Insights into Hepatitis B Virus DNA Integration-55 Years after Virus Discovery

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Hepatitis B virus (HBV), which was discovered in 1965, is a threat to global public health. HBV infects human hepatocytes and leads to acute and chronic liver diseases, and there is no cure. In cells infected by HBV, viral DNA can be integrated into the cellular genome. HBV DNA integration is a complicated process during the HBV life cycle. Although HBV integration normally results in replication-incompetent transcripts, it can still act as a template for viral protein expression. Of note, it is a primary driver of hepatocellular carcinoma (HCC). Recently, with the development of detection methods and research models, the molecular biology and the pathogenicity of HBV DNA integration have been better revealed. Here, we review the advances in the research of HBV DNA integration, including molecular mechanisms, detection methods, research models, the effects on host and viral gene expression, the role of HBV integrations in the pathogenesis of HCC, and potential treatment strategies. Finally, we discuss possible future research prospects of HBV DNA integration.

KEY WORDS: HEPATITIS B VIRUS; INTEGRATION; NON-HOMOLOGOUS END JOINING; INSERTIONAL MUTAGENESIS; HEPATOCELLULAR CARCINOMA
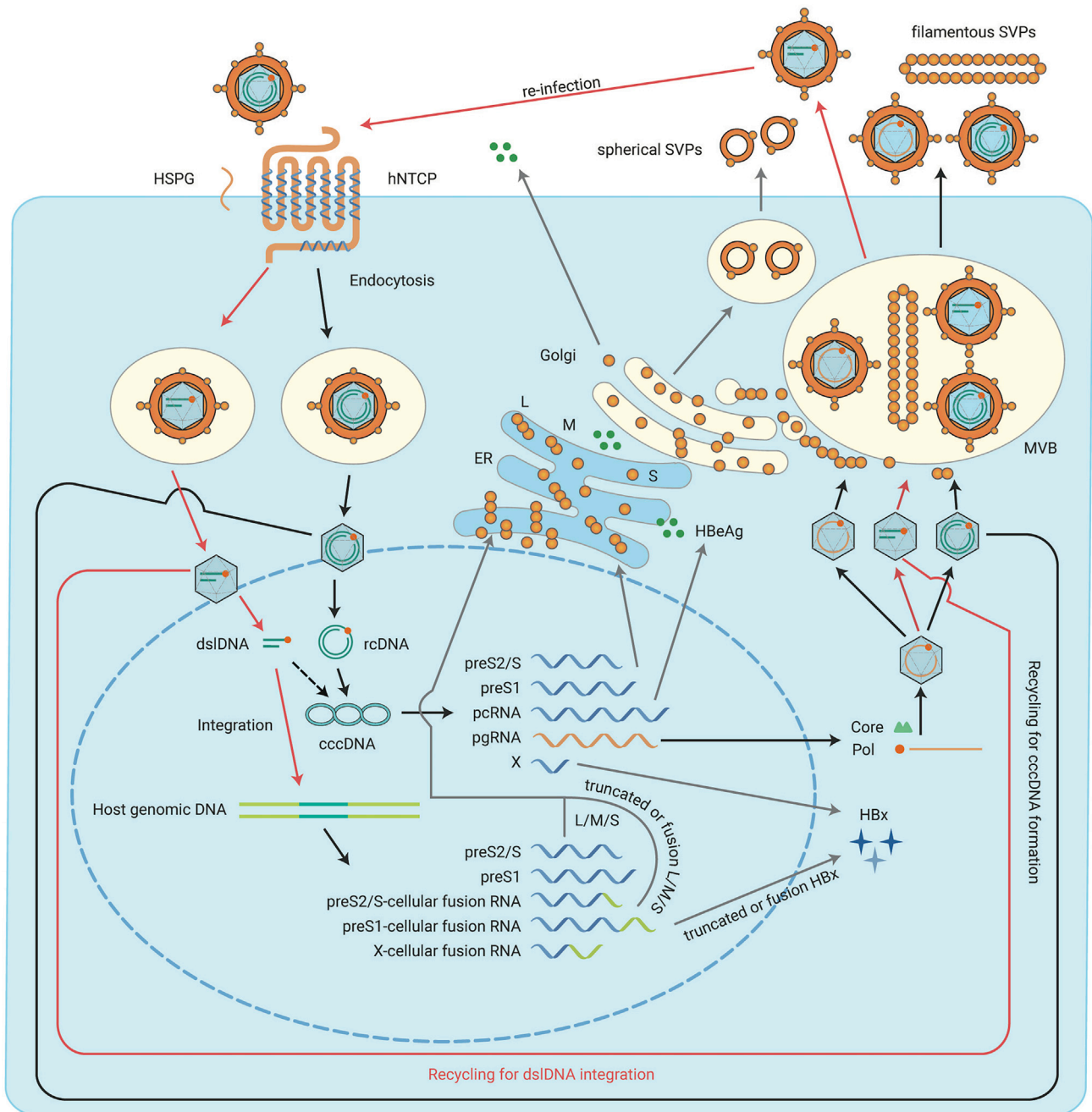
Introduction

In 1965, Dr. Baruch Blumberg made the discovery of the "Australia antigen," subsequently identified as the hepatitis B virus (HBV) surface antigen (HBsAg).[1] Because of this work, Dr. Blumberg was awarded the Nobel Prize in Physiology or Medicine in 1976. HBV infection chronically infects about 250 million individuals worldwide and is a major global health burden.[2,3] Chronic HBV infection is recognized as a high risk factor for developing liver cirrhosis and hepatocellular carcinoma (HCC).[4] After infection, the viral genome is established as a minichromosome, called covalently closed circular DNA (cccDNA). The cccDNA is essential to establishing persistent HBV infection as a stable intracellular HBV replication intermediate, which is localized to the nucleus of infected cells as an episomal plasmid-like molecule that can produce progeny virus.[5] Stably integrated HBV DNA in the host genome is another stable form of viral DNA. Although no progeny virus is produced, integrated HBV DNA can produce viral RNAs and proteins.[6–9] HBV DNA integration was first reported in 1980 in human HCC.[10,11] Since then, technologies used in HBV integration research include Southern blots, *in situ* hybridization, polymerase chain reaction (PCR), and next-generation sequencing (NGS).[7,12–16] HBV DNA integration events occur at a rate of ~1 integration per 10^3–10^4 infected cells in animal models[17–19] and ~1 integration per 10^4 infected cells in the early viral life cycle in an *in vitro* infection model.[20] HBV DNA integration has a close relationship with HCC and many studies have been done to explore its role in the development of HCC. In the genome of hepatic cancer cells, HBV DNA integration occurs more often (86.4%) than in normal liver tissues (30.7%).[21] Observed mechanisms leading to tumorigenesis include insertional mutagenesis acting in *cis* of key HCC-associated genes, induction of chromosomal instability, and the expression of mutant HBV genes from integrated HBV DNA.[21–24] Here, we review and discuss current research advances in HBV DNA integration, including molecular mechanisms, detection methods, research models, and roles in disease and potential treatment strategies.

HBV Life Cycle

HBV belongs to the *Hepadnaviridae*, a family of small enveloped hepatotropic DNA viruses. Unlike other DNA viruses, HBV replicates its DNA through reverse transcription, which also determines the complexity of the HBV life cycle. The HBV life cycle is complex, including processes, such as viral entry, cccDNA formation, transcription, replication, assembly, secretion, and integration (Figure 1). HBV enters hepatocytes from the basolateral (sinusoidal) membrane, where the viral receptor, sodium taurocholate cotransporting polypeptide (NTCP), is localized.[25] This process can be subdivided into adsorption and entry. Adsorption is mediated by glycosaminoglycans, such as heparan sulfate proteoglycans, that can recruit HBV to the cell surface through non-covalent binding.[26,27] As the functional receptor for HBV, NTCP interacts with the preS1 domain of HBV large surface protein (L) to complete the HBV entry process.[28] Mediated by NTCP, the relaxed circular DNA (rcDNA) containing nucleocapsids enter the cytoplasm and are released in the nucleus,[29] where the rcDNA is repaired to form cccDNA with the assistance of host factors, including TDP2, DNA polymerase (POL) κ, POLα, DNA ligase 1 and 3, and flap endonuclease 1.[5,30–36] As a template for HBV transcription, cccDNA transcribes five kinds of HBV RNAs (0.7 kb, 2.1 kb, 2.4 kb, longer and shorter 3.5 kb RNAs) under the action of host RNA polymerase II.[37] Transcription of cccDNA is controlled by four promoters (the Core, preS1, preS2, and X promoters) and two enhancers (enhancers I and II).[38,39] The 0.7-kb RNA can be translated to HBV X protein (HBx) which acts as a transcriptional regulator. The 2.1-kb RNA can be translated to HBV small surface protein (S) and middle surface protein (M). The 2.4-kb RNA can be translated to HBV large surface protein (L). L, M, and S can self-assemble to form empty subviral particles (SVPs) (including spherical SVPs and filamentous SVPs) that are secreted,[40–43] with only filamentous SVPs and virions containing significant amounts of L protein. The spherical SVPs are secreted through the constitutive secretory pathway. The filamentous SVPs are secreted by the endosomal sorting complex required for transport (ESCRT) machinery through multivesicular bodies (MVB).[43] The longer 3.5-kb RNA is termed pre-Core RNA (pcRNA) and can be translated to pre-Core protein, better known as HBV e antigen (HBeAg).[44–46] The shorter 3.5-kb RNA is pre-genomic RNA (pgRNA) that has two roles, as the translation template for HBV polymerase (Pol) and Core proteins and as the replication template for intra-capsid (formed by Core protein polymerization) reverse transcription by Pol to form HBV rcDNA.[45–48] These nucleocapsids can then be enveloped by HBV surface proteins (L, M, and S) to form mature virions and secrete through the ESCRT/MVB pathway.[40] Alternatively, these nucleocapsids can also be transported to the nucleus to form cccDNA.

Other enveloped nucleocapsids containing double-stranded linear DNA (dslDNA), HBV RNA, or the enveloped empty capsid can also form in the HBV life cycle.[49–52] These particles are secreted out of the cells after which they can conduct viral entry. In both *de-novo*-infected nucleocapsids and in cytoplasm-produced nucleocapsids dslDNA can be circularized by the non-homologous end joining (NHEJ) DNA repair pathway into cccDNA-like molecules.[5,53] As NHEJ is error-prone, many of these molecules are functionally defective.[5] Furthermore, dslDNA is the dominant substrate for integration into the host genome.[18] A study performed in an *in vitro* infection model
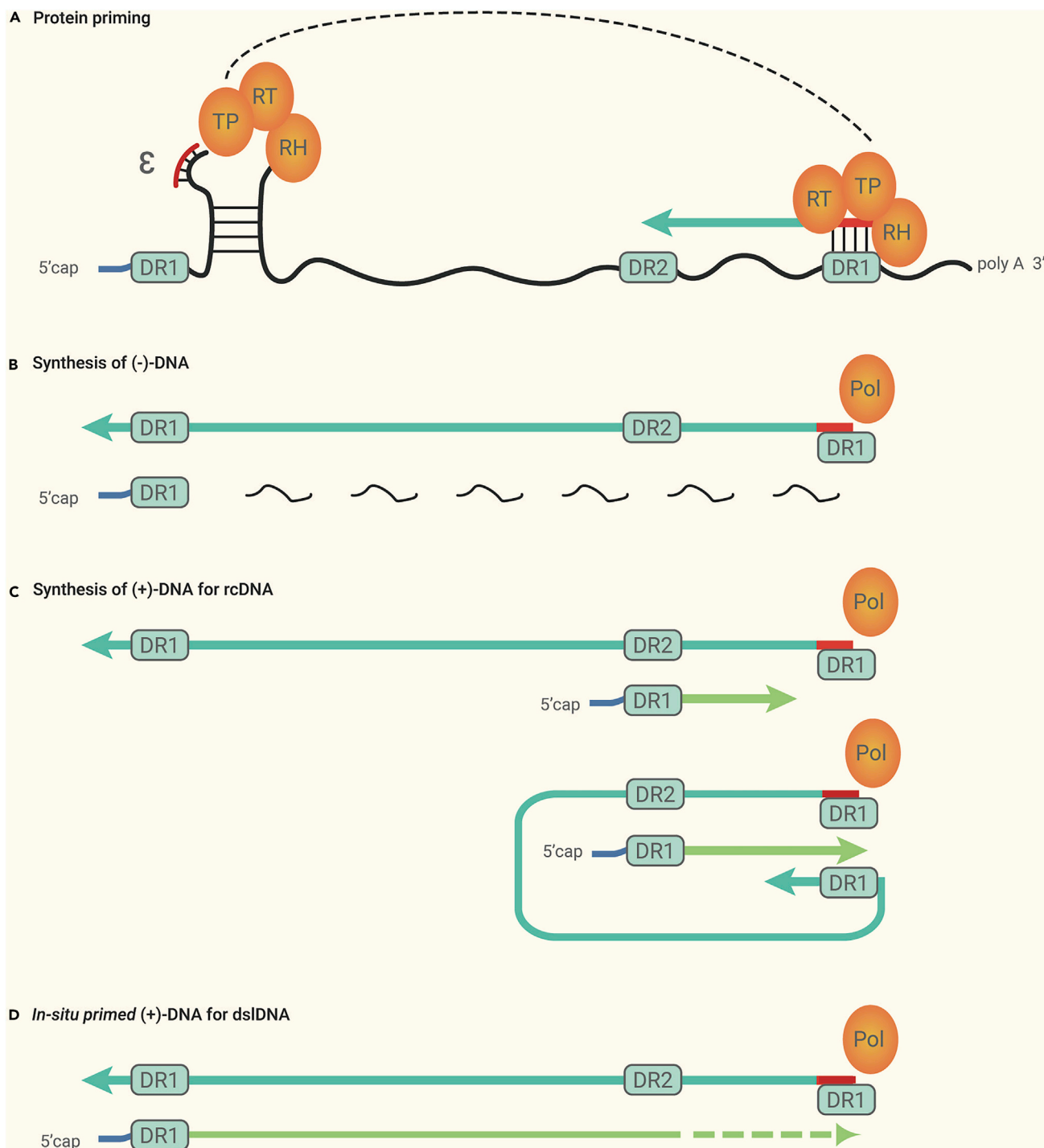
Figure 1. HBV Life Cycle. The HBV life cycle, including viral entry, cccDNA formation, transcription, replication, assembly, secretion, and dslDNA integration is shown. The red arrows indicate the routes of dslDNA integration from both the *de-novo*-infected nucleocapsids and the cytoplasm-produced nucleocapsids. The black dotted arrow indicates the formation of cccDNA-like molecules from dslDNA. See text for detailed description. ER, endoplasmic reticulum; MVB, multivesicular body.

demonstrated that the dslDNA in *de-novo*-infected nucleocapsids was the main contributor of HBV DNA integration.[20] However, some NGS studies report that a few virus-cell junctions are not correlated to the dslDNA termini, suggesting a place for other HBV DNA forms.[21,54,55] Cohesive end linear DNA is another HBV DNA form thought to occur caused by denaturation at the 5′ cohesive overlap region of HBV rcDNA and subsequent extension of the recessed 3′ ends to create terminal redundancies between direct repeat 1 (DR1) and DR2 which may play this role in integration.[18,53,56] HBV RNA in the nucleocapsids may be an additional candidate for viral integration along with HBV spliced variants, as HBV spliced variants lose the *cis*-acting signal for circular DNA and thus support dslDNA.[20,57] The integrated HBV DNA can transcribe viral mRNAs or viral-cellular fusion mRNAs, which is discussed below.

Molecular Mechanisms of HBV DNA Integration

The dslDNA is synthesized from pgRNA like the rcDNA (Figure 2). First, a short DNA oligo templated by the sequence of the ε packaging signal internal bulge, which is phosphodiester-linked to the terminal protein (TP) domain of the Pol protein, is generated by protein priming at the 5′ ε of pgRNA mediated by Pol protein.[58,59] The DNA oligo then is transferred to proximal DR1 at the 3′ terminal of the pgRNA, which subsequently primes the synthesis of the negative strand DNA ((−)-DNA) mediated by the reverse transcriptase domain of the Pol protein. During (−)-DNA extension, most of the pgRNA is degraded by the RNase H domain of the Pol protein, except for the capped 5′ end, and the undegraded RNA oligo serves as the positive strand DNA ((+)-DNA) primer. Subsequently, the RNA primer is transferred to DR2 of (−)-DNA, which primes the synthesis of (+)-DNA and generates rcDNA. However, in 5%–20% of
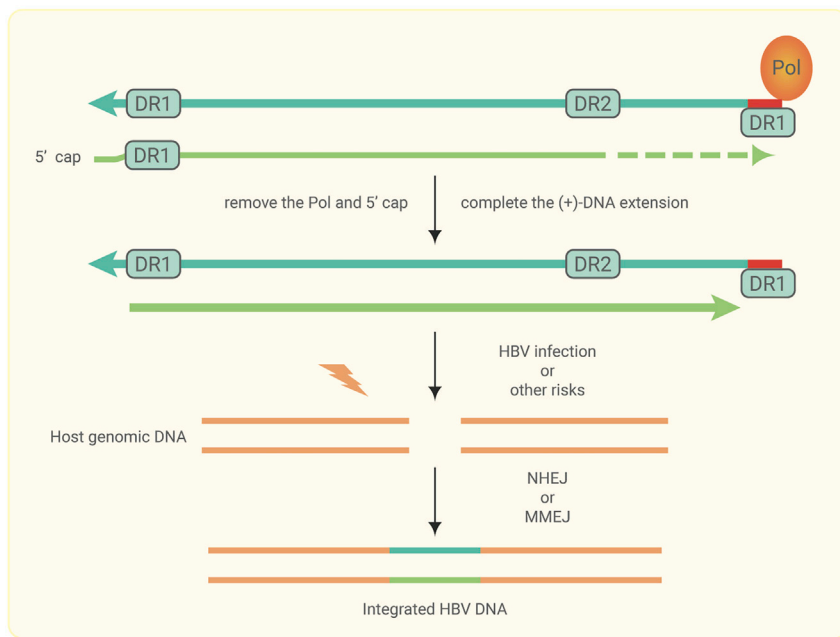
Figure 2. Synthesis of rcDNA and dslDNA. As the template, pgRNA is preformed for protein priming (A) mediated by the TP domain of Pol, and the (−)-DNA is subsequently synthesized (B). For the synthesis of rcDNA (C), the RNA primer is transferred to DR2 of (−)-DNA and primes the synthesis of (+)-DNA. For the synthesis of dslDNA (D), the RNA primer performs the direct (*in situ*) extension yielding (+)-DNA. See text for detailed description.

cases, the RNA primer performs the direct (*in situ*) extension yielding dslDNA, also known as the *in-situ*-primed linear DNA.[5,52,60]

HBV dslDNA is ~10 nucleotides (nt) longer than the genome length (e.g., genotype A, serotype adw2, GenBank accession number X02763.1)[61,62] and contains several features (1) a capped RNA oligomer at the 5′ end of (+)-DNA, (2) Pol protein, which is covalently linked to the 5′ end of the (−)-DNA, (3) DR1 at 1,826−1,836 nt and DR2 at 1,592−1,602 nt which have identical sequences (ttcacctctgc), and (4) DR1 at the 5′ end of (−)-DNA, which has only four nucleotides (tgaa) and the nucleotide "t," which

is covalently linked to the Pol protein. Like rcDNA, the plus strand of dslDNA is incomplete. HBV integration occurs early in the viral life cycle as demonstrated in *in vitro* infection models[20,63] and appears to have the same timeline as the formation of cccDNA, indicating a similar process between the two events. Thus, the first step of HBV integration is likely to remove or repair these structures to remove the RNA oligomer and Pol protein and repair the incomplete plus strand.

The second step of HBV integration is to integrate HBV DNA into the host genome. Although some aspects of this process remain unknown, much

Figure 3. Conceptual Model for HBV DNA Integration. The dslDNA is first repaired by removing the Pol protein and the structure of the 5' cap, after which the (+)-DNA extension is completed. Host genomic DNA double-stranded breaks can result from HBV infection or other factors. Finally, the mature dslDNA is randomly integrated into the host genome by NHEJ or MMEJ.

progress has been made. As a member of the *Hepadnaviridae*, the biological characteristics of duck HBV (DHBV) are very similar to HBV. Most present knowledge of HBV is derived from DHBV models. A study using recombinant restriction enzyme-induced host genomic double-stranded breaks in an *in vitro* DHBV infection model showed that integration preferentially occurs at these sites.[56] A series of studies on HBV or DHBV reported that the integration breakpoints in the viral DNA frequently occur near the DR1 or DR2 sites (1,600–1,800 nt in HBV genome).[18,21,22,54,55,63–67] The sizes of integrated viral DNA fragments range from 28 base pairs (bp) to the full-length viral DNA sequence,[68] with deletions at the termini of up to 200 bp from the integrated viral DNA being common.[18,56,64,65]

The NHEJ DNA repair pathway is typically associated with deletions and sometimes insertions of sequences at the termini.[69] As there is almost no sequence homology between the viral DNA and the cellular DNA in the majority of virus-cell junctions,[20,55,56] NHEJ is proposed as a mechanism used for HBV DNA integration. However, a few integration junctions have short homologous sequences between the viral DNA and the cellular DNA,[20,54,70,71] suggesting that the microhomology-mediated end joining (MMEJ) (also known as non-classical or alternative NHEJ) DNA repair pathway[72] is the mechanism for these cases.

The insertion sites of HBV DNA integration occur throughout the whole host genome.[21,22,54,55,65,66,68] Earlier studies using PCR-based techniques suggested a random process with no sites of preferential integration.[73] More robust high-throughput sequencing studies have found various recurrent sites of insertion in the genes encoding human telomerase reverse transcriptase (*hTERT*),[21,74] mixed-lineage leukemia 4 (*MLL4*),[21,75] cyclin e1 (*CCNE1*),[21] extra spindle pole bodies like 1 (*ESPL1*),[76] and in structures such as telomeres and CpG islands.[54] Recurrent sites of integration are preferentially observed in tumor cells and greater enrichment of integration into specific genes or features has been detected in HCC tissue compared with non-tumor tissues.[21,22,54,77,78] A plausible suggested mechanism is that, after random integration of viral DNA occurs, those hepatocytes carrying recurrent sites of integration experience clonal expansion as they have a selective advantage during tumorigenesis.[24] Thus, in a recent study more virus-integrated hepatocytes are observed in HCC tumors when compared with matched control tissues.[55] However, recurrent sites of integration, such as the retrotransposon sequences long interspersed nuclear elements 1 and 2 have been detected in HepaRG cells, a human hepatocyte stem cell line and an *in vitro* HBV infection model, which has no clonal expansion during

HBV infection.[63] A similar study in a non-clonally expanding *in vitro* HBV infection model via NTCP-dependent uptake of HBV found no obvious genomic recurrent sites of integration, although HBV DNA integration occurred across the whole genome.[20]

A conceptual model is thus proposed to describe the process of HBV integration during HBV infection (Figure 3): (1) host chromosomal DNA double-stranded breaks are formed by HBV infection[79] or endogenous metabolism.[80] (2) The dslDNA in *de-novo*-infected nucleocapsids or in cytoplasm-produced nucleocapsids is repaired by viral proteins or host proteins. (3) The mature dslDNA is randomly integrated into the host genome. The use of the NHEJ or MMEJ DNA repair pathways depends on the sequence characteristics at the ends of the viral DNA and the host double-stranded break DNA. (4) During hepatocarcinogenesis, recurrent sites of integration are a result of positive selection and clonal expansion.

Detection Methods for HBV DNA Integration

HBV DNA integration has been studied for 40 years since the first reports in 1980.[10,11] Knowledge of HBV DNA integration has grown rapidly, largely limited by detection capabilities. From the earliest Southern blot hybridization to the current high-throughput sequencing technologies, each method has unique capabilities and limitations to further the study of HBV DNA integration.

Southern Blot Hybridization. Southern blot hybridization was first used to report the presence of HBV DNA integration both in tumor tissue and in HCC cell lines.[10,11] Total DNA extracted from the cells or tissues infected by HBV is digested with a restriction enzyme, after which the ^{32}P-labeled HBV DNA used as the hybridization probe binds to the digested DNAs, which are separated through gel electrophoresis. The autoradiograph is subsequently presented on film, from which the HBV DNA integration can be judged.[10,11,81] However, this method cannot detect cells below a detection limit of 10^3–10^5 copies of the HBV genome, thus biasing it toward hepatocytes that have undergone clonal expansion.[7] In addition, the integrated sequence information cannot be determined.

In Situ Hybridization. *In situ* hybridization can identify the location of genes in cells and has been used to investigate the chromosomal integration sites of HBV DNA.[82] Cellular DNA fragments are isolated from the DNA of the integrant clones and then radiolabeled with [^{3}H]dTTP for use as a probe for *in situ* hybridization. The target metaphase chromosomes are then extracted and hybridization is performed. Like Southern blot hybridization, the

sensitivity of this technique is low, but improved *in situ* hybridization techniques with higher sensitivity, such as fluorescence *in situ* hybridization (FISH), have been used for the detection of HBV DNA integration in the chromosomes.[83] However, FISH faces drawbacks, such as a high off-target rate resulting in a high noise-to-signal ratio thus necessitating high specificity and careful design of the probe.

Cloning and Nucleotide Sequencing. Cloning and nucleotide sequencing was the earliest technique used to identify the sequences of integrated HBV DNA and the adjacent cellular DNA.[84–86] Although the detailed integrated sequence information can be identified, this method is laborious and inefficient. A cellular genomic library must be constructed through a specific restriction enzyme digest and then screened with the HBV probe. The positive clones are subsequently sequenced.[84] During this process, many integrated sequences may be lost due to the choice of restriction enzyme and the amplification of the genomic library. This method is not suitable to screen a large number of unknown virus-cell junctions.

PCR is a molecular biology technique used to amplify specific DNA fragments. PCR can greatly increase the amount of target DNA making it a suitable method to detect HBV integration from samples that are always obtained in small amounts. Common PCR strategies used for HBV integration are *Alu*-PCR and inverse-nested PCR (invPCR).

Alu-PCR. *Alu*-PCR is a "DNA fingerprinting" technique based on using *Alu* elements as target loci, *Alu*-repeated sequences being one of the most abundant repetitive elements interspersed in the human genome at a mean interval of about 4 kb and accounting for more than 10% of the human genome.[13,87–90] *Alu* elements are frequently mapped within or adjacent to HBV integration fragments.[84] The primers specific for HBV DNA and *Alu*-repetitive sequences are used to amplify virus-cell DNA junctions[13] and many HBV DNA integrations in patients with HBV infection have been detected.[91–93] *Alu*-PCR is a very sensitive method for the detection of HBV DNA integration. However, this method can only effectively detect the integration near *Alu* sequences, thus losing other integration information.

invPCR. invPCR is used to amplify the unknown cellular DNA adjacent to an integrated sequence. Restriction enzymes are used to digest DNA, after which circularization of cleavage products through self-ligation and amplification using specific outward facing primers based on the HBV sequence is done.[90,94] Normally, a restriction enzyme that has only one cutting site in the HBV sequence is chosen. In this situation, the left, right, or both ends of the virus-cell junction can be detected because the cleavage site in the genomic DNA can be either upstream or downstream of the integrated HBV DNA or both.[65,66,95,96] Nevertheless, in one study two restriction enzymes were used to specially detect the right end of the virus-cell junction.[20] This method is highly sensitive; some studies suggest that single copy virus-cell junctions can be detected.[20,97] However, the use of restriction enzymes may limit the detection of some integration junctions due to the deficiency of the corresponding cutting sites upstream and downstream of the virus-cell DNA junctions.

Some uncommon PCR technologies also used to detect HBV DNA integration are fluorescence *in situ* PCR[15] and adaptor-ligation/suppression-PCR.[75]

High-Throughput Sequencing Technology. High-throughput sequencing technology, also known as NGS technology can sequence millions of reads in a single run and does not require previous knowledge of the target sequences.[98] Depending on the strategy and target, NGS can be used for whole-genome sequencing (WGS), whole-exome sequencing, RNA sequencing (RNA-seq), single-cell sequencing, and captured/enriched NGS among others. Many of these technologies have been used for the detection of HBV DNA integration.[16,21,22,54,55,68,70,99–102] WGS and RNA-seq were first used to detect HBV integration in HCC patients in 2012[16,21,55] and are both sensitive and comprehensive at the identification and quantification of viral integrants across the human genome and transcriptome. RNA-seq allows for analysis of differential gene expression and can be more sensitive in reporting sub-clonal HBV integrations than WGS due to a greater sequencing coverage, especially if the sub-clonal genomic integration greatly alters the produced transcript.[103] However, those integrations resulting in no RNA transcription cannot be detected by RNA-seq.

WGS is a powerful method to detect HBV integration in HCC genomes limited by the high cost of performing population-scale studies. Thus, high-throughput viral integration detection (HIVID), a sensitive low cost method, was created[99] and has been used widely.[22,54,68,100,101] HIVID uses a set of HBV probes to enrich HBV sequence fragments, which are then sequenced using a high-throughput platform. Compared with WGS, HIVID is cost-effective while still providing high specificity and sensitivity to detect viral integration throughout the human genome.[99]

Research Models for HBV DNA Integration

Currently, most knowledge about HBV DNA integration is acquired from liver tissues of patients infected with HBV[65,96,104] or non-primate animal models of other hepadnaviruses, such as woodchuck infected with woodchuck hepatitis virus and duck infected with DHBV.[18,64,65] Studies of HBV DNA integration in HBV models have been stymied due to the lack of models for HBV infection. With the discovery of the HBV receptor NTCP, a series of HBV *in vitro* infection models have been established,[105] which is of use in the study of HBV DNA integration. HBV DNA integration occurs in the early stages of infection,[20,63] so here we introduce models that support HBV infection.

HBV *in vitro* infection models consists of primary human hepatocytes, HepaRG/HepaRG-human NTCP (hNTCP) cells, and hNTCP-expressing hepatoma cells (such as HepG2-hNTCP cells and Huh7-hNTCP cells). Studies using these models show that HBV DNA is integrated quickly after infection with a variety of host sequences which often consist of tandemly repeating non-coding DNAs; the HBV genotypes and various cell lines may affect the detection of HBV DNA integration.[20,63]

HBV *in vivo* infection models consists of chimpanzee, macaque expressing hNTCP, *Tupaia* and human liver chimeric mice.[105] Chimpanzees remain the only immunocompetent non-human hosts fully susceptible to infection with HBV.[106] In the livers of chimpanzees with chronic HBV infection, clonally expanded hepatocytes carried integrated viral DNA.[66] Human liver chimeric mice harbor primary human hepatocytes incorporated into the murine liver parenchyma and can support HBV infection for extended periods of time.[19,105] Despite HBV DNA integration was detected in this model, integrated viral DNA was not observed to contribute to antigen production.[19] Macaques expressing hNTCP are a non-human primate that can support HBV infection,[107] while *Tupaia* are the only non-primate animals experimentally susceptible to HBV infection.[108,109] Although infection of adult *Tupaia* results in acute self-limited infection, neonate *Tupaia* can be infected with HBV for chronic infection of the liver with moderate levels of viremia and histopathological changes, such as mild fibrosis and HCC development.[108,109] The *Tupaia* model may be a suitable animal model for studying the relationship between HBV DNA integration and HCC, but at the time of writing the literature is silent on HBV DNA integration using either macaque expressing hNTCP or *Tupaia*.

The Roles of HBV DNA Integration in Disease

HBV DNA integration is a common phenomenon during the HBV life cycle and has been widely reported, from cell lines infected with HBV,[20,63] chimpanzees chronically infected with HBV,[8,66] and patients chronically infected with HBV with and without HCC.[54,65] HBV DNA integration is an early event in HBV infection,[20,63] which can run through all stages of chronic HBV infection. Chronic HBV infection can cause a series of liver diseases, including chronic hepatitis, cirrhosis, and HCC, with HBV DNA integration playing a role in the progression of these diseases.

HBV-related liver diseases are mainly driven by sustained inflammation[110] modulated by immune cells. As an important general phenomenon in liver diseases, HBV DNA integration appears to have some relationship with the immune response in the liver microenvironment. Chronic inflammation associated with HBV infection can increase the exposure of hepatocytes to oxidative stress, leading to greater levels of double-stranded DNA breaks which form the substrate for HBV DNA integration.[56,111] It has been shown that HBV DNA integration is increased under oxidative stress.[79] Furthermore, the immune system in the liver microenvironment may facilitate HBV DNA integration through clonal hepatocyte expansion. HBV-specific T cells, HBV

DNA integration, and clonal hepatocyte expansion has been detected in chronic hepatitis B patients considered immune tolerant.[96] HBV-specific T cells selectively kill hepatocytes undergoing HBV virus replication, leading to clonal expansion of HBV DNA-integrated hepatocytes as they bypass the HBV-specific T cell immune response. Meanwhile, HBV DNA integration also affects the immune response and leads to inflammation in the liver microenvironment. High levels of HBsAg can lead to T cell exhaustion, resulting in limited or weak T cell responses.[112,113] Integrated HBV DNA has been reported as another source of HBsAg apart from cccDNA[8] and may serve as a persistent factor impairing the HBV-specific T cell response. In addition, hepatic CD205⁺ macrophages from HBsAg-transgenic mice exhibited an activated phenotype and expressed higher levels of inflammatory cytokine, chemokine, and phagocytosis-related genes,[110] suggesting that HBsAg produced from integrated HBV DNA may induce inflammation through the immune response in the liver microenvironment. The relationship between HBV DNA integration and the immune system in the liver microenvironment is complex and lacks direct evidence, thus perhaps serving as an important future direction of study.

HBV DNA integration is more common in HBV-related HCC than in the non-tumor tissue,[21,67] and is associated with hepatocarcinogenesis via multiple mechanisms, including insertional mutagenesis acting in *cis* of HCC-associated genes, induction of chromosomal instability, and the expression of mutant HBV genes from the stably integrated HBV DNA.[21–24]

Insertional Mutagenesis. Insertional mutagenesis caused by HBV DNA integration is common as the insertion sites of HBV DNA integration occur throughout the whole host genome.[21,22,54,55,65,66,68] In HCC patients with HBV infection, many recurrent integration sites that may cause mutation of HCC-associated genes have been identified. The first gene that was reported to be a recurrent HBV integration site was *hTERT* in two HCC tumor samples.[114,115] The telomerase reverse transcriptase encoded by *hTERT* plays an important role in overriding cellular senescence and its dysregulation in somatic cells is linked to carcinogenesis.[116] The activation of telomerase and its role in maintenance of telomere length are key events to drive hepatocarcinogenesis.[117–119] A recent study reported that the E74-like ETS transcription factor 4 (ELF4), which normally drives HBV gene transcription, can bind to the chimeric HBV enhancer I at the *TERT* promoter, resulting in the activation of telomerase.[74] A histone methyltransferase encoded by *MLL4* plays an important role in epigenetics and gene expression in cancer cells[75] and is a part of the ASC-2 complex implicated in the p53 tumor suppressor pathway.[120] Other members of the *MLL* family are also frequently mutated in solid tumors.[121,122] Viral integration within the *MLL4* gene results in a >20-fold increase in the transcript level of *MLL4*.[55] As the key cell-cycle regulators in eukaryotic cells, Cyclins are the major targets for oncogenic signals. Cyclin E1 is encoded by *CCNE1* and is required for the cell-cycle G1/S phase transition. The expression of *CCNE1* was increased by approximately 30-fold in tumors with HBV integration compared with normal controls.[21] HBV integration at *CCNE1* suggests a mechanism of dysregulation of the G1/S phase transition leading to HCC;[123] however, a study of overexpressed *CCNE1* in transgenic mice found chromosomal instability in addition to dysregulated cell-cycle control through accelerated S phase entry in *CCNE1* overexpressed mice liver tissues.[124] HBV integration in the ATPase sarcoplasmic/endoplasmic reticulum Ca²⁺ transporting 1 (*SERCA1*) gene in HCC tissue was reported to result in an HBx/SERCA1 fusion protein, which drove oncogenesis by inducing apoptosis.[119]

Chromosomal Instability. Chromosomal instability, a hallmark of cancer cells, is another possible consequence of HBV DNA integration and may contribute to hepatocarcinogenesis.[125–129] It has been hypothesized that viral DNA integration may cause chromosomal instability, which is recognized as one of the factors leading to tumorigenesis.[130] This hypothesis was subsequently tested by a WGS study, in which the somatic copy number variations (CNVs) near the 344 HBV integration sites in tumors were examined. The results indicated that somatic CNVs were positively correlated with the number of HBV integration reads, suggesting that HBV integration might impair chromosomal stability and cause the copy number to change.[21] A similar study reported the same result.[55]

Telomeres are repetitive DNA regions at the ends of chromosomes and maintain chromosomal stability by protecting them from degradation and fusion events. HBV integration sites are markedly enriched in the vicinity of telomeres in HCC compared with matched control tissue,[54] suggesting a mechanism involving chromosomal stability. Furthermore, as discussed above, HBV integrations into *hTERT* and long non-coding RNAs involved in telomere maintenance and chromosome localization have been identified.[131,132]

Mutant HBV Genes. Mutant HBV genes are also the products of HBV DNA integration and can stably express mutant HBV proteins, which may promote hepatocarcinogenesis. The sequence of dslDNA determines that the integrated HBV DNA can at most transcribe one intact 2.4/2.1-kb HBV mRNA (coding HBsAg), with integrated HBV DNA reported as another source of HBsAg apart from cccDNA.[8,67] Because deletions at the termini of up to 200 bp from the integrated viral DNA are common,[18,56,64,65] truncated HBsAg can also be produced. Intact HBsAg and some truncated HBsAg are associated with endoplasmic reticulum stress responses, thereby increasing the risk of HCC.[23,133,134] Moreover, the HBsAg derived from integrated HBV DNA can participate in the assembly and release of hepatitis delta virus (HDV), a satellite virus of HBV that requires HBsAg for production of new virions[135] *in vitro*.[136] Chronic co-infection of HBV and HDV in patients often causes severe necroinflammation and is associated with a higher risk of cirrhosis and HCC development compared with patients with only HBV infection.[137–139]

Furthermore, as enhancer I is recognized to be active in the integrated form, the incomplete HBx open reading frame (ORF) can be transcribed to produce truncated HBx,[140,141] which is functional in transcriptional transactivation.[142] Overexpression of the truncated HBx can induce stem cell-like properties,[143] transformation and inhibition of apoptosis,[144,145] and tumor invasion.[146,147] Meanwhile, HBx cellular transcripts can be produced due to the lack of a stop codon in the 3′ end of the HBx ORF.[70,75,148,149]

HBV DNA transcription and stability are further regulated by epigenetic effects, along with both viral and host factors.[150–152] Thus, integrated HBV DNA in the host genome may also recruit viral or host factors which perform epigenetic modifications, affecting either integrated HBV DNA expression or the host genome. Indeed, as described above, E74-like ELF4 can bind to the chimeric HBV enhancer I at the *TERT* promoter, resulting in telomerase activation.[74] Clarification of the influence of integrated HBV DNA regulated by transcription factors or epigenetic modifications may be an important issue.

Despite the hepatocarcinogenic effects of HBV DNA integration, in some cases it can be utilized for HCC treatment. A study which aimed to detect and use the HBV-host-integrated DNAs in plasma samples as potential circulating biomarkers to detect the tumor load in HBV-related HCC patients may help to monitor the residual tumor and recurrence clonality after tumor resection.[101]

The current goal of HBV antiviral therapy is to achieve a "functional cure" consisting of persistently undetectable HBV DNA in serum, loss of HBsAg, preferably with seroconversion (from HBsAg to antibody to HBsAg, anti-HBs), and normal liver enzymes and histology after stopping treatment.[39,153] Persistently undetectable HBV DNA in serum can commonly be achieved through current antiviral therapies (using nucleos(t)ide analogs or PegIFNα), but the loss of HBsAg is rare,[154] perhaps because current treatments cannot clear cccDNA, the main source of HBsAg. Furthermore, integrated HBV DNA can express HBsAg, making the loss of HBsAg more difficult.[8,154,155] In this way, HBV DNA integration will seriously hinder the achievement of a functional cure.

Potential Therapeutic Strategies for HBV DNA Integration

Many therapeutic approaches for HBV infection have been developed and used to cure HBV infection.[39,156,157] Currently, there are no treatment strategies targeting the integrated HBV DNA. This may be due to the complexity of HBV DNA integration, as the HBV DNA sequence integrated host genome is variable, consists of many different integration sites, which are mostly unable to produce RNAs. Potential treatment strategies to target integrated HBV DNA may be through gene therapies or RNA interference (RNAi)-based

therapeutic targeting transcripts derived from integrated HBV DNA. In a human clinical trial with an RNAi-based therapeutic targeting HBV transcripts, ARC-520 strongly reduced HBsAg levels in treatment-naïve patients positive for HBeAg but the reduction in HBsAg levels was significantly less in patients who were HBeAg-negative, indicating that ARC-520 could not target HBsAg mRNA transcribed from integrated HBV DNA.[8,158] Improved RNAi therapies that can target viral transcripts regardless of origin could be a promising treatment strategy.

Gene therapies mainly depend on gene-editing tools, such as CRISPR-Cas9, zinc-finger nucleases, and transcription activator-like effector nucleases, which have been used to target HBV cccDNA.[36,157,159–164] Gene therapy is still in its early stages of exploration and has many issues to be solved before clinical application is viable. The most basic requirement of gene editing in clinical use is to ensure both accuracy and safety, with off-target effects being troubling. The sequence and site diversity of HBV DNA integration will be problematic in the application of gene therapies. It should be noted that most of the impact of HBV DNA integration comes from the destruction of the continuity of the host genome by insertion, and it is difficult for current gene-editing techniques to remove the inserted HBV DNA sequence. Overall, the use of gene therapies to target integrated HBV DNA is still in its early stages.

Possible Future Research Prospects of HBV DNA Integration

HBV cccDNA is the primary reason that HBV continues to lack a functional cure. Therefore, many studies are devoted to removing or inactivating cccDNA. However, HBV DNA integration also plays an important role in HBV-related diseases, especially HCC. Although research on HBV DNA integration has made great strides, problems remain.

The Role of HBV Itself in HBV DNA Integration. HBV DNA integration occurs early in the viral life cycle[20,63] and integration sites are at double-stranded breaks of the host genome.[56] Can HBV infection cause double-stranded breaks in the host genome? The role, if any, that viral protein plays in the integration process needs to be clarified.

Generation of Mature HBV DNA Integrated Fragment dslDNA. Similar to cccDNA, HBV dslDNA contains several features: (1) a capped RNA oligomer at the 5' end of (+)-DNA , (2) Pol protein, which is covalently linked to the 5' end of the (−)-DNA , and (3) incomplete plus-strand dslDNA. Does HBV dslDNA need to be repaired before integration? Is the mechanism similar to the repair mechanism of HBV rcDNA? What virus or host proteins are involved in this process?

The Integration Mechanisms of HBV DNA. The NHEJ or MMEJ DNA repair pathways are used to perform HBV DNA integration. Why are these two cellular processes hijacked? What is the mechanism of integration, and are there other ways of integration? Moreover, a determination of whether it is random integration into the host genome needs to be investigated.

Appropriate Animal Research Models. The role of HBV DNA integration in HBV-related liver diseases is complex. Appropriate animal models that can simulate the pathogenesis of HBV will help us understand more clearly the role of HBV DNA integration in disease and assist related drug development.

The Relationship between HBV DNA Integration and the Immune Response in the Liver Microenvironment. This relationship is closely related to the occurrence and development of HBV-related diseases. Understanding this will prove useful for the monitoring and treatment of the disease.

Mapping HBV DNA Integration in the Host Genome. This map should include HBV DNA integration sites, integration sequences, transcripts, protein expression, and related pathological changes. This will enable the development of the full picture that HBV DNA integration plays in the occurrence of disease and provide more precise targets for targeted gene therapies. Furthermore, this will support the location of HBV DNA integration sites that cause disease through analysis of HBV DNA integration in individual patients, allowing for precision medicine.

Summary

During the past four decades, great strides have been made in the HBV DNA integration field, although some key aspects remain muddled, such as the molecular mechanism of integration and the role of HBV integration in HCC initiation and development. It remains hopeful that continual progress in the development of detection methods and research models, such as NTCP-expressing hepatoma cell lines and animals will result in a comprehensive and detailed understanding of HBV DNA integration. As a part of the cellular genome, integrated HBV DNA is more stable than cccDNA. While many direct-acting antiviral and host-targeting agents aimed at cccDNA destruction or silencing have been developed, they do not address the problem of integrated HBV DNA. It should be recognized that, in addition to cccDNA, integrated HBV DNA plays an important role in HBV-related diseases, especially HCC. HBV DNA integration should be used as a clinical indicator for disease monitoring and treatment of patients with HBV infection. Even if cccDNA can be cleared in the future, a functional cure may not be feasible because of the fundamental challenge integrated HBV DNA will continue to pose. Thus, the exact role of integrated HBV DNA in disease remains a question of interest in the development of curative therapeutics and to gain a clearer picture of HBV biology.

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DECLARATION OF INTERESTS

The authors declare no competing interests.