Reverse Transcriptase Activity of Hepatitis B Virus (HBV) DNA Polymerase within Core Capsid: Interaction with Deoxynucleoside Triphosphates and Anti-HBV L-Deoxynucleoside Analog Triphosphates

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ABSTRACT

The use of L(−)SddC [β-L-2′,3′-dideoxy-3′-thiacytidine (lamivudine, 3TC)] for the treatment of Herpes B virus (HBV) infection is hindered by the emergence of drug-resistance associated with the L526M, L550V, and L526M/M550V mutations of the viral DNA polymerase (DP). The interactions of the anti-HBV compounds 2′,3′-dideoxy-2′,3′-didehydro-β-L(−)-5-fluorodeoxycytidine and 2′-fluoro-5-methyl-β-L-arabinofuranosyluracil triphosphate with HBV DP and its L(−)SddC-associated mutants have not been studied. The e antigen-negative variant of HBV associated with the G1896A mutation in the precore region has a high prevalence. Its effect on HBV DP is unclear. Because HBV DNA synthesis occurs in the nucleocapsid, we examined the kinetics of the reverse transcriptase activity from wild-type (wt) and mutated DPs with the wt or G1896A-mutated RNA template in the nucleocapsid. The effects of this template mutation on the activities of these L-nucleoside triphosphates were also examined. Results indicated that these DP mutations increased the K_m values of deoxy-NTPs and decreased the efficiencies (V_{max}/K_m) of DPs. The additional L526M mutation increased the efficiency of the M550V-mutated DP but no more than that of the L526M-mutated DP. The G1896A mutation had impacts on the interactions between different DPs and deoxy-NTPs, except dCTP. It also had different impacts on the actions of the L-nucleoside triphosphates toward DPs. The L526M and M550V mutations caused a greater decrease in the V_{max} using the wt RNA template compared with the G1896A-mutated template. The L526M, M550V, and L526M/M550V mutations caused varying degrees of resistance to the different L-nucleoside triphosphates.

HBV infection, associated with the risk of developing liver cirrhosis and hepatocellular carcinoma, is a worldwide public health problem (Kao and Chen, 2002). Different serological strains of HBV have been identified; the adr strain is the most common type found in Asia (Liu et al., 2002). Chronic HBV infection can be classified as e antigen (HBeAg)-positive and HBeAg-negative (Hadziyannis and Vassilopoulos, 2001). The median prevalence of HBeAg-negative HBV is about 33% in the Mediterranean area, 15% in Asia Pacific, and 14% in the United States and Northern Europe (Funk et al., 2002). The HBeAg-negative disease is usually associated with a mutation of the HBV genome in the precore region. The most common precore mutation that prevents e antigen production is a change from G to A at nucleotide 1896 (G1896A). This

creates a novel translational stop codon at precore codon 28, leading to the premature termination of the translation of the precore protein (Carman et al., 1989). This nucleotide is located in the stem loop of viral RNA, which is required to initiate DNA synthesis by the viral DP. The significance of this mutation in the replication of HBV and the behavior of HBV DP is unknown.

L(−)SddC (3TC, lamivudine) was discovered independently in our laboratory (Doong et al., 1991; Chang et al., 1992) and others (Tyrell et al., 1993) as a potent anti-HBV drug. Clinical studies using 100 mg/day orally were found to be effective for the treatment of HBV. Long-term treatment is required because the virus rebounds after a short-term treatment protocol (Tyrrell et al., 1993; Dienstag et al., 1995; Honkoop et al., 1997; Lai et al., 1997). The development of viral resistance to L(−)SddC emerges typically after the first 6 months of a period of treatment. Its frequency increases

ABBREVIATIONS: HBV, hepatitis B virus; HBeAg, HBV e antigen; L(−)SddC, β-L-2′,3′-dideoxy-3′-thiacytidine; DP, DNA polymerase; L(−)SddCTP, β-L-2′,3′-dideoxy-3′-thiacytidine triphosphate; wt, wild type; L(−)Fd4C, 2′,3′-dideoxy-2′,3′-didehydro-β-L(−)-5-fluorodeoxycytidine; L(−)FMAU, 2′-fluoro-5-methyl-β-L-arabinofuranosyluracil; L(−)FMAUTP, 2′-fluoro-5-methyl-β-L-arabinofuranosyluracil triphosphate; L(−)Fd4CTP, 2′,3′-dideoxy-2′,3′-didehydro-β-L(−)-5-fluorodeoxycytidine triphosphate.

progressively to more than 50% after 3 years of treatment in both HBeAg-positive and -negative patients (Hadziyannis et al., 2000; Lau et al., 2000; Liaw et al., 2000; Leung et al., 2001). The viral resistance to L(−)SddC is always associated with mutations of the DP, including L526M and M550V. Clinically, the strain with a double mutation at L526M and M550V/I represents the most common form of L(−)SddC resistance (Ling et al., 1996; Bartholomew et al., 1997). Cell culture studies showed that M550V and L526M mutations decreased the viral replication efficiency by about 100- and 6-fold, respectively (Fu and Cheng, 1998). The additional mutation of L526M to M550V, however, had a compensatory effect of increasing the replication rate of the M550V-mutated virus. A similar observation was made for both adr and ayw strains of HBV (Fu and Cheng, 1998; Ladner et al., 1998; Melegari et al., 1998). The relative degree of L(−)SddC resistance (by comparison of IC_{50}) was as follows: L526M/M550V = M550V > L526M > wt. The interactions of L(−)SddCTP, the active metabolite of L(−)SddC, with purified HBV (ayw) DP using calf thymus DNA, with or without L526M and/or M550V mutations, were studied (Xiong et al., 1998). The results indicate that L(−)SddCTP is an inhibitor of HBV DP, with its potency in the following order: wt > L526M > M550V > L526M/M550V. This finding is consistent with the known action of L(−)SddC against HBV and its mutants in cell culture. Recently, the impacts of the G1896A precore mutation on wt, L526M, M550V, and L526M/M550V-mutated viruses were studied in cell culture (Chen et al., 2003). The G1896A mutation was shown to enhance the replication rate of mutant viruses without changing their sensitivities to L(−)SddC (Chen et al., 2003). However, the impacts of the G1896A precore mutation on the kinetic behavior of HBV DPs and their sensitivities to L(−)SddCTP remain to be elucidated.

To combat the emergence of drug resistance during therapy, anti-HBV compounds with a high potency and/or a unique mechanism of HBV inhibition are needed. L(−)Fd4C and L(−)FMAU (Clevudine) were discovered in our laboratory in collaboration with others. These compounds create potent and unique activities against HBV. In cell culture and in vivo models, L(−)Fd4C has demonstrated a much greater potency than L(−)SddC against HBV (adr and ayw strains) DNA synthesis (Zhu et al., 1998; Fu and Cheng, 2000; Le Guerhier et al., 2000, 2001). L(−)Fd4C is also active against the replication of the L(−)SddC-resistant virus at noncytotoxic concentrations. There are no inhibitory effects on nuclear DNA and mitochondrial DNA synthesis at concentrations at least 100 times higher than the concentration at which the HBV DNA level in cells decreased by 50% (Zhu et al., 1998). L(−)Fd4CTP also has a longer half-life than L(−)SddCTP (Zhu et al., 1998). It is therefore conceivable that L(−)Fd4C could benefit the patients who are resistant to or are unable to tolerate L(−)SddC. L(−)FMAU, a thymidine analog, was found to be a potent inhibitor of HBV and Epstein-Barr virus (Balakrishna Pai et al., 1996; Yao et al., 1996; Ma et al., 1997; Kukhanova et al., 1998). The spectrum of antiviral activity of L(−)FMAU differs from those of L(−)SddC and L(−)Fd4C, which are active against both HBV and HIV-1. One novelty of L(−)FMAU is that it can be phosphorylated by the cellular thymidine kinase as well as deoxycytidine kinase to its monophosphate metabolite and subsequently to the di- and triphosphate metabolites (Bal-

akrishna Pai et al., 1996). L(−)FMAUTP is not a substrate for any human DP examined, but it is an inhibitor of HBV DP activity associated with the HBV virion (Balakrishna Pai et al., 1996; Kukhanova et al., 1998). One unexpected aspect of L(−)FMAU activity was its activity against woodchuck hepatitis virus in vivo. Unlike L(−)SddC and L(−)Fd4C, there was no subsequent rebound of woodchuck hepatitis virus in the serum of animals after a 4-week treatment with L(−)FMAU (Peek et al., 2001). Our previous studies have shown that L(−)Fd4CTP and L(−)FMAUTP could inhibit (+)-strand DNA synthesis in HBV particles secreted from the HepG2 subclone, 2.2.15 cells (Balakrishna Pai et al., 1996; Zhu et al., 1998). There is currently no detailed information about the inhibitory effect of L(−)Fd4CTP and L(−)FMAUTP on the reverse transcriptase activity [(−)-strand DNA synthesis] of HBV DP.

Most published studies of HBV have been done using the ayw strain. Because the adr strain of HBV is the most prevalent one in Asia, where the population has the highest rate of HBV infection among all continents, we chose to focus on the kinetic behavior of the HBV DPs (wild-type and its L(−)SddC resistance-associated mutants with mutations of L526M, M550V, and L566M/M550V) from the adr strain. We studied the impact of the G1896A precore mutation of the RNA template on the reverse transcriptase activities of these DPs. The inhibitory effects of L(−)SddCTP, L(−)Fd4CTP, and L(−)FMAUTP on the reverse transcriptase activity [(−)-strand DNA synthesis] of these DPs were also evaluated. Furthermore, we also assessed the impact of the G1896A mutation on the sensitivities of these DPs to different antiviral nucleotide triphosphates. Because HBV DNA synthesis catalyzed by a DP within a capsid could be different from that catalyzed by a naked DP, we expressed and purified the complex of the HBV core capsid containing the DP and the viral RNA template for our study. This may more closely reflect the natural behavior of HBV replication. The core-DNA complex nucleocapsid system was validated to be suitable for studying the early priming and reverse transcriptase activity of HBV DP (Seifer et al., 1998).

Materials and Methods

Nucleotide Analogs. L(−)SddCTP, L(−)Fd4CTP, and L(−)FMAUTP were synthesized in our laboratory as described previously (Ruth and Cheng, 1981; Zhu et al., 1998).

Constructs. HBV DP and its mutant (L526M, M550V, and L526M/M550V) DNA fragments, from the starting codon of DP to the epsilon region, were amplified by polymerase chain reaction from pBS-adr-HBV plasmid DNA (Fu and Cheng, 1998) and then cloned into the vector pVL1393. Site-directed mutagenesis (QuikChangeXL; Stratagene, La Jolla, CA) was used to change the nucleotide at 1896 from G into A. The HBV core gene was cloned separately into another vector of pVL1393. Recombinant baculoviruses were generated according to the manual provided by BD Biosciences Pharmingen (San Diego, CA).

Cells and Baculovirus. Core-HBV DP complexes, nucleocapsids, were expressed in *Spodoptera frugiperda* cell line Sf9 as described by Seifer et al. (1998). Briefly, 1-liter suspension cultures (2×10^6 cells/ml) were kept in a serum-free medium (SF-900 SFM; Invitrogen, Carlsbad, CA) at 27°C. Cells were typically coinfected with core- and Pol-expressing baculoviruses (at multiplicity of infection of 2–2.5 and 8–10, respectively). To block DNA synthesis in nucleocapsids in vivo, insect cells were fed with 1.5 mM sodium phosphonoformate (Sigma, St. Louis, MO), starting at 1 h after infection and at 24-h

intervals during the course of infection. The infected cells were harvested approximately 3.5 days after infection by low-speed centrifugation (700g, 10 min, 4°C). After one rinse with phosphate-buffered saline, the cell pellet was stored at -80°C until use.

Isolation of Nucleocapsids by Sucrose Gradient. Crude lysate from insect cells was prepared by resuspending the cell pellet in one tenth the original volume of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM EDTA, and 0.75% Nonidet P-40). After 15 min on ice, the lysate was clarified by low-speed centrifugation (2000g, 4°C, 15 min). Nucleocapsids were pelleted by centrifuging the crude lysate at 44,000 rpm using a rotor (SW50; Beckman Coulter, Fullerton, CA) for 2 h. The pellet was resuspended using the lysis buffer. The lysate was treated with 100 μg/ml DnaseI for 5min at 37°C, followed by 50 μg/ml RNaseA for 5min at 37°C. EDTA was added to the lysate for a final concentration of 25 mM. The lysate was centrifuged at 10,000g for 15 min at 4°C. The supernatant was subjected to a 10–40% sucrose gradient by centrifuging at 44,000 rpm (SW50) for 4 h. Fractions containing nucleocapsids were collected and then dialyzed against the dialysis buffer (50 mM Tris-HCl, pH7.4, 50 mM NaCl, and 1 mM EDTA) at 4°C overnight. The nucleocapsids were stored at -80°C.

Endogenous Polymerase Assay. Kinetic experiments on HBV DP were carried out by mixing 12.5 μl of nucleocapsids (10 μg) and 12.5 μl of reaction mixture [50 mM Tris-HCl, pH 7.4, 200 mM NaCl, 20 mM MgCl$_2$, 0.2% (v/v) β-mercaptoethanol, 0.027–1 μM [α-^{32}P]dNTPs (300 Ci/mmol; Amersham Biosciences), and 5 μM concentrations of three other dNTPs]. To determine K_i, different concentrations of nucleotide analogs were added to a reaction mixture that contained 1 μM radiolabeled competing dNTP and 5 μM concentrations of other three dNTPs. After the desired time, 75 μl of stop buffer (1.25% SDS and 5 mM EDTA) and 25 μl of 330 mM KCl were added to the reaction mixture. The mixture was boiled at 100°C for 5 min and then placed on ice for 15 min. The protein-DNA linked product was pelleted by centrifugation at 3000g for 20 min. After removing the supernatant, 100 μl of 50 mM Tris, pH 8.0, and 100 mM KCl was added to the pellet. The pellet was dissolved by boiling it at 100°C for 5 min. The protein-DNA linked product was precipitated again on ice for 15 min and then pelleted by centrifugation. Two volumes of 2$\times$ protein loading buffer [0.8% (v/v) SDS, 125 mM Tris-HCl, pH 6.8, 10% (v/v) glycerol, 5% 2-mercaptoethanol, and 0.0025% bromphenol blue] were added to the pellet. The protein-DNA linked product was electrophoresed on an 8% SDS-polyacrylamide gel and visualized by autoradiography after drying. The product was quantified by scanning the film with a densitometer.

Enzyme Kinetics. K_m values were derived from a Lineweaver-Burk plot. V_{max} values were calculated using the Michaelis-Menten equation. K_i values were calculated by using the equation from Cheng and Prusoff (Cheng and Prusoff, 1973), $K_i = $ IC$_{50}$/(1 + [S]/K_m), where IC$_{50}$ is the nucleotide analog concentration resulting in 50% enzyme inhibition. Values are presented as mean ± S.D. from at least three independent experiments.

Results

General Properties of the Nucleocapsids. Using a baculovirus expression system, we expressed and isolated nucleocapsids in the core-DP (adr strain) complex form, containing the viral RNA template with or without the G1896A mutation. The conditions for HBV DP reaction were optimized by changing the salt and magnesium concentrations. MgCl$_2$ (10 mM) and 125 mM NaCl were found to create the maximum activity. The addition of Nonidet P-40 to the purified nucleocapsids did not enhance the DP activity. The formation of product increased linearly over 10 min (Fig. 2). The activity of the HBV DP nucleocapsid was stable during long-term storage, as described by other investigators as well (Seifer et al., 1998). Western blotting analysis was employed

to determine whether equal amounts of DP were present in core protein capsids among different DPs. A similar ratio of DP to core protein was observed among nucleocapsid preparations having different DPs (data not shown). The ratios of RNA content to DP in different nucleocapsid preparations were found to be similar by using reverse transcription-polymerase chain reaction. This enabled us to compare the relative activities among different DPs. To separate the DNA product from radioactive nucleotides, we attempted to use immunoprecipitation to pull down the HBV DP-DNA complex. However, this method was labor-intensive and yielded data that were not reproducible. Because HBV DP is covalently linked to its product, we adapted the method of potassium-SDS precipitation, which is widely used in the precipitation of protein-linked DNA complexes, to pull down the HBV DP-DNA complex. This method was faster and resulted in the removal of the nonspecific signals in the gel after SDS-polyacrylamide gel electrophoresis. Results from samples prepared by potassium-SDS precipitation were more consistent than results from immunoprecipitation.

Kinetic Properties Using the Viral RNA with or without the G1896A Mutation as a Template. The kinetic properties of the reverse transcriptase activity of the wt DP and its L($-$)SddC resistance-associated mutants with respect to their four natural dNTP substrates were studied. As shown in Table 1, using a wt RNA template, K_m values of four dNTPs for DPs with L526M or M550V mutation were generally higher than the corresponding values for the wild-type DP. A lower K_m of substrate for the DP with the L526M/M550V mutation than for that with the M550V mutation was observed; however, this difference was not significant ($P > 0.05$). The V_{max} values of the mutated DPs with any dNTP were lower than those of the wt DP. When the relative efficiency (V_{max}/K_m) was compared, it was in the order of wt > L526M = L526M/M550V > M550V. This difference in efficiency is consistent with the rate of viral replication in cells transfected with HBV DNA with or without those mutations (Fu and Cheng, 1998).

When the G1896A mutation was introduced into the RNA template, the K_m values of the dATP, dGTP, and dTTP of the wt DP increased, except for the dCTP. The K_m values of the purine nucleotides, dATP and dGTP, for the DPs with L526M, M550V, and L526M/M550V mutations were also higher. However, compared with the results of the wt RNA template, the G1896A mutation did not affect the V_{max} of the wt DP except for the dTTP. The V_{max} of the mutated DPs was higher when the G1896A-mutated template was used than when the wt RNA template was used. The G1896A mutation
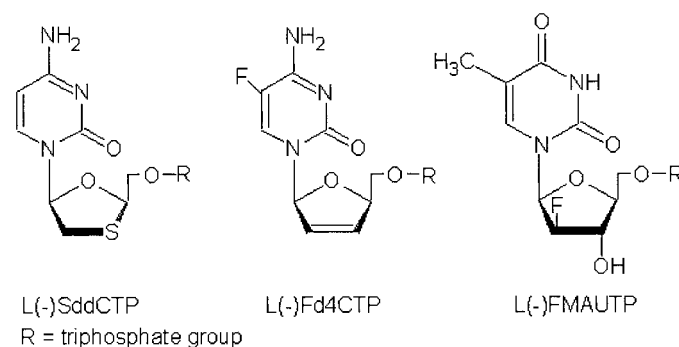

Fig. 1. Chemical structures of L-nucleoside analogs.

did not change the order of the relative efficiencies of DPs compared with the wt RNA template. However, the relative efficiencies of the L526M and M550V-mutated DPs were much higher for the G1896A-mutated template than for the wt template. The L526M/M550V-mutated DP showed a significant increase in efficiency using the G1896A-mutated template only when dATP or dGTP was used as the limiting substrate.

Inhibition by L(−)SddCTP and L(−)Fd4CTP. Both L(−)SddCTP and L(−)Fd4CTP are competitive inhibitors with respect to dCTP. The K_i values of both drugs for the wt and G1896A-mutated templates were estimated as shown in Table 2. Using the wt RNA template, the L526M, M550V, and L526M/M550V-mutated DPs were more resistant to L(−)SddCTP compared with the wt DP. The K_i values increased 14.5-, 76-, and 41-fold and K_i/K_m (dCTP) increased 7-, 19-, and 20-fold, respectively. When the G1896A-mutated template was used, all the mutated DPs were slightly more susceptible to L(−)SddCTP inhibition by reduction in K_i and K_i/K_m compared with the data when the wt RNA template was used.

L(−)Fd4CTP was a less potent inhibitor of the wt DPs than was L(−)SddCTP. Also, in contrast to L(−)SddCTP, the L526M mutation of HBV DP did not have much impact on its interaction with L(−)Fd4CTP. When the HBV DP with the

M550V mutation was used, the K_i value of L(−)Fd4CTP increased more than that of L(−)SddCTP. The introduction of the L526M mutation to the M550V-mutated DP decreased the K_i value of L(−)Fd4CTP toward the M550V0-mutated DP substantially but not that of L(−)SddCTP. When the G1896A-mutated template was used, L(−)Fd4CTP was found to be more inhibitory, as reflected by the K_i or K_i/K_m (dCTP) values, than when the wt RNA template was used.

Inhibition by L(−)FMAUTP. L(−)FMAUTP was found to be a competitive inhibitor with respect to dTTP for all the HBV DPs studied, with K_i/K_m values of 32, 40, 254, and 350 for wt, L526M, M550V, and L526M/M550V-mutated DPs, respectively (Table 2). The L526M, M550V, and L526M/M550V mutations of HBV DP increased the K_i values by 3-, 15-, and 16-fold, respectively. Similar to L(−)SddCTP but unlike L(−)Fd4CTP, introducing the L526M mutation to the M550V-mutated DP did not alter the K_i or K_i/K_m (dTTP). Using the G1896A-mutated RNA template, the K_i values of L(−)FMAUTP for the wt and mutated HBV DPs were about 2- to 3-fold lower than that using the wt RNA template. In addition, there was a decrease in the K_i/K_m values by approximately 2- to 4-fold using the G1986A-mutated template compared with the wt RNA template.

Discussion

HBV DNA replication occurs within the nucleocapsid, in which HBV DP uses its reverse transcriptase activity to start (−)-strand DNA synthesis by using the viral RNA as an initial template in the cytoplasm of the host cells. The activity of HBV DP depends on its interaction with the template and other proteins such as the core protein, as well as the amount of dNTP. Because the content of the four dNTPs in the cytoplasm of hepatocytes could be less than or equal to the K_m values (0.1 to 0.6 μM) of HBV DP, the replication rate of HBV is likely to be dictated by the efficiency of DP.

Our results suggest that an additional L526M mutation can increase the efficiency of the M550V-mutated DP to incorporate deoxyribonucleotides into DNA, particularly when the wt viral RNA is used as the template. This is
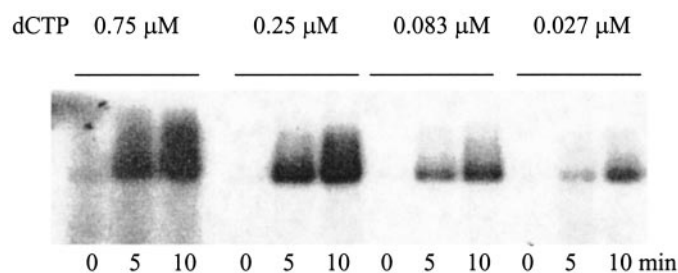

Fig. 2. Reverse transcriptase activity of HBV DNA polymerase. K_m study of wt HBV DP. Autoradiograph of HBV DNA linked complexes separated by SDS polyacrylamide gel electrophoresis. The rest of procedures were as described under *Materials and Methods*.

TABLE 1

K_m and V_{max} values of dNTPs for HBV DNA polymerases with a wild-type RNA template and a G1896A-mutated RNA template
Procedures were as described under *Materials and Methods*. Values are presented as mean ± S.D. of at least three independent experiments.

Variable Substrate	DP	K_m		V_{max}		Relative Efficiency (V_{max}/K_m)	
		W[a]	M[b]	W	M	W	M
		nM		10^{-6} *pmol/min/μg*			
dATP	WT	130 ± 50	240 ± 10	4.20 ± 0.14	3.67 ± 0.72	100	100
	L526M	290 ± 40	330 ± 100	0.56 ± 0.09	2.35 ± 0.78	5.9	47
	M550V	390 ± 180	650 ± 10	0.32 ± 0.17	0.69 ± 0.35	2.5	9.2
	L526M/M550V	220 ± 10	360 ± 120	0.42 ± 0.12	0.88 ± 0.37	5.9	16
dCTP	WT	100 ± 7	110 ± 50	5.34 ± 2.48	5.50 ± 2.1	100	100
	L526M	220 ± 80	170 ± 20	1.34 ± 0.09	3.45 ± 0.92	11	40
	M550V	430 ± 150	270 ± 40	0.39 ± 0.14	0.56 ± 0.14	1.7	4.2
	L526M/M550V	170 ± 100	280 ± 140	0.71 ± 0.14	1.44 ± 0.76	7.9	10
dGTP	WT	50 ± 10	230 ± 70	4.29 ± 0.95	3.12 ± 0.39	100	100
	L526M	130 ± 60	330 ± 60	0.27 ± 0.1	1.31 ± 0.6	2.4	28
	M550V	220 ± 60	550 ± 120	0.11 ± 0.01	0.50 ± 0.14	0.6	6.6
	L526M/M550V	160 ± 50	380 ± 90	0.21 ± 0.09	0.56 ± 0.21	1.5	11
dTTP	WT	130 ± 40	260 ± 40	7.90 ± 1.2	2.98 ± 0.72	100	100
	L526M	350 ± 90	310 ± 100	0.65 ± 0.21	2.19 ± 0.5	3.1	61
	M550V	230 ± 110	280 ± 120	0.13 ± 0.03	0.82 ± 0.18	0.9	2.5
	L526M/M550V	190 ± 20	280 ± 60	0.58 ± 0.10	1.21 ± 0.39	5.1	3.7

[a] Wild-type RNA template.
[b] G1896A-mutated RNA template.

consistent with the suggestion that the L526M mutation can compensate for the loss of viral replication caused by the M550V mutation in cell culture studies (Fu and Cheng, 1998; Ladner et al., 1998; Melegari et al., 1998). The G1896 is located opposite to the T1858 in the stem loop and a few nucleotides upstream of the 3′-ACUU-5′ sequence of the epsilon region of the viral RNA, the sequence for initial DNA priming during viral DNA synthesis. The G1896A mutation could stabilize the stem loop (Lok et al., 1994). The efficiency (V_{max}/K_m) of wt HBV DNA synthesis apparatus with the wt RNA template was higher (2- to 6-fold) than that with the G1896A-mutated template when dATP, dGTP, and dTTP were used as the limiting substrates. The fact that dCTP is not required in the primer formation explains the lack of impact of the G1896A mutation on the efficiency of wt HBV DNA synthesis when dCTP is the rate-limiting substrate. When the L526M- and M550V-mutated DPs with the G1896A-mutated template were used, the K_m and V_{max} were increased to different extents, thus increasing the efficiencies of (−)-strand DNA synthesis. This observation is consistent with the results obtained from cell culture studies (Chen et al., 2003), which suggests that the interactions between the RNA template and the wt or mutated DPs are different. The rate-limiting step between DNA primer formation and (−)-strand DNA synthesis for the wt DP is DNA primer formation, but that for the mutated DPs could be (−)-strand synthesis. The stem loop region would continue to play a role in (−)-strand DNA synthesis catalyzed by the mutated DPs after DNA primer formation because the V_{max} for dCTP was also different.

Compared with our previous results obtained using the isolated virus (Chang et al., 1992), the K_i/K_m (dCTP) for the inhibitory effects of L(−)SddCTP on the (−)-strand DNA synthesis is 8-fold higher than that on the (+)-strand DNA synthesis [K_i/K_m (dCTP), 200 nM/100 nM versus 12 nM/47 nM]. This suggests that the (+)-strand DNA synthesis might be the primary target, rather than the (−)-strand DNA synthesis. Studies by others using the naked HBV DP and activated calf thymus DNA as the template, which may mimic the (+)-strand DNA synthesis, reported the K_i of L(−)SddCTP to be 250 nM when competing with dCTP (K_m,

140 nM). The differences in the K_i/K_m values obtained by using the isolated virus versus the naked DP raise the possibility that the behavior of HBV DP within the capsid could be different. This requires further investigation.

Compared with the wt DP, the L256M, M550V, and L526M/M550V0-mutated DPs showed resistance to L(−)SddCTP with K_i values increased by 7-, 19-, and 20-fold, respectively. Our results are similar to the results obtained by using the naked HBV DPs and activated calf thymus DNA as the template, in which the relative resistance was 2.6-, 19.6-, and 25.2-fold, respectively (Xiong et al., 1998). We therefore conclude that these mutations of DP have a similar degree of impact on both HBV DP systems, although the K_m and K_i values are different. M550 of YMDD motif is in the active site of DP (Das et al., 2001). The steric hindrance between the methyl group of Val in replacing M550 and the sulfur atom of the oxathiolane ring of L(−)SddC could account for the M550V resistance phenotype. The L526M mutation is near but not at the HBV DP active site. The structural model could not explain the role of L526 in altering the interaction between L(−)SddCTP and HBV DP. The L526M mutation could alter the structure of the active site, thus increasing the K_m of dCTP by 2-fold and the K_i of L(−)SddC by 14.5-fold. This mutation may render the active site less tolerable to the bulky sulfur group of L(−)SddCTP. The introduction of L526M into M550V-mutated DP could cause the enzyme to be more efficient and more susceptible to the action of L(−)SddCTP. When the impact of the G1896A mutation of the viral RNA as the template was assessed using HBV DPs with or without the L526M and/or M550V mutations, it was observed that this mutation of the template could not make the mutated DPs more resistant; if anything, it was slightly more sensitive to L(−)SddCTP. This is consistent with the observation in cell culture, in which the G1896A template mutation of HBV has no effect on its sensitivity toward L(−)SddC (Chen et al., 2003).

Compared with our previous results (Zhu et al., 1998), the K_i/K_m (dCTP) for the inhibitory effect of L(−)Fd4CTP on the HBV (−)-strand DNA synthesis is 6-fold higher than on the HBV (+)-strand DNA synthesis [K_i/K_m (dCTP), 900 nM/100 nM versus 69 nM/47 nM]. Similar to L(−)SddCTP, HBV

TABLE 2
K_i of L(−)SddCTP, L(−)Fd4CTP, and L(−)FMAUTP for HBV DNA polymerases in vitro and IC$_{50}$ of L(−)SddC, L(−)Fd4C, and L(−)FMAU for HBV in cell culture

Cell culture results were adapted from our previous report (Fu and Cheng, 2000). Procedures were as described under *Materials and Methods*. Values are presented as mean ± S.D. of at least three independent experiments.

| Substrate | DP | K_i | | K_i/K_m | | Cell Culture |
		W[a]	M[b]	W	M	IC$_{50}$
		μM				nM
L(−)SddCTP	WT	0.2 ± 0.02	0.4 ± 0.2	2 (1)[c]	4 (1)	5 (1)
	L526M	2.9 ± 0.2	1.3 ± 0.3	14.5 (7)	7.6 (2)	35 (7)
	M550V	15.2 ± 1.8	7 ± 1	38 (19)	26 (8.5)	
	L526M/M550V	8.2 ± 0.9	6.7 ± 0.5	41 (20)	24 (6)	8000 (1600)
L(−)Fd4CTP	WT	0.9 ± 0.2	0.6 ± 0.3	9 (1)	6 (1)	0.9 (1)
	L526M	1.4 ± 0.5	1 ± 0.4	7 (0.7)	5.8 (1)	2.7 (3)
	M550V	42.7 ± 7	20 ± 2.5	106 (12)	74 (12)	
	L526M/M550V	7.1 ± 3	5 ± 2.2	35.5 (4)	18 (3)	210 (233)
L(−)FMAUTP	WT	4.2 ± 2	1.3 ± 0.2	32 (1)	5 (1)	20 (1)
	L526M	14.1 ± 4	6.4 ± 0.5	40 (1.3)	20 (4)	80 (4)
	M550V	58.5 ± 13	22 ± 5	254 (7.9)	78 (15.6)	
	L526M/M550V	66.6 ± 8	25 ± 3.5	350 (11)	89 (17.8)	50,000 (2500)

[a] Wild-type RNA template.
[b] G1896A-mutated RNA template.
[c] The relative K_i/K_m or resistance as compared to wt.

(+)-strand DNA synthesis may be the preferred target of L(−)Fd4CTP. L(−)Fd4C was previously shown to be more potent than L(−)SddC in inhibiting HBV replication in cell culture (Table 2) (Fu and Cheng, 2000). However, L(−)Fd4CTP is less potent than L(−)SddCTP in inhibiting HBV (−)-strand DNA synthesis. It is possible that the metabolites of L(−)Fd4C accumulate faster than those of L(−)SddC (Zhu et al., 1998). L(−)Fd4CTP was less influenced by the L526M and M550V mutations, possibly because of the planar structure of the sugar moiety of L(−)Fd4CTP, which gives less steric hindrance (Fig. 1). Cell culture studies also indicates that these DP mutations do not decrease the potency of L(−)Fd4C as much as that of L(−)SddC against HBV replication (Table 2) (Fu and Cheng, 2000).

Unlike L(−)SddCTP and L(−)Fd4CTP, L(−)FMAUTP is a TTP analog, and TTP is involved in the primer formation. The G1896A mutation may help stabilize the stem-loop structure for the primer formation and therefore sensitizes HBV DP toward L(−)FMAUTP, suggesting that L(−)FMAU is more active against HBV bearing a mutation of G1896A. The inhibitory effects of L(−)FMAUTP on the wt and mutated DPs were quite different from the effects of L(−)SddCTP and L(−)Fd4CTP. First, higher K_i/K_m values were found for L(−)FMAUTP than for L(−)SddCTP and L(−)Fd4CTP. Second, the K_i value of L(−)FMAUTP against the L526M/M550V-mutated DP was similar to that of the M550V-mutated DP, but the K_i values of L(−)SddCTP and L(−)Fd4CTP against the L526M/M550V-mutated DP were lower than that of the M550V-mutated DP. The disparities could be a result of different inhibitory mechanisms of these three analogs. Based on our previous report, L(−)FMAUTP is not a substrate for human DPs, including DP α, β, δ, γ, and Epstein-Barr virus DP (Kukhanova et al., 1998). Recently, the molecular dynamics simulation of the HBV-polymerase-DNA-L(−)FMAUTP complex was constructed, demonstrating that L(−)FMAUTP acted as a competitive inhibitor by binding to the active site of HBV DP but did not serve as a substrate of DP because the α-phosphate of L(−)FMAUTP was too far away from the 3′-hydroxyl group of the viral DNA chain (Chong and Chu, 2002). However, they used DNA as the template in their model, whereas our system used RNA as the template for (−)-strand DNA synthesis. Whether L(−)FMAUTP is a substrate for HBV DP remains to be proven. Compared with previous results (Balakrishna Pai et al., 1996), the K_i/K_m (dTTP) for the inhibitory effect of L(−)FMAUTP on HBV (−)-strand DNA synthesis is 16-fold higher than on HBV (+)-strand DNA synthesis [K_i/K_m (dTTP), 4200 nM/130 nM versus 120 nM/62 nM]. A recent study also suggested that L(−)FMAUTP could be a good inhibitor on the (+)-strand DNA synthesis of HBV and duck HBV in cell culture but not on the (−)-strand DNA synthesis that was performed by the reverse transcriptase of duck HBV (Seigneres et al., 2002).

In summary, the reverse transcriptase activities of HBV DPs with wt, L526M, M550V, and L526M/M550V sequences in the core-DP complex system are consistent with the amount of HBV DNA in cells harboring the wt or mutated viruses. The additional L526M mutation has a compensatory effect of increasing the incorporation rate of dNTPs into DNA by the M550V-mutated DP. The G1896A mutation in the RNA template has impacts on the behaviors of HBV DPs in terms of interactions with different dNTPs, except for dCTP, which is not involved in the primer formation. The G1896A mutation sensitizes the HBV DPs to L(−)FMAUTP but not to L(−)SddCTP and L(−)Fd4CTP. Mutations of L526M and M550V cause a greater decrease in the V_{max} with the wt RNA template than with the G1896A-mutated RNA template. This result may help explain why L(−)SddC-resistant HBV (M550V and L526M/M550V) with the G1896A precore mutation replicates faster than that with the wt precore HBV in cell culture (Chen et al., 2003). It would be interesting to know whether this phenomenon is applicable in HBeAg-negative patients. It is worthy to investigate whether the G1896A mutation can increase the frequency of L(−)SddC resistance occurrence in L(−)SddC-treated HBV patients who are HBeAg-negative. The L526M and/or M550V mutations of DP could cause DP to be resistant to the L-nucleotides regardless of using the wt or G1896A-mutated template. When the K_i values of L(−)SddCTP, L(−)Fd4CTP, and L(−)FMAUTP against HBV DP catalyzing the (−)-strand and the (+)-strand DNA synthesis are considered, the (+)-strand DNA synthesis may be the preferred target of these L-nucleotide analogs. The synthesis of both strands, however, is likely to be inhibited because the differences in their sensitivities are only 6- to 16-fold. This requires further investigation.

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