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Molecular Mechanisms Underlying Occult Hepatitis B Virus Infection

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INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a major global problem despite the availability of an efficacious vaccine. In chronic HBV infection, liver cirrhosis and hepatocellular carcinoma (HCC) are associated with considerable morbidity and mortality. The detection of hepatitis B virus surface antigen (HBsAg) in serum remains the mainstay in the diagnosis of chronic HBV infection and screening for HBV in most developing countries. The majority of individuals positive for HBsAg are also positive for HBV DNA in the serum. Occult HBV infection is characterized by the presence of HBV DNA in the absence of detectable HBsAg. Occult HBV infection is a complex clinical entity documented worldwide. Significant advances in understanding the pathogenesis of occult HBV infection have been reported in the last decade. This review is aimed at providing a detailed account of the molecular mechanisms leading to occult HBV infection.

HBV VIROLOGY

HBV contains a 3.2-kb partially double-stranded DNA genome with 4 open reading frames encoding 7 proteins. The presence of partially overlapping open reading frames (151) and the absence of noncoding regions (134) allow for compact organization of the HBV genome. The biological functions of HBV proteins and their role in the pathogenesis of HBV infection are summarized in Table 1.

Replication begins with the attachment of mature virions to the host cell membrane to enter the cell. The pre-S proteins mediate the entry of HBV into hepatocytes (200). The HBV receptor on hepatocytes still remains elusive. Once inside the cell, the viral genome is uncoated to release relaxed circular DNA (RC-DNA). This RC-DNA is transported to the nucleus (126) and converted into covalently closed circular DNA (cccDNA) by cellular enzymes (14). The mechanism for the transport of RC-DNA is not

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doi:10.1128/CMR.00018-11

TABLE 1 HBV ORFs and proteins

ORF	Protein	Function/role in pathogenesis of HBV infection
P	Viral polymerase	DNA polymerase, reverse transcriptase, and RNase H activity; contains functional domains essential for encapsidation of the pregenomic RNA
S	HBV surface proteins	Pre-S1 protein plays a key role in attachment of the virus to the hepatocyte (200)
C	Core protein	Forms the capsid and encapsidates HBV nucleic acids
	HBeAg	Biological role is poorly understood; however, strong epidemiological associations between HBeAg and HBV DNA level (53) and the risk of progression to hepatocellular carcinoma (290) are well documented
X	HBx protein	Transactivator of viral regulatory elements and host gene promoters (60, 248), essential for establishment of infection *in vivo* (300), potential role in hepatocarcinogenesis (60)

clearly understood. The cccDNA is a stable form of the viral genome that is associated with proteins in the nucleus in the form of viral minichromosomes (201), and it also serves as a template for the production of progeny genomes. Genomic transcripts, including pregenomic RNA (pgRNA), precore RNA, and subgenomic HBV RNAs, are transcribed from HBV cccDNA by the host cell enzyme RNA polymerase II. The pgRNA serves as a template for the synthesis of HBV DNA and also as the mRNA of core protein and polymerase. The pgRNA and the HBV polymerase are first packaged into the HBV core protein. Subsequently, pgRNA is reverse transcribed to HBV DNA by the viral polymerase. Subgenomic transcripts serve as mRNAs for surface proteins (i.e., large HBsAg, middle HBsAg, and small HBsAg) and the hepatitis B virus x (HBx) protein. Nucleocapsids are packed into envelope glycoproteins in the cytoplasm and pass through the endoplasmic reticulum and the Golgi apparatus prior to secretion (167). Alternatively, the nucleocapsids can reenter the nucleus, resulting in the amplification of the nuclear cccDNA pool. HBV replication is regulated by 4 promoters, 2 enhancers, and a negative regulatory element (189). Recent studies have demonstrated the role of epigenetic regulation of HBV replication by acetylation of H3/H4 histones (215) and the methylation of HBV DNA (271, 272).

HBV INFECTION AND CLINICAL DISEASE

The incubation period for acute hepatitis B ranges from 1 to 6 months. Acute HBV infection can be either asymptomatic or symptomatic. Asymptomatic acute HBV infection associated with mild or subclinical disease often goes undiagnosed. Clinically inapparent or asymptomatic acute HBV infections are more common in children less than 4 years of age than in adults over 30 years of age (182). Clinically apparent cases have a prodromal phase with nausea, vomiting, malaise, anorexia, fever, and flu-like symptoms. The prodromal phase may be followed by an icteric phase with jaundice, clay-colored or pale stools, discomfort in the right upper quadrant, and hepatomegaly (172). Symptoms in acute HBV infection are clinically indistinguishable from those in other acute viral hepatitis infections. Several HBV markers, including HBV DNA, HBsAg, hepatitis B virus e antigen (HBeAg), and anti-hepatitis B virus core IgM (anti-HBc IgM), are detectable in the serum during acute HBV infection. Biochemical abnormalities such as increased transaminase levels and increased bilirubin levels may also be detected. Seroconversion to antibody to HBsAg (anti-HBs) indicates resolution from acute HBV infection. Fulminant hepatitis B virus infection is associated with high mortality (20, 156). Mutations in the precore region of the HBV genome have been associated with small outbreaks of fulminant hepatitis B (156).

Extrahepatic manifestations occur in up to 20% of HBV-infected individuals (30) and may involve the gastrointestinal, renal, and nervous systems (68). The mechanisms leading to extrahepatic manifestations of hepatitis B virus infection are poorly understood. Circulating immune complexes containing HBV have been demonstrated in patients with extrahepatic manifestations of chronic HBV infection (102, 213). Circulating immune complexes and complement-mediated injury are implicated in extrahepatic manifestations of HBV infection, including arthralgia, serum sickness-like syndrome, polyarteritis nodosa, and glomerulonephritis (2, 30, 163, 263).

Chronic HBV infection is defined as the persistence of HBsAg for 6 months or longer. Chronic HBV infections are more common following anicteric disease (283). The risk of developing chronic HBV infection decreases with age at infection; about 80 to 90% of neonates, 30% of children less than 6 years of age, and fewer than 5% of adults infected progress to chronicity. In addition to age at infection (182), host genetic factors such as polymorphisms in the interleukin-18 gene (47), tumor necrosis factor alpha promoter polymorphisms (136), and human leukocyte antigen-DP gene variants (100) have been linked to persistence of HBV. It is well accepted that $CD8^+$ T cells play a crucial role in the clearance of acute HBV infection (173, 257).

Chronic HBV infection may be classified into 3 phases, consisting of the immune tolerant phase, the immune active phase, and the inactive carrier phase (180). The immune tolerant phase is associated with the presence of HBsAg, HBeAg, and high levels of HBV DNA in the serum. Alanine transaminase (ALT) levels are normal, and no major pathological changes are observed in liver biopsy specimens. The immune tolerant phase may last for several years and is characterized by mild disease. The progression of chronic liver disease is very slow in the immune tolerant phase despite high HBV DNA levels (122); this phase is also referred to as the "highly replicative phase." The immune active phase is associated with elevated ALT levels, detectable HBV DNA, and inflammation of the liver with or without fibrosis. The "tolerance" to HBV is lost, and damage to the hepatocytes is mediated by the host immune responses to HBV; for this reason, the immune active phase is also referred to as the "immune clearance" phase. The immune active phase is seen in patients with or without HBeAg. The HBV DNA levels are generally higher in HBeAg-positive patients than in HBeAg-negative patients in the immune active phase. Patients with an HBeAg-negative status in the immune active phase are also classified as having "HBeAg-negative chronic hepatitis B." The loss of HBeAg may be associated with mutations in the precore region or the core promoter regions (97, 161). About 70% of chronic HBV patients seroconvert from HBeAg to antibody to HBeAg (anti-HBe) within 10 years of diagnosis. How-

TABLE 2 Factors associated with increased risk of developing HBV-related HCC

Factor	Reference(s)
Demographics	
Male sex	42, 82
Age (increased risk with advancing age)	42, 82
Race	5
Social and environmental	
Alcohol	42, 82
Smoking	42
Aflatoxin	285
HBV factors	
HBV DNA levels	41, 42
Presence of HBeAg	42
Genotype (C more than B)	289
Core promoter mutations	289, 292
Liver damage	
Cirrhosis	42, 82
Increased transaminase levels	42
Miscellaneous	
Coinfection with HCV	82, 142
Superinfection with HDV	82

TABLE 3 Role of HBx protein in hepatocarcinogenesis[a]

Mechanism	Comment	Reference(s)
Inhibition of p53	Inhibition of sequence-specific binding of p53	279
Centrosome abnormalities	Induction of multipolar spindle formation	84
Telomerase activity	Increases telomerase activity and expression of hTERT	164
Inhibition of apoptosis	Activates p38/MAPK pathway and upregulates survivin, an antiapoptotic protein	143
Jak1/STAT signaling	Activates Jak1/STAT pathway	152
TGF-β	Shifts TGF-β signaling from tumor suppression to oncogenesis	194
Defective DNA repair	Defective nucleotide excision repair	217
Hypermethylation of TSGs	Induction of DNA methyltransferases and suppression of TSGs	150, 211

[a] Abbreviations: hTERT, human telomerase reverse transcriptase; TGF-β, transforming growth factor β; MAPK, mitogen-activation protein kinase; TSG, tumor suppressor gene.

ever, reversion to HBeAg-positive status occurs in a small proportion of HBeAg-negative individuals (181). The inactive carrier phase is characterized by an HBeAg negative- and anti-HBe positive status with low HBV DNA levels (typically <2,000 IU/ml) and minimal or no fibrosis. The rates of spontaneous seroclearance of HBsAg among inactive carriers range from 0.5% per year to as high as 40% in 25 years of follow-up (55, 181). Symptoms in chronic HBV infection may range from mild nonspecific symptoms such as fatigue and right upper quadrant discomfort in patients with minimal liver damage to ascites, peripheral edema, and encephalopathy in patients with advanced liver disease.

Progression to cirrhosis is associated with multiple episodes of severe acute exacerbations, hepatic decompensation, and reversion to HBeAg-positive status from an anti-HBe positive status (158). The 5-year survival rate in chronic HBV patients with cirrhosis is less than 60% (282).

Hepatocellular carcinoma is a major global problem. In areas with a high prevalence of HBV infection such as Southeast Asia, higher rates of HCC are documented (209). The risk of developing HCC may be increased up to 100-fold in patients with chronic HBV infection (12). The roles of host, viral, and extraneous factors associated with progression to HBV-related HCC are summarized in Table 2. Hepatocellular carcinoma is a leading cause of cancer-related deaths. The morbidity and mortality associated with HBV-related HCC necessitate improved surveillance measures for early diagnosis and newer therapeutic options.

Direct and indirect mechanisms of hepatocarcinogenesis have been identified in HBV-related HCC. The inflammation associated with chronic active hepatitis is a major contributor in hepatocarcinogenesis (13). Integration of HBV DNA fragments into chromosomal DNA is detected in the majority of HBV-related HCCs (192). The precise time of HBV DNA integration remains unclear, though it may occur as early as the acute phase of HBV infection (193, 137). Integration of subgenomic fragments of HBV DNA into host chromosomal DNA was initially thought to be a random process (176). Subsequent studies have identified recurrent HBV DNA integration sites on human chromosomes, including the human telomerase reverse transcriptase (RT) gene (212). The other HBV DNA integration sites identified include cancer-related genes, tumor suppressor genes, and genes involved in key signaling pathways (192). Integration of HBV DNA could potentially modulate expression profiles of genes involved in carcinogenesis, including tumor suppressor genes and oncogenes. Furthermore, HBV DNA integration could be associated with chromosomal aberrations such as translocations, inversions, and deletions leading to chromosomal instability (176).

Interestingly, the expression of HBx protein (a protein that is not an integral part of HBV) is preferentially retained in HBV-related HCC tissue compared to the expression of HBsAg and hepatitis B virus core antigen (HBcAg) (251). The role of HBx protein in HBV-related HCC has been extensively studied and is summarized in Table 3.

It is generally accepted that HBV is not directly cytopathic and that liver injury is immune mediated. However, direct cytopathic effects of HBV have been demonstrated in individuals with an impaired immune system (184). Increased production of the large surface protein is associated with severe hepatic injury, triggering a cascade of events, including dysregulation of cellular genes and secondary genetic events leading to hepatocarcinogenesis in transgenic mice (50, 72). Defective HBV particles are associated with singly spliced HBV RNA that encodes the hepatitis B virus splice-generated protein (HBSP), a novel protein associated with a frameshift during splicing in HBV (256, 245). An increased relative abundance of defective HBV particles is associated with inflammation and fibrosis of the liver (246). HBSP modulates apoptosis and secretion of inflammatory cytokines (247), suggesting a potential role for the protein in hepatocarcinogenesis.

HBV EPIDEMIOLOGY

It is estimated that over 2 billion people have been exposed to HBV infection, of which about 350 million people remain chronically

infected (http://www.who.int/mediacenter/factsheets/fs204/en/). The prevalence of chronic HBV infection varies greatly worldwide. Regions of the world may be classified into areas of high (≥8%), intermediate (>2% and <8%), or low (≤2%) prevalence (http://www.who.int/mediacenter/factsheets/fs204/en/, 149). The majority of HBV infections in areas of high and intermediate endemicity are acquired through vertical transmission or horizontal transmission during the preschool years (149, 151). In contrast, infection in areas of low endemicity is acquired by adolescents or adults predominantly through parenteral or sexual routes (104). Percutaneous transmission among intravenous-drug abusers (IVDA) sharing contaminated needles remains the predominant mode of transmission in developed countries (151, 162). Transmission of HBV through sexual modes is well established; sexual transmission is particularly efficient among homosexual men (146) and in individuals with multiple partners (87). While intrauterine infections are infrequent (286), vertical transmission is primarily perinatal. Horizontal transmission through close nonsexual contact among family members (160) and from one child to another in the community is well documented (63). Inadequate infection control measures are associated with nosocomial transmission (3); higher rates of HBV infection have been reported in health care workers than in the general population (258). Hemodialysis patients represent a high-risk group for acquiring HBV infection (77). In addition, lower rates of response to HBV vaccination among patients with renal insufficiency (138) make this group of patients particularly susceptible to HBV infection. Transmission of HBV from surgeons to patients has also been reported (106, 249).

The correlation between the presence of HBeAg in serum and infectivity was recognized within a few years of the discovery of the virus (240). HBV DNA testing gradually replaced HBeAg as a sensitive and accurate indicator of infectivity (58). The presence of maternal serum HBeAg has been identified as a major risk factor for transplacental transmission of HBV (286). High HBV DNA levels in mothers are associated with failure of hepatitis B virus immune globulin (HBIG) and vaccination in infants (243, 280).

HBV vaccination is now a part of the national immunization programs of over 175 countries (http://www.who.int/immunization/topics/hepatitis_b/en/index.html). The mass HBV vaccination program has led to marked reduction in HBV infection and also in the incidence of HCC (35).

The classical serotyping of HBV (134) has been replaced by genotyping since the early 1990s. Hepatitis B virus genotypes A to I have been described, based on the divergence of the complete genome sequences by 8% or more. Subgenotypes within many HBV genotypes have been described (140). Genotype D is the most widespread genotype (207), while the other HBV genotypes are geographically restricted (8, 134). Infection with multiple genotypes, though infrequent, has been documented (275). An increased prevalence of infection with multiple genotypes has been reported among drug users (40).

DIAGNOSIS

Testing for hepatitis B virus surface antigenemia remains the mainstay in the diagnosis of acute hepatitis B virus infection. Anti-HBc IgM is a useful marker during the "core window," a short period in resolving acute HBV infection between the loss of serum HBsAg and the appearance of anti-HBs. HBV DNA is the earliest detectable marker in acute HBV infection. HBV DNA testing is particularly useful in the detection of the early phase of acute HBV infection prior to the appearance of serum HBsAg; for this reason HBV DNA is tested using nucleic acid amplification technology (NAT) in blood and blood products in resource-rich countries (66, 118). The appearance of anti-HBe followed by the appearance of anti-HBs is characteristic of acute resolving HBV infection (172). The anti-HBs response remains detectable for several years following recovery from acute HBV infection, and it indicates protective immunity. Anti-HBc IgG persists for several decades, if not for life, following acute HBV infection. In areas of low HBV endemicity, anti-HBc screening of blood and blood products in addition to HBsAg testing is performed to identify past exposure to HBV (118).

Chronic HBV infection is defined by the persistence of serum HBsAg for more than 6 months. The presence of HBeAg correlates strongly with HBV DNA levels and an aggressive course of liver disease in chronic HBV infection (105). The loss of HBeAg in chronic HBV carriers generally represents a late phase in the course of chronic HBV infection (89). Low levels of anti-HBc IgM are frequently detected in patients with chronic HBV infection, albeit without any diagnostic significance (244). Estimation of HBV DNA levels is one of the most useful markers in the management of chronic hepatitis B. Estimation of HBV DNA and ALT levels in serum, the presence of HBeAg, and the assessment of liver histology help in the evaluation and identification of patients requiring antiviral treatment for chronic hepatitis B (268). Loss of HBeAg, ALT levels within the upper limit of normal, and reduction in HBV DNA levels are indicators of response to antiviral therapy. In addition, periodic assessment of HBV DNA levels is performed during antiviral treatment to determine therapeutic endpoints and identify the emergence of both drug resistance and primary/secondary treatment failure. Highly sensitive nucleic acid amplification-based methods are commercially available for estimation of HBV DNA levels (274). With the availability of a variety of therapeutic options for chronic hepatitis B, testing for antiviral resistance is being increasingly used. Genotypic (159), phenotypic, and virtual phenotyping methods are used for antiviral resistance testing (274). Clinical differences among HBV genotypes are gaining relevance (294, 289). However, HBV genotyping is not a part of the routine management of chronic hepatitis B.

REACTIVATION OF HBV

Resolution of acute HBV infection is marked by the appearance of anti-HBe in the serum followed by the appearance of anti-HBs. A pool of HBV cccDNA persists in hepatocytes after resolution of infection (153, 221). Reactivation of HBV is characterized by a sudden rise in HBV DNA and serum transaminase levels in patients with past exposure to HBV infection (117). Reactivation of HBV is frequently associated with immunosuppressive therapy (18), cancer chemotherapy (123), sudden withdrawal of antiviral therapy (70), and progressive immunodeficiency in human immunodeficiency virus (HIV) infection (22). In addition, reactivation of HBV may be spontaneous (117). Hepatitis B reactivation following liver transplantation is linked to past HBV exposure of the organ donor (223). The reappearance of HBV DNA in liver transplant recipients with past exposure to HBV is best described as reinfection. Clinical manifestations of HBV reactivation are variable and may include liver dysfunction (168). The rate of HBV reactivation varies greatly across different clinical groups (18, 174, 291). Reactivation of HBV occurs in individuals who are HBsAg

TABLE 4 Factors influencing estimated prevalence of occult HBV infection

Factor	Comment	Reference(s)
Population studied		
Risk group	Prevalence rates vary between high-risk groups and low-risk groups	29, 259
Sampling		
No. of samples	Occult HBV DNA may be intermittently detected in serum; serial sampling is ideal	113
	HBV DNA may be detected in liver but not in serum	108, 238
Testing		
HBsAg testing	Some commercial assays are better suited for detection of HBsAg mutants	147
HBV DNA testing	Targeting multiple regions of the HBV genome improves detection; differences between amplification methods for HBV DNA testing exist	108, 260
Geographical region		
HBV prevalence	Endemicity of HBV infection correlates with prevalence of occult HBV infection	166
Coinfections	HCV/HIV coinfections are associated with higher rates of occult HBV infection	29, 170

positive (168) and also in HBsAg-negative individuals with occult HBV infection (107). Among HBsAg-positive patients on chemotherapy for hematological malignancies, the prophylactic use of lamivudine in preventing reactivation of HBV infection is well recognized (227). Reactivation of occult HBV infection leading to clinical hepatitis may occur in HBsAg-negative patients undergoing chemotherapy (123); however, the need for early identification of occult HBV infection in this group of patients is not widely appreciated. The role for anti-HBV prophylaxis in occult HBV patients undergoing chemotherapy needs further investigation. Emergence of drug resistance following prophylactic antiviral treatment among occult HBV-infected chemotherapy recipients is another potential problem.

HBV DNA contains a glucocorticoid response element (264), and patients receiving corticosteroids had a demonstrable increase in HBV DNA levels (145). The use of glucocorticoids in chemotherapeutic regimens is linked to increased risk of HBV reactivation (46).

Reactivation of HBV in HIV-infected patients with serological markers of past HBV infection is well documented (22, 103). Occult HBV infection is reported in 0.6% of HIV-infected patients with isolated anti-HBc reactivity (197). Reactivation of both overt HBV infection (70) and occult HBV infection (4, 22) has been reported in HIV-infected patients, especially after cessation or interruption of antiretroviral therapy. Recurrent monitoring of HBV DNA levels in HIV-infected patients with markers of past HBV infection may assist in early diagnosis and better management of HBV reactivation in this group of patients.

OCCULT HBV INFECTION

The loss of HBsAg or the presence of detectable anti-HBs indicates resolution from acute HBV infection. Both HBsAg and HBV DNA are detected in the majority of chronically infected patients (276). However, a small proportion of individuals have detectable HBV DNA in the serum and/or the liver in the absence of circulating HBsAg (218, 219, 259). Occult hepatitis B virus infection is defined as the presence of HBV DNA in the liver (with or without detectable HBV DNA in the serum) in HBsAg-negative individuals (219, 259). Seropositive occult hepatitis B virus infection is characterized by the presence of anti-HBc and/or anti-HBs, while neither anti-HBc nor anti-HBs is detected in seronegative occult hepatitis B virus infection (219). HBV antibodies, including anti-HBc, anti-HBs, and anti-HBe, are frequently detected in occult HBV infection (259). In many instances, individuals with occult HBV infection lack hepatitis B virus "e" antigenemia and have virus loads of $<10^3$ copies/ml (259, 295), though exceptions have been reported (26). HBV DNA levels can vary considerably in occult HBV infection among individuals positive for anti-HBs (9, 111). Occult HBV infection was not recognized as a clinical entity until the early 1990s (229, 284). With the extensive use of sensitive molecular techniques for HBV DNA detection there have been an increasing number of studies in the last decade investigating various aspects of occult HBV infection.

Prevalence

Occult HBV infection has been reported from several parts of the world, including areas of low HBV endemicity (187). The prevalence of occult HBV infection varies greatly across the globe, with higher rates being reported from Asia than from the rest of the world (259). Despite high HBV endemicity, a very low prevalence of occult HBV infection among low-risk populations such as blood donors has been confirmed by various groups in Asia and Africa (15, 74, 296, 299). Prevalence rates for occult HBV infection ranging from less than 1% to as high as 87% have been reported from different parts of the world (132, 266, 296). However, these prevalence rates need to be interpreted with caution. Several factors that could potentially influence estimated rates of occult HBV infection are summarized in Table 4. Unfortunately, multicenter studies using identical methods of recruitment of subjects, sampling, and testing methods are lacking. The geographical distribution of occult HBV infection worldwide warrants further investigation. However, it is well known that certain groups of patients are at a much higher risk of having occult HBV infection regardless of the geographical location. The groups with high risk for occult HBV infection are summarized in Table 5.

Clinical Relevance

Apart from posing diagnostic challenges, occult HBV infection may often be associated with a variety of clinical conditions. The role of occult HBV infection in chronic hepatitis C virus (HCV) infection is perhaps the most extensively studied. Cacciola et al. (29) not only found higher rates of occult HBV infection among patients with HCV-related chronic liver disease but also found liver cirrhosis more frequently among chronic HCV patients with

TABLE 5 High-risk groups for occult HBV infection

Patient group	Reported prevalence (%)[a]	Reference(s)
Chronic HCV infection	15–33	29, 128
HIV infection	10–45	103, 169
Injection drug users	45	260
Hemodialysis	27	69
Hepatocellular carcinoma	62	108
Cryptogenic liver cirrhosis	32	34
Liver transplant	64	93

[a] Rounded to the nearest integer.

occult HBV infection (33%) than among monoinfected patients (19%). In another study, transient lower response rates during interferon (IFN) therapy were seen in anti-HBc-positive chronically HCV-infected patients. Only a fraction of the anti-HBc-positive individuals had demonstrable HBV DNA (65). Other studies have failed to demonstrate an association between occult HBV infection and lower rates of response to anti-HCV therapy in coinfected patients (44, 241). A functional assessment of IFN response was done by Fukuda et al. (86) by estimation of intrahepatic mRNA expression of the type I IFN receptor gene. Chronic hepatitis C patients with occult HBV infection had lower expression of the IFN receptor gene and a poorer response to IFN than monoinfected patients. It still remains unclear how occult HBV infection affects the treatment of chronic HCV infection (76, 91, 128, 133, 191, 241).

Occult HBV infection has been associated with liver enzyme flares during chronic HCV infection (235) without changes in HCV RNA levels (127). Occult HBV infection in chronically HCV-infected patients has been associated with higher histological activity and advanced fibrosis (191), while other studies failed to demonstrate this association (76, 132). Differences in geographical region, infecting HBV and HCV genotypes, and environmental cofactors could partially explain these contrasting findings.

Occult HBV infection is frequently detected in cryptogenic chronic liver diseases, including chronic hepatitis and cirrhosis (17, 38, 116). In individuals with chronic hepatitis, the presence of HBV proteins and HBV DNA has been confirmed by immunostaining and *in situ* hybridization (38). The HBV DNA level in individuals with cryptogenic liver disease is generally less than 10^4 copies/ml (38). While the causal role of occult HBV infection in cryptogenic liver disease and chronic hepatitis is still debated (37, 116, 144), the usefulness of monitoring liver enzymes and HBV DNA levels in the management of occult HBV infection has been demonstrated (39). However, specific guidelines for the management of occult HBV-related liver diseases are yet to evolve.

Sequence analysis identified mutations in the surface gene, core gene, and polymerase gene of occult HBV genomes from patients with chronic liver diseases. In addition to sequence changes, differences in the ratios of the large and small surface proteins were also attributed to the loss of HBsAg in serum (37). Another study identified mutations in the X gene which reduced viral replication in occult HBV cases with chronic hepatitis (228). Occult HBV infections have also been reported in patients with nonalcoholic steatohepatitis (NASH) (17) and autoimmune hepatitis (116). Despite detection of HBV DNA and HBV proteins in patients with chronic liver diseases of unknown etiology, the causal role of occult HBV infection and the underlying pathogenic mechanisms remain elusive.

Occult HBV infections are detected in as many as 73% of HCV-related HCC patients (188, 250) and at a lower frequency (18%) in non-B non-C HCC (NBNC HCC) (144). The occult HBV sequences from NBNC HCC lack core promoter mutations that are frequently detected in HBsAg-positive HCC. In addition, diabetes and obesity are frequently detected in the NBNC HCC group compared to the HBsAg-positive HCC group. The authors argue that a higher incidence of nonalcoholic steatohepatitis in the NBNC HCC group weakens the causal association of occult HBV infection in the development of HCC (144). The presence of core promoter mutations in occult HBV genomes in HCV-related HCC argues for a contributory role of occult HBV infections in the pathogenesis of HCC (188). The detection of transcriptionally active and replication-competent episomal HBV in addition to integrated HBV sequences in occult HBV infection-related HCC further strengthens the causative role of occult HBV infection in HCC (214).

The risk of acquiring posttransfusion HBV infection is low with implementation of improved screening procedures. Occult HBV infections and to a lesser extent window-period infections contribute to the risk of transfusion-transmitted HBV infection (296, 299). The increased prevalence of occult HBV infection in high-risk groups was discussed above and is summarized in Table 5. Increased rates of occult HBV infection within the high-risk groups do not necessarily imply transmissibility of occult HBV. Nonetheless, it may not be speculative to anticipate transmission of HBV within these groups, considering the risk of multiple exposures in individuals within the high-risk groups. However, large-scale studies confirming transmission rates in high-risk groups from index cases with occult HBV infection are lacking. Intrafamilial horizontal transmission (61) and vertical transmission (228) from individuals with occult HBV infection have been reported.

Methods for Diagnosis of Occult HBV Infection

Chronic HBV infection is generally ruled out in the absence of detectable HBsAg in the serum. Differences among commercial assays in their ability to detect HBsAg associated with mutations in the "a" determinant are well recognized (281). Assays using polyclonal tracer antibodies for the detection of HBsAg vary in their ability to detect mutant HBsAg (281). However, assays using a polyclonal tracer antibody outperform assays using a monoclonal tracer for the detection of mutant HBsAg (124). Furthermore, HBsAg assays differ in their lower limits of detection (231). Highly sensitive commercial assays for HBsAg that consistently detect frequently encountered "a" determinant mutants (56, 57) should be preferentially used for HBsAg testing.

Detection of HBV DNA from serum or liver samples is considered the gold standard for the diagnosis of occult HBV infection. Experts have recently recommended the use of highly sensitive nested PCR or real-time PCR assays that can detect fewer than 10 copies of HBV DNA for the diagnosis of occult HBV infection (219). In addition, testing for multiple targets on the HBV genome increases HBV DNA detection rates (108) in patients with occult HBV infection. Intermittent viremia can occur in occult HBV infection, and periodic testing of HBV DNA will improve detection of occult HBV infection (127, 113). Periodic sampling for HBV DNA testing may be particularly appropriate for clinical

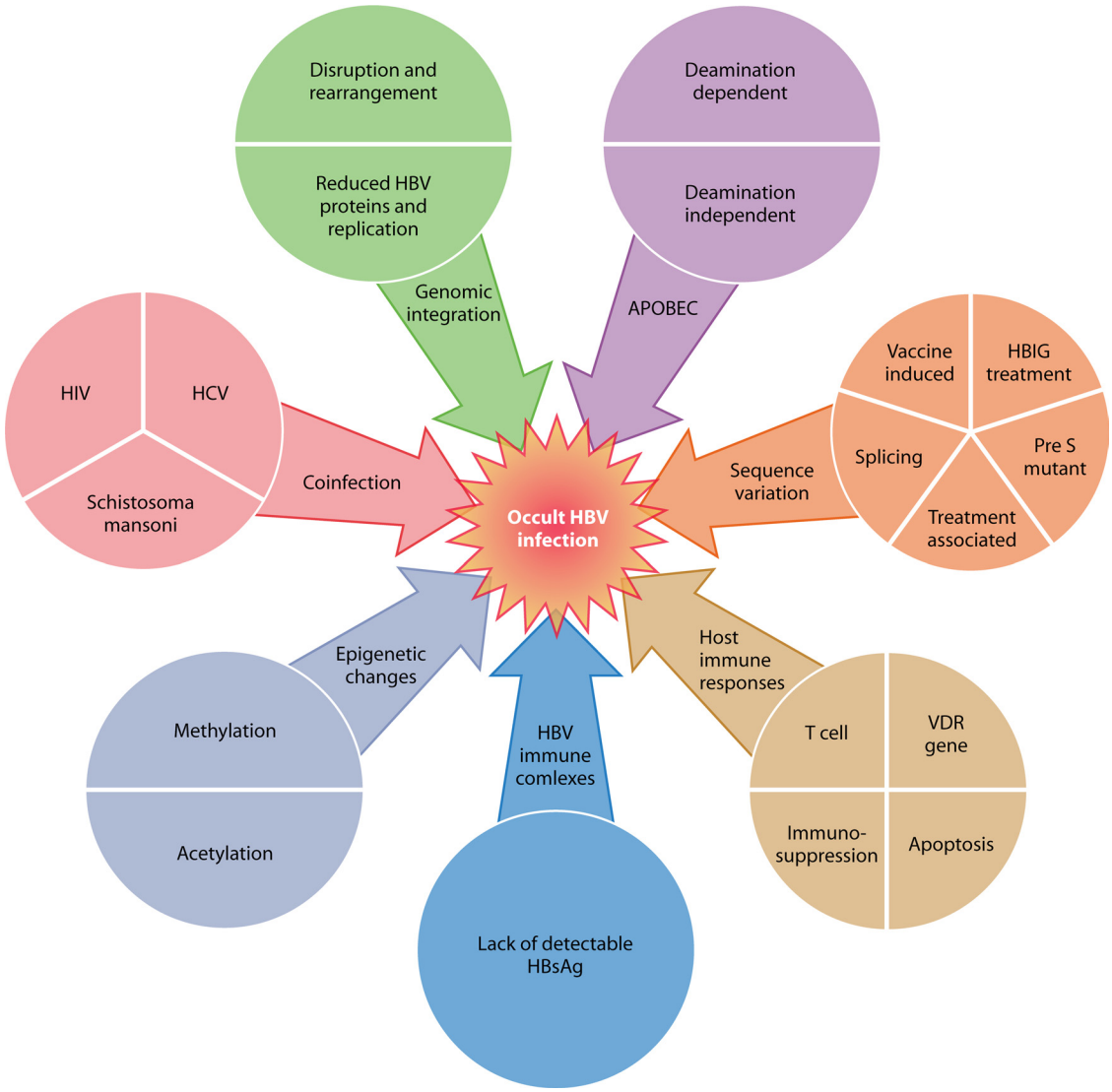

FIG 1 Overview of mechanisms leading to occult hepatitis B virus (HBV) infection.

groups at risk for occult HBV infection (Table 5). When available, testing of a liver biopsy specimen for HBV DNA will further augment the diagnosis of occult HBV infections, as HBV DNA is frequently detected in the liver in the absence of HBV DNA in the serum (108). Different screening methods are used across the world for screening blood and blood products (36, 141, 190). Individuals with occult hepatitis B virus infection can potentially transmit the infection through blood transfusions (154). Occult HBV infection is a major cause of posttransfusion hepatitis B (165). The prevalence of chronic HBV infection and availability of resources are major determinants of the screening methods used for HBV. For example, HBsAg with anti-HBc (total) and/or NAT is used for screening in resource-rich areas with a low prevalence of chronic HBV infection (208, 233). A good proportion of blood donors with occult hepatitis B virus infection have anti-HBc as the only serological marker of HBV infection (129, 242). Blood donors with isolated anti-HBc status are more infectious than those with low titers of anti-HBs (1). However, high rejection rates in areas of high HBV endemicity preclude anti-HBc screening of blood donors (36, 67). Although NAT testing for HBV DNA will reduce the risk of HBV transmission, its cost-effectiveness is still being questioned (125). The suitability of anti-HBc testing and NAT for screening blood and blood products is determined by (i) endemicity of HBV infection, (ii)) rates of anti-HBc detection in the population, and (iii) availability of resources.

MOLECULAR MECHANISMS LEADING TO OCCULT HBV INFECTION

There have been significant advances in understanding the molecular mechanisms underlying occult HBV infection in the last decade; an overview of these mechanisms is shown in Fig. 1. Each of these mechanisms is reviewed in detail below.

Mutations and Deletions in the HBV Genome

Sequence variation in HBV genomes, including (i) mutations in the "a" determinant of HBsAg, (ii) treatment-associated mutations, (iii) splicing, and (iv) mutations in the pre-S region have been linked to occult HBV infection.

Mutations in the "a" determinant of HBsAg. A mutation in the "a" determinant of the surface antigen was one of the earliest recognized mechanisms leading to occult HBV infection. Mutations in HBsAg lead to conformational changes rendering the protein undetectable by some of the commercially available HBsAg assays (219). Recently, the term "false" occult hepatitis B virus infection has been used to describe occult hepatitis B virus infections in individuals with HBV DNA levels comparable to those in individuals with overt HBV infection. "False" occult hepatitis B virus infections are usually associated with surface gene mutants that are not detectable by some commercial HBsAg assays (219, 165). Individuals with isolated anti-HBc-positive status with virus loads of greater than 10^4 copies/ml frequently harbor HBsAg mutants (148). The "a" determinant of HBsAg is a 2-loop structure that includes amino acids (aa) 124 to 147 (31, 32). It is rich in cysteine residues which are involved in disulfide bond formation and maintain the conformation of this region. Carman et al. first reported the sG145R mutation in the "a" determinant of HBsAg in a child who became infected with HBV despite active and passive immunoprophylaxis (31). This sG145R mutant has lower binding affinity to monoclonal antibody against HBsAg. Subsequently, several other mutations within the "a" determinant (49) and mutations in the surface gene outside the "a" determinant (32) were reported to have reduced binding affinity to monoclonal anti-HBs. Oon et al. (210) detected mutations in the "a" determinant among children born to HBsAg- and HBeAg-positive mothers who received both HBIG and the HBV vaccine at birth.

Mutations in the "a" determinant of the surface protein are associated with HBV reinfection following liver transplantation despite HBIG prophylaxis. Withdrawal of HBIG after liver transplantation led to reversion of the mutant to wild type in majority of the patients, indicating the role of HBIG-induced immune pressure in leading to "a" determinant mutants (92). Schilling et al. found that anti-HBs IgG is endocytosed by hepatocyte cell lines, leading to the intracellular accumulation of wild-type HBsAg while the sG145R-associated HBsAg was unaffected. HBIG was also found to inhibit the secretion of HBsAg into the serum without affecting HBV DNA replication, thus making HBsAg inaccessible to antibody used for detection of the surface antigen by some commercial assays (232). A recent study has demonstrated that in addition to blocking HBsAg release, anti-HBs can also partially block virion release from infected hepatocytes, contributing to HBV clearance from circulation (199).

The inability of some but not all commercial assays to detect HBsAg from samples associated with mutations in the "a" determinant is well documented (147). The emergence of "a" determinant mutants is a serious health concern not only because they are not detectable by some commercial HBsAg assays but also because they can infect both unvaccinated and vaccinated individuals.

Treatment-associated mutations. Double mutations in the HBV polymerase associated with the emergence of a mutation in YMDD motif during lamivudine treatment result in amino acid changes in both the HBV polymerase and the surface gene (Q563S in the polymerase and sS207R in the surface gene) (278). In addition, other lamivudine-induced mutations that result in synonymous changes in the polymerase gene open reading frame (ORF) but nonsynonymous changes in the surface gene ORF were reported by Wakil et al. (278). They also reported (i) another novel mutation (V539I) in the "C" domain of the HBV polymerase that was associated with a premature stop codon in the surface gene and (ii) the emergence of a substitution within the "a" determinant of HBsAg (sS143L) in lamivudine-treated patients.

Lamivudine-associated polymerase gene mutations M204I and L180M/M204I, corresponding to sI195M and sW196S in HBsAg, have been shown to be associated with reduced binding to anti-HBs antibodies, suggesting that these mutants may escape detection in some of the commercially available assays for HBsAg (262); these mutants may potentially escape neutralization by vaccine-induced anti-HBs. Mutations that confer resistance to lamivudine also reduce the affinity of the HBV polymerase to natural deoxynucleoside triphosphate (dNTP) substrates, resulting in reduced replication competence (88). Therefore, lamivudine-selected mutants have reduced replication fitness compared to wild-type HBV (64). Lamivudine is used in combination with HBIG in HBV-related liver transplantation. Interestingly, HBIG-induced vaccine escape mutants (sP120T, sT123N, and sG145R) restored the replication fitness of lamivudine-resistant mutants (261). Therefore, a combination of lamivudine-resistant mutations and vaccine escape mutations renders HBsAg undetectable in some commercial assays and also confers replication competence comparable to that of the wild type in addition to resistance to lamivudine and HBIG. These mutants are of particular public health concern because of their ability to escape detection by some commercial HBsAg assays, infect vaccinated individuals, and resist treatment with lamivudine.

RNA splicing. Splicing has been shown to have a significant effect on gene expression in HBV (107). Hass et al. demonstrated that a G-to-A mutation at position 458 of the surface gene altered the splicing of the S gene mRNA. Nucleotide (nt) 458 is close to the 5′ splice site of the S gene mRNA, and a mutation at this site interferes with the splicing of S gene mRNA. This mutation acts through a co-/posttranscriptional mechanism affecting the S gene mRNA export or RNA folding and is associated with a lack of HBsAg expression and a low-replication phenotype (107). Another mechanism of loss of HBsAg expression related to splicing has been reported in genotype D strains (269). A group of genotype D strains that represented a separate evolutionary branch was associated with positive selection (due to accumulation of substitutions) and had an acceptor site at nt 202. The authors report that the acceptor site at nt 202 and the donor site at nt 2986 are involved in a splice event resulting in the loss of the spacer region from the viral polymerase gene while retaining the original reading frame. The functions of the HBV polymerase are retained, while the expression of the small, middle, and large surface proteins are affected by this splice event. Therefore, it appears that alteration of splicing in surface gene mRNA results in reduced HBsAg secretion, thus leading to occult HBV infection (269).

Pre-S mutants. Mutations in the pre-S region, especially deletions, have also been associated with a lack of detectable HBsAg in the serum. Deletions in the pre-S region are associated with reduced expression of HBV surface proteins and also help in viral persistence by eliminating HLA-restricted B-cell and T-cell epitopes. Pre-S1/pre-S2 mutations are frequently detected in occult HBV infection (37, 277). Mutations in the pre-S2/S promoters were detected in patients with occult HBV-related chronic liver disease; serum HBsAg was not detectable in these patients (37). In another study, a 183-bp deletion (nt 3019 to 3201) in the pre-S1 region was detected in occult HBV patients. The deletion covered the CCAAT element that is required for transcription

factor binding. Other point mutations in the pre-S genes were also detected. The association of mutations and deletions in the pre-S gene with a lack of secreted HBsAg and low levels of HBeAg and HBV DNA was demonstrated using functional analysis by transfection into hepatocyte cell lines (80).

Xu and Yen (288) demonstrated that a 129-bp in-frame deletion in the S promoter region is associated with reduced levels of middle and small surface protein transcripts, resulting in a marked reduction in the expression of the two proteins. This S promoter deletion mutant, despite being replication competent, is associated with large amounts of intracellular retention of nonsecretable surface proteins. Melegari et al. (183) found deletions in the pre-S1 and pre-S2 regions following interferon therapy. Many of these pre-S deletion mutants selected after interferon therapy are associated with reduced HBsAg secretion in cell culture systems. Furthermore, extensive study of pre-S1 deletion mutants in Huh7 cells suggests that the loss of HBsAg titers is associated with the loss of the SP1 transcription factor binding site. Interestingly, despite retaining polymerase activity, the pre-S1 mutants required help from the wild-type HBV for secretion of encapsidated defective genomes into the serum. Fan et al. (79) showed that deletions in the pre-S region are detected in a significantly higher proportion of individuals with low HBV DNA levels than in those with higher virus loads. Among individuals with low HBV DNA levels, the deletions ranged from 70 to 141bp in the pre-S1 region and from 54 to 72 bp in the pre-S2 region.

Coinfection with Other Microbes

Coinfection with HCV. Coinfection of HBV and HCV is well documented (27, 33, 43, 81, 236). Numerous studies have demonstrated decreased numbers of HBV replicative intermediates in HCV-coinfected individuals. Cacciola et al. found that about a third of the patients with chronic hepatitis C liver disease have detectable HBV DNA but no HBsAg in serum. They established a significant association between occult HBV infection and cirrhosis among HCV-infected patients. Their study also found a trend toward a response to interferon therapy among patients with chronic HCV infection and concurrent occult HBV infection (29). Occult HBV infection in chronically HCV-infected patients has been associated with an increased risk of HCC (177). The predominance of certain HBV genotypes in concurrent chronic HCV infection and occult HBV infection and their potential role in determining the clinical outcome have also been suggested recently (108). Higher rates of occult hepatitis B virus infection were detected in patients infected with HCV genotype 1b than in those with genotype 2a (85). Increased HBV DNA levels with occult HBV infection among HCV-infected patients have been associated with a simultaneous increase in liver enzymes (transaminases), suggesting a plausible mechanism leading to liver injury (127). In contrast, a lack of occult HBV infection-related clinical implications among patients with HCV-related liver diseases has been reported by other investigators. Kao et al. found that the prevalence of occult HBV infection did not correlate with the severity of HCV-related liver disease. Additionally, therapeutic responses to combination therapy (interferon and ribavirin) against HCV infection were comparable in patients with and without occult HBV infection (128).

Rates of HCV coinfection in patients with chronic HBV infection vary considerably depending on the geographical location. Occult HBV infection in up to 52% of patients with chronic HCV infection has been reported (21, 85, 96, 116, 157). Several studies have shown that HBV and HCV coinfection results in lower levels of HBV replication (54, 59, 83) and decreased expression of HBsAg in the liver (54). Furthermore, superinfection with HCV in HBV-infected chimpanzees led to decreased HBsAg levels in serum (24). Similarly, coinfection with HCV was associated with significantly higher spontaneous HBsAg clearance rates than infection with HBV alone (237), strongly suggesting an interplay between the two viruses/viral proteins. Studies further investigating the inhibition of HBV replication and HBV protein production by HCV have demonstrated several interesting underlying mechanisms (Fig. 2).

(i) Colocalization of HBV and HCV genomes in the nucleus. Interference with HBV replication by HCV can take place if both viruses coexist in the same hepatocyte. Double fluorescent *in situ* hybridization was used to understand the role of HCV in inhibiting HBV replication in 6 liver biopsy specimens from patients with chronic HCV and occult HBV infection (224). The intensities of fluorescence signals corresponding to HBV DNA and HCV RNA were measured in singly infected and doubly infected hepatocytes using digital image analysis. The analysis showed that fluorescence in hepatocytes infected only with HBV was 1.6 ± 0.16 times higher than that in hepatocytes coinfected with HBV and HCV, although the difference was not statistically significant.

In contrast, another study using cell culture-propagated HCV and replication-competent HBV genomes in Huh-7 cells demonstrated a lack of strong interactions between the two viruses in coinfected hepatocytes in cell culture, suggesting a role for host responses in establishing viral dominance in coinfected cells (75). Nonetheless, they found extensive colocalization of HCV and HBV envelope proteins.

(ii) HCV core protein-mediated HBV suppression. The HCV core protein is a part of the viral capsid, and it inhibits HBV replication and gene expression and suppresses activity of HBV enhancers (45, 234). Chen et al. elucidated several mechanisms of HCV core protein-mediated suppression of HBV (45). They cotransfected full-length or truncated versions of HCV structural genes (core and envelope 1) with cloned HBV DNA into Huh-7 cells and showed that the HCV core protein is required for the suppressive activity of HCV on HBV replication as well as the expression of HBsAg. Their findings also suggested impairment in HBV pregenomic RNA encapsidation as a possible mechanism of HBV suppression.

In another study, Shih et al. showed that phosphorylation at Ser-116 and Ser-99 of the HCV core protein is required for suppressive activity of HCV core protein. The replacement of Ser-99 and the Ser-116 with alanine or aspartate residues did not affect HCV core protein expression or nuclear localization; however, it resulted in loss of HBV suppression activity (239).

The package signal (ε) is present at the 5′ end of HBV pregenomic RNA; the HBV core and polymerase proteins interact with this signal to initiate the encapsidation process (10, 112). Chen et al. demonstrated that HCV core protein binds with HBV polymerase and inhibits binding of the polymerase to its package signal, preventing the encapsidation of HBV pregenomic RNA into its capsid (45). Given that HBx is a transactivator and activates HBV promoters and enhancers (52, 78, 195), Chen et al. hypothesized that HCV core protein inhibits HBV gene transcription by directly interacting with HBx. They cotransfected HBx null plasmid or wild-type HBV plasmid along with plasmids en-

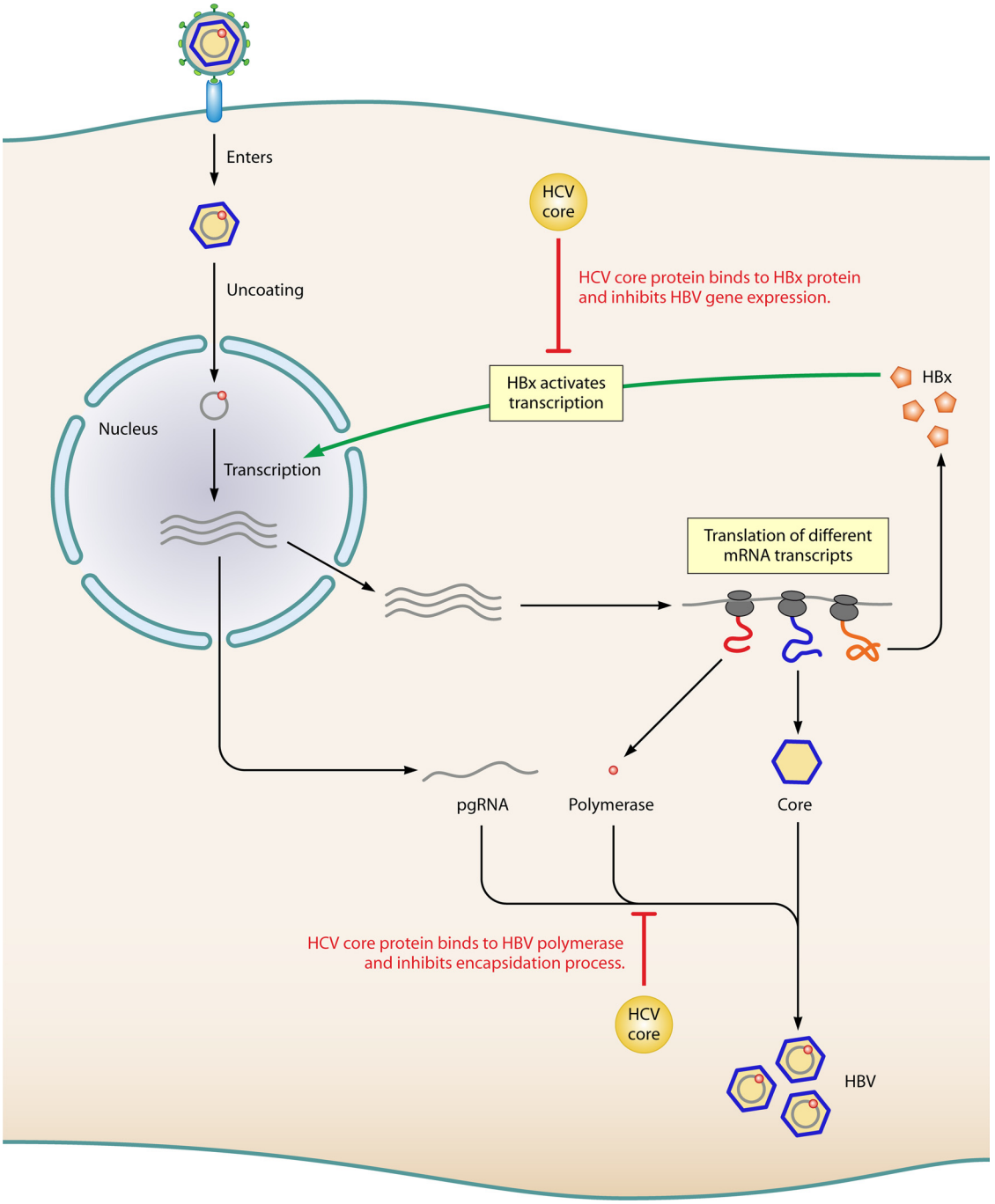

FIG 2 HCV coinfection in occult HBV infection. HCV core protein inhibits HBV gene expression by directly interacting with HBx protein and suppresses HBV replication by interfering with the binding of core and polymerase to package signal "ε" present in pregenomic RNA (pgRNA) within the hepatocyte, thereby preventing HBV encapsidation.

coding HCV core protein. The HCV core protein-mediated inhibition of HBsAg, HBeAg, and HBV transcripts is dependent on the presence of HBx protein colocalized with the HCV core protein in both the nucleus and the cytoplasm. However, the ability of the HCV core protein to inhibit HBV replication is independent of HBx protein expression. Chen et al. further delinked the HCV core protein-mediated suppression of HBV gene expression and HBV replication. They demonstrated that a 22-amino-acid segment (from aa 101 to aa 122) of the C terminus of the HCV core protein plays a vital role in suppressing HBV gene expression, while the HCV core in its entirety is required for the suppression of HBV replication. In an attempt to understand the role of arginine residues in the HCV core protein, Chen et al. performed site-directed mutagenesis experiments on six arginine residues. The mutagenesis studies showed that Arg-101, Arg-113, Arg-114, and Arg-115 are required for the inhibitory activity of HCV core

protein on both HBV gene expression and HBV replication. Arg-104 inhibits HBV encapsidation, but it has no effect on HBV gene expression.

Schuttler et al. showed that HCV core protein suppresses the activity of HBV enhancers I and II (234). They identified nucleotides 1115 to 1236 of HBV enhancer I and nucleotides 1730 to 1822 of HBV enhancer II as regions that are crucial for HCV core protein-mediated suppression of HBV. Interestingly, the regions from enhancers I and II essential for HBV suppression by HCV core protein also represent binding sites for members of the nuclear receptor family such as HNF4 and RXR. These nuclear factors play important regulatory roles in HBV replication by interacting with HBV enhancer I (90). Schuttler et al. also showed that HBV with mutations in the binding sites for nuclear receptors within HBV enhancers is less susceptible to repression by HCV core protein, and hence they proposed that the nuclear receptors bind to a leucine zipper motif present at the C terminus of the HCV core protein and suppress HBV enhancers. They also found that the repressor effect of the HCV core protein is more pronounced on HBV enhancer I than on HBV enhancer II. In their study, Schuttler et al. found differences among HCV core proteins from different HCV genotypes in their ability to suppress HBV enhancer I (234).

(iii) HCV NS2 protein-mediated HBV suppression. NS2 is a nonstructural protein of HCV with autoproteolytic activity (62, 198). Dumoulin et al. (71) cotransfected NS2-encoding plasmids with HBV dimers and demonstrated that the HCV NS2 protein inhibited HBsAg and HBeAg secretion into the supernatant and also inhibited HBV replication. This inhibitory activity of the HCV NS2 protein is associated with its amino terminus. In addition, they demonstrated the role of the HCV NS2 protein in inhibiting various cellular promoters and viral promoters, including gene expression from HBV liver-specific and non-liver-specific promoters.

Coinfection with HIV. Human immunodeficiency virus (HIV) and HBV share modes of transmission. Occult HBV infection in HIV-infected individuals is well recognized. The reported prevalence of occult HBV infection among HIV-positive patients varies based on the methods used for the detection of HBV DNA, the endemicity of HBV in the geographical region studied, and the history of antiretroviral treatment. In a longitudinal follow-up study, HBV DNA was detected at least once in almost 90% of a cohort of Swiss HIV-positive patients, compared to 25% detection rates for HBsAg (113). This finding suggests that (i) occult HBV infections are common among HIV-positive individuals and (ii) HBV DNA is intermittently detected in HIV-positive individuals, necessitating multiple sampling for HBV DNA in this group of patients. Another study, by Gupta et al. (103), found occult HBV infection in treatment-naïve HIV-infected patients; interestingly, about a fifth of the patients with occult HBV infection had detectable anti-HBs. However, the specific mechanisms leading to occult HBV infection in HIV-infected individuals still remain unknown.

Coinfection with *Schistosoma mansoni*. *Schistosoma mansoni* is a parasite that affects over 200 million people worldwide, particularly in Asia and Africa (226). Coinfection with HBV and *Schistosoma* occurs frequently in areas where both agents are endemic (19, 73). When transgenic mice supporting HBV replication were infected with *Schistosoma mansoni*, a Th1-type response was followed by both Th1 and Th2 responses. HBV replication levels remained suppressed during both the Th1 response and the subsequent Th1 and Th2 responses (179). In gamma interferon (IFN-γ) knockout mice coinfected with *Schistosoma mansoni*, the suppression of HBV replication was minimal, suggesting that IFN-γ is the major antiviral cytokine in *Schistosoma mansoni* infection. HBV replication remained inhibited when both Th1 and Th2 responses were detected, suggesting that Th2 cytokines do not interfere in the antiviral activity of IFN-γ (179).

Apolipoprotein B mRNA-Editing Enzyme Catalytic Polypeptide and Occult HBV Infection

The physiological function of apolipoprotein B mRNA-editing enzyme catalytic polypeptides (APOBECs) is cytidine deamination (196, 255). The role of APOBECs in inhibiting and editing HIV replication has been well studied (114, 115). Subsequently, the ability of APOBEC3G to inhibit HBV replication was demonstrated. The expression of APOBEC3G in cells replicating HBV resulted in a 50-fold reduction in HBV DNA levels (265). Both deamination-dependent and deamination-independent mechanisms of inhibition of HBV replication have been reported for APOBECs (Fig. 3).

Deamination-dependent inhibition of HBV replication. Initially it was believed that only the cytidines in the minus strand of the HBV genome are edited, which is reflected as G-to-A mutations in the plus strand of HBV (204). However, Suspene et al. demonstrated that the plus strand of HBV is also deaminated by APOBEC enzymes (253). The overexpression of APOBEC3G is associated with hyperedited HBV genomes and a reduction in the replicative intermediates of HBV. Regions of the HBV genome encoding the surface proteins, polymerase, and the HBx protein are hyperedited by APOBECs (204). Normal human livers express low levels of APOBEC proteins (23). Several APOBEC3 genes are upregulated in HBV-related cirrhotic livers compared to cirrhotic tissue from alcoholic liver disease (270). It has been demonstrated that up to 35% of the HBV genomes in the liver may be edited by APOBECs (270). Of note, common mutations leading to the loss of HBeAg (G1896A) and lack of detectable HBsAg (sG145R) result from G-to-A mutations; these mutations have also been linked to APOBEC editing of HBV (270).

APOBEC hyperedited sequences have been reported from both occult and nonoccult cases of chronic HBV infection (271). It remains clear that the majority of HBV genomes in occult HBV infection are not hyperedited (271). However, minimal editing by APOBEC proteins, for example, the sG145R mutation that results due to a G-to-A mutation, is sufficient to cause occult HBV infection. Therefore, APOBEC-mediated occult HBV infection can occur in the absence of extensive HBV DNA editing. A growing body of literature in the last few years has demonstrated the role of APOBEC proteins in noncytolytic clearance of HBV infection. An increase in serum ALT levels is linked to an increase in hyperediting of the HBV genome, loss of HBeAg, and a reduction in HBV DNA levels, suggesting that hyperediting and the associated reduction in virus loads represent a strong host immune response (205).

Deamination-independent inhibition of HBV replication. Although deamination-inactive APOBEC3G lacked the ability to hyperedit the HBV genome, it still retained the ability to inhibit HBV replication (206), suggesting the presence of deaminase-independent mechanisms inhibiting HBV replication. Rosler et al. reported increased nuclease susceptibility of HBV core-protein-

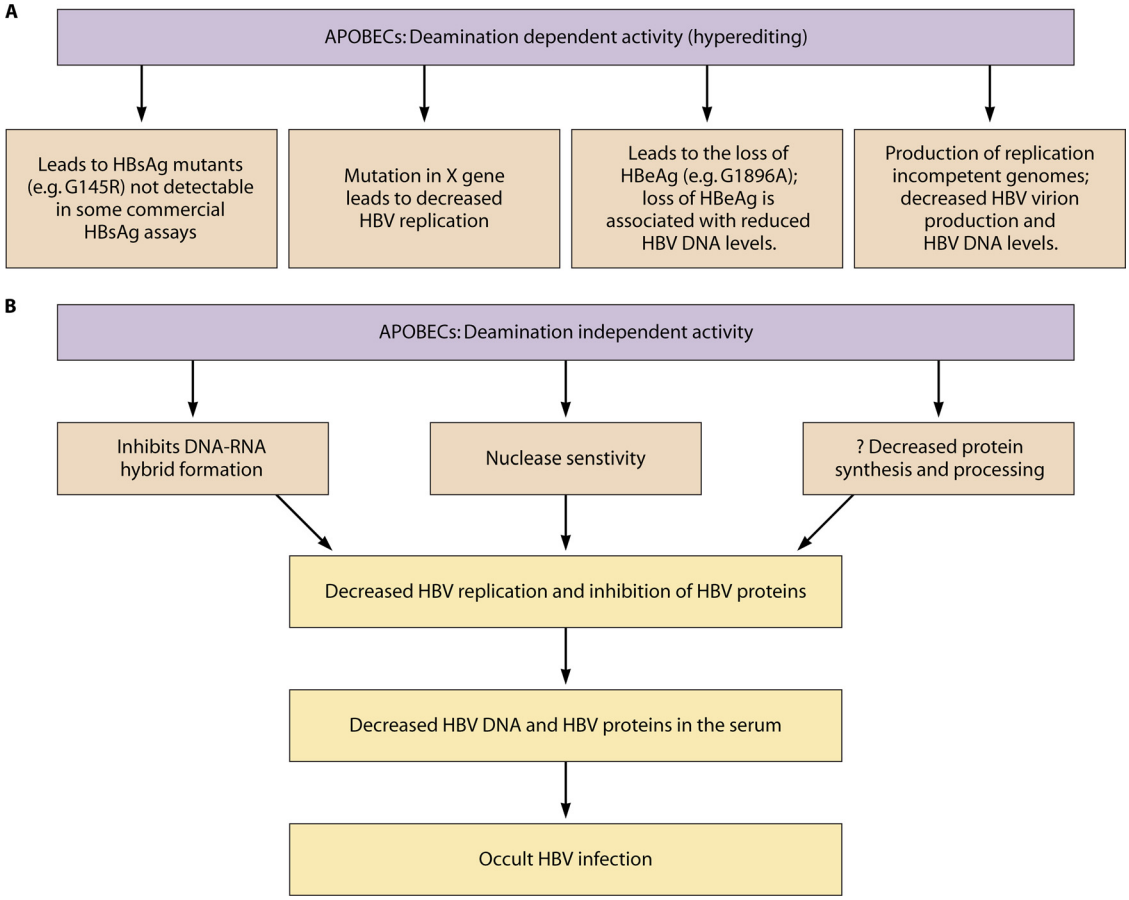

FIG 3 Role of APOBEC deaminases in occult HBV infection. (A) Deamination activity of APOBEC protein converts cytosine to uracil in the HBV genome, leading to various mutations associated with occult HBV infection. (B) Deamination-independent activity of APOBEC deaminases inhibits DNA-RNA hybrid formation, increases susceptibility to nuclease digestion, and decreases protein processing, eventually leading to occult HBV.

associated pregenomic RNA (225). In another study, Nguyen et al. demonstrated the inhibition of HBV reverse transcriptase (RT) activity by APOBEC3G. They also showed that APOBEC3G inhibited early stages of HBV DNA synthesis by targeting HBV DNA-RNA hybrids and single-stranded HBV DNA (203). APOBEC3G copellets with HBV capsids, and encapsidation of APOBEC3G by replication-competent HBV nucleocapsids renders it resistant to proteinase K digestion. Further, it has been suggested that multiple copies of APOBEC3G may be packaged into HBV nucleocapsids by its interaction with HBV RT and the HBV RNA packaging signal (ε) (202). APOBEC proteins bind to the HBV core protein and hinder HBV capsid formation (11). Apart from APOBEC3G, other APOBEC proteins, including APOBEC3F and APOBEC3B, can inhibit HBV replication (23). In addition to inhibiting HBV replication, APOBEC3B can inhibit secretion of both HBsAg and HBeAg. The mechanisms underlying APOBEC-mediated inhibition of HBV proteins remain unclear, but interference with protein synthesis or processing has been suggested as a potential mechanism (23, 298). While the inhibitory mechanisms of APOBEC proteins that could produce occult infection are being increasingly recognized *in vitro*, large-scale studies comparing patients with and without occult infection are required to confirm and better understand the role of these proteins *in vivo*.

Host Immune Responses and Occult HBV Infection

Virus-host interactions play a crucial role in determining the outcome of hepatitis B virus infection. Host immune responses are involved in viral clearance, viral persistence, and immunopathogenesis of HBV infection. Interestingly, several host immune response-related mechanisms, such as apoptosis, cytolytic and noncytolytic T-cell responses, and vitamin D receptor (VDR) polymorphisms, have been linked to modulation of HBV replication and HBV protein synthesis (Fig. 4). However, only a few studies have implicated modulation of the host immune response as a stand-alone mechanism leading to occult hepatitis B virus infection. Martin et al. compared the serum cytokine expression profiles in HIV-infected patients with chronic HBV infection or with occult HBV infection. Lower soluble Fas (sFas) levels were detected in occult HBV infection than in chronic HBV infection ($P = 0.01$) (175). The Fas expression system is known to modulate apoptosis of infected hepatocytes and also plays a key role in the removal of aged hepatocytes and maintenance of normal liver homeostasis (110). Martin et al. argued that their finding of lower sFas levels in occult HBV infection indicates decreased apoptotic inhibition in occult HBV infection and could be one of the mechanisms for clearance of HBsAg and downregulating HBV replication in occult HBV infection (175). It has been suggested that

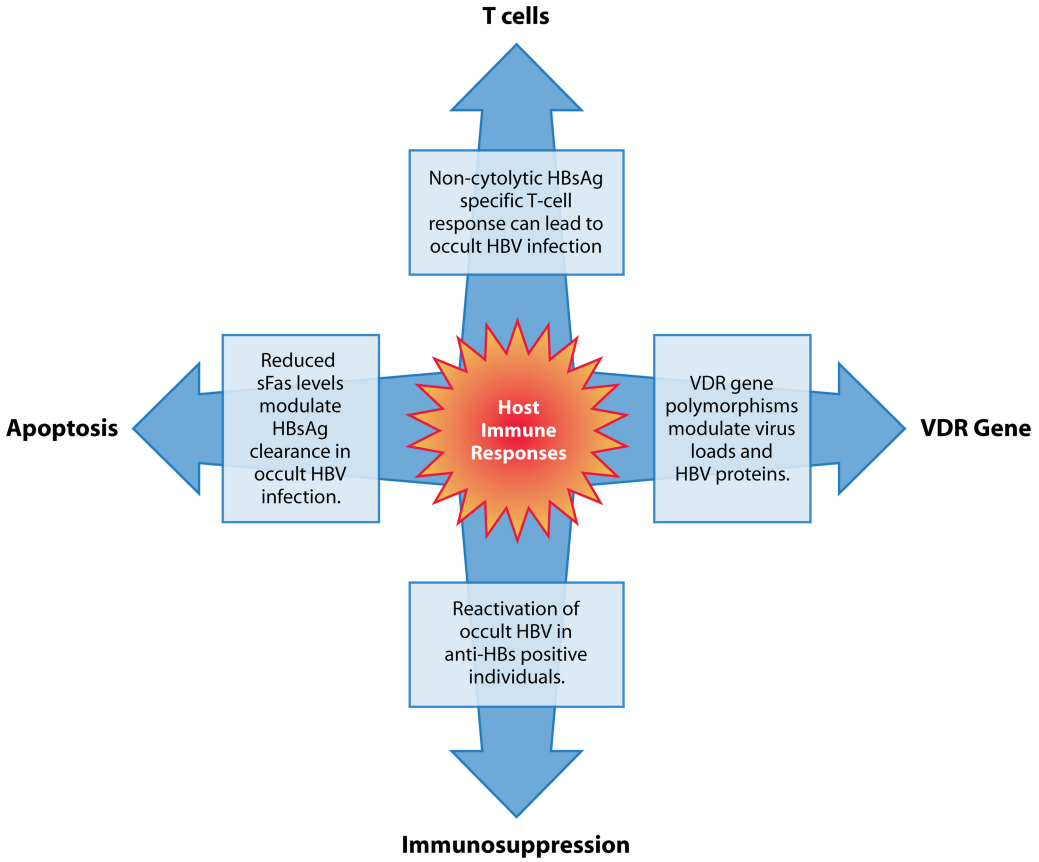

FIG 4 Host immunological responses leading to occult HBV infection. Host-derived responses, including a noncytolytic T-cell response, VDR polymorphism, immunosuppression, and differential regulation of apoptosis, contribute to the etiopathogenesis of occult HBV infection.

reduced expression of CXCL12, a chemokine that modulates apoptosis, may play a role in occult HBV infection (109). Additional studies are required to evaluate the role of apoptosis in modulating the course of HBV infection.

Differences in the HBV-specific cell-mediated immune response have been described in occult HBV infection. Anti-HBc-positive occult HBV patients had T-cell responses concurrent with protective memory, while anti-HBc-negative occult HBV patients had inadequacies in maturation of protective memory (297). The presence of HBV-specific $CD8^+$ T cells in occult HBV infection without anti-HBc was demonstrated by staining with class I major histocompatibility complex tetramers (297). Guidotti et al. (98) demonstrated that clearance of more than 90% of HBV DNA in chimpanzees does not require destruction of HBV-infected hepatocytes, suggesting that some noncytolytic immune responses are critical in the clearance of acute HBV infection. Additionally, they showed that even HBV cccDNA is susceptible to these noncytolytic mechanisms. A noncytolytic HBsAg-specific T-cell response has been suggested as the potential mechanism for occult HBV infections associated with very low and undetectable levels of HBsAg (26).

Woodchucks inoculated with a low, non-liver-pathogenic dose of woodchuck hepatitis virus (WHV) developed occult infection but did not have surface antigenemia or antibody responses to viral proteins (99). Of note, the woodchucks with occult infection had WHV-specific T-cell responses against WHV proteins, and the secondary T-cell proliferative responses were comparable to those seen after primary WHV infection. These findings make occult infection with WHV an immunologically unique and recognizable entity. The authors also suggested that assessment of HBV-specific T-cell responses may be helpful in estimating the burden of occult HBV infection in high-risk groups such as health care workers and family members of infected individuals, who are likely to be exposed to low doses of HBV.

In a long-term follow-up study to assess the cytotoxic-T-lymphocyte (CTL) response following acute HBV infection, HBV-specific CTLs persisted in the blood for decades after clinical and serological recovery from acute HBV infection. Additionally, the strength of the CTL response directly correlates with the presence of HBV DNA in the serum. Furthermore, it is suggested that small quantities of virus may remain after recovery from acute HBV infection and help maintain the CTL response for decades; the CTL responses in turn play a significant role in keeping active viral replication under control (222). It appears that both humoral and cell-mediated responses are likely to play significant roles in the development of occult hepatitis B virus infection.

Vitamin D3 and the VDR regulate several cytokines (139) and are important determinants of the anti-HBV response (120). Polymorphisms in the VDR gene have been linked to the outcome of chronic HBV infection (120). In addition, differences in HBV DNA levels (252) and loss of HBeAg (120) have been linked to

certain VDR genotypes. Recently, polymorphisms in the VDR gene have been detected in occult HBV infections (7).

Reactivation of occult HBV infection in unvaccinated patients with anti-HBs-positive status associated with past exposure to HBV may result in vaccine escape mutants (9). The inability of some commercial assays to detect the HBsAg associated with vaccine escape mutants despite high virus loads further complicates the picture (48).

Epigenetic Changes

Methylation. In the human genome, regions rich in CpG dinucleotides are referred to as CpG islands. Methylation of cytosines in CpG dinucleotides within CpG islands in gene promoters leads to gene silencing (216). Methylation is a key mechanism for regulation of transcriptional activity. Methylation of HBV DNA represents a novel epigenetic mechanism that impairs HBV proteins, HBV replication, and HBV virion production, leading to occult HBV infection (Fig. 5). Nearly 2 decades ago it was demonstrated that HBV DNA integrated into the host genome is methylated. Methylation of HBV DNA encoding the HBV core protein leads to loss of HBV core protein in PLC/PRF/5, a human hepatoma cell line with integrated HBV DNA sequences (186). It was generally accepted that only integrated HBV DNA sequences are methylated. However, the observation of chromatin-like minichromosomes during replication prompted the search for CpG islands in episomal DNA, and three were recently identified (271). Interestingly, key regulatory elements of the HBV genome were located within or adjacent to the 3 CpG islands (271). Episomal HBV DNA from human liver tissue and from cell culture can be methylated. Of note, methylation of CpG island 2 in the HBV genome is frequently detected in occult HBV infection (271). HBV cccDNA is frequently methylated in human liver tissues (272). Transfection of *in vitro*-methylated HBV DNA constructs into hepatocyte cell lines was associated with a >90% decrease in secreted HBsAg. In addition, HBeAg and HBcAg expression was markedly reduced by methylation of HBV, clearly demonstrating the role of CpG islands in regulating HBV gene expression (272). HBV replication in cell culture induced the expression of DNA methyltransferases (DNMTs), enzymes vital for DNA methylation. The HBV-induced DNMTs could methylate HBV DNA, resulting in the inhibition of HBV transcription and HBV replication (273). Hypermethylated HBV DNA sequences are frequently detected in HCC patients with occult HBV infection (130). Methylation of cccDNA is associated with low serum HBV DNA levels and decreased virion production in patients with liver cirrhosis (135). The association between HBeAg and high virus loads is well known (53, 119). It was recently demonstrated that a higher ratio of methylated cccDNA to total cccDNA is detected in HBeAg-negative individuals than that in HBeAg-positive individuals. In addition, methylation of cccDNA correlated with reduced HBV replication (101). Recent studies have demonstrated a pivotal role for HBV methylation in occult HBV infection. However, additional studies will throw more light on the role of this recently identified epigenetic mechanism in occult HBV infection.

Acetylation. The role of acetylation of histones bound to HBV DNA in regulating HBV replication and transcription has been convincingly demonstrated in several studies. In the mid 1990s, Newbold et al. (201) discovered that the hepatitis B viral nucleoprotein complex is arranged in the form of a minichromosome composed of nucleosomes, suggestive of transcriptional regulation of HBV DNA. Hyperacetylation of cccDNA-bound histones is associated with increased HBV replication in cell culture. In the presence of histone deacetylase inhibitors (valproic acid or trichostatin A), high HBV transcript levels and increased HBV replication are correlated with an increase in acetylated histones bound to cccDNA. Furthermore, acetylation of H3 and H4 bound to cccDNA in liver tissues from patients with chronic hepatitis B virus infection correlated with serum HBV DNA levels. Hypoacetylation of histones bound to cccDNA seen in liver tissue from patients with low virus loads is linked to recruitment of histone deacetylase (215). Subsequently, the recruitment of HBx protein to the cccDNA minichromosome along with histones has been demonstrated (16). Interestingly, an HBx mutant with a single nucleotide substitution is associated with rapid hypoacetylation of histones bound to cccDNA, impairing recruitment of p300, a transcriptional coactivator. The HBx mutant was associated with reduced HBV pregenomic RNA and reduced HBV replication, suggesting a key role for the HBx protein in regulating HBV replication. Recently, remodelling of the HBV minichromosome by phosphorylation and methylation of histones has been shown to regulate HBV replication (95). However, this recently reported mechanism and its potential role in occult HBV infection have not yet been investigated by use of molecular epidemiological studies.

Genome Integration

Integration of HBV DNA sequences into the host genome is frequently detected in patients with chronic HBV infection and is found to precede HCC (267). Disruption and rearrangement of genes during integration into chromosomal DNA can result in (i) loss of HBsAg in the serum, (ii) reduction in virion production, and (iii) loss of detectable HBV DNA in serum. Therefore, integration of HBV DNA represents a key mechanism underlying occult HBV infection, especially following several years of chronic HBV infection. Integrated HBV DNA rather than episomal HBV is frequently detected in HBV-related HCC (220). Integrated HBV DNA is often defective (25), and integration of certain HBV genes is observed more frequently than that of others (267). While high rates of HBV DNA integration have been reported from HBsAg-positive HCC (131), HBV DNA integration in HBsAg-negative HCC is also widely reported (178, 188, 254), especially among anti-HCV-positive patients. Specific integration patterns disrupting the expression of HBsAg have not been reported in HBV-HCV dual infection. The inhibition of HBV replication and HBV proteins has been discussed above.

The HBV core gene may often be lost during integration of HBV DNA, resulting in reduction or loss of the HBV core protein. The loss of the HBV core protein is associated with suboptimal virus assembly and the accumulation of unencapsidated HBV DNA within the hepatocyte. This may explain why patients with HCC related to HBV lack detectable HBV DNA in blood though HBV DNA may be readily detected in the liver (220).

Overexpression of large HBs protein prevents secretion of all forms of surface protein and leads to intrahepatic accumulation of surface proteins as granules (51). The large HBs protein constitutes a small proportion of total surface proteins. Huang and Yen (121) studied the role of disruptions and rearrangements in HBV DNA that occur during integration in regulating HBsAg expression. Replacement of a region downstream of the S gene ORF

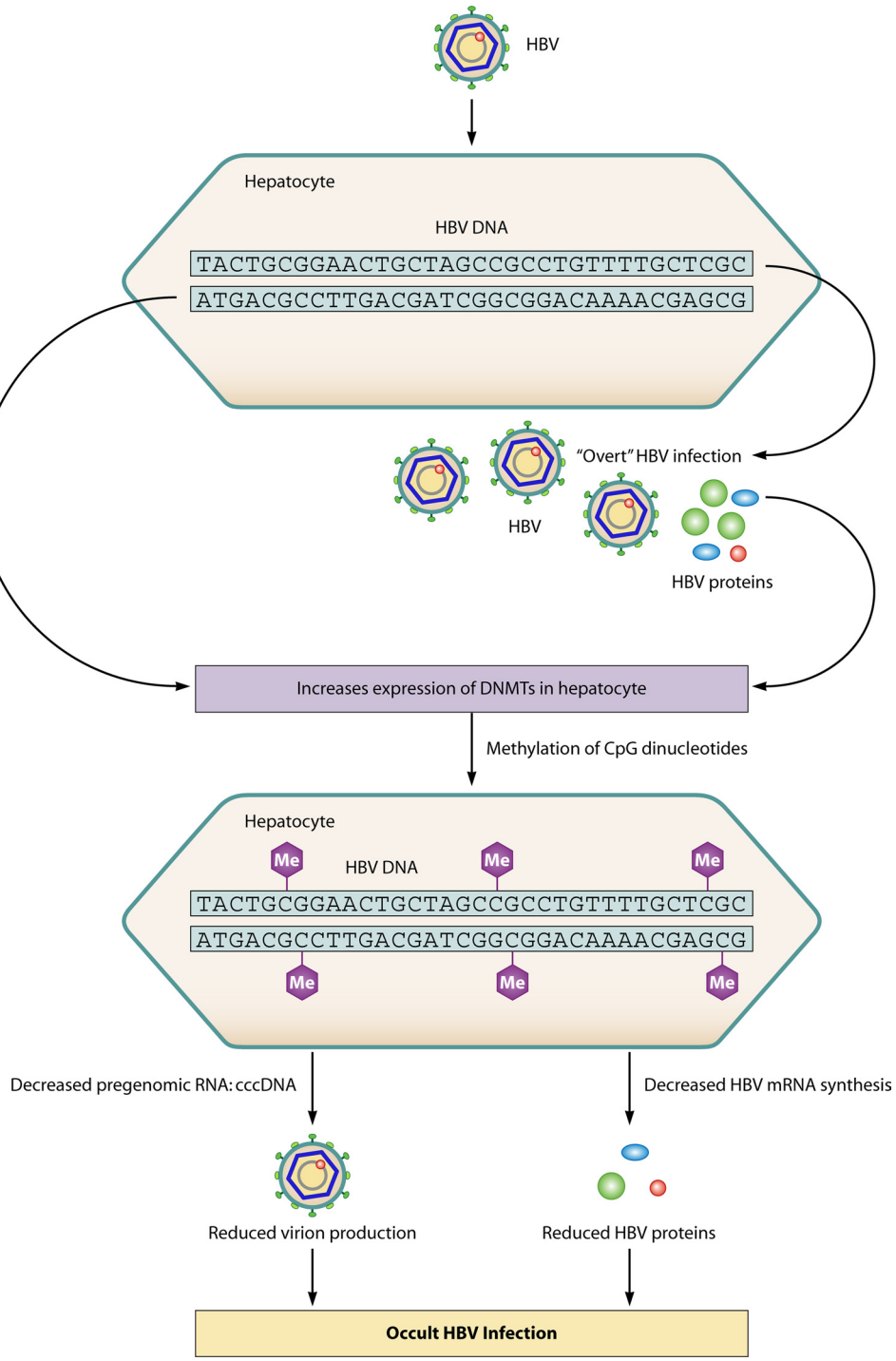

FIG 5 Mechanism of occult HBV infection via methylation of HBV DNA. HBV induces expression of cellular DNA methyltransferases (DNMTs), leading to methylation of CpG dinucleotides in the HBV genome. Hypermethylation impairs HBV replication, virion production, and HBV protein levels, leading to occult HBV infection.

containing enhancer I and II with the pre-S1 promoter resulted in decreased S gene transcripts without affecting the levels of pre-S transcripts. Changes in the ratios of S gene transcripts to pre-S1 transcripts blocked the secretion of S protein. Similarly, other rearrangements resulting in the loss of HBV enhancers lead to intracellular retention of surface proteins that remain undetectable in serum.

Immune Complexes in Occult Hepatitis B Virus Infection

Entrapment of HBsAg in immune complexes with anti-HBs can impair HBsAg detection by conventional serological assays. In general, the appearance of detectable anti-HBs correlates with a decrease or complete disappearance of HBsAg-containing immune complexes (213). Interestingly, immune complexes con-

taining HBsAg have been detected in HBsAg-negative occult HBV infection among patients with HCC (28). HBV DNA was detected in about 40% of HBsAg-negative but anti-HBc total positive blood donors, with HBV-containing immune complexes present in a majority of the HBV DNA-positive donors (293). Sequencing of HBV DNA from these donors demonstrated the lack of nucleic acid changes that alter major epitopes of HBsAg, confirming the role of circulating immune complexes in occult HBV infections. Michalak et al. demonstrated that HBV DNA from convalescent-phase sera cosedimented with HBsAg in a sucrose gradient column representing naked core particles or intact virions in circulating immune complexes. These data suggest that hepatitis B virus can persist despite recovery from acute infection (185). Circulating HBsAg-containing immune complexes have been demonstrated in patients with acute HBV infection, in asymptomatic HBsAg carriers, and also in chronic HBV infection (6). HBsAg-containing immune complexes have also been found in the presence of detectable anti-HBs (230). Madalinski et al. found anti-pre-S1 antibodies to be frequently involved in the formation of HBV-containing immune complexes in patients with chronic hepatitis B virus infection (171). Levya et al. correlated the presence of HBsAg-containing immune complexes and the course of hepatitis. Interestingly, they found that HBsAg-containing immune complexes are consistently present in patients with chronic hepatitis B virus infection compared to those with acute resolving hepatitis (155). Other studies have shown the opposite finding (6). Differences in the methods used could in part account for these contrasting observations. Increases in levels of immune complexes containing woodchuck hepatitis virus surface antigen (WHsAg) and anti-WHs correlated with the peaks of serum viremia and antigenemia, indicating the presence of surface antigen-containing complexes in WHV similar to those in HBV (94). It is clear that circulating HBsAg-containing immune complexes can be associated with occult HBV infection; however, the magnitude of the problem in the settings of acute resolving hepatitis and chronic hepatitis remains poorly understood.

EMERGING ISSUES

The persistence of HBV cccDNA in the liver tissues of both occult and nonoccult HBV cases has been demonstrated (277). Host immune responses play a critical role in controlling HBV replication. Differences, if any, in host immune responses between occult and nonoccult HBV cases in controlling the replication of HBV from the intrahepatic pool of cccDNA are poorly understood. The role of host immune responses in occult HBV infection remains elusive; large-scale studies focusing on this area may provide novel insights on the mechanisms leading to occult HBV infection.

Functional studies on full-length HBV genomes from occult HBV cases did not identify a predominant role for any particular mechanism leading to occult HBV infection, suggesting that occult HBV infection is often a result of multiple mechanisms (37, 80). With a few exceptions, it is likely that the mechanisms discussed above act in tandem, leading to occult HBV infection. Additional studies on full-length HBV genomes from occult HBV cases may throw more light on the interplay between the mechanisms leading to occult HBV infection.

Some of the recently identified molecular mechanisms leading to occult HBV infection play a direct role in carcinogenesis. Increased expression of APOBEC3B in HCC tissue conferred a selective clonal growth advantage to preneoplastic hepatocytes (287). Similarly, HBV-induced DNMTs led to aberrant methylation of the host genes, including tumor suppressor genes, apart from contributing to methylation of the HBV genome (273). Additional studies investigating the mechanisms underlying occult HBV infections and their contribution to hepatocarcinogenesis will help us better understand the causative role of occult HBV in HCC.

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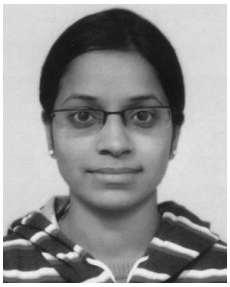

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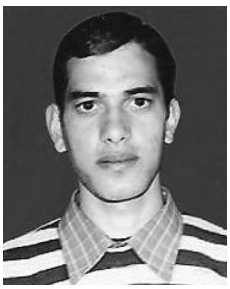

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