

Emergence of a Novel Lamivudine-Resistant Hepatitis B Virus Variant with a Substitution Outside the YMDD Motif[∇]

Hiromi Yatsuji,^{1,2} Chiemi Noguchi,^{1,2} Nobuhiko Hiraga,^{1,2} Nami Mori,^{1,2} Masataka Tsuge,^{1,2}
 Michio Imamura,^{1,2} Shoichi Takahashi,^{1,2} Eiji Iwao,³ Yoshifumi Fujimoto,^{2,4} Hidenori Ochi,^{2,4}
 Hiromi Abe,^{1,4} Toshiro Maekawa,⁴ Chise Tateno,^{2,5} Katsutoshi Yoshizato,^{2,5,6}
 Fumitaka Suzuki,⁷ Hiromitsu Kumada,⁷ and Kazuaki Chayama^{1,2,4*}

Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, 1-2-3, Kasumi, Minami-ku, Hiroshima-shi, 734-8551, Japan¹; Liver Research Project Center, Hiroshima University, Hiroshima, Japan²; Pharmaceuticals Research Unit, Mitsubishi Pharma Corporation, 1000 Kamoshida-cho, Aoba-ku, Yokohama 227-0033, Japan³; Laboratory for Liver Disease, SNP Research Center, The Institute of Physical and Chemical Research (RIKEN), Yokohama 230-0045, Japan⁴; Yoshizato Project, CLUSTER, and Hiroshima Prefectural Institute of Industrial Science and Technology, Higashihiroshima, Japan⁵; Developmental Biology Laboratory, Department of Biological Science, Graduate School of Science, Hiroshima University, Higashihiroshima, Japan⁶; and Department of Gastroenterology, Toranomon Hospital, Tokyo, Japan⁷

Received 24 February 2006/Returned for modification 21 April 2006/Accepted 1 September 2006

Lamivudine is a major drug approved for treatment of chronic hepatitis B virus (HBV) infection. Emergence of drug-resistant mutants with amino acid substitutions in the YMDD motif is a well-documented problem during long-term lamivudine therapy. Here we report a novel lamivudine-resistant strain of HBV with an intact YMDD motif, which included an amino acid substitution, rtA181T, in the reverse transcriptase (RT) domain of HBV polymerase. The substitution also induced a unique amino acid substitution (W172L) in the overlapping hepatitis B surface (HBs) protein. The YMDD mutant strains were not detected even by using the sensitive peptide nucleic acid-mediated PCR clamping method. The detected nucleotide substitution was accompanied by the emergence of an additional nucleotide substitution that induced amino acid change (S331C) in the spacer domain. The rtA181T mutant strain displayed a threefold decrease in susceptibility to lamivudine in *in vitro* experiments in comparison with the wild type. *In vivo* analysis using human hepatocyte-chimeric mice confirmed the resistance of this mutant strain to lamivudine. We developed a method to detect this novel rtA181T mutation and a previously reported rtA181T mutation with the HBs stop codon using restriction fragment length polymorphism PCR and identified one patient with the latter pattern among 40 patients with lamivudine resistance. In conclusion, although the incidence is not high, we have to be careful regarding the emergence of lamivudine-resistant mutant strains with intact YMDD motif.

Hepatitis B virus (HBV) is a small, enveloped DNA virus that causes chronic hepatitis and often leads to cirrhosis and hepatocellular carcinoma (4, 12, 33). To date, interferon and three nucleoside and nucleotide analogs (lamivudine, adefovir dipivoxil, and entecavir) have been approved by the United States Food and Drug Administration for the treatment of chronic HBV infection. Lamivudine, an oral cytosine nucleoside analogue, potently inhibits HBV replication by interfering with RNA-dependent DNA polymerase (10, 16, 22). Lamivudine therapy suppresses HBV replication in most patients and improves transaminase levels and liver histology (16, 22, 25, 30). However, prolonged therapy results in the emergence of drug-resistant mutants in 24% and 70% of patients after 1 and 4 years of therapy, respectively, followed by increases in viral load and re-elevation of transaminase levels (18).

Most lamivudine-resistant strains show amino acid substitutions in the YMDD (tyrosine-methionine-aspartate-aspartate) motif in the C domain of HBV polymerase. In addition to the emergence of the YMDD mutation, rtL180M and rtV173L mutations in the B domain of HBV polymerase are frequently observed (1, 9). *In vitro* analyses have confirmed that the rtL180M mutation augments the level of lamivudine resistance and enhances viral replication, while the rtV173L mutation enhances only viral replication (9, 23). On the other hand, only a few uncommon mutations associated with lamivudine resistance have been reported so far (3, 7, 24, 34). In the C domain of HBV polymerase, rtM204S and rtD205N were detected in patients with lamivudine resistance (3, 7). In the B domain, rtL180C and rtA181T were associated with lamivudine resistance (7, 24, 34). Yeh et al. (34) reported the emergence of rtA181T mutants in 4 of 23 patients who received long-term lamivudine therapy. The mutant appeared concomitantly with or after emergence of YMDD motif mutants and persisted thereafter. The nucleotide substitution in the FLLA motif resulted in early termination of the overlapping HBs gene transcription by creating a stop codon (TGG to TGA). Yeh et al. (34) demonstrated that the mutation reduced the

* Corresponding author. Mailing address: Department of Medical and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Science, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan. Phone: 81-82-257-5190. Fax: 81-82-255-6220. E-mail: chayama@hiroshima-u.ac.jp.

[∇] Published ahead of print on 18 September 2006.

susceptibility to lamivudine *in vitro*. They also detected such mutations in virus from a patient with leukemia and speculated that truncated HBs gene might be related to the development of leukemia (7).

Analyzing nucleotide and amino acid sequences of HBV in patients who developed a breakthrough, we identified a novel mutant that showed nucleotide substitutions in the B domain of the reverse transcriptase. The G residues of nucleotides 669 and 670 were mutated to T and A, respectively, and associated with the amino acid substitution rtA181T. The substitutions also induced the amino acid substitution W172L in the overlapping HBs protein. Since the nucleotide substitution was associated with nucleotide and amino acid substitutions in the putative spacer region of the polymerase, we checked the importance of these substitutions for resistance to lamivudine *in vitro*. We also analyzed the resistance of this new strain *in vivo* using a human hepatocyte-chimeric mouse (27, 31). Furthermore, we analyzed the susceptibility of the mutant strain to adefovir and entecavir. When used alone or in combination with lamivudine, these drugs are known to be effective against wild-type as well as lamivudine-resistant HBV (2, 5, 14, 17, 32). Infrequent emergence of resistance compared with lamivudine resistance has been reported for both of these two drugs (2, 5). We also developed a detection system to identify the novel and previously reported (7, 34) nucleotide substitutions to study the incidence of such mutations.

MATERIALS AND METHODS

Antiviral compounds. Lamivudine [(−)-β-L-2',3'-dideoxy-3'-thiacytidine] was provided by GlaxoSmithKline (Stevenage, Herts, United Kingdom). Adefovir {9-[2-(phosphonomethoxy)ethyl]-adenine} was provided by Gilead Sciences (Foster City, CA), and entecavir {2-amino-1,9-dihydro-9-[(1S,3R,4S)-4-hydroxy-3-(hydroxymethyl)-2-methylenecyclopentyl]-6H-purin-6-one, monohydrate} was provided by Bristol-Myers Squibb Pharmaceutical Research Institute (Wallingford, CT).

Analysis of virological markers. Hepatitis B surface antigen (HBsAg), hepatitis B envelope antigen (HBeAg), and antibody against HBeAg (anti-HBe) were quantified by enzyme immunoassay kits (Abbot Diagnostics, Chicago, IL). HBV-DNA was measured by real-time PCR using a Light Cycler (Roche, Mannheim, Germany). The primers used for amplification were 5'-TTTGGGCATGGACA TTGAC-3' and 5'-GGTGAACAATGTTCCGGAGAC-3'. The amplification condition included initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 5 s, and extension at 72°C for 6 s. The lower detection limit of this assay was 300 copies.

Cloning of HBV DNA and plasmid construction. HBV DNA was extracted from 100 μl of each serum sample by SMITEST (Genome Science Laboratories, Tokyo, Japan) and was dissolved in 20 μl H₂O. Full-length HBV DNA was amplified using the above HBV DNA samples by the method of Gunther et al. (13). Nucleotide sequence positions were numbered from the unique EcoRI site. The 1.4-genome-length HBV DNA amplified from the serum of a patient who showed lamivudine resistance was cloned into plasmid vector pTRE (Takara Bio, Tokyo, Japan) (patient strain). In brief, the PCR product amplified using serum from the patient was cleaved with BamHI and ApaI (HBV positions 1400 to 2600) and cloned into pcDNA3 (Invitrogen, San Diego, CA), and the resulting construct was named pcDNA3-1. Similarly, the PCR product was cleaved with ApaI and BamHI (HBV positions 2600 to 3215 and 1 to 1400) and cloned into pBlueScript SK+ (Stratagene, La Jolla, CA), and the resulting construct was named pB-1. The KpnI-BamHI fragment from pB-1 and the KpnI-ApaI fragment from pcDNA3-1 were cloned into pcDNA3-1. Finally, the plasmids were cleaved with HindIII and NotI within the multicloning site and cloned into plasmid vector pTRE. As a laboratory strain, we employed a plasmid containing a 1.4-genome-length wild-type genotype C HBV (wild-type strain; GenBank accession number AB206816) (31). To introduce the nucleotide substitutions into the S331C/rtA181T patient and wild-type strains, site-directed mutagenesis was performed with a QuikChange site-directed mutagenesis kit (Stratagene).

TABLE 1. *In vitro* susceptibility of the S331/rtA181 mutant to lamivudine^a

Source	Strain Type	S331/rtA181 mutation	Lamivudine IC ₅₀ (μM)	Resistance (fold)
Patient	WT	-/-	0.19 ± 0.01	1
	S331C	C/-	0.23 ± 0.01	1.2*
	rtA181T	-/T	0.58 ± 0.08	3**
	S331C/rtA181T	C/T	0.57 ± 0.06	3**
Laboratory	WT	-/-	0.23 ± 0.04	1
	S331C	C/-	0.3 ± 0.05	1.3*
	rtA181T	-/T	0.88 ± 0.2	3.9**
	S331C/rtA181T	C/T	0.98 ± 0.12	4.3**

^a Experiments were performed in triplicate. Values are expressed as means ± SD. WT, wild type. *, not significant; ** *P* < 0.001 compared to the wild type.

The eight plasmids with and without amino acid substitutions in the spacer and reverse transcriptase domain are listed in Table 1.

Cell culture, transfection, and determination of IC₅₀. HepG2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal bovine serum at 37°C in 5% CO₂. Cells were seeded to semiconfluence in six-well tissue culture plates. Transient transfection of the plasmids into HepG2 cells was performed using TransIT-LT1 (Mirus, Madison, WI) according to the instructions provided by the supplier. To determine 50% inhibitory concentrations (IC₅₀s) for each antiviral drug, various concentrations of lamivudine, adefovir, and entecavir were added after 24 h to the culture plate containing the cells, and cells were harvested after 5 days. The medium containing the drugs was changed on days 1, 3, and 4. A plasmid encoding β-galactosidase (β-Gal) was cotransfected to adjust the transfection efficiency. The β-Gal enzyme assay was performed with a β-Gal enzyme assay system (Promega, Madison, WI). All experiments were performed in triplicate. GraphPad Prism software (GraphPad Software, Inc.) was used to determine the best-fit values for individual dose-response equations.

Analysis of replicative intermediate of HBV by Southern blot hybridization and quantitation. The cells were harvested at 3 or 5 days after transfection and lysed with 250 μl of lysis buffer (10 mM Tris-HCl [pH 7.4], 140 mM NaCl, and 0.5% [vol/vol] NP-40) followed by centrifugation for 2 min at 15,000 × *g*. The core-associated HBV genome was immunoprecipitated by mouse anticore monoclonal antibody 2A21 (Institute of Immunology, Tokyo, Japan) and subjected to Southern blot analysis after sodium dodecyl sulfate-proteinase K digestion followed by phenol extraction and ethanol precipitation. The DNA was detected with a full-length HBV-DNA probe labeled by the DIG DNA labeling and detection kit (Roche Diagnostics, Basel, Switzerland) according to the instructions provided by the manufacturer. Quantitative analysis was performed by real-time PCR with SYBR green using a Light Cycler. The HBV-specific primers used for amplification were 5'-TTTGGGCATGGACATTGAC-3' and 5'-GGTGAACAATGTTCCGGAGAC-3'. The amplification conditions included initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 5 s and extension at 72°C for 6 s. The lower detection limit of this assay was 300 copies.

Evaluation of effects of antiviral drugs on mutant strains using human hepatocyte-chimeric mice. Human hepatocyte-chimeric mice were generated and used in the drug evaluation studies as described previously (27, 31). Briefly, human hepatocytes were transplanted into urokinase-type plasminogen activator-transgenic SCID mice, which are immunodeficient and develop liver failure. The transplanted cells were characterized in terms of *in vivo* growth potential and function. The human hepatocytes progressively repopulated the murine host liver and were susceptible to cultured-cell-line-produced HBV. All animal protocols were performed in accordance with the guidelines of the local committee for animal experimentation. The mice were inoculated with 50 μl of serum samples containing wild-type and newly identified drug-resistant strains. Serum samples obtained from mice were stored at -80°C before further analyses. After stable high-level HBV viremia was confirmed, the mice were administered food containing 30 mg of lamivudine/kg of body weight/day. The nucleotide sequences of wild-type and mutant strains were confirmed by sequencing analysis.

Detection of rtA181T mutants by PCR with restriction fragment length polymorphism (RFLP). HBV DNA extracted from serum samples were amplified by

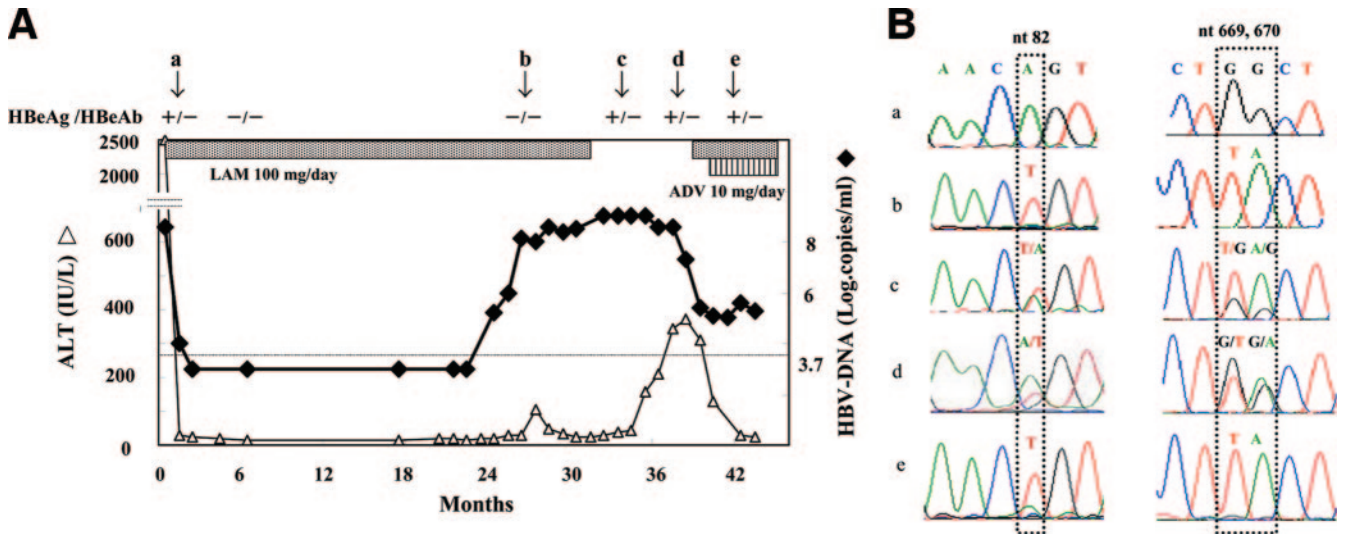


FIG. 1. (A) Clinical course of a patient who developed breakthrough without emergence of YMDD mutants during lamivudine therapy. Arrows a to e indicate time points of serum sampling for direct sequencing and RFLP PCR. (B) Nucleotide sequence analysis of the reverse transcriptase/polymerase gene of hepatitis B virus by direct sequencing. Time points of serum sampling (see panel A) were as follows: (a) just before lamivudine treatment, (b) after breakthrough, (c) after cessation of lamivudine treatment, (d) just before readministration of lamivudine, and (e) during adefovir and lamivudine therapy. Note that the wild type reappeared during the cessation of therapy (c and d), but it disappeared after readministration of the drug (e).

PCR using the primers 5'-GCCCCGTTGTCCTCTACTTCCA-3' and 5'-ACCACTGAACAAATGGCACTAGTAAGCTGA-3'. The reverse primer was designed to introduce an EspI site (GCTCAGC) into only wild-type sequences. The PCR was performed in a total volume of 25 μ l, consisting of a reaction buffer (100 mmol/liter Tris-HCl [pH 8.3], 50 mmol/liter KCl, and 15 mmol/liter MgCl₂), 0.2 mmol/liter of each deoxynucleoside triphosphate, 1 μ l of the DNA solution, 10 pmol of each primer and 1 U of *Taq* DNA polymerase (Gene Taq; Wako Pure Chemicals, Tokyo, Japan) with 0.2 μ g of anti-*Taq* high (Toyobo Co., Osaka,

Japan). The amplification conditions included an initial denaturation at 94°C for 2 min, 35 cycles of amplification (denaturation at 94°C for 1 min, annealing of primer at 58°C for 1 min, extension at 72°C for 2 min), and final extension at 72°C for 7 min. Two μ l of PCR products was digested with 5 U of EspI and subjected to electrophoresis in a 3.5% agarose gel.

Statistical analysis. Data are expressed as means \pm standard deviations (SD). Group comparisons were performed using the Student *t* test. A *P* value of less than 0.05 was considered statistically significant.

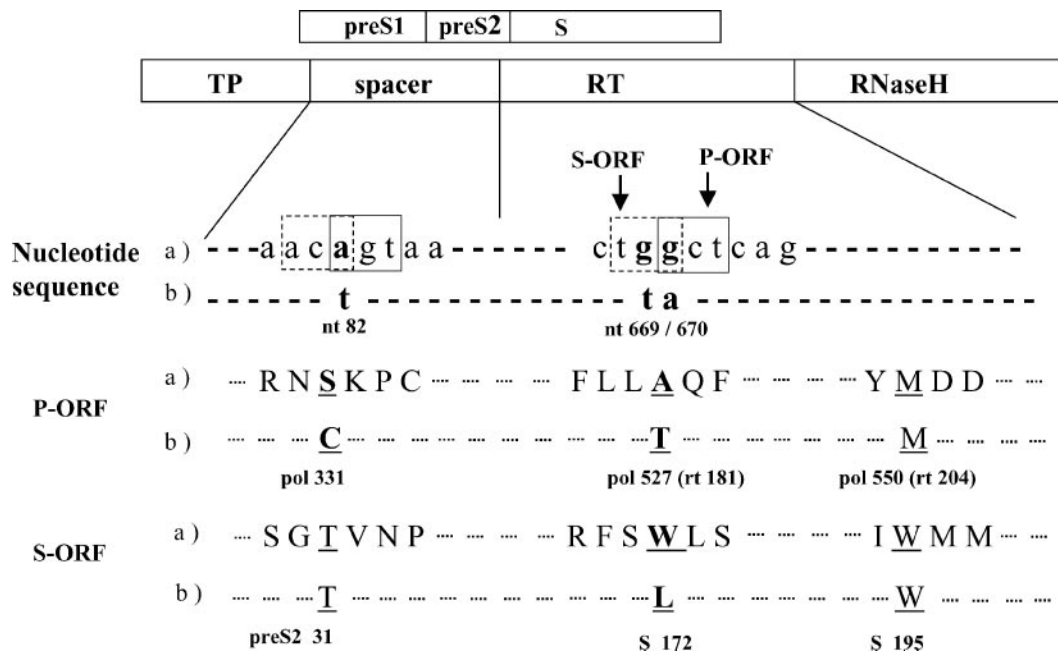


FIG. 2. Comparison of nucleotide sequences and amino acid sequences of two overlapping open reading frames, reverse transcriptase/polymerase and the HBs gene of the hepatitis B virus, before and after viral breakthrough. Sequences obtained from serum samples before (a) and after (b) breakthrough were compared. See Fig. 1A for time points of serum sampling. Nucleotide sequence numbers are those of typical HBV (e.g., accession no. AB206816 [31]), which starts from a unique EcoRI site.

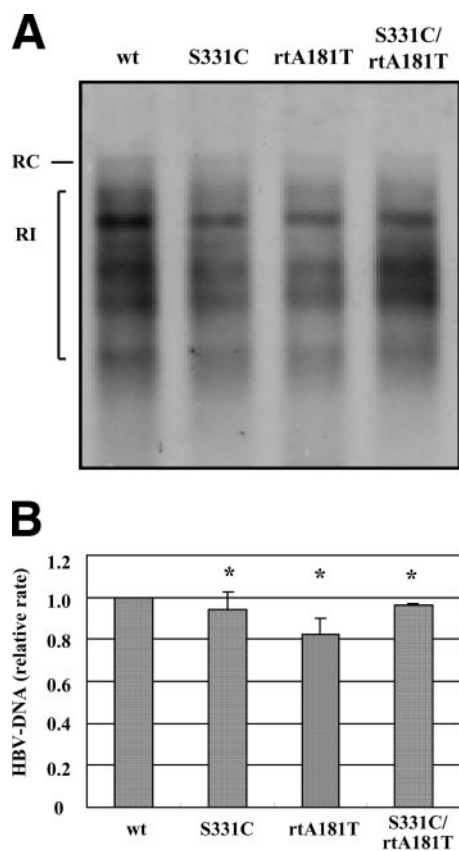


FIG. 3. Replication ability of wild-type HBV and three mutants (S331C, rtA181T, and S331C/rtA181T). Plasmids containing 1.4-genome-length HBV were transiently transfected into HepG2 cells. (A) The replicative intermediates were analyzed by Southern blot hybridization. Core-associated replicative intermediates of HBV DNA were isolated from HepG2 cells at 3 days after transfection. The positions of relaxed circular DNA (RC) and replication intermediates (RI) are indicated. (B) Quantitative analyses of core-associated intermediates of HBV. Experiments were performed in triplicate. Values are relative to those of the wild type and are expressed as means \pm SD. *, not significant compared to the wild type.

RESULTS

Isolation of a novel lamivudine-resistant strain with an intact YMDD motif. The novel lamivudine-resistant strain of HBV was isolated from a 44-year-old Japanese man with chronic HBV infection (Fig. 1A). In this patient, lamivudine successfully reduced the HBV level at the initial stage of treatment, but viral breakthrough was observed at 24 months after the beginning of therapy. The patient was very punctual and confirmed that he took lamivudine with perfect compliance. The HBV viral load reached up to 8.5 log copies/ml, but nucleotide sequence analysis showed no YMDD mutation. The YIDD and YVDD mutants were not detected even with a peptide nucleic acid-mediated PCR clamping method sensitive for detection of YMDD mutants (6). The analysis also showed that this isolate belonged to genotype C of HBV. Comparison by the direct sequence method of nucleotide sequences obtained before and after the viral breakthrough showed three nucleotide substitutions that induced two amino acid substitutions in both spacer (polS331C) and reverse transcriptase

(polA527T or rtA181T) domains of the polymerase (Fig. 1B and 2). The latter nucleotide substitutions induced an amino acid change in the overlapping HBs protein (W172L) (Fig. 2). Twelve HBV genomes were cloned from the serum of this patient after viral breakthrough, and eleven of them showed the above amino acid substitutions. Only one clone showed the wild-type sequence. The new strain of HBV became undetectable when lamivudine therapy was discontinued, and this strain outcompeted the wild-type strain upon administration of the drug (Fig. 1B). These results prompted us to study the significance of each of these mutations.

Effect of substitutions on HBV replication. To assess the effect of nucleotide substitutions on HBV replication, four plasmids containing 1.4-genome-length patient-specific HBV genome (Table 1) were generated and transfected into HepG2 cells. In comparison with the patient's wild-type strain, the replication capacities of the S331C, rtA181T, and S331C/rtA181T mutants were not different (94%, 82%, and 96%, respectively), suggesting that these mutants can replicate at almost the same rate as the wild-type strain (Fig. 3).

Susceptibility of mutants to lamivudine in vitro. To analyze the role of the polS331C and rtA181T mutations in lamivudine resistance, four patient-specific strains and four laboratory strains were transfected into HepG2 cells (Fig. 4; Table 1). A single amino acid substitution in the spacer region did not contribute to resistance in either patient or laboratory strains. In contrast, an amino acid substitution in the polymerase (rtA181T) induced resistance that was 3.0 and 3.9 times greater than that of patient and laboratory strains ($P < 0.001$), respectively. The presence of both of these amino acid changes induced 3.0 and 4.3 times greater resistance in each of the above strains. Thus, the spacer mutation had little effect on the susceptibility to lamivudine (Table 1).

We also compared the rtA181T mutant identified in this study with the rtA181T mutant reported previously, which had premature termination in the HBs protein (7, 34), for replication ability and susceptibility to lamivudine. Although the HBs antigen produced to culture supernatant was different between the two strains (52.5 ± 8.2 and 4.4 ± 0.6 IU/ml, respectively), there was no noticeable difference in replication ability and lamivudine sensitivity between the two mutants (data not shown).

Assessment of drug resistance of novel mutations in vivo using human hepatocyte-chimeric mice. To confirm the lamivudine resistance of the novel mutant strain, two human hepatocyte-chimeric mice were each inoculated with a serum sample obtained from the patient who developed breakthrough without mutations in the YMDD motif (Fig. 1A). The serum was obtained during breakthrough while the patient was still taking the drug. Twelve weeks after the inoculation of the serum samples, both mice developed high-level viremia (7.8 and 6.6 log copies/ml, respectively). Direct sequence analysis showed that the nucleotide sequence of the virus that replicated in the chimeric mice was in accordance with the mutant strain. Cloning and sequencing analysis showed that only 1 of 12 clones obtained from the inoculum was wild type, while the remaining 11 clones were rtA181T mutants with an intact YMDD motif. We also analyzed the serum of the two infected mice before and after lamivudine therapy. All 11 and 15 clones before and all 11 and 12 clones during therapy had the

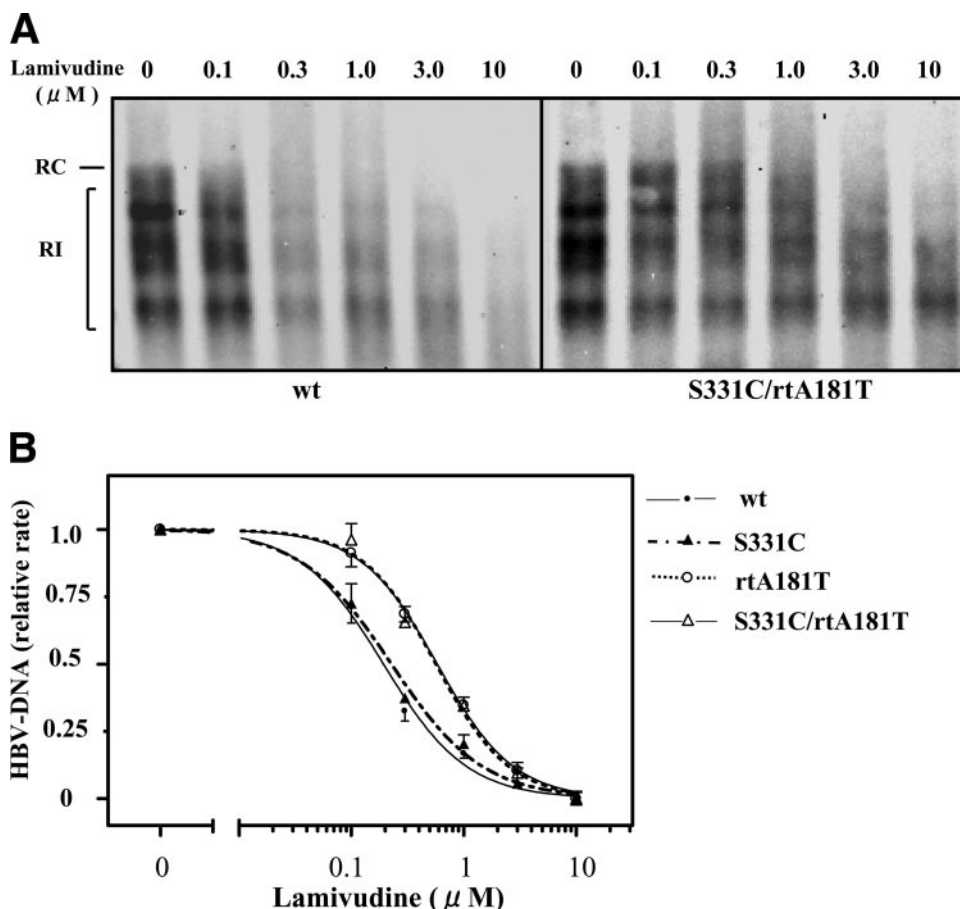


FIG. 4. In vitro analyses of susceptibility of wild-type HBV and three mutants (S331C, rtA181T, S331C/rtA181T) to lamivudine after transient transfection into HepG2 cells. Cells were transiently transfected with plasmids containing 1.4-genome-length HBV and treated with the indicated amount of lamivudine. (A) Southern blot analysis of replicative intermediate. Representative results for the wild type (wt) and the S331C/rtA181T mutant are shown. The positions of relaxed circular (RC) and replication intermediate (RI) forms of HBV DNA are indicated. (B) Dose-response curves of the four HBV strains against lamivudine. The curves were used to estimate the lamivudine IC_{50} s for each HBV strains. Values are relative to no-lamivudine controls for each strain. Experiments were performed in triplicate. Values are expressed as means \pm SD.

rtA181T mutation (data not shown). Two other mice were inoculated with wild-type HBV obtained from a patient not treated with lamivudine as a control, and both mice also developed high-level viremia (8.3 and 9.3 log copies/ml, respec-

tively). Thirteen weeks later, the viremia reached plateau and the mice were fed food containing lamivudine. After 6 weeks of treatment, the mean viral load decreased by 2.8 log copies/ml in the wild type, whereas it decreased by only 0.39 log copy/ml in the mutant ($P < 0.001$) (Fig. 5).

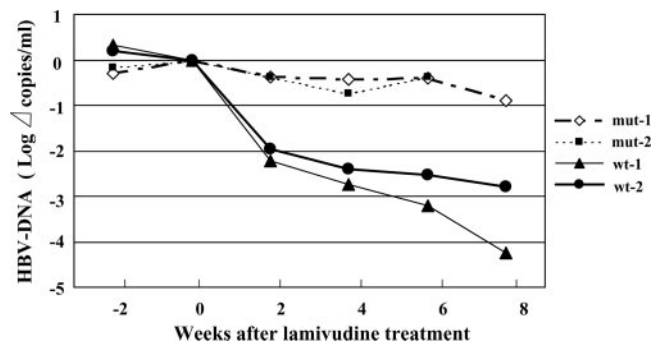


FIG. 5. In vivo analyses of the effect of lamivudine on wild-type and S331C/rtA181T mutant HBV. Four human hepatocyte-chimeric mice were inoculated with serum samples containing wild-type or mutant HBV. One of the animals fed with lamivudine died 6 weeks after the beginning of therapy.

Susceptibility of mutants to adefovir and entecavir in vitro. We also analyzed the effects of adefovir and entecavir against the S331C/rtA181T mutant using a transient-transfection assay with HepG2 cells. The IC_{50} s of these drugs for the mutant strain and wild type were almost the same (Table 2).

Detection of rtA181T mutant in patients treated with lamivudine. In this study, we developed a RFLP PCR method to detect the rtA181T mutants, by which we were able to detect mutant strains even when they were mixed with the wild type (Fig. 6). The system also detected the rtA181T (HBs stop) mutant reported by Chien et al. (7) and Yeh et al. (34). Using this method, we analyzed 40 patients who showed viral breakthrough (increase in viral load equal to or more than 1 log) during lamivudine therapy. We found that only one of these patients was positive (Fig. 6A). Nucleotide sequence analysis of serum samples obtained from this patient showed that the

TABLE 2. In vitro susceptibility of the S331/rtA181 mutant to lamivudine, adefovir, and entecavir^a

Patient strain	S331/rtA181	Lamivudine		Adefovir		Entecavir	
		IC ₅₀ (μM)	Resistance (fold)	IC ₅₀ (μM)	Resistance (fold)	IC ₅₀ (nM)	Resistance (fold)
WT	-/-	0.19 ± 0.01	1	0.37 ± 0.1	1	0.19 ± 0.02	1
S331C/rtA181T	C/T	0.57 ± 0.06	3**	0.36 ± 0.08	0.98*	0.23 ± 0.05	1.2*

^a Experiments were performed in triplicate. Values are expressed as means ± SD. WT, wild type. *, not significant; ** $P < 0.001$ compared to the wild type.

mutant strain had the rtA181T mutation with a truncated HBs antigen, as reported previously (7, 34). The YMDD motif of HBV detected in this patient was of the wild type. All 39 remaining patients with viral breakthrough were positive for YIDD and/or YVDD mutants. The RFLP PCR analysis of these 39 samples showed that four contained a small amount of rtA181T mutants (Fig. 6B). Nucleotide sequence analyses of these samples showed that they contained only a small amount of rtA181T mutants with a truncated HBs antigen (Fig. 6C).

Finally, we examined the presence of YMDD or rtA181T mutants in eight patients who showed a poor response with lamivudine treatment (HBV viral load above 6.0 log copies/ml after 6 months of treatment). None of these patients tested positive for both of these mutations (data not shown).

DISCUSSION

In this study, we identified a novel lamivudine-resistant strain of HBV with an intact YMDD motif in a patient who received long-term lamivudine therapy. YMDD mutants were

not detected even by a sensitivity-enhanced detection method, which was reported previously by our group (6). The double nucleotide substitutions (GG to TA) induced amino acid substitutions in both polymerase (rtA181T) and HBs antigen (HBs W172L). One might assume that the compliance of the patient was poor. However, the patient was very punctual and confirmed that he took lamivudine with perfect compliance.

Our study demonstrated that the rtA181T mutation reduced the susceptibility to lamivudine 3.0- to 3.9-fold in vitro (Table 1). Furthermore, we also confirmed lamivudine resistance of this mutant strain in vivo using human hepatocyte-chimeric mice. The amino acid substitution in the reverse transcriptase (RT) domain is similar to that reported previously (7, 34). However, in contrast to our results, the mutant strains in the latter reports emerged with or after those with the mutation in the YMDD motif (YIDD or YVDD) and took over them (34). There are two additional differences between the substitutions we identified and those described by Yeh et al. (34), as detailed below.

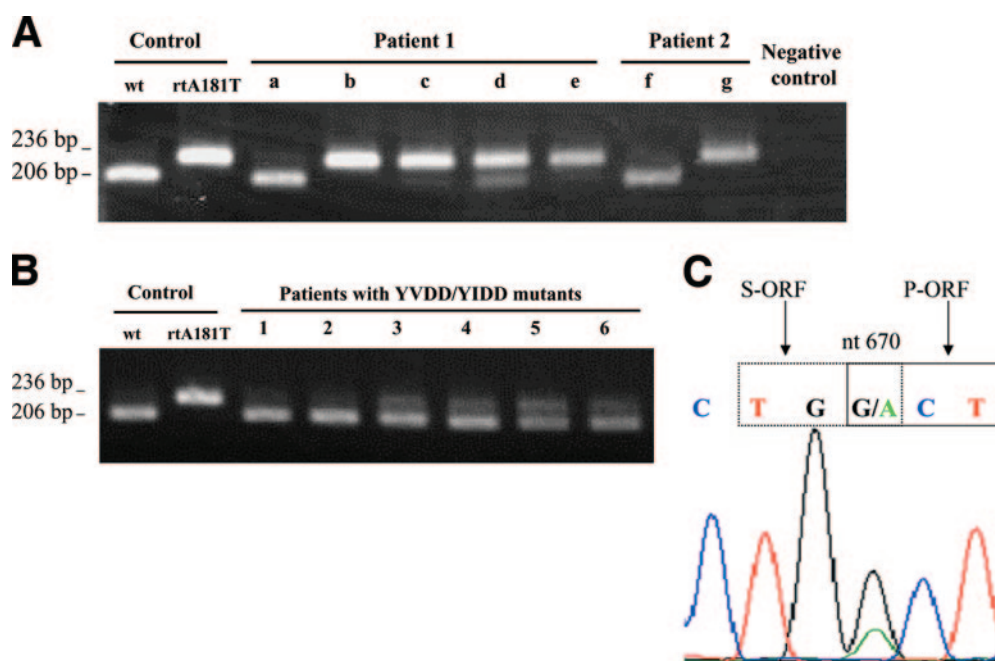


FIG. 6. Detection of the rtA181T mutant by RFLP PCR assay. PCR-amplified DNA fragments were treated with *EspI*, which digests only wild-type sequences, and separated in a 3.5% agarose gel. (A) Agarose gel electrophoresis of RFLP PCR products. Wild-type and rtA181T mutant plasmids were used as controls. See Fig. 1A for the time points of serum sampling (a to e) for patient 1 and see Fig. 1B for a comparison with nucleotide sequence analyses. f and g indicate the time points before and after viral breakthrough for patient 2. (B) Agarose gel electrophoresis of RFLP PCR products using HBV DNA samples obtained from 39 patients who showed lamivudine breakthrough. Of the 39 samples, 35 were wild type (lanes 1 and 2). The remaining four samples (lanes 3 to 7) showed partial digestion, suggesting a mixture of wild-type and mutant strains. (C) Nucleotide sequence analysis of a sample by RFLP PCR suggested the presence of a wild-type-mutant mixture (lane 5 of panel B).

Firstly, the HBs antigen was prematurely terminated in the mutant strain reported by Yeh et al. (34). In this regard, a similar amino acid substitution of the B domain of the polymerase FLLA motif in woodchuck hepatitis virus (WHV) treated with lamivudine was reported (15, 28). The HBs antigen in these WHV mutant strains also had premature stop codons. These findings suggest that the mutant strains of HBV and WHV cannot replicate and spread by themselves because of the lack of HBs antigen. Such strains are thought to replicate by using in vivo-supplied HBs antigen from wild-type strains as helper antigens. In contrast, the novel strain identified in this study had no premature termination of the HBs gene. The in vitro study suggested that the strain had a replication ability similar to that of the wild type. Furthermore, we also showed that the strain infected and reached a high viral load in human hepatocyte-chimeric mice. Although the inoculum contained only a small amount of wild-type strain (one of 12 clones), all clones obtained from mouse serum were mutant strains (rtA181T). Considering these results and the fact that the index patient showed high viral titers after breakthrough (more than 7.6 log copies/ml), this mutant strain can spread and replicate by itself and has strong replicative ability.

Secondly, the substitutions identified in this study appeared with nucleotide and amino acid substitutions in the spacer region of the polymerase (S331C). There are only a few studies that reported the function of the spacer domain (19–21, 28), leaving the biological significance of this region unknown. The substitution in the spacer region reappeared with the A181T mutation in the RT domain in the index patient after the patient restarted lamivudine therapy. Although our study showed no significant contribution of this mutation to drug resistance (Fig. 3 and 4; Table 1), the significance of the mutation in this region (fingers in the HBV polymerase homology model [8]) should further be investigated.

Recently, the amino acid substitutions rtA181T and rtA181V were reported to emerge with resistance against adefovir (11, 32). Tillmann et al. (29) reported one case in which the virus developed the rtA181T mutation during famciclovir breakthrough. The A556T mutation of WHV, analogous to the rtA181T mutation of HBV, has been reported to be associated with lamivudine resistance (15, 28). These results indicate that the amino acid substitutions at position 181 may associate with resistance against many nucleoside analogues, including lamivudine, famciclovir, and adefovir. Although our in vitro study indicated that the rtA181T mutant had no resistance against adefovir and the animal study showed that combination therapy with lamivudine and adefovir effectively reduced the virus load in woodchucks (15), such combination therapy did not produce sufficient suppression of HBV in the index patient (Fig. 1A). The amino acid substitution at position 181 has to be further analyzed with regard to resistance to anti-HBV drugs.

The rtA181T mutation detection system using RFLP PCR developed in this study is a useful tool, as we were able to distinguish the wild type from all mutants with nucleotide substitutions in a given region. The system also enabled us to monitor the fluctuation of the wild-type/mutant ratio during therapy against HBV (Fig. 1 and 6). The incidence of rtA181T mutants with an intact YMDD motif is rare in Japanese patients with chronic HBV infection treated with lamivudine. Interestingly, 4 of the 39 (10%) patients who developed lamiv-

udine breakthrough and were positive for YMDD mutants were found to have small amounts of rtA181T mutant strains. Different from the previous report (34), the mutants did not take over another strain and were not preceded by exacerbation. We have to monitor these patients carefully for further population change of mutants and for exacerbation of hepatitis.

A recent study reported that the prevalence of genotype A HBV infection is increasing in Japan and that the incidence of disease chronicity is higher than for other genotypes (26). It is thus expected that an increasing number of the sexually active population will receive nucleoside analogue therapy against HBV and multiple mutant strains can potentially emerge and spread along with long-term treatment. There is an increasing possibility of emergence of novel mutants resistant to multiple anti-HBV drugs. The importance and significance of the rtA181 mutations, including the novel mutant strain identified in this study, should be investigated further to develop more useful treatment strategies.

ACKNOWLEDGMENTS

This work was carried out at the Research Center for Molecular Medicine, Faculty of Medicine, Hiroshima University. We thank Hiromi Ishino, Asako Kozono, Kana Kunihiro, Rie Akiyama, Yoshiko Seo, Yoshiko Nakata, Eiko Miyoshi and Kiyomi Toyota for their excellent technical assistance.

This work was supported in part by grants-in-aid for scientific research and development from the Ministry of Education, Sports, Culture, and Technology and the Ministry of Health, Labor and Welfare.

REFERENCES

- Allen, M. I., M. Deslauriers, C. W. Andrews, G. A. Tipples, K. A. Walters, D. L. Tyrrell, N. Brown, L. D. Condearey, et al. 1998. Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. *Hepatology* 27:1670–1677.
- Angus, P., R. Vaughan, S. Xiong, H. Yang, W. Delaney, C. Gibbs, C. Brosgart, D. Colledge, R. Edwards, A. Ayres, A. Bartholomeusz, and S. Locarnini. 2003. Resistance to adefovir dipivoxil therapy associated with the selection of a novel mutation in the HBV polymerase. *Gastroenterology* 125:292–297.
- Bozdayi, A. M., O. Uzunalimoglu, A. R. Turkyilmaz, N. Aslan, O. Sezgin, T. Sahin, G. Bozdayi, K. Cinar, S. B. Pai, R. Pai, H. Bozkaya, S. Karayalcin, C. Yurdadin, and R. F. Schinazi. 2003. YSDD: a novel mutation in HBV DNA polymerase confers clinical resistance to lamivudine. *J. Viral Hepat.* 10:256–265.
- Bruix, J., and J. M. Llovet. 2003. Hepatitis B virus and hepatocellular carcinoma. *J. Hepatol.* 39(Suppl. 1):S59–S63.
- Chang, T. T., R. G. Gish, S. J. Hadziyannis, J. Cianciara, M. Rizzetto, E. R. Schiff, G. Pastore, B. R. Bacon, T. Poynard, S. Joshi, K. S. Kleszczewski, A. Thiry, R. E. Rose, R. J. Colonna, and R. G. Hines. 2005. A dose-ranging study of the efficacy and tolerability of entecavir in lamivudine-refractory chronic hepatitis B patients. *Gastroenterology* 129:1198–1209.
- Chayama, K., Y. Suzuki, M. Kobayashi, M. Kobayashi, A. Tsubota, M. Hashimoto, Y. Miyano, H. Koike, M. Kobayashi, I. Koida, Y. Arase, S. Saitoh, N. Murashima, K. Ikeda, and H. Kumada. 1998. Emergence and takeover of YMDD motif mutant hepatitis B virus during long-term lamivudine therapy and re-takeover by wild type after cessation of therapy. *Hepatology* 27:1711–1716.
- Chien, R. N., C. T. Yeh, P. N. Wang, M. C. Kuo, S. Y. Hsieh, L. Y. Shih, and Y. F. Liaw. 2004. Acute leukaemia in chronic hepatitis B patients with lamivudine therapy. *Int. J. Clin. Pract.* 58:1088–1091.
- Das, K., X. Xiong, H. Yang, C. E. Westland, C. S. Gibbs, S. G. Sarafianos, and E. Arnold. 2001. Molecular modeling and biochemical characterization reveal the mechanism of hepatitis B virus polymerase resistance to lamivudine (3TC) and emtricitabine (FTC). *J. Virol.* 75:4771–4779.
- Delaney, W. E., IV, H. Yang, C. E. Westland, K. Das, E. Arnold, C. S. Gibbs, M. D. Miller, and S. Xiong. 2003. The hepatitis B virus polymerase mutation rtV173L is selected during lamivudine therapy and enhances viral replication in vitro. *J. Virol.* 77:11833–11841.
- Doong, S. L., C. H. Tsai, R. F. Schinazi, D. C. Liotta, and Y. C. Cheng. 1991. Inhibition of the replication of hepatitis B virus in vitro by 2',3'-dideoxy-3'-thiacytidine and related analogues. *Proc. Natl. Acad. Sci. USA* 88:8495–8499.

11. Fung, S. K., P. Andreone, S. H. Han, K. Rajender Reddy, A. Regev, E. B. Keeffe, M. Hussain, C. Cursaro, P. Richtmyer, J. A. Marrero, and A. S. Lok. 2005. Adefovir-resistant hepatitis B can be associated with viral rebound and hepatic decompensation. *J. Hepatol.* **43**:937–943.
12. Ganem, D., and A. M. Prince. 2004. Hepatitis B virus infection—natural history and clinical consequences. *N. Engl. J. Med.* **350**:1118–1129.
13. Gunther, S., G. Sommer, F. Von Breunig, A. Iwanska, T. Kalinina, M. Sterneck, and H. Will. 1998. Amplification of full-length hepatitis B virus genomes from samples from patients with low levels of viremia: frequency and functional consequences of PCR-introduced mutations. *J. Clin. Microbiol.* **36**:531–538.
14. Hadziyannis, S. J., N. C. Tassopoulos, E. J. Heathcote, T. T. Chang, G. Kitis, M. Rizzetto, P. Marcellin, S. G. Lim, Z. Goodman, J. Ma, S. Arterburn, S. Xiong, G. Currie, and C. L. Brosgart. 2005. Long-term therapy with adefovir dipivoxil for HBeAg-negative chronic hepatitis B. *N. Engl. J. Med.* **352**:2673–2681.
15. Jacob, J. R., B. E. Korba, P. J. Cote, I. Toshkov, W. E. t. Delaney, J. L. Gerin, and B. C. Tennant. 2004. Suppression of lamivudine-resistant B-domain mutants by adefovir dipivoxil in the woodchuck hepatitis virus model. *Antiviral Res.* **63**:115–121.
16. Lai, C. L., R. N. Chien, N. W. Leung, T. T. Chang, R. Guan, D. I. Tai, K. Y. Ng, P. C. Wu, J. C. Dent, J. Barber, S. L. Stephenson, D. F. Gray, et al. 1998. A one-year trial of lamivudine for chronic hepatitis B. *N. Engl. J. Med.* **339**:61–68.
17. Lai, C. L., M. Rosmawati, J. Lao, H. Van Vlierberghe, F. H. Anderson, N. Thomas, and D. Dehertogh. 2002. Entecavir is superior to lamivudine in reducing hepatitis B virus DNA in patients with chronic hepatitis B infection. *Gastroenterology* **123**:1831–1838.
18. Lai, C. L., J. Dienstag, E. Schiff, N. W. Leung, M. Atkins, C. Hunt, N. Brown, M. Woessner, R. Boehme, and L. Condreay. 2003. Prevalence and clinical correlates of YMDD variants during lamivudine therapy for patients with chronic hepatitis B. *Clin. Infect. Dis.* **36**:687–696.
19. Lanford, R. E., L. Notvall, H. Lee, and B. Beames. 1997. Transcomplementation of nucleotide priming and reverse transcription between independently expressed TP and RT domains of the hepatitis B virus reverse transcriptase. *J. Virol.* **71**:2996–3004.
20. Lanford, R. E., Y. H. Kim, H. Lee, L. Notvall, and B. Beames. 1999. Mapping of the hepatitis B virus reverse transcriptase TP and RT domains by transcomplementation for nucleotide priming and by protein-protein interaction. *J. Virol.* **73**:1885–1893.
21. Lin, X., Z. H. Yuan, L. Wu, J. P. Ding, and Y. M. Wen. 2001. A single amino acid in the reverse transcriptase domain of hepatitis B virus affects virus replication efficiency. *J. Virol.* **75**:11827–11833.
22. Nevens, F., J. Main, P. Honkoop, D. L. Tyrrell, J. Barber, M. T. Sullivan, J. Fevery, R. A. De Man, and H. C. Thomas. 1997. Lamivudine therapy for chronic hepatitis B: a six-month randomized dose-ranging study. *Gastroenterology* **113**:1258–1263.
23. Ono, S. K., N. Kato, Y. Shiratori, J. Kato, T. Goto, R. F. Schinazi, F. J. Carrilho, and M. Omata. 2001. The polymerase L528M mutation cooperates with nucleotide binding-site mutations, increasing hepatitis B virus replication and drug resistance. *J. Clin. Investig.* **107**:449–455.
24. Pai, S. B., A. M. Bozdayi, R. B. Pai, T. Beker, M. Sarioglu, A. R. Turkyilmaz, J. Grier, C. Yurdaydin, and R. F. Schinazi. 2005. Emergence of a novel mutation in the FLLA region of hepatitis B virus during lamivudine therapy. *Antimicrob. Agents Chemother.* **49**:2618–2624.
25. Suzuki, Y., H. Kumada, K. Ikeda, K. Chayama, Y. Arase, S. Saitoh, A. Tsubota, M. Kobayashi, K. Koike, N. Ogawa, and K. Tanikawa. 1999. Histological changes in liver biopsies after one year of lamivudine treatment in patients with chronic hepatitis B infection. *J. Hepatol.* **30**:743–748.
26. Suzuki, Y., M. Kobayashi, K. Ikeda, F. Suzuki, Y. Arase, N. Akuta, T. Hosaka, S. Saitoh, M. Kobayashi, T. Someya, M. Matsuda, J. Sato, S. Watabiki, Y. Miyakawa, and H. Kumada. 2005. Persistence of acute infection with hepatitis B virus genotype A and treatment in Japan. *J. Med. Virol.* **76**:33–39.
27. Tateno, C., Y. Yoshizane, N. Saito, M. Kataoka, R. Utoh, C. Yamasaki, A. Tachibana, Y. Soeno, K. Asahina, H. Hino, T. Asahara, T. Yokoi, T. Furukawa, and K. Yoshizato. 2004. Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am. J. Pathol.* **165**:901–912.
28. Tatti, K. M., B. E. Korba, H. L. Stang, S. Peek, J. L. Gerin, B. C. Tennant, and R. F. Schinazi. 2002. Mutations in the conserved woodchuck hepatitis virus polymerase FLLA and YMDD regions conferring resistance to lamivudine. *Antiviral Res.* **55**:141–150.
29. Tillmann, H. L., C. Trautwein, T. Bock, K. H. Boker, E. Jackel, M. Glowienka, K. Oldhafer, I. Bruns, J. Gauthier, L. D. Condreay, H. R. Raab, and M. P. Manns. 1999. Mutational pattern of hepatitis B virus on sequential therapy with famciclovir and lamivudine in patients with hepatitis B virus reinfection occurring under HBIG immunoglobulin after liver transplantation. *Hepatology* **30**:244–256.
30. Tsubota, A., Y. Arase, S. Saitoh, M. Kobayashi, Y. Suzuki, F. Suzuki, K. Chayama, N. Murashima, K. Ikeda, M. Kobayashi, and H. Kumada. 2001. Lamivudine therapy for spontaneously occurring severe acute exacerbation in chronic hepatitis B virus infection: a preliminary study. *Am. J. Gastroenterol.* **96**:557–562.
31. Tsuge, M., N. Hiraga, H. Takaishi, C. Noguchi, H. Oga, M. Imamura, S. Takahashi, E. Iwao, Y. Fujimoto, H. Ochi, K. Chayama, C. Tateno, and K. Yoshizato. 2005. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. *Hepatology* **42**:1046–1054.
32. Villeneuve, J. P., D. Durantel, S. Durantel, C. Westland, S. Xiong, C. L. Brosgart, C. S. Gibbs, P. Parvaz, B. Werle, C. Trepo, and F. Zoulim. 2003. Selection of a hepatitis B virus strain resistant to adefovir in a liver transplantation patient. *J. Hepatol.* **39**:1085–1089.
33. Wright, T. L., and J. Y. Lau. 1993. Clinical aspects of hepatitis B virus infection. *Lancet* **342**:1340–1344.
34. Yeh, C. T., R. N. Chien, C. M. Chu, and Y. F. Liaw. 2000. Clearance of the original hepatitis B virus YMDD-motif mutants with emergence of distinct lamivudine-resistant mutants during prolonged lamivudine therapy. *Hepatology* **31**:1318–1326.