Hepatitis B Virus Mutants Associated With 3TC and Famciclovir Administration Are Replication Defective

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Hepatitis B virus (HBV) variant strains may develop during therapy for chronic infection with the nucleoside analog 2′,3′-dideoxy-3′-thiacytidine (3TC). HBV mutants result from isoleucine (I) or valine (V) substitutions in the methionine (M) of the YMDD motif in the viral reverse-transcriptase catalytic domain. In addition, other mutations in the reverse-transcriptase "B domain" involving either a phenylalanine (F)-to-leucine (L) at amino acid 501 (F501L) or an L-to-M substitution at amino acid 515 (L515M) have been observed during 3TC and Famciclovir therapy as well. To determine the biologic consequences of these mutations on viral replication, variant viral genomes were constructed and transiently transfected into hepatocellular carcinoma (HCC) and HEK 293 human embryo kidney-derived cell lines. In transiently transfected HCC cells, the viruses bearing the YI/VDD or F501L mutations had greatly impaired replication as compared to wild-type virus, whereas the virus carrying the L515M substitution showed the least defect. Double mutants with the L515M substitution showed intermediate defect between the YI/VDD or F501L and the L515M single-mutant strains. In contrast, when transfected into HEK 293 cells, the viruses bearing the YI/VDD or L515M mutation replicated as wild-type. However, under conditions of deoxynucleotide depletion produced by hydroxyurea treatment of HEK 293 cells, all mutants but not the wild-type virus exhibited a reduced replication phenotype similar to that observed in HCC cells. In both HCC and HEK 293 cells, the mutant viruses carrying the F501L substitution showed a decreased pregenomic RNA encapsidation level, suggesting that the defect in HBV DNA synthesis occurs at the RNA packaging level. These findings show that 3TC and Famciclovir selected mutations alter the properties of the HBV reverse transcriptase, resulting in impaired viral replication within the cell. (HEPATOLOGY 1998;27:628-633.)

Abbreviations: HBV, hepatitis B virus; HCC, hepatocellular carcinoma; 3TC, 2′,3′-dideoxy-3′-thiacytidine; HIV-1, human immunodeficiency type 1 virus; CMV I.E., cytomegalovirus immediate early promoter; HEK, human embryo kidney; HU, hydroxyurea; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B surface antigen.

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Persistent hepatitis B virus (HBV) infection may lead to significant liver diseases such as chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC). Interferon alpha has been shown to be an effective drug, but long-lasting antiviral effects are observed in a minority of individuals.[1] Recently, the nucleoside analog 2′,3′-dideoxy-3′-thiacytidine (3TC) has been shown to be a potent inhibitor of HBV replication both *in vitro*[2] and *in vivo*.[3] Preliminary studies suggest that there are few or no adverse side effects from the use of this potent antiviral agent. However, the levels of viremia have been shown to return to pretreatment values in the majority of individuals following discontinuation of therapy.[4] Therefore, long-term administration of this agent is required to maintain suppression of viral replication, and under these circumstances, the emergence or selection of drug-resistant HBV strains with different biologic properties from the wild-type virus may occur.

3TC is currently used in combination with other agents to reduce viral replication of the human immunodeficiency type 1 virus (HIV-1).[5] It is well established that 3TC, when used as a single agent, leads to the rapid development of 3TC-resistant HIV-1 strains. These mutant viral genomes are characterized by selective amino acid changes in the catalytic domain, as represented by the YMDD motif,[6] of the viral reverse transcriptase. Indeed, the methionine (M) has been found to be replaced by either an isoleucine (I) or a valine (V) residue, and such mutant viral strains display a processivity defect of the reverse transcriptase following infection of primary cells.[7] Furthermore, the 3TC-induced mutations in the YMDD motif alter the reverse-transcriptase activity following deoxynucleotide depletion within the cell.[7]

Hepadnaviruses as well as retroviruses replicate through a reverse-transcription mechanism,[8] and all share a common and highly conserved YMDD motif in the catalytic domain of the reverse transcriptase.[6] It is of interest that 3TC treatment of immunosuppressed HBV carriers following liver transplantation results in the emergence of the same type of drug-resistant strains as characterized by YM^{539}DD to YIDD[9,10,11] or YVDD[9,11] mutations in the catalytic site of the reverse transcriptase.[9,10] Furthermore, the YVDD point mutation in the duck HBV polymerase has been shown to be necessary and sufficient to generate a 3TC-resistant virus[12] *in vitro,* and an HBV strain containing the YVDD mutation was found to be resistant to inhibition of viral replication by 3TC in primary hepatocyte cultures.[11]

Moreover, another mutation in the "B domain"[6] of the reverse transcriptase has been found to occur at positions 508 (V508L) and 515 (L515M), independent of changes in the YMDD motif, following treatment of chronic HBV infection

with Famciclovir.[13] Famciclovir is another nucleoside analog with biologic effects similar to 3TC. Such "B-domain" substitutions have also been described in association with the YI/VDD mutation following 3TC treatment.[9,11] These mutant viral strains appear to reach a high titer in the sera of immunosuppressed patients, indicating that they are replication competent in this setting. Nevertheless, there is no information available on the "fitness" of these mutant strains as compared to wild-type virus. It is therefore important to determine if these drug-induced mutations will alter the viral phenotype with respect to their competence for replication within human hepatocyte-derived cell lines and under conditions of limited deoxynucleotide availability.

MATERIALS AND METHODS

Plasmid Constructs

The payw1.2 construct contains more than one HBV genome length and carries the sequence between the *Aat*II (nucleotide (nt.) position 1411, where nt. 1 is by convention located at GAATTC in the unique *Eco*RI site) and the *Bsp*EI restriction enzyme sites (nt. 2327).[14] This plasmid expresses the HBV pregenomic and subgenomic RNAs under the control of the endogenous viral promoters and permits HBV replication to occur in well-differentiated HCC cells. The pCMVHBV construct expresses the HBV pregenomic RNA from the cytomegalovirus immediate early promoter (CMV I.E.).[15] This vector was used because it permits HBV replication in cells of nonhepatic origin,[16] such as human embryo kidney (HEK) 293. These two vectors are therein considered to express wild-type virus. The polymerase gene mutations were introduced into HBV DNA by means of the Altered Site II *in vitro* mutagenesis systems (Promega Corp., Madison, WI). The following primers were used: M539I [5′ GCTTTCAGTTATATCGATGATGTGGTATTGGG 3′ (this primer will change tryptophan[196] into serine in the overlapping envelope open reading frame)]; M539V [5′ GCTTTCAGTTATGTGGATGATGTGG-TATTGGG 3′ (this primer will change isoleucine[195] into methionine in the envelope gene)]. The primer F501L (5′ CCCATCATCCT-GGGCCTTCGGAAAATTCCTATGGGAG 3′) was used to change phenylalanine[501] into leucine in the polymerase protein. The primer L515M (5′ CTCAGCCCGTTTCTCATGGCTCAGTTTACTAGTGCC 3′) was used to change leucine[515] into methionine. These latter mutations will not alter the coding sequence in the overlapping envelope protein.

The following plasmids were generated by an exchange of DNA fragments into payw1.2 and pCMVHBV vectors and were designated as follows: paywYIDD, paywYVDD, paywF501L, paywL515M, paywF501L/YIDD, paywL515M/YIDD, paywF501L/YVDD, paywL515M/YVDD; pCMVHBVYIDD, pCMVHBVYVDD, pCMVHBVF501L, pCMVHBVL515M, pCMVHBVL515M/YVDD, and pCMVHBVF501L/YIDD. The DNA fragments were sequenced to verify that the introduced mutations were correct. The payw-derived constructs were transfected into HepG2 and HuH-7 HCC cells, whereas the pCMV-derived constructs were transfected into both HCC and HEK 293 cell lines.

Cellular Transfection Studies

The HepG2 and HuH-7 cell lines were used because they will support viral replication of wild-type HBV following transient transfection. The HEK 293 cells will also support high levels of HBV replication when transfected with plasmids expressing the HBV pregenome under the control of the CMV I.E. promoter.[14] The cells were grown in DMEM supplemented with 10% fetal bovine serum. Approximately 1×10^7 cells were seeded into a 10-cm dish and transiently transfected with 10 µg of the wild-type or mutated HBV DNA constructs by the calcium phosphate procedure ($CaPO_4$ transfection Kit, 5′-3′, Inc., Boulder, CO). Cells were harvested 2 or 5 days later for RNA and DNA analysis, respectively, and lysed in TLB buffer (50 mmol/L TRIS-Cl, pH 8.0, 100 mmol/L NaCl, 1 mmol/L EDTA, 1% NP40) for DNA and pregenome RNA encapsidation analysis. Hydroxyurea (HU) was added to HEK 293 cells at concentrations ranging from 0.1 to 0.5 mmol/L at the time of cellular transfection. At these high concentrations, cellular morphology appeared intact by light microscopy. Transfection efficiency was monitored by adding 1 µg of the pCMV luciferase (pLuc) to the transfected DNA and 1/100 of the cell lysate was subsequently subjected to a luciferase assay.[17] Transfection experiments were disregarded if luciferase activity varied more than 20% within the samples, and Southern and Northern blot loading was adjusted according to the luciferase activity of the samples.

Analysis of Viral DNA and RNA

HBV DNA replication was determined by Southern blot analysis of viral DNA extracted from purified nucleocapsid particles as described.[18] Moreover, in order to examine encapsidated pregenomic HBV RNA, viral nucleocapsids were immunoprecipitated from lysates prepared from transfected cells by a polyclonal anti-HBc antibody (DAKO Corp., Carpinteria, CA), followed by the addition of Sepharose Protein A beads to bind the immune complex. The complex was washed three times with TNE (10 mmol/L Tris-Cl pH 7.5, 100 NaCl mmol/L, 1 mmol/L EDTA)/0.5% NP40 buffer. The encapsidated viral RNA was extracted from the nucleocapsids with D solution, as described;[19] the same method was used to purify total cellular RNA.

Viral DNA was fractionated on 1.25% agarose gels in 1×TAE buffer and transferred onto Hybond N+ (Amersham International, Little Chalfont, UK), whereas for Northern blot analysis, total and encapsidated RNA was fractionated on a 1.5% agarose/0.66 mol/L formaldehyde gel in 1× MOPS buffer (10× MOPS: 0.2 mol/L MOPS, 50 mmol/L Na acetate pH 7.0, 10 mmol/L EDTA). HBV DNA and RNA were detected by hybridization with a random-primed, ^{32}P-labeled, 3.2-kb HBV probe (specific activity: 1×10^9 cpm/µg DNA). Prehybridization, hybridization, and washing of the blots were performed as previously reported,[19] followed by autoradiography of the blots on Reflexion films (NEN, Boston, MA) with an exposure time of 2 hours to overnight at −70°C.

Detection of HBV Antigens Following Transient Transfection of Cells

Measurement of the hepatitis B e antigen (HBeAg) in cell culture supernatants was performed according to the manufacturer's instructions (EBK ^{125}I RIA Kit, Incstar Corp., Stillwater, MN). Hepatitis B surface antigen (HBsAg) concentrations in the cell culture supernatant were determined using a radioimmunometric assay as described.[20]

RESULTS

HBV Genomes Carrying Mutations in the Reverse Transcriptase Display a Reduced Replication Phenotype

The design of the plasmid vectors utilized in this study is depicted in Fig. 1. The payw1.2 series of constructs expresses the HBV pregenomic and subgenomic RNAs under the control of the endogenous viral promoters and permits HBV replication to occur in well-differentiated HCC cells. Viral genomes carrying the YMDD and the "B-domain" mutations were transfected into HCC cells and the replicative intermediate forms of the viral DNA were extracted from purified nucleocapsid particles. Southern blot analysis showed that viral genomes carrying the single amino acid substitution of F501L, L515M, YIDD, and YVDD in the reverse transcriptase had reduced levels of viral replication as compared to the wild-type HBV (Fig. 2A). The F501L substitution produced

FIG. 1. Schematic representation of the constructs used in this study. (A) The genomic organization of the HBV open reading frames (ORF) is shown. The C ORF encodes for the nucleocapsid protein and with the extended preC ORF, for the HBeAg, respectively. The P ORF encodes for the polymerase reverse transcriptase, and the preS1, preS2, and S ORFs encode for the envelope proteins. The X ORF encodes for a putative transcription transactivator. The HBV pregenomic RNA (*wavy line*) is transcribed from the endogenous viral promoter in the payw- or from the CMV I.E. promoter in the CMV-series of constructs. Both promoters are identified by the large arrow. (A) $_n$ indicates the polyadenylation site. (B) Amino acid sequence of the "B domain" and the YMDD motif of wild-type HBV reverse transcriptase. The mutant constructs bearing a single or a double amino acid substitution are described.

the most potent suppression of viral replication, followed by the YIDD and YVDD single mutations. The L515M exchange had the least effect on the replication phenotype. The F501L and YI/VDD double mutations produced a replication phenotype similar to the F501L mutant strain. On the other hand, the L515M and YI/VDD double mutations resulted in a partial restoration of viral replication that appeared intermediate between the levels observed with the YI/VDD and the L515M single mutant HBV strains. These transfection studies were repeated four times with similar results, and transfection efficiency was comparable in all experiments.

Properties of Mutant Viral Strains

The concentrations of HBeAg and HBsAg secreted into the culture supernatants following transient transfection of HCC cells with wild-type and mutant viral genomes were equivalent as measured by RIA. Thus, mutations introduced into the overlapping envelope gene by site-directed mutagenesis of the polymerase gene did not alter either the synthesis or the binding properties of HBsAg, as shown in Fig. 2B. No change in the levels of viral mRNA synthesis was observed between wild-type and mutant viral strains carrying the YI/VDD mutations, as shown by Northern blot analysis of total cellular RNA in Fig. 3A. Similar results were observed with the variant HBV genomes carrying the F501L and L515M single mutations as well as those strains bearing the F501L/YIDD and L515M/YVDD double mutations (data not shown). It is noteworthy that the Northern blot analysis of the encapsidated pregenomic RNA (Fig. 3B) showed that the viruses carrying the F501L mutation (namely, the single F501L mutant and the double F501L/YIDD mutant strains) displayed a significantly reduced level of pregenomic RNA packaged in the nucleocapsids, even though the amounts of total viral RNA and secreted antigens were similar to those found with wild-type virus. These experiments were performed three times in both HuH7 and HEK 293 cells with similar results. None of the other mutant viral strains showed differences in the levels of encapsidated pregenomic RNA. Taken together, these studies suggest that the decrease in HBV DNA replicative forms following transient transfection of HCC cells was due to a defect in the activity of the reverse transcriptase as well as reduced packaging of viral RNA in the case of the F501L mutant.

Sensitivity of the Viral Mutants to Deoxynucleotide Depletion

When transient transfection experiments were performed using the CMV I.E.-based vectors in HEK 293 cells, we observed that the viral strains bearing the single YI/VDD and L515M as well as the L515M/YVDD double mutations were almost indistinguishable from wild-type HBV virus with respect to replication capacity. In contrast, the F501L single mutant genome showed a greatly reduced level of HBV DNA replicative forms, as depicted in Fig. 4. Moreover, HBV DNA synthesis was almost completely abolished by the F501L and YIDD double mutation. To determine if the mutant viral strains had an altered sensitivity to deoxynucleotides depletion as has been previously reported with 3TC-induced resistant HIV-1 strains, transfected cells were treated with increasing concentrations of HU.[7,21] As shown in Fig. 4B and C, wild-type HBV replication was least affected by intracellular depletion of deoxynucleotides, whereas the mutant HBV genomes showed variable reductions in viral replicative intermediates. In particular, the YVDD and the YIDD mutant strains showed reduced levels of viral DNA synthesis after 0.1 mmol/L of HU was added to the culture medium. In contrast, viral replication exhibited by the L515M single and the L515M/YVDD double mutant strains was relatively unaffected and was least affected by further increases in the concentrations of HU. These experiments suggest that the 3TC-induced mutations in the viral reverse transcriptase render the enzyme sensitive to intracellular deoxynucleotide depletion that leads to or contributes to a reduced replication phenotype.

DISCUSSION

The hepadnaviruses replicate their DNA through reverse transcription of an RNA intermediate. This critical phase of the life cycle is performed by a virally encoded RNA-dependent DNA polymerase/reverse transcriptase found inside the nucleocapsid particles.[8] The reverse transcriptases of hepadnaviruses share functional as well as structural features with the reverse transcriptases of retroviruses. For example, the YMDD motif has been proposed to be the active catalytic site as determined by sequence comparison, by genetic criteria, and by protein crystallography.[6] Indeed, mutations introduced into the YMDD motif of DHBV[22] and HBV[23] specifically abolish the enzymatic activity of the reverse transcriptases without affecting other polymerase functions such as encapsidation of pregenomic RNA.

Retroviral and hepadnaviral infections are characterized by rapid emergence of variant viral strains, particularly during chronic infection. This phenomenon most likely results from the lack of proofreading activity of the reverse-transcriptase enzyme. Some genetic mutations may confer biologic advantages to HBV, such as escape from the host's antiviral immune responses and/or the development of resistance to drug therapy. Thus the emergence of mutations in the viral reverse transcriptase is believed to account for most failures of antiviral drugs during chronic therapy.[24]

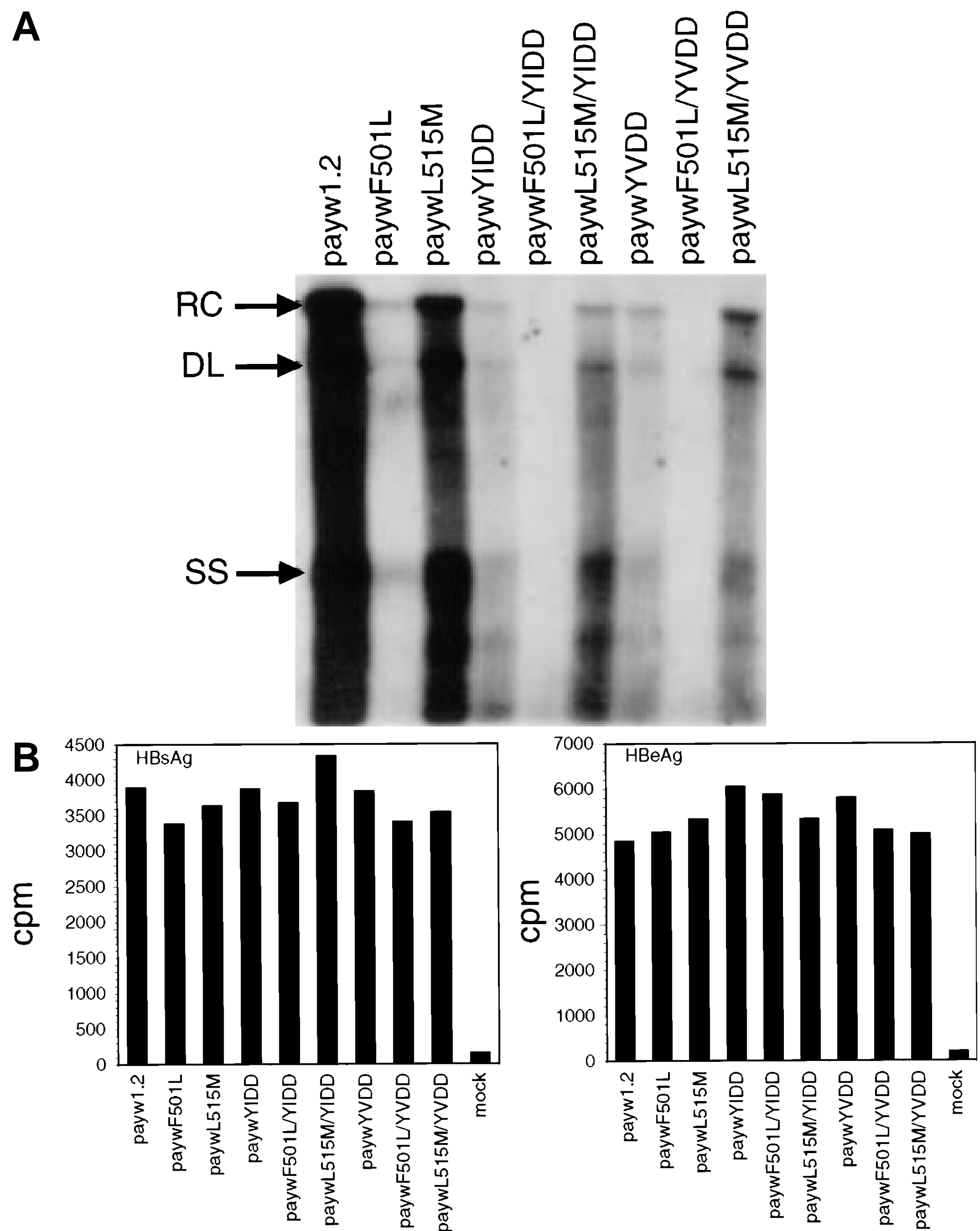

FIG. 2. (A) Replication capacity of HBV genomes carrying mutations in the reverse transcriptase following transfection of HCC cell lines. Southern blot analysis of HBV DNA extracted from intracellular nucleocapsid particles is presented. The arrows on the left indicate the relaxed circular (RC), double-stranded linear (DL), and the single-stranded (SS) HBV DNA species. (B) Mutant viral genomes secrete wild-type levels of HBsAg (*left*) and HBeAg (*right*) in the culture following transfection of HepG2 cells.

Recently, it has been reported that 3TC will strikingly inhibit HBV DNA replication in chronically infected individuals as measured by viral titers in serum.[3] However, prolonged 3TC administration has been associated with virologic relapses while on therapy.[9-11] In addition, the prolonged administration of Famciclovir to HBV-infected individuals appears to cause mutations in the "B domain" of the viral reverse transcriptase.[13] One such "B domain" mutation may also occur in combination with substitutions in the YMDD motif after 3TC treatment.[9]

Therefore, it was of interest to investigate the biologic effects on the reverse transcriptases of HBV that were produced by these mutations. These studies provide the first evidence for a diminished replication capacity of HBV strains bearing the 3TC and Famciclovir selected mutations following transfection into HCC cell lines. There appear to be at least two explanations for the reduced level of DNA replicative intermediates exhibited by the mutant strains. In the case of the F501L and F501L/YIDD mutants, either the U-to-C mutation at nt. 623 in the pregenomic RNA or the F501L substitution in the HBV polymerase protein significantly decreased the level of packaged pregenomic RNA in both HuH7 and HEK 293 cells compared to wild-type virus despite similar levels of secreted antigens and total viral RNA synthesis. The other mutant strains packaged wild-type levels of pregenomic RNA, and therefore the decreased replication of HBV DNA is consistent with the generation of a defective polymerase protein. However, in HEK 293 cells, the viral strains harboring the YI/VDD mutations replicated at levels comparable to wild-type HBV. The difference in the promoters driving the synthesis of the pregenome RNA in HCC and HEK 293 cells appears an unlikely explanation for the wild-type levels of replication in the HEK 293 cells, since the nucleocapsid particles would still presumably contain the

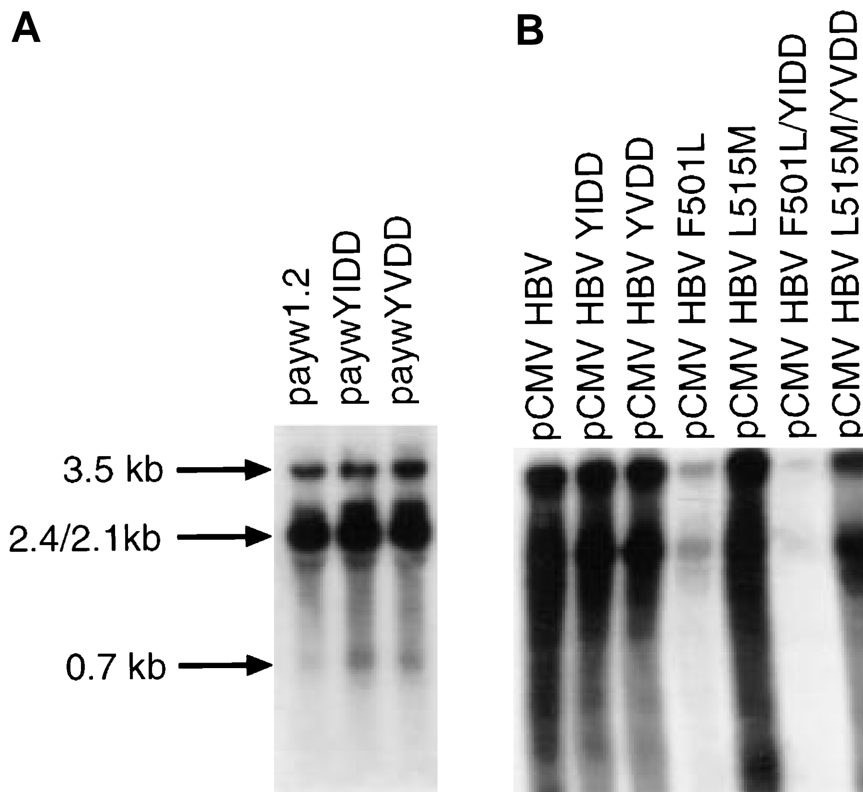

FIG. 3. (A) Northern blot analysis of total cellular RNA extracted from HepG2 cells transfected with wild-type (payw1.2) and constructs with the YIDD and YVDD mutations. The arrows indicate the 3.5-kb pregenomic and 2.4/2.1- and 0.7-kb subgenomic RNA species as revealed by hybridization to a full-length 3.2-kb HBV DNA probe. Note that no reduction in viral transcripts was observed between wild-type and mutant viral strains. (B) Northern blot analysis of encapsidated pregenomic RNA derived from HEK 293 cells transfected with wild-type and mutant constructs after adjusting for transfection efficiency. Note that the two constructs containing the F501L mutation, as either a single or a double mutant strain, exhibited a decreased level in encapsidation of pregenomic RNA. Similar results were obtained in transfected HuH7 cells.

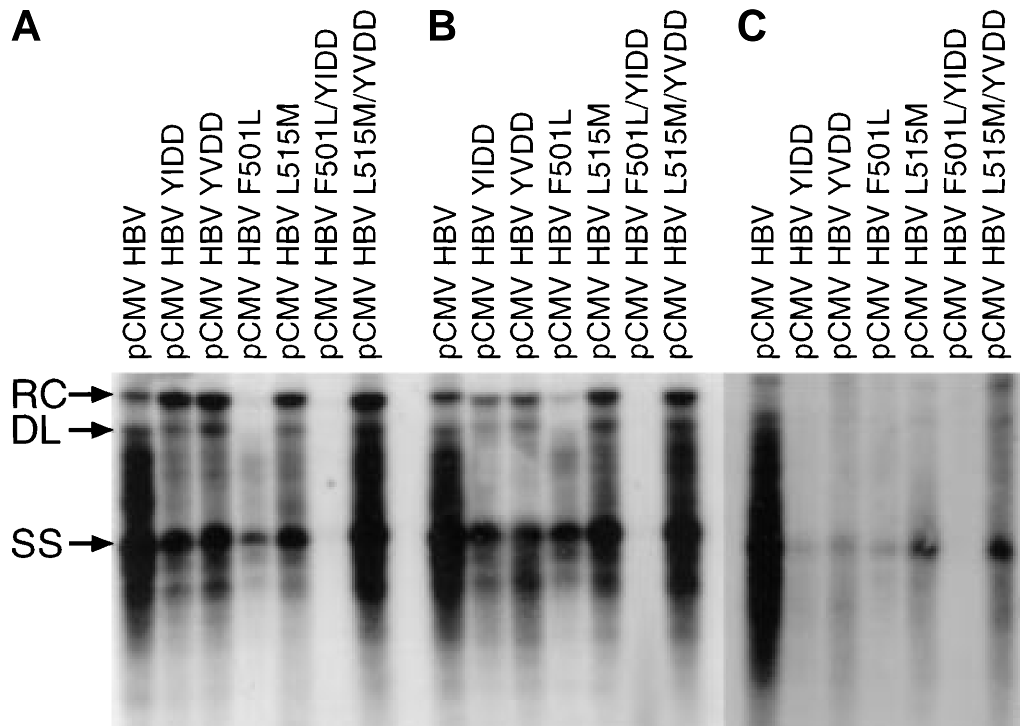

FIG. 4. Replication capacity in HEK 293 cells exhibited by 3TC- and Famciclovir-resistant HBV mutant strains in the presence of increasing concentrations of hydroxyurea. Southern blot analysis of HBV DNA extracted from intracellular nucleocapsids particles is presented. (A) Untreated cells; (B) cells cultivated in the presence of 0.1 mmol/L HU; (C) cells cultivated in the presence of 0.5 mmol/L HU. Cells in C are derived from a different blot. The blots were exposed for the same time period and therefore (A, B, and C) can be compared with one another. The *arrows* indicate the positions of relaxed circular (RC), double-stranded linear (DL), and the single-stranded (SS) HBV DNA species.

mutated polymerase. This phenotype seems more likely to be due to a cell-specific factor(s) that allows the mutated polymerase enzyme to perform more efficiently in the HEK 293 cells. Indeed, the 3TC-resistant HBV mutants also displayed a reduced replication phenotype in HEK 293 cells after deoxynucleotide depletion that was comparable in degree to the levels observed in untreated HCC cells. Therefore, similar to what has been previously proposed for drug-resistant HIV-1 strains,[7] the YI/VDD induced mutations in HBV appear to affect the interaction between deoxynucleotides and the active catalytic site of the reverse transcriptase.

It was also important to determine if the mutations induced by Famciclovir[13] in the "B domain" of the reverse transcriptase, either alone or in combination with the YI/VDD mutations, resulted in a change in the levels of viral replication.[9] The L515M mutation had little effect on the biologic activity of the reverse transcriptase, and in combination with the YI/VDD mutations, appeared to confer a replication advantage under conditions of low deoxynucleotide availability. Finally, another V508L mutation accompanying the L515M has been described in immunosuppressed patients following Famciclovir[13] or 3TC[11] treatment.

It is noteworthy that 3TC-resistant HBV strains have emerged thus far only in immunosuppressed patients following liver transplantation. Such variant viral strains appear to reach high titers in serum, and this finding suggests that even with an impaired reverse-transcriptase enzyme, viral replication is very active within the liver. However, immunosuppressive drugs such as corticosteroids are known to promote HBV replication,[24,25] and under these circumstances even drug-resistant viral strains may replicate at high levels. It is not known whether such mutant viral strains will show different biologic properties in individuals with chronic HBV infection. Additional large-scale studies of immunocompetent patients with chronic HBV infection under 3TC therapy will be required to clarify this issue.

Finally, viral protein synthesis exhibited by the mutant viral strains was no different from wild-type HBV as determined by HBsAg and HBeAg levels measured in the culture supernatants following transfection of HCC cell lines. It will be of interest to determine if the emergence of 3TC-resistant strains *in vivo* is associated with HBsAg levels in serum similar to that observed with wild-type HBV infection. The replication activity of the mutant genomes suggests that the use of combination antiviral therapy with agents intended to block other steps in viral morphogenesis may ultimately be necessary to eradicate viral infection from the liver.[14]

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