**Intracellular Transport and Egress of Hepatitis B Virus**

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**Summary**

Replicating its genomic information in the nucleus via transcription hepatitis B virus (HBV) has to deliver its partially double stranded DNA genome into the nucleus. Like other viruses with a nuclear replication phase HBV genomes are transported inside the viral capsids first through the cytoplasm towards the nuclear envelope. Following the arrival at the nuclear pore, the capsids are transported through them using classical cellular nuclear import pathways. Unique, however, is the arrest of nuclear import at the nucleoplasmic side to the nuclear pore, where the capsids efficiently disassemble leading to genome release. In the later phase of the infection nucleocapsids newly formed in the cytosol have to move to budding sites at intracellular membranes carrying the three viral envelope proteins. Capsids containing single stranded nucleic acid are not enveloped in contrast to empty and double-stranded DNA containing capsids. A small linear domain in the large envelope protein and two areas on the capsid surface have been mapped where point mutations strongly block nucleocapsid envelopment. Possibly, these domains are involved in envelope - capsid interactions driving the budding process. Like other enveloped viruses also HBV uses the cellular ESCRT machinery for catalyzing budding through a membrane away from the cytosol.

**Introduction**

Hepatitis B viruses (HBV) exhibit an extraordinary infectivity. This was shown by chimpanzee infections in which nearly a single virus as determined by Ullrich and colleages was sufficient for causing infection (80 – 90%) [1–3]. Such high relationship between physical and infectious particles suggests that the numerous steps needed for infection and virion production are efficient and coordinated. This involves i) the arrival at the hepatocytes, ii) the entry into the cell, iii) transport of the viral genome to the compartment where multiplication of the genomic information occurs but also iv) a quality control of virions. The latter is of particular importance as neither the host RNA polymerase II transcribing the viral RNA pregenome from the viral DNA genome nor the viral polymerase, which facilitates reverse transcription of the viral RNA pregenome and subsequent DNA synthesis lack a proof reading activity.

**Intracellular cytoplasmic transport after entry**

Like all retro- and DNA viruses (exception Poxviridae), the genomic information of HBV is multiplied within the nucleus [4]. In consequence, the HBV genome has to be transported from the place where the capsid is released into the cytosol to the nuclear envelope and subsequently into the nucleus. The cytoplasm exhibits a high viscosity [5,6] making diffusion inefficient. Microinjection experiments of Luby-Phelps using fluorescent latex beads show that particles up to 50 nm can diffuse, although to limited extent [6]. Most viruses – or their subviral structure containing the genome – use active directed transport via microtubules. Up to date, only baculoviruses and vaccinia virus were described using polymerizing actin filaments as intracellular bacteria do [7–9]. The latter transport is however undirected and must be considered to be less efficient. Microtubules-mediated transport is also used for organelle translocation and the motor protein complex to which the cargos attach determines the direction [10]. For centripetal transport to the nuclear envelope, cytoplasmic dynein is used, which comprises 13 chains. A dynein-bound multiproteic complex, termed dynactin, which modulates dynein activity, however frequently mediates cargo attachment.

Little is known of how HBV displaces its genome. Lipofection experiments, a technique which was used to circumvent infection and which allows high efficient infection/transduction revealed that the genomes were certainly transported within the 32 nm-measuring capsids [11]. This was concluded from fluorescence *in situ* hybridization under native conditions, which only detects capsid-release genomes. The absence of cytosolic signals however does not exclude that a small minority of capsids partially disintegrate, which could evoke detection by sensors involved in innate immune response. In fact, cytoplasmic cores show evidence of proteolysis of the encapsidated polymerase, implying at least transient opening of the capsids [12].

Treatment of cells with paclitaxel, a microtuble-depolymerizing agent used in breast cancer therapy [13,14], showed that capsid arrival at the nuclear envelope was microtubule-dependent. These experiments further showed that genome release from the capsids was dependent upon capsid translocation to the nucleus. In agreement with dynein-mediated transport velocity, which varies between 800 and 100 nm/s [15,16] the first released genomes were observed 1 hour post lipofection but exclusively intranuclear. This shows that intracytoplasmic transport of HBV capsids and genome release is rapid, directed and coordinated at least with regard to genome liberation. These observations are also in agreement with unpublished results of us, showing that capsids arrival at the nuclear envelope after microinjection in much larger *Xenopus laevis* oocytes was observed after less than 1 hour (Osseman, Panté, Kann, unpublished). Of note, microtubule-mediated transport is highly conserved between species; even plant cells translocate cargos of other eukaryotes [17]. Recent observations of us showed that HBV capsids bind exclusively to one particular dynein chain, which is conserved between *Xenopus laevis* and man and for which no polymorphisms are known (Osseman, Kann, unpublished). This argues that the intracytoplasmic transport is neither host nor organ-specific. It further leads to the conclusion that this step of the hepadnaviral life cycle is likely no target for therapy without risking severe side effects. However, drugs as HAPs modifying the capsid structure have eventually the potential of interfering with cytosolic transport. Like picornaviruses,HBV capsids have an imminent instability also known as capsid breathing [18,19], an innate immune response could be promoted. This assumption is supported by observations linking inflammation with cytoplasmic capsids [20–22].

**Nuclear transport and genome release**

Having a diameter of 36 nm [23,24] HBV capsids are small enough to pass the nuclear pores [25]. Such passage must be concluded from the facts that HBV infects non-dividing cells and that the nuclear pore complexes (NPC) form the only aqueous channels between nucleo- and cytoplasm. There are 400 – 18500 NPCs per cell (oligodrendrocytes, Purkinje cells)[26], dependent upon cells’ metabolic activity [27]. NPCs allow rapid exchange of ions but also macromolecules, the latter reaching a rate of up to 800 to 1000 transport events per second [28]. While ions and other small molecules traverse the nuclear pores by diffusion, macromolecules depend on cytoplasmic transport receptors. Transport receptors can be separated into exportins and importins, the latter comprising 10 members acting on cargos exposing different nuclear localization signals [29]. E.g. transportin – a member of the importin beta superfamility – binds to so-called M9 domains, which are glycine-rich, and which are present on nuclear housekeeping proteins. Better known is the nuclear import via importin alpha and importin beta, which bind to classical nuclear localization signals (NLS) and which consisting of four to six basic amino acids [30]. In this transport, importin alpha serves as an adaptor protein binding to the cargo, while importin beta facilitates nuclear translocation after binding to an importin beta binding domain (IBB) on importin alpha. Like NLS, IBB is composed of basic amino acids but is with 39 amino acids much longer. IBBs, which comprised 13 basic amino acids in 7 clusters [31] do not only exist on importin alpha but also on karyophilic proteins, which are then directly imported by importin beta without the need of importin alpha [32,33]. The higher number of interacting amino acids compared to NLSs make the binding more stable than the one of importin α – NLS (NLS-importin α µM [34] IBB-importin α nM [31]).

NPCs are composed of c. 30 different proteins collectively called nucleoporins (Nups). NPCs have an octagonal symmetry and Nups exist in eight to 56fold copies. Many Nups bearing FXFG or GXFG repeats are essential and are involved in nuclear transport [35,36]. Despite more than two decades of research, the exact mode of translocation is not known. However, the current models show significant similarity to conclude that the cargo import receptor complex attaches to cytoplasmic fibers extruding from the NPCs, and which comprise Nup358 and Nup214, being important in adenoviral capsid docking to the nuclear pore and to genome liberation [37]. The import complex then passes the central channel of the pore, which is filled with a hydrophobic mesh, limiting diffusion to small molecules of 2 nm. Noteworthy there is no clear size or molecular weight cut as other factors including charge and shape are important. After passing the pore, the import complex binds to nuclear filaments, which form the nuclear basket. This interaction leads to arrest of the import complex unless the small GTPase Ran in its GTP-bound form dissociates importin beta or transportin from the cargo or adaptor molecule [31,38]. The reversibility of cargo import receptor binding is also reflected by the high Km. Upon classical nuclear import, the cargo subsequently diffuses deeper in the karyoplasm while RanGTP in complex with the import receptor is exported into the cytoplasm followed by recycling of Ran.

The primary amino acid sequence of the HBV core protein from which 240 copies form the capsid, comprises an arginine rich C terminal domain containing nuclear localisation signals [39–41]. This domain also comprises a putative IBB-like sequence, which could allow capsid transport if entirely exposed. In RNA containing capsids, at least after their expression in *E. coli* the C termini localize in the lumen of the capsid as shown by Zlotnick et al. using nano gold labeling [42]. This is in agreement with data from permeabilized cells showing that RNA-containing capsids do not interact with the nucleus even in the presence of nuclear import factors. Lowering the affinity between RNA and C termini of the core protein by serine phosphorylation resulted in Importin alpha/beta mediated interaction with the NPCs and allowed capsid pull-down by these import factors [41] but not by importin beta alone. However, the extent of capsid phosphorylation, the position of phosphorylated serines are not fully understood and data that are more recent suggest that after initial phosphorylation further genome maturation coincidences with dephosphorylation [43,44]. This allows the conclusion that capsids from infection are likely non phosphorylated but these data also indicate that parts of the C termini can flip to the capsids surface [45,46]. This hypothesis in turn is in agreement with observations by tryptic cleavage of capsids, showing that in capsids with mature double stranded DNA – to which the C termini bind poorly - the C termini are exposed outside the capsid and do not interact with the NPCs after cleavage (Rabe et al. 2003). Accordingly, these mature capsids interact with importin alpha/beta and with the NPCs in permeabilized hepatocyte cell lines used to circumvent inefficient entry but also after microinjection into the cytosol of *Xenopus laevis* oocytes. It must thus be considered that the C termini have a dual topology, which depends upon their affinity to the encapsidated nucleic acids. Likely, the structural changes lead to presentation of a short NLS but do not comprise the entire C terminus. This can be concluded from experiments with mature and *in vitro* phosphorylated RNA capsids as they interact with importin beta only in the presence of importin alpha. Of note, this structural changes, which are required for nuclear delivery of the genome are genetically separable from those allowing envelopment by surface proteins [47].

Investigations of the fate of mature capsids fate upon infection are difficult as the number of capsids entering the cell or at least the cytoplasm is limited [48–51]. As mentioned before lipofection and permeabilized cells were used, both allowing to follow the capsids and eventually genomes by microscopy. For nuclear import studies permeabilized cells further show the advantage that defined amounts of capsids can be applied. Using this system and adding virus-derived capsids either from HepG2.2.15 cell culture supernatant or from HBV-infected patients plasma, nuclear import of capsids was observed, which was combined with the appearance of nuclear released viral DNA [11,52,53]. Quantification revealed that each nucleus released the genomes from hundreds of capsids within minutes supporting the speed of the reaction [54]. These studies further showed that – within the detection limits – all capsids released their genome. This is in agreement with the very low particle to infectious unit - ratio. Interestingly, genome liberation was combined with the dissociation of the capsids to core protein dimers, which is similar to adenoviral disassembly [55,56] but the dimers were released into the nucleus where they reassembled to capsids. This observation allows the conclusion that entry of capsids into cells upon infection is not combined with irreversible structural changes by low pH or proteolytic cleavage as it is the case for most other viruses.

Investigating the capsid transport in more detail by electron microscopy showed that intact capsids passed the nuclear pore intact [25] but that they became arrested within the nuclear basket by interaction with Nup153 [53]. While artificially cross-linked capsids stayed in the basket, the physiological non-linked ones dissociated. Disassembly was further shown to be genome maturation dependent, which supports the importance of this trafficking pathway during infection. Of note, the underlying mechanisms were observed in different cultured cells indicating that no liver-specific factor is required. While this is the case for attachment of the capsids with the NPC, the current data do not exclude that genome release is more specific.

Of interest for clinics is the potential to intervene by disturbing the nuclear import pathway and genome release. However, the latter is poorly understood making it difficult to draw conclusions. Regarding nuclear import, one has to realize that this pathway is well conserved through all eukaryotes indicating severe side effects. This assumption becomes even more plausible when considering that Nups are not only important for nuclear trafficking but also in cell division, in particular in reformation of the nuclear membranes [57–60].

**Post entry trafficking**

As outlined before there are probably no irreversible capsid changes upon entry into the cell. In consequence, progeny capsids – as far as they contain the mature DNA like in the virion-derived capsids – should be identical to entering ones. Accordingly, progeny capsids are subject to the same trafficking than capsids upon infection. In fact, progeny capsids can thus deliver their DNA into the nucleus. This is well documented for duck hepatitis B virus (DHBV) capsids exhibiting more than 19 fold increased concentration of nuclear viral DNA in the absence of the viral surface proteins (420 to 630 copies/cell respectively for 1165A and 1S mutants vs WT 22 copies/cells (10-50 copies/cell) [61]. Single cell analysis reveal a significant difference even in the presence of functional surface proteins: 90% of nuclei contained between 1 and 17 cccDNA molecules and 10% more [62].

For HBV the situation is less clear but artificial surface protein deficient mutants exhibited a 1.5 to 6 fold increased cccDNA copy number [63,64,12,65,66]. In particular, the low numbers sometimes reported to be below one copy per cell show that these numbers are mostly derived from calculations of DNA molecules per total number of cells and not per number of infected cells.

Core proteins are expressed in excess with regard to the quantity needed for progeny virus synthesis. Dependent upon the individual cell but also the inflammation state of the patient, these core proteins localize as assembled capsids either in the cytoplasm or in the nucleus [67–70]. The latter capsids were found to be empty [71] or at least devoid of the viral genome. The thousands of copies apparently not reduce hepatocytes life span , which seems rather to be extended as indicated by clonal expansion of infected cells in Chimpanzees [72]. Evidently, these capsids have not been derived from infecting capsids but from progeny core proteins. This raises the question on their transport. The current knowledge allows two hypotheses: First, an import as non- or preassembled capsids. This likely involves core protein dimers as dimerization occurs rapidly [19,73–75]. In dimers, the entire C termini are exposed so that nuclear import could be mediated either by their NLS but also directly by their IBB. The latter idea is supported by the higher affinity of importin beta to IBBs (nM range) compared to importin alpha – NLS interaction (µM range) [31]. The second model argues for a transport based on cytosolically formed empty capsids. This hypothesis is justified by the conclusion that there is no nucleic acid in these capsids fixing the C termini in the capsid lumen. A structural change of the topology similar to mature capsids is further supported by findings showing that empty capsids can be encapsidated by the viral surface proteins leading to empty virions [76,77].

**Virus egress**

During the productive phase of the HBV infection at least a fraction of newly formed cytoplasmic nucleocapsids has to escape from the nuclear transport pathway and move towards budding sites for envelopment and egress. These buddings sites are formed at intracellular membranes carrying the three viral envelope proteins S, M, and L (for small, middle, and large) as transmembrane polypeptides. How capsids are transported from their site of formation in the cytosol to budding sites is unknown.

**HBV envelope proteins**

The viral envelope proteins are encoded by a single open reading frame and translated by the usage of three start codons in this frame [78]. Therefore, the sequence of the 226 aa long S protein is present at the C termini of the M and L proteins. The 119 or 108 aa long sequence (depending on the virus genotype) unique at the N terminus of L is called preS1, and the 55 aa long sequence in the M protein N-terminal to its S domain is referred to as preS2. After translation the S and M protein span the membrane probably four times with transmembrane domains (TM) in the S region. N and C termini are oriented to the lumen of the endoplasmic reticulum (ER) [79]. The preS1 domain of L contains a sequence between aa 70 to 94 binding to the cytoplasmic chaperone Hsc70 which blocks the cotranslational translocation of preS1 and preS2 (together referred to as preS) into the ER lumen [80]. Therefore, preS of L initially stays in the cytoplasmic compartment and TM1 of its S domain does not traverse the membrane while TM2 – 4 anchor the L protein in the lipid bilayer [81–83]. All three proteins form disulfide-linked homo- and heterodimers with each other laterally floating in the membrane [84,85].

Independent of the viral capsid the HBV envelope proteins form higher oligomers which can bud into the lumen of a post-ER/pre-Golgi compartment and form two morphologically distinct populations of subviral lipoprotein particles of 20 nm diameter: spherical particles containing relatively little L protein (up to 10 % of total protein) and filamentous particles of various lengths carrying more L. Overexpression of the L protein blocks subviral particle release in a dose dependent fashion [86,87]. N-terminal truncation of the L protein which also abolishes myristylation releases this suppressive effect of L. Subviral particles are released from cells by the constitutive secretion pathway [88,89]. Their formation is not depending on other viral functions or liver specific factors since efficient subviral particle secretion can be achieved in a wide variety of eukaryotic cells expressing the envelope proteins.

**Budding sites**

The viral envelope proteins are required for budding of the HBV capsid [90]. This is different from type C retroviruses where myristylated gag proteins bind independent of env proteins to the plasma membrane via the N-terminally attached fatty acid moiety, and oligomerization of the gag proteins drives budding even when no viral envelope proteins are present [91]. Accordingly, the envelope of type C retroviruses often is not tightly covered with viral surface proteins and contains in addition membrane proteins from the host which are passively incorporated. Therefore, retroviruses can be pseudotyped just by overexpression of foreign membrane proteins in the plasma membrane of the virus producing cell. In contrast, the HBV L and S protein are both necessary for virus formation while the M protein is not essential [90,92]. The current model for HBV budding proposes that similar to alphaviruses one or two cytoplasmic domains (matrix domains) of the HBV surface proteins contact the capsid, that these contacts order the envelope protein in the membrane into a tightly packed array and that the envelope protein – capsid interactions drive the budding process [93]. As a consequence, host membrane proteins cannot be found in the envelope of HBV particles and pseudotyping with non-related viral envelope proteins was not successful. However, the L protein from the woodchuck hepatitis virus could substitute the L protein from the human virus whereas this was not possible with the less related L protein of the duck hepatitis B virus [94].

One 22 aa long matrix domain (MD) at the boundary of preS1 and preS2 (aa 103 to 124) in L (MD1) [95,96] and one domain in the C-terminal half of the cytoplasmic loop between TM1 and TM2 in S (MD2) [97–99] was defined genetically by phenotypic characterization of deletions, substitutions, and point mutants. A spacer with a minimal length of 26 aa between MD1 and the first transmembrane domain of L (TM2 in the S domain) was required for nucleocapsid envelopment [100]. Therefore, MD1 can potentially reach binding sites on the entire capsid surface and is not sterically restricted to bind to or close to the tip of spikes protruding from the capsid. Indeed, a genetic screen using a large number of single alanine mutations located at the capsid surface mapped two small areas at the ground of the spikes and between two spikes where point mutations strongly blocked nucleocapsid envelopment [101,102]. The function of these areas is very sensitive to mutations because single exchanges to alanines in these areas usually blocked virion formation very strictly and some amino acids could not be exchanged by any other amino acid without losing function. These areas might represent binding partners of matrix domains (matrix binding domains, MBD). However, it is also possible that they are involved in other steps of the virion morphogenesis pathway like capsid maturation (see below) or transport of the capsid to budding sites. It has been tried to use MBDs as targets for small molecules to inhibit virion formation. Indeed, a DNA aptamer selected for binding to one MBD partially blocked virus production in transiently transfected cells [103]. However, also peptides that bind to the tip of the capsid spike inhibit nucleocapsid envelopment [104] and electron microscopic studies showed an interaction of the spike tip with the viral envelope [105].

It seems possible that MD1 in the L protein is an important factor in redirecting nucleocapsids from nuclear transport to budding sites. For DHBV it has been shown that the absence of L causes a strong enhancement of nuclear cccDNA formation [106,107]. A possible model explaining this observation suggests that without L no budding sites are generated and nucleocapsids therefore move into the nucleus delivering the viral genome for cccDNA formation. If a certain threshold of budding sites is reached nucleocapsids are redirected for egress.

Like many other enveloped viruses [108] also HBV utilizes the ESCRT complexes of the host cell to catalyze the membrane fission that has to occur as the last step of budding [109]. These host factors are involved in the formation of vesicles that bud away from the cytosol into the lumen of multivesicular bodies. Many viruses like HBV also bud away from the cytosol into the lumen of a cellular compartment or into the extracellular space and they hijack these factors for this task. So called late domains in viral capsid proteins interact with factors of the fission machinery. The HBV capsid protein contains the late-domain like motif PPAY and binding of the cellular Nedd4 ubiquitin ligase to this motif could be demonstrated [110]. Nedd4 is proposed to escort cargos to the ESCRT machinery. Up to now it could not be shown by mutational analysis that the PPAY motif is required for envelopment because alterations in this site have additional effects on capsid assembly and maturation.

**Nucleocapsid maturation**

The synthesis of the HBV DNA genome occurs within the lumen of the capsid by reverse transcription of an RNA pregenome generating a single stranded DNA molecule of (-) polarity. Subsequently, this DNA is the template for DNA (+) strand synthesis. Capsids with all intermediates of genome synthesis can be found within the cell. However, secreted virions carry no capsids with single stranded nucleic acid, they contain partially double stranded DNA. This observation led to the hypothesis that early nucleocapsids are immature and that a maturation signal is generated on the capsid surface during second strand DNA synthesis that can be sensed by the envelopment machinery and that excludes immature capsids from budding [111]. This model is supported by the observation that a virus with a mutation in the reverse transcriptase blocking DNA synthesis so that the nucleocapsids are frozen in an immature state is unable to form virions [112]. Recently however, it was found, that empty capsids containing no nucleic acid are incorporated into virus like particles. This prompted the proposal of a different paradigm, the single strand blocking model [77]. According to this model the single stranded RNA pregenome and the single stranded DNA (-) strand induce a signal in capsids blocking their envelopment and the synthesis of the second DNA strand releases this block. The nature of the accompanying signal in the capsid is not clear. Possibly, differential phosphorylation of serine residues in the C-terminal domain of the core protein plays a role or a conformational change in the core protein [113]. Also it is unclear at which step of the virion formation pathway the envelopment competent and incompetent capsids are separated. This may e.g. happen at the level of transport of capsids to budding sites, at the level of interaction with viral envelope proteins, or at the level of interaction with cellular factors necessary for membrane fission. For DHBV it could be demonstrated that mature capsids associated with intracellular membranes in the absence of viral envelope proteins in contrast to immature capsids [114]. The basis for this binding, however, is unknown.

In virions a conformation of the L protein can be found that is different from the initial conformation: Newly synthesized L displays its preS domain at the cytosolic side of the ER membrane. This site is equivalent to the inner side of the viral envelope. However, in virions L proteins expose their preS domain to the outside [81] and this is important for the virus because the preS1 sequence is involved in binding to the host cell during infection [115]. Apparently, the L protein can switch from an i-preS to an e-preS conformation. How this posttranslational translocation of preS across the membrane is achieved is unclear. Also it is not known when this switch happens. Investigation of the L topology in virions showed that approximately half of the L chains has the i-preS topology and the other half has the e-preS folding. It is possible that in each virus particle both conformations are present in a roughly 1:1 ratio or that e.g. half of the virions carry L with i-preS and the other half with e-preS. The later possibility is supported by the observation of two different kind of virions with compact and gapped appearance in the electron microscope [105]. This would imply that the topological switch of L is triggered more or less simultaneously in a virus particle.

Transfected HBV expressing cells release naked capsids (capsids not surrounded by an envelope) in addition to subviral lipoprotein particles and virions. It is unclear how the capsids leave the cell without disruption of the plasma membrane. The pathway is independent of the ESCRT machinery but depends on Alix, a multifunctional protein with key roles in membrane biology [116,117]. However, in vivo naked capsids could not be found in the serum of virus carriers negative for antibodies against capsids [118].

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