HBV load in treated and untreated individuals

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Chapter

1

General Introduction


**Viral Hepatitis**

Viral hepatitis is a general term describing liver infection caused by viruses actively replicating in the liver. Like hepatitis caused by other agents, such as alcohol and drug abuse or metabolic disorders, a typical feature of the disease is jaundice.

Acute hepatitis is a transient episode of inflammatory liver disease. During this phase of up to six months, the disease may be clinically inapparent, or accompanied by clinical features ranging from mild symptoms such as jaundice, nausea and malaise to severe symptoms such as liver failure or death.

Chronic hepatitis, in which markers of the disease can be found for a period longer than six months, has the same range of clinical outcomes seen in acute hepatitis. Furthermore, even if the process of prolonged liver inflammation is initially not accompanied by clinical apparent disease, chronic hepatitis may lead to liver fibrosis, cirrhosis and eventually liver cancer.

Although other viruses, such as cytomegalovirus, adenoviruses and Epstein-Barr virus may incidentally cause hepatitis as well, at least five truly hepatotropic viruses have been identified so far. They have been designated A-E, in order of their discovery. Another candidate hepatitis virus, hepatitis G virus, has been identified but was recently classified as a non-hepatotropic virus.

Although they all share their hepatotropism, the hepatitis viruses are completely unrelated to one another in structure and mode of replication despite the similarity in the acute syndrome produced by each virus:

- Hepatitis A virus is a single-stranded RNA virus that has a very stable capsid and whose proteins are derived from a single polyprotein
- Hepatitis B virus is a DNA virus that replicates through an RNA intermediate
- Hepatitis C virus is a labile single-stranded RNA virus whose proteins are derived from a polyprotein
- Hepatitis D virus is a defective RNA virus related to viroids that encodes a capsid antigen, delta antigen, and requires the envelope protein of HBV for its propagation
- Hepatitis E virus is a labile RNA virus that is unrelated to other known viruses.

The hepatitis A and E viruses, or HAV and HEV, are enterically transmitted viruses that produce acute disease only. The hepatitis B, C and D viruses, or HBV, HCV and HDV, are
most efficiently transmitted by infected blood, but also can be transmitted by exposure to other infectious bodily fluids. These three viruses can cause both acute and chronic hepatitis.

**History of Hepatitis B Virus**

MacCallum *et al.* first introduced the terms hepatitis A and hepatitis B in 1947 in order to categorize two differently transmitted forms of hepatitis. Type A hepatitis was considered to be predominantly transmitted via the fecal-oral route while type B hepatitis was believed to be primarily transmitted parenterally. These terms were eventually adopted by the World Health Organization Committee on Viral Hepatitis.

In 1968 it was first established by Prince, and Okochi and Murakami that the antigen first described as the “Australia (Au) antigen” by Blumberg *et al.* in 1963, could only be found in the serum of patients with type B hepatitis. Therefore, the Au antigen was renamed into hepatitis B surface antigen (HBsAg).

In 1970, Dane *et al.* described virus-like particles detected by electron microscopy in the serum of patients suffering from type B hepatitis. These so-called Dane particles were designated as the hepatitis B virus (HBV). The viral nature of these particles was confirmed by Kaplan *et al.*, who detected an endogenous DNA-dependent DNA polymerase in the core of the viral particle. Discovery of this polymerase allowed Robinson to detect and characterize the HBV genome. This event was followed shortly by the discovery of hepatitis core and early antigen (HBcAg and HBeAg, respectively).

When it became apparent that antibody to HBsAg (anti-HBs) was the neutralizing antibody for HBV, hepatitis B vaccines could be developed. Early vaccines utilized plasma-derived, highly purified HBsAg particles, while more recent vaccines use biotechnically engineered recombinant yeast-derived HBsAg particles. The safety and protective efficacy of these vaccines have been well established, and provide important means for controlling the spread of HBV infection. However, it is good to realize that vaccination programs aimed at risk groups are important for individual protection, but will not eliminate hepatitis B transmission in Europe. This is caused by the fact that 70% of acute hepatitis B cases in Europe and the United States are either acquired by
sexual activity or are of unknown origin\textsuperscript{1,29,30}.

Despite the development of a protective vaccine, hepatitis B therefore remains an important public health problem. In the United States and Europe, an incidence of about 6 chronic hepatitis B cases per 100000 population was reported in 1993\textsuperscript{31,32}. However, it is well known that many hepatitis cases are so mild that they are not recognized as hepatitis, or fail to be reported by physicians even when the disease is identified. Therefore, it has been estimated that the actual number of hepatitis B cases may be up to 10 times larger than reported\textsuperscript{1}. In many other countries, the prevalence is much higher (see figure 1) and HBV has been estimated by the World Health Organization (WHO) to have infected over two billion people worldwide. More than 350 million are chronic carriers\textsuperscript{1,33}. This makes hepatitis B a globally significant health threat in terms of affected individuals and public costs involved in treatment and prevention.

\textbf{Figure 1.} Global distribution of hepatitis B infection. Distribution is expressed in percentage of chronically HBV infected individuals of the total population in a given geographic area. Modified with permission from Robert Garces\textsuperscript{34}. 

- >8\% - High
- 2\% - 7\% - Intermediate
- <2\% - Low
**Structural and Functional Organization of HBV**

**Virus Structure**

The hepatitis B virus is a protein coated, partially double-stranded DNA virus with a diameter of 42 nm and is comprised of an outer surface coat and a 27 nm core\(^1,16,35\) (see figure 2).

![Figure 2. Electron microscopic picture of the infectious HBV Dane particle (magnification 1:300000). The majority of particles are empty 22 nm spheres. Slightly below the center of the picture, two Dane particles can be seen.](image)

The coat of HBV contains HBsAg, while the core or nucleocapsid contains HBcAg. A unique DNA-dependent DNA polymerase is associated with the core of the virus. The core also contains a circular DNA, the HBV genome that contains both double-stranded and single-stranded regions.

In addition to the infectious particle, also known as the Dane particle, two other types of particles can be detected in the blood of most infected individuals\(^{16,36,37}\) (see figure 2 and 3). These are denoted hepatitis B filament and hepatitis B sphere, respectively. They both have a diameter of 22 nm and contain hepatitis B surface proteins. They do not contain a nucleocapsid, polymerase or DNA genome and are therefore considered to be non-infectious. However, it is uncertain if all defective particles lack DNA. Furthermore, in HBV-infected liver cells, and in both culture medium and cells of HBV-transfected cell lines, 27 nm electron dense nude core particles can be found. These particles do contain
DNA.
The sphere is composed of the small and middle hepatitis B surface proteins and always has approximately the same size, whereas the filament also includes the large hepatitis B surface protein and can be of various lengths\textsuperscript{38}.

![Figure 3](image)

\textit{Figure 3.} Schematic view of HBV particle types present in patient sera. Picture taken with permission from Robert Garces\textsuperscript{34}.

The hepatitis B filaments and spheres can be found in HBV infected patients in an up to 10000-fold higher titer than the infectious Dane particles. It is believed that the presence of these high titers of non-infectious particles may distract the host’s immune system, allowing the infectious viral particles to circulate in the blood stream undetected by neutralizing antibodies\textsuperscript{39}. In addition, it has been shown that non-infectious particles enhance HBV infection in the duck hepatitis B system\textsuperscript{40}. Binding of non-infectious particles to potential host cells appears to activate the cells, possibly by priming the cells for infection. Whether this could also be true for the human hepatitis B virus remains to be proven.

\textit{The Hepatitis B Virus Genome}
The HBV genome is unique in the world of viruses due to its compact nature, use of overlapping reading frames, and dependence on a reverse-transcriptional step, though the virion contains primarily DNA with protein covalently linked to the 5’ end of the (-) DNA strand\textsuperscript{41,42}. In the recent past, many related viruses have been found in a wide range of animal species, each particular virus being species specific. With human HBV as the archetype, the members of the so-called \textit{hepadnaviridae} family include duck hepatitis B virus (DHBV)\textsuperscript{43}, ground squirrel hepatitis virus (GSHV)\textsuperscript{44}, woodchuck hepatitis virus
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(WHV)\textsuperscript{45}, chimpanzee hepatitis B virus (chHBV)\textsuperscript{46,47}, gibbon hepatitis B virus and orangutan hepatitis virus\textsuperscript{48}, along with others\textsuperscript{49}.

The hepatitis B genome is approximately 3200 nucleotides long. Numbering of basepairs on the HBV genome is most frequently based on the cleavage site for the restriction enzyme EcoRI or at homologous sites if the EcoRI site is absent. Other methods of numbering based on the start codon of the core protein or at the first base of the RNA pregenome are also used.

Four serotypes of HBV have been identified, based on peptide differences in the hepatitis B surface antigen (HBsAg). These subtypes are adw, ayw, adr, and ayr. They are defined by two mutually exclusive antigenic determinant pairs d/y and w/r with a common determinant 'a'\textsuperscript{50-52}.

Six genomic groups of HBV were later referred upon as genotypes designated with A-F, based on the nucleotide variability of the S-gene. This classification has been confirmed with full genome sequencing. Contrary to serotyping, genotyping provides information about geographic origin of a virus strain because different HBV genotypes are found in different geographic regions\textsuperscript{52-56} (see table 1).

<table>
<thead>
<tr>
<th>HBV genotype</th>
<th>Geographic origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Northern Europe, Sub-Saharan Africa</td>
</tr>
<tr>
<td>B</td>
<td>Eastern Asia, mainly China</td>
</tr>
<tr>
<td>C</td>
<td>Far East, mainly Japan</td>
</tr>
<tr>
<td>D</td>
<td>Mediterranean countries, Near and Mid East, South Asia</td>
</tr>
<tr>
<td>E</td>
<td>Western Sub Saharan Africa</td>
</tr>
<tr>
<td>F</td>
<td>North and South America</td>
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</tbody>
</table>

Table 1. Overview of the different HBV genotypes and their geographic origin.
Structural and Functional Organization of the HBV Genome

Regulatory elements

The HBV genome contains many promoter and signal regions necessary for viral replication to occur, the regulation of levels of transcription, and determination of the site of polyadenylation. There are also signal regions involved in marking a specific transcript for encapsidation into the nucleocapsid (see figure 4).

![Figure 4. Schematic overview of the organization of the HBV genome. Waved lines indicate the various mRNA transcripts, boxes denote the various open reading frames, and the lines mark the HBV DNA strands. Note the dotted line representing the partial (+) strand of the genome, which is of various length among individual hepatitis B viruses.]

All HBV transcripts share a common adenylilation signal located in the region spanning 1916-1921 in the genome. The resulting transcripts range from approximately 3.5 to 0.7 kb in length. Due to the location of the core/pregenomic promoter, the polyadenylation site is differentially utilized. The polyadenylation site is a hexanucleotide sequence (TATAAA) as opposed to the polyadenylation signal sequence commonly seen in eukaryotic organisms (AATAAA). The TATAAA is known to work less efficiently, suitable for its differential use by HBV.

The enhancer I element spans a region from nucleotides 970-1240. Many transcription
factors are believed to interact with this region of the HBV genome. These molecules include HNF-3 HNF-4, EF-C and NF-1. Both enhancer I and enhancer II can activate heterologous promoters despite their position or orientation. Deletion of either enhancer region results in a strong reduction of viral transcripts.

The epsilon-stem loop region (ε) spans nucleotides 1847-1907 in the HBV genome and plays a key role in HBV DNA encapsidation. The location of the ε sequence was determined by fusing heterologous genes to various regions of the HBV genome and subsequently looking for encapsidation of these foreign genes. Despite the terminal redundancy of the pregenomic RNA, only the 5' ε sequence was shown to retain functionality. Because of this, only pregenomic RNAs are encapsidated despite the fact that all HBV transcripts have the ε stem loop coding region at their 3' ends. Sequence analysis of ε shows a series of inverted repeats that are predicted to fold into a three-dimensional stem-loop structure. This stem-loop structure is conserved among all hepadnaviruses despite differences in the primary sequence. It is believed that the polymerase protein recognizes and interacts directly with the ε-stem-loop structure. Interaction of the hepatitis B polymerase and ε initiates both encapsidation as well as reverse transcription of the HBV pregenomic RNA.

There are at least four defined overlapping open reading frames (ORFs) in the HBV genome which result in the transcription and expression of the seven different hepatitis B proteins by use of varying in-frame start codons. Every base pair in the HBV genome is involved in encoding for at least one of the HBV proteins, making its coding organization extremely compact (see figure 4). The four open reading frames are controlled by four promoter elements (preS1, preS2, core and X) and two enhancer elements (Enh I and Enh II).

**HBV Core antigen**

ORF C codes for the HBe protein, which is the main constituent of the HBV core particle, and the HBe protein. HBeAg is generated by proteolytic cleavage from HBCAg, resulting in soluble HBe protein which can be found circulating in the serum of infected individuals. Its function remains unknown, but it is used as a marker for replication.

The core/pregenome promoter controls the transcription of multiple mRNAs which have 5' heterogeneity (see figure 5). These include the core antigen, e antigen, polymerase and the pregenomic RNA.

These transcripts are also regulated by both viral enhancer elements and a negative regulatory element (NRE). The core/pregenome promoter has yet to be clearly defined, but
has been mapped loosely to a region spanning nucleotides 1591 to 1851\textsuperscript{35}. Positive regulation of this region involves many transacting cellular factors. CCAAT/enhancer binding protein (C/EBP), HNF-3, HNF-4 and an ubiquitous cellular factor, Sp1, all appear to assist in activation\textsuperscript{70-72}. Studies have also indicated that members of the nuclear receptor family may be involved in downregulation of the core/pregenome promoter\textsuperscript{73}.

**Figure 5.** Features of the HBc protein and gene. Picture taken with permission from Robert Garces\textsuperscript{34}. See text for description.

**HBV surface antigen**

ORF S contains three in-frame start sites which direct synthesis of the three distinct hepatitis B surface proteins (see figure 6). There are two promoter regions which control the expression of these proteins, namely the preS1 promoter and preS2 promoter. The preS1 promoter controls transcription of a single 2.4kb RNA molecule which includes the entire ORF S region\textsuperscript{35}.

However, the preS2 promoter controls transcription of a family of transcripts of 2.1kb in length. The preS2 promoter has been shown to be stronger than the preS1 promoter. This results in more middle and small surface proteins being expressed as compared to the large surface protein\textsuperscript{74}. Cellular transcription factors such as Oct-1, HNF-1 and HNF-3, as well as HBV enhancer elements have been shown to be involved in preS1 promoter activity\textsuperscript{75}.

Downregulation of the preS1 promoter appears to be dependent on a region within the preS2 promoter (nucleotides 3160 to 3221). The mechanism remains poorly understood.

The preS2 promoter appears to be upregulated by a CCAAT element, which is also involved in the downregulation of the preS1 promoter. NF-Y, the CCAAT-binding factor, appears to be involved in the preS2 activation as well as both HBV enhancers\textsuperscript{76}. Also, stress on the endoplasmic reticulum due to the presence of hepatitis B large surface
protein appears to result in increased activation\textsuperscript{77}.

ORF S encodes the three surface proteins, which form the antigenic determinants found on the surface of the infectious Dane particle and the empty 22 nm spheres and filaments\textsuperscript{35,78}. Furthermore, it has transactivating activity\textsuperscript{79,80}.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure6.png}
\caption{Features of the HBs protein and gene. Picture taken with permission from Robert Garces\textsuperscript{34}. See text for description.}
\end{figure}

**HBV pol**

The largest ORF in the HBV genome encodes for the hepatitis B polymerase protein (HBp)\textsuperscript{81}. The polymerase coding region does not appear to have a promoter element directly upstream. Its expression is believed to be the result of ribosome scanning of the pregenomic RNA transcript\textsuperscript{35,82}.

HBp has been divided into four characterized domains (see figure 7).

ORF P occupies the majority of the genome and encodes for the hepatitis B polymerase protein. The protein is 90Kd in size and has RNA and DNA dependent polymerase activity. HBp plays a key role in HBV genome replication as well as pregenomic RNA encapsidation. HBp is packaged together with pregenomic RNA within HBV nucleocapsids. Based on sequence homologies and studies on the mechanism of viral genome replication, most parts of HBp are indispensable. The N-terminus portion of the protein acts in priming (-) DNA strand synthesis and ends up covalently linked to the 5' end of the (-) DNA strand. This domain is termed primase.
The next domain does not appear to have any enzymatic function but acts as a spacer between the first and third domain.

The third domain gives HBp its name. It occupies approximately 40% of the protein and encodes for the RNA and DNA dependent polymerase activity. However, HBp also requires the presence of metal ions and the presence of the ε-stem-loop structure for polymerase/reverse transcriptase activity to occur.

The fourth domain of HBp possesses its RNase H activity. This domain also plays a key role in HBV genome replication.

Also, HBp expression has been found to be important for packaging of the pregenomic mRNA into core particles.

**HBV X antigen**

ORF X, encoding a 17 kd protein known as the hepatitis B X Protein (HBx), has its own promoter controlling the transcription of a 0.8kb RNA (see figure 8). However, due to the proximity of the X promoter to the Enhancer I region, the precise borders remain controversial. The promoter is believed to lie within the region spanning nucleotides 1230 to 1376. ORF X encodes the hepatitis B X protein.

The function of the HBx protein remains unclear. However, it has been shown that it possesses trans-activating capacity, downregulates cellular DNA repair, and is thought to be involved in HBV-related liver carcinogenesis. Despite the fact that ORF
X is lacking in the DHBV genome, it is present in all other hepadnaviridae, and has been shown to be important for efficient HBV replication\textsuperscript{35}.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure8.png}
\caption{Features of the HBx protein and gene. Picture taken with permission from Robert Garces\textsuperscript{34}. See text for description.}
\end{figure}

**Replication of HBV**

Although the liver cell is the most effective cell type for replicating HBV, other extrahepatic sites have been found to be able to support replication to a lesser degree. HBV Dane particles and/or viral transcripts have been found in mononuclear, bile duct epithelial, endothelial, and pancreatic cells as well as in tissue smooth muscle of adrenal glands, gonads, cultured bone marrow, kidneys, lymph nodes, spleen and thyroid glands of hepatitis B infected patients. However, the finding of HBV replicative intermediates does not necessarily mean that HBV really replicates in these sites. Therefore, the importance of these findings is still disputed\textsuperscript{1,91-93}.

Many cellular proteins have been found associated with the various hepatitis B surface proteins. Attempts to define the receptor for HBV have yielded a plethora of candidates such as apolipoprotein H, human serum albumin, fibronectin and interleukin-6 (IL-6). More recently, a protein (gp180) has been identified to interact with the preS1 domain in DHBV. Also, an 80kd protein has been found to bind onto human HBV which has yet to be identified\textsuperscript{94}. Despite these findings, the mechanism of and cellular receptor for HBV entry into cells has yet to be identified and characterized\textsuperscript{94-103}. A schematic overview of our current understanding of the HBV lifecycle is given in figure 9.

The immediate steps following HBV entry are also yet poorly characterized. The
nucleocapsid is released from the envelope proteins upon entry. Some studies suggest that a proteolytic event must occur on the large surface protein to expose a membrane fusion domain\textsuperscript{104}. However, it is believed that this occurs at the plasma membrane and not within an acidic vesicle\textsuperscript{105}.

After removal of the envelope, the nucleocapsid is believed to be transported to the nuclear membrane\textsuperscript{106}. The DHBV system has suggested that HBV genome uncoating occurs at the nuclear membrane.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure9.png}
\caption{Schematic overview of the HBV life cycle. Picture taken with permission from Robert Garces\textsuperscript{34}. See text for description.}
\end{figure}

The HBV DNA is transported into the nucleus where it is repaired to form a covalently closed-circle (cccDNA). Repair requires completion of the dsDNA form, removal of the 5' terminal RNA primer and the polymerase protein, as well as covalent ligation of the strands. The hepatitis B polymerase protein is not required for these steps to occur. Integration of HBV DNA into the host cell genome is known to occur regularly. However, it is not required for replication, and may result in disruption of one or more HBV ORFs and prevent transcription of functional pregenomic RNA\textsuperscript{35}. On the other hand, integration of HBV DNA into the host genome may lead to disturbance of the normal function of some host genes, for instance oncogenes. This may therefore eventually be one of the processes which lead to the development of cancer.
Once recircularized, enhancer and promoter activities start producing the various HBV transcripts required for HBV protein synthesis and pregenomic RNA generation. The pregenomic RNA lacks the ATG start codon for the E protein, and is selected specifically by the HBV polymerase protein for packaging into core particles. It is hypothesized that ribosome-mediated suppression may account for the inability of HBV to package genomes with the additional e start codon, because mutations which disable this ATG allow the long messages to be encapsidated\textsuperscript{107,108}.

Binding of the polymerase protein to the ε-stem-loop results in pregenomic RNA encapsidation\textsuperscript{109,110}. Deletion of the C-terminus of the polymerase protein still allows for binding of the polymerase onto the ε-stem-loop but prevents encapsidation. In hepadnaviruses, only polymerase proteins bound into ε-stem-loops are believed to be packaged by core proteins. Expression of core and polymerase proteins alone does not result in the encapsidation of polymerase proteins\textsuperscript{61,65,108,111}.

Reverse-transcription of the pregenomic RNA only occurs after core proteins bind to the polymerase, when pregenomic RNA encapsidation is started\textsuperscript{64}. Then, the pregenomic RNA is converted to a partially double-stranded DNA by a complex pathway, while the RNA template is degraded by the HBV polymerase RNAse H activity (see figure 10).

The completed nucleocapsid is thought to associate with areas of the Golgi high in HBV surface protein content. LHBs (see figure 6) is believed to interact with the HBe protein at the cytoplasmic face of the Golgi, pulling the nucleocapsid into the forming vesicle, resulting in the 42 nm enveloped particle which is secreted by the cell via exocytosis, ready to infect other liver cells\textsuperscript{35}. 
Figure 10. Conversion of pregenomic RNA into double stranded DNA.

Binding of the HBV polymerase to the 5' epsilon stem-loop triggers packaging of pregenomic RNA into core particles, and RNA encapsidation is believed to occur immediately thereafter. The polymerase serves as a primer when initiating reverse transcription, and upon binding of the polymerase to ϵ, the polymerase begins to reverse transcribe the pregenomic RNA template for three to four bases. The polymerase and covalently attached nascent (-) DNA are then transferred to the 3' copy of the DR1 region. Once there, the (-) DNA is extended by the HBV polymerase while the RNA template is degraded by the HBV polymerase RNase H activity. The 15 to 18 capped oligoribonucleotides at the 5' end of the pregenomic RNA remain intact once the (-) DNA strand is completed. This RNA oligomer is then translocated and annealed to the DR2 region at the 5' end of the (+) DNA, and serves as the primer for (+) DNA strand synthesis. It appears that the reverse transcription as well as plus strand synthesis may occur in the completed core particle. Typically, the (+) DNA strand is not completed until re-entry of the virion into a host cell. This yields the characteristic single-stranded gap seen in packaged hepadnaviral DNA. One proposed theory is that steric hindrance within the nucleocapsid prevents further reverse-transcription/replication events. Another suggests that it may be lack of deoxyribonucleic acids in extracellular space which prevents further genome replication. At any rate, incomplete dsDNA/RNA genomes can be readily found in the secreted virions. Picture modified with permission from Robert Garces. 
HBV RNA in serum

HBV has always been described as a DNA virus. This means that no HBV RNA should be detectable in serum. However, an increasing number of anecdotal reports exist which describe the detection of HBV RNA in the serum of HBV infected individuals. A similar phenomenon has been described in Epstein-Barr infection. The data available suggest that HBV RNA may be residing in peripheral blood monocytes, or circulating in the serum. Also, a link with chronic infection has been suggested.

It remains to be clarified if the presence of HBV RNA in serum is an artifact, and if not so, what its clinical significance is.

HBV diagnostic markers

A variety of serological assays may be employed to differentiate the type of viral infection as well as discriminate between chronic and acute hepatitis B virus (HBV) infection (see figure 11). The most sensitive and specific methods used commercially in diagnosing are enzyme-linked immunosorbent assays (ELISA) for detection of specific antibodies against the various HBV proteins. Nucleic acid detection techniques, such as PCR, bDNA and NASBA are used in detecting HBV DNA present in both blood and liver tissue samples. This HBV DNA may be directly related to the presence and quantity of viral particles when measured in cell free compartments like plasma and serum.

In the case of analyzing cellular fractions, integration of the HBV genome into the host cells must also be considered. During acute infection an incubation phase of 4 till 12 weeks, in which there are no detectable HBV antigens or DNA, is followed by an acute phase during which HBsAg, HBeAg and HBV DNA are detectable in high titers. During this phase, anti-HBc becomes detectable, which may then be followed by the occurrence of anti-HBe and the clearance of HBsAg, HBeAg and HBV DNA. 3 – 6 months later, this is followed by the occurrence of anti-HBs. At this stage, the patient is considered to have cleared the HBV infection.

During chronic infection, initially the same pattern can be seen as in acute infection. However, when the markers typical for the acute phase continue to be detectable for more than 6 months, the patient is considered to be chronically infected. The chronic phase of infection is then entered. After several months till years, two serologic patterns are possible. First, and most commonly, all markers except for anti-HBc IgM may remain
detectable. Second, more rarely, HBeAg and HBV DNA may become undetectable, followed by the occurrence of anti-HBe. However, the patient’s blood is still considered to be infectious at this stage.

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**Figure 11. A:** Serological patterns of HBV markers in acute infection.

**Figure 11. B:** Serological patterns of HBV markers in chronic infection. Both the usual serological pattern without HBe seroconversion and the less common pattern with anti-HBeAg seroconversion are shown.

Most of these patients with HBeAg-negative chronic hepatitis B harbor HBV strains with mutations in the precore or core promoter region. The most common precore mutation, G1896A, inhibits production of HBeAg by introducing a premature stop codon in the precore region. This variant is commonly found in association with HBV genotype D, which is prevalent in the Mediterranean basin. It is rarely detected in association with HBV genotype A, which is prevalent in the United States and North-West Europe. Patients with HBeAg-negative chronic hepatitis B tend to have lower serum HBV DNA levels and are more likely to run a course of infection characterized by persistently elevated or fluctuating ALT levels. For an overview of possible combinations of markers present in HBV infection, see figure 11 A and B.
**NASBA Technology for detection of HBV DNA**

**NASBA**

NASBA is a nucleic acid amplification technique using the simultaneous activity of reverse transcriptase (RT), T7 RNA polymerase and RNase H\(^{128-130}\). The process occurs at one temperature (41°C) without the need of adding intermediate reagents, and results in the exponential accumulation of single-stranded RNA products.

The specificity of the reaction is determined by two oligonucleotide primers that are specific for the sequence of interest. The antisense primer (P1) has a 5'-overhang which encodes the T7 polymerase promoter, while the sense primer (P2) is specific for a region upstream of the P1 annealing site. The reaction for amplification of RNA is started by the single addition of all ingredients except the enzymes, a 5 minutes 65°C incubation to denature any secondary structure in the RNA, addition of the enzyme mix at 41°C and a 90 minutes incubation at 41°C. A schematic presentation of the order of biochemical reactions in the amplification reaction is given in figure 12.

During the early stages of the reaction, a cDNA copy of the target area is generated through the P1 by the activity of RT. RNase H hydrolysis the RNA in the resulting RNA:DNA hybrid leaving a single-stranded cDNA to which P2 anneals. The RT enzyme then synthesizes the second DNA strand. The resulting dsDNA encodes a functional double stranded T7 RNA polymerase promoter at the 5'-end. T7 RNA polymerase binds to the promoter and generates antisense RNA copies of the target molecule (see figure 12). Each new RNA molecule is now available as template for RT in the so-called cyclic phase of the NASBA amplification process. In this phase, P2 binds to the template first,
resulting in a single-strand cDNA copy through the coordinated activities of RT and RNase H. After subsequent annealing of the P1, the T7 promoter becomes double stranded by 5′-extension of the cDNA to which the P1 annealed. T7 RNA polymerase can now again generate RNA copies that can be used as a template. The final NASBA product thus consists of single-stranded copies of antisense RNA representing the area targeted by the primers.

**Amplification of HBV nucleic acids with NASBA**

Because of its ease of use and other advantages like smaller likelihood for false-positive results and suitability for high-throughput testing, NASBA was considered by our lab as a method to amplify HBV nucleic acids. As a result of this, the isothermal amplification of HBV DNA (Yates et al., Journal of Clinical Microbiology) and HBV RNA (Penning et al., submitted to PNAS) by NASBA has been described by us recently.

![Figure 13. Schematic representation of the template switch in HBV DNA NASBA.](image)

In contrast to the amplification of RNA templates, the mechanism of the isothermal DNA amplification by NASBA is yet only partially elucidated. For HBV DNA, the efficiency of the HBV DNA amplification by NASBA can be enhanced by a single denaturation step at 95°C to make single stranded DNA before the amplification starts. From that point, it is unclear how the newly synthesized DNA strand denatures from the template strand to allow the extension of the second primer that renders the double stranded T7 RNA polymerase promoter sequence, which is part of primer P1.

One hypothesis is that there is a switch of template strand by the AMV-RT from the newly made dsDNA to the P1 primer that is present in high excess amounts (see figure 13) (Bob van Gemen, personal communication). According to this theory, the T7 RNA polymerase starts from the P1 sequence and subsequently switches its template to make a double stranded T7 promoter sequence, which is part of the P1.
**Detection of nucleic acids with molecular beacons**

Detection of the amplified nucleic acid during the amplification reaction results in real-time detection of a positive signal, thereby minimizing the involved hands-on time compared to other detection methods, which require post amplification handling of the amplified nucleic acid. Some clear examples of the combination of amplification and detection in the same reaction are bDNA\(^{132}\) (signal amplification method) and the PCR-Taqman system\(^{133}\), which is probably the first homogeneous real-time amplification method described. Additional strength of homogeneous real-time detection can be accomplished by the addition of extra features in the probes that are being used. The molecular beacon probes described by Tyagi and Kramer\(^{134,135}\) have those features due to the folding structure of the probe.

![Figure 14. Schematic presentation of molecular beacon hybridizing to a target sequence.](image)

The folding structure of the molecular beacon probe has proven to be useful in several ways. First, the stem structure of the probe can be labeled with a fluorescent dye at one end and a quencher of fluorescence at the other end. Due to the structure there is no fluorescence in the folded state. Upon hybridization to the target sequence the probe unfolds and the label and quencher are physically separated allowing the label to emit light (see figure 14). In addition, Tyagi et al. described the added value of molecular beacons in the detection of point mutations real-time in PCR reactions\(^{135}\).

The isothermal NASBA amplification technology and real-time detection with molecular beacons have been integrated in the development of the HBV DNA NASBA, leading to an easy-to-use assay with good sensitivity and specificity.

The generated RNA amplicons in the NASBA process are detected with sequence-specific 6-FAM labeled molecular beacons. DABCYL was used as quencher. Due to the continuous measurement of fluorescence in the real-time NASBA, the time point at which a reaction reaches the threshold of detection and thus becomes positive is easily determined. In the methods applied for the detection of HBV DNA or HBV RNA, there is an inverse correlation between the time it takes for a reaction to become positive, the so-called Time
To Positivity (TTP) and the initial amount of HBV DNA copies used as input in the reaction. The higher the input of HBV, the shorter the time the reaction needs to amplify to a detectable level.

Real-time monitoring of NASBA reactions gives fluorescence plots with typical initial exponential rates followed by a plateau phase. The initial exponential rate is consistent with a cyclic phase of the NASBA reaction in which the products of amplification function as templates. With the slope and Y-axis intercept of the logarithmically plotted exponential phase, the expected amount of DNA or RNA can then be calculated.

**Epidemiology**

The hepatitis B virus is primarily found in the blood and liver of infected individuals. Virus titers as high as $10^9$ virions per milliliter of blood have been reported in HBe-positive carriers. However, HBV has also been detected in other bodily fluids including tears, urine, saliva and nasopharyngeal fluids, semen and menstrual fluids.

Transmission of HBV occurs primarily by blood exchange (e.g., by shared needles during injection drug use) and by sexual contact. For comparison, approximately 8-16 million HBV, 2.3-4.7 million HCV and 80,000-160,000 HIV infections may result every year from unsafe injections.

Persons infected early in life are much more likely to become chronically infected than those infected during adulthood: as many as 90% of infants infected perinatally develop chronic infection and up to 25% will die of HBV-related chronic liver disease as adults. Acutely HBV-infected adults may develop chronic infection in 5-10% of cases.

Pre-exposure vaccination results in protective antibody levels in almost all infants and children (> 95%) and healthy adults younger than 40 years of age (> 90%). Transmission of HBV is most efficient via parenteral pathways such as transfusions with infected blood and re-usage of infected syringes. Due to the fact that in most industrialized countries donated blood is routinely screened for the presence of HBV, the major risk group in these countries are the intravenous drug users who often share needles or syringes. Sexual transmission is also possible, though less efficient. Children of mothers with active HBV are also at risk of acquiring HBV perinatally.

Uninfected individuals living with an HBV carrier are at greater risk of contracting HBV than those not living with a carrier.
HBV infection among homosexual men

As discussed before, HBV is in general associated with indicators of sexual activity such as number of lifetime or recent sexual partners, years of sexual activity and a history or serological marker of other sexually transmitted infections.

In epidemiological studies it has been shown that homosexual men are a major risk group for HBV infection. An early study by Dietzman et al. showed that the prevalence rates of serum HBsAg and anti-HBs were 5.6% and 34% in 144 homosexual men in Seattle. However, prevalence rates were only 0.9% and 3.6% in 111 heterosexual male venereal disease clinic patients, and also 0.9% and 3.6% in 111 healthy men undergoing routine physical examinations. Therefore, previous exposure to hepatitis B virus (HBV) was estimated to be 8.8 times greater for homosexual men than for heterosexual men in this study.

Another, larger, study showed that of 3,816 homosexual men examined in five sexually transmitted disease clinics in the United States, 6.1% had HBsAg, 52.4% anti-HBs, and 3.0% of the men who had no other indicator of infection with hepatitis B virus (HBV) had anti-HBc. The rate of seropositivity for HBV indicated by the presence of one or more of these serologic markers was 61.5%, which is higher than in the previously discussed study but may be explained by the higher sensitivity of the methods used for detection of HBV markers.

Data suggest that HBV is transmitted 8.6-fold more efficiently than HIV-1 among homosexual men.

In homosexual men, HBV seropositivity is significantly related to insertive anal intercourse, the duration of regular homosexual activity, and to the number of non-steady male sexual contacts. Asymptomatic rectal bleeding is frequent in homosexual men with persistent HBV infection, and HBV can be detected in semen, rectal mucosa, feces, and anal canal mucosa in high titers. These circumstances are believed to create the setting for sexual transmission in homosexual men. Interestingly, it is possible that the HBV involved with sexual transmission is a different type of HBV than the one involved with parenteral infection. For instance, Dietzman et al. found that the HBV strain found in homosexual men, ad, was different from the ay subtype found among intravenous drug abusers.
Pathogenesis of HBV infection

Biochemical markers of HBV infection

Besides measuring HBV-specific antigens and/or antibodies, it is also possible to estimate the stage and severity of HBV infection by measuring biochemical markers. When HBV-induced liver damage arises, enzymes or proteins specifically found in the liver are released from damaged cells into the blood circulation. This results in the detection of liver specific enzymes and proteins in the blood. Therefore, these biochemical markers have in common that they give an indication of the severity of HBV-induced liver disease. However, it should be noted that other events, such as alcohol and drug abuse might also lead to elevated levels of liver specific enzymes and proteins in the circulation.

Aspartate aminotransferase (AST) is an enzyme found mainly in heart, kidney, liver, muscle and pancreatic tissue. Tissue damage releases this enzyme and elevated levels can then be detected in the blood. Vitamin B deficiency and pregnancy may decrease levels of this enzyme found in the blood.

Alanine aminotransferase (ALT) is another enzyme found mainly in the liver, but can also be seen in lower amounts in heart, muscle and other tissues. Increased levels of this enzyme in the blood can be attributed to liver damage, kidney infection, chemical toxins or a cardiac infarction (heart attack).

Lactose dehydrogenase LDH is an enzyme primarily found in brain, heart, kidney, liver, lung and skeletal muscle tissue. Increased levels are associated with cell death. Decreased levels can be associated with malnutrition or low tissue/organ activity.

Albumin is a protein synthesized in the liver and is involved in maintaining blood protein base levels. Liver damage may result in low levels of albumin produced. When albumin levels drop to extremely low levels, fluid from the blood may leak into surrounding tissues resulting in swelling known as edema.

Bilirubin is a byproduct of red blood cell breakdown. It is formed when the hemoglobin ring is opened through other enzyme activities. Bilirubin is typically excreted into the bile, giving bile its pigmentation. Increased levels are associated with liver disease, mononucleosis, and toxicity due to some types of drugs and hemolytic anemia.

It may be clear from the above that these clinical markers may not always be specific for HBV-caused disease, and should therefore always be interpreted with care and the additional measurement of HBV-specific antigens and antibodies.
Pathogenesis

The immune response to HBV-encoded antigens is responsible both for viral clearance and for disease pathogenesis during infection. While the humoral antibody response to viral envelope antigens contributes to the clearance of circulating virus particles, the cellular immune response to the envelope, nucleocapsid, and polymerase antigens eliminates infected cells\textsuperscript{158}. The class I- and class II-restricted T cell responses to the virus are vigorous, polyclonal, and multi-specific in acutely infected patients who successfully clear the virus, and the responses are relatively weak and more narrowly focused in chronically infected patients who do not\textsuperscript{159-162}.

Although neonatal tolerance probably plays an important role in viral persistence in patients infected at birth, the basis for poor responsiveness in adult-onset infection is not well understood and still requires further analysis. Viral evasion by epitope inactivation and T cell receptor antagonism may contribute to the enhancement of viral persistence in the setting of an ineffective immune response, as can the incomplete downregulation of viral gene expression in, and the infection of immunologically important tissues\textsuperscript{163}. Chronic liver cell injury and the accompanying inflammatory and regenerative responses create the mutagenic and mitogenic stimuli for the development of DNA damage, that can eventually cause hepatocellular carcinoma\textsuperscript{158,164}.

Intracellular HBV primarily causes little or no damage to the infected cell, which is an excellent strategy of viral survival\textsuperscript{163,165,166}. However, viral oligo-peptides of 8-15 amino acids are loaded on host cell MHC-class 1 molecules and are transported to the cell surface. This enables HBV-specific T-lymphocytes to detect infected cells and destroy them. However, this cell deletion triggered by inflammation cells and subsequent inflammatory events may become clinically apparent as acute hepatitis. If HBV is not eliminated, a delicate balance between viral replication and immune defense prevails which may lead to chronic hepatitis and liver cirrhosis\textsuperscript{158}. The pathogenic and antiviral potential of the cytotoxic T lymphocyte (CTL) response to HBV has been demonstrated recently\textsuperscript{167,168}. First, by the induction of a severe necroinflammatory liver disease following the adoptive transfer of HBsAg-specific CTL into HBV transgenic mice. Second, by the non-cytolytic suppression of viral gene expression and replication in the same animals by a posttranscriptional mechanism mediated by interferon gamma, tumor necrosis factor alpha, and interleukin 2.

For reasons poorly understood, HBV may become partly cytopathogenic in chronically infected cells, and the viral DNA may integrate into the host cell DNA through the viral transcriptase\textsuperscript{158}. If integration leads to activation of some and/or inactivation of other
host genes, hepatocellular carcinoma may result.

The severity of liver damage depends on the occurrence of the events described above, as well as other factors. HBeAg defective mutants appear to be involved in the loss of virus tolerance and therefore in the pathogenesis of acute hepatitis B\textsuperscript{169}. In addition, HBeAg mutants appear to be positively selected for by the host antiviral immune response and behave as escape mutants\textsuperscript{170-172}. The combination of these characteristics explains the relative prevalence of this mutant over wild-type HBV in patients with severe acute hepatitis B and in chronic HBsAg carriers during anti-HBe seroconversion and/or hepatitis B exacerbations\textsuperscript{170,172}. However, the absence of HBeAg defective mutants in some cases of severe and fulminant hepatitis B as well as its detection in asymptomatic carriers of HBsAg strongly suggests that HBeAg mutants are not necessary for HBV to become virulent, but may be sufficient in some cases\textsuperscript{173,174}.

Other factors influencing the severity of hepatitis are the number of virus infected cells, the competence and genetic heterogeneity of the immune system, the vigor and extent of non-specific inflammatory response, and the killing of hepatocytes caused by other diseases or other hepatotropic viruses.

\textbf{Course of HBV infection in HIV coinfected persons}

The natural history of hepatitis B virus (HBV) infection is modified by coinfection with the human immunodeficiency virus (HIV). HIV infects the CD4 positive cells involved in the regulation of the immune system and may eventually lead to the acquired immune deficiency syndrome (AIDS). HIV largely shares its route of infection and risk populations with HBV.

The outcome of chronic hepatitis B depends on a balance between HBV infection, which is not directly cytopathic, and the host's immunologic response to it (see elsewhere in this chapter). Coinfection with HIV may therefore modify the natural history of the HBV infection. Despite the consensus reached on these topics, the precise effects of HIV coinfection on the natural history of HBV are still under debate.

After initial HBV infection, both development and persistence of chronic HBV infection are more frequent among people with prior HIV infection\textsuperscript{175-177}. Furthermore, higher HBV load\textsuperscript{177}, and reactivation of HBV infection in chronic carriers who have lost detectable serum HBeAg\textsuperscript{178-180} are well described among people with HIV coinfection. However, some studies have suggested that in anti-HIV–positive patients, HBV replication may be not different or enhanced as compared with anti-HIV–negative patients\textsuperscript{175,181-183}. Some
studies have also indicated that liver disease activity as assessed with serum transaminase activities\textsuperscript{178,184} or histology activity index\textsuperscript{181,183,185} is not influenced or diminished in anti-HIV-positive patients compared with anti-HIV-negative patients. Other studies have indicated that cirrhosis was more common among anti-HIV-positive patients than in anti-HIV-negative patients\textsuperscript{176}. It is possible that in some patients a progression to extensive fibrosis and cirrhosis occurs with only minimal inflammatory process, as observed in other immunosuppressed populations such as transplant recipients\textsuperscript{186,187}.

The high level of HBV replication and the low serum transaminase levels in HIV-positive patients may be related to decreased function of cytotoxic T (CD8\textsuperscript{+}) lymphocytes, which may be caused by the impairment of CD4\textsuperscript{+} T lymphocytes and monocytes by HIV infection\textsuperscript{188}. These findings are consistent with an immunological pathogenesis of the liver lesions in chronic hepatitis B, and may also explain the poor response to interferon-alpha therapy usually observed in these patients compared with immunocompetent patients\textsuperscript{189-192}. The exacerbation of chronic hepatitis B observed with the restoration of the immunity during protease inhibitor treatment is also consistent with immune-mediated pathogenesis of HBV-induced liver disease\textsuperscript{193}.

Finally, it is not excluded that reactivation episodes of chronic hepatitis B occur more frequently among anti-HIV-positive patients than among anti-HIV-negative patients as suggested previously\textsuperscript{179,194}.

On the other hand, some studies suggested that coinfection with HBV may be associated with a more rapid course of HIV disease\textsuperscript{195,196}. In AIDS patients, Ockenga et al.\textsuperscript{197} reported a reduced survival in HBV coinfected patients. However, actuarial survival in HIV infected patients was not found to be altered by the presence of HBV coinfection\textsuperscript{196}.

The altered natural history of HBV infection and recent improvements in HIV survival through combination antiretroviral therapy emphasize the need to address HBV-related morbidity in people with coinfection.

In HBV/HIV coinfected persons, combination therapy with lamivudine and protease inhibitors produced pronounced and sustained increases in CD4 cells, HIV load reductions, reduction in HIV disease progression\textsuperscript{31,185}, and HBV DNA suppression below the lower limit of detection of the assay\textsuperscript{198}.
Treatment of HBV infection

A number of chemical treatments have been used with varying success in the treatment of chronic HBV. The goals of treatment are three-fold: to eliminate infectivity and transmission of HBV to others, to arrest the progression of liver disease and improve the clinical prognosis, and to prevent the development of hepatocellular carcinoma.

Treatment with interferon-α of chronic HBV infection has become widely accepted, resulting in up to 30 to 40% of cases in the elimination of the virus. The success of therapy is measured by sustained clearance of HBV DNA and/or HBeAg and HBsAg from the serum. Long-term follow-up of patients who respond to interferon treatment with clearance of HBeAg indicates that the majority ultimately clear HBsAg as well, and have persisting remission of liver disease, although low levels of HBV DNA can commonly be detected in liver tissue.

Treatment is laborious and expensive, may have serious adverse effects, and the mechanism of action is still poorly understood. The combination of interferon-α with lamivudine has recently proven to be much more powerful than the use of these two agents separately, increasing the success rate up to 80% of cases. Lamivudine (3'-thiacytidine, or 3TC) results in marked reduction of HBV DNA levels and improvement in serum aminotransferases and hepatic histology in the majority of patients. When stopped, however, most patients relapse. The shortcomings of long-term therapy have been the development of viral resistance in up to one-quarter of patients within a year, and a higher percentage with more prolonged therapy.

Lamivudine resistance has been shown to occur in > 15% of patients with chronic HBV infection and in 27% of liver transplantation patients. It is clearly associated with certain patterns of mutations, all of which include either a Met->Ile or a Met->Val mutation at codon 552 of the HBV polymerase gene. These codon 552 mutations, which are similar to the mutations detected in lamivudine-resistant human immunodeficiency virus (HIV) strains, lead to a change in the YMDD motif of the HBV polymerase, and apparently constitute the molecular basis of lamivudine resistance.

Nucleoside analogues such as famciclovir, adefovir, ganciclovir and lobucavir are accepted or currently being investigated as potential therapeutic agents, either alone or in combination with interferon-α. This may on the one hand enlarge the rate of sustained viral eradication, and on the other hand prevent the emergence of therapy resistant HBV
mutants\textsuperscript{219,220}. However, the toxic effect on the liver caused by the therapy regimens described above may also be an important factor, and has not been properly investigated currently\textsuperscript{221,222}.

When all therapy fails, liver transplants may be required for those patients with extensive liver damage due to HBV infection. However, there are many factors to consider before a liver transplant is considered as therapeutic option. First and foremost, transplantation is a complex operation that requires a suitable donor. Moreover, transplanting a liver into a chronically hepatitis B infected individual might result in the situation that the newly transplanted liver becomes re-infected. Finally, liver transplant operations tend to be expensive procedures, putting an enormous pressure on the healthcare budget. Despite these problems, for some individuals liver transplants may offer the only hope for a healthy life.
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