Oligomer synthesis by priming deficient polymerase in hepatitis B virus core particle

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Abstract

Hepadnavirus DNA polymerase functions in DNA synthesis and encapsidation, and acts as a primer for minus-strand DNA synthesis. Through protein priming reaction, a short DNA oligomer synthesized from the bulge of epsilon (ε) as template is covalently attached to the Tyr residue in the terminal protein (TP) domain of DNA polymerase. Using endogenous polymerase assays and native agarose gel analysis, we detected endogenous polymerase activity in priming-deficient mutant core particles, but not in reverse transcriptase (RT) reaction- or P protein-deficient mutant core particles. In addition, priming-deficient mutant core particles incorporated radiolabeled [32P-dATP, [32P-TTP, and [32P-dGTP, but not [32P-dCTP. Our results suggest that the priming-deficient mutant P protein has the ability to synthesize oligomers (presumably nascent minus-strand DNA) in the absence of covalent linkage between TP and the first deoxynucleotide. We propose that the priming-deficient mutant may be defective in minus-strand DNA translocation to direct repeat (DR) 1 at the 3’ end of pregenomic RNA (pgRNA) that leads to the elongation of minus-strand DNA.

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Introduction

Hepadnaviruses are small, enveloped DNA viruses that replicate preferentially in liver cells and are involved in hepatocellular carcinoma (Wands and Blum, 1991). Hepatitis B virus (HBV), a prototype of hepadnavirus, has a partially duplex relaxed circular DNA genome, which is replicated by reverse transcription of the RNA genome to produce DNA. Pregenomic RNA (pgRNA) serves as an mRNA for capsid (C) and polymerase (P) proteins and as a template for reverse transcription (Ganem and Schneider, 2001). Additionally, pgRNA is selectively encapsidated into core particles with P protein (Bartenschlager and Schaller, 1992; Bartenschlager et al., 1990; Hirsch et al., 1990).

Hepadnavirus P, a multifunctional protein, plays a role in HBV DNA synthesis and encapsidation of pgRNA (Bartenschlager et al., 1990; Hirsch et al., 1990), and acts as a primer for minus-strand DNA synthesis (Wang and Seeger, 1992; Weber et al., 1994; Zoulim and Seeger, 1994). The P protein has four domains, specifically, terminal protein (TP), spacer, reverse transcriptase (RT), and RNase H. The TP domain is covalently linked to minus-strand DNA upon protein-primed initiation of reverse transcription (Bartenschlager and Schaller, 1988; Radziwill et al., 1990; Wang and Seeger, 1992; Weber et al., 1994; Zoulim and Seeger, 1994). The spacer region connects the TP and RT/RNase H domains. This region is highly divergent between hepadnaviruses and appears to be nonessential because substitution, deletion, and insertion mutations are tolerated (Bartenschlager and Schaller, 1988; Chang et al., 1990). RT and RNase H regions of HBV polymerase display homology with RT of retrovirus, which has an active and conserved motif for RNA-dependent DNA polymerase (YMDD) and a conserved RNase H motif (Chang et al., 1990; Hirsch et al., 1990).

Hepadnavirus P protein recognizes the epsilon (ε) sequence, which is the encapsidation signal of pgRNA. A P-ε ribonucleoprotein (RNP) complex is formed via structural
altered in both pgRNA and P protein (Beck and Nassal, 1998; Tavis and Ganem, 1996; Tavis et al., 1998). It is believed that chaperones and ATP assist the RNP complex to adopt the right conformation for encapsidation, and the priming reaction (initiation of reverse transcription) occurs at this stage (Hu and Seeger, 1996; Hu et al., 1997). Before or after the priming reaction, the RNP complex is recognized and encapsidated by dimers of C proteins to form core particles. Reverse transcription occurs inside the core particle. Phosphodiester bonds are observed between the hydroxyl group of Tyr in the TP domain and the first nucleotide involved in the priming of reverse transcription (Lanford et al., 1997; Weber et al., 1994; Zoulim and Seeger, 1994). Three or two nucleotides are subsequently copied from the bulge region of ε to make TGAA or GAA, the nascent minus-strand DNA. Following the synthesis of nascent minus-strand DNA, the oligomer covalently linked to the P protein translocates from ε to direct repeat (DR) 1 at the 3′ end of pgRNA, leading to the elongation of minus-strand DNA (Tavis and Ganem, 1995; Tavis et al., 1994).

Mutations in the YMDD motif abolish reverse transcriptase activity with no effect on pgRNA packaging (Bartenschlager and Schaller, 1992; Bartenschlager et al., 1990; Chang et al., 1990; Hirsch et al., 1990; Radziwill et al., 1990; Roychoudhury et al., 1991). In DHBV, a priming-deficient P protein (Tyr96 Phe mutant) forms a P-ε ribonucleoprotein (RNP) complex (Wang et al., 1994) and encapsidates pgRNA into core particles in vivo (Weber et al., 1994). The mutant DHBV P protein displays polymerase activity and synthesizes DNA from exogenous RNA in vitro (the trans reaction) in an epsilon- and template-dependent manner (Tavis et al., 1998; Wang et al., 1994), although HBV DNA synthesis is completely abolished (Tavis et al., 1998; Weber et al., 1994). In view of these findings, it is suggested that DNA synthesis occurs in the absence of the protein priming reaction (Tavis et al., 1998; Wang et al., 1994). It is additionally suggested that the P protein (particularly the TP region) is involved in minus-strand translocation (Gong et al., 2000).

Using a new assay system that combines endogenous polymerase assay (EPA) and native agarose gel analysis, we demonstrate that a priming-deficient mutant P protein inside core particles can synthesize oligomers from the bulge region of ε as a template in the absence of protein-priming reaction. Our results indicate that priming-deficient mutant P protein has polymerase activity. We suggest that the covalent linkage between TP and the first nucleotide is not a prerequisite for initiating oligomer synthesis. However, this linkage is required for the elongation of minus-strand DNA synthesis via guiding nascent minus-strand DNA to DR1 at the 3′ end of pgRNA. Thus, it appears that the priming-deficient mutant P protein is defective in nascent minus-strand DNA translocation to DR1.

Results and discussion

HBV expression and pregenomic RNA encapsidation

To investigate the initiation of HBV DNA synthesis, we prepared a HBV wild-type (wt) construct of subtype adw R9 (Blum et al., 1991) in which transcription of pgRNA is controlled by a cytomegalovirus immediate early (CMV IE) promoter. Next, a priming reaction-deficient TP Y65F mutant involving the alteration of Tyr65 in the TP domain of P protein to Phe was generated (Fig. 1). Additionally, P protein-deficient and RT reaction-deficient RT YMHA mutants were constructed in which the AUG start codon was changed and frame-shifted, and the conserved YMDD reverse transcriptase motif was modified to YMHA, respectively (Fig. 1).

The in vitro priming reaction was abolished in the TP Tyr96 Phe mutant of the DHBV P protein and Tyr96 has been identified as the amino acid with which self-priming occurs in DHBV P protein (Wang et al., 1994; Weber et al., 1994; Zoulim and Seeger, 1994). Tyr63 of the HBV subtype ayw P protein is involved in the in vitro priming reaction with HBV P protein expressed using the baculovirus system in insect cells (Lanford et al., 1997). Tyr65 of subtype adw of the HBV P protein is homologous to Tyr63 of HBV ayw and Tyr96 of DHBV.

We initially analyzed HBV RNA expression in wt or mutant-transfected HuH7 cells. Total RNA was analyzed by Northern blot analysis and the RNase protection assay (RPA). Upon transfection, pgRNA was expressed by the action of the CMV promoter. Subgenomic RNA sequences were synthesized under control of their authentic promoters. The 3.5-kb pgRNA, and 2.1- and 2.4-kb mRNA sequences of surface proteins were detected from wt or mutant-transfected cells (Fig. 2A). There were no significant differences in RNA expression levels between wt and mutant-transfected cells (Fig. 2A).

We attempted to detect core particles by native agarose gel electrophoresis and Western blotting using the anti-HBc antibody (Koschel et al., 2000). Core particles were detected from wt or mutant-transfected HuH7 cells that displayed similar mobility on a native agarose gel (Fig. 2B). This finding indicates that the P-deficient mutant can form core particles in the absence of P protein and pgRNA. We additionally show that wt, and priming and RT-deficient mutants form core particles with similar rates. A greater number of core particles were consistently detected from P-deficient mutants (Fig. 2B). We speculate that the P protein or RNP complexes downregulate core particle formation for encapsidation.

RPA was conducted to detect cytoplasmic and encapsidated pgRNA from wt, TP Y65F, or P-deficient mutant-transfected HuH7 cells. Similar levels of cytoplasmic pgRNA were observed in wt or mutant-transfected cells (Figs. 2A and 2C, lower panel). The pgRNA from isolated core particles were analyzed with RPA. Wt and TP Y65F
mutants encapsidated pgRNA (Fig. 2C, upper panel), while the P-deficient mutant was unable to encapsidate pgRNA (Fig. 2C). This finding confirms that both P and C proteins are required for encapsidation of pgRNA (Bartenschlager et al., 1990; Hirsch et al., 1990). Our data (Fig. 2C) indicate that priming-deficient mutant P proteins can encapsidate
pgRNA (Bartenschlager and Schaller, 1992; Weber et al., 1994). In view of earlier data that P proteins with mutations at the conserved YMDD motif in the RT domain encapsidate pgRNA (Bartenschlager and Schaller, 1992; Chang et al., 1990; Radziwill et al., 1990; Roychoudhury et al., 1991), we did not examine the RT YMHA mutant for encapsidated pgRNA.

To investigate HBV DNA replication, cytoplasmic core particles were isolated from HuH7 cells transfected with HBV wt or mutants. Following extraction of HBV DNA from isolated core particles, Southern blot analysis was performed to detect replicating HBV DNA. Replicating HBV DNA was only observed in wt core particles with single-stranded, double-stranded, and partially double-stranded relaxed circular HBV DNA (Fig. 2D). We did not detect HBV DNA in TP Y65F, RT YMHA, or P-deficient mutant-transfected cells (Fig. 2D). Theoretically, the P-deficient mutant should be a dead virus. Due to the loss of ability of priming-deficient mutants to form covalent linkages with nucleotides (Lanford et al., 1997; Wang and Seeger, 1992; Weber et al., 1994; Zoulim and Seeger, 1994), the initial stage of replication was blocked in the DHBV Y96F mutant, and consequently DNA replication did not occur (Weber et al., 1994). Therefore, the HBV TP Y65F mutant is deficient in both the priming reaction and DNA replication. Mutation of the conserved reverse transcriptase motif YMDD to YMHA results in a dead virus that does not support HBV DNA synthesis in vivo (Chang et al., 1990; Hirsch et al., 1990).

Endogenous polymerase activity of priming-deficient mutant P protein

Previous studies show that the DHBV priming-deficient mutant P protein synthesizes DNA from exogenous RNA in vitro, suggesting that this P protein still displays polymerase activity (Tavis et al., 1998; Wang et al., 1994), although HBV DNA synthesis is completely abolished in vivo (Tavis et al., 1998; Weber et al., 1994). In view of these results, it is proposed that DNA synthesis can occur in the absence of protein priming (Tavis et al., 1998; Wang et al., 1994).

To determine whether the priming-deficient mutant P proteins of HBV have polymerase activity, we performed EPA by incubating the isolated wt and mutant core particles with $^{32}$P-dATP. Our rationale for this experiment is that free nucleotides should pass through core particles freely (which makes it possible to conduct EPA), but DNA or oligomers cannot pass through core particles easily. Consequently, radioactively labeled DNA or oligomers containing core particles after EPA should be detected by autoradiography following native agarose gel electrophoresis, but not core particles with free radioactive $^{32}$P-dATP. After the EPA reaction, reaction mixtures were electrophoresed on 1% native agarose gels (Koschel et al., 2000) and subjected to autoradiography. As expected, wt core particles were labeled with strong intensity (Fig. 3A). Surprisingly, TP Y65F mutant core particles were labeled with signals that were weak compared to wt, but strong enough to be detected by autoradiography (Fig. 3A). We did not detect radioactively labeled P-deficient or RT YMHA mutant core particles (Fig. 3A). This finding is inconsistent with previously reported results showing that the priming-deficient mutant has no endogenous polymerase activity (Bartenschlager and Schaller, 1992; Tavis et al., 1998; Weber et al., 1994). However, upon extracting $^{32}$P-labeled DNA from core particles after EPA, we detected single-, double-stranded, and partially double-stranded relaxed circular forms of HBV DNA only from HBV wt, but not from TP Y65F, P-deficient, and RT YMHA mutants (Fig. 3B). This finding is in agreement with data from our Southern blot analyses (Fig. 2D) and previous reports. We propose that the priming-deficient mutant P protein synthesizes the short oligomer by incorporating $^{32}$P-dATP into core particles. Our data collectively indicate that the TP Y65F mutant P protein synthesized DNA from RNA in vitro, suggesting that this P protein still displays polymerase activity (Tavis et al., 1998; Wang et al., 1994), although HBV DNA synthesis is completely abolished in vivo (Tavis et al., 1998; Weber et al., 1994). In view of these results, it is proposed that DNA synthesis can occur in the absence of protein priming (Tavis et al., 1998; Wang et al., 1994).
inside core particles has polymerase activity for oligomer synthesis.

*Nascent minus-strand DNA synthesis by priming-deficient mutant P protein*

Because the priming-deficient mutant P protein is defective in HBV DNA synthesis due to loss of covalent linkage between the first nucleotide and P protein, this mutant was excluded in the analysis of HBV DNA synthesis initiation. Previous studies attempted to identify HBV DNA from core particles by Southern blotting, EPA, or primer extension analyses.

Based on data on $^{32}$P-dATP-labeled TP Y65F mutant core particles (Fig. 3A), we hypothesize that the short oligomer synthesized from the bulge region of $\varepsilon$ template by the priming-deficient mutant P protein may be TGAA or GAA, despite the absence of covalent linkage between the P protein and the first nucleotide. To confirm our theory, we conducted another EPA of isolated core particles with different $^{32}$P-labeled nucleotides and unlabeled dNTP mixtures. Next, we analyzed labeled core particles on a native agarose gel (Koschel et al., 2000). There were no significant signaling differences among different $^{32}$P-labeled nucleotides from wt core particles (Fig. 4A). We easily detected priming-deficient mutant core particles labeled with $^{32}$P-dATP, $^{32}$P-TTP, and $^{32}$P-dGTP, but not $^{32}$P-dCTP (Fig. 4A).

In one experiment, $^{32}$P-dCTP-labeled priming-deficient mutant core particles were detected as a very faint band following longer exposure times (data not shown). We speculated that dCMP could be incorporated into the synthesized oligomer with very low efficiency by copying the bulge region of $\varepsilon$ (Fig. 5) because the oligomer synthesis was arrested at this stage (Schaaf et al., 1999). In the case of priming-deficient mutant, this arrest should be permanent because this mutant could not elongate HBV DNA. We did not detect labeled P-deficient and RT YMHA mutant core particles with different $^{32}$P-labeled nucleotides (Fig. 4A).

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**Fig. 4.** Endogenous polymerase assay (EPA) to identify nucleotides in a short oligomer synthesized by the TP Y65F mutant P protein. (A) Isolated core particles from HBV wt or mutant-transfected HuH7 cells were incubated with EPA reaction buffer supplemented with different $^{32}$P-labeled nucleotides and unlabeled dNTP mixtures at 37 °C overnight. Reaction mixtures were electrophoresed on a 1% agarose gel and subjected to autoradiography. (B) Isolated wt and priming-deficient mutant core particles were incubated with EPA reaction buffer supplemented with different $^{32}$P-labeled nucleotides and unlabeled dNTP mixtures with a certain dNTP omission at 37 °C overnight. To label the core particles, $\alpha$-$^{32}$P-dGTP (3000 Ci/mmol) was used. When we omit dGTP, $\alpha$-$^{32}$P-TTP (3000 Ci/mmol) was used. Reaction mixtures were electrophoresed on a 1% agarose gel and subjected to autoradiography.
This result supports the theory that oligomers synthesized by priming-deficient mutant P protein may be TGAA or GAA, presumably nascent minus-strand DNA (Fig. 5).

Next, we performed another EPA reactions with wt and priming-deficient mutant core particles with different 32P-labeled nucleotides and unlabeled dNTP mixtures with omission of a certain dNTP to demonstrate that the synthesized oligomer by priming-deficient mutant P protein was TGAA or GAA. Because it was not possible to extend wt HBV DNAs in EPA reactions with one nucleotide omission, the radiolabeled intensity of wt core particles was decreased significantly (Fig. 4B). When we omitted dCTP, the radiolabeled intensity of priming-deficient mutant core particles was not decreased (Fig. 4B). Without dCTP, the radiolabeled intensity of wt and priming-deficient mutant core particles was similar (Fig. 4B). This result strongly supports that the synthesized oligomer by priming-deficient mutant P protein would be TGAA or GAA, the nascent minus-strand DNA. As expected, we detected very faintly labeled priming-deficient mutant core particles when we omit TTP after EPA reaction (Fig. 4B). We could not exclude the possibility that these faintly labeled core particles without TTP might be labeled by the endogenous dNTPs (see below). We expected that we could not detect any radiolabeled priming-deficient mutant core particles when we omitted dATP or dGTP. But when we omitted dATP or dGTP, we often detected the priming-deficient mutant core particles as a very faint band (Fig. 4B), which was a little bit controversial to our hypothesis. In one experiment, we did not detect the priming-deficient mutant core particles when we omitted dATP or dGTP (data not shown). Then again, these core particles were isolated from HBV wt and mutant-transfected HuH7 cell lysates which contained the high concentration of endogenous dNTP pool (Beck and Nassal, 1998), and it was not possible to completely eliminate endogenous dNTP from the lysate. By utilizing endogenous dNTPs, these core particles could be labeled very faintly (Fig. 4B). But the labeled intensity of these priming-deficient mutant core particles was negligible compared to that of dCTP-omitted core particles (Fig. 4B), which support our hypothesis.

Because priming-deficient mutant P protein can encapsidate pgRNA, it was generally admitted that the tertiary structure of RNP complexes by wt and priming-deficient mutant P protein would be the same. Therefore, it would be unlikely to think that the oligomer was synthesized from the other region of pgRNA as a template. After priming reaction, the DNA synthesis is arrested (Schaaf et al., 1999), then the translocation occurs to elongate HBV DNA. During this process, it had been suggested that the conformation of P protein or RNP complex might be changed from a priming mode to an elongation mode (Gong et al., 2000). Because priming-deficient mutant P protein of DHBV could elongate DNA from exogenously added template RNA (Tavis et al., 1998; Wang et al., 1994), it would be reasonable to speculate that the priming-deficient mutant P protein of HBV may still retain the ability to elongate DNA.

Experiments with in vitro-expressed DHBV P protein show that the protein-priming reaction is divided in two stages, specifically, deoxynucleotide addition and elongation to synthesize nascent minus-strand DNA (Wang and Hu, 2002). These two stages are independent processes and possibly require different domains of the P protein (Wang and Hu, 2002).
Because newly synthesized oligomers are not covalently linked to TP, we propose that the priming-deficient TP Y65F mutant is defective in minus-strand translocation. Moreover, the P protein covalently linked with the oligomer functions to guide this nascent minus-strand DNA to DR1 at the 3' end of pgRNA for elongation of minus-strand HBV DNA (Fig. 5). It is reported that the DHBV TP region deletion mutant P protein has reduced ability in minus-strand translocation (Gong et al., 2000). Our data suggest that the covalent linkage through the Tyr residue of P protein and oligomer is a key element in this process.

In this study, we show that priming-deficient mutant HBV P protein can synthesize oligomers without covalent linkage between the Tyr residue of TP and the first deoxynucleotide. Moreover, covalent linkage may not be a prerequisite for the initiation of oligomer synthesis. This raises a very important question whether this P protein may have features of the RNA polymerase. This issue is beyond our reach, but it had been suggested that the DHBV P protein has several features that are parallel to T7 RNA polymerase (Schaaf et al., 1999). Also, it had been shown that the RT of DHBV P protein could bind NTP into its dNTP-binding pocket even with a single amino acid change that the RT of DHBV P protein could bind NTP into its dNTP-binding pocket even with a single amino acid change (Blum et al., 1991), was used to generate the partially redundant HBV genome. In another PCR reaction, the oligonucleotides used included reverse mutagenic primer HBV14 (complementary to HBV15) and forward primer HBVPS1 (5'-GAATTCCAGGGGTGGAAC-3') that binds nt 2907–2927 of the HBV genome. The resulting 0.26- and 0.44-kb products were combined for fusion PCR. The fusion PCR product was digested with BspEI and BstEII, and cloned among the corresponding restriction sites of pPB, yielding the priming-deficient HBV TP Y65F mutant.

To generate a P protein-deficient mutant of HBV, the P gene was frame-shifted and the AUG start codon was modified to ACG with a silent mutation in C ORF. To modify AUG to ACG, PCR was performed with the forward mutagenic primer HBV24 (5'-GGTCCGGAATGTTGA-TAAGATAGGGCGTTGGTGTC-3') specific for nt 2301–2339 of HBV and reverse primer PC3 (5'-CGAGTA- CATCAAGTGTAT-3') which binds to the CMV promoter region of pcDNA3. The PCR fragment was digested with BspEI and SnaBI, and cloned among the corresponding restriction sites of pPB, resulting in pPB ACG. To introduce a frame-shift mutation, pPB ACG was digested with BstEII and incubated with T4 DNA polymerase to fill in the 5' end, yielding a HBV P protein-deficient mutant.

For constructing the RT reaction-deficient RT YMHA mutant of HBV, the conserved YMDD motif was modified to YMHA by site-directed mutagenesis using a fusion PCR technique. The oligonucleotides employed were HBV15 (5'-GGTACTGAGGGCGCCAGTAAAAAGCC- CAGTAAAG-3'), a forward mutagenic primer that targets nt 2488–2517 and the reverse primer HBVc (5'-GGTCTCT-TTTGGAGTGTGGG-3') that binds nt 2259–2277 of the HBV genome. Another PCR reaction, the oligonucleotides used included reverse mutagenic primer HBV14 (complementary to HBV15) and forward primer HBVPS1 (5'-GAATTCCAGGGGTGGAAC-3') that binds nt 2907–2927 of the HBV genome. The resulting 0.26- and 0.44-kb products were combined for fusion PCR. The fusion PCR product was digested with BspEI and BstEII, and cloned among the corresponding restriction sites of pPB, yielding the priming-deficient HBV TP Y65F mutant.

A novel assay system has been developed to detect the initiation of HBV DNA replication inside core particles by combining EPA and native agarose gel analysis (Koschel et al., 2000). Using this system, it is possible to analyze numerous hepadnavirus DNA replication-deficient mutants that are reported to have no polymerase activity by conventional EPA.

**Materials and methods**

**DNA construction**

HBV subtype adw R9, 1.3 length of the HBV genome (Blum et al., 1991), was used to generate the partially redundant HBV construct. The 1.3 length of HBV adw R9 construct was cloned into pGEM4Z, yielding pGEM HBV. Next, the 5' end of HBV (from nucleotides 1418–1818) was deleted by PCR-derived mutagenesis. The 3' end (a total of 225 nucleotides) was additionally deleted by PCR-derived mutagenesis. To express HBV under the cytomegalovirus immediate early (CMV IE) promoter, partially redundant 3.37 kb of HBV was cloned into pcDNA3 (Invitrogen). For increasing encapsidation efficiency, 55 nucleotides of the pcDNA3 sequence were deleted (Jeong et al., 2000) by PCR-derived mutagenesis. The resulting construct encompassing wild-type (wt) HBV subtype adw R9 was designated pPB.

Tyr65 (TAT) in the TP domain of P protein was modified to Phe (TTT) by site-directed mutagenesis using a fusion PCR technique. The oligonucleotides employed were HBV15 (5'-GGTACTGAGGGCGCCAGTAAAAAGCC- CAGTAAAG-3'), a forward mutagenic primer that targets nt 2488–2517 and the reverse primer HBVc (5'-GGTCTCT-TTTGGAGTGTGGG-3') that binds nt 2259–2277 of the HBV genome. In another PCR reaction, the oligonucleotides used included reverse mutagenic primer HBV14 (complementary to HBV15) and forward primer HBVPS1 (5'-GAATTCCAGGGGTGGAAC-3') that binds nt 2907–2927 of the HBV genome. The resulting 0.26- and 0.44-kb products were combined for fusion PCR. The fusion PCR product was digested with BspEI and BstEII, and cloned among the corresponding restriction sites of pPB, yielding the priming-deficient HBV TP Y65F mutant.

To generate a P protein-deficient mutant of HBV, the P gene was frame-shifted and the AUG start codon was modified to ACG with a silent mutation in C ORF. To modify AUG to ACG, PCR was performed with the forward mutagenic primer HBV24 (5'-GGTCCGGAATGTTGA-TAAGATAGGGCGTTGGTGTC-3') specific for nt 2301–2339 of HBV and reverse primer PC3 (5'-CGAGTA-CATCAAGTGTAT-3') which binds to the CMV promoter region of pcDNA3. The PCR fragment was digested with BspEI and SnaBI, and cloned among the corresponding restriction sites of pPB, resulting in pPB ACG. To introduce a frame-shift mutation, pPB ACG was digested with BstEII and incubated with T4 DNA polymerase to fill in the 5' end, yielding a HBV P protein-deficient mutant.

For constructing the RT reaction-deficient RT YMHA mutant of HBV, the conserved YMDD motif was modified to YMHA by site-directed mutagenesis using a fusion PCR technique. The following oligonucleotides were used in the PCR reaction: HBV40 (5'-CTTTGGCCCAATACCA-CAGCATGCTATATAGCTGAAAGCCAAAC-3'), forward mutagenic primer specific for nt 722–765 and reverse primer HBV2 (5'-GCGGGTCACCATATTCTTGG-3') that targets nt 2259–2277 of the HBV genome. In another PCR reaction, the oligonucleotides used were reverse mutagenic primer HBV14 (complementary to HBV40) and forward primer HBV9 (5'-GGGAGGAGGACGACAATTG-3') which targets nt 1335–1354 of the HBV genome. The resulting 1.17- and 0.63-kb PCR products were combined for fusion PCR. The fusion PCR product was digested with EcoRI and EcoRV, and cloned into the corresponding restriction sites of pPB, yielding a HBV RT YMHA mutant. All constructs were sequenced to confirm the presence of specific mutations and to ensure that no extraneous mutations were introduced during PCR.

**Cell culture and transfection**

HuH7 hepatoma cell lines were used for the transfection of HBV wt and mutant clones. HuH7 cells were maintained in Dulbecco's modified eagle's medium supplemented with penicillin and streptomycin, and 10% fetal bovine serum. HBV wt and mutant plasmids (8 µg) were transfected into...
HuH7 cells on a 10-cm plate using Lipofectamine (Invitrogen) according to the manufacturer’s instructions.

Isolation of core particles

Three days after transfection, HuH7 cells were lysed in 1 ml TNE (10 mM Tris–HCl [pH 8.0], 50 mM NaCl, 1 mM EDTA)–1% Nonidet P-40. The clarified lysate was adjusted with 10 mM MgCl2 and 8 mM CaCl2 solution, and incubated overnight at 37 °C with 20 U DNase I (Sigma) and 60 U micrococcal nuclease (Calbiochem). Cytoplasmic core particles were precipitated with 6.5% polyethylene glycol.

RNase protection analysis (RPA)

To analyze encapsidated pgRNA, core particles were isolated as described above. pgRNA from core particles was extracted following proteinase K (100 µg/ml) and DNase I (20 U) treatment. To analyze cytoplasmic pgRNA, total RNA was extracted with RNA stat 60 (Tel-test, Inc.). To prepare riboprobe for RPA, part of the HBV sequence from pRPAFD. From this construct, 446 nt of radiolabeled anti-sense probe was synthesized in vitro using SP6 RNA polymerase with α-32P-UTP (specific activity, 800 Ci/mmol). The RPA procedure was performed using the manufacturer’s protocol (RPA II, Ambion). The protected pgRNA was 369 nt long.

Northern and Southern blotting

Total RNA (10 µg) was denatured and electrophoresed on a 1% agarose gel containing formaldehyde and blotted onto a nylon membrane. RNA on the membrane was hybridized to a 32P-labeled random-primed probe specific for the HBV sequence or GAPDH cDNA. To analyze HBV DNA synthesis by Southern blotting, core DNA was extracted, separated by agarose gel electrophoresis, and hybridized to a 32P-labeled random-primed probe specific for the HBV sequence.

Western blotting

Isolated core particles were electrophoresed on a 1% native agarose gel (Koschel et al., 2000). Core particles were transferred to polyvinylidene fluoride (PVDF) membranes as described for Northern blot analysis. Immunoblotting was performed using an anti-HBc antibody (DAKO). Horseradish peroxidase-conjugated anti-rabbit secondary antibody and enhanced chemical luminescence (ECL) were employed to visualize HBV core particles.

Endogenous polymerase assay (EPA)

Isolated core particles were incubated at 37 °C overnight with EPA reaction buffer (50 mM Tris–HCl [pH 7.5], 75 mM NH4Cl, 1 mM EDTA, 25 mM MgCl2, 0.1% β-mercaptoethanol, 0.5% Nonidet P-40) supplemented with 0.5 mM each of dCTP, dGTP, and TTP, and 10 µCi α-32P-dATP (3000 Ci/mmol). 32P-labeled reaction mixtures were electrophoresed on a 1% agarose gel and subjected to autoradiography. 32P-labeled DNA was extracted following the EPA reaction and separated by 1% agarose gel electrophoresis. EPA was additionally performed using 10 µCi α-32P-dCTP, α-32P-dGTP, α-32P-TTP, or α-32P-dATP with unlabeled dNTP mixtures. EPA was also performed using 10 µCi α-32P-dGTP or α-32P-TTP with unlabeled dNTP mixtures with a certain nucleotide omission.

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