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Review

Molecular biology of hepatitis B virus infection

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ABSTRACT

Human hepatitis B virus (HBV) is the prototype of a family of small DNA viruses that productively infect hepatocytes, the major cell of the liver, and replicate by reverse transcription of a terminally redundant viral RNA, the pregenome. Upon infection, the circular, partially double-stranded virion DNA is converted in the nucleus to a covalently closed circular DNA (cccDNA) that assembles into a minichromosome, the template for viral mRNA synthesis. Infection of hepatocytes is non-cytopathic. Infection of the liver may be either transient (<6 months) or chronic and lifelong, depending on the ability of the host immune response to clear the infection. Chronic infections can cause immune-mediated liver damage progressing to cirrhosis and hepatocellular carcinoma (HCC). The mechanisms of carcinogenesis are unclear. Antiviral therapies with nucleoside analog inhibitors of viral DNA synthesis delay sequelae, but cannot cure HBV infections due to the persistence of cccDNA in hepatocytes.

Contents

1. Introduction

It has been known, since at least the 1940s, that blood and plasma samples may contain a virus(es) that causes transient and chronic hepatitis (MacCallum, 1947), also known as post-transfusion hepatitis. Moreover, a possible association with hepatocellular carcinoma (HCC) was noted by the 1950s (Edmondson and Steiner, 1954; Higginson et al., 1957). The unknown infectious agent was named hepatitis B virus (HBV), to distinguish it from another infectious agent transmitted primarily by a fecal/oral route, hepatitis A virus (HAV) (MacCallum, 1947). HAV does not cause chronic hepatitis. The nature of hepatitis B virus was unknown, as were means for testing donor blood and plasma for its presence. Thus, post-transmission hepatitis was common following transfusion, afflicting up to 30–40% or more of recipients (Shimizu and Kiamoto, 1963).

This changed in the mid-1960s, when Blumberg and colleagues discovered that a serum antigen, previously identified in their lab and found to be common in leukemia patients treated via blood transfusion, was actually the component of the infectious agent, HBV (Blumberg, 1977; Blumberg et al., 1967). This serum antigen, known as Australia antigen because of its identification in an Australian Aborigine, was found as 22 nm rods and spheres in the blood of infected individuals. These particles were subviral and comprised of the three HBV envelope proteins, L, S and M, now known collectively as hepatitis B surface antigen (HBsAg) (Bayer et al., 1968; Dane et al., 1970). The enveloped virus, 42 nm in diameter, is much less abundant

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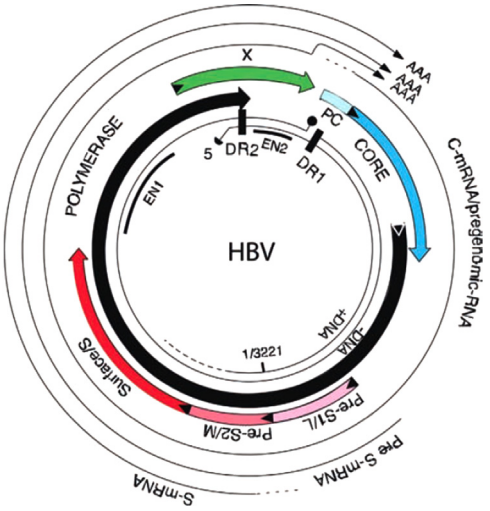

Fig. 1. Genome structure, genes and mRNAs of hepatitis B virus. The relaxed-circular DNA genome of HBV with a complete minus strand and incomplete plus strand is shown (inner circle), along with the major mRNAs, all of which end at a common polyadenylation signal located in the core open reading frame. Dashed lines at the 5′ end of mRNAs indicate the use of staggered starts sites for preCore/core and M/S, as described in the text. All open reading frames have a clockwise direction. The single stranded gap in the plus strand can be filled in *in vitro* by the viral RT, which is covalently attached to the 5′ end of minus strand DNA. In avihepadnaviruses, the plus strand is generally complete except for DR2, which remains as an RNA/DNA hybrid (Lien et al., 1987). DR1 and DR2 represent ~11 base direct repeats that have an important role in viral DNA synthesis. Minus strand synthesis exhibits a short terminal redundancy created during reverse transcription of pregenomic RNA (Fig. 2).

in serum than HBsAg. Virions were named Dane particles after the first author of the electron microscopy study in which they were first visualized (Dane et al., 1970). HBV is transmissible not just among humans but also in the great apes (Bancroft et al., 1977; Barker et al., 1973; Linnemann et al., 1984; Warren et al., 1999; Zuckerman et al., 1978), including the chimpanzee, which was used until recently as an experimental model. HBV can also infect the macaque (*Macaca fascicularis*)(Dupinay et al., 2013) and, in a laboratory setting, the tree shrew (*Tupaia belangeri*)(Walter et al., 1996).

The ability to assay for HBsAg led to screening of blood banks and a 2–3-fold reduction in the incidence of post-transfusion hepatitis, after discounting cases of hepatitis following transfusion of blood from paid blood donors (Alter et al., 1972; Senior et al., 1974). Many of the remaining cases, including from paid donors, were thought due to an agent referred to as NonANonB hepatitis virus(es). A major cause of NonANonB hepatitis, hepatitis C virus (Engle et al., 2014), was identified in 1989 (Choo et al., 1989, 1990). The HBsAg assay also confirmed the suspected association between chronic HBV infection and HCC in humans (Beasley et al., 1981; Blumberg et al., 1975; Prince et al., 1975). This link has not so far been reported in great apes chronically infected with HBV. HBsAg, purified from HBV carriers, became the first vaccine to prevent HBV infection (Buynak et al., 1976; Purcell and Gerin, 1975) and, consequently, HBV-associated HCC.

The studies of the molecular biology of HBV carried out by Robinson and colleagues showed that HBV contained a double-stranded DNA genome about 3 kbp in length, as well as an endogenous DNA polymerase activity that could incorporate nucleotides into the viral DNA genome in an *in vitro* reaction of virions in which the viral envelope was disrupted with non-ionic detergent (Kaplan et al., 1973; Robinson et al., 1974; Robinson and Greenman, 1974). Following up on this work, Summers and colleagues showed that virion DNA was actually a partially double-stranded DNA held in a relaxed circular conformation by a short cohesive overlap between the 5′ ends of the two DNA strands. One strand was complete, the other incomplete (Fig. 1), leaving a single stranded gap that could be repaired, *in vitro*, by the endogenous DNA polymerase (Summers et al., 1975). The endogenous reaction carried out in the presence of P-32-labeled deoxynucleotides was used by Summers and others to screen serum samples of different host species for a hepatitis B like virus. This approach rapidly led to the discovery of HBV-like viruses in eastern woodchucks (woodchuck hepatitis virus) (Summers et al., 1978), domestic ducks (duck hepatitis B virus) (Mason et al., 1980), and Beechey ground squirrels (ground squirrel hepatitis virus) (Marion et al., 1980). Chronic infection leads to HCC in woodchucks and ground squirrels (Marion et al., 1986; Summers et al., 1978; Tennant and Gerin, 2001), but not in ducks. Collectively, the HBV family of viruses is now known as the hepadnaviridae, with two genera, orthohepadnaviruses (infecting mammals), of which HBV is the prototype, and avihepadnavirus (infecting birds), of which DHBV is the prototype (Fauquet et al., 2005).

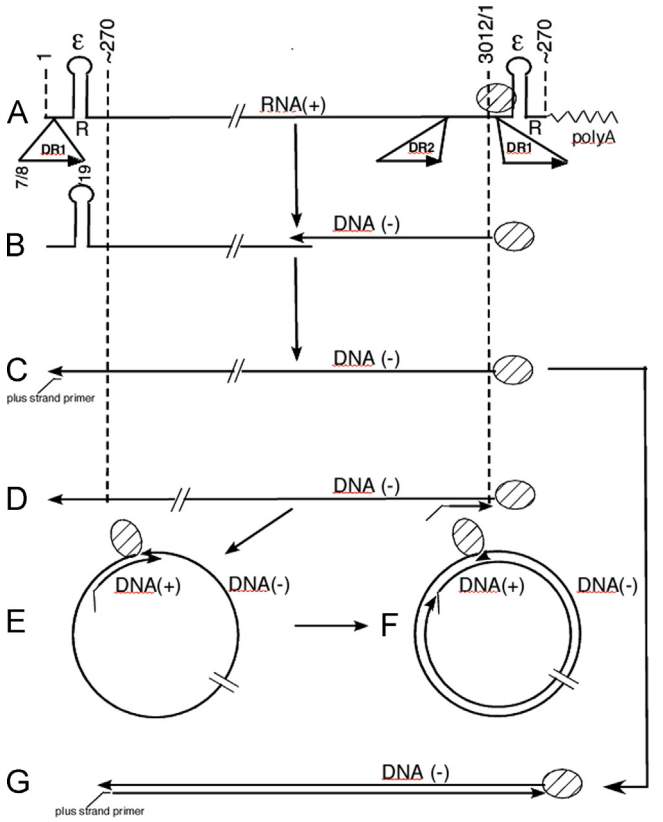

Fig. 2. Replication of HBV DNA. Reverse transcription begins with binding of the pol protein to the 5′ copy of a stem–loop structure epsilon (ε) located in the terminal redundancy of pregenome RNA (A), which facilitates packaging into viral nucleocapsids. Then, following copying of 3 nucleotides from a bulge in the side of epsilon, the polymerase translocates to the copy of the 11 nt sequence, DR1, located in the 3′ terminal redundancy. The basis for the translocation is unclear but is sufficient to align the nts copied from epsilon with a complementary sequence in DR1. Reverse transcription then continues to the 5′ end of the pregenome (B, C), with the template being degraded by an RNase H encoded within the pol protein. The terminal 18 nts including the CAP and DR1 are not degraded. Normally this oligonucleotide translocates to DR2, where it can anneal because of the identity of DR1 and DR2 (D). Plus strand synthesis then initiates and extends to the 5′ end of the minus strand. Circularization then occurs, facilitated by the short terminal redundancy on the minus strand (E). Plus strand synthesis stops before completed (F), perhaps because nucleocapsids containing incomplete plus strands are packaged into viral envelopes, preventing access to dNTPs. For avihepadnaviruses, the plus strand is generally complete except for DR2. About 10% of the time, the RNA primer of plus strand synthesis fails to translocate, resulting in *in situ* priming to produce double stranded linear DNA (dsl DNA) (G). Both rcDNA and dslDNA can be converted to cccDNA when transferred to the nucleus, though the pathways are different and, for dsl DNA, generally lead to defective cccDNA. Dsl DNA is a preferred substrate for integration into host DNA.

Animal models, in particular the duck and woodchuck, were essential for early investigations on viral replication and for testing of antiviral drugs. With the help of the duck model, Summers, Mason and Taylor discovered that hepadnaviruses replicate via reverse transcription (Mason et al., 1982; Summers and Mason, 1982) in a pathway similar to that of retroviruses, except that hepadnavirus DNA is converted into a minichromosome, covalently closed circular DNA (cccDNA) (Fig. 2), that is the template for synthesis of the viral RNAs (Bock et al., 2001; Newbold et al., 1995). The woodchuck and duck also provided systems to investigate the course of transient and chronic infections and the influence of nucleoside-based antiviral therapies. Moreover, the models provided an important source for primary hepatocyte cultures, the only cell culture-based systems permissive for hepadnavirus infections until the early 2000s (Gripon et al., 2002). Starting in the mid 1980s, functional analyses of viral genomes using recombinant DNA technology and testing of reverse transcriptase (RT) inhibitors for antiviral therapies became easier with the identification of liver tumor cell lines that supported HBV replication from transfected DNA (Condreay et al., 1990; Sells et al., 1987; Shih et al., 1989; Sureau et al., 1986; Tsurimoto et al., 1987; Yaginuma et al., 1987).

Early studies in ducks and woodchucks, as well as chimpanzees, had indicated that transient hepadnavirus infections could be cleared even after infection of apparently the entire hepatocyte population (Barker et al., 1973; Berquist et al., 1973; Hoofnagle et al., 1978; Jilbert et al., 1992; Kajino et al., 1994; Ponzetto et al., 1984). This was surprising, since no mechanism was known that would clear viral DNA from the nucleus of hepatocytes, which constitute a self-renewing population with a very low turnover rate. Thus, it seemed *a priori* that cccDNA elimination would require killing of all infected hepatocytes, leading to liver failure and death, whereas most patients recover uneventfully. Histologic analyses did not support the notion that hepatocytes were being replaced by proliferation and differentiation of liver stem/progenitor cells. This led to the idea that there must be non-cytopathic mechanism(s) helping, along with hepatocyte killing, to eliminate cccDNA and cure transient infections, and that these might eventually be exploited as therapy for chronic infections.

To look for mechanisms of HBV clearance that were not solely dependent on the death of infected hepatocytes, Chisari and colleagues carried out experiments with HBV transgenic mice during the 1990s. These studies showed that HBV DNA replication intermediates (Fig. 3) could be non-cytolytically cleared from the cytoplasm of hepatocytes in response to certain cytokines (e.g., interferon γ, TNFα) (Guidotti et al., 1996; Guidotti and Chisari, 1996). However, the mechanism of cccDNA clearance from the nucleus remained mute, as their mice did not make cccDNA. Studies in primary, non-dividing, duck hepatocyte cultures infected with DHBV failed to detect a cytokine driven clearance of cccDNA (Schultz and Chisari, 1999; Schultz et al., 1999).

Thus, further efforts to understand recovery from transient infections in chimpanzees and woodchucks were carried out (Mason et al., 2009b; Murray et al., 2005; Summers et al., 2003; Wieland et al., 2004b, 2005). These studies had two principle questions: (1) Are any hepatocytes actually cured during recovery from HBV infection? (2) If so, how is this achieved? While there is now strong evidence from these studies for hepatocyte curing (as well as abundant killing), the mechanism for cccDNA elimination remains elusive. These questions, as well as studies of chronic

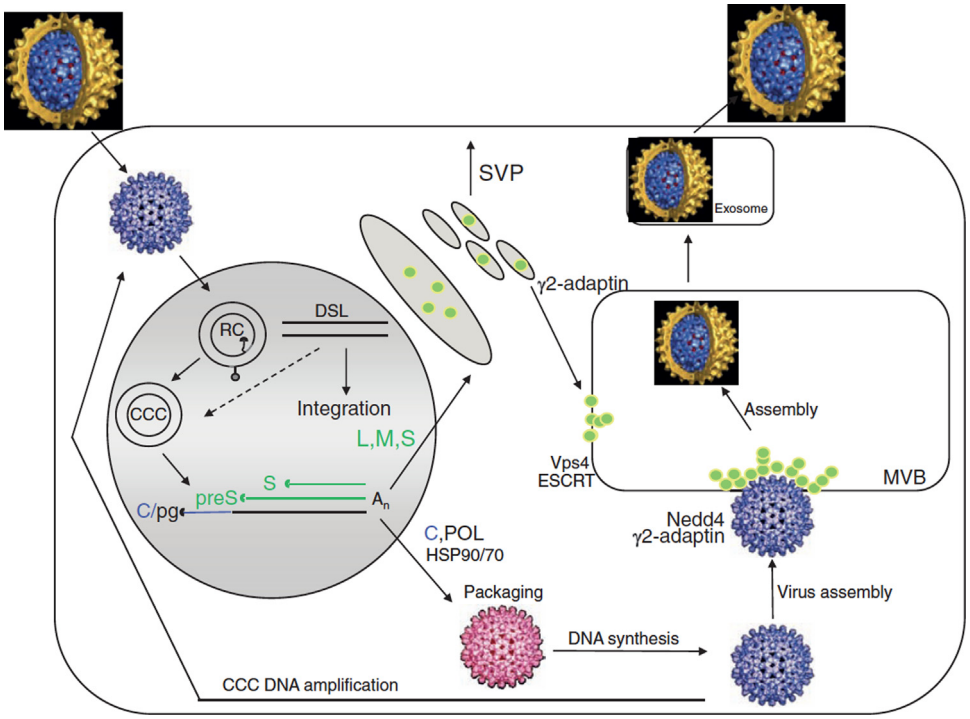

Fig. 3. HBV replication cycle. The figure shows a model for the lifecycle of hepadnaviruses, as described in the text. Envelope proteins are shown in yellow, DNA-containing capsids in blue and RNA-containing capsids in red. Upon infection mediated by the entry receptor, NTCP, virus nucleocapsids are transported to the nucleus, where rcDNA is converted to cccDNA. cccDNA does not undergo semiconservative DNA synthesis (Tuttleman et al., 1986a). Early in infection, when envelope protein concentrations are low, newly made nucleocapsids, with their enclosed viral DNA, are transported to the nucleus to amplify cccDNA copy number to up to 50 per hepatocyte (Tuttleman et al., 1986b). At the same time, envelope proteins enter the ER and assemble into subviral particles (SVP) or transfer to MVBs where virion assembly is believed to occur. When sufficient envelope is present, nucleocapsids are directed to their secretory pathway and cccDNA amplification ceases. Mature virions might exit cells through exosomes (for details and references see the text). Virus with dsl DNA can also infect hepatocytes. cccDNA is formed from dsl DNA by non-homologous recombination, resulting in a loss of sequences and, generally, rendering this cccDNA unable to support virus replication (Yang and Summers, 1995). Dsl DNA may also integrate into host DNA via non-homologous recombination; this pathway does not appear to have a role in the virus life cycle. The figure does not show "empty" HBV virions produced by infected hepatocytes because the pathway for their assembly and secretion is not yet known (Ning et al., 2011).

infection discussed later, need to be understood first of all in the context of the current view of hepatocyte replacement, which was until recently thought to include, at least under conditions of extreme stress to the liver, stem/progenitor cell differentiation.

Under normal conditions, the hepatocyte population is self-renewing, with most hepatocyte able to divide to maintain liver mass. The hepatocyte turnover rate is uncertain. The PCNA labeling index of S-phase hepatocytes in normal liver is about 0.05% (Mancini et al., 1994); however, the daily turnover may be higher, depending on the duration of S-phase in these cells. When hepatocyte proliferation is inhibited, such as by exposure to a toxic chemical (e.g., 2-acetylaminofluorene), presumptive hepatocyte progenitor cells (sometimes called facultative stem cells) are observed to proliferate (Fig. 4) (Evarts et al., 1987; Hsia et al., 1992). These cells are thought to be associated with bile ductules in the liver, or with the associated Canals of Hering, which form a bridge between bile ductules and the plates of hepatocytes in which bile is formed (Fig. 4). Their proliferation seeds the hepatic lobule, especially near the portal tracts, with ovoid cells (oval cells) that have been thought to then differentiate into hepatocytes. However, recent studies question the role for such a distinct hepatocyte progenitor cell population in hepatocyte regeneration. In particular, hepatocyte replacement during injuries historically attributed to hepatocyte progenitor cell proliferation, in rats, could not be replicated in mice using various means to trace cell lineages (Schaub et al., 2014; Yanger et al., 2014). It remains unclear if this finding is specific to mice, or also extends to rats, from which most data on progenitor cell populations during liver injury had historically been derived (Grompe, 2014); recent data support the possibility they are similar (Marongiu et al., 2014). In view of these recent data, it is not clear if hepatocyte progenitor cells exist in humans, or play a role in hepatocyte replacement in the face of the excessive long-term destruction of mature hepatocytes as seen in chronic hepatitis B (Hoare et al., 2010; Kordes and Haussinger, 2013; Riehle et al., 2011). Serial transplant studies in mice with mouse and with human hepatocytes suggest that both young cells, as well as mature hepatocytes with markers of senescence, are able to proliferate *in vivo* to restore liver mass (Wang et al., 2014). That is, that senescence is reversible. To make the picture more complicated, recent data suggest that oval cells can form directly from mature hepatocytes (metaplasia), proliferate, and re-differentiate to mature hepatocytes. That is, so-called progenitor cells (oval cells), seen when the hepatocyte population is stressed, are first formed from mature hepatocytes (Tarlow et al., 2014).

Based on these recent data, as well as historic observations, we take the view here that hepatocyte replacement during both transient and chronic infections is accomplished by proliferation of existing hepatocytes, which may include an intermediate step of progenitor/oval cell formation. One implication of this, discussed below, is that HCC ultimately arises from hepatocytes, not from a stem cell precursor originating in the bile ducts and/or Canals of Hering. However, recent findings leave open the possibility that HCC may arise from mature hepatocytes via pathways that include progenitor cell formation.

Finally, like many retroviruses, mammalian hepadnaviruses sometimes cause cancer in association with activation of host oncogenes resulting from nearby insertion of viral DNA (reviewed in Buendia and Neuveut, in press). Integration of hepadnavirus DNA into chromosomes occurs by illegitimate recombination, predominately of double stranded linear (dsl) DNA, which constitutes about 10% of virion DNA and is formed as an aberrant by-product of the

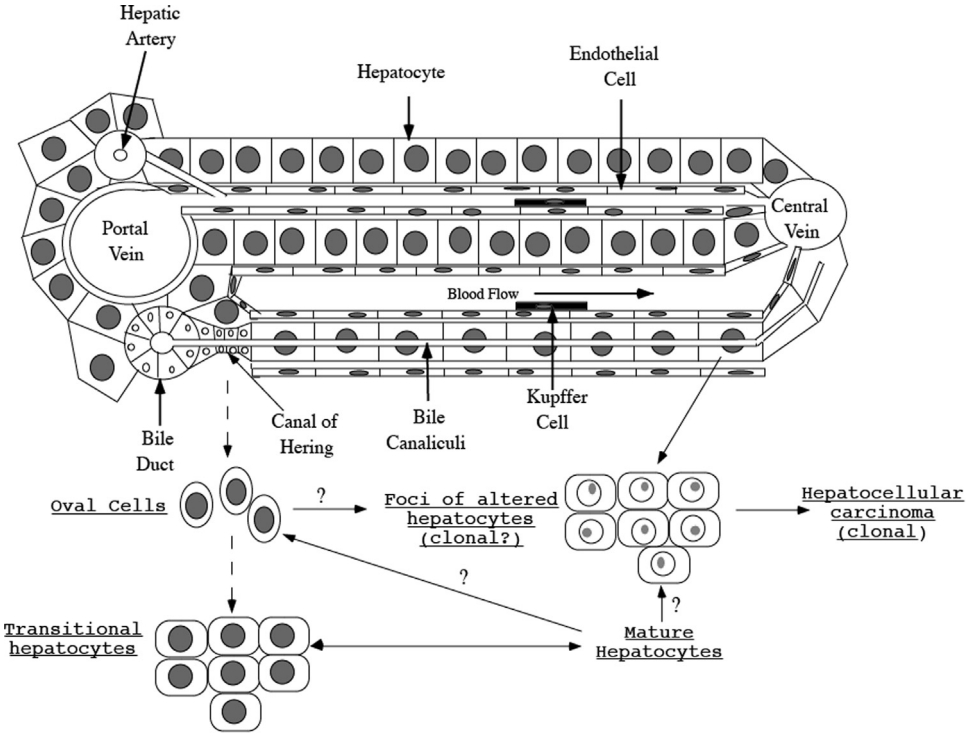

Fig. 4. Liver structure. A highly idealized 2D representation of a portion of a 3D liver lobule is shown representing some of the major cell types that are present. Blood enters through the portal vein and hepatic artery and exits at the central vein, after flowing through sinusoids in which hepatocytes are separated from blood via a fenestrated epithelium though which virus can pass. Bile produced by hepatocytes is released into bile canaliculi formed by the apical interfaces of hepatocytes and flows to the bile ducts via the Canals of Hering. Bile eventually flows to larger ducts before leaving the liver. Historically, progenitor cells residing in bile ducts and/or the Canals of Hering were thought to emerge under conditions of stress as ovoid cells (oval cells) and to differentiate into small transitional hepatocytes and finally mature hepatocytes (paths shown as dashed lines), as well as to contribute to formation of foci of altered hepatocytes, some of which appear to be preneoplastic. Recent studies questioned the role of oval cells in hepatocyte replacement, suggesting instead that hepatocytes themselves are able to evolve and repopulate the liver even under extreme stress to the normal hepatocyte population. And, by inference, to give rise to foci of altered hepatocytes and HCC. A possible resolution of some of these divergent observations is suggested by the very recent report that mature hepatocytes themselves give rise to oval cells that have the capacity to re-differentiate to hepatocytes (Tarlow et al., 2014). Thus, at present, some of the illustrated pathways are still uncertain (indicated with ? mark). (Figure adapted from Seeger et al. (2013).)

process normally leading to rcDNA (Fig. 2G). Integration is not orchestrated by viral proteins (Bill and Summers, 2004; Gong et al., 1995, 1999; Yang and Summers, 1995, 1999). However, it is still not clear how important insertional activation of oncogenes is in most HCCs occurring in HBV patients, and whether hepadnavirus proteins expressed from integrated DNA have a direct role in initiation or maintenance of cancer cells.

2. Molecular biology of hepadnavirus replication

Infection of hepatocytes is productive and, for the most part, non-cytopathic. Duck hepatitis B virus, an avihepadnavirus, has been documented to infect, in addition, bile duct epithelium of the liver, rare exocrine cells and alpha and beta islets of the pancreas, proximal tubular epithelium of the kidney, and possibly a subset of splenic cells (Guo et al., 2005; Halpern et al., 1983, 1984; Jilbert et al., 1987). In contrast to DHBV, the evidence that the orthohepadnaviruses infect cells other than hepatocytes has been controversial, and it has not always been clear that reported infections distinguish passive association of virus with a cell population from its actual infection, as conventionally defined by the presence of cccDNA, the viral transcriptional template.

The life cycle of hepadnaviruses resembles that of most retroviruses with the distinction that genome conversion to DNA occurs prior to the releases of virus particles (Figs. 2 and 3). Hence, hepadnaviruses are classified as DNA viruses. HBV itself encodes 7 proteins, preCore, core, pol, X (HBx), and the three envelope proteins, L, M, and S, which form a carboxy-terminal nested set, S being the smallest, M the next largest, and L the largest. The extra domains in M and L are named PreS2 and PreS1, respectively (Fig. 1). Core is the subunit of the viral nucleocapsid and pol encodes the enzymes needed for synthesis of viral DNA (reverse transcriptase, RNaseH, and primer), which takes place in nucleocapsids localized to the cytoplasm of infected hepatocytes. HBx is required for efficient transcription of cccDNA; it is not known to be a virus structural protein. preCore is the core protein with an N-terminal signal peptide and is proteolytically processed at its N and C termini before secretion from infected cells, as the so-called hepatitis B e-antigen (HBeAg). Its role in the virus life cycle is uncertain, but thought to inhibit or delay antiviral immunity in some way. The organization of the genes on the viral genome, or more particularly on the pregenome (the template for reverse transcription) is somewhat reminiscent of retroviruses (Fig. 1), but differs in at least two important ways. First, all the viral proteins have their own mRNA except for core and pol, which share an mRNA, the pregenome (Fig. 1). No polyproteins are produced. Second, with the exception of the secretory protein, preCore, none of the viral proteins are subject to post-translational proteolytic processing; in addition, hepadnaviruses do not encode a proteinase.

Hallmarks of hepadnaviruses, hepatotropism and species specificity are controlled on at least two levels: virus entry and transcription of viral RNAs. The discovery of hepatitis delta virus (HDV), a viroid-like satellite virus of HBV, provided an excellent model system for investigations of the viral determinants required for entry of HBV, because HDV depends on HBV envelope proteins for infection (Taylor, 2006). However, once in the cell, HDV replicates with much higher efficiency than HBV. Reverse genetic experiments with HDV led to the identification of a 75 aa-long domain in the N-terminus of the preS1 domain of the large envelope protein (L) (Fig. 1) that was required for infection of primary human hepatocytes (PHH) and of differentiated HepaRG cells, a hepatocarcinoma-derived cell line that is propagated in an undifferentiated form, but induced to differentiate by changing cell culture conditions (Blanchet and Sureau, 2007; Gripon et al., 2002). Experiments with DHBV infected ducklings showed that N-myristylation of L was essential for infectivity, but not virus assembly, an observation subsequently confirmed with HBV (Gripon et al., 1995; Macrae et al., 1991). Lipopeptides derived from the first 48 aa of preS1 can bind to hepatocytes permissive for HDV and HBV infections and inhibit infection (Gripon et al., 2005). Building on this information, Li and colleagues (Yan et al., 2012) used the myristylated preS1-derived peptide to isolate receptor candidates expressed in primary hepatocytes cultures derived from tree shrews, which can be infected with HBV and HDV. They identified the bile acid transporter, sodium taurocholate cotransporting polypeptide (NTCP) and demonstrated that it is the *bona fide* HBV/HDV receptor. NTCP is expressed on the basolateral membrane of hepatocytes, which is exposed to the space of Disse and separated from the blood stream by a fenestrated endothelium. Following ectopic expression, NTCP confers susceptibility to HBV and HDV infections of hepatoma cell lines that are otherwise resistant to these agents. Besides determining hepatotropism, preS1 binding to NTCP also controls species specificity (Ni et al., 2014; Yan et al., 2013; Zhong et al., 2013), consistent with observations of PreS function made earlier with avihepadnaviruses (Ishikawa and Ganem, 1995). While HDV can infect mouse cells expressing human NTCP, HBV cannot, indicating that other factors must play a role in determining species specificity of HBV (Ni et al., 2014; Yan et al., 2013). Uncoating, migration of virus nucleocapsids to the nuclear membrane and conversion of rc to cccDNA (Fig. 3) are the steps subsequent to viral entry that might depend on host specific factors. As noted earlier, cccDNA cannot be detected in transgenic mice expressing HBV in hepatocytes from a transgene, also pointing to species-specific determinants that play a role in the HBV lifecycle subsequent to viral entry.

The mechanisms for disassembly of nucleocapsids and cccDNA synthesis are not well understood. Purified HBV nucleocapsids added to digitonin-permeabilized hepatoma cells accumulate in nuclear baskets in a process that depends on the transport factors importin alpha and beta (Rabe et al., 2003, 2009). RcDNA is released into the cell nucleus and subsequently repaired to yield cccDNA (Fig. 3). However, the nature of the cellular enzymes that execute the DNA repair reaction remains elusive. A cellular polymerase and/or the RT are required to extend the gap in plus stand DNA (Fig. 1). Nucleoside analogues that inhibit the RT cannot prevent cccDNA formation of DHBV, underscoring the requirement for cellular factors in its formation (Fourel et al., 1994b). Cellular endonucleases are required to remove a short terminally redundant sequence in rcDNA minus strands created during viral DNA synthesis and to remove the RT and the RNA primer on minus and plus strand DNA, respectively (Figs. 1 and 2). Finally, a ligation reaction is required to close the gaps in both DNA strands. This model implies that rcDNA is the direct precursor of cccDNA. An alternative model (not shown) predicts that a polymerase extends the 3′ ends of both DNA strands and, through DNA strand displacement synthesis, creates a double-stranded linear DNA with a large terminal redundancy (LTR) spanning the cohesive overlap region in rcDNA (Yang et al., 1996). Homologous recombination between the LTRs would then lead to cccDNA formation without the requirement of an endonuclease to process the ends of rcDNA. Defective cccDNAs have been detected that could have been created by non-homologous recombination between the LTRs of such dsl DNAs (Yang and Summers, 1995). So far, however, dsl DNA intermediates with these terminal redundancies have not been detected, suggesting that they are either a very short-lived intermediate or that cccDNA is formed directly from rcDNA.

CccDNA is detected in the liver within the first day of infection of ducklings with DHBV (Tagawa et al., 1986). CccDNA binds to histones and forms a mini-chromosome (Bock et al., 2001; Newbold et al., 1995). For HBV, cccDNA serves as the template for transcription of six viral RNAs from four separate promoters, core, PreS, S and X. The mRNAs are termed precore/core, preS, M/S and X in orthohepadnaviruses, with preCore/core and M/S transcription directed by the core and S promoters, respectively. The extra mRNAs result from the use

of staggered initiation sites immediately upstream and downstream of an AUG that is in frame with a downstream AUG directing translation of core and S proteins. Two enhancers exhibiting binding sites for liver specific transcription factors, including HNF4 and HNF3, are required for the activity of the promoters and represent additional determinants for the marked hepatotropism of hepadnaviruses (reviewed in Kosovsky et al., 1998; Moolla et al., 2002).

HBx is also required for transcription from cccDNA in HepaRG cells and most likely in HepG2 cells infected with HBV (Lucifora et al., 2011; Seeger and Sohn, 2014). However, the exact mechanism of HBx action is still obscure. Evidence for direct binding of X to the mini-chromosome has been obtained with chromatin immunoprecipitation experiments (Belloni et al., 2009). However, the best-documented interaction of X is with DNA damage-binding protein 1 (DDB1) and the cullin4A-RING DDB1-ubiquitin ligase (Becker et al., 1998; Li et al., 2010). This scenario is reminiscent of Cullin4A-DDB1 binding of the V protein of paramyxoviruses, which leads to the ubiquitination and degradation of Stat1 and hence, inhibition of the IFN response (Leupin et al., 2003). While the interaction of HBx with DDB1 has been firmly established with biochemical, genetic and structural studies, the search for the HBx binding partner targeted for ubiquitination and degradation has not yet provided firm answers. If the Cullin4A-DDB1-HBx complex is indeed controlling transcription from cccDNA, it might play a role in the regulation of acetylation of histones, to activate transcription, because it has been observed that in the absence of HBx, histones of the mini-chromosome are hypoacetylated (Belloni et al., 2009). However, these observations have to be interpreted with some caution, because they were derived with a mimic of cccDNA derived from transfected plasmid DNA. Nevertheless, the current hypothesis about a role of HBx in transcription of cccDNA is exciting and consistent with the fact that this protein is required for natural infections of woodchucks by WHV (Zhang et al., 2001) and, by inference, for infection by the other orthohepadnaviruses. Interestingly, many avihepadnaviruses do not encode an X gene; for those that do, the protein may not be necessary for infection of their host (Meier et al., 2003). All known avihepadnaviruses express a larger core protein than orthohepadnaviruses; this larger core protein might exert an activity similar to X of HBV and WHV (Feitelson and Miller, 1988).

The six unspliced mRNAs transcribed from HBV cccDNA encode the seven proteins described above (for a more detailed description, see Seeger et al., 2013). (Spliced mRNAs have been reported but their functional significance is still uncertain (e.g., Obert et al., 1996; Soussan et al., 2003.) They are transported to ribosomes through a transport mechanism that depends, at least in part, on a posttranscriptional cis-acting regulatory element, termed PRE, overlapping the N-terminal portion of HBx (Huang and Liang, 1993; Huang and Yen, 1994). Core and polymerase (RT) proteins are transcribed from pgRNA, the latter by internal initiation. Pre-core (e-antigen) is transcribed from a pre-core RNA, which is colinear with pgRNA, except for a short extension at the 5′ end permitting translation from the pre-core AUG. Similarly, the middle (M) and small (S) envelope (surface) proteins are derived from colinear mRNAs, termed preS2 and M/S, respectively, with distinct 5′ends bracketing the AUG codon of M. Large envelope (surface) protein and HBx are translated from PreS1 and X transcripts, respectively. HBx might be produced immediately after formation of the cccDNA mini-chromosome resulting in activation of the other three promoters by direct or indirect mechanism as explained above. In infected liver tissue, the HBx transcript accumulates to much lower levels compared with the other viral mRNAs, and often cannot be detected at all, explaining the lack of consistent evidence of HBx expression in infected hepatocytes.

Translation of core and polymerase proteins is regulated to account for the stoichiometric imbalance required for morphogenesis of icosahedral particles consisting of 240 subunits of core and one molecule of polymerase bound to pgRNA. (The reader is referred to recent reviews for a detailed description of the mechanism for protein priming and reverse transcription (Hu and Seeger, in press; Seeger et al., 2013).) Binding of the polymerase to the copy of the stem–loop structure termed epsilon, in the 5′ terminal redundancy of the pgRNA (Fig. 2), triggers packaging and hence, the transition of the core/pol mRNA to pgRNA for DNA replication (Bartenschlager and Schaller, 1992; Hirsch et al., 1991). This binding reaction, which can be reproduced *in vitro*, requires heat shock proteins believed to stabilize a conformation of the polymerase for binding to the epsilon structure (Hu and Seeger, 1996; Hu et al., 1997; Wang and Seeger, 1992). Once bound to RNA, the polymerase primes reverse transcription from a tyrosine residue in the N-terminal domain of the polymerase, termed the terminal protein (TP) domain (Weber et al., 1994; Zoulim and Seeger, 1994). A major gap in knowledge concerning DNA replication is the lack of complete structural information about the polymerase, and in particular its TP domain, which does not share any apparent similarities with known proteins. Finally, in DHBV-infected ducks the majority of expressed polymerase appears to accumulate in cytoplasmic lipid-containing structures (Yao et al., 2000). However, a function for non-encapsidated polymerase remains obscure, although, sporadically, studies suggesting a role in inhibiting innate immunity have been reported (Foster et al., 1991; Wang and Ryu, 2010).

The final product of DNA replication in cytoplasmic nucleocapsids is the partially double stranded rcDNA still attached to the RT at the 5′ end of minus strand DNA and attached to an RNA oligomer derived from the 5′ end of pgRNA at the 5′ end of plus strand DNA (Figs. 1 and 2). The latter fulfills a role in the priming of plus strand DNA synthesis (Lien et al., 1986; Seeger et al., 1986; Will et al., 1987). An intricate aspect of the hepadnavirus life cycle is the fact that DNA, but not RNA containing nucleocapsids, can assemble with envelope proteins residing in ER membranes. Yet the signals responsible for this transition are not known. Biochemical studies revealed differences in the phosphorylation of core proteins isolated from RNA and DNA containing particles, respectively. DNA containing particles are hypo-phosphorylated in a region near the C-terminus of the core protein that contains several serine residues (Perlman et al., 2005). While the observed reduction in phosphate might compensate for the net increase of phosphate, a consequence of DNA synthesis, it could also create the binding sites for envelope proteins by changing a conformation of the C-terminal region. Unfortunately, structural information about the C-terminal domain of nucleocapsids is not yet available to explain distinct properties of nucleocapsids in assembly (Crowther et al., 1994; Watts et al., 2002).

The fate of DNA containing nucleocapsids depends on the stage of the infection of a hepatocyte. Early, when the concentration of envelope proteins is low, nucleocapsids enter a retrograde transport and deliver their DNA cargo into the cell nucleus leading to amplification of cccDNA copy number (Fig. 3) (Summers et al., 1990). Later in the infection, nucleocapsids bind to envelope proteins and mature into infections virions that are secreted into the blood stream.

Investigations on the assembly of HBV have been hampered by the low amount of virus produced in an infected cell, which has been estimated as 1–10 virions per cell per day (Nowak et al., 1996). Because envelope proteins accumulate in the endoplasmic reticulum–Golgi intermediate compartment, a model has been proposed where DNA-containing capsids bind to envelope proteins at the ER, translocate into the lumen and exit the cells via the secretory pathway. Hence, virion formation and secretion would follow the pathway for assembly and secretion of the much more abundant subviral spherical and filamentous particles (SVP) consisting predominantly of M and S, and to a lesser part L envelope proteins and lipids. However, subsequent biochemical studies revealed that virions might use a different pathway that depends

on proteins normally associated with the endosomal sorting complex required for transport (ESCRT) and with the formation of multivesicular bodies (MVPs) (Piper and Katzmann, 2007; Watanabe et al., 2007). The MVB machinery is also used for budding by other enveloped viruses including HIV (Sundquist and Krausslich, 2012). Evidence for MVB function in HBV assembly and budding is largely derived from DNA co-transfection studies with HBV DNA and plasmids expressing dominant negative mutants of proteins known to be associated with the MVB machinery such as Vsp4 and AIP1 (Kian Chua et al., 2006; Watanabe et al., 2007). A caveat with these experiments is that the dominant negative mutants could disturb cellular homeostasis and hence, make interpretation of the data difficult. For example, overexpression of wild-type Vsp4 causes reduced accumulation of intracellular and extracelluar forms HBV DNA (Kian Chua et al., 2006), while expression of dominant negative Vsp4 increases the release of "naked" nucleocapsids from transfected cells (Watanabe et al., 2007). Assuming that MVBs play the predicted role in virion maturation and budding, there are several gaps in knowledge that need to be filled to complete our understanding of HBV assembly and release. For example, what are the signals that direct HBV into the ESCRT pathway? Where in the infected hepatocyte does budding occur?

Whatever the site and exact mechanism for virus assembly might be, a critical event during morphogenesis of virus particles is the novel conformational arrangements of the N-terminal pre-S1 domain of L, the large envelope protein (Bruss, 2007; Bruss et al., 1994; Guo and Pugh, 1997; Ostapchuk et al., 1994), such that about half of the PreS1 domains are on the cytoplasmic side and half on the cisternal side of the membrane as virions form via budding. The PreS1 located on the cytosolic side is required for binding to viral nucleocapsids. The pre-S1, which had translocated across the viral membrane, is exposed on the surface of infectious virus particles where it functions as binding sites for NTCP, the viral receptor, as described above. Finally, it should be noted that so-called "empty" virus particles accumulate in sera of HBV infected patients (Ning et al., 2011; Luckenbaugh et al., 2015. The pathway for their assembly and the conformation of pre-S1 in these particles is not yet known.

3. Biology of transient infections

Infection of hepatocytes appears to be non-cytopathic and histopathology a consequence of the adaptive immune reaction to infection, in particular, the production of virus-specific cytotoxic T cells (CTLs). Transient infections are generally less than 6 months in duration. Events occurring during transient infections of humans are mostly inferred from studies of woodchucks and chimpanzees. Virus appears to spread in the first several weeks post-exposure without producing more than a transient activation of the innate immune response, lasting a few days (Guy et al., 2008; Wieland et al., 2004a), or any apparent induction of virus neutralizing antibodies or antiviral-CTLs (Wieland et al., 2004b). During this phase, virus spreads through the entire hepatocyte population. This may be followed by several weeks of productive infection, with no evidence of inflammation or other overt liver damage (Kajino et al., 1994; Summers et al., 2003; Wieland et al., 2004b). *A priori*, an antiviral-CTL response arising at this stage of infection might be expected to destroy the entire liver, leading to death of the host. However, such an outcome, termed fulminant hepatitis, is rare. Instead, activation of the CTL response and the associated inflammation kills some, perhaps a majority of hepatocytes over a period of a few weeks, but the remainder survive, are cured of their infection, and repopulate the liver, as shown by hepatocyte lineage tracking using viral DNA randomly integrated into host DNA during the infection (Summers et al., 2003). Resolution of infection often occurs without overt symptoms. A small fraction of hepatocytes may remain infected, as evidenced by short-term follow-up studies in ducks and woodchucks and deduced from long-term studies in humans (Reaiche et al., 2010; Rehermann et al., 1996; Summers et al., 2003). In these cells, replication appears to be suppressed by the immune response, but has been observed to rebound in some patients when the immune system is compromised (Hoofnagle, 2009); for example during chemotherapy of cancer patients.

Antiviral cytokines appear to play a major role in recovery, but surprisingly, neutralizing antibodies do not appear to be required (Kajino et al., 1994; Ponzetto et al., 1984; Wieland et al., 2004b). A direct function of cytokines is to induce elimination of virus replication intermediates from the cytoplasm of infected hepatocytes by mechanisms that are not well understood, although it has been demonstrated that in DHBV infected duck hepatocyte cultures, and HBV transgenic mice, IFNα interferes with packaging of pregenomic RNA into capsids (Guo et al., 2003; Schultz et al., 1999; Wieland et al., 2005). Clearance of cccDNA, which defines the infected state, appears to be delayed compared to clearance of DNA replicative intermediates (RI) in cytoplasmic nucleocapsids (Wieland et al., 2004b). Computer modeling studies and hepatocyte lineage studies using randomly integrated viral DNAs as cell markers led to the deduction that at least some cccDNA may be cleared non-cytolytically (Mason et al., 2009b; Murray et al., 2005); i.e., in a process dependent neither on hepatocyte death nor compensatory division. As noted, early studies showed that cccDNA was not reduced by interferon alpha or gamma treatment of primary duck hepatocyte cultures infected by DHBV (Schultz and Chisari, 1999; Schultz et al., 1999), which appears to fit with the observation that cccDNA is not lost at the time of clearance of the cytoplasmic viral DNA (RI DNA) present in nucleocapsids (Fig. 3) (Wieland et al., 2004b). A more recent cell culture study presented evidence that interferon alpha, gamma and other cytokines can induce APOBEC3, which then targets cccDNA for depurination and degradation, and might explain non-cytolytic clearance (Lucifora et al., 2014). This study remains controversial, however, as depurination appeared specific to the minus strand of cccDNA. Editing of the minus strand of HBV DNA replication intermediates is already well-described (e.g., Noguchi et al., 2005; Rosler et al., 2005; Suspene et al., 2005), raising the possibility that the editing observed in cccDNA actually occurred in the cccDNA precursor, RI DNA (Figs. 2 and 3). In brief, while there is a basis from cell culture and *in vivo* studies to further investigate non-cytolytic mechanisms for cccDNA clearance, acquiring convincing experimental data so far remains elusive.

One alternative model, not requiring non-cytolytic loss of cccDNA, stipulates that cccDNA is stable in infected hepatocytes and can only be lost by cell death. Thus, curing of a hepatocyte lineage would require elimination of RI DNA by cytokine-mediated processes, followed by one or more rounds of mitosis to dilute out cccDNA and produce virus-free progeny. Another model is that cccDNA is lost when a cell divides, so hepatocyte division produces two progeny free daughter cells (Mason and Litwin, 2002). Current *in vivo* data may be consistent with a role for the latter mechanism, which necessitates killing of about 70% of hepatocytes (based on a model, which assumes hepatocytes divide at random to maintain liver mass), or with this together with non-cytopathic mechanisms for elimination of cccDNA during recovery from transient infections (Mason et al., 2009b; Summers et al., 2003). The data do not support a model in which cccDNA survives cell division during immune clearance of the virus and is lost solely via cell death (Mason et al., 2009b). Further experiments, perhaps following up on the idea that APOBEC3 targets cccDNA, may help resolve the role of cytokines versus cell division in cccDNA elimination.

The possibility that cccDNA can survive mitosis, at least in some situations, was based on estimates of hepatocyte turnover during

antiviral therapy of chronically infected woodchucks with a nucleoside analog inhibitor of WHV DNA replication. In particular, there was an initial cccDNA decline, consistent with estimates of hepatocyte turnover. However, an equivalent decline in the fraction of infected hepatocytes was not observed early in treatment, suggesting that hepatocytes containing multiple copies of cccDNA were eventually cured by diluting out cccDNA through multiple rounds of cell division (Zhu et al., 2001). CccDNA survival through cell division was also inferred from a study of infected hepatocytes induced to divide in the presence of an antiviral nucleoside analog (Dandri et al., 2000). Relevant to this latter study, cccDNA appears stable in non-dividing primary hepatocyte cultures in which viral DNA synthesis is blocked by nucleoside analogs (Moraleda et al., 1997; Zhu et al., 2001). These results may suggest that cccDNA loss during recovery from transient infections, to the extent that it is mediated by mitosis, is dependent on cytokine effects that destabilize cccDNA during mitosis. However, interpretation of these antiviral-studies depends on the assumption that the antiviral nucleosides completely inhibited new RI DNA synthesis, and thus cccDNA synthesis throughout the cell cycle. This assumption has recently been called into question based on antiviral studies with DHBV infected ducklings in which hepatocytes were proliferating at a predictable rate (Reaiche-Miller et al., 2013). In brief, as with determining if cytokines mediate cccDNA loss in non-dividing hepatocytes, the fate of cccDNA in cycling cells also remains elusive.

4. Chronic infections

As with transient infections, most chronic infections are thought to begin with spread of virus through the entire hepatocyte population. However, the immune response is ineffective and the virus is not cleared. Approximately 90% of human HBV infections at less than 1 year of age become chronic compared to only 5% in adults. Most chronic infections occur at birth, from infected mothers, or during the first year of life. A similar inverse relationship between age and frequency of chronic infection was found using the duck and woodchuck models (Cote et al., 1991; Jilbert et al., 1998). Passive immunization and/or vaccination of newborns at risk for perinatal infection reduces the risk of chronic infection >10-fold (Chen et al., 2011) and represents a major advance in reducing the worldwide burden of chronic hepatitis B, estimated at 300–400 million. Unfortunately, this regimen is less effective when virus titers were high in maternal blood (Wen et al., 2013).

Although chronic infections may clear spontaneously, most are life-long. At least early, chronic infection is associated with persistently high virus titers, of the order of 10^9–10^{10} per ml of serum. In humans, virus titers often decline with age, especially after 30 years, sometimes dropping to less than $\sim 10^{2-3}$ per ml (Chen and Yang, 2011). This decline has not been documented in ducks or woodchucks.

An idealized view of chronic HBV infection, for individuals infected early in life, was published about 10 years ago by Yim and Lok (2006), and is summarized in Fig. 5. In brief, once established, chronic infection is characterized by an immune tolerant phase, which may last up to 30 years or more. The concept of immune tolerance is based on high virus titers (e.g., the order of 10^9–10^{10} per ml), the absence of persistently elevated alanine aminotransferase (ALT) levels in the blood stream, indicative of hepatocyte killing and, where liver biopsies are available, the absence of inflammation and/or fibrosis (Lok and McMahon, 2007, 2009). However, recent immunological studies have called into question the concept of immune tolerance as well as the conclusion that liver injury, albeit low level, is not ongoing in these patients (Bertoletti and Kennedy, 2014; Kennedy et al., 1995). In any case, patients may then enter an immune clearance phase, characterized by one or more bouts of acute hepatitis, during each of which virus titers may decline and afterwards rebound. Ultimately, this phase may end with a stable reduction of virus titers to a low level, presumably reflecting immune control of virus replication, possibly as seen after resolution of transient infections. Unlike recovery from transient infections, generally associated with anti-HBsAg IGs, HBsAg expression may persist, perhaps from integrated DNA and residual cccDNA. Such patients are not considered to have resolved their infections in the sense seen after transient infections or when HBsAg disappears and is

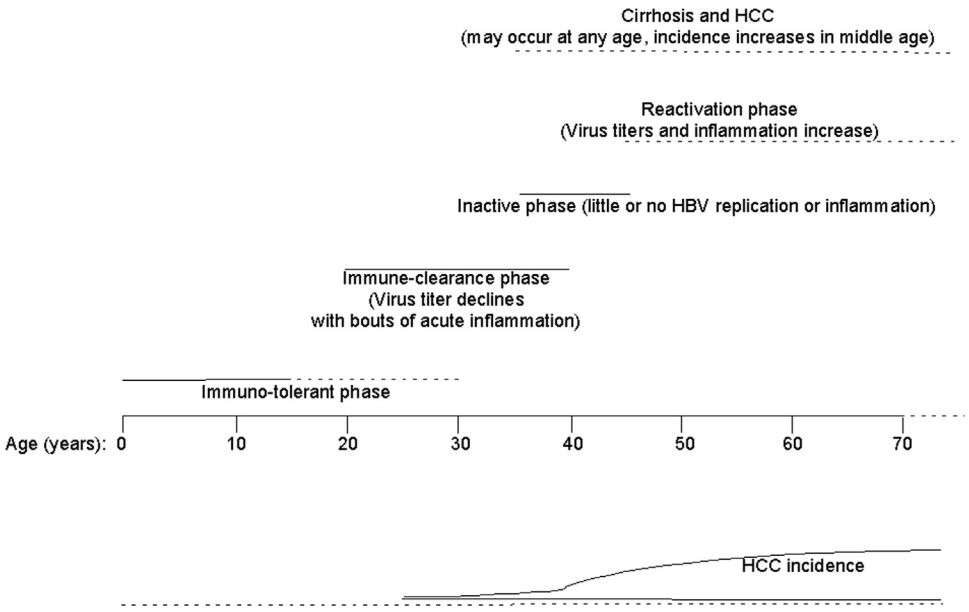

Fig. 5. Stages of chronic HBV infection. Chronic HBV infection is thought to go through immune tolerant, immune clearance, and immune control (no disease progression) phases. In this latter phase, the degree of ongoing liver damage can vary from none to significant, depending on how completely virus replication is blocked (see text). HCC incidence generally rises after the immune clearance phase, which may not be clinically apparent but can still be inferred to have occurred because of the greatly reduced virus titers that come after it, reflecting immune control or restriction of virus replication. Loss of immune control may also occur after a period of disease inactivity, leading to enhanced virus replication and exacerbation of liver damage. (Figure adapted from Yim and Lok (2006)).

replaced by anti-HBsAg IGs. As in transient infections, symptoms may not be severe enough, during the immune clearance phase, to lead the patient to seek medical care. Nonetheless, the immune clearance phase can initiate extensive damage to the liver resulting in fibrosis and cirrhosis. In a prospective study, the rate of subsequent disease progression to cirrhosis and HCC was found to depend on virus titers at study entry. For example, the long-term risk of HCC increased several fold for patients with titers $\geq 10^5$ per ml, as compared to patients with lower titers, suggesting some sort of ineffective balance between immune control, virus replication, and immune killing of hepatocytes in these patients (Chen and Yang, 2011).

Long-term infection is often associated with evolution of the virus population, probably resulting from immune selection against viral epitopes that lead to efficient destruction of infected hepatocytes by the antiviral immune response. One possible example is the loss of HBeAg, either due to stop codon mutations in the signal peptide domain or to upstream mutations in the promoter for the HBeAg mRNA (Brunetto et al., 1999; Okamoto et al., 1994; Parekh et al., 2003; Sato et al., 1995; Yu and Mertz, 1996). How these mutants emerge to become a dominant virus genotype in the liver is elusive.

One possibility is that mutants that emerge have higher *in vivo* replication rates than wildtype HBV and spread through the infected liver by super-infection to become the dominant genotype. However, work with the duck and woodchuck models does not favor this hypothesis, as super-infection resistance seems high (Rodrigues et al., 2014; Zhang and Summers, 1999). An alternative scenario is that virus evolution after the liver is fully infected is largely vertical, division of an infected hepatocyte producing two infected daughter cells via retention of nuclear cccDNA and/or cytoplasmic RI DNA. Thus, commonly occurring mutations might emerge because they give the infected hepatocyte a survival advantage to escape anti-viral CTLs (Frelin et al., 2009), allowing these cells to slowly repopulate the liver (Mason et al., 2008). To our knowledge, the data needed to resolve these scenarios does not yet exist. The distinction may be important, however, because selection at the cellular level would enhance genetic narrowing of the hepatocyte population and might facilitate, indirectly, the clonal expansion of hepatocytes destined to evolve to HCC (see below).

Interestingly, wild type virus re-emerges as the dominant genotype during transmission to a new host, probably because it has a selective advantage in spreading through the large population of uninfected hepatocytes ($\sim 5 \times 10^{11}$ in adults). This selection may be partially due to a higher replication rate, but may also be due to an ability of wt virus to suppress the host immune response. For instance, HBeAg, the secreted product of preCore (Fig. 1), has been reported to suppress anti-viral immunity to core protein (Chen et al., 2004), a capacity that is presumably transient, but adequate to delay the process until the liver is fully infected. Thus, immune selection seems to be a major factor, driving emergence of mutant virus as dominant species late in an infection.

An issue related to virus evolution, but also impinging on the question of hepatocarcinogenesis, is whether hepatocytes stably evolve genetically or epigenetically in response to pressures placed upon infected cells by antiviral CTLs. It has been clear since the earliest studies of HBV infected human livers that a majority of hepatocytes are not productively infected late in the disease (Burrell et al., 1982; Gowans et al., 1981). This has also been observed in chronically infected chimpanzees even in the face of persistent viremia (Mason et al., 2009a). The vast majority of HBV negative hepatocytes in non-cirrhotic livers of chimpanzees and of HCC patients have normal histology (e.g., Mason et al., 2009a, 2010). Evolution of HBV-resistant hepatocytes may be an early step in neoplastic progression, allowing hepatocytes destined to give rise to tumors to escape elimination by the antiviral immune response. This idea is consistent with the notion that the hepatocyte population is entirely self-renewing.

5. Chronic hepadnavirus infection and hepatocellular carcinoma

Many cancers, including HCC, are likely the end result of pathologies that evolve over a lifetime (Farber and Sarma, 1987). Studying a life-long process in chronic HBV infection is intrinsically difficult, not only because of the time factor, but also because liver biopsies are seldom taken until patients show clinical signs of chronic liver disease (e.g., abnormally high liver enzymes in the circulation for 6 months or more as a sign of chronic hepatocyte destruction). Such patients are no longer in the immune tolerant phase, and often 30 years or more in age. An unfortunate consequence is that while a great deal is known about late stages of infection, as defined by signs of clinically overt disease, almost nothing is known about evolution of liver pathology earlier in infection. An implicit assumption in the clinical setting has been that the immune tolerant phase is essentially apathogenic, though recent data may challenge this perspective (Bertoletti and Kennedy, 2014; Zoulim and Mason, 2012).

The pathogenic processes leading to HCC, as for many cancers, are believed to involve at least three stages: initiation, promotion, and progression (Farber and Sarma, 1987). Initiation is probably the fixation of a mutation(s) in host DNA via cell division. Promotion would correspond to clonal expansion of mutated hepatocytes that have an increased risk of oncogenic transformation, while progression includes the steps by which members of these clonal populations evolve to become cancer cells. Additional mutations would presumably occur during these latter steps.

As suggested above, emergence of a large fraction of normal-appearing hepatocytes that are free of replicating virus may be a step in tumor promotion, since it presumably involves clonal expansion of virus-resistant hepatocytes. In addition, HCCs generally do not support HBV replication: for example, core protein, which is required for replication, is detected in only about 15% of HCCs and even in these cases, only in a minor fraction of tumor cells (Hsu et al., 1989). This also seems to be true of preneoplastic lesions (Govindarajan et al., 1990). Presumably related phenomena are seen in genetic diseases of the liver that predispose to HCC such as alpha-1-antitrypsin deficiency (Rudnick and Perlmutter, 2005) or in tyrosinemia Type I, in which liver disease may be ablated due to partial repopulation of the liver by hepatocytes in which the defect in fumarylacetoacytase (involved in tyrosine degradation) is corrected via spontaneous mutation (Demers et al., 2003; Grompe et al., 1998; Kvittingen et al., 1994; Marongiu et al., 2008). Hence, immune selection for hepatocytes deficient in HBV replication may be an additional basis for tumor promotion in HBV patients.

A possible basis for progression is less evident. The problem here, as evident from recent high throughput studies of HCC genomes, is that vast differences between tumors, and between tumors and normal liver, obscure identification of genetic events that are essential, and in what order they have occurred. For instance, does viral DNA integration near an oncogene reflect initiation, promotion, or progression, and is this distinction important for HCC development? Integration of viral DNA is a low frequency event occurring in only 0.1–0.01% of hepatocytes during transient infections (Summers et al., 2003). However, it is an ongoing process and is found in ~10–100% of hepatocytes by the time HCC develops in chronic carriers. Early studies suggested that the HBV DNA integration site in each tumor was distinct, not even confined to a single chromosome and, with a few exceptions, not near a known oncogene (Dejean et al., 1986; Wang et al., 1990), The apparent complexity of HCC development in humans appeared to be in stark contrast to the scenario in woodchucks, where early studies suggested that a much simpler picture might eventually emerge.

6. Evidence that integration of hepadnaviral DNA can be pro-oncogenic

In the woodchuck model, transcriptional activation of the N-myc2 pseudogene is found in about 80% of HCCs, often in association with WHV integration just 5′ or 3′ of the ORF of the gene, or at the distal loci *b3n* and *win*, ~10 kbp and 150 kbp, respectively, from N-myc2 (Bruni et al., 1999; Fourel et al., 1990, 1994a). The existence of additional integration sites distal to N-myc2 that activate its transcription has also been suggested (Bruni et al., 2006).

N-myc2 expression is common in preneoplastic foci of altered woodchuck hepatocytes (FAH) (Yang et al., 1993), but not in normal appearing hepatocytes. It is not known if expression in these preneoplastic FAH is due to WHV integration. In any case, this result suggests that N-myc2 is important in neoplastic progression, but that additional mutations are needed to produce an HCC. What these are remains unknown. In a small fraction of woodchuck HCCs, proximal integration of WHV DNA along with transcriptional activation may be found instead at N-myc1 or C-myc (Fourel et al., 1990; Hsu et al., 1988).

To address issues related to the oncogenicity of C-myc and N-myc2, Buendia and colleagues made transgenic mice containing either WHV/Nmyc2 or WHV/Cmyc DNA fragments cloned from woodchuck HCCs (Renard et al., 2000; Terradillos et al., 1997). With WHV/C-myc, virtually all mice developed HCC by one year of age. In the WHV/N-myc2 transgenic mice, the tumor frequency was much lower (about 55% between 11 and 30 months of age), perhaps due to lower transgene expression. In both transgenic models, normal hepatocytes, preneoplastic lesions and HCCs were found. It remains unclear if this heterogeneity reflects secondary events (e.g., mutations) needed for emergence of preneoplastic lesions and HCC (Renard et al., 2000), or heterogeneity in transgene expression within the hepatocyte population, particularly in the adult mouse in which endogenous expression of C-myc is low. In brief, while these studies showed that myc activation is a critical step in carcinogenesis in the woodchuck, they also re-enforced the conclusion that HCC formation, even in this host, is a multistep process in which most steps remain elusive.

In humans, the relationship between HBV DNA integration and gene activation (or inactivation) and hepatocarcinogenesis are much less clear. Genomic analyses revealed some preferences of HBV integration near genes encoding, for example, telomerase (TERT), the histone H3K4 methyltransferase MLL4 and cyclin E1 (CCNE1) (Sung et al., 2012). However, none of these and other recurrent integrations have been found in more than a few percent of HCCs, making their overall importance unclear (Ding et al., 2012; Jiang et al., 2012; Murakami et al., 2005; Sung et al., 2012), As alluded to above, it has not been possible to identify genes that are uniformly activated as a direct consequence of HBV integration.

7. Role of HBV proteins in hepatocarcinogenesis

Although most human HCCs contain integrated viral DNA, expression of core protein has been found in only about 15% of HCCs, and then, only in a minority of cells in the tumor (Hsu et al., 1989). Envelope proteins are detected in about 30% of HCCs and, in a larger fraction of tumor cells than typical of core, which might reflect stringent requirement of the core promoter for hepatocyte-specific transcription factors (Hsu et al., 1989). HBx expression has been observed in ~20–50% of HCCs (Seo et al., 1997; Su et al., 1998) but, like core, only in a small fraction of cells in a tumor (Su et al., 1998). Thus, there does not appear to be an obvious role for virus proteins in maintaining the transformed state.

Evidence that HBx may, however, contribute to hepatocarcinogenesis comes from studies with HBx transgenic mice (Koike et al., 1994). While HBx does not increase the risk of HCC in mice with a low spontaneous incidence, it does potentiate HCC development when these mice are treated with DEN, a liver carcinogen, at least in part by inhibiting DNA excision repair (Becker et al., 1998; Madden et al., 2001; Minor and Slagle, 2014). HBx may also promote expression of pro-carcinogenic genes in late stage liver disease, as illustrated by faster HCC formation when the WHV/C-myc transgenic mice described above were crossed with HBx transgenic mice. While it seems reasonable that HBx may be important in formation of human HCC, it remains unclear at what stage this protein would exert its effects. It is also unclear how expression levels in these mice compare to HBx expression in HBV infected hepatocytes.

L protein (Fig. 1) is also speculated to have a role in hepatocarcinogenesis. Over expression of wt L protein of HBsAg in the liver of transgenic mice led to severe liver damage and HCC, apparently due to a massive, persistent hepatocyte killing as a result of excessive L mediated accumulation of HBsAg in the ER (Chisari et al., 1989). While this massive-overexpression of L is not a feature of HBV infection *per se*, it might occur late in chronic HBV infection when some hepatocytes overexpress L giving them a histologic appearance known as ground glass hepatocytes (Gerber et al., 1975), perhaps for the same reason (over-expression of L in some hepatocytes). More recently, HBsAg, particularly with mutations affecting the PreS2 domain, has been suggested to contribute to oncogenesis, possibly via induction of an ER stress response and oxidative DNA damage due to ER accumulation of this aberrant L protein (Pollicino et al., 2014). Ground glass hepatocytes with a PreS2 mutant often appear in focal clusters that have been suggested to be preneoplastic (Su et al., 2008). One of these mutant L proteins was expressed in the hepatocytes of transgenic mice prone to develop liver cancer. Twenty-five percent of these mice developed HCC by 30 months of age, starting at 22 months of age (non-transgenic sibs were not shown for comparison). In contrast, 100% of HBx transgenic mice in the same study developed HCC between 14 and 22 months, while double transgenics developed HCC slightly faster, at younger ages than the HBx transgenics. In the absence of non-transgenic littermate controls, it is not clear if the mutant L protein caused HCC; however, the results argue that in this mouse lineage, at least, HBx is carcinogenic and that the mutant L potentiated the effects of HBx.

In summary, there is circumstantial evidence that HBx and possibly mutant L protein contribute to hepatocarcinogenesis. As with insertional activation of oncogenes, it is not yet clear when or how often, in the process of cellular transformation, these proteins actually play a vital and direct role.

8. Antiviral therapy and HCC

In most HBV carriers, liver disease progresses through cirrhosis to HCC, though HCC may also occur in the non-cirrhotic liver. Thus, the goals of antiviral therapy are to halt the progression of cirrhosis, or even lead to a reversion, and to block the appearance of HCC. Two approaches are used, interferon therapy and treatment with nucleoside analog inhibitors of HBV reverse transcription. Interferon therapy aims to induce permanent immune control of infection via stimulation of the hosts' antiviral immune response and, not unexpectedly, is most successful during the immune clearance phase of the infection; i.e., in patients with significant immune mediated liver damage when therapy is initiated (Hoofnagle, 1990). The main goal of nucleoside analog therapy is to block viral DNA synthesis and thereby reduce the number of infected hepatocytes, as cccDNA is lost via hepatocyte turnover. As with interferon

therapy, nucleoside analog therapy may also potentiate immune control. Combination therapy has not so far provided a major improvement is this regard, probably because many patients responsive to an interferon induced cure are also those responsive to nucleoside analog mediated cure (Wong et al., 2014).

An obvious problem with nucleoside therapy in the majority of cases, in which immune control is not achieved, is the necessity of life-long treatment to prevent virus rebound. For this and other reasons, antiviral therapy has not been recommended for patients still in the immune tolerant phase of infection (Lok and McMahon, 2009). With nucleoside analogs, reasons to not treat immune tolerant patients include potential drug toxicity, cost and, until recently, the potential emergence of drug resistant strains of HBV. With the more recent FDA-approved agents, Tenofovir and Entecavir, the emergence of drug-resistant variants in treatment naïve patients (no history of therapy with nucleosides with a lower resistance threshold) is, so far, almost absent.

Following the current treatment guidelines, it has been found that therapy reduces short-term risk (e.g., five year) of HCC several fold (Hosaka et al., 2013). Nucleoside analog therapy can also inhibit and sometimes reverse the progression of cirrhotic and fibrotic liver injury (Marcellin et al., 2013; Tana and Hoofnagle, 2013), which can be life threatening.

What remains unclear is whether the risk of HCC can be reduced to background levels by starting treatment earlier, in the immune tolerant phase (Hosaka et al., 2013). While there is evidence that the immune tolerant phase is actually associated with antiviral immunity targeting infected hepatocytes (Bertoletti and Kennedy, 2014; Wang et al., 2010), it is not yet clear if this poses an HCC risk. Indeed, it remains uncertain why HCC is not totally prevented even following current treatment guidelines. Is this because an HCC risk is set early, during the immune tolerant phase, or because of the inherent difficulties in identifying patients until well after they have progressed to late stages of infection where preneoplastic lesions are already present? For this among other reasons, a short-term curative therapy, as now available for HCV carriers (Afdhal et al., 2014), is highly desirable, especially one that can be applied at any stage of chronic hepatitis B including the immune tolerant phase.

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