Journal of General Virology (1997), **78**, 2055–2065. Printed in Great Britain

Effects of a frequent double-nucleotide basal core promoter mutation and its putative single-nucleotide precursor mutations on hepatitis B virus gene expression and replication

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The basal core promoter (BCP) of hepatitis B virus (HBV) directs the transcription of both precore RNA and core RNA which code for e antigen (HBeAg) and core antigen, respectively. A double mutation in the BCP which converts nucleotide (nt) 1762 from A to T and nt 1764 from G to A is frequently observed in patients with chronic hepatitis B. We recently demonstrated that this double mutation prevented the binding of a liver-enriched factor (LEF) to the BCP, suppressed only precore RNA transcription (and hence HBeAg expression), and enhanced progeny virus production. In order to understand the mechanism for the selection of this frequent double mutation, we have extended our previous studies to further characterize LEF and to compare the effects of this double-nucleotide mutation (M1) with each single-nucleotide mutation at nt 1762 (M2) and nt 1764 (M3). Our results indicate that LEF is likely composed of a heterodimer formed between the transcription factor chicken ovalbumin upstream promoter-transcription factor (COUP-TF) and an unidentified liver-enriched factor. Further studies reveal that both M1 and M2 prevent the binding of LEF to the BCP, suppress only precore RNA transcription, and increase the efficiency of progeny virus synthesis. In contrast, M3 retains some LEF binding activity, does not suppress HBV RNA transcription, and reduces slightly the efficiency of virus progeny synthesis. The reduced ability of M3 to replicate indicates that it has no selection advantage in itself at the level of the infected hepatocyte. In spite of its enhanced replication rate, M2 is rarely detected in HBV patients. This indicates the involvement of factors other than intracellular replication rates in the selection of these virus variants in the infected individual.

Introduction

Hepatitis B virus (HBV) can cause acute and chronic hepatitis and is also a major cause of hepatocellular carcinoma. This virus contains a 3·2 kb, partially double-stranded, circular DNA genome. This genome, which is converted to a covalently closed circular DNA molecule after infection, contains four open reading frames which code for at least seven viral gene products. The expression of HBV genes is regulated by four different promoters (for a review see Yen, 1993). The core promoter controls the transcription of both the core RNA and precore RNA.

The core RNA codes for the core protein, which is the viral capsid protein, and for the DNA polymerase. In addition, the core RNA also serves as the pregenomic RNA, which is packaged with the viral DNA polymerase into the core particle and subsequently reverse transcribed by the polymerase into a DNA copy. The precore RNA codes for the precore protein which contains the entire coding sequence of the core protein plus a leader sequence (the 'precore sequence') of 29 amino acids. This leader sequence contains a signal peptide which directs the precore protein to the endoplasmic reticulum (ER) for secretion (Bruss & Gerlich, 1988; Garcia *et al.*, 1988; Ou *et al.*, 1989). After removal of the signal peptide by signal peptidase located in the ER lumen, the precore protein is

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further cleaved at multiple sites at its carboxy terminus by aspartyl peptidase located in a post-ER compartment (Jean-Jean *et al.*, 1989; J. Wang *et al.*, 1991). The secreted precore protein derivatives are collectively called e antigen (HBeAg). HBeAg is not required for HBV replication as mutations preventing it from being expressed do not obviously affect HBV replication in animal models (Chang *et al.*, 1987; Chen *et al.*, 1992; Schlicht *et al.*, 1987; Tong *et al.*, 1991). Studies of babies born to HBeAg-negative ($HBeAg^-$) and HBeAg-positive ($HBeAg^+$) mothers suggest that HBeAg is important for the establishment of persistent infection following neonatal infection (Okada *et al.*, 1976).

The loss of HBeAg expression ($HBeAg^-$ phenotype) and the concomitant appearance of antibodies directed against it (anti-HBeAg) are frequently observed in chronically infected individuals (Pignatelli *et al.*, 1987). The most frequently detected genomic mutation which leads to the $HBeAg^-$ phenotype is the mutation of nucleotide (nt) 1896 from G to A (G → A). This mutation converts codon 28 of the precore sequence to a termination codon and thus prevents HBeAg from being expressed. Other mutations which can also cause the $HBeAg^-$ phenotype include other nonsense and frameshift mutations in the precore region (Carmen *et al.*, 1989; Hawkins *et al.*, 1994; Raimondo *et al.*, 1994; Tong *et al.*, 1990) and/or various mutations in the basal core promoter (BCP) (Kaneko *et al.*, 1995; Kidd-Ljunggren *et al.*, 1995; Kurosaki *et al.*, 1996; Laskus *et al.*, 1995; Okamoto *et al.*, 1994; Sato *et al.*, 1995; Takahashi *et al.*, 1995), which has been mapped between nt 1744 to 1804 (Yuh *et al.*, 1992; Zhang & McLachlan, 1994). The $HBeAg^-$ mutants often become the dominant virus quasispecies in the virus population present in HBV-infected individuals after the appearance of anti-HBeAg (Carmen *et al.*, 1993). These $HBeAg^-$ mutants may be selected for during chronic infection by the immune system (Carmen *et al.*, 1993) or by other mechanisms.

In addition to $HBeAg^-$ mutants, another group of HBV mutants with a reduced level of HBeAg expression has been recently reported. These 'HBeAg-suppressive' mutants contain a double-mutation of nt 1762 from A → T and nt 1764 from G → A in the BCP and are frequently the predominant HBV quasispecies observed in patients with chronic hepatitis symptoms (Kaneko *et al.*, 1995; Kidd-Ljunggren *et al.*, 1995; Kurosaki *et al.*, 1996; Laskus *et al.*, 1995; Okamoto *et al.*, 1994; Sato *et al.*, 1995; Takahashi *et al.*, 1995). Our recent studies revealed that this double mutation reduced the level of transcription of the precore RNA and hence the level of expression of HBeAg, yet had no effect on the expression of other HBV transcripts (Buckwold *et al.*, 1996). This indicates that the transcription of the precore RNA and core RNA can be uncoupled. Further analysis revealed that this double mutation prevented the binding of a liver-enriched factor(s) (LEF) to nt 1756 to 1766, suggesting a specific role for LEF in precore RNA transcription. Interestingly, the reduced HBeAg production due to this double mutation is associated with an increased efficiency of virus progeny production (Buckwold *et al.*, 1996). This increase of virus replication efficiency could be reversed in part by a precore expression plasmid supplied *in trans* in complementation experiments. These results indicate that the precore gene suppresses the replication of HBV and are in agreement with similar results reported by others (Guidotti *et al.*, 1996; Lamberts *et al.*, 1993). The precore gene most likely suppresses HBV progeny virus production at the encapsidation step because the level of pregenomic RNA packaged in the intracellular core particles of the HBV double mutant was found to be increased while the levels of pregenomes transcribed were similar between wild-type (WT) and mutant. The increase in replication rate may provide a selection advantage for this HBV double mutant during chronic infection (Buckwold *et al.*, 1996).

In this report, we describe our continuing research to characterize LEF, which apparently regulates precore RNA transcription, and an investigation into the mechanism that leads to the selection of the HBV nt 1762 A → T and nt 1764 G → A double-nucleotide mutant (M1 mutant) during chronic infection. To conduct these studies, we have created the nt 1762 A → T (M2 mutant) and the nt 1764 G → A (M3 mutant) single nucleotide HBV mutants. These two mutants, which are presumably intermediates for the generation of the M1 double mutant, are rarely detected in HBV patients. Our results show that the transcription factor chicken ovalbumin upstream promoter-transcription factor (COUP-TF), or a protein factor closely related to it, is a component of LEF. The M2 mutation, similar to the M1 double mutation, can prevent binding by LEF, reduce precore RNA transcription, and enhance progeny virus production. The M3 mutation, in contrast, retains some LEF binding activity, slightly enhances precore RNA transcription, and suppresses progeny virus synthesis. These results provide an explanation why M3 is not selected for over wild-type virus, and indicate that other factors are involved in the selection of M1 over M2 in infected patients.

Methods

■ **Plasmids.** The WT HBV construct pWTD contains a head-to-tail dimer of 3·2 kb HBV adw2 DNA (Valenzuela *et al.*, 1980) subcloned into the *Eco*RI site of the pUC19 vector, while the M1 dimer (pM1D) contains the same adw2 sequences with mutations of nt 1765 A → T and nt 1767 G → A, which correspond to the nt 1762 A → T and nt 1764 G → A mutations previously described in other HBV strains (Kaneko *et al.*, 1995; Kidd-Ljunggren *et al.*, 1995; Kurosaki *et al.*, 1996; Laskus *et al.*, 1995; Okamoto *et al.*, 1994; Sato *et al.*, 1995; Takahashi *et al.*, 1995). Our samples will be labelled as being nt 1762 and nt 1764 to avoid confusion. The construction of these two plasmids has been described (Buckwold *et al.*, 1996). Mutant dimers pM2D and pM3D were constructed in the same manner by M13-based site-directed mutagenesis (Ou *et al.*, 1989), using oligonucleotide M2 (5′ GAGGAGATTAGGTTAATGGTCTTTGTAT 3′) and oligonucleotide M3 (5′ GAGGAGATTAGGTTAAAGATCT-TTGTAT 3′), respectively. The former generates a nt 1762 A → T mutation, while the latter generates a nt 1764 G → A mutation. Plasmids were verified to be authentic mutant head-to-tail dimers by restriction digest analysis and direct sequencing.

pWTH contains the WT HBV sequence with a *Hin*dIII site engineered at nt 1820 for efficient end-labelling. pXGH5 contains a human growth hormone (HGH) reporter whose expression is under the control of the mouse metallothionein promoter. This plasmid was used as an internal control for monitoring transfection efficiency by measuring the levels of secreted HGH (Nichols Institute).

The plasmids used for *in vitro* transcription were as follows: pCMX-COUP (unpublished) and pMX-hRXRα(Kpn) (Mangelsdorf *et al.*, 1990), which contain the coding sequences of COUP-TF and human retinoid X responsive receptor (RXRα), respectively, were a gift of R. M. Evans (Salk Institute). These two plasmids were linearized with *Bam*HI and *Eco*RI, respectively, followed by transcription with RNA polymerase T7 (Promega). pSP64-HNF4 contains the hepatocyte nuclear factor-4 (HNF-4) coding sequence (Guo *et al.*, 1993). This plasmid was linearized with *Sph*I followed by transcription with RNA polymerase SP6 (Promega). pT7HMSHP (Seol *et al.*, 1996) and pT7lac-his-myc-MB67 (Baes *et al.*, 1996) contain the small heterodimer partner (SHP) and MB67 coding sequences, respectively. These two plasmids, which were a gift of D. D. Moore (Massachusetts General Hospital), contain both phage T7 promoter and terminator sequences, and were transcribed directly using RNA polymerase T7.

■ Cell culture and DNA transfections. Huh7 human hepatoma cells were grown in a 1:1 ratio of Dulbecco's modified Eagle's medium (DMEM) and F12 medium plus 5% foetal bovine serum (FBS). Cells were transfected with CsCl gradient-purified plasmid DNA by the calcium phosphate co-precipitation method (Guo *et al.*, 1993).

■ Electrophoretic mobility shift assays. Electrophoretic mobility shift assays (EMSA) were conducted as before (Buckwold *et al.*, 1996) with nuclear extracts or with protein factors synthesized *in vitro* using rabbit reticulocyte lysates (Promega). The probe used was either a [γ-^{32}P]ATP end-labelled DNA fragment purified from pWTH (nt 1688 to 1820, labelled at nt 1820), or an end-labelled double-stranded oligonucleotide containing one of the following sequences:

WT: 5′ GAGGAGATTAGGTTAAAGGTCTTTGTAT 3′

3′ CTCTAATCCAATTTCCAGAAACATAATC 5′

M1: 5′ GAGGAGATTAGGTTAATGATCTTTGTAT 3′

3′ CTCTAATCCAATTACTAGAAACATAATC 5′

M2: 5′ GAGGAGATTAGGTTAATGGTCTTTGTAT 3′

3′ CTCTAATCCAATTACCAGAAACATAATC 5′

M3: 5′ GAGGAGATTAGGTTAAAGATCTTTGTAT 3′

3′ CTCTAATCCAATTTCTAGAAACATAATC 5′

The WT sequence listed above corresponds to the HBV adw2 sequence from nt 1749 to nt 1776 for the positive strand and nt 1752 to nt 1779 for the negative strand. M1, M2 and M3 sequences are identical to the WT sequence except that M1 contains the nt 1762 A → T and nt 1764 G → A double mutation, M2 contains only the nt 1762 A → T single mutation, and M3 contains only the nt 1764 G → A mutation. The sequence of a nonspecific competitor (gift of S. Tahara and T. Dietlin) for EMSA was as follows:

5′ AGCTTAGGCCTATAAGAGTGATTGTCTAGAGTAC 3′

3′ ATCCGGCTATTCTCACTAACAGATCTCATG 5′

Individual reactions contained 5 μg of Huh7 nuclear extract or 1 μl *in vitro* translated proteins, 2·5 μg poly(dI–dC), 4 μl of 5 × Stefan's binding buffer (Buckwold *et al.*, 1996) and H_2O to 20 μl. These were incubated on ice for 10 min, followed by addition of the probe and incubation on ice for another 20 min. For the antibody-supershift assays, 1 μl of antibody was added next and the reactions were placed on ice for an additional 20 min. The antibodies used were: rabbit anti-human RXRα #5142 against amino acids 214 to 229 (gift of R. M. Evans, Salk Institute; Kliewer *et al.*, 1992); rabbit anti-N-terminal amino acids 2 to 21 of the human RXRα receptor [anti-RXRαD20 (Santa Cruz Biotechnology)]; rabbit anti-HNF-4 against the very C terminus of the protein (gift of F. M. Sladek; Sladek *et al.*, 1990); rabbit anti-COUP-TF (gift of M.-J. Tsai; Cooney *et al.*, 1992); and rabbit anti-X protein, which was used as a control. Samples were run on a 4% nondenaturing polyacrylamide gel for the full length BCP probe (nt 1688 to 1820) or on a 5% gel in the case of oligonucleotide probes.

■ Analysis of the effects of the mutations on HBV. RNA was isolated using RNeasy spin columns (Qiagen) according to the manufacturer's instructions. The probe used for Northern blots was a randomly primed, [^{32}P]dCTP-labelled X-gene probe (nt 1386 to 1988). Primer extension was performed as before (Buckwold *et al.*, 1996) using RNA extracted with RNAzolB (Biotecx) and the antisense oligonucleotide primer 5′ GGTGAGCAATGCTCAGGAGACTCTAAGG 3′ corresponding to nt 2051 to 2024 of the HBV genome (Zheng & Yen, 1994). The primer 5′ GCCACTGCAGCTAGGTGAGCGTCC 3′ was used for the primer extension for the HGH mRNA (Zheng & Yen, 1994). Southern blot analysis of excreted viral particles was performed as before (Buckwold *et al.*, 1996; Lenhoff & Summers, 1994). The polyethylene glycol-precipitated virus particles electrophoresed on a 1% agarose gel were transferred to Duralon-UV nylon membranes (Stratagene) and hybridized with a 3·2 kb random-primed, ^{32}P-labelled HBV genomic DNA fragment. All experiments were performed at least three times.

Results

Effects of the BCP mutations on protein factor binding

Our previous studies indicated that the nt 1762 A → T and nt 1764 G → A double mutation prevented LEF from binding to nt 1756 to 1766 in the BCP (Buckwold *et al.*, 1996). In order to understand why this double mutation is preferentially selected for over the individual nt 1762 A → T and nt 1764 G → A single mutations during chronic infection, we have used EMSA analysis to investigate the effect of these mutations on LEF binding to the BCP. The structure of the BCP and the mutations used in this study are shown in Fig. 1. As shown in Fig. 2(*a*), a major shifted band (labelled LEF) and one or more minor bands which migrated faster on the gel were detected when Huh7 hepatoma nuclear extract and an oligonucleotide containing the WT LEF binding sequence were used in the binding reaction. The major band was removed by competition with specific, but not by nonspecific competitor, indicating the specificity of this binding. This result is consistent with our previous finding which indicated that a liver-enriched protein factor bound to nt 1756 to 1766 (Buckwold *et al.*, 1996). As expected, the LEF band was not observed when we used an oligonucleotide containing the nt 1762 A → T and nt 1764 G → A double mutation (M1 mutation) (Fig. 2*b*). This signal was also not seen with an oligonucleotide containing the nt 1762 A → T single mutation (M2 mutation), but a weak shifted

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WT  gaggagattaggttaaAgGtctttgtat
M1                  T A
M2                  T G
M3                  A A
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Fig. 1. Structure of the BCP and the mutations used in the study. The structure of the BCP is shown with the transcriptional start sites of precore and core RNAs. The locations of the translation initiation ATG codons of precore and core proteins are also indicated. Below are the sequences of the mutants used in this study. The underlined sequence shows the binding site for LEF determined previously using methylation interference analysis (Buckwold *et al*., 1996). Only nt 1762 and nt 1764 are shown in capital letters. The sequence is that of HBV adw2 (Valenzuela *et al*., 1980). WT contains the wild-type sequence A (nt 1762) and G (nt 1764), M1 contains the double mutation to T (nt 1762) and A (nt 1764), M2 contains the single mutation of nt 1762 to T, and M3 contains the single mutation of nt 1764 to A. The sequences shown are those corresponding to the sense strand of the double-stranded oligonucleotides used in this study (see Methods). These mutations were also individually introduced into cloned head-to-tail dimeric constructs of HBV genome which were named pWTD, pM1D, pM2D and pM3D.

band with the same mobility was observed when the oligonucleotide containing the nt 1764 G → A single mutation (M3 mutation) was employed. Interestingly, a new group of shifted bands of low intensity was observed only with the M1 mutant. This binding event was also found to be specific in competition assays (data not shown), indicating that the double mutation created a new protein factor binding site(s).

COUP-TF is a component of LEF

As shown in Fig. 3(*a*), the LEF binding site has a high degree of sequence homology with the DR1 binding site of the steroid–thyroid hormone receptor superfamily (Williams & Franklyn, 1994). This DR1 element has an AGGTCA direct repeat separated by one base pair. Members of the steroid–thyroid hormone receptor superfamily including COUP-TF, liver-specific HNF-4, liver-specific MB67 (Baes *et al*., 1994), liver-enriched RXRα (Mangelsdorf & Evans, 1995; Tsai & O'Malley, 1994) and liver-specific SHP (Soel *et al*., 1996) can bind to DR1 or related sequence elements as homodimers or heterodimers.

An EMSA analysis using antibodies directed against COUP-TF, RXRα and HNF-4 was conducted to examine whether LEF contains any of these transcription factors. As

Fig. 2. Effects of the BCP mutations on protein factor binding. (*a*) Specificity of the LEF bandshift. WT oligonucleotide end-labelled with [γ-^{32}P]ATP was incubated without nuclear extract (lane 1), or with 5 μg Huh7 nuclear extract (lanes 2 to 8) and increasing concentrations of either unlabelled WT oligonucleotide as a specific competitor (10, 50 and 100 ng for lanes 3 to 5, respectively) or a nonspecific oligonucleotide (10 , 50 and 100 ng for lanes 6 to 8, respectively) and electrophoresed on a 5% native polyacrylamide gel. LEF marks the LEF-specific bandshift. Probe indicates the location of the free probe. (*b*) Effects of the BCP mutations on LEF binding. Double-stranded oligonucleotides containing the LEF binding site with WT, M1, M2, or M3 sequences were used as probes and incubated without (−) or with (+) 5 μg Huh7 nuclear extract. 'A' marks the M1-specific bandshifts; the asterisk indicates a nonspecific band; 'P' marks the location of free probe.

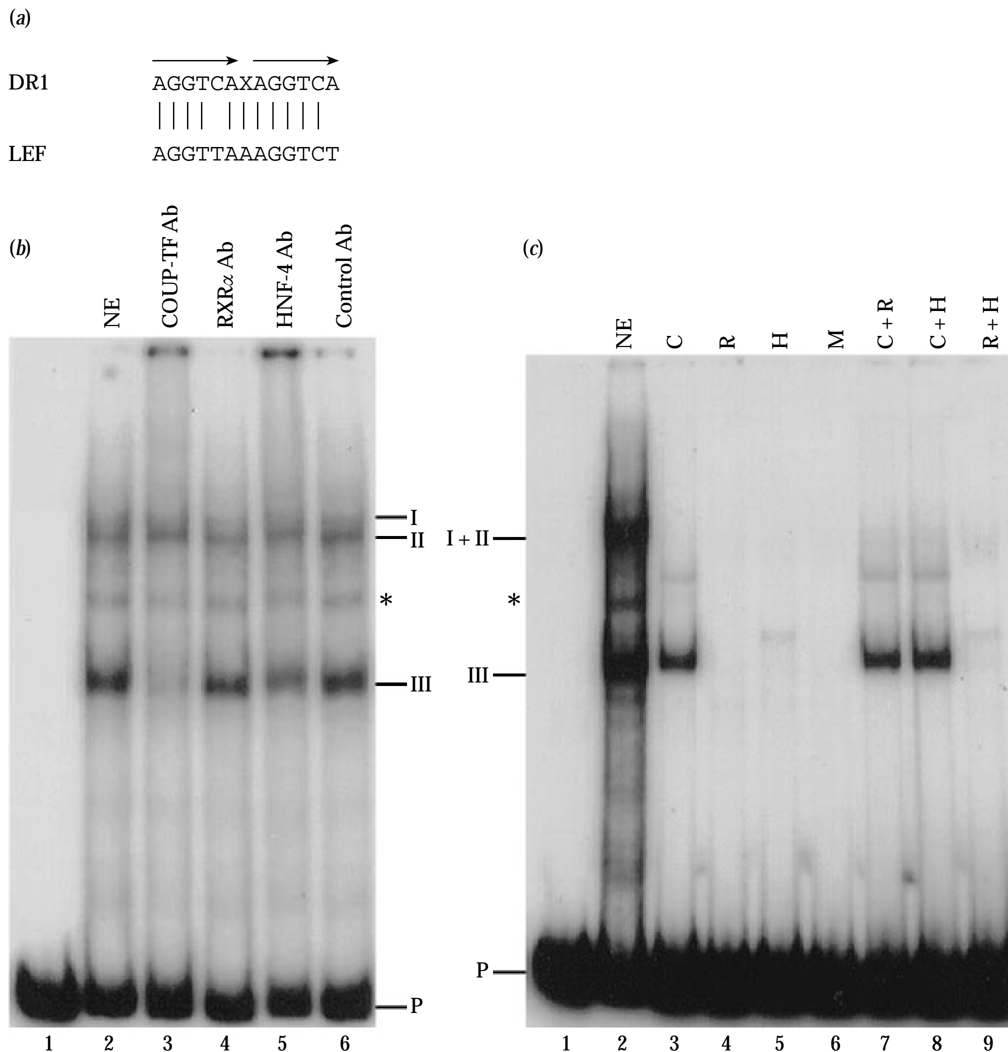

Fig. 3. COUP-TF is a component of LEF. (*a*) Sequence homology between DR1 and LEF. The steroid–thyroid hormone receptor superfamily consensus binding sequence DR1 direct repeat sequence AGGTCA with a single nucleotide spacer is shown. X indicates any nucleotide. Arrows show the repeat sequences. Below is the LEF sequence including the binding site determined previously using methylation interference analysis (underlined in Fig. 1). Sequence identities are indicated by vertical lines. (*b*) EMSA experiment using antibodies directed against various steroid hormone receptor superfamily members. EMSA was performed using an end-labelled whole BCP fragment from WT HBV (nt 1688 to 1820 of pWTH) and electrophoresed on a 4% native polyacrylamide gel. Lane 1, probe alone without nuclear extract; lanes 2 to 6, with 5 μg Huh7 nuclear extract; lane 3, with antibody (Ab) against COUP-TF; lane 4, with Ab against RXRα; lane 5, with Ab against HNF-4; lane 6, with a control Ab. I, II and III mark the locations of specific bandshifts while the asterisk denotes a nonspecific band (Buckwold *et al*., 1996). P marks the location of free probe. (*c*) BCP binding ability of *in vitro* translated steroid hormone receptor superfamily members. EMSA was performed as in (*b*) using 5 μg Huh7 nuclear extract or 1 μl of *in vitro* translated products. Lane 1, probe alone; lane 2, with Huh7 nuclear extract; lane 3, with *in vitro* translated COUP-TF (C); lane 4, with *in vitro* translated RXRα (R); lane 5, with *in vitro* translated HNF4 (H); lane 6, with a mock *in vitro* translation reaction (M); lane 7, co-translation of COUP-TF and RXRα (C+R); lane 8, co-translation of COUP-TF and HNF-4 (C+H); lane 9, co-translation of RXRα and HNF-4 (R+H).

shown in Fig. 3(*b*), multiple shifted bands were detected when the full-length wild-type BCP was used as the probe. Previously we demonstrated that complexes I, II and III were specific binding events, that the band denoted by an asterisk was due to non-specific binding, and that complex III was due to LEF binding (Buckwold *et al*., 1996). Most of the complex III (LEF) signal was removed in the lane containing antibody against COUP-TF (lane 2), indicating that COUP-TF or a closely related protein is a component of LEF. The antibodies against HNF-4 or RXRα did not significantly reduce the signals of complex III, indicating that these two protein factors are not major constituents of LEF. The specificities of these antibodies directed against COUP-TF (Cooney *et al*., 1992), HNF4 (Sladek *et al*., 1990) and RXRα (Kliewer *et al*., 1992) have previously been demonstrated. None of the antibodies affected the signals of complexes I and II.

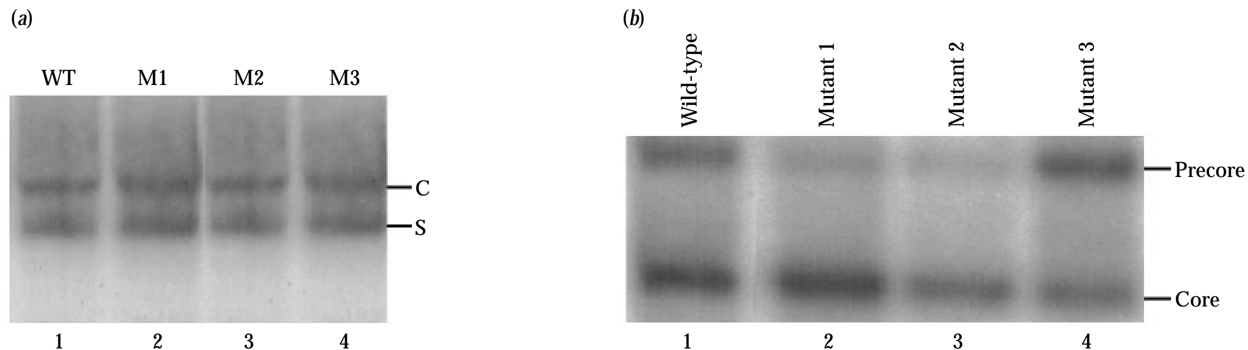

Fig. 4. Effects of the BCP mutations on HBV RNA transcription. (*a*) Northern blot analysis of WT and mutant HBV RNAs. Plates (100 mm) of Huh7 were transfected with 20 µg of pWTD (lane 1), pM1D (lane 2), pM2D (lane 3) or pM3D (lane 4) and 0·2 µg pXGH5 as an internal transfection control. RNA was collected and electrophoresed on a 1% agarose gel, blotted to nitrocellulose membrane, and probed with a ^{32}P-labelled X gene fragment. The locations of the core (C) and surface (S) gene transcripts are indicated. (*b*) Primer extension analysis of the effects of the introduced mutations on precore RNA transcription. RNA was collected from Huh7 cells transfected as in (*a*) and analysed by primer extension for precore and core transcripts. The positions of precore and core transcripts are indicated.

To further investigate whether COUP-TF and other members of the steroid–thyroid hormone receptor family bind to the LEF site in the BCP, we have performed an EMSA experiment using a variety of these hormone receptors synthesized *in vitro* with rabbit reticulocyte lysates. The synthesis and the size of these protein factors were verified by labelling these proteins with [^{35}S]methionine followed by gel electrophoresis (data not shown). As shown in Fig. 3(*c*), COUP-TF could indeed bind to the WT BCP probe while RXRα could not. HNF4 bound weakly to the probe and produced a weak shifted band that migrated slower than the band produced by Huh7 nuclear extract. No binding was observed with SHP or MB67 (data not shown). The mobility of the COUP-TF bandshift was slightly slower than that of complex III. This difference was more obvious when the BCP probe was replaced with the oligonucleotide probe containing the LEF site (data not shown). These results indicate that COUP-TF is related but not identical to LEF. To examine whether COUP-TF and other members of the steroid–thyroid hormone receptor family bind as heterodimers to the BCP, different combinations of these protein factors synthesized *in vitro* were used for the EMSA analysis. Among all the possible combinations of factors, only those that included COUP-TF could bind to the BCP. Some of the EMSA results of these combination experiments are shown in Fig. 3(*c*). Since the mobility and the intensity of the shifted signals generated by the combined factors that contained COUP-TF were identical to those generated by COUP-TF alone, it appears that COUP-TF does not bind to the BCP as heterodimers with these factors. Similar results were obtained when the full-length BCP probe was replaced with the oligonucleotide probe that contains the WT LEF site (data not shown). Since LEF is a liver-enriched factor (Buckwold *et al*., 1996) and COUP-TF is a ubiquitous factor (L. H. Wang *et al*., 1991), the results shown in Fig. 3(*b*) indicate that if COUP-TF is indeed a component of LEF, it must interact with other liver-enriched factor(s) to bind to the BCP.

Effect of the BCP mutations on HBV transcription

Since the double and single mutations all affected binding by LEF to the BCP, they may all affect precore RNA transcription. To investigate this possibility, head-to-tail dimers of the HBV genome containing the WT (pWTD), M1 (pM1D), M2 (pM2D) or M3 (pM3D) sequence were constructed and transfected into Huh7 human hepatoma cells. As shown in Fig. 4(*a*), Northern blot analysis of HBV RNA extracted from transfected cells revealed no gross changes in the expression of viral transcripts. Primer extension analysis was next employed to specifically analyse the transcription of precore and core RNA. As shown in Fig. 4(*b*), primer extension products of 260 nt and 230 nt corresponding to nt 1791 for the start site of precore RNA and nt 1821 for the start site of core RNA, respectively, were detected. These transcription start sites were the same as reported previously (Buckwold *et al*., 1996; Zheng & Yen, 1994). The levels of core RNA were not significantly different between different samples as were the levels of the HGH RNA transcribed from the plasmid pXGH5 (data not shown), which was co-transfected to monitor transfection efficiency. In agreement with our previous observation, the precore RNA level was reduced to about one-third that of WT for the M1 double mutant (Buckwold *et al*., 1996). There was a similar degree of reduction in precore RNA with the M2 mutant. In contrast, M3 showed no reduction and perhaps even a slight increase in precore RNA synthesis. There was no change in the transcription start sites for precore and core RNAs for all the DNA constructs tested.

Effect of the BCP mutations on virus replication

Next we examined the effects of the mutations on virus progeny synthesis by performing Southern blot analysis of the

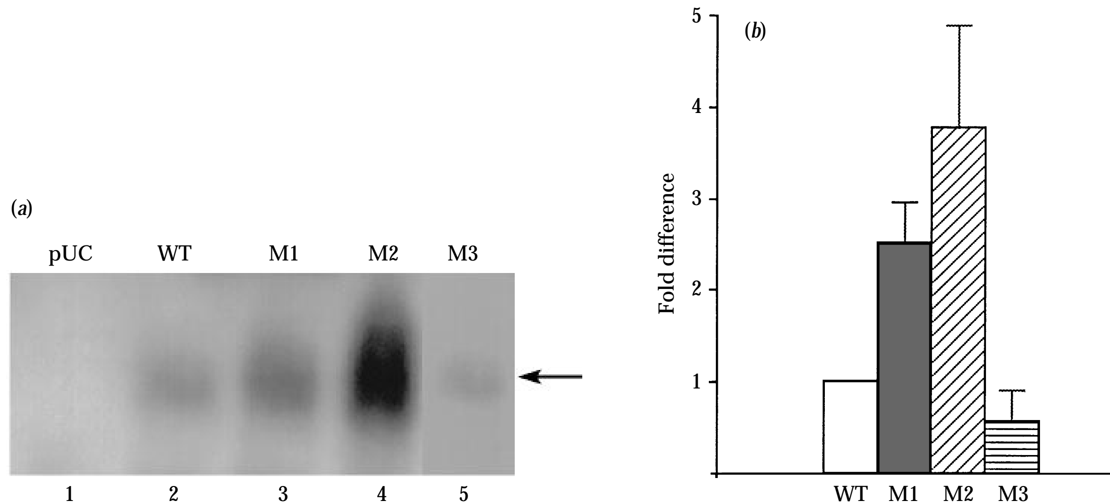

Fig. 5. Quantitative analysis of virus particles secreted from WT- and mutant-transfected cells. The media from 60 mm plates of Huh7 cells transfected 48 h previously with either 10 μg pUC19 (control), pWTD (wild-type), pM1D (mutant 1), pM2D (mutant 2) or pM3D (mutant 3) were precipitated with polyethylene glycol, resuspended, electrophoresed on a 1% agarose gel, transferred to nylon blotting membrane, and probed with a ^{32}P-labelled WT HBV whole genome fragment.
(*a*) Representative result of Southern blotting. The position of HBV virion DNA is indicated by an arrow. For lane 5, a longer exposure of the autoradiogram was used to show that a small amount of the progeny virus was also produced by pM3D.
(*b*) Statistical analysis of the results. Autoradiograms as in (*a*) were analysed with an LKB Broma ULTROSCAN XL laser densitometer, and the fold difference determined by dividing the density of the mutant virus signals with that of the WT signal following adjustment of the signal relative to transfection efficiency, which was monitored by measuring the HGH levels secreted into the media of transfected cells with an HGH radioimmunoassay kit (Nichols). The results shown are the mean and standard deviation of at least three independent transfection experiments.

HBV particles secreted by Huh7 cells transfected with pUC19 control, pWTD, pM1D, pM2D and pM3D (Fig. 5*a*). A statistical analysis of multiple, independently performed Southern blotting experiments is shown in Fig. 5(*b*). As reported previously, an approximately 2-fold increase in the production of virus particles relative to WT HBV was observed for the M1 double mutant (Buckwold *et al*., 1996). pM2D-transfected cells displayed a reproducible and significantly more pronounced 3- to 4-fold increase of virus progeny production, while pM3D-transfected cells consistently produced less virus progeny relative to WT HBV-transfected cells.

Discussion

The nt 1762 A → T and nt 1764 G → A double mutation (M1 mutant) in the BCP of the HBV genome frequently appears during chronic infection (Kaneko *et al*., 1995; Kidd-Ljunggren *et al*., 1995; Kurosaki *et al*., 1996; Laskus *et al*., 1995; Okamoto *et al*., 1994; Sato *et al*., 1995; Takahashi *et al*., 1995). Our previous studies indicated that this double mutation prevented the binding of LEF to the BCP, suppressed only precore RNA transcription and hence HBeAg synthesis, and enhanced HBV progeny virus production. The increase of HBV replication rate may provide an explanation why HBV carrying this double mutation often becomes the dominant quasispecies during chronic infection. The increase of virus progeny production likely occurs at or immediately prior to the pregenome encapsidation step of the virus life-cycle, as the packaging efficiency of the pregenomic RNA was found to be increased by the double mutation. This effect could be partially reversed if a precore expression plasmid was used in a *trans*-complementation experiment, suggesting a negative role for the precore gene in HBV replication. This suggestion is also supported by a recent study which indicated that precore gene suppressed HBV replication in transgenic mice (Guidotti *et al*., 1996).

In spite of the likelihood that nt 1762 A → T (M2 mutant) and nt 1764 G → A (M3 mutant) single-nucleotide mutations are the two intermediates that lead to the eventual appearance of the M1 double mutant, both M2 and M3 single mutants are rarely detected in HBV patients. In order to understand the mechanism that regulates precore gene expression and the process that leads to the selection of the M1 double mutant, we have compared the effects of the M1 double mutation with M2 and M3 single mutations on precore gene expression and HBV replication. We found by EMSA that both M1 and M2 mutants lacked LEF binding activity while M3 displayed a weak bandshift signal likely due to residual LEF binding (Fig. 2*b*). A group of new, albeit weak, and specific bandshift signals (marked with 'A') were detected only with the M1 mutant. The protein factor(s) responsible for these new binding activities were not identified. These new binding activities do not seem to be important for precore and core RNA

transcription as the M2 mutant, which did not generate these new binding activities, had a similar expression pattern of precore and core RNA as the M1 mutant (see below).

The LEF binding site on the BCP and the consensus DR1 binding site of the steroid–thyroid–retinoid hormone receptor superfamily share a high degree of sequence homology (Fig. 3*a*), suggesting that LEF may be composed of members of this receptor superfamily. We have investigated this possibility by examining the binding of several of these protein factors to the BCP using specific antibodies. As shown in Fig. 3(*b*), only anti-COUP-TF was able to significantly remove the LEF binding activity, indicating that COUP-TF or a protein closely related to it is a component of LEF. This result was supported by a different EMSA experiment in which COUP-TF synthesized *in vitro* was found to bind to the BCP and to an oligonucleotide probe containing the LEF binding site (Fig. 3*c*). The mobility of the shifted BCP probe due to binding by the *in vitro* synthesized COUP-TF was slightly different from that due to binding by LEF (Fig. 3*c*). This difference was even more obvious when the oligonucleotide containing the LEF site was used as the probe (data not shown). These results indicate that COUP-TF is not identical to LEF. COUP-TF is known to bind DNA as a homodimer or heterodimer (Cooney *et al.*, 1992). The difference in mobility between COUP-TF and LEF bandshifts raises the possibility that LEF is a heterodimer formed between COUP-TF and another factor. Since COUP-TF is a ubiquitous factor (L. H. Wang *et al.*, 1991) and LEF is liver-enriched, the heterodimeric partner of COUP-TF must be a liver-enriched factor. This unknown liver factor is not RXRα, because the antibody directed against RXRα did not affect the signal of the LEF bandshift (Fig. 3*b*). It is also not likely to be MB67 or SHP, because these two protein factors synthesized *in vitro* did not affect COUP-TF binding to the LEF site (data not shown). Although HNF4 synthesized *in vitro* binds weakly to the BCP probe (Fig. 3*c*) and to the oligonucleotide probe containing the LEF site (data not shown; Yu & Mertz, 1996), it seems unlikely that HNF4 was the other component of LEF because anti-COUP-TF antibody almost entirely removed the LEF bandshift signal, but anti-HNF4 antibody only marginally affected the LEF signal (Fig. 3*b*). Furthermore, an HNF4 site-containing oligonucleotide could only partially compete away LEF binding activity while authentic HNF4 binding activity was completely competed away under these conditions (data not shown), suggesting that HNF4 is likely not the heterodimeric liver-specific partner of LEF activity. Thus, the identity of the liver-enriched component of LEF remains unknown.

Co-transfection experiments utilizing WT and mutant HBV DNA constructs and a COUP-TF expression vector (gift of R. M. Evans, Salk Institute) were uninformative due to the overall suppression of HBV RNA transcription by COUP-TF (data not shown), possibly due to the known repressive effects of this transcription factor on HBV enhancer I activity (Garcia *et al.*, 1993). The effects of COUP-TF on the transcription of precore and core RNAs by primer extension utilizing constructs which lacked the HBV enhancer I were also uninformative as the transcription of precore and core RNA in the absence of the enhancer I sequence was beyond the sensitivity limit of our assay (data not shown).

In support of our previous observation that the binding of LEF to the BCP is important solely for the transcription of precore RNA, but not core RNA (Buckwold *et al.*, 1996), we find that both the M1 and M2 mutations, which prevent LEF from binding to the BCP, reduce the precore RNA transcription to approximately one-third that of WT (Fig. 4*b*). These two mutations do not grossly affect the transcription of other HBV RNAs including the core RNA. In contrast, the M3 mutation does not reduce precore RNA transcription. Rather, it seems to slightly increase the precore RNA level (Fig. 4*b*). Thus the reduced, but detectable, LEF binding ability of M3 appears to be sufficient for the production of precore RNA, at least in these transfection experiments using Huh7 cells. Note that M1, M2 and M3 mutations also generate different mutations in the overlapping X protein coding sequence (see below). Thus, it is possible that these mutated X proteins also participate in the regulation of precore RNA transcription.

Southern blot analysis of HBV particles secreted from WT- or mutant HBV-transfected Huh7 cells demonstrates that M1 and M2 display an increase in progeny virus production, while M3 shows a decreased efficiency of virus production relative to that of WT (Fig. 5). Previously, we demonstrated that the increase in progeny synthesis by the M1 double mutation could be partially reduced by co-transfection *in trans* with a precore expression plasmid (Buckwold *et al.*, 1996). This finding is in agreement with previous studies demonstrating that precore RNA transcription can suppress HBV progeny synthesis (Guidotti *et al.*, 1996; Lamberts *et al.*, 1993). Our current finding that there is a correlation between the level of precore RNA transcribed and the efficiency of virus progeny production (Figs 4*b* and 5) is also in support of a negative role for the precore gene in HBV replication. Previously, we demonstrated that the suppression of virus progeny production by the precore gene occurs at or immediately prior to the encapsidation step (Buckwold *et al.*, 1996). Since a small amount of precore protein can be released into the cytosol (Garcia *et al.*, 1988; Ou *et al.*, 1989), it is conceivable that this cytosolic precore protein, which is structurally similar to the core protein, may act as a dominant-negative regulator to interfere with the encapsidation process. This hypothesis has also been proposed by Guidotti *et al.* (1996), who found that precore protein could suppress the formation of core particles in the hepatocytes of transgenic mice.

The M1 mutant frequently becomes the dominant quasi-species in HBV patients with chronic hepatitis (Kaneko *et al.*, 1995; Kidd-Ljunggren *et al.*, 1995; Kurosaki *et al.*, 1996; Laskus *et al.*, 1995; Okamoto *et al.*, 1994; Sato *et al.*, 1995; Takahashi *et al.*, 1995), indicating that there is a selective advantage for it over WT HBV in these patients. This selective advantage may be provided in part by its increased replication rate. The single-

nucleotide mutant M3 replicates slightly less efficiently than the WT in Huh7 cells. This would explain why it is not selected for at the hepatocyte level and consequently during chronic infection. The M2 mutant, however, produces progeny virus at a slightly higher rate than that of M1. In the absence of other factors, M2 should have a selection advantage over M1 in patients due to its increased replication efficiency. This, apparently, is not the case, as the M2 mutation, similar to the M3 mutation, is rarely detected in HBV patients. Thus, our results indicate that other factors are also involved during the course of HBV infection *in vivo* to give M1 a higher competitive advantage over M2. This inference is also supported by the observation that the M1 mutant is often found in conjunction with other mutations which produce an $HBeAg^{-}$ phenotype (Kurosaki *et al*., 1996; Laskus *et al*., 1995; Okamoto *et al*., 1994; Sato *et al*., 1995; Takahashi *et al*., 1995), such as the nt 1896 G → A mutation which produces a stop codon in the precore coding sequence (Lok *et al*., 1994; Yuan *et al*., 1995). As the fixation of either of these two distinct types of mutations does not appear to precede the other, the selective advantages brought about by the two mutation types should be additive or at least distinctive in that they are both selected for simultaneously *in vivo*. This observation indicates that the levels of precore gene expression and virus replication rates as well as other factors are involved in the selection of these HBV mutants in patients.

Another viral genetic element that is affected by the M1 mutation is the X open reading frame and it is possible that the changes introduced into HBx may be responsible for the selective advantage of M1 over M2 (or M3) in infected patients. The HBV X protein (HBx) functions as a transcriptional transactivator of viral and cellular genes (Yen, 1996) and is required for the establishment of hepadnaviral infection in animals (Chen *et al*., 1993; Zoulim *et al*., 1994). The M1 BCP double mutation also changes amino acids 130 and 131 of the overlapping HBx coding region from Lys to Met (nt 1762 A → T, A$\underline{A}$G → A$\underline{T}$G) and Val to Ile (nt 1764 G → A, $\underline{G}$TC → $\underline{A}$TC), respectively. The change of these two codons together may alter the functions of X protein to provide a selection advantage for the M1 mutant during natural infection. Alternatively, the simultaneous change of these two codons may be compensatory and essential for restoring HBx activity, which may otherwise be deleteriously affected in the case of the M2 or M3 single mutation. It is also possible that the coding changes introduced into HBx may create altered epitopes which are recognized differently by the immune system of patients.

In conclusion, our data show that while increased virus replication associated with decreased precore protein expression may be one factor that favours the *in vivo* selection of the M1 mutant over the M3 intermediate form of this mutation, other factors possibly related to the coding changes introduced into HBx are likely involved in the selection of the M1 mutant over the M2 mutant.

We thank Ray-Chang Wu and the members of J.O.'s laboratory for helpful discussions. We thank Dr R. M. Evans (Salk Institute) for the gifts of pCMX-COUP, pMX-hRXRα(Kpn) and anti-RXRα antibodies; Dr D. D. Moore (Massachusetts General Hospital) for pT7HMSHP and pT7lac-his-myc-MB67; Dr F. M. Sladek (University of California, Riverside) for the anti-HNF-4 antibody; Dr M.-J. Tsai (Baylor College of Medicine) for anti-COUP-TF antibody; and Dr S. Tahara and T. Dietlin (University of Southern California) for the nonspecific oligonucleotide. This work was supported by grants from the Council for Tobacco Research (to J.O.) and the Department of Veteran Affairs (to T.S.B.Y.).

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Received 11 February 1997; Accepted 10 April 1997