# Hepatitis B Virus Morphogenesis

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## 1 Introduction

Hepatitis B viruses, or hepadnaviruses (*hepa*totropic *DNA* viruses), comprise a family of small enveloped DNA viruses that replicate through reverse transcription of an RNA intermediate; their replication cycle is hence a cyclic permutation of that of retroviruses which are RNA viruses replicating through a DNA intermediate. Hepadnaviruses are characterized by narrow host range and pronounced liver tropism. The type member, and causative agent of B-type hepatitis

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in man, hepatitis B virus (HBV), infects only humans and higher primates; related viruses have been found in other mammals like the woodchuck (WHV), or the ground squirrel (GSHV), and also in birds like the Pekin duck (DHBV) or the grey heron (HHBV).

In addition to their unique replication properties hepadnaviruses are of considerable medical importance, as HBV is still one of the major human pathogens (Lok 1994): in adults some 5%–10%, in young children up to 90% of acute infections progress into chronic hepatitis, with a highly increased risk for liver cirrhosis and eventually primary liver carcinoma. The number of chronic virus carriers is estimated to exceed 300 million worldwide. Since currently no generally applicable therapy is available, understanding the HBV life-cycle in detail is a vital prerequisite for the development of new antiviral strategies, including inhibitors of virus morphogenesis.

One of the main experimental obstacles for such an analysis is the lack of infectable cell lines. Apart from the much restricted possibility of employing in vitro infection of primary human hepatocytes (Gripon et al. 1993; Galle et al. 1994), the major tools available are the separate expression of those viral gene products that can be obtained in biologically active form from heterologous systems, transfection of cloned HBV DNA into suitable liver cell lines (yielding complete virions), and the use of the animal hepadnaviruses as models. Each of these systems has yielded valuable information regarding the individual viral components and some of their interactions in the process of hepatitis B virus morphogenesis; however, we are still far from a complete molecular understanding of how the infectious hepatitis B virion is formed.

Below I will focus on the progress that has been made during the last few years concerning the supramolecular structures of viral capsids, envelopes and virions. Before that I will briefly survey the major differences between hepadna- and retroviruses, and give a short update on the basic replication cycle of hepatitis B viruses; this appears adequate as hepadnaviral replication and assembly are extremely tightly coupled. The reader interested in the general molecular biology of hepatitis B viruses is referred to previously published reviews by Ganem and Varmus (1987) and monographs edited by McLachlan (1991) and, in this series, Mason and Seeger (1991), which also contain chapters covering the topic of this review; more recent articles concerning replication (Nassal and Schaller 1993a), capsid assembly (Nassal and Schaller 1993b) and the core protein (Seifer and Standring 1995) are also available.

## 2 Hepatitis B Viruses Versus Retroviruses – A Short Overview

Reverse transcription as replication principle, and the presence and to some extent order of the genes for the principal components Gag-Pol-Env (termed Core-P-preS/S in hepadnaviruses) are common characteristics shared by hepa-

titis B and retroviruses. However, many aspects are distinctly different in both virus families (NASSAL and SCHALLER 1993a; ROTHNIE et al. 1994), partly due to the extremely small size of the hepadnaviral genome (3.0–3.2 kb), and the need to efficiently exploit this restricted genetic space as evidenced, for instance, by the largely overlapping arrangement of both coding regions and regulatory elements (Fig. 1A). In the hepadnaviruses, extracellular virions contain DNA rather than RNA; integration is not an obligatory step in replication; functional mRNAs are produced from several internal promoters on the circular DNA genome rather than one LTR-based promoter, and RNA splicing does not appear to play a critical role in the basic replication cycle of at least the mammalian viruses. Although another distinction, that hepadnaviruses could synthesize their first DNA strand in a continuous fashion from a 3′-proximal origin on the RNA template, had to be given up in favour of a mechanism more closely resembling retroviral reverse transcription (see below), the differences are not confined to the genetic level; rather, they extend to the strategies employed to bring together the viral gene products that make up the infectious enveloped virion, i.e. the capsid, P and envelope proteins, and the genomic RNA. First, there is no evidence that any of the protein products requires processing; second, envelopment appears to be necessary for export of viral capsids; third, this process occurs at an internal rather than the plasma membrane; fourth, P protein, not core protein, mediates specific RNA pregenome packaging; fifth, this protein/RNA interaction rather than a protein/protein interaction is also responsible for P encapsidation. Finally, all hepadnaviruses secrete empty envelopes, termed subviral or S particles.

## 3 Genetic Organization of Hepadnaviruses

Figure 1A shows the genome organisation of HBV and DHBV. The HBV genome contains four major open reading frames (ORFs): preC/C, P, preS1/S2/S and X. The X gene product encodes a pleiotropically acting transcriptional activator which in the WHV model has been shown to be required for the establishment of infection (CHEN et al. 1993). Despite intensive efforts, its mode of action has not been finally established.

The preC/C ORF codes for two distinct products: one is the core protein forming the protein shell of the nucleocapsid, the other, made by translation of the joint preC/C ORF, is the precore protein which is targeted into the cell's secretory pathway, processed at both ends and eventually found in the serum of infected individuals as HBeAg. Both products are translated from genomic, terminally redundant 3.5-kb transcripts with slightly different 5′-ends. The longer precore mRNAs contain the preC initiation codon, the shorter core mRNA lacks it. The P-ORF covers some 80% of the genome and encodes the viral replication enzyme P, which is also an indispensable component in the assembly process (see below). P protein is translated from the same genomic RNA that directs

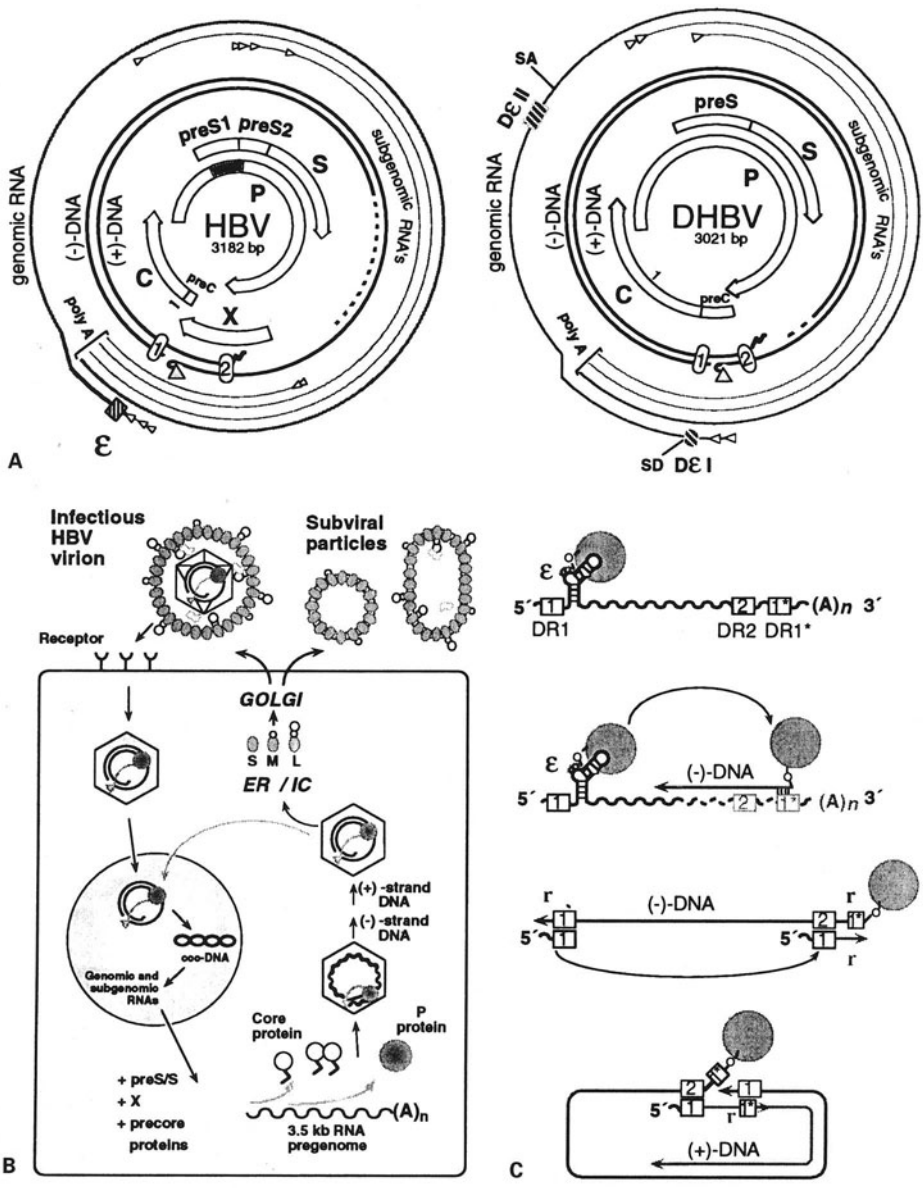


**Fig. 1A–C.** Fundamental aspects of hepadnavirus biology. **A** Genetic organization of HBV and DHBV. *Inner circles* represent the partially double-stranded circular DNA genomes found in extracellular virions. *Boxes marked DR1 and DR2* represent the direct repeats, the *triangle* the TP domain of P protein which is covalently attached to the (–)-DNA, and the *zigzag line* the RNA primer at the 5′-end of (+)-DNA. Open reading frames are indicated by *arrows*. *Hatched region* in the HBV P-ORF represents a non-essential spacer. *Numbers* are nt positions; for HBV, the numbering system of PASEK et al. (1979) is used, which starts with the initiator codon of the C gene; in DHBV, nt position 1 is defined by a unique *Eco*RI site inside the C gene. *Outer lines* represent the viral transcripts, *arrowheads* the approximate start sites. ε, HBV encapsidation signal; *D*εI, *D*εII, regions I and II of DHBV encapsidation signal; *SD, SA*, splice donor and acceptor sites. **B** Basic infectious cycle of hepatitis B virus. The HBV

core protein synthesis and later serves as RNA pregenome. The *env* gene consists of three in-phase ORFs, termed in 5′- to 3′-direction preS1, preS2 and S. S can be separately expressed to give the small S, or S protein; cotranslation of preS2/S yields the middle S, or M protein, that of the entire preS1/preS2/S gene the large S, or L protein. Thus the S domain is common to all three forms of Env protein. As for the preC/C ORF, this is achieved by the generation of mRNAs with staggered 5′-ends in which the initiator codons of the preS1, the preS2 or the S region are the first to be encountered by translating ribosomes. L protein is translated from a 2.4-kb mRNA, M and S from a set of 2.1-kb transcripts. Due to the more favourable context of the S-AUG, S protein may also be translated from mRNAs containing the preS2 initiator codon (SHEU and LO 1992). All viral transcripts are 3′-terminally colinear, ending after a unique polyadenylation signal located in the C gene.

The slightly smaller DHBV genome is similarly organized, but has some unique features: the X-ORF is absent; the preC/C gene is substantially larger than in HBV, coding for a core protein of 262 aa rather than 183 or 185 aa as in HBV; the *env* gene consists of only two regions, preS and S, and consequently only two products, the large and the small surface protein, are made from corresponding mRNAs of 2.35 and 2.13 kb (BÝSCHER et al. 1985); finally, recent data indicate that a second preS mRNA is produced from the pregenome by splicing (OBERT et al. 1996); hence the avian hepadnaviruses may be more closely related to retroviruses than their mammalian counterparts.

## 4 Basic Replication Cycle of Hepadnaviruses

The fundamental aspects of the hepadnaviral life-cycle are outlined in Fig. 1B (adapted from NASSAL and SCHALLER 1993a). The infectious DNA-containing virion binds to its target cell via interaction of the L protein (NEURATH et al. 1986,

virion attaches to its target cell via interactions between the L protein and (a) still unidentified cellular receptor(s). After delivery into the nucleus, the partially dsDNA genome is repaired into cccDNA from which genomic and subgenomic RNAs are transcribed. The RNA pregenome interacts with both its translation products, core and P protein, to form a replication-competent nucleocapsid in which the RNA is reverse transcribed into DNA. At the ER, or a subsequent compartment, capsids acquire their envelope of surface proteins and are exported; alternatively, they may recycle the viral genomes to the nucleus. The surface proteins alone form spherical and filamentous S particles. *Irregularly shaped symbols* shown in filaments and virions represent the cytosolic chaperone *Hsc*70, which is associated with DHBV S particles, and probably complete virions. See text for details. **C** Simplified model of hepadnaviral replication. (–)-DNA synthesis initiates by P-protein-catalysed production of a short DNA primer with part of ε serving as template. The covalent P/primer complex is translocated to DR1* and extended to the 5′-end of the pregenome. The RNA template is degraded, except for a short oligonucleotide from the 5′-end containing the DR1 sequence, which is then transferred to DR2 and extended to the 5′-end of (–)-DNA. Due to a short terminal redundancy (''r''), the 3′-end of the (+)-DNA can use the 3′-end of (–)-DNA to continue (+)-strand synthesis

1992) with (a) still unidentified cellular receptor(s); for DHBV, the importance of the preS domain was directly demonstrated by infectivity competition (KLINGMÜLLER and SCHALLER 1993). Probably by a pH-independent mechanism (RIGG and SCHALLER 1992), the nucleocapsid is delivered into the cytoplasm of the host cell; the viral genome gains access to the nucleus and is transformed into covalently closed circular (ccc) DNA by cellular enzymes (KÖCK and SCHLICHT 1993). Subgenomic and genomic RNAs are transcribed from the cccDNA, exported into the cytoplasm and used as templates for translation of the various gene products. The *env* gene products are targeted to the endoplasmic reticulum (ER), and most of them are exported in the form of empty S particles via the constitutive secretory pathway. Core and P protein remain in the cytoplasm, where they interact specifically with the one species of genomic transcript from which they were translated, forming the nucleocapsid with its core protein shell and incorporated P protein and RNA pregenome. Inside the capsid, P protein reverse transcribes the RNA, yielding the characteristic circular, partially double-stranded DNA molecule with P protein covalently attached to the 5′-end of the first DNA strand (Fig. 1C). These replication-competent nucleocapsids either leave the cell by interaction with the *env* gene products still present in internal membranes, or they may cycle their nucleic acid back into the nucleus, replenishing the pool of cccDNA (TUTTLEMAN et al. 1986; WU et al. 1990). It is unlikely that major revisions to this replication scheme will be required in the future; however, our current knowledge of many details and even some fundamental cell biological aspects of the assembly of nucleocapsids, S particles and especially complete virions is still limited.

## 5 Supramolecular Hepatitis B Virus Structures

During hepatitis B virus infection three kinds of particulate, supramolecular structures are formed: infectious enveloped virions ("Dane particles" of about 42 nm diameter) containing an inner core, empty subviral particles (spherical and filamentous forms about 20–22 nm in diameter), and naked isometric capsids, or core particles (about 28 nm in diameter). Virions and S particles are found in serum; core particles can be liberated from virions by detergent treatment, or can be found as such inside infected hepatocytes. Electron micrographs of these particles as seen by negative staining are shown in Fig. 2A,B.

### 5.1 The Nucleocapsid (Core Particle)

The protein shell of the hepadnaviral nucleocapsid consists, as far as we know, exclusively of multiple subunits of a single species of core protein. Inside it harbours the viral genome, initially in the form of probably one copy of the RNA

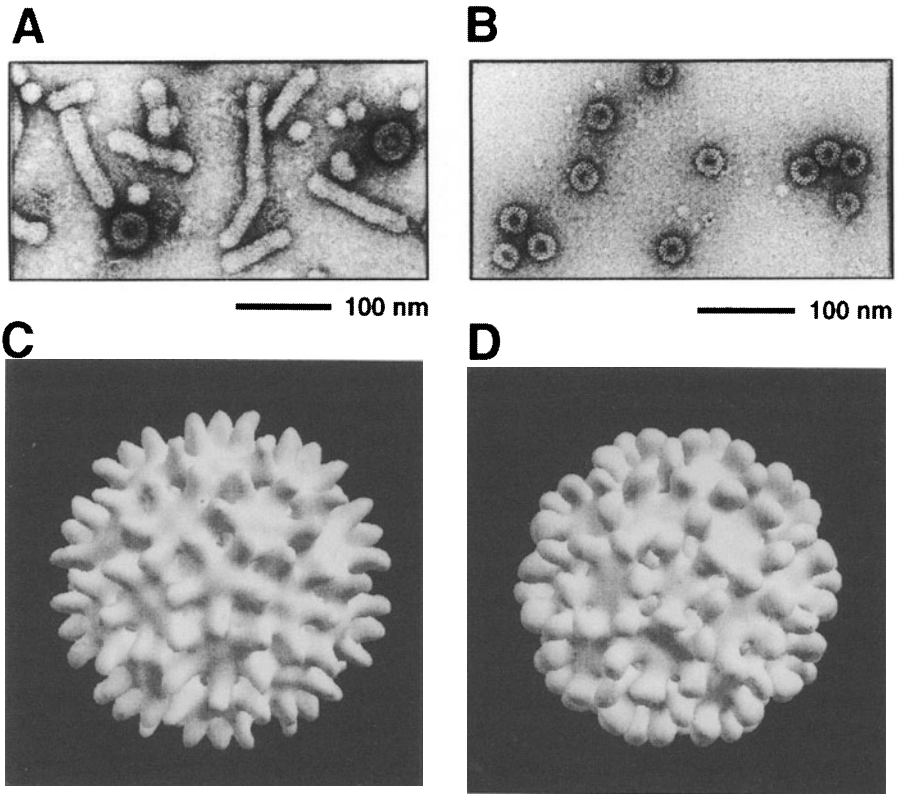


**Fig. 2A–D.** Ultrastructure of hepatitis B virus particles. Electron micrographs of negatively stained hepatitis B virions and S particles from patient serum (**A**) and recombinant HBV core particles produced in *E. coli* (**B**) *Larger round objects* in **A** represent complete virions (42- nm Dane particles), *smaller structures* are spherical and filamentous forms of S particles (Courtesy of Dr. H. Zentgraf, Applied Tumor Virology, German Cancer Research Center, Heidelberg, Germany.) Image reconstructions of HBV (**C**) and DHBV (**D**) core particles produced by *E. coli*. Reconstructions are based on electron micrographs from frozen-hydrated samples. The particles shown, viewed along a fivefold axis, exhibit T = 4 symmetry; smaller particles with T = 3 symmetry are also present in the preparations (Adapted from KENNEY et al. 1995)

pregenome, which is then reverse transcribed, by the co-encapsidated P protein, into the DNA genome found in extracellular virions. Most of the structural results referred to below have been obtained using the intrinsic capability of the core protein to self-assemble even in the absence of other viral components. However, P protein and RNA pregenome have a profound influence on the assembly pathway of core particles. This is not unexpected as virus propagation requires efficient co-encapsidation of these two essential replication components. Data pertaining to the assembly of such replication-competent cores are discussed separately (Sect. 5.1.5).

### 5.1.1 The Core Protein

The primary sequence length of the core proteins (see Fig. 3A) from the mammalian hepadnaviruses varies between 183 (HBV subtype ayw) and 188 aa (WHV); the avian hepadnavirus core proteins are substantially larger (262 aa for DHBV). The sizes of the proteins isolated from core particles correspond to

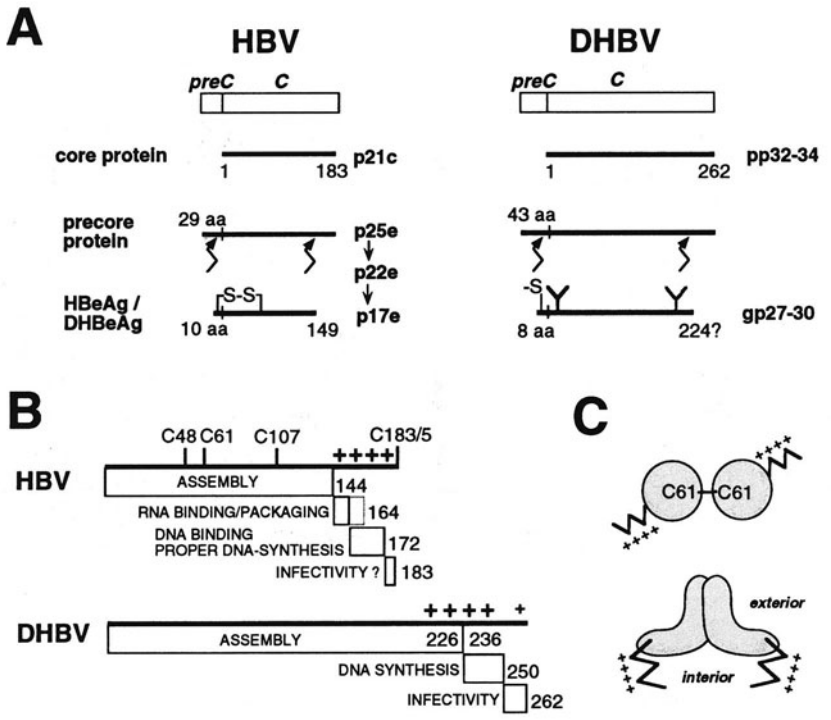


**Fig. 3A–C.** Hepadnaviral core proteins. **A** Correlation of preC/C genes and their products. *Open bars* represent genes, *filled bars* proteins. The capsid proteins are the primary translation products of the C genes. The secretory precore proteins arise by translation of the complete preC/C ORFs, and hence contain N-terminal extensions. During export, they are proteolytically processed at both ends (*arrows*); the cleavage sites for the DHBV protein have not been experimentally determined. In HBeAg, an intramolecular S-S-bridge blocks the assembly competence of the resulting p17e; in DHBeAg a similarly located Cys residue forms an intermolecular S-S-bridge (M. Nassal and A. Rieger, unpublished data). *Numbers* are aa positions; *designations to the right* show the apparent molecular masses in kilodaltons; *p*, protein; *pp*, phosphoprotein; *gp*, glycoprotein. *Y-shaped symbols represent* glycan residues; *filled symbols* indicate complete, *stippled symbols* partial glycosylation. **B** Functional domains in hepadnaviral core proteins. *Bars* represent the assembly domains, the strongly basic C-terminal regions are indicated by +. Cys residues are indicated by their aa position. Diagram is a composite of data obtained in heterologous expression systems and by transfection of cloned viral genomes. **C** Topological models for the HBV core protein dimer. *Upper model* is derived from cross-linking data. *Spheres* represent the assembly, *zigzag lines* the nucleic acid binding domain. *Lower model* is compatible with the electron densities observed in cryo-EM studies, which suggest a hammerhead-like structure in which the protruding domains from two tightly associated monomers form one of the visible spikes. See text for details

those of the primary translation products; hence there is apparently no processing. The only known chemical modification is phosphorylation, which might be involved in regulating the different functions of the core proteins in the viral life-cycle (see Sect. 5.1.6). All core proteins can be relatively efficiently expressed in various heterologous systems, and they retain the ability to self-associate into regular particulate structures. However, attempts to produce crystals suitable for an X-ray analysis have so far failed. Hence neither the overall architecture of the core particle nor the three-dimensional structure of the core protein itself is known at atomic resolution. Theoretical predictions have suffered from the lack in the structural data base of capsid proteins with strong sequence similarity to the hepadnaviral core proteins. The Mengovirus VP3 based model for the HBV core protein proposed by ARGOS and FULLER (1988) may be correct, but it has not been further substantiated by additional experimental evidence. Thus the major tools for the study of core protein structure and function are site-directed mutagenesis and analysis of the phenotypic consequences by expressing the variant core proteins either alone or in the context of a complete viral genome; accessibility of certain regions to antibodies and proteases; and, for complete particles, electron microscopy.

Mutational studies have provided convincing evidence for the two-domain structure of the HBV core protein, which is already reflected in the marked accumulation of basic residues in the 34 C-terminal aa residues, most of them clustered in four blocks (indicated by +'s in Fig. 3B), of which three contain the motif SPRRR(R). The self-assembly capability resides in the 144 N-terminal aa residues of the HBV core protein; mutants comprising this primary sequence are assembly competent (GALLINA et al. 1989; BIRNBAUM and NASSAL 1990); those lacking the sequence following residues 138 and 139 are not. N-Terminal deletions of more than a few aa are also deleterious, in both the HBV (e.g. CHANG et al. 1994) and DHBV (YANG et al. 1994) core protein. The basic C-terminal region constitutes an apparently non-sequence-specific nucleic acid binding domain (HATTON et al. 1992) and mediates RNA encapsidation even in *E. coli*, apparently with some preference for the mRNA the protein was translated from (BIRNBAUM and NASSAL 1990). This preference might be explained by a local concentration phenomenon; certainly it is mechanistically different from the specific pregenome encapsidation process mediated by P protein and the RNA encapsidation signal $\varepsilon$ described below. Cross-linking studies (NASSAL et al. 1992; ZHENG et al. 1992; ZHOU and STANDRING, submitted) confirmed the two-domain topology, since except for those involving the C-terminal Cys residue C183 identical cross-links between the internal Cys residues 48, 61 and 107 (see Fig. 3B) were found in a C-terminally truncated and the full-length core protein. The most prominent covalent link observed was a homologous S-S-bridge between two Cys 61 residues, suggesting that these two residues are in close spatial proximity, most likely in a symmetrical dimeric arrangement (Fig. 3C). While a complete dissociation and reassociation of particles from the full-length core protein has not yet been reported, this is possible with the C-terminally truncated variants, indicating a stabilizing influence of interactions between the basic core protein tail

and RNA. In such experiments, the dissociated subunits were always found to migrate as dimers during gel filtration under native conditions, regardless of the presence or absence of Cys 61 (NASSAL et al. 1992). These data strongly support the view that the core protein has a high intrinsic propensity to form dimers. The C-terminal Cys residues are also easily oxidized; in particles produced in *E. coli*, the resulting S-S-bridges cross-link neighbouring dimers into a polymeric network (NASSAL et al. 1992; ZHENG et al. 1992); in *X. laevis* oocytes homologous cross-links within one dimer appear to be preferred (ZHOU and STANDRING, submitted). The basis for this difference is currently not clear; it may be related to the time course of oxidation relative to the progress of assembly which itself is dependent on the core protein concentration (see below), or the use of core proteins from different HBV subtypes with slightly differing primary sequences.

Further confirmation for the two-domain model comes from the observation that engineered fusion proteins with the foreign sequence attached to the extreme N-terminus, or after aa 144 of the HBV core protein, are still assembly competent (e.g. SCHÖDEL et al. 1992; BORISOVA et al. 1993). Such hybrid core particles hold promise as potent subunit vaccines.

An alignment of the DHBV core protein (see Fig. 3A) with the mammalian hepadnavirus core proteins shows a markedly higher homology in the N- and C-terminal parts than in the centre (SPRENGEL et al. 1985; ARGOS and FULLER 1988; STANDRING 1991). Possibly, the homologous sequences form the assembly domain while the extra sequences present in the DHBV protein are looped out, but this prediction has not yet been directly tested. In general, even small sequence alterations in the N-terminal and central regions of the DHBV core protein appear to severely compromise its self-assembly competence (YANG et al. 1994). However, the two-domain structure (Fig. 3B) seems to be conserved since deletions of the C-terminal region do not inhibit self-assembly, either in *E. coli* (YANG et al. 1994) or in transfected mammalian cells (SCHLICHT et al. 1989a; YU and SUMMERS 1991). This region is also highly basic and involved in nucleic acid binding although the positively charged residues are not all present in similarly ordered clusters as in the mammalian virus proteins.

### 5.1.2 The Secretory Core Gene Product HBeAg

A characteristic feature of all hepadnaviruses is that they use the core gene, in conjunction with the short in-phase preC-ORF, to produce a second, non-particulate and antigenically distinct gene product known as HBeAg. The function of the antigen which is used as a serological marker for ongoing viral replication is still obscure. The discovery in patients of mutant $HBeAg^-$ viruses and their apparent selection under conditions of immune surveillance or interferon treatment (for review: BLUM 1993; MISKA and WILL 1993) has roused considerable interest in the medical community. Obviously, HBeAg is not essential for the basic viral life-cycle, but it may be a modulator of the host response to viral infection. HBeAg biosynthesis (see Fig. 3A) involves translation of the joint preC/C ORF to produce the precore precursor protein, its targeting into and

processing in the secretory pathway of the cell, and finally export as a soluble, non-particulate protein which appears to be tightly associated with serum proteins. In HBV, the final product differs from the core protein by the presence of a 10-aa N-terminal extension and the absence of the basic C-terminus. Hence, the entire assembly domain of the core protein is present in HBeAg; yet its properties are fundamentally different. The solution to this structural puzzle is the formation of a specific intramolecular disulphide bridge connecting a Cys residue in its unique N-terminal extension with Cys61 (Fig. 3A), which in core protein resides at the dimer interface. The net result is the prevention of dimerization and subsequent assembly (WASENAUER et al. 1993; NASSAL and RIEGER 1993; SCHÖDEL et al. 1993).

### 5.1.3 Architecture and Structure of Core Particles

Various reports describing the electron microscopic appearance of negatively stained, heterologously produced HBV core particles have been cited in previous reviews; these data showed that HBV cores have an approximate diameter of 28 nm (Fig. 2B); in addition, slightly smaller particles can be seen on a number of the published electron micrographs (COHEN and RICHMOND 1982; ONODERA et al. 1982), but were usually not explicitly discussed. A powerful method bridging the gap between conventional electron microscopy and X-ray diffraction is the combination of cryo-electron microscopy and computer-assisted image reconstruction techniques (DUBOCHÉT et al. 1988). The principal advantage of cryo-electron microscopy is that the biological specimen is not dehydrated but rather embedded in vitreous (non-crystalline) ice; hence the authentic structures should be preserved much better than by conventional techniques. Applying this method to the full-length and a variant HBV core protein lacking the Arg-rich C-terminus expressed in *E. coli*, CROWTHER et al. (1994) have recently obtained strong evidence that the majority of particles (diameter approximately 34 nm) from both proteins have icosahedral symmetry (triangulation number $T = 4$) and are built from 240 subunits of the core protein (cf. Fig. 2C). A fraction of the particles have a smaller diameter (approximately 30 nm) and consist of only 180 subunits ($T = 3$). Possibly it is this heterogeneity that has so far prevented the formation of sufficiently ordered crystals for an X-ray analysis, in particular since the overall packing of subunits is extremely similar in both types of particles. In accord with the biochemical and genetic data outlined above, the reconstructions revealed a tight association between pairs of subunits. Thus, the particles are more accurately described as consisting of 120, or 90, respectively, core protein dimers. The electron density maps can be interpreted to indicate a hammerhead-like structure of the dimer (Fig. 3C), in which part of the proteins form a surface protrusion, similar to the "protruding" P domain in many RNA virus capsids. Also in accord with the biochemical data, most of the particles from the truncated variant had no detectable inner contents, while those of the full-length protein showed an internal density, probably corresponding to packaged RNA.

We have recently confirmed these data and extended the analysis to HBV cores isolated from infected human liver, and DHBV cores expressed in *E. coli* (KENNEY et al. 1995). The T = 4 protein shell of the liver-derived HBV cores appears very similar to that of the *E. coli* material shown in Fig. 2C, corroborating earlier inferences from their antigenic similarity; also this preparation contained smaller particles with apparent T = 3 symmetry. Despite the much larger size of its core protein, the basic architecture of the DHBV capsid is very similar to that of HBV and hence an evolutionarily conserved feature: the majority of particles also consist of 240 subunits, which are arranged in hammerhead-like dimers (Fig. 2D). Some of the extra mass is apparently located in the spikes, which are more oblong-shaped than in HBV. Smaller capsids with T = 3 symmetry are also found.

HBV and DHBV cores appear to have holes, in accord with the permeability of the core shell for small molecules like nucleotides. This is obviously important for reverse transcription inside the particle and is experimentally utilized in the "endogenous polymerase reaction", which provides a sensitive assay for replication-competent core particles: if naked cores are incubated with dNTPs, the endogenous (i.e. encapsidated) P protein will elongate the initiated DNA strands and convert them into more or less completely double-stranded molecules. Another structural feature may also relate to the fact that the core particle is not only a protective container for the hepadnaviral genome but a transcription machine: RNA-containing cores from *E. coli*, and liver-derived particles which should contain the complete RNA pregenome and/or various amounts of partially double-stranded DNA (and P protein), show an inner electron density, well separated from the peak density of the protein shell, except for apparently icosahedrally ordered contacts. Thus it appears that the basic C-terminal regions of the core protein keep most of the nucleic acid apart from the outer shell to allow sufficient flexibility for the P protein catalysed reverse transcription process inside the particle.

Two major unresolved issues are the nature of the molecular switch that determines formation of the complex 240 subunit T = 4 structure rather than the simple T = 1 arrangement of 60 identical subunits, and the significance of the T = 3 and T = 4 particles. In many T = 3 plant RNA viruses, T = 1 structures form in vitro if the basic nucleic acid binding regions of the capsid proteins are proteolytically removed. It is believed that interaction with the RNA mediates establishment of the T = 3 arrangement, which requires the capsid protein to be able to adopt three similar but not identical ("quasi-equivalent") conformations (for a review: ROSSMANN and JOHNSON 1989). In hepadnaviruses, the information to properly fold into distinct, only quasi-equivalent conformations possibly resides in the primary sequence of the assembly domain, as even C-terminally truncated core protein variants form predominantly particles with T = 4 symmetry. However, a potential influence of RNA on the arrangement of subunits in the particle still cannot be excluded, as even the particles from the truncated core protein contain some RNA (about 1/10th to 1/20 the amount found in particles from the full-length protein; BIRNBAUM and NASSAL 1990; HATTON et al. 1992); an analysis

of core particles reconstituted in vitro after complete digestion of any nucleic acid should reveal such a potential influence. A possibly interesting finding is that the shortest of a series of C-terminally truncated DHBV core proteins that is still assembly competent appears to form smaller particles of about 20 nm (YANG et al. 1994); if confirmed, this might indicate that at least part of the information to build the complex T = 3 and T = 4 structures resides in the very C-terminal residues of the assembly domain.

Whether the T = 4 or T = 3 particles represent the biologically relevant form remains to be determined. The higher abundance of the T = 4 particles in *E. coli* and, more importantly, in cores from infected human liver suggests that these larger, more complex particles are the relevant species. Applying the above-described techniques to complete virions should reveal whether the two particle classes are discriminated between during envelopment.

The resolution of the EM data outlined above is still at the level of protein subunits. However, additional topological information can be derived from studies analysing the accessibility of certain regions of the core protein to either antibodies or proteases. The major HBcAg epitope (''c'') is located around aa 80 (SALFELD et al. 1989; SÄLLBERG et al. 1991), and hence surface exposed; in addition, the region encompassing residues 127–133 was found to react with site-specific monoclonals while regions 9–20 and 133–145 were only exposed after denaturation (BICHKO et al. 1993; PUSHKO et al. 1994). Protease sensitivity studies have shown that cores produced in *E. coli* (e.g. DALSEG 1990) or in *Xenopus* oocytes (SEIFER and STANDRING 1994) have only one major sensitive site located between aa 145 and 150, i.e. the region connecting the assembly and the basic nucleic acid binding domain; interestingly, only one-third to one-half of the subunits are cleaved by trypsin or Asp-N, possibly a reflection of the only quasi-equivalent arrangement of subunits in a T = 3 or T = 4 particle. The entire assembly domain, by contrast, is highly resistant to protease and hence likely to be compactly folded.

The accessibility of the region around aa 150 to protease, and the ability of an antipeptide antiserum directed against the very terminal aa residues of DHBV core protein (SCHLICHT et al. 1989a), have been taken as arguments for a surface exposure of the entire basic region. However, a monoclonal antibody against (phosphorylated) aa 165–175 fails to react with intact HBV cores (MACHIDA et al. 1991); HBV cores from *E. coli* with at least part of the basic region contain RNA which is protected from RNAse (BIRNBAUM and NASSAL 1990; HATTON et al. 1992), and the only major difference visible in the cryo-EM studies between particles from full-length and the variant core protein lacking the basic region is the internal electron density presumably representing RNA; finally, in cores produced in animal cells proper reverse transcription of the viral RNA pregenome requires the presence of the basic region (NASSAL 1992). Hence the nucleic acid binding domain is, at least in its greater part, buried inside the particle. Combining the large panel of monoclonal antibodies with known linear epitopes and well-designed core protein mutants with the cryo-EM techniques should help to further refine the structural model of the hepadnaviral core particle.

In summary, HBV core particles consist of an outer protein shell made from the tightly folded assembly domains of 120, or 90, respectively, dimeric core protein subunits, in which regions around aa 80, 130 and 150 are surface exposed; at least the majority of the basic nucleic acid binding regions face the interior of the capsid, where it is available for binding, and possibly shaping, the viral RNA pregenome for the complex transformation into partially dsDNA catalysed by P protein (see below).

### 5.1.4 Core Particle Assembly

Two systems are currently used to study the dynamics of core particle assembly: direct injection of core mRNA into *Xenopus laevis* oocytes, and in vitro transcription/translation; informative data on the reconstitution of particles from purified, dissociated core protein subunits are not yet available. The advantage of both the *Xenopus* and in vitro translation systems is that the concentration of core protein produced can be controlled by the amount of input mRNA. The major results with the oocyte system were first that dimers, but not higher aggregates, are the main intermediates in assembly (ZHOU and STANDRING 1992). Thus, unlike many other viral systems, there is no evidence for the accumulation of, for instance, pentameric or hexameric intermediates (which might more easily explain the formation of the complex T = 3 and T = 4 structures). These data corroborate the importance of the dimeric interaction between core protein subunits described above. Second, assembly requires a critical threshold concentration of core protein, which in this system is in the micromolar range (SEIFER et al. 1993). Protease accessibility experiments are fully consistent with the notion that in the dimers the assembly domain is resistant to many proteases while the basic region is easily clipped off; in the assembled particle, by contrast, the C-terminal region is in its greater part shielded from protease attack (SEIFER and STANDRING 1994). Co-expression of hepadnavirus core proteins from different species demonstrated that the core proteins from closely related mammalian hepadnaviruses form mixed particles (CHANG et al. 1994). Hence their lateral interaction surfaces must be very similar in structure, while the DHBV core protein was not incorporated into such mosaic cores. Interestingly, the dimeric intermediates were preferentially formed from monomers of the same species; at least in part this *cis*-preference appears to be due to a very fast, perhaps cotranslational, dimerization process. Very likely, the pool of core protein from which capsids are formed in an HBV-infected cell consists also of dimers rather than monomers.

While the information to form a symmetrical capsid is encoded in the primary sequence of the core protein, cellular factors may be involved in the assembly process. Using in vitro translation of HBV core protein, LINGAPPA et al. (1994) have recently presented evidence for the association of a bona fide assembly intermediate with a cytoplasmic chaperone of the *Hsc*60/*Gro*EL family (BRAIG et al. 1994; for reviews: GEORGOPOULOS and WELCH 1993; MARTIN and HARTL 1994). This high molecular weight intermediate has sedimentation char-

acteristics distinct from dimers and complete particles, and, importantly, it can be chased into complete cores with concomitant release of the chaperonin. That chaperones may be involved in the assembly process comes as no surprise given that *Gro*EL was originally characterized as a host factor in *E. coli* required for proper assembly of phage lambda particles. However, whether the observed association of *Hsc*60 with core protein in the reticulocyte system reflects a mechanistic necessity for core particle assembly in an infected hepatocyte remains to be determined. Given the ubiquitous existence of chaperones and their, by necessity, broad interaction specificities, they might also be involved in core particle assembly in the other systems used; hence it would be even more important to set up an assembly system comprising just the purified core protein monomers or dimers.

### 5.1.5 Replication-Competent Nucleocapsids

For the virus it is absolutely essential that the core particles produced are true replication-competent nucleocapsids. Thus specific incorporation of the correct RNA, and of the specialized enzyme able to transform this RNA into DNA, is of utmost importance for virus propagation. In retroviruses, protein-protein interactions between Gag, and the Gag-domain in the Gag-Pol fusion proteins, provide the fundamental mechanism for Pol incorporation into capsids, at the cost of a requirement for subsequent processing of the fusion proteins; RNA encapsidation is apparently mediated by Gag recognizing genomic viral RNA. That hepadnaviruses have evolved a completely different strategy became obvious with the discovery that, although the P ORF overlaps with the 3′ -part of the core ORF as in retroviruses, P protein is expressed as a separate entity (SCHLICHT et al. 1989b; CHANG et al. 1989); the exact mechanism of P expression from the RNA pregenome is still not established (CHANG et al. 1990; LIN and LO 1992). P protein consists of three domains (Fig. 4A) in the order terminal protein (TP), which is covalently linked to (–)-strand DNA, polymerase and RNAseH (RADZIWILL et al. 1990). As outlined above, the nucleic acid binding capacity of core protein is rather broad and unlikely to account for the specific encapsidation of the hepadnaviral RNA pregenome. In retrospect, therefore, it came as no surprise that it is a specific interaction between P protein and the RNA pregenome that is responsible for the co-encapsidation of both components (BARTENSCHLAGER and SCHALLER 1992). The details of this interaction are the subject of ongoing studies in several laboratories; however, many of the basics of the process have been established (NASSAL and SCHALLER 1993a). I will only summarize these fundamental aspects, and add new findings that corroborate the tight coupling of assembly and replication in hepadnaviruses.

Two key observations were that P protein and pregenome encapsidation are mutually dependent (BARTENSCHLAGER et al. 1990; HIRSCH et al. 1990), and that a relatively short region close to the 5′-end of the RNA pregenome mediates specific RNA packaging, even if fused to foreign RNA molecules (JUNKER-NIEPMANN et al. 1990). This region, termed the encapsidation signal $\varepsilon$, was

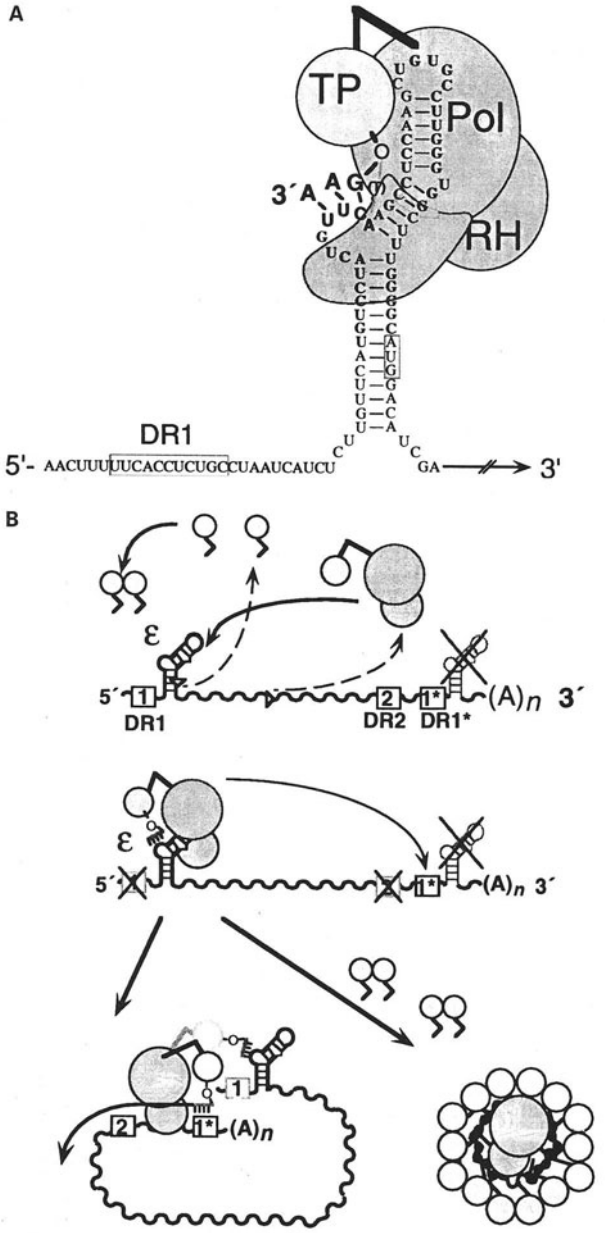


**Fig. 4A,B.** Central role for P protein/ε interaction in the hepadnaviral life-cycle. **A** Model for the interaction between P and ε. The secondary structure of ε and the three-domain structure of HBV P protein have been experimentally determined. The arrangement of P protein on the RNA is compatible with mutational studies analysing the competence of mutant ε-sequences for encapsidation and (–)-DNA initiation. Possibly, Hsp90 and other chaperones are associated with this complex (Hu and SEEGER 1996) **B** Events mediated by P/ε interaction. Core and P protein are translated from the pregenome. The binding of P to ε in *cis* triggers formation of a short DNA primer for (–)-DNA synthesis from the ε-bulge, but also addition of core protein dimers, initiating capsid assembly. The temporal order of events is not yet known; however, P protein mutants incapable of primer synthesis also mediate RNA encapsidation

subsequently shown to act via a characteristic bipartite stem-loop structure (Fig. 4A), which is necessary and sufficient for the process (POLLACK and GANEM 1993; KNAUS and NASSAL 1993). The interaction between P and ε may already be initiated during translation, as in competition experiments a preferential encapsidation of the RNA used as template for P production was observed. In the preC RNAs serving as mRNAs for the precore protein, translating ribosomes compete with P protein binding, and hence prevent encapsidation of these transcripts which would not support viral replication (NASSAL et al. 1990). Further mutational analyses of ε using defined (POLLACK and GANEM 1993; KNAUS and NASSAL 1993), or pools of partially randomized, ε variants (NASSAL and RIEGER 1995a) revealed that in particular the upper part of the ε structure is important for encapsidation; the apical loop tolerates only a few nt exchanges, while in the bulge region separating the two base-paired stems especially positions 1 and 2, but not the following unpaired residues, are critical. Thus, as in other RNA-protein interactions, a specific protein-binding site is created by a combination of structural and sequence-specific features on the RNA (Fig. 4A). The biological relevance of the ε structure is strongly supported by sequence data from HBV variants isolated from infected patients in all of which the characteristic ε structure is preserved (LASKUS et al. 1994). Why the 3′-copy of the ε sequence, present on all viral transcripts, is functionally silent, is still an unresolved issue. However, the selective interaction of P with the 5′-copy of ε which contains the initiator codon for core protein, may provide an advantage for packaging the correct RNA: P binding should suppress translation of core (and P) protein, and in effect deprive the RNA from ribosomes which might interfere with encapsidation.

In summary, binding of P to ε appears to provide a nucleation centre for core particle assembly (Fig. 4B), most likely by providing a binding site for the first one or few core protein dimers; the high cooperativity of core protein association plus the increased substrate quality of the ribosome-deprived pregenome may then be sufficient to ensure the highly selective encapsidation of the correct viral transcript. This principle is known from other viral systems: for example, in vitro assembly of phage R17 capsids is relatively inefficient without any RNA; the critical concentration for self-assembly of coat protein is lowered by the presence of unspecific RNA, and further reduced by the presence of the specific operator fragment acting as encapsidation signal (BECKETT et al. 1988). The analogy should not be drawn too far, as in the phage it is the many subunits of coat protein that mediate RNA encapsidation (PEABODY 1993; OLSTHOORN et al. 1994), and copackaging of the viral replicase is not required. An alternative view with the same outcome would be that P protein binding to ε creates a high-affinity binding site on the RNA for core protein. Quantitative data on the role of RNA and/or chaperones as potential assembly mediators are, however, not yet available.

In DHBV, principally the same mechanism for specific co-encapsidation of P protein and pregenome is operating. However, the sequence homologous to HBV ε is not sufficient for encapsidation; a second region located inside the P

ORF is also required ("region II"; CALVERT and SUMMERS 1994), possibly due to the relatively low stability of the DHBV ε structure, which has not yet been experimentally analysed. According to computer predictions it is quite different from that in HBV while phylogenetic arguments (JUNKER-NIEPMANN et al. 1990) as well as functional homologies (POLLACK and GANEM 1994) suggest that an HBV ε like structure can form. Interestingly, the lack of region II in the recently found major DHBV transcript that arises from splicing of the genomic RNA would prevent its encapsidation, similar as for subgenomic retroviral transcripts.

The general picture emerging from these data is that P protein interacts specifically with a structured RNA element on the RNA pregenome, forming a preassembly complex which lowers the critical concentration of core protein required to initiate self-assembly (Fig. 4B). This dependence favours the selective co-encapsidation of both the RNA substrate and the reverse transcriptase required to convert it into DNA, i.e. formation of replication-competent cores.

Intriguingly, the very same P/RNA interaction that triggers capsid assembly is also directly involved in viral replication. Until recently, it was believed that reverse transcription of the hepadnaviral pregenome (cf. Fig. 1C) would initiate, via protein priming, de novo at the 3′-proximal DR1*, and then proceed continuously, in contrast to the discontinuous mechanism employed by retroviruses and most other retro-elements. However, data obtained with an in vitro translation system capable of producing enzymatically active DHBV P protein (WANG and SEEGER 1992, 1993; TAVIS and GANEM 1993) suggested that part of region I of the DHBV encapsidation signal would represent the replication origin, by serving as template for a short DNA primer which is subsequently translocated to 3′-DR1*. We have recently obtained direct sequence evidence that indeed the 3′-part of the HBV ε bulge region is used as template for the synthesis of such a short DNA primer (NASSAL and RIEGER 1996). Exclusively the 5′-copy of ε and the 3′-copy of DR1 are involved (RIEGER and NASSAL 1996). Possibly, selective usage of the individual ε and DR copies is made possible by a close spatial proximity of the 5′- and the 3′-terminal regions of the RNA pregenome. In summary, the very same *cis*-acting RNA element is crucial for both encapsidation and hence nucleocapsid assembly, and for virus replication, as indicated in Fig. 4B.

The use of a *cis*-acting structured, 5′-proximal RNA element to correctly position the polymerase over the initiation site is a unique strategy which places the evolutionary origin of hepadnaviruses between that of primitive retro-elements like the Mauriceville plasmid from *Neurospora mitochondria*, which likewise uses a *cis*-acting, but 3′-proximal RNA structure to position its reverse transcriptase (WANG and LAMBOWITZ 1993), and modern retroviruses in which the specificity of (–)-DNA initiation is determined by a *trans*-acting tRNA primer, its basepairing with the 5′-proximal primer-binding site and the affinity of the retroviral RT for tRNA, in particular the one used as primer (MAK et al. 1994). However, the discontinuity of (–)-DNA synthesis as such brings hepadnaviral replication into closer accord with the vast majority of retro-elements than previously thought.

While experiments in different heterologous systems have shown that the only viral gene products required for replication initiation are P protein and RNA (WANG and SEEGER 1992; SEIFER and STANDRING 1993; TAVIS and GANEM 1993), proper formation of the viral DNA genome has not been demonstrated in the absence of core protein. This suggests that the complex nucleic acid transformations during reverse transcription depend on the auxiliary functions of core protein.

Where in the cell do replication-competent core particles assemble? All available evidence suggests that this process takes place in the cytoplasm: P protein interacts probably cotranslationally with the pregenome, and encapsidation exclusion of the preC RNAs is translationally controlled; after transfection of an intron-containing $\varepsilon$ reporter construct the spliced cytoplasmic but not the unprocessed nuclear transcript was encapsidated (RIEGER and NASSAL 1995). Hence, the major events during core particle assembly have to occur in the cytoplasm. In keeping with this, many immune fluorescence data show a cytoplasmic stain for core protein. Sometimes, however, staining is also observed in the nucleus, particularly with HBV (e.g. GUIDOTTI et al. 1994). Core protein does indeed contain nuclear localization signals (YEH et al. 1990; ECKHARDT et al. 1991) which might act in a cell cycle dependent manner (YEH et al. 1993). In transgenic mice expressing HBV core protein, the antigen is strictly nuclear in resting cells, becomes detectable in the cytoplasm during mitosis, and then remains cytoplasmic, indicating that intact core particles are unable to cross the nuclear membrane (GUIDOTTI et al. 1994). This suggests that if the net rate of synthesis is faster than that of nuclear transport assembly will occur in the cytoplasm; in the reverse case, unassembled core protein subunits will accumulate in the nucleus and assemble there. The exclusive cytoplasmic localization of the DHBV core protein (PUGH et al. 1989) may then be explained by a more efficient synthesis and/or lower critical concentration for assembly. The more general implication of these data is that after infection, or during the postulated intracellular cycling (TUTTLEMAN et al. 1986), the core particles delivering the viral genome will have to disassemble at the nuclear membrane.

### 5.1.6 Covalent Modifications of the Core Protein

The only known covalent modification of hepadnaviral core proteins is phosphorylation. Core particles isolated from infected livers contain, in addition to viral nucleic acid and P protein (see below), an endogenous kinase activity (ALBIN and ROBINSON 1980; GERLICH et al. 1982) that, upon addition of ATP, phosphorylates the core protein at several sites in the basic C-terminal region; similarly, a kinase activity is associated with cores produced from recombinant baculovirus (LANFORD and NOTVALL 1990). Heterologously expressed cores from *Xenopus* oocytes are also heavily phosphorylated; those from *E. coli* or yeast are not (MACHIDA et al. 1991; HATTON et al. 1992). Studies with specific kinase inhibitors, and an in vitro reconstitution system KANN and GERLICH (1994), have recently suggested that the major fraction of the associated kinase activity is

attributable to protein kinase C, estimated to be present in about two copies per particle. A general conclusion from these studies is that core protein phosphorylation at the multiple SPRRR clusters decreases or abolishes nucleic acid binding, similarly to the known effect of phosphorylation at the SPKK motifs in histones (HILL et al. 1991); it may hence be involved in replication of the viral genome and/or uncoating after infection. However, only recently have functional data been obtained with the DHBV system, revealing a complex pattern of preferences for phosphorylated or non-phosphorylated Thr or Ser residues during the steps of core particle maturation; importantly, intracellular DHBV capsids are heterogeneously phosphorylated while capsids isolated from extracellular virions appear homogeneous with a mobility corresponding to unphosphorylated protein (YU and SUMMERS 1994). It therefore seems conceivable that different activities of the capsid in the viral life-cycle are regulated by sequential phosphorylation dephosphorylation.

## 5.2 Subviral Particles

Envelopment of hepadnaviruses differs in several respects fundamentally from that of retroviruses: (1) all hepadnaviruses generate more than one envelope protein; (2) there is no evidence for proteolytic processing; (3) the surface proteins by themselves, i.e. without requiring interactions with the capsid, form empty, non-infectious lipoprotein particles, usually in vast excess over complete virions (cf. Figs. 1B, 2A); (4) hepadnaviral S proteins do not accumulate to any appreciable extent at the plasma membrane of the host cell; (5) the S proteins are required for export of capsids, i.e. core particles cannot, by themselves, leave their host cell. This latter statement is based on the absence of naked capsids in serum, even of immune-compromised patients (POSSEHL et al. 1992); however, the supernatants of cells transfected with efficient expression plasmids for core protein often contain such naked cores (BRUSS and GANEM 1991b; NASSAL 1992; LENHOFF and SUMMERS 1994). It is believed but not proven that these capsids are released from dead cells.

After summarizing earlier data on the composition of S particles, I will emphasize recent findings that in particular concern the influence on membrane topology of the additional N-terminal domains in the L protein. The reader interested in additional information on the biochemistry of S particles should consult the reviews by HEERMANN and GERLICH (1991) and GANEM (1991).

### 5.2.1 S Particle Composition

Earlier electron microscopic studies, using negative staining, of serum samples from HBV-infected individuals showed abundant coreless spherical particles about 22 nm in diameter (from preliminary cryo-EM data, however, it appears that for HBV the round objects may represent disks rather than spheres; S.D. Fuller, personal communication), fewer filamentous structures with the same

diameter but of variable length, and even fewer virions with their characteristic inner core (Fig. 2A). Typical particle numbers in the serum of chronic carriers are in the range of $10^{13}$/ml for the spheres, $10^{10}$/ml for filaments and up to $10^{9}$/ml for virions. These values, however, are extremely variable for different patients, and within one patient during the course of infection (HEERMANN and GERLICH 1991). The 22- nm particles of HBV are of historical interest as they represent the overwhelming majority of the classical "Australia antigen" (BLUMBERG et al. 1965) now known as HBsAg; also the first-generation HBV vaccine was derived from S particles purified from chronic carriers but has now been superseded by recombinant S protein preparations isolated from yeast (MCALEER et al. 1984). For DHBV, the empty envelopes appear, at least by negative staining, as less-ordered "bags" of similar, or larger size as complete virions (diameter approximately 40 nm), and filamentous forms are absent. The biological function of S particles is not clear; it is generally believed that they represent decoys that trap neutralizing antibodies in unproductive interactions.

The three envelope proteins produced by mammalian hepadnaviruses (L, M and S) and the two avian proteins (L and S) are schematically represented in Fig. 5A. All HBV, but not the DHBV, surface proteins occur also in glycosylated form. For HBV, the apparent molecular masses of the proteins are p24/gp27 (S), p30/gp33/ggp36 (M) and p39/gp42 (L); the DHBV S protein appears as p17, the L protein as p35, sometimes as doublet p35/p36. While for HBV the overall ratio of HBV S, M and L proteins in serum is approximately 1000:10:1, the individual particles, enriched from serum by biochemical fractionation, differ substantially in their protein composition, in particular regarding L (HEERMANN and GERLICH 1991). In all of them, the small S protein is the predominant species, and M constitutes some 5%–10% of the total protein mass. However, spheres, estimated to consist of about 100 protein subunits (PETERSON 1987), contain very little L protein (up to maximally 5%); by contrast, L is enriched in filaments (approximately 10%–20% by weight) and even more abundant in virions (from 20% up to 50%); the exact composition is difficult to measure and may vary with the source of S particles (W.H. Gerlich, personal communication). In DHBV, the difference in composition between S particles and virions is much less pronounced (both contain some 10%–25% of L protein; SUMMERS et al. 1991) and, owing to the very similar properties of empty and core-containing particles, a complete separation of virions and S particles has not yet been achieved.

S particles from patient serum contain some 25% total lipid by weight, with about 67% phospholipids (PL), 15% free cholesterol, 14% cholesterol esters and 4% triglycerides (TG; GAVILANES et al. 1982). As the lipid composition of internal membranes is different from that of the plasma membrane (VAN MEER 1989; ALLAN and KALLEN 1994), the high proportion of phosphatidylcholine and relatively low proportions of phosphatidylserine and sphingomyelin suggest that the S particle lipid is derived from the ER; however, phosphatidylinositol (PI) is usually high in the ER and lower in the plasma membrane, yet PI could not be detected at all in HBsAg. Even more striking is its high content of total cholesterol. Typical ratios of cholesterol/phospholipid are about 0.08 for ER membrane,

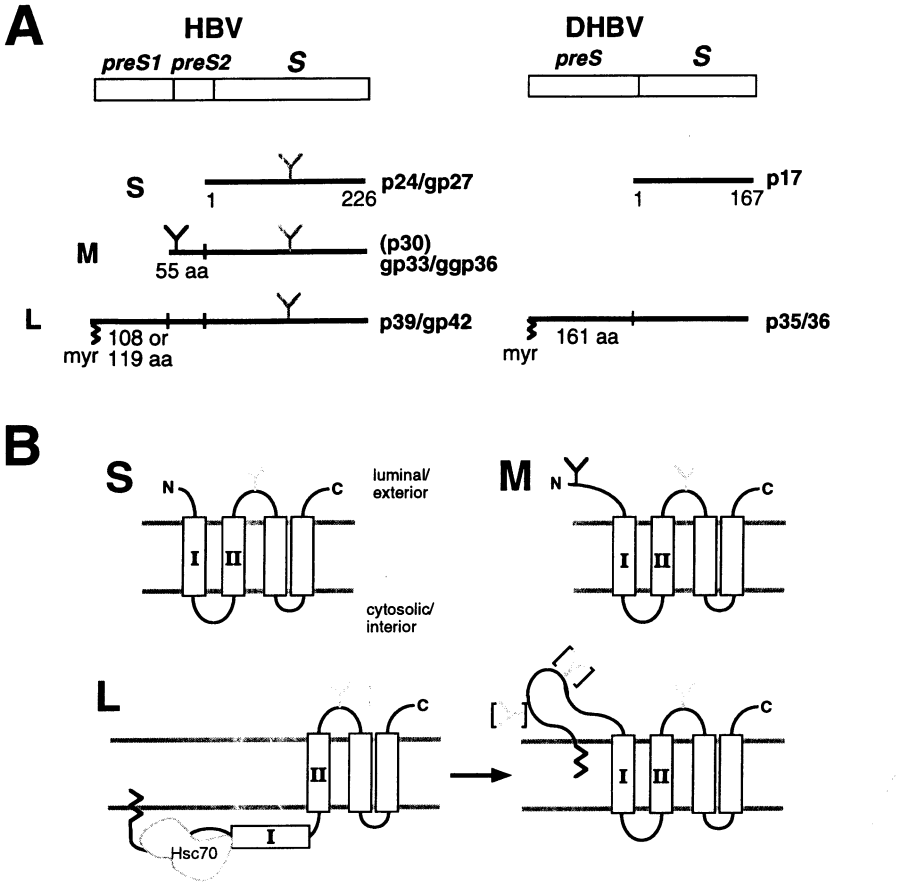


**Fig. 5A,B.** Hepadnaviral surface proteins. **A** Correlation of preS/S genes and their products. The length of the individual domains is given in numbers of aa; apparent molecular masses of glycosylated and unmodified proteins are indicated *on the right. Y-shaped symbols represent* glycan residues; *filled symbols* indicate complete, *stippled symbols* partial glycosylation; *myr*, myristic acid residue covalently attached to the N-terminal residues of HBV and DHBV L protein. **B** Membrane topology of the HBV surface proteins. *Horizontal lines* represent the borders of the membrane, *bars* transmembrane regions. Regions I and II have been experimentally characterized; the topology of the following two transmembrane domains is not finally settled. S and M adopt their similar topology in a cotranslational fashion; part of the initially cytoplasmically disposed preS domains of L are apparently post-translationally translocated across the membrane. Note that in L the glycosylation sites in the preS1 and preS2 domain are only used in artificial fusion proteins with a cleavable N-terminal signal sequence, while authentic M is completely glycosylated in its preS2 domain. The overall organization of the DHBV S and L proteins is probably very similar. The presence of *Hsc*70 at the indicated region is inferred from data obtained with the DHBV L protein. See text for details

and between 0.40 and 0.76 for plasma membrane; in HBsAg a ratio of 0.48 was found. The lipid composition of S particles from permanent cell lines (i.e. relatively freshly synthesized particles, in part obtained from cells grown in serum-free medium) with PL 91%, 4% TG and, strikingly, only about 5% cholesterol, is

in much better agreement with ER derivation (SATOH et al. 1990). Altogether, however, the variability of the data suggests that lipids may be easily exchanged, and that lipid composition is of limited value for determining the site of S particle or virion biogenesis. It should be noted that the overall lipid content of S particles is very low, and that it has not been demonstrated that the lipids are arranged in a typical unit membrane bilayer.

### 5.2.2 Structure and Function of the Surface Proteins

As for the core protein, transfection of genetically altered preS/S genes, either alone or in the context of complete genomes as well as in in vitro experiments, has shed light on the roles of the individual S proteins in S particles, and virion assembly. In the DHBV system, infectivity is also a relatively easily addressable parameter. Before describing in more detail the newer data, focused on the surprising multifunctionality of the L protein, the major functions of the different surface proteins will be briefly summarized.

S alone is able to form 22- nm particles that are efficiently secreted, at least in cells from higher eukaryotes (not in insect cells or yeast; LANFORD et al. 1989). The same is true for M protein (SHEU and LO 1992) but not for L, which is retained in the endoplasmic reticulum (ER), or a later compartment, unless S protein is also present; in fact, excess L protein *trans*-inhibits S secretion (PERSING et al. 1986). This suggests that normal L export is mediated by formation of mixed particles, and that the ratio of S to L protein is a critical factor determining the fate of L protein. Retention of excess L protein in the ER, or a subsequent compartment, can explain the extreme dilation of membranous structures observed in liver cells from transgenic mice overexpressing L, and may be one factor in hepatocellular carcinogenesis (CHISARI et al. 1989). While, except for one report (UEDA et al. 1991), all other studies agree that M protein has no vital functions (e.g. BRUSS and GANEM 1991b; FERNHOLZ et al. 1993), L, and in particular its preS1 domain, is absolutely essential for two critical steps in the viral life-cycle: attachment to the target cell, and formation of complete virions, i.e. envelopment of the capsid (see below). Intriguingly, this dual function requires the preS1 domain to be located once on the exterior and once in the interior of the virion. Recent studies on the membrane topology of the hepadnaviral surface proteins may hold a clue to this structural puzzle.

### 5.2.3 Membrane Topology of the Hepadnaviral Surface Proteins

All hepadnaviral surface proteins are integral membrane proteins; as in their host cell counterparts, topology, i.e. the orientation of individual polypeptide chain segments relative to the membrane, is of utmost functional importance (Fig. 5B). In contrast to most viral glycoproteins (DOMS et al. 1993), they contain more than one transmembrane region. N-Linked glycosylation provides clear biochemical evidence that all HBV surface proteins are initially targeted to the ER membrane, in accord with ultrastructural data (PATZER et al. 1986). The characteristic mod-

ifications of the glycan side chains demonstrate that surface protein export follows the normal secretory pathway. Initial ER targeting is mediated by an internal signal-anchor sequence ("signal I" in Fig. 5B) close to the N-terminus of S (aa residues 4–28), presumably in a signal recognition particle (SRP) dependent fashion. Such signal-anchor sequences usually consist of some 10–20 hydrophobic aa residues, mediating membrane targeting, and flanking hydrophilic regions. Combinations of signal anchor and so-called stop-transfer sequences allow multispanning membrane proteins to adopt a very specific topology (for review: HIGH and DOBBERSTEIN 1992). In the S protein, a second hydrophobic region ("signal II" in Fig. 5B; aa residues 80–100), separated from signal I by a cytoplasmic loop of about 50 aa, has also been shown to be embedded into the membrane, and is followed by a stretch of some 70 luminally exposed residues which carry the major S-specific epitope and a glycosylation site (EBLE et al. 1987), which for as yet unknown reasons is only used in about half of the S protein molecules. The following 50 residues are again hydrophobic and may be arranged in two more transmembrane domains (STIRK et al. 1992; but see also PRANGE and STREECK 1995). Signal II, in the absence of signal I, is able to mediate ER-targeting of corresponding N-terminal deletion variants and to correctly orient the remaining C-terminal part of S; such proteins are, however, secretion incompetent (EBLE et al. 1987; BRUSS and GANEM 1991a).

The topology of the M protein appears to be essentially the same as that of S; its larger N-terminal PreS2-containing domain is obviously efficiently translocated into the ER lumen by the downstream signals in the S-domain (EBLE et al. 1990), as demonstrated by its complete glycosylation at Asn-4 of the preS2 domain.

### 5.2.4 Special Role of the L Protein

Although L protein contains the complete M and S domains, its properties are distinctly different from those of the smaller surface proteins: it is not by itself secreted and instead retained in the ER, or a subsequent compartment, and it is required for both virion formation and for infectivity. These characteristics are obviously correlated with the preS1 domain, and possibly with the myristic acid modification present at the N-terminal Gly residue in mature L (PERSING et al. 1987). Deletion studies had indicated that the retention signal in L (subtype adw) is exclusively located in the very N-terminal region of the PreS1 domain (aa 2–19; KUROKI et al. 1989), and independent of myristoylation; other studies, however, concluded that both myristoylation (PRANGE et al. 1991) and the primary sequence of the carboxyl-proximal preS1 amino acids are important (YU 91; NEMECKOVA et al. 1994). Probably, these conflicting data are due to the different HBV subtypes used in these studies; e.g. subtype adw contains an insert of 11 aa at its N-terminus. While L protein retention may enhance virion production in vivo, it is not a prerequisite for virion formation in transfected cells (BRUSS and

THOMSSEN 1994); however, the fatty acid modification is certainly essential for infectivity of both DHBV (MACRAE et al. 1991) and HBV (GRIPON et al. 1995).

Previous data on the accessibility to antibodies and proteases of the preS1 domain in virions and filaments showed that, in accord with the proposed function of the domain as viral attachment protein, virions can be precipitated with preS1-specific antibodies and that the preS2 domain in the L protein is accessible to protease (HEERMANN et al. 1984), indicating an exterior location. Surprisingly, however, and in contrast to M, the L protein is only glycosylated in its S part (to about 50%, as also observed for S), i.e. neither the preS2-specific nor the preS1-specific (Asn-4 of preS1) sites are used. Also, the requirement for L protein in capsid envelopment (see below) is hard to understand if all of its extra sequence were located on the outside of the virion.

Recent data from three groups indicate now that HBV L protein is initially synthesized with a cytoplasmic disposition of the preS1 domain, and that a fraction of the preS1 domains is post-translationally translocated across the membrane (Fig. 5B). Thus it appears that hepadnaviruses exploit an unusual mechanism which allows them to use the same protein domain for functions requiring a luminal/exterior (host cell attachment) as well as a cytoplasmic/interior location (capsid envelopment).

In a coupled in vitro translation/translocation system, which is incompetent for particle formation, most if not all of the preS1 and preS2 domains of L remained accessible to protease, while the preS2 domain of M was protected, indicating that the N-terminal region of M, but not that of L, was efficiently translocated (OSTAPCHUK et al. 1994). Similar experiments with microsomes from in vivo labelled transfected cells showed that also in vivo the preS1/preS2 domains of L are initially cytoplasmically exposed whereas in secreted particles derived from transfected cells and patient serum about half of the preS domains of L are accessible on the particle exterior and hence must have traversed the membrane (BRUSS et al. 1994). Translocation is likely to occur in a post-translational fashion, since the protease-resistant fraction of L molecules increases slowly with time; however, it is not accompanied by glycosylation of the preS1 and/or preS2-specific glycan attachment sites (PRANGE and STREECK 1995). By contrast, these sites are efficiently used if cotranslational translocation is artificially brought about by fusing a cleavable N-terminal signal sequence to L (BRUSS et al. 1994). The region responsible for the unusual topology of L appears to be located in the very C-terminal part of the preS1 domain since an internal deletion of the last 38 preS1 aa gave an L protein whose preS domains are efficiently translocated and glycosylated (PRANGE and STREECK 1995). Complementary, cotranslational translocation was prevented in N-terminally truncated L variants as long as at least the last 17 preS1 encoded aa were present, while further deletions led to an M-like topology (BRUSS and THOMSSEN 1994). Interestingly, the same region preventing cotranslational translocation is also essential for interaction with the capsid.

What could be the mechanism for the delayed and apparently to some extent regulated translocation of about half of the preS1 domains? The answer is

not yet clear; however, recent observations with the DHBV system may hold some clues. Although the topology of the DHBV L protein has not been studied in detail, the available data on accessibility to antibodies (YUASA et al. 1991) and proteases (KLINGMÜLLER 1992) suggest a similar organization to its HBV counterpart. Pulse-chase experiments indicate that the proportion of protease-protected and hence luminally disposed preS domains of intracellular L protein increases with time, and in secreted virions about one-half of the preS domains is accessible to protease (I. Swameye and H. Schaller, personal communication). By ortho-phosphate labelling of DHBV-infected primary duck hepatocytes it was shown that a fraction of the L protein is phosphorylated (GRGACIC and ANDERSON 1994). The exact phosphorylation sites are not yet known but may be located in the preS domain, which in HBV L protein can face either the interior or the exterior of the particle. Conceivably, in the phosphorylated molecules the extra charges would interfere with membrane translocation and could thus account for the initial cytoplasmic disposition of the preS domain. As the electrophoretic band pattern changes with time after infection one might envisage regulation of translocation by sequential phosphorylation/dephosphorylation events. A number of obvious mutational experiments should soon clarify this point.

However, post-translational translocation of an already folded protein domain is not a trivial task. An interesting observation is therefore that a cellular chaperone is associated with DHBV particles (I. SWAMEYE, C. KUHN, M. HILD, U. KLINGMÜLLER and H. SCHALLER, in preparation). The protein of about 72 kDa consistently copurifies with DHBV S particles and is also present in preparations enriched for virions; a similar protein is found in preparations of HBV filaments and virions. Direct sequencing followed by molecular cloning showed the duck protein to be the homologue of the constitutively expressed heat shock protein Hsc70, which is highly conserved between species. The major roles for the cytosolic heat shock proteins of the Hsp70/DnaK family, at least in bacteria usually performed in cooperation with DnaJ, are the prevention of premature folding of nascent polypeptides, inhibition of unproductive aggregation, and maintaining proteins in a translocation-competent state, likely mediated by the chaperones recognizing exposed hydrophobic protein regions; this interaction is released by ATP (for reviews: GEORGOPOULOS and WELCH 1993; MARTIN and HARTL 1994). Co-immunoprecipitations suggest that it is the preS domain that interacts with Hsc70 (Fig. 5B). Proteolytic cleavage and ATP-mediated release of the particle-associated Hsc70 require the presence of detergent, indicating that the protein is located in the interior of the particle.

Whether a causal relationship exists between Hsc70 association and the unusual topology of the L protein remains to be established; however, it is tempting to speculate that both are mechanisticly linked. That Hsc70 remains associated with the preS domain despite the relatively high ATP concentration in the cytoplasm is per se remarkable. One explanation could be that the primary sequence of PreS contains (a) region(s) of very high affinity to the chaperone (the C-terminal part of HBV preS1 preventing cotranslational translocation would be a candidate); the chaperone would then keep the preS domain competent for later

translocation. Such an explanation is consistent with the finding that replacing preS by the tightly folding globin domain abrogates secretion competence, which is restored if an N-terminal signal sequence mediating cotranslational translocation is added to the artificial protein (BRUSS and GANEM 1991a). Alternatively, the preS domain might be unable to fold properly in the reducing environment of the cytoplasm and hence remain associated with the chaperone; those chains that manage to be translocated might be stabilized by disulphide bonds inside the ER lumen (or later compartment) with its much higher redox potential (DOMS et al. 1993). All S proteins contain abundant Cys residues, which are indeed extensively used for intra- and intermolecular disulphide bridges (PETERSON 1987). Correct disulphide bonding is not only important for further secretion but also for maintaining the antigenic, and hence presumably structural, integrity of the proteins (MANGOLD and STREECK 1993). Further experiments are required to elucidate the functional significance of Hsc70 in the hepadnaviral life-cycle; however, the principal parallel to the recent finding that another cytosolic chaperone, cyclophilin A (peptidyl-prolyl-*cis-trans*-isomerase), is associated with and required for the infectivity of HIV-1 virions (FRANKE et al. 1994; THALI et al. 1994) is certainly remarkable.

### 5.2.5 S Particle Assembly

The data presented above demonstrate that all hepadnaviral surface proteins are initially synthesized as transmembrane proteins at the ER (SIMON et al. 1988), with S and M acquiring their final topology in a cotranslational fashion, while in at least a fraction of L the preS domains are post-translationally reoriented. The absence of detectable surface proteins on the plasma membrane indicates that somewhere during their intracellular journey the surface proteins must bud, as S particles, into the lumen of one of the compartments of the secretory pathway, or, in the case of excess L protein, be retained in the membrane of such a compartment. Usually, the ER itself is considered the budding compartment, based on EM studies that show S particles in the lumen of smooth intracellular membranes (PATZER et al. 1986), and the observation that the glycans on all intracellular surface proteins are sensitive to endoglycosidase H while on the extracellular forms they are resistant. The enzyme digests only immature, high-mannose-type glycans (PATZER et al. 1984). This indicates that the rate-limiting step of export must lie prior to the medial Golgi.

Meanwhile, there is accumulating evidence for (an) additional ''intermediate'' compartment(s) between the ER and the Golgi complex (for review: HENDRIKS and FULLER 1993); thus, budding into this compartment would also be compatible with the glycosylation data. Double immunofluorescence of stably transfected, HBV S protein producing cells indeed showed a fraction of S in a compartment devoid of resident ER markers like BiP and protein disulphide isomerase (PDI) but containing Rab2, an established intermediate compartment marker (HUOVILA et al. 1992). When disulphide formation was followed by pulse chase experiments, initially only dimer cross-links were observed; these dimers

were then slowly chased into heavily cross-linked multimers. At least in vitro, the oligomeric but not the dimer cross-links were unstable in the presence of PDI. Brefeldin A, a drug that via interaction with ARF (ROTHMAN 1994) blocks exit from the ER and leads to redistribution of *cis* and medial Golgi markers to the ER (LIPPINCOTT-SCHWARTZ et al. 1990), led to accumulation of dimers but prevented oligomer formation, suggesting that their generation requires exit from the ER. The PDI-excluding compartment, but not the ER contained the typical S particles. Thus, S particle formation appears to proceed by a two-step mechanism (Fig. 6A), in which S-S-linked S protein dimers are generated in the ER in the presence of PDI, but assemble into particles in a later, PDI-deficient compartment which is discontinuous with ER and Golgi (HUOVILA et al. manuscript submitted). Oligomerization, and stabilization of the multimers by oxidative cross-linking, could facilitate budding into the lumen of the compartment by excluding host components that mediate normal transport vesicle formation towards the cytoplasmic side (ROTHMAN 1994).

As the half-times of oligomer-cross-link formation, carbonate extractability (indicating transition from a membrane-bound into the soluble, particulate form) and particle secretion are very similar (SIMON et al. 1988), the budding process in this compartment appears to be the rate-limiting step in spherical S particle secretion. Whether the same holds true for the L-containing filaments (as implied in Fig. 6C) has not yet been established, and it is not clear where translocation of the preS domains occurs. The lack of glycosylation in the translocated preS domains as well as the inability of isolated microsomes to support translocation suggests that the process takes place in a post-ER compartment, possibly the intermediate compartment mentioned above. Determining the preS topology in cells kept at low temperature, or treated with brefeldin A, could help to address this question.

## 5.3 Virion Formation

Envelopment of the cytoplasmically produced nucleocapsids is the least understood of the morphogenetic events in the hepadnaviral life-cycle. Attempts to directly visualize budding virions by EM, i.e to show profiles in which the viral envelope is contiguous with the unit membrane of the corresponding compartment, have not led to convincing results, possibly because budding is very rapid. Based on analogies to S particle formation, it is generally assumed that budding occurs into the ER, as with flavi- and pestiviruses; in view of the newer data on empty envelope formation, the intermediate compartment may as well be the site of virion formation, as seen, e.g. for corona viruses (KRIJNSE-LOCKER et al. 1994; for reviews: PETTERSON 1991; GRIFFITHS and ROTTIER 1992). However, it is by no means proven that empty envelopes and virions are generated by exactly the same mechanism (as speculated in Fig. 6D). One might expect that capsid-triggered budding is in fact a more efficient process than S particle formation, otherwise only empty envelopes may be generated.

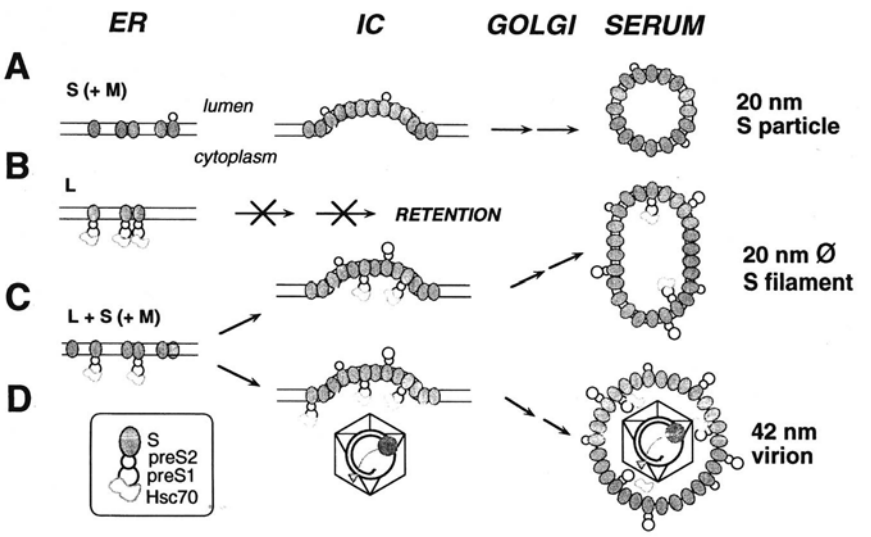


**Fig. 6A-D.** Model for the formation of S particles and virions. **A** Spherical S particles. S protein plus a small amount of M dimerize in the ER and oligomerize in the intermediate compartment (*IC*). Presumably triggered by interactions between subunits, spherical particles bud into the lumen of the IC and are exported through the subsequent compartments of the secretory pathway. **B** L protein retention. L protein is initially synthesized with cytoplasmically disposed preS domains which are probably associated with *Hsc*70. In the absence of S, L is not exported but retained in the ER or IC. **C** Filamentous S particles. If both L and S protein are present, mixed oligomers are formed which bud as filamentous subviral particles. About half of the preS domains are post-translationally translocated, the others probably remain associated with *Hsc*70. The different composition of spherical and filamentous particles suggests that L protein is responsible for their different morphologies. **D** Virions. Cytoplasmic nucleocapsids interact with the non-translocated preS domains of L, possibly mediated by *Hsc*70, while the translocated preS domains in the mature virion are available for later attachment to the host cell. There is good experimental evidence for the two-step assembly mechanism of spherical S particles which contain no or little L protein. The models shown in **C** and **D** assume similar pathways for the L-containing particles; inferred details of the sites of assembly and preS translocation, or the role of *Hsc*70 in HBV filament or virion formation, are compatible with the limited data available from the DHBV system but should be regarded as speculative at present

While it is not clear how capsids are targeted to the membrane containing the surface proteins, and what regions on the capsid are the determinants for interaction with the envelope proteins, recent data have revealed some details for the surface proteins. First, L protein is absolutely required for virion formation (Bruss and Ganem 1991b), implying a matrix protein-like function for the preS1 domain in this interaction. As outlined above, the paradox that this domain is found on the virion exterior has been resolved by demonstrating that about half of the preS domains remain on the interior side of the envelope. As shown using a series of deletions in the preS1 domain, the same relatively short C-terminal region of the preS1 domain that prevents cotranslational translocation is sufficient to produce replication-competent particles that can be immunoprecipitated with anti-S antibodies, as expected from enveloped virions (Bruss and Thomssen 1994). This suggests that an initial cytoplasmic disposition of preS is

important for virion formation, and that the C-terminal one-sixth of preS1 is involved in capsid binding. Both myristoylation and L protein retention in an early secretory compartment are not per se essential for the process in transfected cells (Bruss and Ganem 1991b). However, the fatty acid modification is apparently critical for virion infectivity (see above), possibly reflecting a role in attachment to the host cell, or subsequent steps such as membrane fusion. Also, L protein retention might at least contribute to efficient virion formation in vivo.

In general, the functional roles of the DHBV S and L proteins appear to be similar to those of their HBV counterparts. Both proteins are required to form enveloped virus particles (Summers et al. 1991). Truncated L proteins lacking up to 52 N-terminal aa still support formation of enveloped virions which, however, are non-infectious, like a mutant lacking the myristoylation signal (Macrae et al. 1991). A linker-scan analysis of the preS region (Lenhoff and Summers 1994) showed that the primary sequence between aa residues 117 and 136 is important for capsid envelopment, while almost all of preS is essential for infectivity. A defect for capsid envelopment could also be induced by replacing the authentic aa residues at positions 128 and 131, suggesting that this region comprises a determinant for capsid/envelope interaction. Hence the preS domain appears to be functionally bipartite, with the N-terminal two-thirds being required for interaction with the host cell (i.e. infectivity), and the C-terminal one-third for interaction with the capsid (i.e. envelopment).

The regions directly implicated in these interactions are small compared to the overall size of the preS domains; for HBV, the viral attachment site has been proposed to reside in the preS1 sequence comprising aa 21–47 (Neurath et al. 1986, 1992), for DHBV the corresponding region has been mapped to the preS aa 81–121 (Klingmüller 1992). It should be stressed, however, that infectivity not only requires the physical presence of the viral attachment site but its being exposed on the virion exterior, while for envelopment the C-terminal part of preS must be cytoplasmically disposed. Thus, the information for preS translocation must also be encoded in the preS sequence. Moreover, the time course of the process must be coordinated with export, as neither topological state with all preS domains on one side is compatible with assembly of infectious virions. The as yet mysterious retention of L protein might contribute to this coordination.

Retroviruses are long known to form pseudotypes with unrelated viruses, e.g. vesicular stomatitis virus (cf. Hopkins 1993), indicating that the interaction between the capsid and Env proteins is relatively unspecific. The apparent dependence of core particle export on the presence of L protein implicates that hepatitis B virus envelopment, by contrast, relies on specific recognition events between the capsid and the surface proteins. Phenotypic mixing experiments support this view. Concerning envelope formation as such, only the closely related avian, or the mammalian envelope proteins can substitute for each other, since surface proteins from HBV and WHV, but not DHBV coassemble into mixed S particles. Regarding interaction with the capsid, a chimeric L protein with the preS region of WHV and the S domain of HBV supports virus envelopment, whereas a corresponding DHBV-preS/HBV-S chimera does not al-

though it forms S particles (GERHARDT and BRUSS 1995). These data suggest that even if the lateral interactions in the envelope are undisturbed the preS domains can discriminate between different capsids. Complementary, HHBV cores with a DHBV envelope produced by cotransfection infect duck cells much more efficiently (ISHIKAWA and GANEM 1995) than authentic HHBV virions (SPRENGEL et al. 1988). Hence the preS domains discriminate between the different target cells but not the very similar capsids. Overall it appears that in its specificity the mechanism of hepadnaviral envelopment is more closely related to that of alphaviruses than that of retroviruses (SUOMALAINEN and GAROFF 1994; CHENG et al. 1995; for review: GAROFF et al. 1994).

The interaction between envelope proteins and capsids may also be responsible for the apparent regulation of cccDNA levels in DHBV-producing cells (SUMMERS et al. 1990). Nuclear cccDNA is the transcriptionally active template; the pool of cccDNA is believed to be replenished by an intracellular recycling of cytoplasmic cores to the nucleus (TUTTLEMAN et al. 1986; WU et al. 1990). If no envelope protein, or only S protein, is present, the copy number of cccDNA molecules per infected primary duck liver cell can increase from some 20 to several hundred (SUMMERS et al. 1990; LENHOFF and SUMMERS 1994). Downregulation depends on the L protein, and mutations in L that prevent capsid envelopment upregulate cccDNA levels. This suggests that the interaction of L with the capsid is involved in both processes. According to this model, core particles deliver the partially ds DNA genome to the nucleus by default; once sufficient amounts of L and S protein have accumulated they are instead sequestered into the secretory pathway. Recent in vitro evidence for direct binding between capsid and L protein supports this attractive model (DYSON and MURRAY 1995). Expectedly, higher cccDNA levels result in increased amounts of total viral RNA; however, for unknown reasons the amounts of replicative intermediates are not correspondingly amplified (SUMMERS et al. 1991).

A still unresolved question is whether all capsids, or capsids in all stages of maturation, are equally well enveloped and exported. At least for DHBV, exported virions contain much more mature DNA molecules (i.e. RC and full-length linear genomes) than intracellular DHBV cores. This has been interpreted to indicate the presence of some kind of maturation signal on the core particle surface which would trigger preferential envelopment of capsids with more mature genomes (SUMMERS and MASON 1982). The nature of that signal is not known to date, but it might be related to core protein phosphorylation (see above), as in virions core protein with fewer phosphoryl groups is enriched compared to the intracellular forms (YU and SUMMERS 1994). Genome maturation is, however, not an absolute requirement for HBV envelopment, as for instance HBV cores which contain immature genomes due to a defect in core protein can still be exported as enveloped particles, albeit with lower efficiency (NASSAL 1992).

### 5.4 Hepatitis Delta Virus

The interactions between hepadnaviral cores and envelope proteins appear to be specific; however, hepatitis Delta virus (HDV) provides a naturally occurring, and medically important, example of pseudotyping, using the surface proteins of HBV and, at least experimentally, WHV, but not DHBV (for review: LAZINSKI and TAYLOR 1994). HDV is a subviral agent with a small rod-shaped single-stranded RNA genome of about 1700 nt that bears structural features found in viroids and virusoids from plants, and encodes a nucleic acid binding protein. This delta antigen ( δAg) occurs in two forms which are generated by RNA editing; the larger δAg-L differs from the smaller δAg-S by a 19-aa C-terminal extension. While δAg-S supports genome replication, δAg-L inhibits it and instead promotes genome packaging, a process correlated with isoprenylation of a Cys residue present in the unique C-terminal extension of δAg-L. The hepadnavirus helper is not required for HDV replication but rather for its efficient transmission, by supplying an envelope for the RNA/δAg complex. The resulting virions are spherical with a diameter similar to that of Dane particles (35–41 nm). The RNP complex, thought to consist of one molecule of genomic RNA and some 60–90 molecules of δAg (both forms), appears to be roughly spherical with a diameter of about 18 nm (RYU et al. 1993), and hence is substantially smaller than a hepadnaviral core particle. Other differences exist in morphogenesis: envelopment is independent of L protein since HDV core-containing, secreted particles can be produced in the presence of only S protein; δAg-L can also be packaged into S particles in the absence of viral RNA. Thus specific interactions between δAg-L and surface proteins appear to mediate HDV virion assembly, as in hepadnaviral morphogenesis; however, the contact regions on the surface proteins are apparently different, and the value of HDV as a model for HBV morphogenesis is disputable. By contrast, infectivity of HDV is apparently as dependent on the presence of L protein on the envelope as that of the hepadnaviruses, and independent of the M protein (SUREAU et al. 1993). Hence, the several 100 000 copies of δRNA/infected cell may provide a convenient marker for studies aimed at analysing the role of the hepadnaviral envelope in infection.

## 6 Model for Hepadnavirus Morphogenesis

In combination, the salient features of the individual assembly steps described above lead to a plausible though in some respects still speculative model for hepatitis B virus morphogenesis. The RNA pregenome is translated in the cytoplasm to yield core and P protein (Fig. 4B), while the surface proteins, made from the subgenomic preS/S and S mRNAs, are targeted to the ER. Core protein monomers rapidly dimerize and provide the pool of assembly intermediates. P protein binds to the encapsidation signal on the pregenome (Fig. 4A), forming a

pre-assembly complex that triggers core protein association at a lower critical concentration than in its absence (Fig. 4B). The majority of S protein, plus M and very little L protein, dimerize in the ER and oligomerize and bud into the lumen of a post-ER/pre-Golgi compartment, forming spherical (Fig. 6A) and, in the presence of more L protein, filamentous subviral particles (Fig. 6C). In a relatively slow process, part of the preS1 domains of L are translocated across the membrane while the other part remains in its initial cytoplasmic location, presumably associated with the Hsc70 chaperon. Virion formation initiates, perhaps mediated by Hsc70, via specific interactions between core particles and the cytoplasmically located preS domains of L, and leads to budding into the ER or the intermediate compartment (Fig. 6D). L retention in an early compartment of the secretory pathway (Fig. 6B) may increase, at least in vivo, the efficiency of virus formation; the interaction with the core particle, on the other hand, could relax some sterical constraints that apparently prevent the formation of empty subviral particles with a high L protein content. After budding, the virions are secreted via vesicular transport through the remaining compartments of the secretory pathway, and are eventually released into the blood-stream. Overall, this mechanism ensures the formation of infectious particles: the triggering function of the pre-assembly complex of P protein and pregenome prevents formation of replication-incompetent cores, and the unusual topological properties of L warrant specific envelopment of these cores and the presence of a viral attachment domain for interaction with the target cell. HDV envelopment, by contrast, is L independent, which may be the reason that more than 90% of the HDV virions are non-infectious (Lazinski and Taylor 1994). This less elaborate strategy is compensated for by the 1000- to 10 000-fold higher number of HDV genomes per infected cells.

## 7 Summary and Perspectives

The studies summarized above have established that many steps of hepadnaviral morphogenesis rely on molecular mechanisms that are uniquely distinct from their retroviral counterparts. Prominent examples are the coupled encapsidation of the separately expressed P protein and the RNA pregenome, its concomitant reverse transcription, the lack of protein processing, the production of empty envelopes, the multifunctionality of the large S protein which depends on its most unusual topological properties, or the specificity of the interactions between capsid and surface proteins. Overall, a consistent picture of the apparently unique hepatitis B virus assembly pathway is emerging; however, many details as well some of the basic features are not yet fully understood.

The resolution of the structural data available for the hepadnaviral particles and their constitutent components is still low. Quick results from X-ray diffraction are not in sight, but advanced electron microscopic techniques combined

with mutational studies might reveal the location of individual secondary structure elements in the core protein, the interior organization of the capsid including its nucleic acid contents as well as the architecture of the envelope, along with the arrangement of the lipids. Besides their importance for the further development of core and S particles into suitable carriers for foreign antigenic determinants, such studies might also provide information on some of the dynamic aspects of assembly, e.g. what structural changes accompany reverse transcription of the RNA pregenome into DNA, whether the T = 3 or the T = 4 capsids are the biologically relevant species, and whether the interaction between core and envelope is so tight and specific that the surface proteins are forced into a similar icosahedrally symmetrical arrangement as the underlying core protein subunits. Other biophysical techniques may help to elucidate the three-dimensional structure of the RNA encapsidation signal.

Some of the following open questions may yield to the continued application of reverse genetics and DNA transfection; others will require the development of in vitro systems allowing the reconstitution of the assembly of capsids, S particles and eventually complete virions from purified components. Of particular importance for a systematic study of the early steps of infection, which require a reversal of the assembly process, will be the establishment of a feasible in vitro infection system; in the meantime, microinjection techniques might be helpful. Some of the essential interactions in the assembly process still await experimental confirmation, e.g. between core protein and the preassembly complex of P protein and $\varepsilon$ or between the capsid and the envelope; further studies might then reveal the specific regions involved. It is not clear how formation of the complex T = 4 or T = 3 structures of the capsid is determined or how the selective interaction between P protein and the 5′-copy, but not the 3′-copy of $\varepsilon$, is controlled. The entire process of RNA transformation into dsDNA within the restricted space of the capsid and with a single P protein molecule covalently linked to its substrate is a mechanistic puzzle. The unusual topological properties of L protein need to be further clarified. How and where does translocation of the preS domain occur and how does it influence the shape of S particles? Is the mechanism of virion formation identical to that of S particle production, and where in the cell does virus budding occur? The functional role of the chaperones implied in assembly will have to be elucidated; more than likely, further cellular proteins will be identified that play a crucial role in the virus life-cycle, not only for assembly but also for initiating a new round of infection. The cellular receptor for none of the hepadnaviruses is known. It is unclear where penetration of the capsid into the host cell cytoplasm occurs, what the mechanism of fusion is, where in the surface proteins the fusogenic activity resides and how it is triggered. Similarly, we do not know how capsid disassembly and delivery of the viral genome into the nucleus are regulated. Phosphorylation of the core and surface proteins may be involved, but there are as yet no experimental data to directly support this assumption.

This list of open questions is certainly still incomplete; however, the data collected so far are encouraging enough to initiate studies aimed at interfering

with individual steps of the morphogenetic pathway. The frightening prospects of liver cirrhosis and hepatocellular carcinoma for long-term chronic HBV carriers certainly warrant that the potential of such novel antiviral concepts is explored.

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