ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 1996, p. 1957–1960 Vol. 40, No. 8
0066-4804/96/$04.00+0

Generation of Duck Hepatitis B Virus Polymerase Mutants through Site-Directed Mutagenesis Which Demonstrate Resistance to Lamivudine [(−)-β-L-2′,3′-Dideoxy-3′-Thiacytidine] In Vitro

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Received 12 February 1996/Returned for modification 10 April 1996/Accepted 12 June 1996

Hepatitis B virus replication is very sensitive to lamivudine. A single amino acid change in the human immunodeficiency virus reverse transcriptase is responsible for high-level resistance to this compound. Duck hepatitis B virus mutants were created bearing the analogous amino acid change in the duck hepatitis B virus polymerase. Viral DNA production was reduced 92% for the wild-type virus at 2 μg of lamivudine per ml, while the mutants required 40 μg of lamivudine per ml to inhibit replication by greater than 80%.

The (−) enantiomers of oxathiolane-cytosine analogs like (−)-β-L-2′,3′-dideoxy-3′-thiacytidine (3TC or lamivudine) and its 5-fluoro derivative have been shown to have potent antiviral activities, in vitro and in vivo, against human immunodeficiency virus type 1 (HIV-1) and type 2 (HIV-2) and hepadnaviruses (1, 3–9, 12, 15, 16, 18, 21, 24, 28, 30). The triphosphate forms of these compounds are found to act as chain terminators when tested against purified HIV-1 reverse transcriptase (RT) in vitro (13), and the triphosphate derivative of lamivudine has recently been shown to act as a chain terminator of duck hepatitis B virus (DHBV) DNA when tested in replicating cores (23).

In studies of HIV, there have been reports showing that 3TC-resistant HIV-1 variants can arise in vivo and in vitro and that this resistance is mediated by a single amino acid change in the HIV RT. The methionine of the YMDD motif, characteristic of retroviral polymerases, was changed to a valine residue in the resistant mutants (2, 10, 14, 22, 28). Since this motif is also conserved at the polymerase catalytic site of all hepadnaviruses, it was of interest to determine if an analogous mutation produced in vitro could confer resistance of DHBV to lamivudine. Resistance to lamivudine has not been seen in ducks chronically treated for 1 year (29), or in human studies using lamivudine to treat chronic hepatitis B infection (31). Lamivudine is currently in phase II clinical trials in the treatment of chronic HBV infections (20, 31). In this study we have initiated site-specific mutagenesis of the DHBV polymerase to produce two mutants which demonstrate a markedly decreased sensitivity to lamivudine.

All cloning was performed by standard techniques (19), and all DHBV nucleic acid sequence positions reported in this study are numbered by the numbering system of Mandart et al. (17). The replication-competent construct pCMVDHBV2 (Fig. 1A), which contains a greater-than-genome-length copy of DHBV, was used as a positive control in transfection and served as a recipient for mutagenized fragments. pCMVDHBV2 was constructed as follows: a 2.8-kb *Eco*RI-*Sph*I fragment of DHBV type 16 (DHBV-16) was cloned into similarly digested pcDNA I/Amp (InVitrogen Corporation, San Diego, Calif.) to yield pcES14. The pcES14 construct was digested with *Hin*dIII, blunt ended with Klenow fragment and deoxynucleoside triphosphates, and cut with *Bam*HI. The 5′ end of the DHBV sequence in pCMVDHBV2 was generated by cleaving pSP DHBV 5.1 (2×) (containing a head-to-tail *Eco*RI dimer of DHBV-16, a kind gift from Jesse Summers, University of New Mexico, Albuquerque) with *Afl*II, blunt ended with Klenow fragment and deoxynucleoside triphosphates, and digested with *Bam*HI to yield a 2.15-kb blunt cohesive fragment which was subsequently cloned into the digested pcES14. The DHBV open reading frames (ORFs) of the final pCMVDHBV2 construct are represented in Fig. 1B. For site-specific mutagenesis, the plasmid pAltD2-8 was constructed by cloning an *Eco*RI monomer of DHBV-16 into the *Eco*RI site of phagemid pAlter-1 (Promega, Madison, Wis.). Mutagenesis of the DHBV polymerase at methionine codon 512 was done by using the Altered Sites *in vitro* Mutagenesis Kit (Promega Corporation, Madison, Wis.) according to the manufacturer's protocol. Mutagenic oligonucleotides used in this study are shown in Fig. 1C. Mutants were verified by sequencing prior to mobilizing a 2.1-kb *Bgl*II-*Afl*II fragment (nucleotides 391 to 2526) into similarly digested pCMVDHBV2 to yield pCMVDHBV2-M512V and pCMVDHBV2-MI512VM. All plasmids used for transfection experiments were purified by Quiagen matrix chromatography (Qiagen Inc., Chatsworth, Calif.).

LMH cells (7) were cultured in a combination of Ham's F12 nutrient medium and minimal essential medium (1:1) supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, 100 μg of streptomycin per ml, 50 IU of penicillin G per ml and buffered with sodium bicarbonate. Six-well plates were seeded at 5×10^5 cells per well and were transfected by the calcium phosphate method (19) at 16 to 18 h postplating with 5 μg per plate of pCMVDHBV2 or mutant plasmid DNA. Twenty-four hours posttransfection, the cells were washed with phosphate-buffered saline and reincubated with drug-free medium or medium containing lamivudine (GlaxoWellcome, Stevenage, UK) at the concentrations indicated. After 3 days of incubation, the monolayers were washed, and intracellular virus DNA was isolated as previously described (25). Viral DNAs were resolved on a 1% agarose gel, transferred to a nylon membrane by Southern blotting, and detected with a radioactively labeled DHBV genomic probe. Densitometric analysis was performed on resulting autoradiograms by using the program Image v1.59 (National Institutes of Health).

The M512 mutants of DHBV were found to be competent for replication following transfection into LMH cells, though

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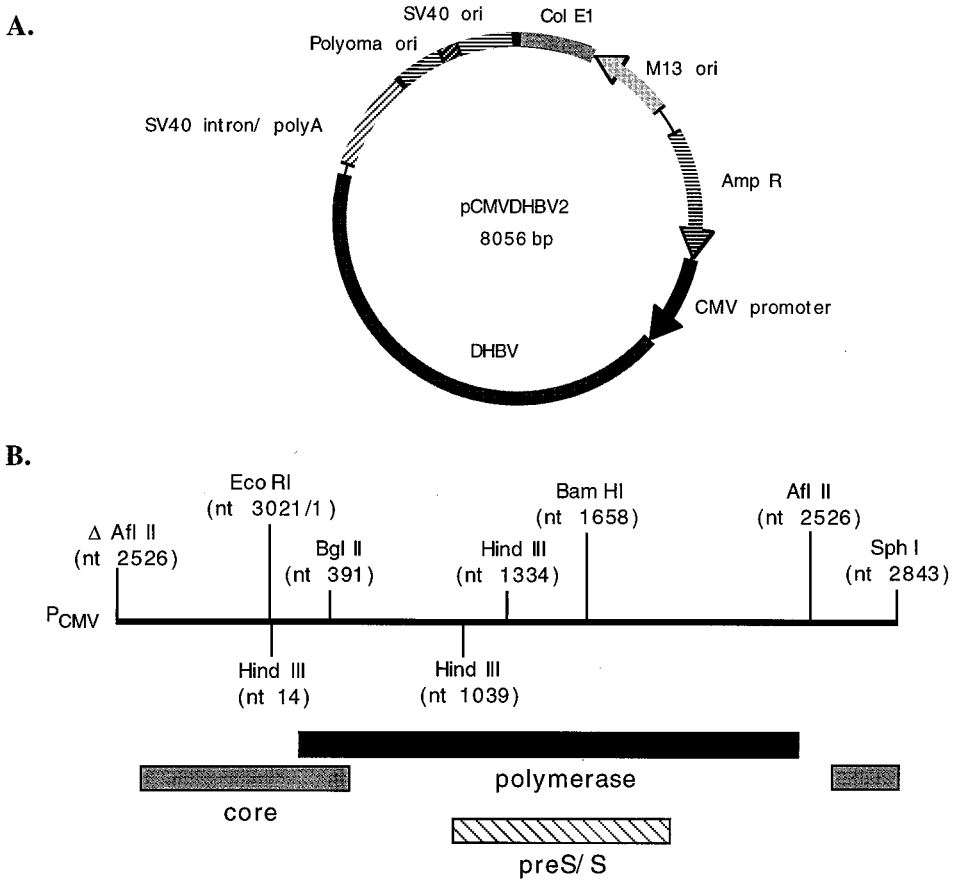

C.

	ACT TTC ACT TAT ATG GAT GAC TTC	Wild-type
	Y M D D	
5'	TC ACT TAC GTA GAT GAC TTC	M 512 V
5'	ACT TAT GTG GAT GAC T	MI 512 VM
	Y V D D	

FIG. 1. (A) Plasmid construct pCMVDHBV2. Abbreviations: SV40, simian virus 40; ori, origin; Polyoma, polyomavirus; Amp R, ampicillin resistance; CMV, cytomegalovirus. (B) DHBV ORFs in the pCMVDHBV2 construct. The Δ symbol represents the loss of the *Afl*II site due to the Klenow fill-in reaction. nt, nucleotides. (C) Mutagenic oligonucleotides for generation of DHBV polymerase mutants with methionine changed to valine at amino acid position 512.

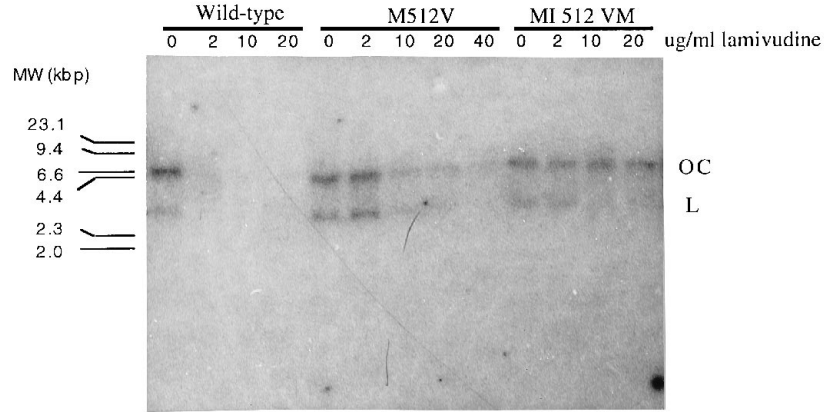

FIG. 2. Mutants with methionine changed to valine demonstrate resistance to lamivudine. Each lane contains intracellular viral DNA extracted from approximately 7×10^5 cells. Autoradiographic exposure was 3 days. OC, open circular DNA; L, linear DNA.

TABLE 1. Resistance of DHBV mutants with the methionine-to-valine substitution to selected concentrations of lamivudine

Virus construct	% Reduction[a] of intracellular viral DNA at the following concn (μg/ml) of lamivudine:		
	2	10	40
Wild-type	92	DND[b]	ND[c]
M512V	48	56	80
MI512VM	26	40	89

[a] Reduction was determined relative to the amount of intracellular viral DNA in the transfected, but untreated, companion culture.
[b] DND, DNA not detected.
[c] ND, not done.

levels of intracellular viral DNA were slightly reduced from that of the wild-type construct (Fig. 2, 0 lanes). The replication level of the M512V mutant (mutant with methionine at amino acid position 512 changed to valine), which has a premature termination of the pre-S/S ORFs (27-amino-acid deletion at the carboxyl terminus), was similar to the MI512VM mutant which contains an isoleucine-to-methionine change in the pre-S/S antigen. Wild-type viral DNA production was markedly reduced by treatment with lamivudine, whereas the DNA levels for the mutants were unchanged from that of the untreated control. A titration of lamivudine showed that intracellular DHBV DNA production for the wild-type construct is inhibited >90% at a concentration of 2 μg/ml, whereas 40 μg/ml was required for a similar level of suppression of the mutants (Fig. 2; Table 1).

To date, the development of resistance to antihepadnavirus agents has not been demonstrated either in vitro or in vivo. While this might be explained by the low mutation rate of hepadnavirus genomes ($<2 \times 10^{-4}$ base substitutions per site per year [11]) compared with those of other viruses that lack polymerase-associated proofreading functions, resistance to antiviral agents in viruses which do have this correcting function is not without precedent—mutations in cytomegalovirus polymerase which confer resistance to ganciclovir which have arisen both in vitro (26) and in vivo (27) have been described. This report of a genetically engineered lamivudine-resistant hepadnavirus serves as a cautionary note of what could occur when lamivudine is used in the prolonged treatment of chronic HBV infections, analogous to the emergence of 3TC resistance in the treatment of AIDS (2, 10, 22, 28).

We acknowledge the financial support of this study by GlaxoWellcome Canada.

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