Biologic Properties of Hepatitis B Viral Genomes with Mutations in the Precore Promoter and Precore Open Reading Frame

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It is now well recognized that mutations in the hepatitis B virus (HBV) genome occur during the natural course of chronic viral infection. Regions of the viral genome that are frequently affected by such mutations, rearrangements, and/or deletions generally involve the precore promoter, precore, and core as well as the preS gene regions. However, little is known regarding the biologic consequences of these mutations on the functional properties of the variant viral strains with respect to effects on viral replication. In this study, we investigated the functional significance of precore promoter and precore gene mutations that reduce or abolish the synthesis of hepatitis B e antigen (HBeAg). We found that precore promoter mutations diminished the expression of HBeAg but did not affect the synthesis of pregenomic RNA. However, these precore mutations were associated with a modest increase in HBV replication. In contrast, a naturally occurring mutant that carries a termination codon in position 28 of the precore open reading frame demonstrated increased encapsidation of pregenomic mRNA into nucleocapsid particles. Consequently, this variant viral strain demonstrated a substantial increase in the level of viral replication compared to “wild-type” HBV and other precore promoter mutant viral strains. These studies suggest that substitutions in the precore promoter and precore gene not only alter the synthesis of HBeAg but also affect the level of viral replication.

INTRODUCTION

The hepatitis B virus (HBV) is the prototype member of the hepadnavirus family (McLachlan, 1991). HBV is a major cause of acute and chronic hepatitis and persistent infection is often associated with the development of liver cirrhosis and hepatocellular carcinoma (Lau and Wright, 1993; Wands and Blum, 1991). The viral genome is made of a partially double-stranded 3.2-kb DNA molecule arranged in a relaxed circular conformation. HBV encodes four partially overlapping open reading frames (ORF) designated as precore/core, polymerase, preS/s, and X. The precore gene product is a 25-kDa protein that contains at the amino terminus a signal peptide sequence that directs it to the secretory pathway within the cell (Ou et al., 1986). An intermediate 22-kDa protein is produced by cleavage of the 19-aa signal peptide and this species is either translocated into the lumen of the endoplasmic reticulum or released back into the cytoplasm (Garcia et al., 1988). The translocated p22 protein is further processed at the carboxy terminus to generate a 17-kDa soluble monomeric product that is secreted from the cell as the HBeAg (Standring, 1991). HBeAg is found in serum during active infection and generally correlates with the degree of viremia. Indeed, HBV DNA levels have been found to decline in serum following the development of an immune response to HBeAg (anti-HBe) (Carman and Thomas, 1993).

It is now recognized that chronic HBV infection is associated with the emergence of mutations throughout the viral genome that result in the generation of diverse viral populations or quasispecies. In this regard, viral genomes defective in HBeAg synthesis (HBeAg− strains) are frequently found during persistent viral infection. The biologic properties of these naturally occurring HBV mutants is controversial. They have been linked to fulminating hepatitis and associated with high levels of viral replication (Carman et al., 1991; Liang et al., 1991; Omata et al., 1991). Moreover, such viral strains have been found in individuals with chronic hepatitis B (Brunetto et al., 1989; Carman et al., 1989; Naoumov et al., 1992; Okamoto et al., 1990; Tong et al., 1990). At the molecular level, the most prevalent mutation described is a transition from TGG to TAG that introduces an amber termination signal at codon 28 in the precore ORF. Some studies have reported that these mutations result in increased levels of viral replication (Lamberts et al., 1993; Scaglioni et al., 1997); however, no effects on HBV DNA synthesis have been reported in other studies (Yuan et al., 1995; Hasegawa et al., 1994; Tong et al., 1992).

Point mutations in the precore promoter (Yu and Mertz, 1996) have been found as an isolated event or in association with the termination codon at position 28 in the pre-
core ORF (Okamoto et al., 1994; Sato et al., 1995; Laskus et al., 1995; Baumert et al., 1996). The most frequent mutations involve an A-to-T and a G-to-A exchange at nucleotides (nts) 1760 and 1762 in the precore promoter. A liver-enriched transcription factor has been found to bind to this region of the precore promoter (Guo et al., 1993; Zhang and McLachlan, 1994). A recent study demonstrated that a HBV genome carrying the mutations at nts 1760 and 1762 displayed reduced levels of HBeAg synthesis in vitro and was associated with enhanced viral replication (Buckwold et al., 1996). It has been reported that another double mutation at nts 1764 and 1766 may affect pregenomic RNA encapsidation, leading to increased levels of viral replication (Baumert et al., 1996). However, it should be noted that different HBV genotypes have been studied and some of the differences found in viral replication may be the result of mutations in other regions of the viral genome. In the present report, we investigated the functional consequences of introducing mutations in the precore promoter/precore gene of a "wild-type" HBV genome (Galibert et al., 1979) characterized as the D genotype (Norder et al., 1994). The biologic effects of these mutations were assessed with respect to viral replication, antigen secretion, viral mRNA synthesis, and pregenomic RNA encapsidation.

MATERIALS AND METHODS

Plasmid constructs

The plasmid vectors used in this study are depicted in Fig. 1. All constructs were derived from the previously described payw1.2 plasmid (Scaglioni et al., 1997). This construct consists of a more-than-one HBV-genome-length sequence of genotype D (Galibert et al., 1979; Norder et al., 1994) from nts 1411 to 2327 (where nt 1 is by convention located at the GAATT of the unique EcoRI site) and has been cloned into the Aattl and Smal sites of the pGEM7 Zf(+) vector (Promega Corp., Madison, WI). The HBV RNA transcripts are expressed under the control of endogenous viral regulatory sequences.

The construct payw*28AB has a 0.9-kb Aattl–BspEI fragment insert from the pC*28 plasmid. The pC*28 construct contains a dimer of a HBV DNA derived from serum of an individual with a naturally occurring HBeAg− mutant HBV infection (Tong et al., 1992). This mutant carries the TGG to TAG nonsense mutation which introduces a termination codon in the precore ORF at codon 28. In addition, this DNA fragment also carries 12 other nucleotide substitutions upstream of and including the precore promoter and other 21 mutations in the precore/core ORF. In particular, this HBV carries the mutated sequence ATGAT (nts 1759–1763) (wild-type D genotype is AAGGT) that has been described in naturally occurring HBV variants, either alone or in combination with the stop codon mutation in the precore ORF at codon 28 (Okamoto et al., 1994). The plasmid paywFB was generated by exchanging the FspI–BspEI (nts 1798–2327) fragment from pC*28 into payw1.2. This plasmid has the stop codon at position 28 in the precore ORF and the other aforementioned precore/core ORF mutations but contains a wild-type precore and core promoter sequence. The paywOM construct was generated by introducing an Aattl–FspI fragment derived from pC*28 into payw1.2. Therefore, this construct has 12 nucleotide substitutions upstream of and including the precore promoter mutations with the sequence ATGAT (nts 1759–1763) but contains the wild-type HBV precore/core genes. The plasmid paywXB was obtained by introducing an Xbal–BspEI (nts 1988–2327) fragment obtained from pC*28 into the payw1.2 construct. This plasmid contains 12 mutations in the core gene.

Specific point mutations were created in the wild-type HBV of D genotype by site-directed mutagenesis (pAlter system, Promega Corp.). In this regard, the paywOMPM carries the double mutation of A to T and G to A at nts 1760 and 1762, respectively. The paywFHPM carries a double mutation of C to T and of T to A at nts 1764 and 1766 in the precore promoter sequence. These mutations have been proposed to be important in the generation of a fulminant hepatitis B viral strain (Baumert et al., 1996). The payw*28PM carries the nonsense TGG to TAG mutation that introduces a stop codon in the precore ORF at codon 28. In addition, the plasmid pCMVHBV*28PM was generated to carry the nonsense TGG to TAG mutation into the pCMVHB vector that expresses the pregenomic RNA under the control of the CMV IE promoter (Fallows and Goff, 1995). This construct was designed as pCMVHBV*28PM. The pZeroDB plasmid was prepared by cloning the DraI–BglII (nts 1720–1982) HBV insert into the EcoRV–BamHI sites of the pZero-2.1 polylinker (Invitrogen, San Diego, CA). The correct design of the plasmids was assessed by restriction enzyme mapping and by direct DNA sequencing. The restriction enzymes were purchased from New England Biolabs (Beverly, MA). Sequence reactions were carried out with the Sequenase Version 2.0 enzyme (U.S. Biochemicals, Cleveland, OH). Plasmids were grown in JM109 Escherichia coli cells and purified by a commercially available kit using the manufacturer's instructions (Wizard maxi-prep kit; Promega Corp.).

Tissue culture

The HepG2 and HuH-7 cell lines were transiently transfected by means of the calcium phosphate method (CaPO4 transfection Kit, 5’-3’, Inc., Boulder, CO) with 10 μg of plasmid expressing wild-type or mutant HBV DNA. The transfection procedure and maintenance of these cell lines in tissue culture have been previously described (Scaglioni et al., 1994). Transfection efficiency was monitored by adding 1 μg of pCMV luciferase construct, and approximately 1/100 of the cell lysate was subjected to a luciferase assay (Ausubel et al., 1989). Experiments were discharged if luciferase activity varied more than 20% between the cell lysates derived from
transfected cells. The quantity of sample to be analyzed was adjusted according to the level of luciferase activity. Moreover, the HBsAg concentration was measured in the culture supernatant and was used as a second independent assay to determine transfection efficiency. Every transfection experiment was repeated at least three times with two different plasmid preparations to ensure reproducibility of the results. Following transfection of cells, viral RNA and DNA was extracted at 2 and 5 days, respectively.

Analysis of viral replication

Levels of DNA replication were determined by Southern blot analysis. HBV DNA was extracted from purified core particles following transient transfection as described (Pugh et al., 1988). Prehybridization, hybridization, and washing of the Southern blots were performed as previously described (Melegari et al., 1994). The bands on the gels were analyzed with a Phospholmager system and the results obtained from at least three independent experiments were averaged (Molecular Imaging System, Model GS-363, Bio-Rad Laboratories, Inc., Hercules, CA).

Measurement of viral antigens

The presence of HBV antigens was measured in the supernatant of cells 5 days after transient transfection with wild-type and mutant viral genomes. The HBeAg assay was performed using a specific radioimmunoassay (RIA) kit (EBK RIA KIT, Incstar Corp., Stillwater, MN). It should be emphasized that the monoclonal antibody used in this kit is specific for HBeAg and does not cross-react with the HBV core protein. Measurement of HBsAg in the cell culture supernatants was performed using a radioimmunometric assay as previously described (Melegari et al., 1994). All measurements were performed in the linear range of the assay so as to obtain quantitative results.

Western blot analysis

Western blot analysis of lysates derived from transiently transfected cells was performed as described (Scaglioni et al., 1997). HBV core protein was revealed by polyclonal rabbit anti-core antiserum (Dako Co., Carpinteria, CA).
Extraction and analysis of viral RNA

Total RNA was extracted 2 days after transient transfection as previously described (Scaglioni et al., 1994). Core particles derived from the cytoplasm of transfected cells were immunoprecipitated by a rabbit polyclonal anti-core antibody (Dako Corp.), followed by protein A-Sepharose precipitation. A digestion with 16 U of RNase-free RQ1 DNase was carried out on the pelleted beads followed by an additional wash in the presence of 50 mM EDTA. After these procedures the encapsidated viral RNA was extracted as described (Scaglioni et al., 1994).

A RNase protection assay was performed on encapsidated viral RNA using a commercially available kit (RPA II-Ribonuclease protection kit, Ambion Inc., Austin, TX). The viral-specific RNA probe was obtained by incubation of the XhoI linearized pZeroDB with SP6 polymerase (Promega Corp.) in the presence of [α-32P]UTP (100 mCi at 3000 Ci/mmol, New England Nuclear, Boston, MA) as described (Ausubel et al., 1989). The resulting RNA probe contains a 262-nt specific antisense HBV sequence and a 57-nt plasmid sequence that will not be protected by digestion after hybridization with the HBV RNAs. This probe is predicted to protect from RNase digestion two segments of the pregenomic RNA: a 166- and a 200-nt species located at the 3’ and 5’ ends, respectively, of the terminally redundant region of the pregenomic RNA (Nassal et al., 1990; McLachlan, 1991). Hybridizations and RNase digestions were performed as previously described using encapsidated pregenomic RNA derived from a 10-cm dish of transiently transfected HuH-7 cells (Scaglioni et al., 1994). The RNA fragments protected by RNase digestion were separated on a 6% polyacrylamide denaturing gel. Northern blot analysis of total cellular RNA was performed as previously described (Scaglioni et al., 1997). The bands on the gels were analyzed by phosphoimaging (Molecular Imaging System Model GS-363, Bio-Rad Laboratories, Inc.).

RESULTS

HBV genomes carrying naturally occurring mutations in the precore promoter and precore gene display an enhanced replication phenotype

Increased levels of HBV replication were observed in HCC cells transiently transfected with a more-than-one-HBV-genome-length vector containing naturally occurring mutations in the precore promoter and the precore gene. Figure 2 shows a representative experiment. Southern blot analysis of purified HBV core particles demonstrated that transfection of payw*28AB in HuH-7 cells resulted in a fivefold increase (average of three experiments) in HBV DNA replicative forms as compared to the wild-type payw1.2 construct (Fig. 2). To characterize the specific mutation(s) responsible for this high-replication phenotype, smaller fragments of the AatII-BspEI DNA insert containing the mutations were exchanged into the parental payw1.2 wild-type construct. The paywFB construct also exhibited a similar high-replication phenotype as compared to the payw*28AB genome (Fig. 2), while the paywOM construct exhibited an average twofold increase in the level of replication when compared to the wild-type payw1.2 HBV. The paywXB construct contains mutations only in the central portion of the core gene. Transient transfection experiments demonstrated that the paywXB displays very similar replication levels to wild-type payw1.2 HBV. Transfection experiments were performed in both HepG2 and HuH-7 HCC cell lines to exclude the possibility that the differences in viral replication were related to cell type and similar results were obtained with both cell lines (data not shown).
mutations in the wild-type payw1.2 HBV genome closely resembled the findings described above with the naturally occurring viral mutants.

We also compared the pattern of HBV replication of paywFHPM to wild-type payw1.2. This plasmid was engineered to contain a double-nucleotide substitution in the precore promoter region that has been recently described as important in conferring a high-replication phenotype in a “fulminant” hepatitis B viral strain (Baumert et al., 1996). This construct directed the synthesis of a twofold (average of three experiments) increase of viral replicative forms as compared to wild-type payw1.2. However, the levels are much lower than those found with payw*28PM (Fig. 3). To ascertain that the introduction of the nonsense mutation resulting in a stop codon at position 28 of the precore gene did not affect the encapsidation efficiency and DNA priming of the pregenomic RNA, this same mutation was introduced into the pCMVHBV construct. This plasmid does not express precore mRNA and as a consequence does not synthesize HBeAg. The pCMVHBV and pCMVHBV*28 exhibited similar levels of HBV replication following transfection into HuH-7 cells (Fig. 3).

Effect of the mutations in the precore promoter and precore ORF on viral antigen production

We determined if point mutations introduced either into the precore promoter region or into the precore gene affected the level of viral antigen synthesis. Figure 4 shows the results obtained in five independent experiments. We have previously found that overexpression of the precore protein did not alter HBsAg secretion (Scaglioni et al., 1997). Thus, as expected, HBsAg secretion was not affected by any of the mutations as assessed in HCC cell lines (Figs. 2 and 3). In addition, HBeAg was undetectable in cell culture supernatant derived from cells transfected with payw*28, pCMVHBV, and pCMVHBV*28PM. HBeAg was greatly reduced in the supernatant of cells transfected with plasmids that contained precore promoter mutations as represented by paywOMPM and paywFHPM. neg. represents cell culture supernatant derived from mock DNA transfected cells.

A stop codon in the precore ORF generates a high-replication viral phenotype

It was necessary to confirm that the above-described mutations were directly responsible for the high-replication phenotype. Therefore, we introduced defined nucleotide substitutions by site-directed mutagenesis. We found that payw*28PM exhibited a 5-fold increase (average of three experiments) in the level of viral replication compared to the wild-type payw1.2 (Fig. 3). In addition, the plasmid aywFB showed a similar replication capacity as represented by a 3.5-fold increase in viral replication illustrated in Fig. 3. In contrast, paywOMPM displayed only a 2-fold increase in viral replication. Therefore, the phenotype exhibited by these artificially created point mutations in the wild-type payw1.2 HBV genome closely resembled the findings described above with the naturally occurring viral mutants.

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Precore promoter and precore ORF mutations result in increased levels of encapsidated pregenomic RNA

We determined if precore promoter and precore ORF mutations affect viral gene expression. Northern blot analysis performed on total cellular RNA revealed no differences in viral gene expression between wild-type payw1.2 and
a fivefold increase in HBV DNA synthesis was associated with a mutant viral strain that contains a precore stop codon mutation at position 28 whereas a twofold increase in HBV DNA replication was found to be associated with specific mutations in the precore promoter region. To confirm that either one of these naturally occurring mutations was responsible for a high-replication phenotype, we engineered the same mutations by site-directed mutagenesis into the wild-type HBV as well.

First, the biologic consequences of these mutations on expression of HBV antigens were explored. It was found that the precore promoter mutation reduced HBeAg synthesis to about 30% in HepG2 and 20% in HuH-7 cells as compared to levels produced by wild-type HBV. There was no effect of these mutations on HBsAg secretion or on the intracellular levels of HBV core protein. As expected, the precore stop codon mutation at position 28 abolished HBeAg synthesis but did not affect the synthesis of pregenomic RNA. These observations demonstrate that the precore promoter and precore gene mutations do not alter the expression of other HBV genes. Our results are in general agreement with recent findings indicating that precore promoter mutations will result in decreased levels of precore mRNA and results in enhanced HBV replication (Buckwold et al., 1992).
al., 1996). Such studies are consistent with a recent observation indicating that the promoter elements used in the synthesis of the precore and pregenomic RNAs are genetically distinct and separately regulated (Yu and Mertz, 1996).

Although the amount of pregenomic RNA present in the cytoplasm was unchanged when comparing the levels produced by wild-type and mutant viruses, the concentration of pregenomic RNA encapsidated into core particles was increased fivefold in the payw*28PM construct that contains the precore stop codon and only twofold in the paywOMP construct that contains the precore promoter mutations. To rule out the possibility that the TGG to TAG transition that introduced the stop codon at position 28 in the precore ORF was responsible for increasing the encapsidation efficiency of the pregenomic RNA, the same mutation was introduced into pCMVHBV, a plasmid that expresses an authentic pregenomic RNA but does not synthesize any precore RNA species (Fallows and Goff, 1994). Our finding indicating that this plasmid does not synthesize any detectable HBeAg following transient transfection in HuH7 and HepG2 cells suggests that the transfected DNA is the major template for viral transcription in this tissue culture system and not covalently closed circular HBV DNA (cccDNA). Indeed, under these experimental conditions, there was no increase in HBV replicative forms, indicating that the stop codon mutation did not alter the encapsidation efficiency of the pregenomic RNA (Nassal et al., 1990; Tong et al., 1992) or the DNA priming activity of the viral polymerase (Wang and Seeger, 1993). It appears that the levels of HBV replication are inversely related to the expression of the precore protein. It has recently been shown that the expression of the precore protein can suppress HBV replication both in transfected HCC cells and in a HBV transgenic mouse model (Lamberts et al., 1993; Guidotti et al., 1996; Scaglioni et al., 1997).

HBeAg+ HBV genomes have been associated with fulminant hepatitis and high levels of viremia as well as with chronic infection. A close association of these mutant genomes with severe acute or chronic liver disease has been observed in the Mediterranean countries and in the Asian Pacific region. It is of interest that in these areas of the world, the most frequent HBV strain is the D genotype, which is prone to develop the precore stop codon mutation at position 28. In the rest of Europe and the United States, the HBV A genotype is the most prominent viral strain and it appears that this viral species rarely develops the precore stop codon at position 28 because such a mutation appears to disrupt the viral encapsidation signal (Li et al., 1993).

Therefore, some mutations appear to be genotype-specific, such as the precore stop codon substitution in genotype D. These mutations may result in different biologic properties of the variant viral strain as shown by this investigation. Alternatively, the same mutation may confer a different phenotype such as that observed with the fulminant hepatitis B mutant strain described by Baumert et al. (1996). In their experiments with a genotype A virus, the precore promoter mutations appeared to produce a high-replication phenotype whereas in our experiments with a genotype D virus the same mutations led to only a twofold increase in viral replication. However, in both viral strains, the trend toward a higher replication phenotype was observed with these precore promoter mutations.

The enhanced replication exhibited by the mutant virus containing the precore stop codon mutation at position 28 described in the present investigation may have important biologic consequences in vivo. The increased level of viral replicative forms may lead to accumulation of viral antigens within infected hepatocytes and promote liver injury through an enhanced host immune response (Chisari and Ferrari, 1995). Increased levels of mutant viral replicative forms may lead to accumulation of cccDNA in the nucleus and result in increased cellular cytopathic effects as well (Lenhoff and Summers, 1994). Finally, in vitro and in vivo studies with well-defined mutations in HBV strains of known genotype will be required to assess the potential pathogenic role of alterations in precore promoter, precore, and preS gene regions with respect to their effects on viral replication either alone or in combination.

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