Structure and molecular virology

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HISTORICAL BACKGROUND

Discovery of hepatitis B virus

Hepatitis B virus (HBV) was the first human hepatitis virus from which the proteins and genome could be identified and characterized. Before discovery of the viruses, two types of hepatitis transmission were differentiated on the basis of epidemiological observations: type A was considered to be predominantly transmitted by the faecal–oral route, while type B was transmitted parenterally. In 1963, B S Blumberg, in a search for polymorphic serum proteins, discovered a previously unknown antigen in the blood of an Australian aborigine (Australia antigen). Soon thereafter, it was recognized that the appearance of this antigen was related to type B hepatitis (Blumberg et al 1967). Using immune electron microscopic methods, D S Dane eventually discovered virus-like particles that carried this antigen on their surface, in the serum of hepatitis B patients (Dane et al 1970). These particles were consequently considered to be 'the' hepatitis B virus (HBV). Further non-related viruses that caused parenterally transmissible hepatitis in humans were discovered subsequently, but HBV retained its name. In 1973 the viral nature of the particles discovered by Dane was confirmed by the detection of an endogenous, DNA-dependent DNA polymerase within their core (Kaplan et al 1973). This enzyme allowed Robinson et al (1974a,b) to detect and characterize the HBV genome as a small, circular DNA that was partially double-stranded and partially single-stranded.

Hepadnaviridae

HBV infection was known to result in acute and chronic liver disease and, based on epidemiological data, it was postulated as early as the 1970s that this virus might represent a cause of liver cancer. This led to a search for an HBV-like agent in woodchucks (marmot-like animals from North America), which have been observed to develop liver cancer. Woodchuck hepatitis virus (WHV) was subsequently discovered (Summers et al 1978), and a similar virus was found in seemingly healthy ground squirrels (GSHV) (Marion et al 1980), a species that is distantly related to marmots. In addition, an HBV-like virus was also found in Pekin ducks, which also occasionally develop liver cancer (Zhou et al 1980), and in grey herons living in their natural wild habitat (Sprengel et al 1988). HBV and its relatives throughout the animal kingdom now comprise an officially recognized virus family termed hepadnaviridae, the name derived from their hepatotropism and DNA genome (Howard 1991). The hepadnaviruses of animals are currently not of particular interest to veterinary medicine, but they serve as important models for human HBV. A highlight was the discovery of reverse transcription of viral RNA into DNA within core particles of duck HBV (DHBV) as an essential step of the viral genome replication (Summers & Mason 1982). This replication strategy is used by all hepadnaviruses. Thus, hepadnaviruses are also termed pararetroviruses, in contrast to orthoretroviruses, which have an RNA genome.

BIOLOGY OF HEPADNAVIRIDAE

Immune pathogenesis

Human hepatitis virus was recognized as a pathogen that causes acute hepatitis. However, in order to understand the biology of hepadnaviruses, it is necessary to keep in mind that the members of this virus family are not highly cytotoxic per se, certainly not to the extent of causing massive cell death. Acute and chronic hepatitis B are the result of the host defence mechanisms against HBV, which result in the death of HBV protein-expressing cells and the neutralization of circulating virus (see Ch. 10). Immunologically immature or deficient individuals do not develop a typical hepatitis, but they become chronic carriers of the virus. In this case, the virus continues to replicate in hepatocytes and, to a lesser extent, in other organs or cell types, without destroying tissues or severely impeding organ function.

Viraemia

The infected hepatocytes continue secreting complete virus particles (virions) until titres typically as high as 10^8–10^{10} particles per ml of serum are reached. The great majority of the virions are not cell bound, but a minor number may exist in certain subpopulations of leucocytes. The significance of the persistent viraemia for the virus appears obvious; if these viruses are unable to replicate in cells of the mucosa, but only replicate further within the organism, they require the bloodstream of the host to leave their site of replication as well as to reach it in a newly infected individual. Blood-to-blood contact is not very frequent between individuals and may lead to exchange of only minute amounts of serum. Thus, long-lasting, high-level viraemia seems to be useful for the spread and survival of hepadnaviruses in the population of its host species.

Host range

Typical for hepadnaviruses is their narrow host range. Human HBV infects only higher primates such as chimpanzees. The hepadnaviruses of marmot-like animals do not infect other rodents, and even the duck virus DHBV does not infect all species of ducks. Important transmission-effective contacts such as sexual activity, squabbles and similar activities would usually occur between individuals of the same species. Thus, a wide host range would not be useful for a virus that is transmitted by blood, unless an insect vector was involved. It must be noted, however, that replication of mammalian hepadnaviruses in insects has never been reported nor have hepadnaviruses been found in insects. Hepadnaviruses are relatively stable against heat and can, therefore, maintain infectivity while circulating in the blood. They do, however, appear to be sensitive to detergents and proteases. Thus, passage through the bile to the faeces, if it occurs at all, would not lead to spread of infections as it does so efficiently for hepatitis A virus.

Antigenaemia

The unusually high level of viraemia is accompanied by antigenaemia. In typical individuals with severe viraemia of all host species, the hepadnaviral surface (HBs) proteins are present at concentrations of 10–1000 μg per ml of serum. The HBs proteins are secreted by the infected cells as particles of variable morphology and size (see below). Moreover, it appears that all hepadnaviruses are able to induce secretion of their core protein (HBc protein) in a non-assembled soluble form that is referred to as HBe protein. This molecule reaches also such high concentrations that it can be detected by a technique as insensitive as agar-gel immunodiffusion, indicating levels of 10 μg/ml and more (Magnius & Espmark 1972). The origin and meaning of the 'e' in the name of this protein is a mystery.

Immune tolerance

It appears that, even in immunologically competent individuals who are able to resolve the infection, the onset of the immune response is delayed by several weeks. Among HBV-infected humans with normal immune systems several per cent never develop an effective cytotoxic immune

response and proceed directly to persistent infection with high-level viraemia and antigenaemia. The mechanisms by which hepadnaviruses suppress or escape immune elimination are not known, but it appears that variability of the surface proteins, as occurs in hepatitis C virus (see Chs 13 and 14) does not play a major role during the high-level viraemic phase. Several serological subtypes and genotypes of HBV are recognized, but variability within these types is very low, even at those genomic sites that are known to vary most between genotypes. Infection of immune cells has been described (Korba et al 1988), but a general decrease in immune functions has not been observed in HBV carriers. Induction of a specific tolerance toward HBs and HBe proteins, however, appears to be a possibility.

Immune complex disease

High-dose tolerance induction may be one possible function of the viral proteins that are excessively produced and secreted. However, this immune tolerance is not complete since circulating immune complexes of HBsAg or HBeAg and their antibodies are often detectable in viraemic carriers (Madalinski et al 1991). Glomerulonephritis, periarteritis nodosa and acrodermatitis are immune complex diseases that are occasionally found in viraemic carriers.

HBe seroconversion

In HBV carriers a state of low-level viraemia may often develop after elimination of HBeAg from the blood while HBsAg persists to circulate in the blood. Disappearance of HBeAg seems to indicate that HBc protein-expressing cells are no longer tolerated by the immune system. Once most of the HBV-producing cells are eliminated, the state of 'healthy' HBsAg carrier is reached. However, some latent HBV genomes seem to remain and are able to become reactivated when the immune system is weakened. Certain variants that cannot synthesize HBeAg may be selected out during immune pathogenesis and lead to considerable viraemia. Such variants are often found in fulminant hepatitis or chronic hepatitis B without HBeAg (see Ch. 7).

Integration of HBV DNA and oncogenesis

As will be pointed out later, integration of its DNA is, in contrast to orthoretroviruses, not an essential step in the replication of HBV. However, integration of HBV DNA in fragments occurs often. In some cases cells may gain an illegitimate growth advantage by this event, if genetic elements of growth control are disturbed by the insertion of viral DNA. Furthermore, viral proteins themselves, if expressed by integrated HBV DNA fragments in a dysregulated manner, may favour uncontrolled growth. There can be no doubt that hepadnaviruses have oncongenic potential, but it varies from species to species: WHV has the highest oncogenicity, DHBV and GSHV the lowest (see Ch. 15).

STRUCTURE OF HEPADNAVIRIDAE

Electron microscopy (EM)

Figure 6.1 shows the three types of virus-associated particles that are present in the blood of HBV-infected subjects. Similar particles are also found in the serum of hepadnavirus-infected woodchucks or ground squirrels.

Virion morphology

The virus is spherical with a diameter of 42 nm. Using negative staining of virions adsorbed to the EM grids the double-shelled structure of the virions becomes apparent. The outer protein shell (or envelope) is formed by the HBs proteins (Dane et al 1970). Apparent surface structure details such as knobs or spikes, as observed on many other enveloped viruses, are not found on HBV. The inner protein shell is referred to as the core particle or capsid (Almeida et al 1971). It is composed of HBc protein. The capsid encloses the viral DNA, which is often positively stained as in Figure 6.1.

HBs particles

The sera of highly viraemic carriers contain very large numbers of non-infectious particles, composed of excessive HBs protein. Most abundant are spherical particles of 17–25 nm that typically

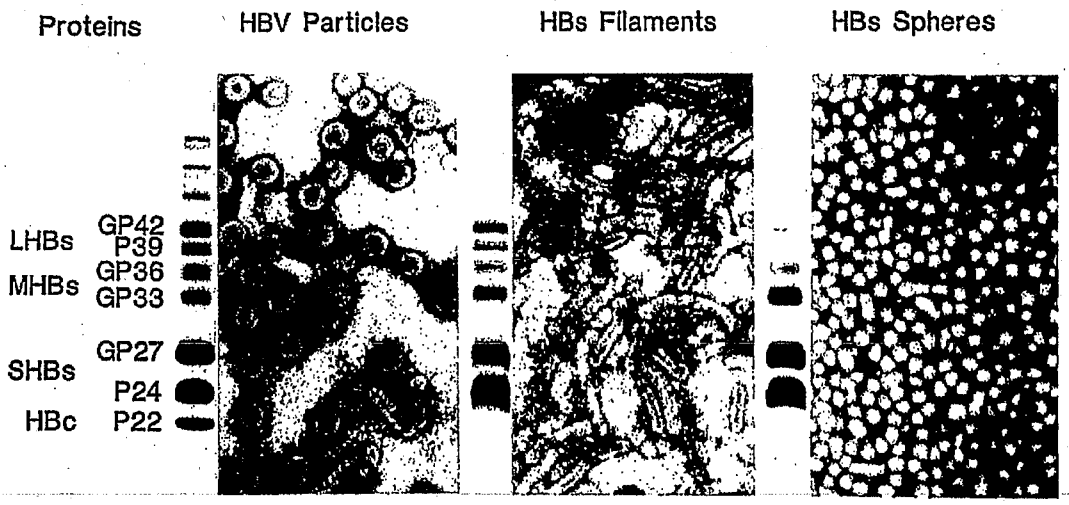


Fig. 6.1 *Morphology and protein composition of purified HBV and HBs particles.* The particles were isolated from high-titred HBV carrier plasma and separated from each other using ultracentrifugation and gel chromatography. The HBV particles were enriched approximately 1000-fold and the filaments approximately 10-fold, while the HBs spheres were slightly diluted for preparation of these three negatively stained electron micrographs. The same purified particle preparations were denatured by SDS/DTT and run through a 13% polyacrylamide gel and stained with colloidal silver. The apparent size and the nomenclature of the viral proteins are shown at the left. The viral surface antigen contains small, middle-sized and large proteins (SHBs, M, L), which appear in variably glycosylated forms. The core protein (HBc) forms a single P22 band in gel electrophoresis.

reach numbers of 10^{13}/ml. The spheres seem to be small vesicles with walls approximately 4 nm thick and an interior diameter of 10–15 nm. Less numerous (up to 10^{11}/ml or 1 μg/ml) are the filamentous (or tubular) particles of 20 nm diameter and variable length. Sera from low-viraemic HBsAg carriers contain up to 10^{12}/ml HBs spheres and few or no HBs filaments.

Avian hepadnaviridae.

DHBV has a similar virion morphology as the mammalian hepadnaviruses (Mason et al 1980). The serum from DHBV-infected ducks also contains excessive HBs particles but their morphology is different in that the vesicles have a variable diameter of 40–60 nm. No filaments are detectable.

HBs particles in the liver

The virion envelope and the HBs particles are synthesized and assembled at the membrane of the endoplasmic reticulum (ER). From the ER, the particles are transported and excreted by vesicles. However, when the largest of the three HBs proteins (LHBs) is overproduced in comparison with the smaller HBs proteins, filamentous particles are formed that are retained within the ER (Ganem 1991). This may lead to the accumulation of HBs protein to the point that the ER becomes dilated (Chisari et al 1987). In light microscopy such LHB-storing hepatocytes appear opaque like ground glass.

Core particles in liver

The core particles are synthesized and assembled independently from the HBs proteins in the cytosol. Thereafter they probably attach to patches of HBs protein in the ER membrane. By enclosing the whole core particle, the HBs proteins would mediate budding of the virions to the ER lumen, but these processes have not yet been studied. Non-enveloped core particles of human HBV are often found in the nucleus where they are obviously stored (Gudat et al 1975). In highly viraemic individuals without high disease activity

virtually all hepatocytes may contain considerable numbers of core particles, which can be extracted, purified and visualized by EM as 27-nm spherical particles. Both empty core particles without nucleic acid and full particles with DNA are found. Non-enveloped core particles are not detectable in the serum. However, a certain HBV-producing cell line, HepG2.2.15, secretes not only virions and HBs particles but also naked core particles (Sells et al 1987).

PROTEIN COMPOSITION OF HBV PARTICLES

Purification of HBs particles

The typical density of HBs particles and virions is between 1.16 and 1.19 g/ml in sucrose gradients or 1.19–1.24 in caesium chloride gradients. This density is only shared by high-density lipoproteins, which are, however, much smaller than the HBs particles. Thus, a combination of isopycnic and rate-zonal centrifugation is suitable to purify virions and HBs particles. Size chromatography using wide-pore gels is also useful for separation of HBs spheres from HBs filaments and virions. Virions can be partially separated from filaments because of their higher density (Heermann et al 1984). The particles shown in Figure 6.1 were separated by this method.

SDS gel electrophoresis of proteins

Virions and large structures like the HBs or HBc particles are built from protein subunits that are held together by non-covalent interactions and, in the case of HBV proteins, by disulphide bonds between cysteines of different protein molecules. These interactions can be disrupted by the anionic detergent sodium dodecyl sulphate (SDS) and disulphide cleaving reagents such as ß-mercaptoethanol or dithiothreitol (DTT). Moreover, the protein subunit is denatured to form a random coil. For analysis of HBs proteins gel electrophoresis after treatment with SDS/DTT was applied to separate them by size. Thereafter, the gel slabs were stained with colloidal silver, as shown in Figure 6.1.

In the HBs filaments, six protein bands are visible ranging from 24 000 to 42 000 daltons apparent molecular weight. All of these proteins can also be stained specifically by the immunoblotting technique with an antibody against the smallest component, P24 (data not shown), which demonstrates that at least one epitope of the smallest protein is present in the larger proteins. Four of the proteins contain an oligosaccharide linked to one or two of their asparagine residues (*N*-linked glycan). These glycoproteins migrate as somewhat larger molecules in electrophoresis than the non-glycosylated forms. Enzymatic removal of the glycan shows that HBs filaments contain only three different HBs polypeptides:

1. The largest (LHBs) is converted by partial glycosylation in vivo from P39 to GP42.
2. A middle-sized protein (MHBs) that is either single or double glycosylated as GP33 or GP36.
3. A small protein (SHBs) that may be glycosylated as GP27 or non-glycosylated as P24.

SHBs is the most abundant polypeptide in all three HBV-associated particles, whereas MHBs is a minor component in all three. LHBs is more prevalent than MHBs in virions and filaments, but less prevalent in HBs spheres. It appears that the proportion of LHBs determines the morphology of the HBs particles (Marquardt et al 1987), while the ratio of SHBs to MHBs does not significantly alter morphology. A model of the HBs particles is shown in Figure 6.2.

Core particles

In addition to the HBs proteins, purified virions possess a non-glycosylated protein, P22, which builds up the core particle. Naked core particles from liver show the same P22 HBc protein in SDS gel electrophoresis (Gerlich et al 1982).

Virion cores are known to contain the viral DNA polymerase, a protein covalently bound to the minus DNA strand (Gerlich & Robinson 1980), and a protein kinase (Albin & Robinson 1980), but the identity of these proteins has not

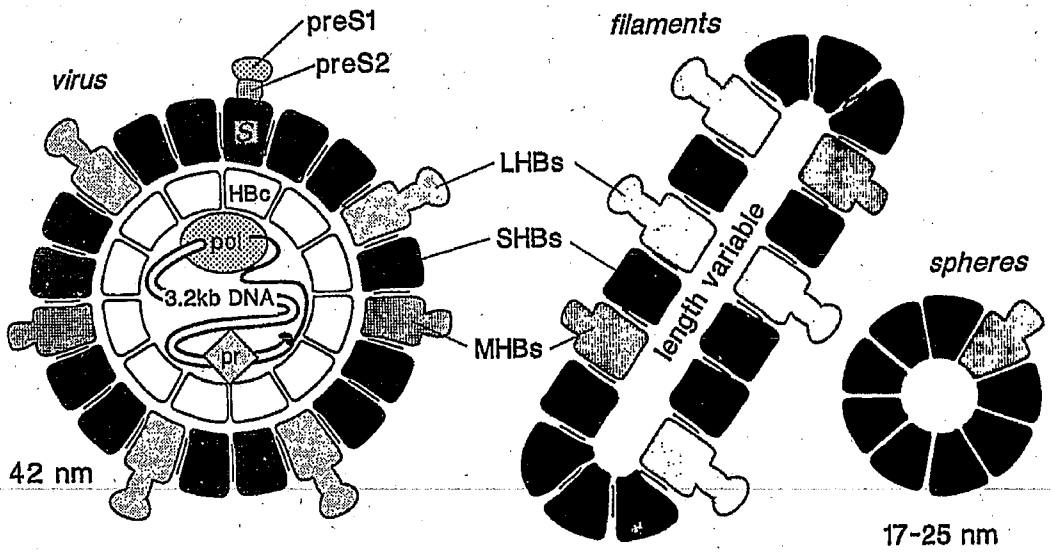


Fig. 6.2 *Schematic model of the HBV and HBs particles.* The SHBs protein of the viral envelope is identical to the S domain of MHBs and LHBs. MHBs contains a small pre-S2 domain facing toward the outside of the particle. LHBs contains, above pre-S2, a pre-S1 domain. The viral envelope encloses a symmetrical capsid consisting of 180 HBc protein subunits. These encapsidate the 3.2- kb-long DNA genome of HBV to which a primase protein (pr) and a DNA polymerase (pol) are associated. It is not completely clear whether the pol and pr are part of one polyprotein or of two separate proteins. The filaments consist of the same proteins as the virion envelope. The spheres contain less LHBs. The virion envelope and the HBs particles contain small amounts of lipid between the protein subunits.

yet been verified by gel electrophoresis. A model of the virions is shown in Figure 6.2.

Host proteins

Numerous human proteins can be found in purified preparations of HBs particles or virions. Well documented is the binding of modified serum albumin to human HBV and HBsAg (Machida et al 1983). Moreover, a small amount of antibodies to HBs antigen may simultaneously be produced with HBsAg, even in high-viraemic carriers. Thus, virions and HBs particles isolated from virus carrier serum often contain immunoglobulin. The significance of the three protein bands in the virion preparation shown in Figure 6.1 is not known.

GENOME OF HBV

Endogenous DNA polymerase of HBV

The first hint regarding the nature of the HBV genome came from the discovery of an endogenous DNA polymerase activity in ultracentrifugation pellets from HBV-containing sera (Kaplan et al 1973). The reaction was dependent on all four desoxynucleotide triphosphates and divalent cations, but no primer or template was necessary. It could be inhibited by actinomycin D, which intercalates into DNA templates. The enzyme was precipitable with antibodies against HBc protein if the HBs envelope was removed from virions by treatment with a non-ionic detergent. The DNA template was not accessible to DNAase unless the core protein was lysed by SDS or digested by proteinase. The existence and the properties of the endogenous DNA polymerase provided evidence that the virus contained a DNA genome.

Structure of the virion DNA

Using electron microscopy, the DNA of HBV was shown to be circular, partially double stranded and 3200 nucleotides in length (Robinson et al 1974). The endogenous DNA polymerase reaction is possible because one of the DNA strands is incomplete (Landers et al 1977). The remaining gap is filled by the viral DNA polymerase if the dNTPs are added in vitro. In vivo, HBV particles

are obviously secreted from the infected cell before the double strand is completed. Thus the incomplete plus strand has a defined 5' end but a variable 3' end, as shown in Figure 6.3 a.

The complete minus stand has defined 5' and 3' ends, with a terminal redundancy of nine bases, in the region in which the genome is triple stranded (Will et al 1987). A protein is covalently bound, probably via a tyrosine-linked phosphodiester bridge to the 5' end of the minus strand. This linkage causes extraction of virion-derived DNA to the phenol phase during classical DNA extraction, unless the sample is thoroughly digested with proteinase (Gerlich & Robinson 1980).

The 5' end of the plus DNA strand is formed by 18 ribonucleotides that are capped in the same manner as an mRNA (Seeger et al 1986). The genome is held in an open circular structure because there is an overlap between the two DNA strands (Sattler & Robinson 1979). The genome contains two directly repeated sequences of bases, DR1 and DR2 (Dejean et al 1984). This genome structure is typical for all hepadnaviridae, but in the case of the avian hepadnaviruses the overlap and the distance between the DRs is shorter.

The structure of the hepadnaviral genome does not abide by the usual classification criteria for viruses (Francki et al 1991) in two respects: it contains both DNA *and* RNA and its genome contains partially single-stranded, double-stranded and even triple-stranded DNA. These unusual features are a direct consequence of its replication mechanism, which will be explained below.

Cloning of HBV DNA

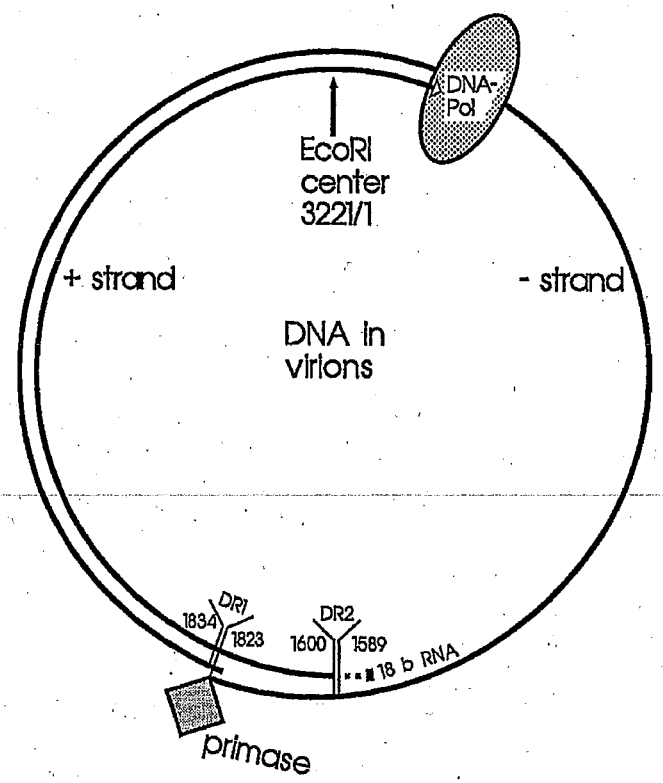
In the late 1970s only minute amounts of virion DNA were available. These samples were used for cleavage with a restriction enzyme (often *Eco*RI) and the DNA was ligated to plasmid DNA. The recombinant DNA was introduced into *Escherichia coli*, and bacterial clones carrying the inserted HBV DNA molecules were identified and multiplied (Galibert et al 1979, Pasek et al 1979, Valenzuela et al 1979). By this technique unlimited amounts of HBV DNA became available. During replication of the recombinant DNA within the bacteria all the unusual features of the HBV DNA were removed by the bacterial enzymes for DNA repair. Thus, covalently closed DNA circles of recombinant plasmids were obtained from the bacteria. From these plasmids the HBV DNA or defined fragments thereof could be recovered by cleavage with suitable restriction enzymes. Such fragments were used to determine the complete DNA sequence and virtually all studies on the HBV genome have subsequently been performed using cloned DNA.

Definition of open reading frames (ORFs)

From the nucleotide sequence of a double-stranded version of the DNA genome it is possible to derive six different sequences of amino acid-encoding triplets of nucleotides (codons). If a sequence of potential codons does not encode a protein, it is randomly interrupted by one of the three possible stop codons, whereas protein-encoding sequences are free of stop codons for a distance of at least 50 codons. These stretches of codons are called open reading frames (ORFs). Protein biosynthesis additionally requires a start codon (AUG) in messenger RNA (mRNA). By applying these rules, all genomes of mammalian hepadnaviruses must contain four ORFs, which are encoded by the same DNA strand (Fig. 6.3b). Virologists define the polarity of viral nucleic acid strands in such a way that the mRNA has plus polarity. Thus, the protein-encoding DNA strand of the HBV genome, which is transcribed into mRNA, has minus polarity. Additional ORFs can be identified both on the minus and plus strands, but they are not conserved and are probably generated by statistical sequence variations.

Compact genome structure of HBV

Most functional ORFs of cellular organisms as well as those from larger viruses are arrayed in a linear fashion on the genomes and encode one protein. Moreover, they are interspersed by large



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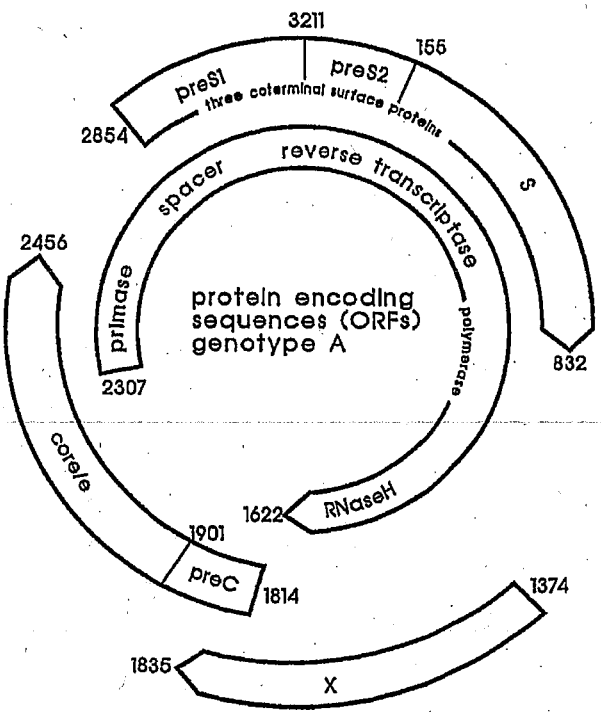

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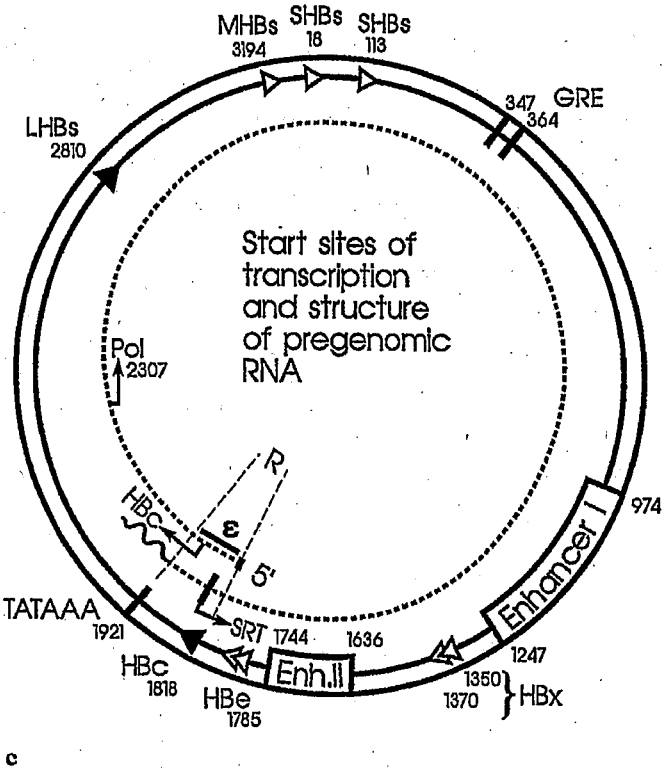

c

Fig. 6.3 *Physical structure (a), open reading frames (b), transcription signals and pregenomic RNA (c) of HBV.* (a) All known complete HBV genomes are circular and between 3182 and 3221 bases long. The figure shows one of the longer examples. Numbering begins at a cleavage site of *Eco*RI. Within the virion, the DNA strand that encodes the viral proteins (i.e. the minus strand) is of full length but is not covalently closed. It has a small redundancy of 9–10 bases at the ends. At its 5' terminus it contains a covalently bound protein known as primase because it is necessary for priming of the minus strand synthesis. The plus strand starts with an 18-base-long RNA piece containing a methylated base (cap) at its 5' end. The plus strand keeps the minus strand in a circular conformation because it bridges the discontinuity of the minus strand. The 3' end of the plus strand is not at a fixed position, and is still connected with the HBV DNA polymerase. Most virion-bound genomes contain a single-stranded gap of 300–2000 bases in length. (b) The ORFs shown here are defined by the first start codon of protein synthesis and the first stop codon. Some internal start codons are also shown. The longest ORF with its four continguous domains encodes the polymerase polyprotein of HBV. (c) The initiation sites of mRNA synthesis for the various HBV proteins in hepatocytes are shown as triangles within the covalently closed genome. Initiation sites that are also used in non-hepatic cells are shown as open triangles. The common termination signal for all mRNAs is the TATAAA box at base 1921. Only the pregenomic HBV RNA is shown as a dashed line. It has a terminal redundancy R, and within the 5' terminal R the encapsidation signal sequence ε. A short sequence within the 3' terminal R is the signal for reverse transcription (SRT). The pregenome is polyadenylated (wavy line) and encodes the HBc and pol protein. Shown are the start sites of these proteins (→).

non-coding regions (introns) that are removed after transcription by splicing. Regulatory and protein-coding regions are well separated. However, the genomes of hepadnaviruses seem to have evolved towards minimal length. Thus, the genome is not much longer than its longest ORF, P, which encodes the viral DNA *p*olymerase and its accessory functions. ORF S is completely located within the ORF P. ORF C and ORF X overlap partially with ORF P. HBV encodes more than one protein from one ORF by using internal AUG codons in an ORF as starting sites for protein biosynthesis. Thus, nested sets of proteins with different amino ends and a common carboxy end are synthesized. ORF S encodes the three HBs proteins (Heermann et al 1984). ORF C encodes the HBe protein and the HBc protein (Ou et al 1986). ORF X also appears to encode more than one protein (Kwee et al 1992). Furthermore the numerous genetic elements that regulate transcription of the viral DNA into RNA are placed within coding regions.

Complete HBV genomes are between 3182 and 3221 bases long (Tiollais et al 1981). The numbering of the bases starts in most publications (as it does here) at the cleavage site for the restriction enzyme *Eco*RI or at homologous sites if a particular genome type does not have such an *Eco*RI site. Other numberings, e.g. beginning at the ATG of the HBc protein or the first base of the RNA pregenome (see below), are also in use.

STRUCTURE OF HBs PROTEINS

Protein sequence of small HBs

The amino acid sequence of small HBs (SHBs) at the amino and carboxy ends has been determined by Edman degradation (Peterson et al 1977, Peterson 1981). The internal sequences could only be partially analysed by this approach. However, together with the protein sequence predicted by the nucleotide sequence, it became clear that the sequence of SHBs begins at the third conserved AUG of ORF S, and that it ends at the stop codon of ORF S. The function of the 5' terminal part of ORF S was originally unknown and was named region pre-S, simply to indicate its location upstream of gene S (Tiollais et al 1981). It is important to note that proteins containing the pre-S sequence are *not* precursors of SHBs.

SHBs are very rich in hydrophobic amino acids. They have many tryptophans but few tyrosines, and, thus, unlike most proteins possess an ultraviolet absorption spectrum similar to tryptophan. Furthermore, they contain the unusually high number of 14 cysteines, which are all cross-linked with each other. At asparagine 146 there is a signal for addition of an *N*-linked glycan, which is present in approximately half of the molecules (Peterson 1981). This glycan has two complex antennas with terminal sialic acids (Heermann & Gerlich 1991). In SDS gel electrophoresis, SHBs have a microheterogeneity, the pattern of which is typical of a virus carrier (Stibbe & Gerlich 1982). The origin of this microheterogeneity is unknown.

Subtypes

SHBs occur in stable subtypes that were originally defined by antibodies. Antigen reactivities that were present on all known HBs isolates were considered as determinant *a*. The best-known subtype determinants are *d* or *y* (Le Bouvier et al 1972) and *w* or *r* (Bancroft et al 1972). Determinant *d* has a lysine at position 122, *y* an arginine (Peterson et al 1984). Likewise, determinant *w* has a lysine at position 160, *r* an arginine (Okamoto et al 1988). Recently, a further subtype allele that has either isoleucine or threonine in position 126 of SHBs has been identified (Ohnuma et al 1993). These type-specific amino acid exchanges may, however, occur in quite divergent HBV genomes. Recently, subtyping has been done by DNA sequencing of the SHBs gene. At least six genotypes, A–F, which differ by more than 8% in the protein sequence, have been identified (Okamoto et al 1988, Norder et al 1992). These possess group-specific amino acids that have not yet been clearly assigned to serological determinants. In contrast, the determinants *d* and *w* occur in four different genotypes. Since all SHBs subtypes are able to induce cross-protection after immunization, the significance of serological or other subtyping is mainly of

epidemiological and phylogenetic interest. However, it cannot be excluded that some of the neutralizing antibodies are subtype specific, as is the case with most other viruses.

Topology of SHBs

Circular dichroism of HBs spheres suggests that 50–60% of the polypeptide chain are folded into α-helices. Computer programs used to model the secondary structure of proteins predict that these α-helices are formed by four hydrophobic stretches of SHBs, as shown in Figure 6.4 (Stirk et al 1992). Biosynthetic studies suggest that α-helix I is inserted co-translationally into the ER membranes and furthermore that it is able to trans-

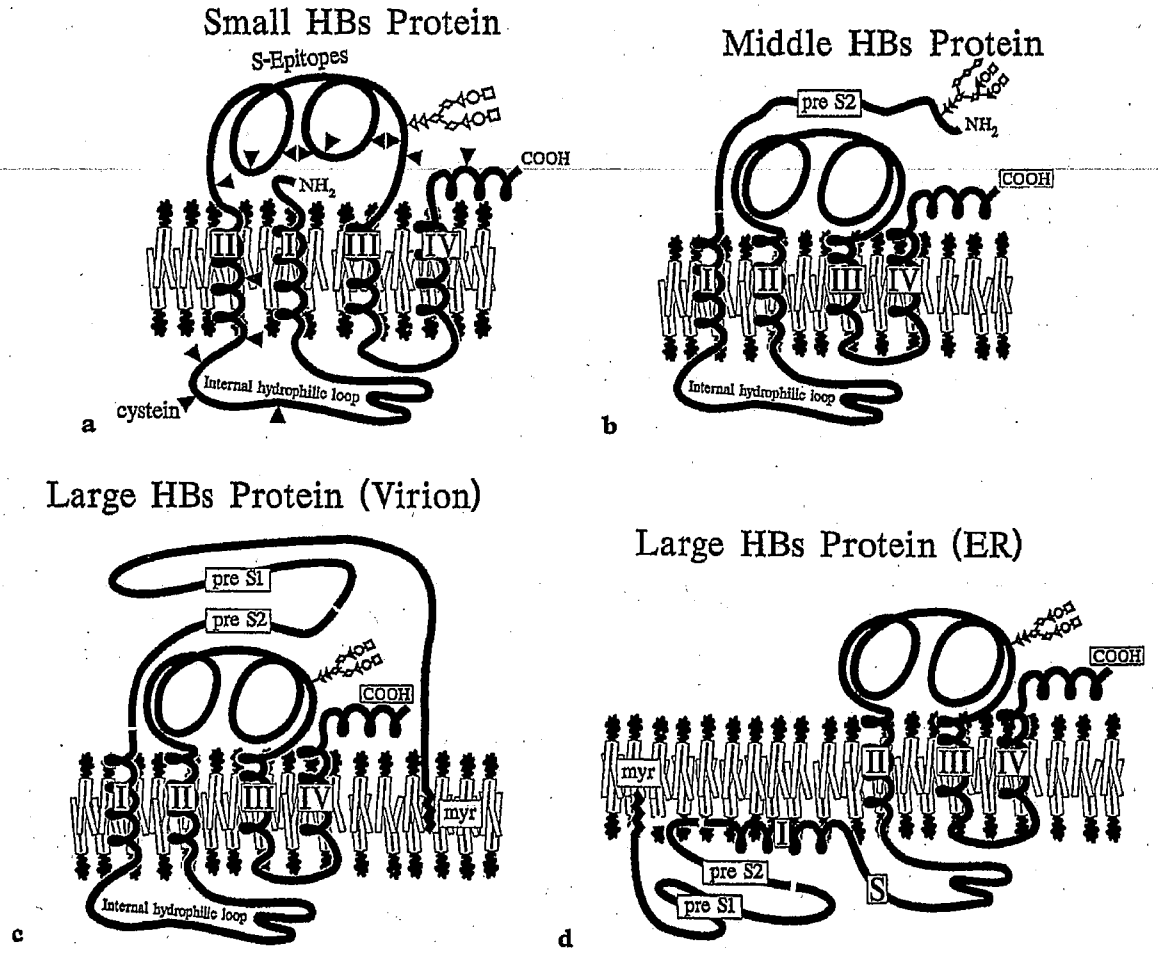

Fig. 6.4 *Topological models of the three HBs proteins.* The polypeptide thread is shown as thick black line. The lipid bilayer of the ER membrane is symbolized by the lipid molecules. The top of the figures corresponds to the lumen of the ER or – after multimerization and budding of the HBs protein – to the surface of the virus particles. The bottom of the figure corresponds to the cytosol or to the interior of the particles. It is assumed that the ER membrane is crossed at least four times by hydrophobic α-helical segments of the S domain. (a) Structure of SHBs. The branched structure at top right is a complex two-antenna glycoside present in GP27 but not in P24. The black triangles represent cysteines that are linked with each other, but the exact nature of the linkage is not known. The HBs epitopes are generated by the complex folding of the external hydrophilic loop between α-helices II and III and are stabilized by cysteine bonds. The amino end seems to be hidden within the molecule. (b) Structure of MHBs. The arrangement of the transmembraneous α-helices of the S domain seems to be slightly altered in that the amino end is more accessible and its hydrophilic loop is less often glycosylated. The pre-S2 domain carries a three-antenna glycoside. (c) Structure of LHBs in the virion. The structure seems to be similar to MHBs, but the pre-S2 domain is *not* glycosylated. The pre-S1 domain partially covers the pre-S2 domain and is linked at methionine 2 with myristic acid (myr). (d) Presumed structure of LHBs soon after its synthesis. At the early phase of LHBs folding it appears that the entire pre-S domain stays in the cytosol. Schematically, the proposed structure would result, but the true folding of the cytoplasmic pre-S domain is not known. At later phases of virion or HBs filament maturation the entire pre-S domain is translocated through the particle membrane to the surface as shown in (c).

locate amino-terminal sequences to the lumen of the ER if they are not too long (Eble et al 1986). Helix II is also inserted into the ER membrane, but it translocates the carboxy-terminal sequences (Eble et al 1987). This topology implies that the hydrophobic sequence between helix I and II remains cytoplasmic. After budding of HBs particles to the lumen of the ER, this sequence is in the interior of the particles. In agreement with this evidence, B-cell epitopes have not been found in this region. The sequence from amino acids 99 to 168 forms the HBs antigen region.

Most of the SHBs epitopes depend upon the presence of disulphide bonds. Mild treatment with DTT first destroys subtype-specific epitopes and at higher concentrations also the *a* epitopes. Completely reduced HBs particles are almost non-immunogenic. Thus, SHBs is virtually undetectable in immunoblots in which reduced and denatured proteins are reacted with antisera against HBs particles (Neurath et al 1984).

Computer modelling of the following hydrophobic regions suggests formation of two membrane-spanning helices III and IV, which may insert post-translationally into the ER membrane. Owing to their polarity with one hydrophobic and one hydrophilic side, these two helices are predicted to multimerize (Stirk et al 1992, A Berting & W H Gerlich, unpublished). Multimerization may possibly also occur between different HBs subunits, and this event would potentially mediate budding of the HBs particles. In agreement with that presumption, truncated HBs proteins without this region no longer form HBs particles, but remain at the ER (Bruss & Ganem 1991a). Genes for such truncated HBs proteins have been found in certain hepatoma cells (Lauer et al 1992). The carboxy-terminal part is dispensable for HBs particle formation (Bruss & Ganem 1991a, Prange et al 1992).

Middle HBs (MHBs)

This minor component of the virion or HBs particles is composed of the S domain, the sequence of which is identical to that of SHBs, and of the 55 amino acid long pre-S2 domain (Stibbe & Gerlich 1983). The pre-S2 domain is hydrophilic and does not contain cysteines. It is very sensitive to proteases, and can be removed selectively from HBs particles without destroying the S domain. Thus, pre-S2 is virtually absent in HBV vaccines that contain protease-treated HBs particles from carrier plasma. The asparagine at position 4 is linked with a glycan of the mixed type (Fig. 6.4). It contains a mannose chain in addition to two complex chains (X Lu & W H Gerlich, unpublished). The amino-terminal part of the pre-S domain is relatively conserved, but sequence 32–54 is highly variable.

The pre-S2 domain is located at the surface and partially covers the S domain of MHBs. It may be slightly more immunogenic than the HBs antigen. Furthermore, the epitopes are not conformation dependent and can (in contrast to HBsAg) easily be generated by synthetic peptides. Peptides with the sequence of the amino-terminal half of pre-S2 were found to induce protective immunity (Itoh et al 1986, Neurath et al 1986a, Neurath & Kent 1988). Pre-S2-containing HBs particles from transfected Chinese hamster ovary cells (CHO) have been introduced in some countries as a vaccine (Tron et al 1989).

The central part of the pre-S2 domain, which also forms the major epitopes, binds a modified form of serum albumin (Machida et al 1984, Krone et al 1990). In vivo, approximately 1 in 10 000 serum albumin molecules is able to bind to pre-S2. HBV carriers with more than 10 μg of HBsAg per ml usually have free albumin binding sites on their particles, while at lower HBsAg concentrations all binding sites are occupied. The nature of the modification is not known, but it can be mimicked by cross-linking of albumin with glutaraldehyde (Yu et al 1985). Serum albumin of non-primate origin does not bind (Machida et al 1984).

Large HBs (LHBs)

The largest HBs protein contains the three domains: pre-S1, pre-S2 and S. In the mature virions or HBs particles the pre-S domains are accessible for antibodies (Heermann et al 1984), receptors (Neurath et al 1992) and proteases (Heermann et al 1987). The S domain and parts

of the pre-S2 domain are hidden by the pre-S1 domain of LHBs (Fig. 6.4). During biosynthesis, the entire pre-S domain of LHBs seems to stay initially at the cytoplasm (V Bruss, personal communication, 1992). Thus, the asparagine 4 of the pre-S2 domain is not glycosylated in LHBs (Heermann et al 1984), because this modification occurs co-translationally in the ER lumen. The pre-S1 domain, however, probably becomes membrane attached by another modification. The amino end of the pre-S1 domain carries the sequence methionine–glycine which, together with other less well-defined neighbouring amino acids, serves as a signal for the replacement of the methionine by the C_{14} fatty acid, myristic acid (Persing et al 1987). During virion or HBs particle maturation, the pre-S1 domain is obviously reconfigured and translocated to the surface of the particle.

Overexpression of LHBs relative to expression of SHBs prevents secretion of HBs particles (Persing et al 1986). Instead, filaments become enriched in the ER (Chisari et al 1987). Expression of MHBs and SHBs is independently regulated from expression of LHBs (see below). Hepatocytes that express and secrete SHBs predominantly appear to exist, whereas others express more LHBs and store it intracellularly. It is known that the degree of hepatic immune staining for HBsAg does not correlate with the level of HBsAg in the serum. Only hepatocytes that express LHBs, SHBs and the other viral components in a well-balanced manner are able to assemble and secrete virions (Bruss & Ganem 1991b).

The pre-S1 domain is one of the most variable regions of the HBV genome. One reason for this may be that the part of the polymerase protein that is encoded by the same DNA region as pre-S1 is not essential for replication. The other reason is that this may be *the* surface structure that is most intensively selected for by immune pressure. However, within a chronically infected person or within a defined chain of infection pre-S1 is not mutated (Uy et al 1992). Thus, it is not similar to the hypervariable domains of human immunodeficiency virus (HIV) or HCV envelope proteins.

STRUCTURE OF CORE PROTEINS

Products of ORF C

The essential product of ORF C is the HBc protein of either 183 or 185 amino acids, depending upon the genotype of the virus. In most isolates from highly viraemic carriers, ORF C has 212 or 214 codons, but translation of HBc protein starts only at the AUG, 29 codons downstream of the first AUG (Ou et al 1986). The region upstream is termed pre-C for historical reasons (Tiollais 1981). This name is misleading because the product of the entire ORF C is *not* a precursor of HBc protein but of the secretory form of the core protein, which is termed HBe protein.

HBc protein

This protein contains many hydrophilic and charged amino acids. It does not contain lipid or glycan, but if expressed in eukaryotic cells it becomes phosphorylated (Roosinck & Siddiqui 1987, Lanford & Notvall 1990). It is synthesized in the cytoplasm of the infected cells. As an essential step in the viral life cycle it packages its own mRNA and the viral polymerase and assembles into core particles. A protein kinase, most likely of cellular origin, is also packaged. These particles are then enveloped by patches of the ER membrane that contain the three HBs proteins.

HBc protein expressed in bacteria such as *E. coli* packages RNA in a non-specific manner. The ability to assemble to particles resides in the first 147 amino acids (Gallina et al 1989), while the four arginine clusters of the last 36 or 38 amino acids are involved in packaging of nucleic acids (Hatton et al 1992, Nassal 1992a). Phosphorylation of serine 170 or 172 between arginine clusters 3 and 4 may interfere with nucleic acid binding (Machida et al 1991).

It appears that a critical concentration of HBc protein is necessary for assembly. First, dimers of HBc protein are formed (Zhou & Standring 1992), which then assemble at 0.8 μM concentration (Seifer et al 1993) to isometric

particles of T3 symmetry, i.e. 180 HBc subunits form one particle (Birnbaum & Nassal 1990). It has been postulated that HBc protein folds in a similar manner to the subunits of other viral capsids with T3 symmetry into two apposed β-sheets, each with four anti parallel strands, but experimental data for this have not yet been obtained (Argos & Fuller, 1988). Once the core particles are assembled, their structure is stabilized by disulphide bonds (Zhou & Standring 1992, Nassal 1992b).

HBe protein

All hepadnaviruses have evolved the ability to express a secretory form of their HBc protein. They achieve this by the 5' terminal part of the ORF C, called the pre-C sequence. The pre-C sequence encodes a hydrophobic α-helix that is a secretion signal and allows for translocation of the HBe protein into the lumen of the ER (Bruss & Gerlich 1988, Standring et al 1988). During that process, 19 of the 29 pre-C amino acids are cleaved off by the signal peptidase (Fig. 6.5). The 10 remaining amino acids of the pre-C sequence prevent assembly of HBe to core particles by interaction with the HBc protein sequence (Wasenauer et al 1992). Thus, HBe protein differs in almost all aspects from HBc protein, although the primary sequence of these two molecules is almost identical. Part of the HBe protein is transported to the plasma membrane (Schlicht & Schaller 1989); another part is further cleaved within the arginine-rich domain by a Golgi protease and then secreted (Standring et al 1988). Another part of the HBe protein does not reach the ER lumen and is not cleaved at all. The P25^e protein exposes a nuclear transport signal (Ye et al 1990, Wang et al 1991). Thus, HBe proteins of variable length are found in practically all compartments of the cell and furthermore are secreted.

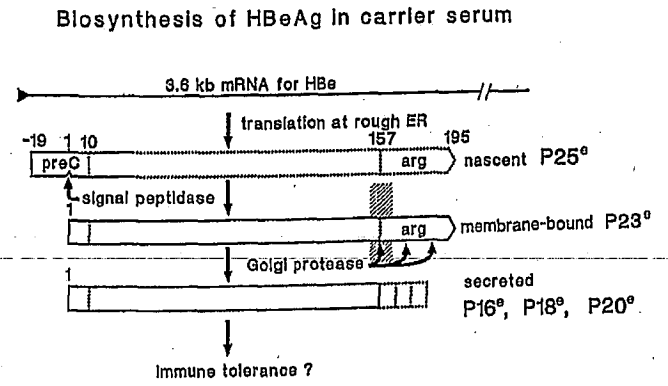

Fig. 6.5 *Biosynthesis of HBe proteins.* When the more-than-genome-length mRNA starts upstream of the HBe start codon, translation of the pre-C region prevents the necessary folding of ε. Thus, the HBe mRNA does not usually function as a pregenome. It is translated to the HBe precursor P25^e, which may be co-translationally cleaved to a membrane-associated P23^e. The 10 remaining amino acids of the pre-C sequence prevent folding of P23^e to core particles. Secretion requires partial or complete removal of the arginine-rich carboxy-terminal domain by a Golgi protease. Thus, secreted HBe protein may be of variable size between 16 and 20 kD. The HBe protein is *not* essential for virion replication or assembly, but may modulate the host's immune response to HBV.

Function of HBe protein

HBe protein is not essential for the viral life cycle. Variants without functional pre-C sequence and HBe protein arise often during acute or chronic HBV infection (see Ch. 7). Nevertheless, all known hepadnaviruses from duck to man have an HBe protein. Using a different expression strategy, murine leukaemia viruses have also developed the ability to produce a secretory form of their nucleoprotein, the glycosylated gag protein.

High levels of secreted HBe protein are found in highly viraemic virus carriers with few symptoms. Elimination of HBeAg is usually accompanied by a flare-up of immune pathogenesis and a decrease of viraemia (see Ch. 10). An HBe-minus variant of woodchuck hepatitis B virus was infectious for newborn woodchucks but it could not induce persistent infection, whereas the HBe-expressing virus resulted in persistent infection (Chen et al 1992). These observations suggest that HBe protein may somehow suppress the immune elimination of HBV-producing hepatocytes. Because HBe protein is probably able to pass the placenta, it may make the T cells of the fetus tolerant of the HBc protein, thus preventing a cytotoxic T-cell response against HBc/e epitopes (Milich et al 1990). However, the tolerogenic effect of HBe protein is not dependent on maternal transmission to the newborn.

PRODUCTS OF ORF P

While the products of ORFs S and C have been identified and well characterized within virions, evidence regarding the products of ORF P is quite circumstantial. There is no doubt that such (a) product(s) (is) are expressed during infection in vivo, but the exact mode of transcription, translation and post-translational processing is not known.

Domains of ORF P

As will be discussed below, mutational analysis of the 834–845 codon-spanning ORF P, sequence homologies with well-studied reverse transcriptases (Toh et al 1983), together with studies on the mechanism of genome replication of HBV, show that most parts of the ORF are indispensable for the virus. These studies suggest that ORF P has four clearly distinguishable domains (Schlicht et al 1991):

1. The amino-terminal domain, which encodes the terminal protein, which is linked to the 5' end of the minus-strand of virion DNA. This part of ORF P is necessary for priming of minus-strand synthesis and is, thus, also termed primase.
2. The next domain has no specific function except as a spacer or tether.
3. The following domain encodes the RNA- or DNA-dependent polymerase, i.e. the reverse transcriptase.
4. The last carboxy-terminal domain is an RNAase H, which cleaves the RNA if it is present in hybrids of RNA and DNA.

The ORF P of hepadnaviruses differs from the ORF P of retroviridae in that it has most likely no protease and integrase domain, but it has a primase domain that is absent in retroviridae.

Products of ORF P in virions

Until now, extraction of ORF P products from virions has only yielded controversial results. Extraction of an active endogenous DNA polymerase from virion core particles has been difficult, because disruption of cores requires harsh treatment. Moreover, it has been noted that a template switch of the viral polymerase to exogenously added templates and primers is difficult or impossible (Radziwill et al 1990).

Bavand & Laub (1988) finally succeeded in extracting DNA polymerase from virions and demonstrated in situ its enzymatic activity together with 90-kD and 70-kD protein bands using so-called activity gels. A 70-kD band was also observed in immune blots using antisera against the conserved active centre of the reverse transcriptase (Mack et al 1988) or against the C-terminal peptide (Bavand et al 1989). These observations suggest either post-translational cleavage of the entire ORF-P product or a separate expression of the DNA polymerase domain without from the amino-terminal part. However, recent data from Bartenschlager & Schaller (1992) suggest that a 90-kD protein encoded by the entire ORF P is predominant in virions.

The polymerase protein is only packaged together with the pregenomic RNA within core particles (Bartenschlager & Schaller 1992). Since the pregenome contains only one packaging signal (Junker Niepmann et al 1990), and core particles seems to have only a packaging capacity for 3300 nucleotides (Melegari et al 1991) probably one polymerase molecule is packaged. It cannot be excluded, however, that the polymerase is packaged as dimer or even oligomer.

HBx PROTEIN

The 154 amino acid-spanning ORF X is conserved in similar form in the hepadnaviruses of woodchucks and ground squirrels. The absence of HBx in the avian hepadnaviruses suggests that it does not participate in the mechanism of genome replication or virion assembly. Furthermore, analysis of codon usage suggests that phylogenetically the HBx gene was introduced quite recently from the eukaryotic genome into the genome of the primordial hepadnaviral genome, which itself seems, in contrast, to be very old (Miller 1991).

Occurrence of HBx

Mutational studies suggest that the HBx protein is dispensable for virus production after transfection of permanent cell cultures in vitro (Blum et al 1992). However, transfection of WHV DNA without a functional X ORF into livers of susceptible woodchucks did not cause infection, which shows that HBx is essential for replication in vivo (Chen et al 1993). Detection of antibodies toward HBx protein in a large number of HBV-infected humans demonstrates that HBx is expressed in vivo, but detection of HBx protein in naturally infected liver specimens or serum presents certain problems because the antibodies used for its detection tend to react non-specifically with other proteins. There is no clear indication that HBx protein is a structural component of virions or core particles. The amino acid sequence suggests that HBx is a cytosolic protein without a specific intracellular transport signal. Thus, reports on the occurrence of HBx protein in the serum of virus carriers (Horiike et al 1991) are difficult to explain.

Function of HBx

In vitro-expressed HBx is highly unstable in animal cells, with a short half-life of 20 min, during which time it becomes phosphorylated (Schek et al 1992). Reports on various biochemical activities of HBx protein, such as protein kinase (Wu et al 1990), dinucleotide kinase (Shaul 1991), protease inhibitor (Arii et al 1992) or squelching factor (Shaul 1991) are preliminary and need confirmation. It is now clear that HBx protein activates transcription of many genes in a somewhat non-specific manner when it is introduced artificially into cells by co-transfection with reporter systems (Rossner 1992). The in vivo function of HBx protein remains, however, completely obscure. The size and number of HBx proteins expressed from ORF in vivo are also unknown, but the conservation of its three start codons and the results of mutational studies suggest that the smaller potential proteins are expressed and transcription activating.

One of the most significant side-effects of HBx protein may be its tumurigenic activity, which has been shown in immortalized mouse hepatocyte cultures (Seifer et al 1992) and transgenic mice (Kim et al 1991). In the immortalized hepatocyte cell line FMH 202, HBx protein is associated with cytoplasmic retention of the tumour-suppressor protein p53 (Höhne et al 1993).

REPLICATION OF HBV

The life cycle of the virus

As with all other viruses, the life cycle of HBV and its relatives in the animal kingdom can be divided into several steps: attachment of the virus to the host cell, virus penetration into the cell, release of the viral genome, expression of viral gene products, replication of the viral genome, formation of virions, release of the virus. A discussion of these various steps follows, and they are schematically shown in Figure 6.6.

Attachment of the virus to the host cell

Virus attachment is one of the crucial steps that determines among other factors the host range and organ tropism of viruses. Furthermore, blocking of attachment by neutralizing antibodies against surface epitopes is a major component of protective immunity and is the basis of many antiviral vaccines. Thus, it is interesting to identify the viral attachment site(s) and the corresponding cellular receptor(s).

Lack of infectivity systems. A great drawback of many of the studies on the attachment of HBs proteins to cell surface proteins is that the target cells were not *susceptible* for HBV infection even if they were *permissive* for HBV replication after transfection. A most striking example is the human hepatoma cell line HepG2, which could not be infected by most – but not all (Bchini et al 1990) – investigators but replicates and secretes HBV quite efficiently after transient or stable introduction of HBV DNA (Sureau et al 1986, Sells et al 1987). Even primary cultures of chimpanzee hepatocytes were found to be non-susceptible to HBV infection (Sureau et al 1992). Somewhat better results were reported with polyethylene glycol-treated primary cultures of human hepatocytes from adult liver (Gripon et al 1988,

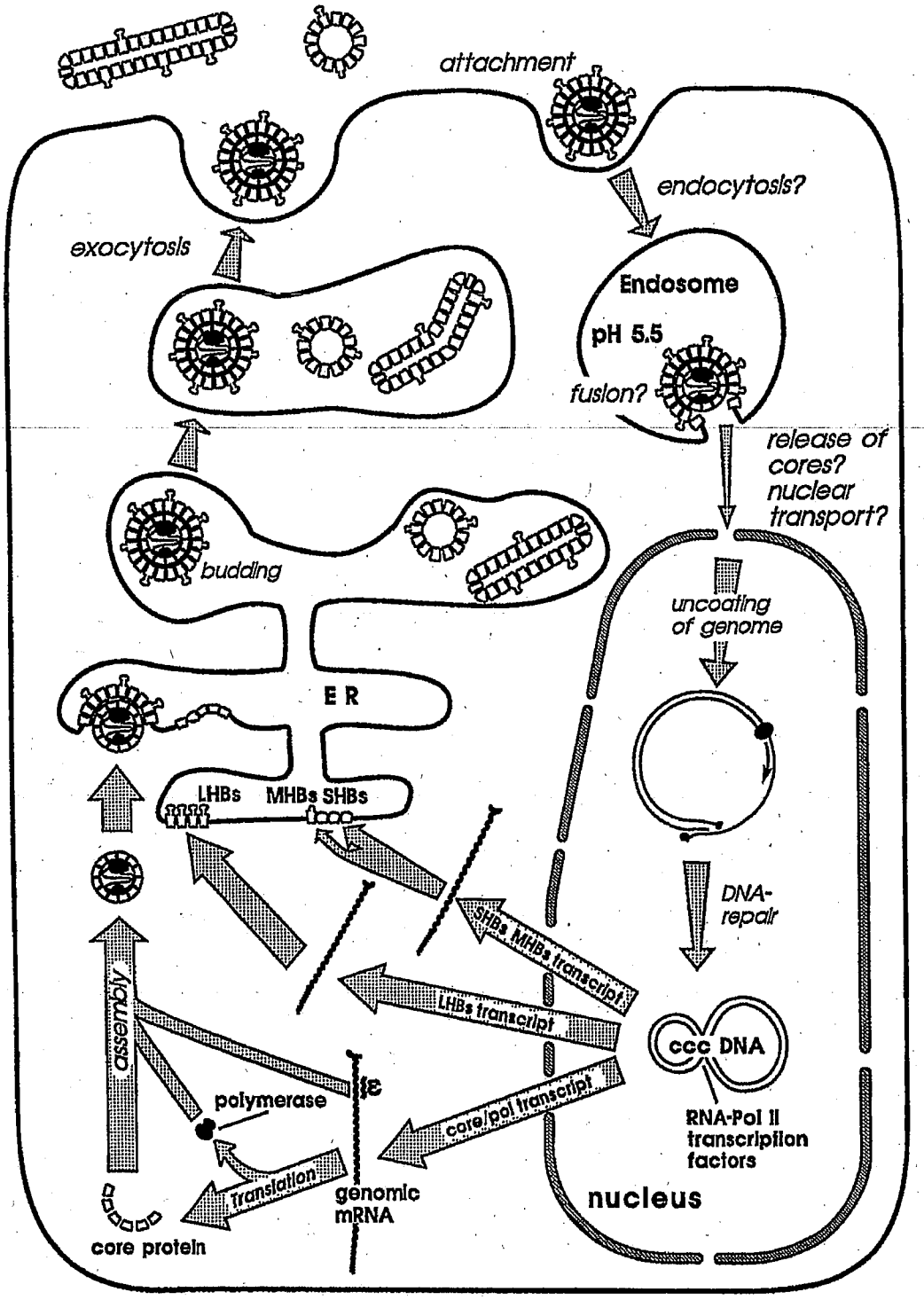

Fig. 6.6 *Schematic view of the life cycle of HBV.* In many details this model is still speculative. It is assumed that the virus is endocytosed after attachment, and the genome or nucleocapsid is released to the cytosol after acidification in the endosome. The genome is repaired in the nucleus and transcribed to three essential classes of mRNA. The non-essential HBe and HBx synthesis is omitted for the sake of simplicity. Translation of the core/pol transcript in the cytosol allows for assembly of core particles that contain the pregenome. The transcripts for LHBs and M/SHBs are translated at the rough ER and the HBs proteins are inserted in that membrane. The HBs particles bud to the lumen of the ER, and at least a part of LHBs-rich ER membrane areas envelopes core particles. The HBV and HBs particles are secreted thereafter by the constitutive pathway.

1993). This relatively high resistance of hepatocytes in culture contrasts sharply with the high infectivity of HBV in vivo.

An effective infectivity system exists for DHBV, for which primary hepatocytes from newly hatched ducklings are a highly susceptible target (Tuttleman et al 1986a). Primary woodchuck hepatocyte cultures are also susceptible for WHV (Aldrich et al 1989). However, these animal hepatocytes also rapidly lose their susceptibility within several days in culture. The reason for the non-susceptibility of long-term hepatocyte cultures to HBV infection is not fully known, but it appears that it is caused by a block in attachment and/or virus entry.

Pre-S1-mediated attachment. An organ- and species-specific attachment of serum-derived HBs particles was found by Neurath et al (1986b) with HepG2 cells, but not with animal liver cells or human carcinoma cells of non-hepatic origin. The binding could be *blocked* by antibodies to the pre-S1 sequence 21–47, and *competed* for by the peptide sequence itself. The relevance of this attachment site was confirmed by studies using natural, purified HBV-particles and plasma membrane preparations from surgically obtained human liver specimens (Pontisso et al 1989). Furthermore, antibodies to peptide pre-S1 (21–47) were able to neutralize in vitro HBV inocula that were no longer infectious (Neurath et al 1989). Monoclonal antibodies to this region could block attachment to HepG2 cells or infectivity for primary human hepatocyte cultures (Petit et al 1991, 1992). Many monoclonal neutralizing antibodies against duck HBV are also directed against the pre-S region of DHBV.

Recently, Neurath et al (1992) suggested that the pre-S1 (21–47) sequence binds to a novel form of membrane-bound interleukin 6, the occurrence of which can be induced on white blood cells. Pontisso et al (1992) observed that IgA competed with pre-S1 (21–47) for the receptor on liver plasma membranes, but they could not identify the receptor. While the pre-S1 sequence 21-47 seems to be essential for the infection process, its attachment to the cell surface is obviously not sufficient for infectivity, or HepG2 cells would be more susceptible to HBV.

Pre-S2-glycan mediated attachment. Three potential candidates for attachment sites have been found in the pre-S2 domain. The pre-S2-linked glycan of MHBs has a weak but distinct affinity for HepG2 cells, but not for permanent mouse hepatocyte cultures or the human carcinoma cell line HeLa. This binding depends on the hybrid structure of the glycan with one mannose antenna and two complex antennas (Y Lu & W H Gerlich, unpublished). With plasma membranes from human liver no such binding was detectable, but it cannot be excluded that in vivo hepatocytes may bind this glycan in certain states of growth or differentiation. Irrespective of this, such binding is probably not sufficient for infectivity because the HepG2 cell is not highly susceptible.

Pre-S2-pHSA-mediated attachment. It is possible that the attachment that can be mediated by modified human serum albumin is more important. Human liver specimens contain receptors for polymerized human serum albumin (pHSA), and pretreatment of liver plasma membranes with pHSA strongly enhances binding of HBV (Pontisso et al 1989). The absence of pHSA receptors on HepG2 cells (Y Lu & W H Gerlich, unpublished) may be one reason why these cells are refractory to infection. The fate of the attached pHSA is probably uptake and digestion within the hepatocyte. HBV may therefore use this altered serum protein as a vehicle for entry into the hepatocyte. Unfortunately, an in vitro system for this possible mode of entry has not yet been established. Recent observations suggest that MHBs may be dispensible for infectivity in vivo and in vitro (H Will, personal communication).

Transferrin-mediated uptake of pre-S2. Binding and uptake of HBs particles has recently been demonstrated with T lymphocytes. It was suggested that these processes are mediated by the transferrin receptor and are dependent upon the amino-terminal portion of the pre-S2 domain (Franco et al 1992). These observations do not explain the hepatotropism of HBV, but may be relevant for the interactions of HBV with the immune systems and for the latency of HBV in liver-transplanted hosts.

SHBs and attachment. Studies with HepG2 cells and liver plasma membranes suggested that

SHBs is not directly involved in attachment (Neurath et al 1986b, Pontisso et al 1989), but Leenders et al (1990, 1992) observed attachment of SHBs particles to primary hepatocytes. Furthermore, a specific attachment of SHBs to Vero cells, a permanent primate kidney cell line, has been reported (Komai & Peeples 1990). While it cannot be excluded that such a cell may accidentally express a relevant receptor for HBV, the significance of that binding remains unclear. The existence of neutralizing antibodies toward SHBs, and of escape mutants to those antibodies (see Ch. 7), suggests that SHBs structures participate in the attachment, entry or both. Alternatively, such antibodies may mediate aggregation and immune elimination of HBV, or sterically hinder the pre-S domains.

Regulation of attachment. One reason for the unsatisfactory understanding of the attachment process may be that it is indeed of low affinity and possibly more non-specific than specific. Since high-titred viraemia and HBs antigenaemia belong to the evolutionary strategy of hepadnaviruses, a highly abundant and avid receptor should theoretically not exist. Otherwise, viraemia and antigenaemia could be never established and the potential target cells would be completely overloaded by regurgitated virus and HBs antigen. Binding to inducible receptors of low abundance would be one possible mechanism, and the membrane-bound interleukin or the pre-S2-glycan binding receptor of HepG2 would be candidates for such receptors. Alternatively, the natural ligand of the receptor would compete with the HBs protein, as may be the case with transferrin or IgA. Binding may also be mediated by a partially degraded serum protein that is destined for recycling in the liver, for example serum albumin. Finally, it may be considered that entry of HBV into hepatocytes is completely non-specific and may be compared to the hepatic uptake and biliary excretion of Indian ink particles. The species specificity of hepadnaviruses would then be more a matter of gene expression and immunotolerance, and the attachment sites mentioned above may be only an auxiliary factor.

Virus penetration into the cell

Two principal mechanisms by which animal viruses can enter a cell are known. The first is only used by certain enveloped viruses that possess fusion proteins. The fusion protein inserts a hydrophobic sequence into the lipid bilayer of the target cell and causes fusion of the viral membrane with the cellular membrane, thereby releasing the viral nucleocapsid into the cytoplasm. However, most enveloped and non-enveloped viruses use the second method, which requires receptor-mediated endocytosis into endosomal vesicles. The physiological acidification and/or proteases in the endosome activate(s) processes that allow(s) transition of the viral nucleocapsid or the genome alone to the cytosol. The mechanism of HBV entry into hepatocytes is not known. As mentioned above, the transferrin receptor allows not only for attachment to T lymphocytes but also for endocytosis and proteolytic processing of HBs particles, but the behaviour of virions and potential genome release have not yet been studied.

In the duck it was originally reported that infection could be blocked by substances that inhibit acidification of endosomes (Offensperger et al 1991), but recent data suggest that this is not the case (Rigg & Schaller 1992). Circumstantial evidence suggests that uptake of human HBV into hepatocytes may require proteolysis and acid pH, because the low-susceptible hepatoma cell HepG2 became more susceptible after proteolytic removal of the pre-S domains and incubation at pH 5.5 (Y Lu & W H Gerlich, unpublished).

Release of the viral genome

Even more obscure than penetration are the further steps of capsid transport and genome release. It is known that 24 h after infection DHBV DNA occurs in the nucleus and is converted to covalently closed circular molecules (Tuttleman et al 1986b). However, it is not known whether the free DNA or core particles are transported to the nucleus. An involvement of

the cytoskeleton during that process appears very likely.

Generation of episomal DNA

Currently, it is presumed that the replication of hepadnaviridae requires conversion of the virion DNA with its gap, terminal protein, RNA sequence and triple-stranded structure into a double-stranded covalently closed circle as shown in Figure 6.3c (Seeger et al 1991). It appears likely that the virion polymerase helps to close the single-stranded gap, but possibly further functions of virus proteins may contribute to this repair process. From the result of the cloning experiments in *E. coli* it is known that bacterial enzymes are able (1) to remove all unusual structural elements from the virion DNA, (2) to fill up the gap, (3) to close the nicks and (4) to supercoil the covalently closed HBV DNA. Analogous enzymes of the natural host cell, e.g. hepatocytes, probably also have this capability. Whether the HBV DNA in eukaryotic cells becomes associated with histones and forms nucleosomes has not yet been shown experimentally. A question of clinical relevance is the half-life of the episomal HBV DNA. In cell culture a half-life of 2–4 days has been found, but in vivo the extended latency period of HBV reactivation after liver transplantation suggests a longer survival time.

In contrast to the retroviridae, an integration of HBV DNA into host cell DNA is not only unneccessary for replication of HBV but even detrimental, because linearization of the circular HBV DNA would disrupt at least one gene and would prevent transcription of a functional RNA pregenome. Such a RNA pregenome can, however, be transcribed from linear DNA that is longer than the genome. Such artificial HBV DNA constructs have often been used to transfect cell lines in vitro, but such integrated replication-competent HBV DNA molecules are not found in HBV-infected liver or hepatocellular carcinomas (HCCs). What is occasionally found are fusions between HBV genes and host cell genes. The resulting neoproteins may acquire novel properties, and have been found in certain HCC as potentially oncogenic factors (see Ch. 15).

Transcription of HBV DNA into RNA

General aspects of transcription. Transcription of double-stranded DNA into RNA is a highly regulated process that controls the expression of all genes – whether from the host or the virus – in a timely and structurally ordered manner. Thus, all genomes including those of the smallest replication-competent DNA elements contain not only structural genes that encode protein sequences but also regulatory elements. These DNA sequences bind a number of cellular or viral proteins that act as positive or negative transcription factors. Positive factors bind the cellular RNA polymerase II at a more or less defined position of the DNA sequence, where it initiates RNA synthesis. The RNA polymerase transcribes one strand of the double-stranded DNA into RNA until a termination signal for this enzyme in the DNA sequence occurs.

There are two types of DNA sequence elements that contribute to the binding of transcription factors. *Promoters* are necessary for binding of essential transcription factors, and bring the RNA polymerase into a position where it begins to transcribe only one DNA strand at a defined site. Many promoters contain the so-called TATA box, which binds transcription factor (TF) IID. This factor binds RNA polymerase II in a way that transcription initiates approximately 30 bases downstream. *Enhancers* are DNA segments that enhance transcription initiation at a given promoter, but they do not need to be in an exactly defined position next to the promoter, and their sequence orientation may even be inverted without impairing their function. Thus, promoters are a clearly defined part of one gene, but enhancers, such as those of HBV, may act on expression of several genes.

The rate of RNA synthesis beginning at a promoter is controlled by the amount, type, and activity of positive transcription factors which are available in the host cells, and by the accessibility of enhancers and promoters. There are some essential ubiquitous transcription factors, others are organ specific and a third group is inducible by intra- or extra-cellular signals. Most regulatory gene sequences also contain negative elements that counteract the effect of promoters and

enhancers by binding negative transcription factors. Such sequence elements are termed *silencers.*

Methods for the study of transcriptional control elements. When studying their function, transcription-regulating DNA elements are usually separated from the other parts of the genome and linked to the structural gene of a protein that is not naturally present in the cell used for the experiment. These 'reporter genes' encode proteins which can be easily detected by their enzymatic activity. Typical reporter genes are the ones for chloramphenicol acetyltransferase (CAT) or bacterial luciferase. The DNA constructs are made in vitro and are transfected into the target cell, which is then analysed for the expression of the reporter gene. For the study of enhancers, these molecules are linked to a basal promoter that has a low activity in the target cell. Enhancers and promoter are called *cis-acting* transactivating elements because they must be on the same DNA strand as the gene that is to be expressed. *Trans-acting* elements are those DNA segments that also activate transcription when they are present on a separate DNA strand. Usually their effect is exerted by expression of a transcription factor.

Enhancers and promoters are usually composed of modular elements consisting of 6–10 bases, sometimes palindromic or a direct repeat. These elements bind single or dimerized transcription factors that need to cooperate for activation or suppression of transcription. Binding of such proteins to their target DNA can be studied by the reduced accessibility of this DNA to modifying reagents such as DNAase or methylating chemicals. They leave a so-called *footprint* in a sequence ladder of a DNA.

Promoters of HBV. Using the above-mentioned techniques, four promoters and two enhancers have been identified in the HBV genome (Siddiqui 1991, Schaller & Fischer, 1991). These promoters direct initation of RNA synthesis upstream of the HBc/e gene, LHBs gene, M/SHBs gene and the HBx gene as shown in Figure 6.3c. Only the LHBs promoter (often referred to as S promoter I, SpI) has a typical TATA box, and only the mRNA encoding LHBs protein has a sharply defined 5' end. The TATA-less promoters usually have multiple initiation sites, and this is indeed the case for the HBc/e, M/SHBs and X mRNAs. As a result the corresponding sets of mRNAs are able to encode nested sets of co-terminal proteins. The two longer of the HBc/e mRNAs encode HBe protein; the shorter starts four bases after the start codon for the the HBe protein. In this case the ribosome can only use the next start codon for protein synthesis, which defines the amino end of HBc protein. A similar phenomenon occurs for the mRNAs encoding MHBs and SHBs. Only the largest of three mRNAs that are initiated at this promoter (known also as SPII promoter) contains the start codon for MHBs; the other two mRNAs contain only the proximal start codon of SHBs. Whether there are mRNAs that encode only smaller HBx proteins but not full-length HBx protein is not clear.

The relative activity of the four promoters and the fine specificity of the heterogenous 5' ends is dependent on the cell type in which the HBV genome or fragments thereof are expressed. It appears that the M/SHBs promoter and the HBx promoter can be active in all mammalian cell lines (Seifer et al 1990a). The M/SHBs promoter contains, for example, four binding sites for ubiquitous constitutive transcription factor SpI (Raney et al 1992). The LHBs promoter requires the hepatic nuclear factor I (HNF I) (Chang et al 1989). The HBc/e promoter (as well as enhancer II) binds other liver-specific transcription factors such as HNF III and C/EBP in order to be activated (Yuh et al 1992).

Because the proteins expressed by the corresponding mRNAs are absolutely necessary for genome replication and virion assembly, the specificity of the transcription signals in the HBV genome may already be sufficient to explain its organ tropism. Replacement of the liver-specific HBc/e promoter by a ubiquitously active promoter such as the immediate-early promoter of cytomegalovirus allows replication of the HBV genome and assembly of complete core particles in non-hepatic cell lines (Seeger et al 1989). Addition of the SV40 enhancer to the HBV genome also allows for generation of virus-like particles in mouse fibroblasts (Seifer et al 1990b).

Termination of transcription. Not only the start but also the stop of transcription is a well-defined and regulated process. The genomes of all mammalian hepadnaviruses contain one TATAAA stop signal shortly after the start of the HBc gene as shown in Fig. 6.3c. It needs an additional sequence element upstream of the HBc/e promoter and, furthermore, it only becomes active if the initiation site of the RNA is more than 400 bases distant (Cherrington et al 1992). Thus, the stop signal is ignored during the first pass of the RNA polymerase II after initiation at the HBc/e promoter. An RNA of supergenomic length is generated, starting upstream or within the pre-C sequence and ending within the core gene. It has redundant ends ('R'), which are essential for the genome replication of hepadnaviridae (see Fig. 6.3c). All other RNAs of HBV are shorter than genome length. Thus, RNAs of 3.5 kb for HBc/e, 2.4 kb for LHBs, 2.1 kb for M/SHBs and 0.8 kb for HBx are formed. They are all co-terminal. The existence of several promoters and one common stop signal is an elegant way to express several different translation products from one very compact genome with overlapping genes. This expression strategy is unique for hepadnaviridae.

Enhancers. Transcription of HBV RNAs would probably be very low without the enhancer I. This DNA sequence element enhances the initiation of transcription by a factor of 10–50 in liver cells. Its effect is much lower in non-hepatic cells but it is not strictly liver specific (Shaul 1991, Siddiqui 1991). The effect of enhancer II is more liver specific (Yuh et al 1992). Extracellular factors may influence transcription as well. Addition of dexamethasone activates the glucocorticoid receptor, which then binds to the glucocorticoid-responsive elements (GREs) of the HBV genome and enhances transcription further by a factor of 2–5. This effect may be also present in vivo, because glucocorticoid-treated patients express very high levels of HBV, HBeAg and HBsAg.

Viral transcription activator proteins. All double-stranded DNA viruses and retroviruses contain positive sequence signals for initiation of transcription, but – at least in mammalian viruses – these cis-acting elements are obviously insufficient to warrant highly efficient expression and replication of the viral genome. For most viruses the first gene products to be expressed are transcription factors, which activate promoters of other viral genes in a time-ordered manner. Such products are called *immediate early (IE)* genes. The time order of HBV gene expression has not yet been fully analysed, but it appears that HBx protein is the first protein to be expressed (Wu et al 1991) and it has been shown that HBx activates many enhancers (Rossner 1992). It links cellular transcription factors together and stabilizes their binding to enhancer I (Maguire et al 1991). Furthermore, HBx protein activates formation of diacylglycerol. This activates protein kinase C, which then phosphorylates the transcription factor AP1 (Kekulé et al 1993). AP1 acts on many promoter/enhancers, including the HBV enhancer and the promoters of cellular proto-oncogenes c-*myc* and c-*fos* (Balsano et al 1991). Furthermore, other transcription-activating pathways can be opened by HBx protein, e.g. one that uses reactive oxygen intermediates for activation of the transcription factor NFKB (Meyer et al 1992).

Oncogenic transcription factors of HBV. In this respect HBx is similar to the IE proteins of oncogenic DNA viruses, such as E6/E7 of papillomaviruses or E1A/B of adenoviruses. These viral transcription activators are also quite non-specific and act also on cellular growth-controlling genes. Furthermore they have the ability to inactivate the cellular tumour-suppressor protein p53 or Rb. HBx inactivates p53 in that p53 is hyperphosphorylated and retained in the cytoplasm (Höhne et al 1993). An HBV protein that inactivates Rb has not yet been observed. However, a further transcription activator of HBV has been discovered in HBV DNA-containing hepatocellular carcinomas. These are truncated LHBs (Caselmann et al 1990) or MHBs (Kekulé et al 1990) proteins that have lost the sequences necessary for budding and secretion of HBs particles. Whether this type of protein has a function in the normal viral life cycle is questionable, because the genes for these proteins are generated only by integration of HBV DNA fragments with a truncated S domain.

Expression of IE proteins is down-regulated by

negative feedback on their transcription by the gene products that are induced by them. It appears that this is also the case in HBV infection. This may explain why in vivo mRNA for HBx is not very abundant during acute infection or is even is undetectable (Will et al 1987). A dangerous situation for the host occurs when viral DNA fragments are integrated into a host genome that is able to express IE proteins but not the genes exerting the negative feedback. Such a situation is found in tumorigenesis by oncogenic adeno- or papillomaviruses and may also occur with HBV, because HBV-associated HCCs often contain HBx genes but no HBc gene, which may represent a candidate for a negative feedback factor on transcription of HBx (Twu & Schloemer, 1989).

Processing of HBV RNAs. After transcription, RNAs are modified by addition of methylated bases to the 5' end (known as a cap) and addition of a poly-(A) tail at the 3' end. Most eukaryotic RNAs are further modified by splicing, i.e. removal of internal non-coding RNA regions (introns) and ligation of the remaining fragments (exons), which finally constitute the mRNA. However, all known HBV genes are contiguous and, thus, the mRNA precursors mentioned above must not be spliced, although numerous potential splice signals are present in the RNA. Spliced HBV RNAs are, however, found in HCCs (Su et al 1989). It appears possible that as yet unrecognized spliced mRNAs lead to fused HBV gene products that may contain portions of different ORFs.

Transport of HBV mRNAs does not seem to be restricted. The half-life of the mRNAs in the plasma has not been well studied, but it has been reported that fusion of viral RNA with a 3' terminal portion derived from host cell DNA may enhance the stability of such a hybrid RNA (von Loringhoven et al 1985).

Translation

General aspects of translation. Protein synthesis in eukaryotic cells is predominantly regulated by the site and frequency of its initiation at the mRNA. The scanning model of this process applies to most mRNAs. The small ribosome subunit binds to the 'capped' 5'-end of the mRNA and thereafter scans the sequence until an AUG start codon for methionine occurs. Protein biosynthesis starts from this site after binding of initiation factors and the large subunit of the ribosomes. The efficiency of protein synthesis at a start codon is influenced by flanking bases. A purine at the +4 and/or -3 position is usually found in initiation codons, but not in internal codons for methionine (Kozak 1989). Synthesis stops at one of the three possible stop codons, UAA, UAG or UGA, that occur first in this particular ORF. Thus, the position of the 5'-ends of the mRNAs in the HBV genome determines which gene product is expressed.

Exceptions to this rule are: internal ribosome entry sites (IRES), leaky scanning for start codons, use of atypical start codons, readthrough of stop codons, frame shifting and reinitiation. Some of these exceptions may apply to the translation of certain HBV proteins.

An important early step in protein biosynthesis is the distribution of the protein synthetic complex between the cytosol and the endoplasmic reticulum (ER). Nascent protein chains that contain a hydrophobic α-helical stretch of 16–20 amino acids bind to a signal recognition particle, which attaches the ribosome to a pore-like structure at the ER. The growing peptide is transported through this pore to the lumen of the ER. The particular structure and number of such hydrophobic signal sequences determines the intracellular distribution or secretion of the protein. Proteins without signal sequence are synthesized by free ribosomes and released after completion to the cytotosol. Enveloped viruses with subgenomic mRNAs, such as HBV, normally use free ribosomes for synthesis of their nucleocapsid proteins, and ER-bound ribosomes for synthesis of the envelope proteins, as shown in Figure 6.6

HBe protein. As mentioned above, the pre-C sequence encodes a signal sequence that makes HBe protein into a secreted protein. As with many secreted proteins, the amino-terminal signal sequence of HBe is cleaved by the cellular signal peptidase at the ER lumen (Standring et al 1988). Further proteolytic processing of the HBe protein probably occurs in the trans-Golgi

apparatus (Wang et al 1991). This cellular compartment contains proteases (e.g. furin) that cleave within the arginine-rich carboxy-terminal domain of the HBe protein. Since the signal peptide of HBe protein is not particularly strong, a significant part of the HBe protein remains cytoplasmic (Yang et al 1992). The amount of synthesized HBe protein relative to other HBV proteins is probably regulated by the level of mRNA containing the start codon of an uninterrupted HBe gene. It is likely that the HBe mRNA may be used for synthesis of the polymerase protein (Seifer et al 1990b).

HBc protein. The start codon of HBc protein has two guanosines at positions -3 and +4, which is a relatively good but not optimal context for initiation. A highly efficient translation of the HBc mRNA is necessary, because at a critical level of HBc protein and polymerase protein this mRNA is encapsidated into core particles and is no longer available for protein synthesis (Bartenschlager & Schaller 1992). Co-translational modifications probably do not occur.

Polymerase. An intensive search for an mRNA containing a 5'-end slightly upstream of the presumed amino end of the polymerase (pol) sequence was not successful. Most orthoretroviridae have developed the strategy of pol expression to induce a frame shift between the ORFs of gag (i.e. the analogue of HBc protein) and pol, thus generating a gag–pol fusion protein. Mutational analysis excluded this possibility for HBV. Instead, an unusual internal initiation of the pol protein synthesis at the HBc mRNA is assumed. This would generate a full-length non-fused HBV polymerase (Schlicht et al 1991). A leaky scanning mechanism appears possible, because an optimal start codon of ORF C reduces the efficiency of pol expression (Lin & Lo 1992). There are, however, several stronger initiation codons upstream in the HBc mRNA. The existence of a weak internal ribosome entry site in proximity to the pol start codon would be another obvious explanation (Jean-Jean et al 1989). Ideally, the relative frequency of initiation at the HBc or pol start should regulate the required proportion of HBc to pol protein, which is probably 180:1. Mutational analysis suggests that pol protein initiates encapsidation of the HBc mRNA and itself into core particles predominantly (but not exclusively) in cis (Hirsch et al 1990). This means that the pol protein binds to its own template directly after its completion.

LHBs. Initiation of LHBs protein synthesis follows the usual rules (Gallina et al 1992). It occurs initially in the cytosol because the pre-S domains do not contain an ER translocation signal peptide. The signal peptides I and II of the S domain insert the growing LHBs into the ER membrane, but possibly the entire pre-S domain is too long to be translocated to the ER lumen during protein synthesis (V Bruss, personal communication). In agreement with this conclusion is the absence of glycoside in the pre-S2 domain of LHBs. However, another co- or post-translational modification occurs with all LHBs molecules. The amino-terminal methionine is replaced by myristic acid, which links it to the cytosolic side of cellular membranes. The central hydrophilic part of the S domain is, however, in the lumen of the ER and more efficiently glycosylated than the S domain of MHBs or SHBs. The mRNA for LHBs is not translated well into MHBs or SHBs (Masuda et al 1990, Gallina et al 1992).

MHBs and SHBs. These two gene products are usually coexpressed because of the common promoter for their mRNAs. It is not clear which factors control the relative ratio of the mRNAs containing a start for MHBs or only for SHBs. Irrespective of this transcriptional regulation, the ratio of MHBs to SHBs is also regulated at the translational level. The start codon of MHBs does not have the optimal flanking bases, whereas that of SHBs is optimal for initiation of protein synthesis. Thus, mRNAs for MHBs also always express some SHBs unless the start codon of SHBs has been mutated away.

Signal I of the S domain is obviously able to translocate the pre-S2 domain of MHBs to the ER lumen rapidly, because MHBs is always glycosylated in secreted HBs particles (Eble et al 1990). However, the folding of the nascent S domains seems to be slightly different in MHBs and SHBs, because the S domain is only rarely glycosylated in MHBs (Heermann & Gerlich 1991).

HBx. Translation of HBx protein(s) may occur also from mRNAs that contain or do not contain the first start codon of ORF X (Seifer et al 1990a), but the size of naturally occurring HBx proteins has not yet been reliably elucidated. Sequence predictions suggest that it is a cytosolic protein. Overexpression of HBx protein using vaccinia vectors suggests that it is a very labile phosphoprotein within the cell, with a half-life of 20 min (Schek et al 1991). The insolubility of HBx protein suggests that it oligomerizes rapidly or binds to other cellular proteins.

Formation of virions

Encapsidation of pregenomic RNA. After translation of sufficient amounts of HBc proteins and at least one polymerase protein molecule, these proteins assemble together with their mRNA to form the core particle, as shown in Figure 6.6. Encapsidation occurs only when the polymerase interacts with a specific RNA sequence present at the 5' end (base 1846 to base 1907) of the HBc mRNA (Junker-Niepmann et al 1990). This signal is termed ε (for encapsidation). It is characterized by a secondary structure consisting of a stem, a bulge, a loop and a non-paired U. These elements and the sequence of the loop are required and sufficient for encapsidation. The signal sequence ε is also present in the mRNA for HBe, but there it is obviously not folded to the required secondary structure because of the movement of the ribosome during synthesis of HBe protein (Nassal et al 1990). Since the sequence of ε is also present in the 3' terminal part of all known subgenomic mRNAs of HBV, an unknown interaction of this ε* with 3' terminal sequences in HBV mRNAs may be postulated which prevents encapsidation.

Empty core particles. Empty or nearly empty core particles are found in the hepatocyte nuclei of patients with severe viraemia (Gerlich et al 1982). They probably do not contain HBV DNA, RNA or polymerase. It is not known whether these empty particles are primary assembly products of HBc protein, or whether they are remnant or reassembled from complete core particles that have migrated to the nucleus and released their nucleic acid. Both complete and empty particles contain phosphorylated HBc protein and an endogenous protein kinase activity. It is likely that the kinase is packaged while it is bound to HBc protein.

Envelopment of core particles. The assembly of core particles occurs in the cytosol, but after assembly HBc protein seems to exhibit an affinity for ER membranes that contain inserted LHBs molecules (Bruss & Ganem 1991b). It appears that only the last 16 carboxy-terminal amino acids of the pre-S1 domain in LHBs are necessary for envelopment (V Bruss, personal communication). This length of the pre-S sequence seems to be sufficient to keep LHBs in a conformation such that it can promote binding of core particles. For secretion of enveloped virions an excess of SHBs protein is necessary. It appears that the envelope is formed by mixed aggregates of LHBs, MHBs and SHBs. For secretion, virions and accompanying HBs particles move from the ER via the Golgi apparatus to the cell surface (Ganem 1991). During this migration within transport vesicles the HBs protein is further modified similar to normal cellular secreted proteins. The glycoside side chains of the HBs proteins are trimmed and modified, and covalent disulphide bridges are formed within and between HBc and HBs subunits. It appears that the release of virions and HBs particles from secretory vesicles does not require any specific signal and follows the constitutive pathway of secretion.

Human hepatoma cell lines such as HepG2 and Huh7 express a suitable ratio of all HBV proteins to allow for virion assembly and secretion. Immortalized mouse hepatocyte cultures (Höhne et al 1990) are also able to produce HBV. In vivo, not all hepatocytes may be able to express all HBV proteins in a suitable ratio. Thus, in histological specimens from persistently infected liver only few cells contain HBV DNA, HBcAg and HBsAg, while many cells contain HBsAg and HBcAg alone.

Replication of the viral genome

General strategy. The actual multiplication of the HBV genome occurs in the nucleus of the infected cell by the cellular RNA polymerase II,

which transcribes the circular HBV DNA to more than genome-length mRNA with redundant ends (see Fig. 6.3 c). HBx protein may possibly support or enhance this process but is not essential in cell culture. All other essential gene products of HBV are used to encapsidate that RNA, transcribe it to a circular DNA and secrete it as an enveloped virion with attachment sites for new target cells. An overview is given in Figure 6.7.

Signal for reverse transcription. In the woodchuck hepatitis virus model it has been shown that the 3' terminal direct repeat 1 (DR*) in the pregenomic RNA is the natural site of initiation. Essential elements of this site are a short sequence UUUC, within the seven bases that extend from -1 to +6 of DRI*. However this signal for reverse transcription needs to be in the context of 1000 bases upstream of DR1* (Seeger & Maragos, 1990, 1991).

Priming of DNA minus strand. Previously, it was believed that reverse transcription would only occur after encapsidation (Schlicht et al 1991), but recently a DHBV polymerase protein translated in vitro was found to reversely transcribe its own mRNA, beginning at the cytosine within DR1* of the RNA. As with virion DNA, the newly synthesized product was linked to the polymerase protein itself. The first step was addition of guanosine monophosphate to the polymerase protein. Previous studies have shown that virion DNA was linked via an alkali-resistant bond to the amino-terminal domain of the polymerase, probably to a tyrosine. The priming of this reaction is resistant to the polymerase inhibitor

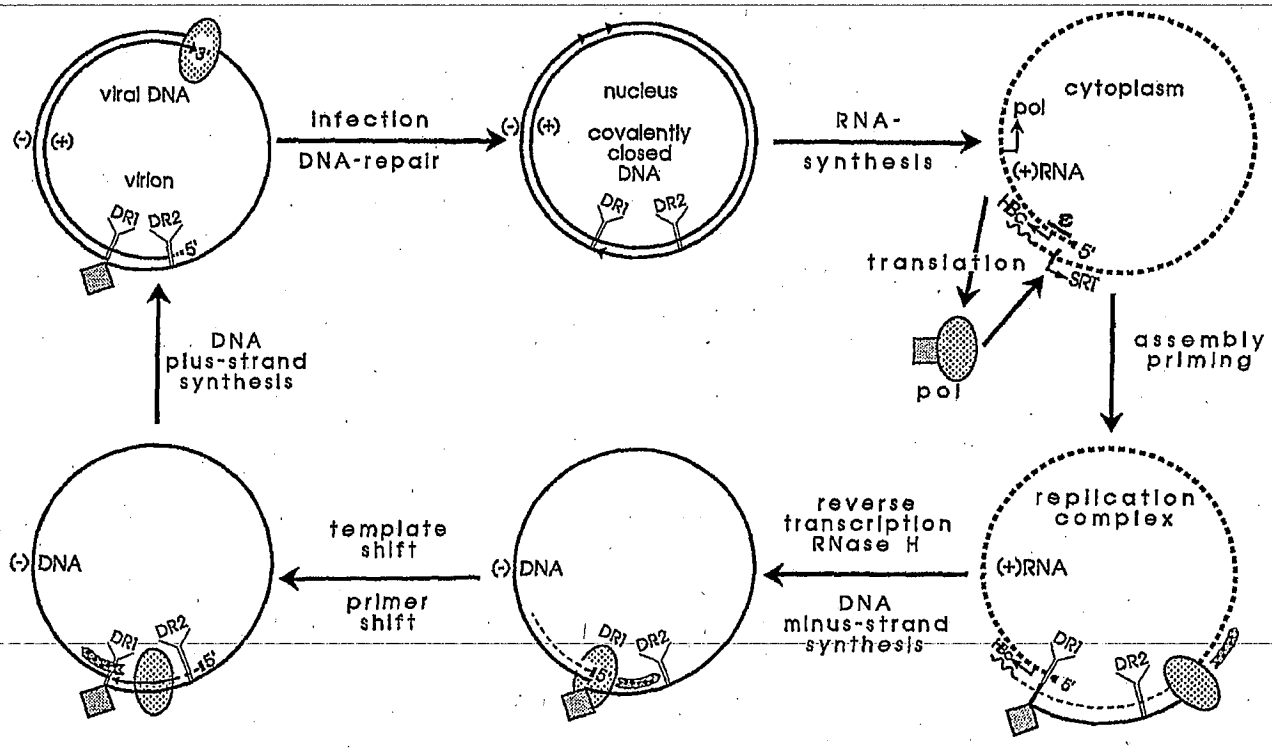

Fig. 6.7 *Replication of the HBV genome.* The virion DNA (top left, see also Fig. 6.3a) is brought by the mechanism shown in Figure 6.6 to the cell nucleus and is converted to covalently closed DNA. This episomal DNA is transcribed to various RNAs (see also Figures 6.3c and 6.6), one ofwhich serves as template for the polymerase protein (→) and HBc protein (→). These two proteins assemble together with their mRNA to the replication complex. The encapsidation signal e at its 5' end governs the packaging of the RNA. The redundant part of the 3' end serves as a signal for reverse transcription (SRT). The primase domain of the polymerase (♦) serves as a primer for the reverse transcriptase. Thus, the growing minus DNA strand (see bottom right) is linked at its 5' end to the primase. The reverse transcription proceeds until the 5' end of the RNA template is reached (bottom centre). Thus, a short redundancy is generated in the minus strand. The RNAase H activity associated with the reverse transcriptase degrades the RNA tempalte, and leaves at its 5' end an 18-base-long capped RNA fragment. This fragment has a six base homology to the direct repeat DR1 and DR2. This weak interaction allows shifting of the fragment and of the reverse transcriptase/DNA polymerase from DR1 to DR2. There, the RNA fragment functions as primer for the plus-strand DNA. The DNA polymerase is able to cross the discontinuity in the minus-strand template owing to its short terminal redundancy. Thereafter, the structure of the virion DNA is reproduced. The multiplication effect originates from the fact that one episomal HBV genome can be transcribed to many pregenome molecules by the cellular RNA polymerase II.

phosphonoformic acid, but elongation of the DNA is not (GH Wang & Seeger 1992).

Elongation of DNA minus strands. DNA minus strands of heterogeneous length with less than 3200 bases are often found as replicative intermediates in HBV-producing cells. Presumably most of these intermediates are within core particles, but in view of the above-mentioned results it cannot be excluded that there are also free intermediates. Normally, the pol-expressing RNA would contain ε and would be encapsidated. In a full cycle of DNA synthesis, reverse transcription will proceed until the pregenome is completely transcribed. This generates a nine-base-long redundancy (r) in the DNA minus strand (Seeger et al 1991).

RNAase H and priming of the DNA plus strand. All reverse transcriptases are associated with an RNAase H activity, which cleaves the RNA of RNA–DNA hybrids into oligoribonucleotides. Mutational inactivation of the RNAase H domain in the HBV polymerase results in a block of DNA plus-strand synthesis (Radziwill et al 1990). Most important for plus-strand synthesis is the generation of an 18-base-long capped RNA fragment from the 5' end of the pregenome. This fragment dissociates for unknown reasons from its DR1 site in the DNA counterstrand and is translocated to DR2 of the same strand. Here it functions as a primer for the DNA plus strand. The DNA begins with the last base of DR2 (Seeger et al 1991).

Circularization of the genome. In Figure 6.7 the pregenome has been drawn as a circle for the sake of simplicity, although the tertiary structure of the pregenome is not known. It is, however, reasonable to assume a structure in which the 5' and 3' short redundances (r) come into close proximity. Otherwise the plus-strand DNA synthesis would stop at the 5' end of the minus strand. However, the polymerase is able to switch at r to the 3' terminal part of the minus strand as template. The circular structure of the non-covalently linked minus strand is then stabilized by the plus strand, which bridges the discontinuity.

Normally, the plus strand is not completed within the core particle or the virion. It is not clear whether this is due to steric hindrance within the core particle, due to inverted repeats within the minus strand acting as pause sites, or due to secretion of the virion to the extracellular space where no deoxynucleotide triphosphates are available.

Conclusion

With minor variations all hepadnaviridae replicate in the same manner. One of the most surprising results in the study of their replication was the finding that all of the seemingly strange properties of these viruses are a logical consequence of their biological strategy as a small persistent virus in the bloodstream.

REFERENCES

Albin C, Robinson W S 1980 Protein kinase activity in hepatitis B virus. Journal of Virology 34: 297–302

Aldrich C E, Coates L, Wu T T, Newbold J, Tennant B C, Summers J, Seeger C, Mason W S 1989 In vitro infection of woodchuck hepatocytes with woodchuck hepatitis virus and ground squirrel hepatitis virus. Virology 172: 247–252

Almeida J D, Rubenstein D, Stott E J 1971 New antigen–antibody system in Australia-antigen-positive hepatitis. Lancet ii: 1225–1227

Argos P, Fuller S D 1988 A model for the hepatitis B virus core protein: prediction of antigenic sites and relationship to RNA virus capsid proteins. EMBO Journal 7: 819–824

Arii M, Takada S, Koike K 1992 Identification of three essential regions of hepatitis B virus X protein for trans-activation function. Oncogene 7: 397–403

Balsano C, Avantaggiati M L, Natoli G, De Marzio E, Will H, Perricaudet M, Levrero M 1991 Full-length and truncated versions of the hepatitis B virus (HBV) X protein (pX) transactivate the cmyc protooncogene at the transcriptional level. Biochemical and Biophysical Research Communications 176: 985–992

Bancroft W H, Mundon F K, Russell P K 1972 Detection of additional antigenic determinants of hepatitis B antigen. Journal of Immunology 109: 842–848

Bartenschlager R, Schaller H 1988 The amino-terminal domain of the hepadnaviral P-gene encodes the terminal protein (genome-linked protein) believed to prime reverse transcription. EMBO Journal 7: 4185–4192

Bartenschlager R, Schaller H 1992 Hepadnaviral assembly is initiated by polymerase binding to the encapsidation signal in the viral RNA genome. EMBO Journal 11: 3413–3420

Bavand M R, Laub O 1988 Two proteins with reverse transcriptase activities associated with hepatitis B virus-like particles. Journal of Virology 62: 626–628

Bavand M, Feitelson M, Laub O 1989 The hepatitis B virus-associated reverse transcriptase is encoded by the viral pol gene. Journal of Virology 63: 1019–1021

Bchini R, Capel F, Dauguet C, Dubanchet S, Petit M A 1990 In vitro infection of human hepatoma (HepG2) cells with hepatitis B virus. Journal of Virology 64: 3025–3032

Birnbaum F, Nassal M 1990 Hepatitis B virus nucleocapsid assembly: primary structure requirements in the core protein. Journal of Virology 64: 3319–3330

Blum H E, Zhang Z S, Galun E, von Weizsäcker F, Garner B, Liang T J, Wands J R 1992 Hepatitis B virus X protein is not central to the viral life cycle in vitro. Journal of Virology 66: 1223–1227

Blumberg B S, Gerstley B S J, Hungerford D A, London W T, Sutnick A J 1967 A serum antigen (Australia antigen) in Down's syndrome, leukemia, and hepatitis. Annals of Internal Medicine 66: 924–931

Bruss V, Ganem D 1991a Mutational analysis of hepatitis B surface antigen particle assembly and secretion. Journal of Virology 65: 3813–3820

Bruss V, Ganem D 1991b The role of envelope proteins in hepatitis B virus assembly. Proceedings of the National Academy of Sciences of the USA 88: 1059–1063

Bruss V, Gerlich W H 1988 Formation of transmembranous hepatitis B e-antigen by cotranslational in vitro processing of the viral precore protein. Virology 163: 268–275

Caselmann W H, Meyer M, Kekule A S, Lauer U, Hofschneider P H, Koshy R 1990 A trans-activator function is generated by integration of hepatitis B virus preS/S sequences in human hepatocellular carcinoma DNA. Proceedings of the National Academy of Sciences of the USA 87: 2970–2974

Chang H K, Wang B Y, Yuh C H, Wei C L, Ting L P 1989 A liver-specific nuclear factor interacts with the promoter region of the large surface protein gene of human hepatitis B virus. Molecular and Cellular Biology 9: 5189–5197

Chen H S, Kew M C, Hornbuckle W E et al 1992 The precore gene of the woodchuck hepatitis virus genome is not essential for vial replication in the natural host. Journal of Virology 66: 5682–5684

Cherrington J, Russnak R, Ganem D 1992 Upstream sequences and cap proximity in the regulation of polyadenylation in ground squirrel hepatitis virus. Journal of Virology 66: 7589–7596

Chen H S, Kaneko S, Girones R et al 1993 The woodchuck hepatitis virus X gene is important for establishment of virus infection in woodchucks. Journal of Virology 66: 1218–1226

Chisari F V, Filippi P, Buras J et al 1987 Structural and pathological effects of synthesis of hepatitis B virus large envelope polypeptide in transgenic mice. Proceedings of the National Academy of Sciences of the USA 84: 6909–6913

Dane D S, Cameron C H, Briggs M 1970 Virus-like particles in serum of patients with Australia-antigen-associated hepatitis. Lancet i: 695–698

Dejean A, Sonigo P, Wain Hobson S, Tiollais P 1984 Specific hepatitis B virus integration in hepatocellular carcinoma DNA through a viral 11-base-pair direct repeat. Proceedings of the National Academy of Sciences of the USA 81: 5350–5354

Eble B E, Lingappa V R, Ganem D 1986 Hepatitis B surface antigen: an unusual secreted protein initially synthesized as a transmembrane polypeptide. Molecular and Cellular Biology 6: 1454–1463

Eble B E, Macrae D R, Lingappa V R, Ganem D 1987 Multiple topogenic sequences determine the transmembrane orientation of the hepatitis B surface antigen. Molecular and Cellular Biology 7: 3591–3601

Eble B E, Lingappa V R, Ganem D 1990 The N-terminal (pre-S2) domain of a hepatitis B virus surface glycoprotein is translocated across membranes by downstream signal sequences. Journal of Virology 64: 1414–1419

Francki R I B, Fauquet C M, Knudson D L, Brown F 1991 Classification and nomenclature of viruses. Springer, Vienna

Franco A, Paroli M, Testa U, Benvenuto R, Peschle C, Balsano F, Barnaba V 1992 Transferrin receptor mediates uptake and presentation of hepatitis B envelope antigen by T lymphocytes. Journal of Experimental Medicine 175: 1195–1205

Galibert F, Mandart E, Fitoussi F, Tiollais P, Charnay P 1979 Nucleotide sequence of the hepatitis B virus genome (subtype ayw) cloned in *E. coli*. Nature 281: 646–650

Gallina A, Bonelli F, Zentilin L, Rindi G, Muttini M, Milanesi G 1989 A recombinant hepatitis B core antigen polypeptide with the protamine-like domain deleted self-assembles into capsid particles but fails to bind nucleic acids. Journal of Virology 63: 4645–4652

Gallina A, De Koning A, Rossi F, Calogero R, Manservigi R, Milanesi G 1992 Translational modulation in hepatitis B virus preS-S open reading frame expression. Journal of General Virology 73: 139–148

Ganem D 1991 Assembly of hepadnaviral virions and subviral particles. Current Topics in Microbiology and Immunology 168: 61–83

Gerlich W H, Robinson W S 1980 Hepatitis B virus contains protein attached to the 5' terminus of its complete DNA strand. Cell 21: 801–809

Gerlich W H, Goldmann U, Müller R, Stibbe W, Wolff W 1982 Specificity and localization of the hepatitis B virus-associated protein kinase. Journal of Virology 42: 761–766

Gripon P, Diot C, Theze N, Fourel I, Loreal O, Brechot C, Guguen Guillouzo C 1988 Hepatitis B virus infection of adult human hepatocytes cultured in the presence of dimethyl sulfoxide. Journal of Virology 62: 4136–4143

Gripon P, Diot C, Guguen-Guilhozo C 1993 Reproducible high level infection of cultured adult human hepatocytes by hepatitis B virus: effect of polyethylene glycol on adsorption and penetration. Virology 192: 534–540

Gudat F, Bianchi L, Sonnabend W, Thiel G, Aenishaenslin W, Stalder G A 1975 Pattern of core and surface expression in liver tissue reflects state of specific immune response in hepatitis B. Laboratory Investigations 32: 1–9

Hatton T, Zhou S, Standring D N 1992 RNA- and DNA-binding activities in hepatitis B virus capsid protein: a model for their roles in viral replication. Journal of Virology 66: 5232–5241

Heermann K H, Gerlich W S 1991 Surface proteins of hepatitis B viruses. In: McLachlan A (ed) Molecular Biology of the hepatitis B viruses. CRC Press, Boca Raton pp 109–144

Heermann K H, Goldmann U, Schwartz W, Seyffarth T, Baumgarten H, Gerlich W H 1984 Large surface proteins of hepatitis B virus containing the pre-S sequence. Journal of Virology 52: 396–402

Heermann K H, Kruse F, Seifer M, Gerlich W H 1987 Immunogenicity of the gene S and pre-S domains in hepatitis B virions and HBsAg filaments. Intervirology 28: 14–25

Hirsch R C, Lavine J E, Chang L J, Varmus H E, Ganem D 1990 Polymerase gene products of hepatitis B viruses are required for genomic RNA packaging as well as for reverse transcription. Nature 344: 552–555

Höhne M, Schaefer S, Seifer M, Feitelson M A, Paul D, Gerlich W H 1990 Malignant transformation of immortalized transgenic hepatocytes after transfection with hepatitis B virus DNA. EMBO Journal 9: 1137–1145

Horiike N, Blumberg B S, Feitelson M A 1991 Characteristics of hepatitis B X antigen, antibodies to X antigen, and antibodies to the viral polymerase during hepatitis B virus infection. Journal of Infectious Diseases 164: 1104–1112

Howard C R 1991 Hepadnaviridae. In: Francki RIB, Fauquet C M, Knudson, P L, Brown F (eds) Classification and nomenclature of viruses. Springer, Vienna, pp 111–116

Itoh Y, Takai E, Ohnuma H et al 1986 A synthetic peptide vaccine involving the product of the pre-S(2) region of hepatitis B virus DNA: protective efficacy in chimpanzees. Proceedings of the National Academy of Sciences of the USA 83: 9174–9178

Jean-Jean O, Levrero M, Will H, Perricaudet M, Rossignol J M 1989 Expression mechanism of the hepatitis B virus (HBV) C gene and biosynthesis of HBe antigen. Virology 170: 99–106

Junker Niepmann M, Bartenschlager R, Schaller H 1990 A short cis-acting sequence is required for hepatitis B virus pregenome encapsidation and sufficient for packaging of foreign RNA. EMBO Journal 9: 3389–3396

Kaplan P M, Greenman R L, Gerin J L, Purcell R H, Robinson W S 1973 DNA polymerase associated with human hepatitis B antigen. Journal of Virology 12: 995–1005

Kekulé A S, Lauer U, Meyer M, Caselmann W H, Hofschneider P H, Koshy R 1990 The preS2/S region of integrated hepatitis B virus DNA encodes a transcriptional transactivator. Nature 343: 457–461

Kekulé A S, Lauer U, Weiss L, Luber B, Hofschneider P H 1993 Hepatitis B virus transactivator HBx uses a tumour promoter signalling pathway. Nature 361: 742–745

Kim C M, Koike K, Saito I, Miyamura T, Jay G 1991 HBx gene of hepatitis B virus induces liver cancer in transgenic mice. Nature 351: 317–320

Komai K, Peeples M E 1990 Physiology and function of the vero cell receptor for the hepatitis B virus small S protein. Virology 177: 332–338

Korba B E, Cote P J, Gerin J L 1988 Mitogen-induced replication of wood-chuck hepatitis virus in cultured peripheral blood lymphocytes. Science 241: 1213–1216

Kozak M 1989 The scanning model for translation and uptake. Journal of Cellular Biology 108: 229–241

Krone B, Lenz A, Heermann K H, Seifer M, Lu X, Gerlich W H 1990 Interaction between hepatitis B surface proteins and monomeric human serum albumin. Hepatology 11: 1050–1056

Kwee L, Lucito R, Aufiero B, Schneider R J 1992 Alternate translation initiation on hepatitis B virus X mRNA produces multiple polypeptides that differentially transactivate class II and III promoters. Journal of Virology 66: 4382–4389

Landers T A, Greenberg H B, Robinson W S 1977 Structure of hepatitis B Dane particle DNA and nature of the endogenous DNA polymerase reaction. Journal of Virology 23: 368–376

Lanford R E, Notvall L 1990 Expression of hepatitis B virus core and precore antigens in insect cells and characterization of a core-associated kinase activity. Virology 176: 222–233

Lauer U, Weiss L, Hofschneider P H, Kekule A S 1992 The hepatitis B virus pre-S/S(t) transactivator is generated by 3' truncations within a defined region of the S gene. Journal of Virology 66: 5284–5289

Le Bouvier G L, McCollum R W, Hierholzer W J J, Irwin G R, Krugman S, Giles J P 1972 Subtypes of Australia antigen and hepatitis-B virus. Journal of the American Medical Association 222: 928–930

Leenders W P, Glansbeek H L, de Bruin W C, Yap S H 1990 Binding of the major and large HBsAg to human hepatocytes and liver plasma membranes: putative external and internal receptors for infection and secretion of hepatitis B virus. Hepatology 12: 141–147

Leenders W P, Hertogs K, Moshage H, Yap S H 1992 Host and tissue tropism of hepatitis B virus. Liver 12: 51–55

Lin C G, Lo S J 1992 Evidence for involvement of a ribosomal leaky scanning mechanism in the translation of the hepatitis B virus pol gene from the viral pregenome RNA. Virology 188: 342–352

Machida A, Kishimoto S, Ohnuma H et al 1983 A hepatitis B surface antigen polypeptide (P31) with the receptor for polymerized human as well as chimpanzee albumins. Gastroenterology 85: 268–274

Machida A, Kishimoto S, Ohnuma H et al 1984 A polypeptide containing 55 amino acid residues coded by the pre-S region of hepatitis B virus deoxyribonucleic acid bears the receptor for polymerized human as well as chimpanzee albumins. Gastroenterology 86: 910–918

Machida A, Ohnuma H, Tsuda F 1991 Phosphorylation in the carboxyterminal domain of the capsid protein of hepatitis B virus: evaluation with a monoclonal antibody. Journal of Virology 12: 1017–1021

Mack D H, Bloch W, Nath N, Sninsky J J 1988 Hepatitis B virus particles contain a polypeptide encoded by the largest open reading frame: a putative reverse transcriptase. Journal of Virology 62: 4786–4790

Madalinski K, Burczynska B, Heermann K H, Uy A, Gerlich W H 1991 Analysis of viral proteins in circulating immune complexes from chronic carriers of hepatitis B virus. Clinical and Experimental Immunology 84: 493–500

Magnius L O, Espmark A 1972 A new antigen complex co-occurring with Australia antigen. Acta Pathologica Microbiologica Scandinavia B 80: 335–337

Maguire H F, Hoeffler J P, Siddiqui A 1991 HBV X protein alters the DNA binding specificity of CREB and ATF-2 by protein–protein interactions. Science 252: 842–844

Marion P L, Oshiro L S, Regnery D C, Scullard G H, Robinson W S 1980 A virus in Beechey ground squirrels that is related to hepatitis B virus of humans. Proceedings of the National Academy of Sciences of the USA 77: 2941–2945

Marquardt O, Heermann K H, Seifer M, Gerlich W H 1987 Cell type specific expression of pre S 1 antigen and secretion of hepatitis B virus surface antigen. Archives of Virology 96: 249–256

Mason W S, Seal G, Summers J 1980 Virus of Pekin ducks with structural and biological relatedness to human hepatitis B virus. Journal of Virology 36: 829–836

Masuda M, Yuasa T, Yoshikura H 1990 Effect of the preS1 RNA sequence on the efficiency of the hepatitis B virus preS2 and S protein translation. Virology 174: 320–324

Melegari M, Bruss V, Gerlich W H 1991 The arginine-rich

carboxyterminal domain is necessary for RNA packaging by hepatitis B core protein. In: Hollinger F B, Lemon S M, Margolis H (eds) Viral hepatitis and liver disease. Williams & Wilkins, Baltimore, pp 164–168

Meyer M, Caselmann W H, Schlüter V, Schreck R, Hofschneider P H, Baeuerle P A 1992 Hepatitis B virus transactivator MHBs^t: activation of NF-kappa B, selective inhibition by antioxidants and integral membrane localization. EMBO Journal 11: 2991–3001

Milich D R, Jones J E, Hughes J L, Price J, Raney A K, McLachlan A 1990 Is a function of the secreted hepatitis B e antigen to induce immunologic tolerance in utero? Proceedings of the National Academy of Sciences of the USA 87: 6599–6603

Miller R H 1991 Evolutionary relationship between hepadnaviruses and retroviruses. In: McLachlan A (ed) Molecular biology of the hepatitis B virus. CRC Press, Boca Raton, pp 227–244

Nassal M 1992a The arginine-rich domain of the hepatitis B virus core protein is required for pregenome encapsidation and productive viral positive-strand DNA synthesis but not for virus assembly. Journal of Virology 66: 4107–4116

Nassal M 1992b Conserved cysteines of the hepatitis B virus core protein are not required for assembly of replication-competent core particles nor for their envelopment. Virology 190: 499–505

Nassal M, Junker Niepmann M, Schaller H 1990 Translational inactivation of RNA function: discrimination against a subset of genomic transcripts during HBV nucleocapsid assembly. Cell 63: 1357–1363

Neurath A R, Kent S B 1988 The pre-S region of hepadnavirus envelope proteins. Advances in Virus Research 34: 65–142

Neurath A R, Kent S B, Strick N 1984 Location and chemical synthesis of a pre-S gene coded immunodominant epitope of hepatitis B virus. Science 224: 392–395

Neurath A R, Kent S B, Parker K, Prince A M, Strick N, Brotman B, Sproul P 1986a Antibodies to a synthetic peptide from the preS 120–145 region of the hepatitis B virus envelope are virus neutralizing. Vaccine 4: 35–37

Neurath A R, Kent S B, Strick N, Parker K 1986b Identification and chemical synthesis of a host cell receptor binding site on hepatitis B virus. Cell 46: 429–436

Neurath A R, Seto B, Strick N 1989 Antibodies to synthetic peptides from the preS1 region of the hepatitis B virus (HBV) envelope (env) protein are virus-neutralizing and protective. Vaccine 7: 234–236

Neurath A R, Strick N, Sproul P 1992 Search for hepatitis B virus cell receptors reveals binding sites for interleukin 6 on the virus envelope protein. Journal of Experimental Medicine 175: 461–469

Norder H, Hammas B, Löfdahl S, Courouce A M, Magnius L O 1992 Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. Journal of General Virology 73: 1201–1208

Offensperger W B, Offensperger S, Walter E, Blum H E, Gerok W 1991 Inhibition of duck hepatitis B virus infection by lysosomotropic agents. Virology 183: 415–418

Ohnuma H, Machida A, Okamoto et al 1993 Allelic subtypic determinants of hepatitis B surface antigen (i and t) that are distinct from d/y or w/r. Journal of Virology 67: 927–932

Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo R I, Imai M, Miyakawa Y, Mayumi M 1988 Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. Journal of General Virology 69: 2575–2583

Ou J H, Laub O, Rutter W J 1986 Hepatitis B virus gene function: the precore region targets the core antigen to cellular membranes and causes the secretion of the e antigen. Proceedings of the National Academy of Sciences of the USA 83: 1578–1582

Pasek M, Goto T, Gilbert W, Zink B, Schaller H, MacKay P, Leadbetter G, Murray K 1979 Hepatitis B virus genes and their expression in *E. coli*. Nature 282: 575–579

Persing D H, Varmus H E, Ganem D 1986 Inhibition of secretion of hepatitis B surface antigen by a related presurface polypeptide. Science 234: 1388–1391

Persing D H, Varmus H E, Ganem D 1987 The preS1 protein of hepatitis B virus is acylated at its amino terminus with myristic acid. Journal of Virology 61: 1672–1677

Peterson D L 1981 Isolation and characterization of the major protein and glycoprotein of hepatitis B surface antigen. Journal of Biological Chemistry 256: 6975–6983

Peterson D L, Roberts I M, Vyas G N 1977 Partial amino acid sequence of two major component polypeptides of hepatitis B surface antigen. Proceedings of the National Academy of Sciences of the USA 74: 1530–1534

Peterson D L, Paul D A, Lam J, Tribby II, Achord D T 1984 Antigenic structure of hepatitis B surface antigen: identification of the "d" subtype determinant by chemical modification and use of monoclonal antibodies. Journal of Immunology 132: 920–927

Petit M A, Dubanchet S, Capel F, Voet P, Dauguet C, Hauser P 1991 HepG2 cell binding activities of different hepatitis B virus isolates: inhibitory effect of anti-HBs and anti-preS1(21–47). Virology 180: 483–491

Petit M A, Capel F, Dubanchet S, Mabit H 1992 PreS1-specific binding proteins as potential receptors for hepatitis B virus in human hepatocytes. Virology 187: 211–222

Pontisso P, Ruvoletto M G, Gerlich W H, Heermann K H, Bardini R, Alberti A 1989 Identification of an attachment site for human liver plasma membranes on hepatitis B virus particles. Virology 173: 522–530

Pontisso P, Ruvoletto M G, Tiribelli C, Gerlich W H, Ruol A, Alberti A 1992 The preS1 domain of hepatitis B virus and IgA cross-react in their binding to the hepatocyte surface. Journal of General Virology 73: 2041–2045

Prange R, Nagel R, Streeck R E 1992 Deletions in the hepatitis B virus small envelope protein: effect on assembly and secretion of surface antigen particles. Journal of Virology 66: 5832–5841

Radziwill G, Tucker W, Schaller H 1990 Mutational analysis of the hepatitis B virus P gene product: domain structure and RNase H activity. Journal of Virology 64: 613–620

Raney A K, Le H B, McLachlan A 1992 Regulation of transcription from the hepatitis B virus major surface antigen promoter by the Sp1 transcription factor. Journal of Virology 66: 6912–6921

Rigg R J, Schaller H 1992 Duck hepatitis B virus infection of hepatocytes is not dependent on low pH. Journal of Virology 66: 2829–2836

Robinson W S, Greenman R L 1974a DNA polymerase in the core of the human hepatitis B virus candidate. Journal of Virology 13: 1231–1236

Robinson W S, Clayton D A, Greenman R L 1974b DNA of a human hepatitis B virus candidate. Journal of Virology 14: 384–391

Roossinck M J, Siddiqui A 1987 In vivo phosphorylation and

protein analysis of hepatitis B virus core antigen. Journal of Virology 61: 955–961
Rossner M T 1992 Review: hepatitis B virus X-gene product: a promiscuous transcriptional activator. Journal of Medical Virology 36: 101–117
Sattler F, Robinson W S 1979 Hepatitis B viral DNA molecules have cohesive ends. Journal of Virology 32: 226–233
Schaller H, Fischer M 1991 Transcriptional control of hepadnaviruses gene expression. In: Mason W S, Seeger C (eds) Hepadnaviruses molecular biology and pathogenesis. Current Topics in Microbiology and Immunology 168: 21–39
Schek, N, Bartenschlager R, Kuhn C, Schaller H 1991 Phosphorylation and rapid turnover of hepatitis B virus X-protein expressed in HepG2 cells from a recombinant vaccinia virus. Oncogene 6: 1735–1744
Schlicht H J, Schaller H 1989 The secretory core protein of human hepatitis B virus is expressed on the cell surface. Journal of Virology 63: 5399–5404
Schlicht H J, Bartenschlager R, Schaller H 1991 Biosynthesis and enzymatic functions of the hepadnaviral reverse transcriptase. In: McLachlan A (ed) Molecular biology of the hepatitis B virus, CRC Press, Boca Raton, pp 171–180
Seeger C, Maragos J 1990 Identification and characterization of the woodchuck hepatitis virus origin of DNA replication. Journal of Virology 64: 16–23
Seeger C, Maragos J 1991 Identification of a signal necessary for initiation of reverse transcription of the hepadnavirus genome. Journal of Virology 65: 5190–5195
Seeger C, Ganem D, Varmus H E 1986 Biochemical and genetic evidence for the hepatitis B virus replication strategy. Science 232: 477–484
Seeger C, Baldwin B, Tennant BC 1989 Expression of infectious woodchuck hepatitis virus in murine and avian fibroblasts. Journal of Virology 63: 4665–4669
Seeger C, Summers J, Mason W S 1991 Viral DNA synthesis. Current Topics in Microbiology and Immunology 168: 41–60
Seifer M, Gerlich W H 1992 Increased growth of permanent mouse fibroblasts in soft agar after transfection with hepatitis B virus DNA. Archives of Virology 126: 119–128
Seifer M, Heermann K H, Gerlich W H 1990a Expression pattern of the hepatitis B virus genome in transfected mouse fibroblasts. Virology 179: 287–299
Seifer M, Heermann K H, Gerlich W H 1990b Replication of hepatitis B virus in transfected nonhepatic cells. Virology 179: 300–311
Seifer M, Höhne M, Schaefer S, Gerlich W S 1992 In vitro tumorigenicity of hepatitis B virus DNA and HBx protein. Journal of Hepatology 13 (suppl. 4): S61–S65
Seifer M, Zhou S, Standring D N 1993 A micromolar pool of antigenically distinct precursors is required to initiate cooperative assembly of hepatitis B virus capsids in *Xenopus* oocytes. Journal of Virology 67: 249–257
Sells M A, Chen M L, Acs G 1987 Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. Proceedings of the National Academy of Sciences of the USA 84: 1005–1009
Shaul Y 1991 Regulation of hepadnaviruses transcription In: McLachlan A (ed) Molecular biology of the hepatitis B viruses. CRC Press, Boca Raton, pp 193–212
Siddiqui A 1991 Transcription of hepadnaviruses. In: McLachlan A (ed) Molecular biology of the hepatitis B viruses. CRC Press, Boca Raton, pp 95–104
Sprengel R, Kaleta E F, Will H 1988 Isolation and characterization of a hepatitis B virus endemic in herons. Journal of Virology 62: 3832–3839
Standring D N, Ou J H, Masiarz F R, Rutter W J 1988 A signal peptide encoded within the precore region of hepatitis B virus directs the secretion of a heterogeneous population of e antigens in *Xenopus* oocytes. Proceedings of the National Academy of Sciences of the USA 85: 8405–8409
Stibbe W, Gerlich W H 1982 Variable protein composition of hepatitis B surface antigen from different donors. Virology 123: 436–442
Stibbe W, Gerlich W H 1983 Structural relationships between minor and major proteins of hepatitis B surface antigen. Journal of Virology 46: 626–629
Stirk H J, Thornton J M, Howard C R 1992 A topological model for hepatitis B surface antigen. Intervirology 33: 148–158
Su T S, Lai C J, Huang J L, Lin L H, Yauk Y K, Chang C M, Lo S J, Han S H 1989 Hepatitis B virus transcript produced by RNA splicing. Journal of Virology 63: 4011–4018
Summers J, Mason W S 1982 Replication of the genome of a hepatitis B-like virus by reverse transcription of an RNA intermediate. Cell 29: 403–415
Summers J, Smolec J M, Snyder R 1978 A virus similar to human hepatitis B virus associated with hepatitis and hepatoma in woodchucks. Proceedings of the National Academy of Sciences of the USA 75: 4533–4537
Sureau C, Romet Lemonne J L, Mullins J I, Essex M 1986 Production of hepatitis B virus by a differentiated human hepatoma cell line after transfection with cloned circular HBV DNA. Cell 47: 37–47
Sureau C, Moriarty A M, Thornton G B, Lanford R E 1992 Production of infectious hepatitis delta virus in vitro and neutralization with antibodies directed against hepatitis B virus pre-S antigens. Journal of Virology 66: 1241–1245
Tiollais P, Charnay P, Vyas G N 1981 Biology of hepatitis B virus. Science 213: 406–411
Toh H, Hayashida H, Miyata T 1983 Sequence homology between retroviral reverse transcriptase and putative polymerases of hepatitis B virus and cauliflower mosaic virus. Nature 305: 827–829
Tron F, Degos F, Brechot C et al 1989 Randomized dose range study of a recombinant hepatitis B vaccine produced in mammalian cells and containing the S and preS2 sequences. Journal of Infectious Diseases 160: 199–204
Tuttleman J S, Pugh J C, Summers J W 1986a In vitro experimental infection of primary duck hepatocyte cultures with duck hepatitis B virus. Journal of Virology 58: 17–25
Tuttleman J S, Pourcel C, Summers J 1986b Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. Cell 47: 451–460
Twu J S, Schloemer R H 1989 Transcription of the human beta interferon gene is inhibited by hepatitis B virus. Journal of Virology 63: 3065–3071
Uy A, Wunderlich G, Olsen D B, Heermann K H, Gerlich W H, Thomssen R 1992 Genomic variability in the preS1 region and determination of routes of transmission of hepatitis B virus. Journal of General Virology 73: 3005–3009
Valenzuela P, Gray P, Quiroga M, Zaldivar J, Goodman H M, Rutter W J 1979 Nucleotide sequence of the gene coding for the major protein of hepatitis B virus surface antigen. Nature 280: 815–819
von Loringhoven A F, Koch S, Hofschneider P H, Koshy R

1985 Co-transcribed 3' host sequences augment expression of integrated hepatitis B virus DNA. EMBO Journal 4: 249–255

Wang G H, Seeger C 1992 The reverse transcriptase of hepatitis B virus acts as a protein primer for viral DNA synthesis. Cell 71: 663–670

Wang J, Lee A S, Ou J H 1991 Proteolytic conversion of hepatitis B virus e antigen precursor to end product occurs in a postendoplasmic reticulum compartment. Journal of Virology 65: 5080–5083

Wasenauer G, Köck J, Schlicht H J 1992 A cysteine and a hydrophobic sequence in the noncleaved portion of the pre-C leader peptide determine the biophysical properties of the secretory core protein (HBe protein) of human hepatitis B virus. Journal of Virology 66: 5338–5346

Will H, Reiser W, Weimer T, Pfaff E, Büscher M, Sprengel R, Cattaneo R, Schaller H 1987 Replication strategy of human hepatitis B virus. Journal of Virology 61: 904–911

Wu J Y, Zhou Z Y, Judd A, Cartwright C A, Robinson W S 1990 The hepatitis B virus-encoded transcriptional trans-activator hbx appears to be a novel protein serine/threonine kinase. Cell 63: 687–695

Wu H L, Chen P J, Lin M H, Chen D S 1991 Temporal aspects of major viral transcript expression in HepG2 cells transfected with cloned hepatitis B virus DNA: with emphasis on the X transcript. Virology 185: 644–651

Yang S Q, Walter M, Standring D N 1992 Hepatitis B virus p25 precore protein accumulates in *Xenopus* oocytes as an untranslocated phosphoprotein with an uncleaved signal peptide. Journal of Virology 66: 37–45

Yeh C T, Liaw Y F, Ou J H 1990 The arginine-rich domain of hepatitis B virus precore and core proteins contains a signal for nuclear transport. Journal of Virology 64: 6141–6147

Yu M W, Finlayson J S, Shih J W 1985 Interaction between various polymerized human albumins and hepatitis B surface antigen. Journal of Virology 55: 736–743

Yuh C H, Chang Y L, Ting L P 1992 Transcriptional regulation of precore and pregenomic RNAs of hepatitis B virus. Journal of Virology 66: 4073–4084

Zhou Y Z 1980 A virus possibly associated with hepatitis and hepatoma in ducks. Shanghai Medical Journal 3: 641–649

Zhou S, Standring D N 1992 Hepatitis B virus capsid particles are assembled from core-protein dimer precursors. Proceedings of the National Academy of Sciences of the USA 89: 10046–10050