer II/basal core, large surface antigen (Pre-S1), major surface antigen (S) and enhancer I/X, respectively (Ganem and Schneider 2001).

2.2.1 Pol ORF

The Pol gene spans approximately 80% of the genome length, and its product, the viral polymerase, is translated from pregenomic RNA. The polymerase is a 90 kDa, multifunctional protein that has four main domains required for viral replication (Ganem and Schneider 2001).

1. The N-terminal domain is known as the terminal protein and is responsible for linking the polymerase to the 5′ end of the minus DNA strand. The terminal protein is also required for priming of minus strand synthesis. This is achieved by provision of a tyrosine substrate at amino acid 96 of the polymerase protein, which covalently binds to the first nucleotide of the minus strand DNA (Zoulim and Seeger 1994).
2. The second domain is the spacer or tether region, and has no specific known function, but overlaps pre-S1 and pre-S2.
3. The third and largest domain is the typical viral polymerase, responsible for RNA- and DNA-dependent reverse transcription. Reverse transcriptase activity is conferred by the essential YMDD sequence motif in domain C (Schlicht et al. 1991).
4. The fourth, C-terminal domain has ribonuclease H activity, which cleaves the RNA-DNA hybrids formed during the process of reverse transcription. It also has a role in viral packaging and in optimizing priming and elongation of minus DNA strand synthesis.

2.2.2 Pre-C/C ORF

The Pre-C/C ORF encodes two main protein end products: the core protein (HBc) P21, which is the major polypeptide of the nucleocapsid and expresses the HBV core antigen (HBcAg), and the soluble early antigen (HBeAg). The HBc protein varies in length depending on viral genotype and is either 183, 185 or 195 amino acids long. Upstream of the core ORF is a short, in-phase highly conserved ORF called the precore region, from which HBeAg is synthesized (Ganem and Schneider 2001).

HBc Protein

The HBc protein has two distinct domains. The first extends from amino acid residues 1–144 and is required for the assembly of the 32-nm nucleocapsid. The second

commences from approximately position 140 to the C-terminus and is rich in arginine clusters, forming a protamine-like domain that mediates nucleic acid binding and is involved in viral encapsidation and DNA replication (Ganem and Schneider 2001). This region includes a putative nuclear localization sequence. The core protein contains many hydrophilic and charged amino acids and becomes phosphorylated when expressed (Kann and Gerlich 2005). Phosphorylation of serine 170–172 between arginine cluster 3 and 4 may block nucleic acid binding, which negatively regulates nuclear localization of the core protein (Kann and Gerlich 2005). The HBc protein is translated from pregenomic RNA and carries the HBcAg epitopes, which are cross-reactive with the HBeAg epitopes (discussed below).

The HBc protein may also play an active role in regulating viral transcription. This hypothesis emerged following experiments showing that HBcAg aggregates in different cellular compartments in different phases of infection (Chu and Liaw 1987). The immunotolerant phase, in which high HBV DNA levels are seen, is associated with a predominant nuclear distribution of HBcAg. Conversely, in the immune clearance phase, HBcAg becomes predominantly cytoplasmic, in conjunction with decreasing viral load. Subsequently, HBcAg becomes undetectable in the immune control phase. In addition, HBcAg exhibits preferential binding for HBV cccDNA and is able to modulate viral transcription by altering nucleosomal packaging (Bock et al. 2001). These studies demonstrate the multifunctional nature of HBV proteins.

The predominant epitopes of HBcAg presented to the host immune system are discussed below in conjunction with the HBe protein.

HBe Protein

The HBeAg is an accessory protein of the virus and is essentially a soluble secretory form of the HBc protein. As mentioned, the precore sequence is upstream of core, and translation produces the precore protein. This protein contains the entire core amino acid sequence plus an additional 29 amino acids at the N-terminal end (P25) (Ganem and Schneider 2001). The bulk of translated precore protein undergoes extensive post-translational modification in the endoplasmic reticulum (ER). The first 19 amino acids of the pre-translational precore protein form a signal that allows translocation into the ER lumen. This signal is then cleaved off by a host cell signal peptidase, leaving the precore protein derivative P22. P22 is then secreted through the ER and Golgi apparatus, with further cleavage of up to 34 amino acids from the C-terminal end. The resulting proteins are a heterogeneous population of approximately 14–17 kDa, serologically defined as HBeAg, and are secreted into the serum (Ganem and Schneider 2001). This extensive modification renders the HBe protein quite different in almost all aspects from HBc protein despite the primary amino acid sequences being almost identical. A quantity of precore protein does not reach the ER lumen, however, and thus exists in an unmodified form. Additionally, the P25 HBe protein also expresses a nuclear transport signal (Kann and Gerlich 2005), meaning forms of HBeAg of variable length are found in practically all compartments of the cell, as well as being secreted. HBeAg and HBcAg are cross-reactive at the B cell level (see below) (Milich 1988).

The HBe protein has functions that target various arms of the host immune system in an effort to subvert and evade detection and elimination. Therefore, whilst not essential for viral replication, it is essential for the establishment of persistent infection (Hadziyannis 1995; Hunt et al. 2000). Mutations in the precore sequence are found commonly during chronic infection, some of which result in reduced or absent HBe production (discussed further below). Study of these mutants reveals the importance of the HBe proteins in chronic infection. As an example, an HBeAg-negative mutant of the woodchuck HBV is infectious for newborn woodchucks but is unable to establish persistent infection (Chen et al. 1992). Similar scenarios have been described in human infection (Hadziyannis and Vassilopoulos 2001), with many possible mechanisms of action including:

- Functioning as an immune tolerogen in vivo (Milich et al. 1990)
- Slowing of HBV DNA replication in vitro by reducing HBcAg dimerization, thereby reducing encapsidation of pgRNA (Scaglioni et al. 1997)
- Possessing anti-apoptotic properties in vitro (Locarnini et al. 2005)
- Downregulating TLR-2 expression on hepatocytes and on monocytes and CD86 on Kupffer cells (Visvanathan et al. 2007)

The HBeAg has two B cell epitopes, one of which is linear (HBe1) and the other conformational (HBe2). The HBe1 epitope is found at core amino acid positions 76–89, and the HBe2 epitope is at positions 130–138 (Salfeld et al. 1989). Two immunodominant core epitopes (HBc) are localized between residues 74 and 83 (HBc1) (Salfeld et al. 1989), which is co-linear with HBe1, and to residues 107–118 (HBc2) (Colucci et al. 1988). Given this overlap, HBcAg and HBeAg are highly cross-reactive at the T cell and B cell level (Chisari and Ferrari 1995; Milich 1987). Two cytotoxic T lymphocyte (CTL) epitopes of HBc can be mapped to residues 18–27 (human leukocyte antigen HLA-A2 restricted) (Bertoletti et al. 1993) and residues 141–151 (HLA-A31 and HLA-Aw68 restricted) (Missale et al. 1993). Three T-helper cell (T_H) epitopes have been found on the core protein at amino acid residues 1–20, 50–69 and 117–131 (Ferrari et al. 1991).

2.2.3 Pre-S/S ORF

The HBsAg contains small (SHBs), medium (MHBs) and large (LHBs) proteins, all of which exist in two forms that differ in the extent of glycosylation. N-linked glycosylation and glucosidase processing through the ESCRT pathway are necessary for virion secretion (Kann and Gerlich 2005; Stieler and Prange 2014; Bruss 2007). Conversely, subviral particles are packaged and released by the endoplasmic reticulum and Golgi complex, a process which is not dependent on glycosylation status.

SHBs

The SHBs is the most abundant of all three HBsAg particles. It is 226 amino acids long and is found in both glycosylated and non-glycosylated forms. It is this component of the HBs protein that houses the major antigenic determinant of HBsAg, the *a* determinant or antigenic loop (AGL) (Carman et al. 1990). The AGL is a conformational loop that contains a high number of cysteine residues that cross-link

with each other, spanning amino acids 99–169 of SHBs. Given its complexity, and conformational mature, a crystal structure of HBsAg has not been described to date. The AGL is highly conserved amongst all known HBsAg isolates, as it is responsible for initiating viral entry to target hepatocytes, by initially interacting with its low-affinity receptor, the cell surface heparan sulphate proteoglycans (HSPG) (Sureau and Salisse 2013). The AGL has the subdeterminants *d* or *y* and *w* or *r*. Determinant *d* has a lysine at position 122, whereas *y* has an arginine; likewise, determinant *w* has a lysine at position 160, but *r* is an arginine (Okamoto et al. 1994). These antigenic subtypes elicit cross-protecting anti-HBs following immunization. The *a* determinant has been renamed the major hydrophilic region (MHR) and is considered the main neutralization domain for anti-HBs in the context of prophylactic (protective) vaccination.

MHBs

The MHBs is constructed of the entire SHBs sequence with the addition of a 55-amino acid N-terminal extension (Ganem and Schneider 2001). This extension is known as the pre-S2 domain and is a minor component of the virion. MHBs can be either single or doubly glycosylated but is not essential for virus assembly and release. The immunogenic epitopes are not conformationally dependent, as is the case for SHBs.

The central part of the pre-S2 domain carries its major antigenic epitope. The region between amino acids 3 and 16 has the ability to bind polymerized human serum albumin (Kann and Gerlich 2005). The significance of this binding is unknown but has been postulated to act as a possible co-receptor or to mask the immunogenicity of this epitope (Sobotta et al. 2000). The MHBs is considerably more immunogenic than SHBs at the B cell level (Milich et al. 1985), and pre-S2-containing HBs particles generated from animal cell lines have been used in some countries as a prophylactic vaccine (Tron et al. 1989).

LHBs

The LHBs has a further N-terminal extension to the MHBs protein of either 108 or 119 amino acids (depending on the subtype/genotype). This extension is known as the pre-S1 domain, meaning that LHBs contains all three domains—pre-S1, pre-S2 and S. It exists solely in glycosylated form and is more prevalent than MHBs in virions and filaments but less prevalent in subviral spheres. In the mature virion and in HBs particles, the pre-S1 domain is exposed on the surface, where it covers both the S domain and parts of the pre-S2 domain (Kann and Gerlich 2005). In contrast to MHBs, the LHBs are essential for infection and viral morphogenesis, as the pre-S1 region encodes the entry receptor-binding domain (see Sect. 3.1 below) (Ni et al. 2014; Urban et al. 2014). The N-terminal end of the pre-S1 domain is myristoylated at glycine 2, a process that is essential to enable the virus particle to interact with the cellular plasma membrane (Urban et al. 2014). Amino acids 2–48 of the pre-S1 sequence specifically interact with the high-affinity entry receptor and sodium taurocholate co-transporting polypeptide (NTCP) and has a highly conserved motif (9-NPLGF(F/L)P-15) that is crucial for binding. Residues 49–75 are also required for infection, but the precise function of this region is not known (Urban et al. 2014).

The LHBs has important antigenic sites for both B and T cells that appear to play critical roles in recovery from viral infection or protection from infection (Milich et al. 1986). The major immunogenic epitopes within the pre-S1 coding region are at amino acids 27–35, 72–78 and 95–107. LHBs is also highly immunogenic for T cells in humans, at residues 21–48 and 81–108.

2.2.4 X ORF

The X ORF encodes a 154 amino acid polypeptide of 17 kDa known as the HBx protein. This is the second accessory protein of HBV and is conserved across all orthohepadnaviridae. The expression of full-length HBx protein is a critical component of the infectivity process in vivo (Lucifora et al. 2011) but can be dispensed of in in vitro culture. HBx acts as a transcriptional transactivator of a number of viral and cellular gene promoters through direct interaction with transcription factors such as the RPB5 subunit of RNA polymerase II, TATA-binding protein and ATB and is also involved in the activation of signal transduction pathways, such as the Ras/Raf/MAP kinase cascade (Kann and Gerlich 2005). It is also involved in epigenetic modification of the cccDNA template, resulting in upregulation of transcription (Levrero et al. 2009). In brief, epigenetic modification controls how tightly wound chromatin is to structural histone proteins. More tightly wound chromatin is not translated, whereas the converse is true; histone acetylation loosens chromatin, but methylation tightens it. This provides a mechanism to regulate gene transcription without acting directly on the coding sequences or their promoters. HBx recruits histone acetyltransferase and deacetylase enzymes in a fashion designed to prevent hypoacetylation (Belloni et al. 2009). Additionally, HBx in vitro inhibits protein arginine methyltransferase 1 (PRMT1), which increases histone methylation (Benhenda et al. 2013).

Another major target of HBx is the ubiquitin-proteasome system (UPS), which is a conserved host cellular pathway responsible for protein ubiquitination and proteolysis (Minor and Slagle 2014). Ubiquitin tagging marks cytosolic proteins for degradation via the proteasome. Many enzymes and proteins contribute to the function of this pathway, but of interest in the context of HBx is the damaged DNA-binding protein-1 (DDB1) and the Cullin4 (CUL4) proteins. HBx has been shown to bind DDB1, integrating itself into the DDB1-CUL4 complex, thus allowing regulation of its function (Guo et al. 2014). The importance of this interaction with regards to HBV replication has been demonstrated in vitro and in WHV models (Hodgson et al. 2012; Tang et al. 2008; Leupin et al. 2005). A particular target of HBx utilizing the DDB1-CUL4 machinery is the structural maintenance of chromosomes (Smc) 5/6 complex (Decorsiere et al. 2016). Smc5/6 acts as a restriction factor that selectively blocks extra-chromosomal DNA transcription. By destroying this complex, HBx relieves this inhibition, thereby allowing productive HBV gene transcription from the minichromosome.

The HBx protein is also a cofactor in HBV-mediated hepatocellular carcinoma (HCC) (Koike et al. 2002). The exact mechanism(s) of the contribution to hepatic carcinogenesis is unknown, although HBx-associated transactivation activity leads

to alterations in cellular gene expression that contributes to transformation (Rossner 1992). Findings in support of this hypothesis include:

- HBx suppression of p53-mediated upregulation of microRNA pathways that enhance the growth and metastatic potential of tumours in a mouse HCC model (Esteller 2011).
- Studies of epigenetic changes show that HBx induces hypermethylation of genes with tumour-suppressing activity (Tian et al. 2013).
- Disruption of signalling pathways critical to maintenance of cellular homeostasis (Zhang et al. 2014).
- Abrogation of p53-dependent apoptosis and cell cycle checkpoint deregulation (Wang et al. 1995).
- HBx targeting of the 26S proteasome complex has been implicated in hepatic carcinogenesis (Minor and Slagle 2014; Huang et al. 1996; Hu et al. 1999), suppressing viral antigen processing, and consequently presentation, thereby assisting evasion of immune detection (Wieland and Chisari 2005).
- Truncated HBx can be produced from integrated HBV DNA sequences (Martin-Vilchez et al. 2011).

3 Viral Life Cycle

There are multiple key events in the complete viral life cycle, which are described below. Detail of the molecular biology of HBV replication has been thoroughly reviewed previously and will not be repeated here (Beck and Nassal 2007).

3.1 Attachment and Penetration

The first stage of infection involves binding of the virion to its receptor on the hepatocyte surface membrane, with subsequent penetration of HBV into the cell cytoplasm. The AGL of HBsAg attaches to hepatocyte-associated heparan sulphate proteoglycans (HSPG) as the initial step in attachment, leading to cellular entry (Fig. 1.1) (Schulze et al. 2007). This is a co-receptor step, facilitating approximation of the pre-S1 receptor-binding domains to the sodium taurocholate co-transporting polypeptide (NTCP), which is the entry receptor for HBV (Ni et al. 2014; Yan et al. 2014). The NTCP normally functions to maintain bile acid homeostasis via the enterohepatic circulation. NTCP is encoded by the human *SLC10A1* gene, which is found on the long arm of chromosome 14. The pre-S1 domain of the HBV envelope protein contains two regions within 75 amino acids at the N-terminal end, which are responsible for binding to NTCP (Ni et al. 2014). These sequences must be myristoylated to permit virion infectivity (Meier et al. 2013; Gripon et al. 1995). After binding, receptor-mediated endocytosis is responsible for the delivery of viral nucleocapsids into the cytoplasm.

3.2 Conversion of Genomic RC DNA into cccDNA and Transcriptional Activation of the Viral Minichromosome

The nucleocapsids are transported to the nuclear membrane, where they uncoat and deliver their genetic load into the nucleus (Rabe et al. 2003). The RC DNA is released and converted into cccDNA using host cell enzymes, resulting in the formation of the viral minichromosome. This is the template of HBV that is used for the transcription of all viral mRNA and is also responsible for viral persistence (Bock et al. 1994; Newbold et al. 1995)(Fig. 1.1). The first step in this conversion is

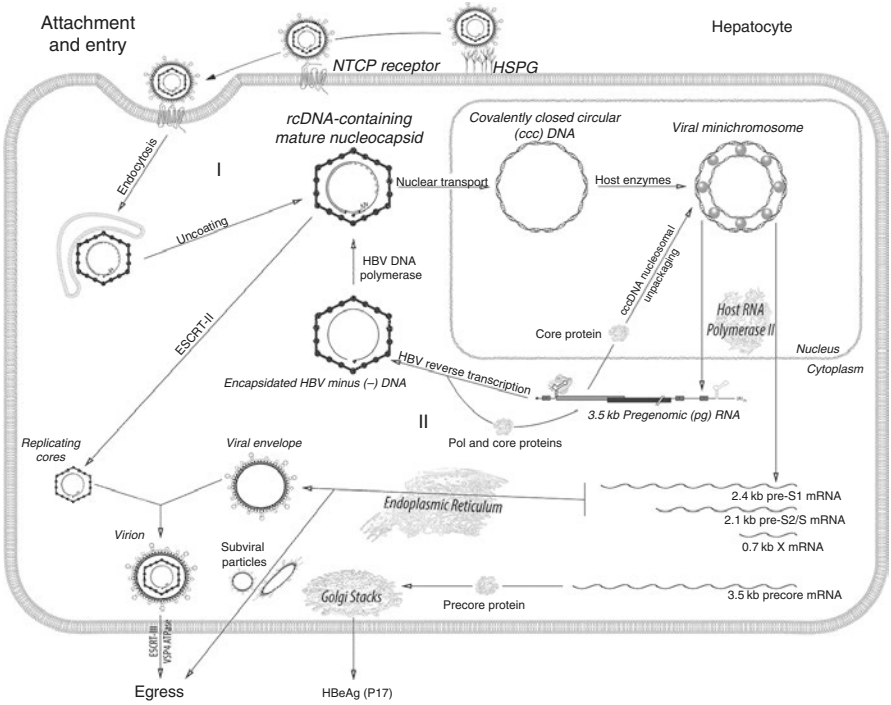

Fig. 1.1 Life cycle of HBV. Following attachment, penetration, and uncoating, the viral nucleocapsid is released into the cytosol and transported to the nuclear pore. The relaxed circular DNA is delivered into the nucleus where it is converted into cccDNA, and the viral minichromosome is generated. Transcription of the viral minichromosome produces the genomic and subgenomic HBV mRNA transcripts. Translation of the pregenomic RNA in the cytosol produces the Core and Pol proteins, and in association with Hsp-60, all are selectively packaged into a replication complex. Within the nucleocapsid, reverse transcription begins. The envelope proteins Pre-S1, Pre-S2 and S are translated at the rough endoplasmic reticulum (ER) and then bud into the lumen of the intermediate compartment. Approximately 50% of the Pre-S1-enriched ER-membrane areas envelope core particles. The HBV virions and subviral particles are then secreted into the extracellular space by usurping the cellular ESCRT pathway. The nucleocapsids can also be transported to the nucleus via an intracellular conversion pathway thereby increasing the number of nuclear cccDNA molecules

the release of the viral polymerase protein from the 5′ end of the minus strand and is achieved by utilizing the host DNA repair enzyme tyrosyl-DNA-phosphodiesterase 2 (TDP2) (Koniger et al. 2014). The subsequent steps in cccDNA generation are yet to be elucidated but probably involve topoisomerase I and II and other host domain modification enzymes.

Genomic length and subgenomic length mRNA transcripts are synthesized from the HBV minichromosome utilizing host RNA polymerase II (Ganem and Schneider 2001). All transcripts are heterogenous, are of positive orientation and are capped at the 5′ end and polyadenylated at the 3′ end. The subgenomic transcripts function exclusively as mRNAs for translation of the envelope protein subunits and X protein. The two genomic transcripts are longer than genomic length and encode the precore, core and polymerase proteins. Generation of the pre-C/C mRNA is an early transcription/translation event and functions in translation of the precore protein, which is processed and then secreted as HBeAg (Ganem and Schneider 2001). It is not involved in reverse transcription. In contrast, the pregenomic RNA (pgRNA) is multifunctional, serving as the template for reverse transcription into the minus DNA strand and for translation of both HBcAg and HBV Pol (Fig. 1.1).

HBx production is needed to maintain the transcriptional activity of the HBV minichromosome, mainly by blocking its hyperchromatinization by Smc5/6 (see above).

3.3 Genomic Replication via Reverse Transcription

HBV genomic replication is initiated following packaging of the pgRNA together with newly translated viral polymerase into subviral core particles, forming replication complexes in the cytosol. Reverse transcription occurs within the HBV nucleocapsid. As the polymerase is being translated off the same pgRNA molecule that it will eventually be packaged with, its terminal protein domain specifically binds to the ‘bulge’ region of a unique RNA stem loop structure, known as the epsilon (ε) loop, at the 5′ end of the pgRNA to prime reverse transcription (Bartenschlager and Schaller 1992) (Fig. 1.2). The epsilon loop is also the encapsidation signal around which cytoplasmic core protein dimers assemble into nucleocapsids. Following completion of translation, the polymerase undergoes a conformational change, which results in enzymatic activation, with the terminal protein domain now actively priming DNA synthesis (Zoulim and Seeger 1994). This Pol-oligonucleotide (Pol-G-A-A) complex then translocates to the complementary sequence of a direct repeat (DR-1) region located at the 3′ end of the pgRNA. Minus DNA strand synthesis then continues until it reaches the 5′ end of the pgRNA molecule (Will et al. 1987), generating a short terminal redundancy of approximately 8–9 nucleotides. Whilst reverse transcription is proceeding, the RNaseH activity of the polymerase degrades the pgRNA but leaves the 5′-capped terminal 18 nucleotides that contains the DR-1 sequence (Ganem and Schneider 2001). This fragment includes a six nucleotide homology to the direct repeat sequence, which allows circularization of the minus strand. The 18 nucleotide-capped RNA structure is then translocated to a second DR

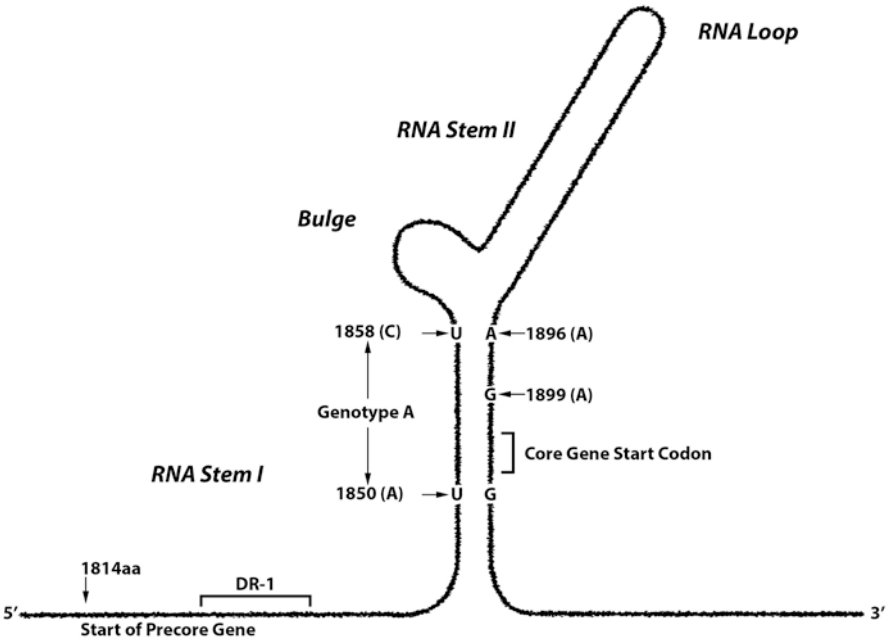

Fig. 1.2 Diagrammatic representation of the epsilon (ε) stem-loop structure of HBV. This is a highly conserved structure within the 10 genotypes of HBV, and the positions of base changes for genotype A2 are shown, as are the common translational precore mutations at G1896A (precore stop codon: UAG) and G1899A

sequence (DR-2) on the 5′-end of the newly made minus strand where it acts as primer for plus strand synthesis, using the minus strand as a template (Will et al. 1987). Synthesis of the plus strand continues until it reaches about 50–70% of the length of the minus strand.

3.4 Virion Assembly and Release

The viral envelope and HBsAg subviral particles are synthesized and assembled at the ER and then bud into its lumen. The HBc protein is synthesized in the cytosol and assembled independently of the envelope protein (Ganem and Schneider 2001). Immature nucleocapsids containing pgRNA-Pol can only commence envelopment once the nucleocapsid is phosphorylated, a process that is coupled to the initiation of minus strand synthesis (Ganem and Schneider 2001). The assembled nucleocapsids containing genomic RC DNA are then selectively enveloped before exiting the cell (Ganem and Schneider 2001). An export bias exists towards genomes that have completed minus strand synthesis and have started plus strand synthesis (Ganem and Schneider 2001). Envelopment of replicating cores requires a critical relative molar ratio of pre-S1 to S, without which abnormal virions result, hampering release (Ganem and Schneider 2001). As with many other enveloped viruses, HBV usurps

the endosomal sorting complex required for transport (ESCRT) network of proteins, which is a cellular network that functions to form multivesicular bodies and is involved in daughter cell cytokinesis (Stieler and Prange 2014; Bruss 2007; Votteler and Sundquist 2013; Lambert et al. 2007). Various components of this system have important roles in the HBV replication cycle. In particular, the formation of mature nucleocapsids requires the ESCRT-II protein (Stieler and Prange 2014), and virion budding and egress require ESCRT-III and the VPS4 ATPase (Lambert et al. 2007).

4 Variant Viruses

The replication strategy of HBV is error-prone due to a lack of proofreading capability. Additionally, all reverse transcription processes have an intrinsic G-to-A hypermutation rate largely attributable to host APOBEC enzymes (Noguchi et al. 2005). Accounting for approximations of daily base-pairing errors and the rate of virion production, it has been estimated that every 24 h, 10^{14} nucleotides are replicated with potentially 10^{7} base-pairing errors (Sheldon et al. 2006). This rate equates to the potential for every nucleotide in the whole viral genome to be substituted daily. However, although mutations can occur randomly along the HBV genome, the overlapping open reading frames constrain the evolution rate, limiting the number and location of mutations that can result in viable virions. Because chronic HBV infection frequently persists for decades, many variants exist within the one host at any given time; the virus therefore exists as a quasispecies, with one variant typically dominating. There are three forms of variant HBV that are encountered commonly in clinical practice:

- HBeAg-negative mutants, which typically emerge after HBeAg seroconversion, are due to the development of mutations in the precore and basal core promoter regions.
- Polymerase mutants that emerge in the setting of nucleos(t)ide analogue (NA) therapy and are responsible for virological breakthrough and treatment failure.
- Envelope mutants, selected by hepatitis B immunoglobulin therapy in the post-transplantation setting, or by vaccines following prophylactic immunization.

4.1 Precore Mutants

The precore mutant results from a mutation at nucleotide position 1896 (codon 28: TGG; tryptophan) of the precore gene. A single-base substitution of G-to-A here gives rise to a translational stop codon (TGG to TAG; TAG = stop codon) in the second last codon (codon 28) of the precore gene located within the epsilon loop structure of the pgRNA. Ordinarily, the G1896 forms a base pair with nucleotide 1858 at the base of the loop. In HBV genotypes B, D, E, G and some strains of genotype C, the 1858 is a thymidine (T). Thus, the stop codon mutation created by G1896A (i.e., from T to A) stabilizes the structure based on conventional Watson-Crick pairing (Fig. 1.2). In contrast, HBV genotypes A, F and some strains of

genotype C have a cytidine (C) at position 1858, making the precore stop codon mutation rare in these genotypes. Other mutations have been found within the precore transcript that block HBeAg production, including abolition of the methionine initiation codon (Bartholomeusz and Schaefer 2004), and the development of stop codons at other positions within the precore/core region of genotype G, but these are far less common.

4.2 Basal Core Promoter Mutants

The basal core promoter (BCP) is the promoter for pre-C/C mRNA. Mutations within the BCP typically occur as a pair at nucleotides 1762 and 1764, resulting in a reduction in transcription rather than complete abolition (Hunt et al. 2000). These mutations, such as A1762T/G1764A, occur in isolation as well as in conjunction with precore mutations. The double mutation of A1762T plus G1764A results in a significant decrease in HBeAg levels and has been associated with an increase in viral load. This pattern of mutation is found in genotype A-infected individuals as the major cause of HBeAg loss (Hunt et al. 2000). A double mutant of BCP and precore is more common in the other, non-genotype A strains (Bartholomeusz et al. 2004). Importantly, these BCP mutations do not affect the transcription of HBV pgRNA or the translation of the core or polymerase protein. Thus, by removing the inhibitory effect of the precore protein on HBV replication, the BCP mutations appear to enhance viral replication by suppressing pre-C/C mRNA relative to pregenomic RNA and may account for the increased pathogenicity of these mutant strains (Hunt et al. 2000). Longitudinal follow-up of a Taiwanese patient cohort infected with genotype B or C HBV confirmed this pathogenicity (Tseng et al. 2015). Patients infected with a BCP mutant virus were shown to have an independent risk factor for the development of cirrhosis. There also appeared to be a dose-response effect, with a significantly higher cumulative incidence if the BCP mutant population exceeded 45% of the quasispecies pool.

4.3 Polymerase Mutants

Drug resistance is associated with the loss of virological, biochemical and eventually histological therapeutic gain. In the setting of advanced liver disease, resistance may lead to hepatitis flares, hepatic decompensation and death (Liaw et al. 2004). Drug-resistance mutations also appear to be archived in the nuclear cccDNA template, highlighting the importance of prior antiviral therapy exposure when considering treatment, and first-line utilization of high-genetic barrier drugs (entecavir or tenofovir) (Zhou et al. 1999; Zoulim and Locarnini 2009).

Primary resistance mutations are those that directly alter drug binding and thus confer drug resistance. These mutations commonly result in reduced viral replicative ability when compared to wild-type virus (Zoulim and Locarnini 2009). Secondary resistance mutations arise in selected variants that have acquired primary resistance mutations and have the effect of causing a compensatory increase in viral replication levels.

4.3.1 L-Nucleoside Analogue Resistance

Primary resistance to lamivudine (LMV) and telbivudine (LdT) has been mapped to mutations in the YMDD locus in the catalytic domain of the HBV polymerase (Stuyver et al. 2001). These occur primarily at rtM204I/V/S ± rtL180M (Stuyver et al. 2001) and rtA181T/V (Yeh et al. 2000). The latter mutation confers cross-resistance to adefovir (ADV) and tenofovir (TDF) (Villet et al. 2008). Subsequent to these primary resistance mutations, multiple secondary resistance mutations can be selected for, which in combination may result in entecavir (ETV) resistance. When they occur individually, these secondary mutations do not significantly impact upon the clinical efficacy of ETV (Villet et al. 2007). The rtM204I substitution has been detected in isolation, but rtM204V and rtM204S are found only in association with other changes (Delaney et al. 2001). The major patterns of substitutions are (1) rtM204I, (2) rtL180M + rtM204V, (3) rtL180M + rtM204I, (4) rtV173L + rtL180M + rtM204V and (5) rtL80V/I ± rtL180M + rtM204I. Genotype influences the dominance of a particular mutation sequence (Zollner et al. 2004).

Resistance to LMV is a result of steric hindrance caused by the branched side group of valine or isoleucine amino acids colliding with the oxathiolane ring of L-nucleosides in the dNTP-binding site (Bartholomeusz et al. 2004). The in vitro sensitivity of such mutants to LMV decreases by between 100-fold and greater than 1000-fold. The rate of LMV resistance increases progressively with duration of therapy from between 14 and 32% per year (Lai et al. 2003). Whilst the rate of LdT resistance is lower than that seen with LMV, an exponential increase is still seen after the first year of therapy. Genotypic resistance was observed at 1 and 2 years of therapy in 4.4%/2.7% and 21.6%/8.6% in HBeAg-positive and -negative patients, respectively (Lok and McMahon 2009).

4.3.2 Acyclic Nucleoside Phosphonate Resistance

Resistance to ADV occurs less frequently than does resistance to L-nucleosides. The described mutations result in a three to eightfold increase in the IC_{50} and confer partial cross-resistance to TDF in in vitro studies. This cross-resistance results from a similar molecular mechanism of resistance, with indirect perturbation of the triphosphate-binding site (Bartholomeusz et al. 2004). Mutations at rtA181T/V/S and rtN236T have been described (Angus et al. 2003). The rtN236T does not significantly affect sensitivity to LMV, LdT or ETV but reduces the in vitro efficacy of TDF (Angus et al. 2003; Brunelle et al. 2005). The rtA181T/V mutation is partially cross-resistant to LMV and LdT (Villet et al. 2008) and reduces sensitivity to TDF by threefold (Qi et al. 2007). The rtA181S mutation appears specifically to affect ADV (Liu et al. 2015).

Resistance to TDF in patients with HIV-HBV co-infection is conferred by the rtA194T mutation in combination with rtL180M and rtM204V (Sheldon et al. 2005). In vitro the presence of rtA194T results in partial resistance to TDF, but no cross-resistance with LdT or ETV. Additionally, these mutants had reduced replication efficiency compared to wild type, but this could be reversed by the introduction of either precore or BCP mutations (Amini-Bavil-Olyaee et al. 2009). Clinical studies have not confirmed an in vivo effect of this mutation. Further in vitro studies have shown a tenfold reduction in TDF sensitivity when the rtN236T and rtA181T/V mutations are found together. It should be noted that

these in vitro results show that TDF resistance is theoretically possible; however, there have been no reported cases of resistance in clinical practice due to these substitutions.

4.3.3 D-Cyclopentane Resistance

Genotypic resistance to ETV is uncommon in NA treatment naïve patients, seen in 1.2% of patients after 5 years of therapy (Tenney et al. 2009). However, patients with LMV resistance at commencement of ETV monotherapy develop resistance rapidly. This is because ETV resistance develops in a stepwise fashion, which means virus with substitutions in the YMDD domain need to develop fewer additional mutations to become ETV resistant (Villet et al. 2007). Therefore, ETV should not be used as monotherapy to treat LMV-resistant patients.

Mutations in the viral polymerase associated with the emergence of ETV resistance have been mapped to multiple domains in the viral polymerase. Thus, at least three mutations are required for resistance to develop, two of which are the YMDD substitutions rtM204V/I and rtL180M (Tenney et al. 2004). In isolation, the rtM250V mutation causes a tenfold increase in IC_{50}, but no clinical resistance is seen (Levine et al. 2002). The molecular mechanism of resistance for the rtM250V change is exerted during RNA-directed DNA synthesis (Walsh et al. 2010). The mutation reduces ETV binding by shifting its target, the dNTP-binding site. The rtT184G + rtS202I ETV resistance-associated substitutions often occur together, but their in vitro effect is modest. The mechanism of resistance is an allosteric change with altered geometry of the nucleotide-binding pocket near the YMDD site (Walsh et al. 2010). A newly recognized mutation at rtA186T confers primary ETV resistance in combination with the LMV resistance substitutions (Hayashi et al. 2015). Importantly, ETV resistance-associated mutations are not cross-resistant with ADV or TDF.

4.3.4 Multidrug Resistance

Multidrug-resistant HBV occurs in patients who receive sequential NA monotherapy (Brunelle et al. 2005; Tenney et al. 2004; Fung et al. 2005; Mutimer et al. 2000; Yim et al. 2006). Such strains have been shown to develop if an 'add-on' therapeutic strategy to treat initial resistance to a single agent does not result in rapid and complete viral suppression (Zoulim and Locarnini 2009). Thus, incomplete viral suppression represents the most important risk factor for the development of antiviral resistance. The rtA181V/T mutation is a marker for multidrug resistance, as it is responsible for reduced susceptibility to both the L-nucleosides and the acyclic phosphonate nucleosides (Villet et al. 2008; Warner and Locarnini 2008).

4.4 Envelope Mutants

The current hepatitis B vaccine contains yeast-derived recombinant HBsAg. The subsequent anti-HBs response to the MHR of HBsAg between residues 137 and 148 induces protective immunity (Urban et al. 2014). Mutations within this