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Increased DNA Polymerase Fidelity of the Lamivudine Resistant Variants of Human Hepatitis B Virus DNA Polymerase

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Although efficient antiviral lamivudine is used for HBV-infected patients, a prolonged treatment with nucleoside analogs often results in lamivudine-resistant variants. In this study, we evaluated the fidelity of the lamivudine-resistant variants. The FLAG-tagged wild-type (FPolE) and Met550 variants (FPolE/M550A, M550V, and M550I) of HBV DNA polymerases were expressed in insect cells, then purified. Like many other reverse transcriptases, no 3'→5' exonuclease activity was detected in the HBV DNA polymerase. Since there is no proofreading activity, then the use of the site-specific nucleotide misincorporation method is beneficial. From the f_{ins} value analysis, it is evident that M550I and M550V exhibit higher fidelity values than the wild-type HBV DNA polymerase, while M550A exhibits similar fidelity values. It is therefore suggested that lamivudine resistance comes from the stringency to dNTP binding and the discrimination of dCTP and lamivudine in M550V and M550I.

Keywords: Fidelity, HBV polymerase, Met550 variants, Lamivudine

Introduction

The hepatitis B virus (HBV) is a member of *hepadnaviridae*, a family of enveloped-hepatotropic DNA viruses. This virus can cause severe liver disease with eventual progression to cirrhosis and primary hepatocellular carcinoma (Beasley *et al*., 1981; Lok, 2000). The number of chronic HBV carriers is estimated to exceed 350 million (De Clercq, 1999). According to the 1997 World Health Organization (WHO) report, HBV chronic infection remains one of the ten most common causes of death worldwide (Ono *et al*., 2001).

Unlike most DNA viruses, HBV replicates via reverse transcription of the RNA intermediate. This process includes polymerization of the minus-strand DNA, degradation of the pre-genome from the RNA-DNA heteroduplex, and synthesis of the plus-strand DNA from the minus-strand DNA template. All of the enzyme activities that are responsible for these steps come from the viral DNA polymerase. In general, retroviruses exhibit a relatively high rate of mutation (Preston *et al*., 1988; Hubner *et al*., 1992; Yu and Goodman, 1992). One reason for its low fidelity is that RT lacks 3' to 5' exonuclease activity (Williams and Loeb, 1992; Bakhanashvili *et al*., 1996), which is considered to be deficient in HBV. DNA polymerases in other organisms generally have this activity, which removes incorrectly added nucleotides by reorganizing the mismatched base with the template sequence. Low fidelity gives HBV a genomic hypervariability, which is a defense device that allows HBV to escape selection pressures, such as those imposed by the host immune system or drug therapies.

Despite the existence of an effective vaccine, no effective antiviral treatment has been developed for patients that are chronically infected with HBV. Recently, an attractive option became the use of reverse-transcriptase inhibitors as an antiviral drug for hepatitis B (Lai *et al*., 1998; Dienstag *et al*., 1999; Jarvis and Foulds, 1999; Santantonio *et al*., 2000). Lamivudine, or (-)-β-L-2',3'-dideoxy-3'-thiacytidine (3TC), is a reverse transcriptase inhibitor that has antiviral activity against the human immunodeficiency virus (HIV) (Coates *et al*., 1992) and HBV. However, it was recently reported that short-term monotherapy is insufficient to clear viral infection, and the prolonged use of lamivudine therapy has caused an increased emergence of lamivudine-resistant HBV (Zoulim, 1999).

Lamivudine-resistant HBV has isoleucine or valine substitutions for methionine in the YMDD (tyrosine, methionine, and two aspartic acids) motif, which is a highly conserved domain in the RNA-dependent DNA polymerase. It is involved in nucleotide binding in the catalytic site of the

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polymerase (Bartholomew *et al*., 1997; Allen *et al*., 1998; Buti *et al*., 1998).

In this study, the FLAG-tagged wild-type and lamivudine-resistant Met550 variants of HBV polymerases were expressed in insect cells and purified by using immunoaffinity column chromatography. DNA polymerase fidelity was then analyzed to evaluate the relationships between lamivudine resistance and DNA polymerase fidelities.

Materials and Methods

Construction and purification of the wild-type and mutant HBV polymerase Four recombinant plasmids were used as follows: wild-type (pFPolE) and M550 variants (pFPolE/M550A, M550V, M550I) containing the entire HBV polymerase gene (subtype *adr*) (Rho *et al*., 1989), and the catalytic mutant HBV polymerase gene with FLAG sequences at the NH_2-terminal region. The FLAG tag was used to isolate the wild-type and mutant HBV polymerases. Each recombinant baculovirus was expressed in Sf9 cells. The proteins were purified as described previously (Park and Jung, 2001). In order to substitute alanine, valine, and isoleucine for methionine, site-directed mutageneses were performed in nucleotides 1648-1650, changing ATG to GCG, GTC and ATA for pFPolE/M550A, M550V, and M550I, respectively (Kim *et al*., 2001; Park and David, 2002).

SDS-PAGE and immunoblot analysis The partially-purified proteins were separated by 7.5% SDS-polyacrylamide gel electrophoresis (PAGE). For the immunoblot analysis, the proteins were electrophoretically transferred to a PVDF blotting membrane. The membranes were probed with a M2 monoclonal antibody and resuspended in PBS containing 0.5% skim milk and 0.3% Tween-20. The immunoblots were then incubated with horseradish peroxidase-conjugated anti-mouse antiserum. The immunoreactive bands were visualized using ECL (Amersham Pharmacia, Buckinghamshire, UK).

DNA polymerase activity The DNA polymerization reaction (total reaction volume of 50 µl) contained 50 ng of the homopolymer template poly(dA) · oligo$(dT)_{12\text{-}18}$ (Amersham Pharmacia) , 2 Ci of [α-^{32}P]dTTP (3,000 Ci/mmol) (NEN Life Science Products, Boston, USA), 50 mM Tris-Cl (pH 7.4), 0.01% NP-40, 10 mM $MgCl_2$, 1 mM DTT, 10 mM KCl, and 50 µM unlabeled dTTP. The reaction was started by adding 90 ng (1 pmol) of either the purified proteins of the wild-type or M550 variants, and incubated at 37°C for 30 min. The reaction was stopped by the addition of 2 µl 0.5 M EDTA. The reaction products were then phenol-extracted and ethanol-precipitated. Two µl of 95% formamide was added and the proteins were immediately denatured by incubating at 95°C for 3 min, then analyzed by electrophoresis in 7 M urea/16% polyacrylamide gels. Next, the gel was dried and exposed to a phosphoimager system (BAS FLA2000, Fuji, Tokyo, Japan).

Template-primers To measure the exonuclease activity, the primer (5'-CCCCTAGAAGAAGAAG-3') and template (5'-CGCTA TCCTAAGAAGAAGATCCCC-3') were synthesized for exonuclease activity, and Primer 5385T (5'-TTT TAG ACA GGA ACG GT-3') was used for the site-specific nucleotide mis-insertion. The primers were end-labeled with [γ-^{32}P]ATP (3000 Ci/mmol) (NEN Life Science Products) using T4 polynucleotide kinase (20 U) (New England Biolabs, Beverly, USA). The reaction was started by adding 100 nM oligonucleotide, incubated at 37°C for 1.5 h, and stopped by adding EDTA to the final concentration of 20 mM. The reaction mixture was then phenol-extracted twice and ethanol-precipitated. To measure the $3' \rightarrow 5'$ exonuclease activity, hybrid molecules between 16-mer oligonucleotide and 24-mer template were made. To measure the site-specific nucleotide mis-insertion, 5385T primers were hybridized to the M13mp18 single-stranded template. A partially double-stranded template-primer structure was created by combining the 740 nM ^{32}P-end-labeled primer with 4 µM template in 50 mM Tris-Cl (pH 7.4), 5 mM $MgCl_2$, 2 mM β-mercaptoethanol, and 17 µg BSA. The mixture was heated at 95°C and allowed to slowly cool to room temperature.

Exonuclease activity The $3' \rightarrow 5'$ exonuclease activity was measured by the removal rate of the mismatched 3'-terminal nucleotides from the 5'-[γ-^{32}P] end-labeled oligonucleotide. The reactions were carried out in a 25 µl reaction mixture containing 300 ng mismatched template-primer, 50 mM Tris-Cl (pH 7.4), 10 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, and 0.01% NP-40. The reaction was started by adding 1 pmol FPolE, 100 mU Klenow fragment of *E. coli* polymerase I as a positive control, or 10 mU HIV-1 RT as a negative control. After incubating for 30 min at 37°C, the reactions were stopped by adding equal volumes of a formamide dye mix. The mixtures were electrophoresed in 7 M urea/16% polyacrylamide sequencing gels and dried. Lastly, the dried gel was exposed to the phosphoimager system.

Site-specific nucleotide mis-insertion Before measuring the kinetic constants of the correct and incorrect nucleotide incorporation, a time course study was carried out to determine the time frame, while the products were accumulated linearly with time and less than 30% of the original primer was extended (Mendelman *et al*., 1990). The reaction time was chosen as 30 min for FPolE, according to the results of the time course experiments (data not shown). The specific activity of the partially-purified enzyme was 40 units/µg (one unit is defined as the amount of enzyme that catalyzes the incorporation of 1 pmol of dTTP into DNA in the poly$(dA)_n$ · oligo$(dT)_{12\text{-}18}$-directed reaction, 30 min at 37°C). The reaction was started by combining the 4 µl enzyme-primer-template (12.5 nM FPolE or 5.3 nM HIV-1 RT, 25 µg BSA and 2 µl of the original annealed primer-template solution) and 4 µl dNTP-salts solutions (52 mM Tris-Cl, pH 7.8, 20 mM $MgCl_2$, 5 mM DTT, 150 µg BSA, and increasing concentrations of single dNTP). It was then incubated at 37°C. The reaction was terminated by the addition of EDTA to the final concentration of 50 mM in a 95% formamide buffer. The reaction products were denatured by incubating at 95°C for 3 min and analyzed by electrophoresis in 7 M urea/16% polyacrylamide gels. An analysis of the deoxynucleotide incorporation assays was done by using a gel based steady-state kinetic assay (Boosalis *et al*., 1987; Mendelman *et al*., 1989, 1990) to determine the mis-insertion efficiency for all the mispairs. The gel band intensities of the substrates and products were quantitated using the phosphoimager system within the linear response range.

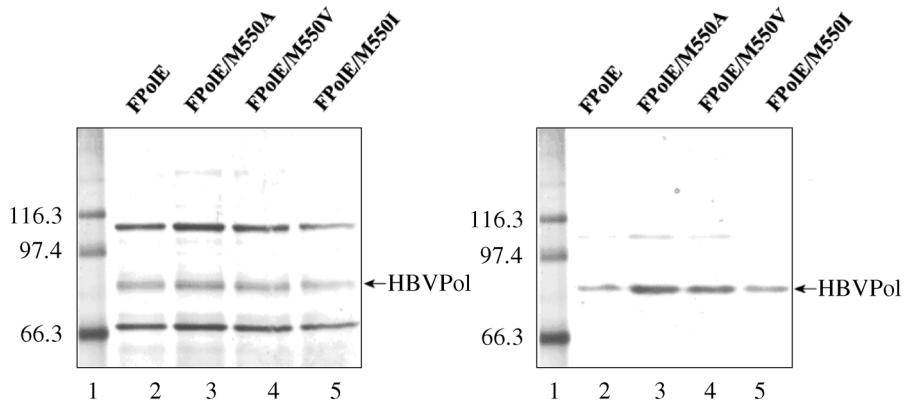

Fig. 1. Expression and purification of FLAG-tagged wild-type (FPolE) and M550 variants (FPolE/M550A, M550V, M550I) HBV polymerases. (A) Sf9 cells were infected with the FLAG-tagged wild-type (vFPolE) or mutant (vFPolE/M550A, M550V, M550I) baculovirus. They were then harvested 48 h post-infection. The FPolE (lane 2) and FPolE/M550A (lane 3), FPolE/M550V (lane 4) and FPolE/M550I (lane 5) proteins were partially purified with an affinity resin containing the M2 monoclonal antibody, separated by 7.5% SDS-polyacrylamide gel electrophoresis (PAGE), and stained with Coomassie blue R250. (B) For the immunoblot analysis, the proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) blotting membrane (Millipore, Billerica, USA), and probed with a M2 monoclonal antibody (Sigma, St. Louis, USA). The immunoblots were then incubated with horseradish peroxidase-conjugated anti-mouse antiserum. Lane 1, the molecular weight standards are indicated in kDa. The arrows indicate the position of FPolE and FPolE/M550A, M550V, M550I on the right.

For each dNTP concentration, the observed rate of deoxynucleotide incorporation (V_{obs}) was determined by dividing the relative amount of the extended product by the incubation time. The observed rate of deoxynucleotide incorporation was plotted as a function of the dNTP concentration. The data were fitted to the Michaelis-Menten equation using the non-linear least-squares method. Apparent K_m and V_{max} steady-state parameters for the incorporation of the correct and incorrect deoxynucleotides were obtained from the fit and used to calculate the frequency of the nucleotide mis-insertion (f_{ins}) (Randall *et al*., 1987).

Results

Construction and purification of FLAG-fused HBV polymerases in insect cells Three single amino acid substitutions were generated at Met550 of this YMDD motif by using site-directed mutagenesis. To express and purify the wild-type (FpolE) and Met550 variants (FpolE/M550A, M550V, M550I), the FLAG-fused HBV polymerases were expressed in insect cells by using the recombinant baculovirus expression system; they were purified using immunoaffinity column containing the M2 monoclonal antibody (Fig. 1). The enzyme preparations were homogeneous, as judged by Fig. 1. The protein expression levels, yields, and chromatographic characteristics of the mutant proteins were identical to those of the wild-type enzyme, suggesting that there is no significant change in the overall structure of the mutant enzyme.

The DNA-dependent DNA polymerase activity of the wild-type HBV DNA polymerase and Met550 variants were performed as described in Materials and Methods. Under the standard reaction conditions that are described in Materials and Methods, the polymerase reactions were conducted with the purified fractions of the wild-type or Met550 variants. The polymerase activity of the Met550 variants is 40-60% of the wild-type polymerase (data not shown).

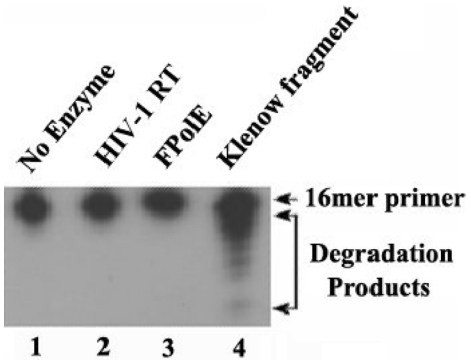

Fig. 2. Electrophoretic analysis of terminal mismatch excision. The reactions for the terminal mismatched (G : A) excision were performed, as described in Materials and Methods, with no enzyme (lane 1), HIV-1 RT (lane 2), FPolE (lane 3), and the Klenow fragment of *E.coli* polymerase I (lane 4). The position of the 16-mer primer is indicated by an arrow. The direction of the electrophoresis is from top to bottom.

Analysis of the 3' → 5' exonuclease activity All of the reverse transcriptases lacked 3' → 5' exonuclease activity. HBV DNA polymerase, displaying similarities to retroviral transcriptases, was analyzed by its exonuclease activity (Fig. 2). Terminal nucleotide excision activity was analyzed in the presence of no enzyme (lane 1), HIV-1 RT (lane 2), FPolE proteins (lane 3), and the Klenow fragment of *E. coli*

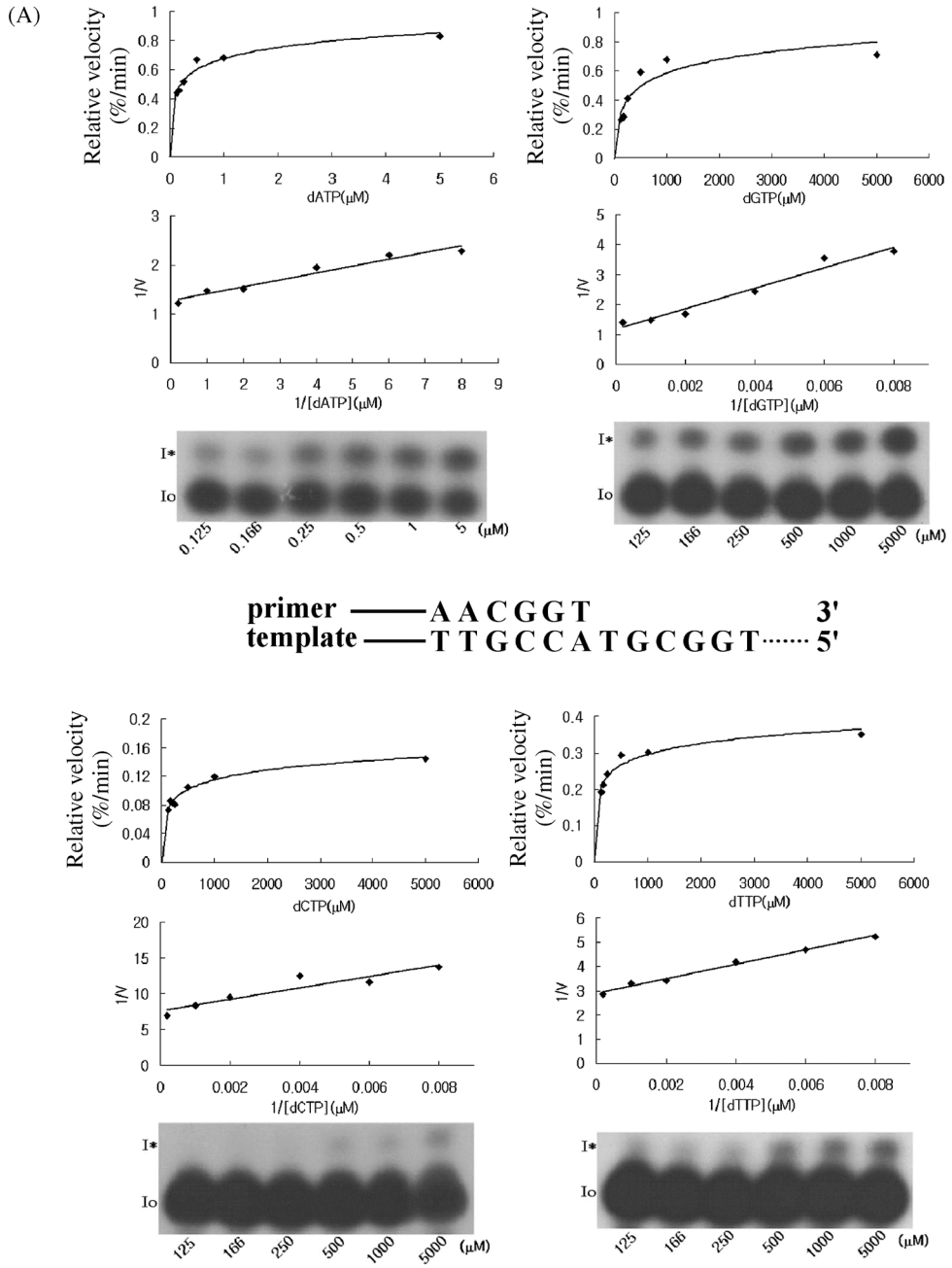

Fig. 3. Kinetic assay for site-specific nucleotide mis-insertion. The 5'-[^{32}P] labeled primer 5385T was annealed to a M13mp18 template strand to produce the 3'-terminal mispairs in the presence of increasing concentrations of single dNTP. These are indicated with (A) FpolE, (B) FPolE/M550A, (C) FPolE/M550V, and (D) FPolE/M550I. Gel band intensities of the substrates (Io) and products (I*) were quantitated using the phosphoimager system. The observed rate of deoxynucleotide incorporation (I*/(Io + I*)) was plotted as a function of the dNTP concentration. The data were fitted to the Michaelis-Menten equation using the non-linear least-squares methods.

polymerase I (lane 4). No 3' → 5' exonuclease activity was found in HIV-1 RT (lane 2). There was also no change in the length of the oligonucleotide primer when FPolE proteins were used (lane 3), the same as with HIV-1. However, the efficient excision of the terminal nucleotide occurred when the Klenow fragment of *E. coli* polymerase I was used as a positive control (lane 4). Thus, HBV polymerase does not have 3' → 5' exonuclease activity, as is the case with many reverse transcriptases.

Site-specific nucleotide misincorporation To evaluate the fidelity of the wild-type and mutant HBV DNA polymerases, we used the site-specific nucleotide misincorporation method. The frequency of the nucleotide mis-insertion, $f_{ins,}$ was

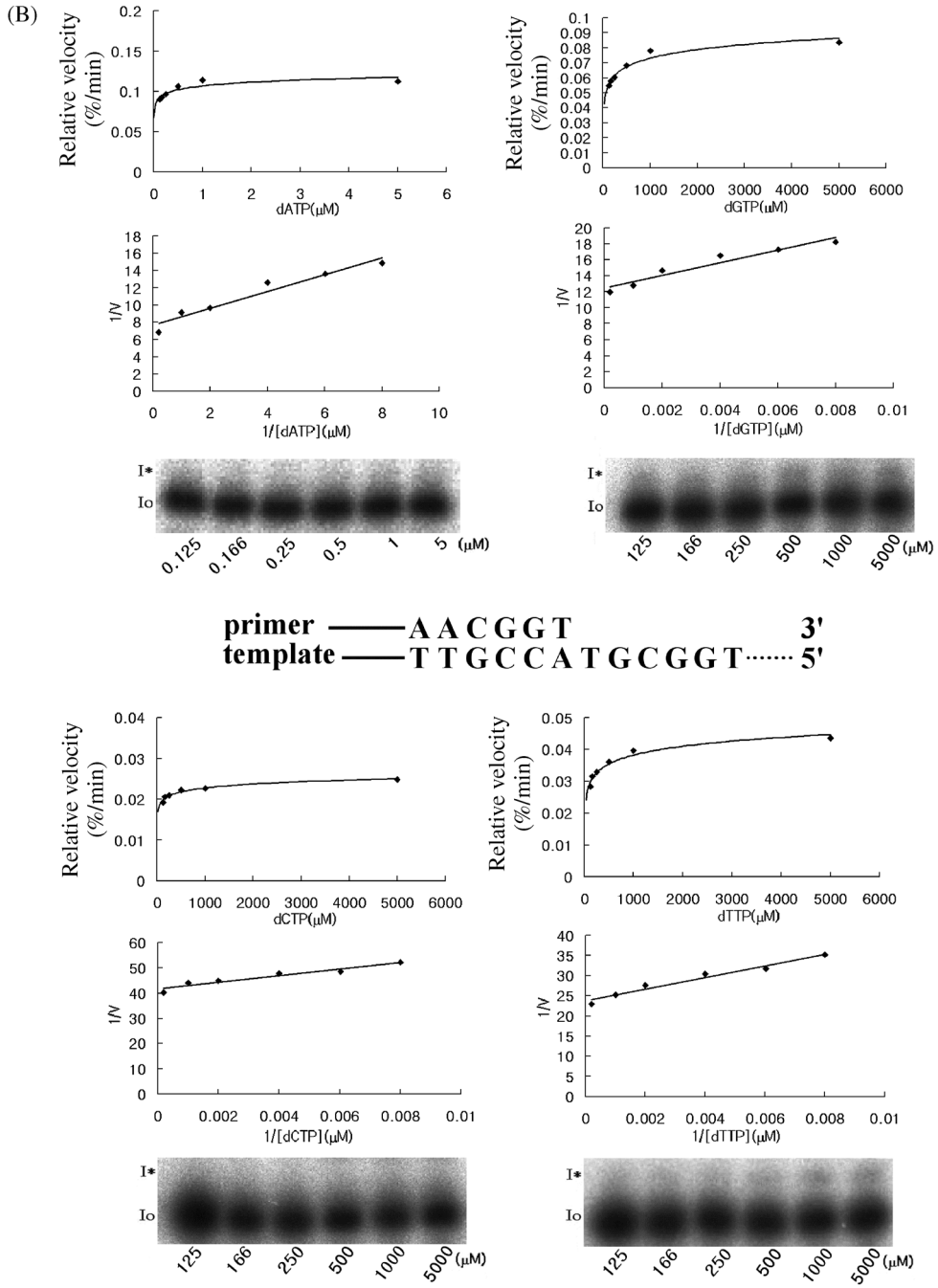

Fig. 3. Continued.

calculated from the *ratio* of the insertion efficiency for wrong (W) *versus* right (R) base pairs, as described in Materials and Methods. The lack of proofreading activity permitted this method. To determine the nucleotide mis-insertion frequency of the wild-type and M550 variants, we measured the V_{max} and K_m steady-state parameters for the incorporation of correct and incorrect deoxynucleotides (G, A, T, and C) opposite the T residues on the native M13mp18 template-strand that was primed with the 5'-^{32}P end-labeled oligonucleotide primers 5385T (Fig. 3). From the quantitation of the unextended and extended primers from each reaction set, the initial velocities of the product formation were plotted against the dNTP concentrations, and double-reciprocal plots for the initial velocities *versus* the substrate concentrations were made. Although some of the extended bands (I*) could barely be seen by the naked eye, the scanner that we used was sensitive enough to read the bands. The f_{ins} values for each of the 4 possible insertion events were then derived from the apparent K_m and V_{max} kinetic values for each dNTP that was calculated from the double-reciprocal plots. They are summarized in

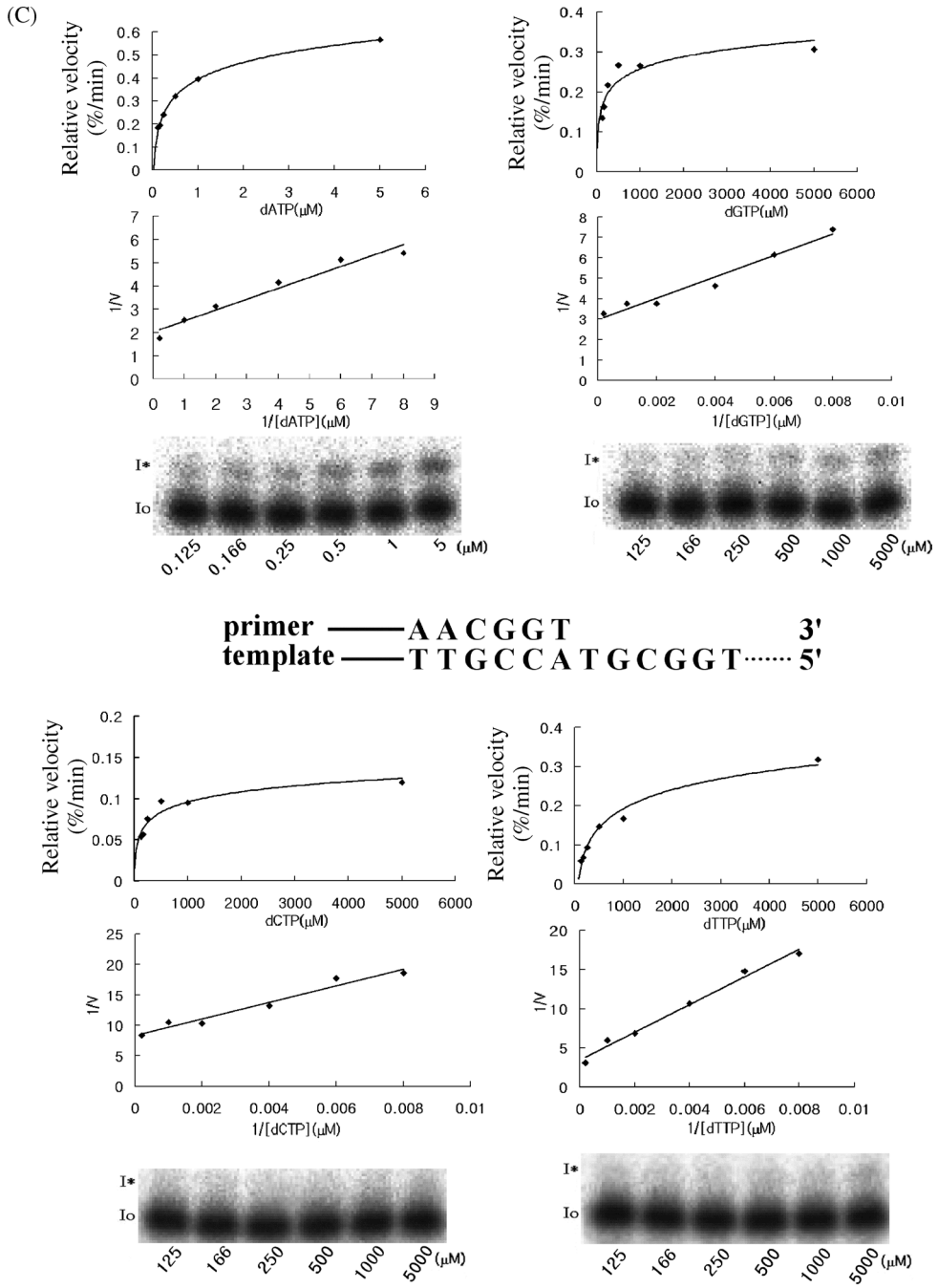

Fig. 3. Continued.

Table 1.

The K_m values of the HBV DNA polymerases for the correct and incorrect pairs ranged as follows: 0.12 to 98.43 mM in the wild-type, 0.13 to 64.48 mM in M550A, 0.23 to 503.76 mM in M550V, and 0.04 to 189.79 mM in M550I. The V_{max} values ranged from 0.357 to 0.749 in the wild-type, 0.024 to 0.103 in M550A, 0.120 to 0.494 in M550V, and 0.031 to 0.114 in M550I.

The range of the f_{ins} values of the wild-type HBV polymerase for all of the mispairs ranged from 5.91×10^{-4} to 1.31×10^{-3}, whereas the ones by M550A ranged from 6.73×10^{-4} to 1.22×10^{-3}, the ones by M550V ranged from 2.67×10^{-4} to 8.96×10^{-4}, and those by M550I ranged from 1.04×10^{-4} to 4.81×10^{-4}. In the fidelities of each variant, T : G is the smallest and T : T is the biggest, except in M550I. As evident from the f_{ins} value analysis, M550I and M550V have higher fidelities than the wild-type HBV DNA polymerase, and M550A has similar fidelities.

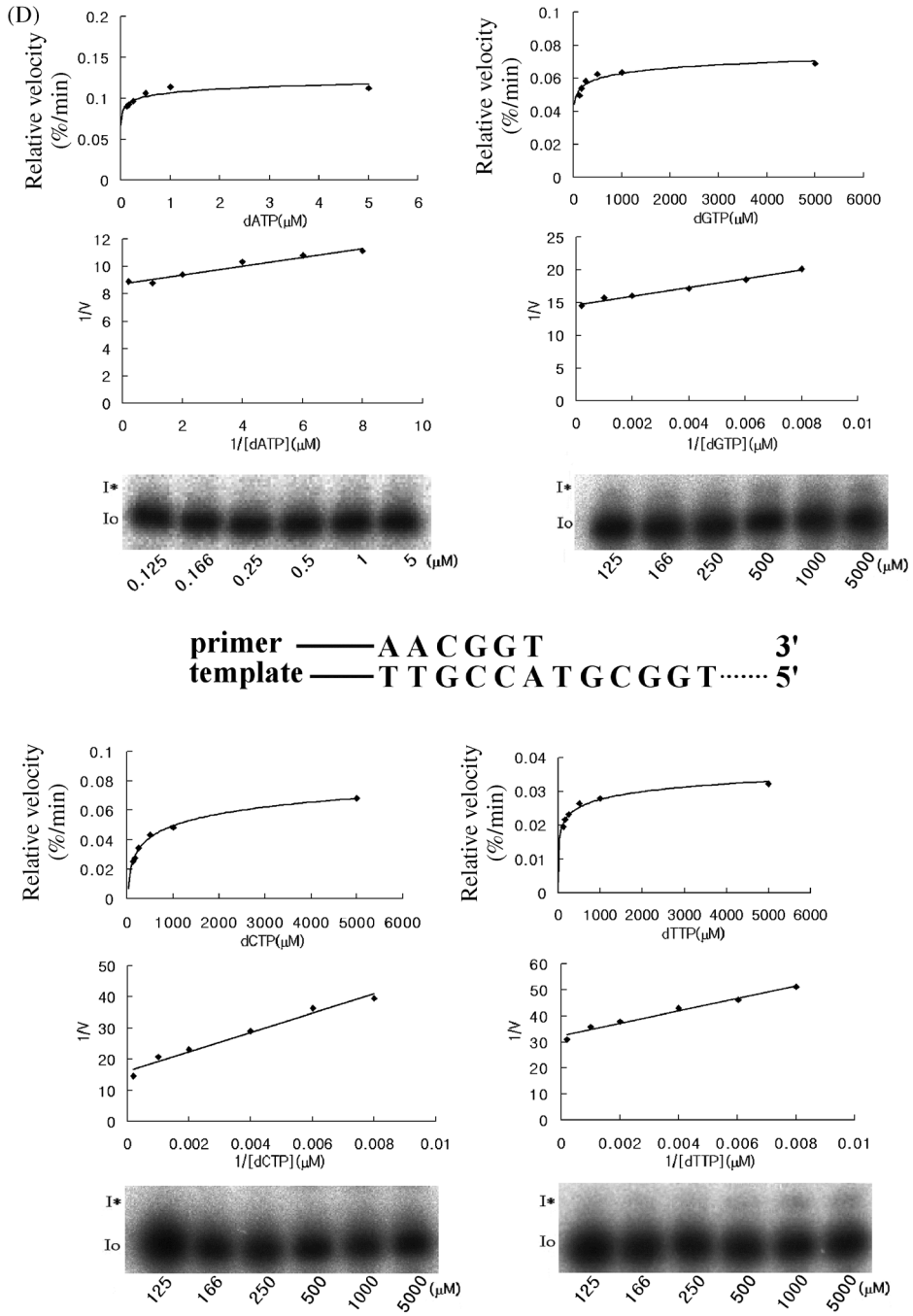

Fig. 3. Continued.

Discussion

The fidelity of DNA synthesis is a major determinant in generating spontaneous mutation. The frequencies of errors during DNA replication in prokaryotic and eukaryotic cells are between 10^{-9} and 10^{-10} substitutions per base pair (Echols and Goodman, 1991). These low mutation rates are achieved by multiple steps in error discrimination. These include base selection by DNA polymerase, $3' \rightarrow 5'$ exonucleolytic proofreading, and post-replicative repair (Kunkel, 1992). Because all of the reverse transcriptases, including HBV DNA polymerase, lack $3' \rightarrow 5'$ proofreading exonuclease activity, then the error-prone mutation rates are 10^{-4}-10^{-5}. Nucleotide mis-insertion rates are important parameters that contribute to the overall reverse transcriptase fidelity. In the wild-type isolates of HBV, the sequence of the genome may vary up to 10%, despite conservation of the open-reading frame and function (Harrison, 1996). These genetic variations are possibly related to the infidelity of the HBV DNA polymerase and reverse transcription strategy of HBV.

Table 1. The apparent K_m, V_{max} values and mis-insertion frequency (f_{ins}) were for the wild-type HBV polymerase (FPolE) and HIV-1 RT

	Base pair (T : dNTP)	K_m (μM)	V_{max} (%/min)	f_{ins}	Fold decrease
Wild-Type	T : A	0.12 + 0.01	0.749 + 0.041	1	-
	T : G	75.26 + 3.75	0.606 + 0.031	1.31×10^{-3}	-
	T : C	96.54 + 15.42	0.391 + 0.036	6.60×10^{-4}	-
	T : T	98.43 + 14.51	0.357 + 0.035	5.91×10^{-4}	-
M550A	T : A	0.13 + 0.02	0.103 + 0.025	1	
	T : G	64.48 + 5.52	0.080 + 0.013	1.22×10^{-3}	1.07
	T : C	31.37 + 7.63	0.024 + 0.004	7.47×10^{-4}	0.88
	T : T	61.01 + 9.91	0.042 + 0.009	6.73×10^{-4}	0.88
M550V	T : A	0.23 + 0.02	0.494 + 0.026	1	
	T : G	176.61 + 15.21	0.337 + 0.023	8.96×10^{-4}	1.46
	T : C	163.36 + 23.32	0.120 + 0.036	3.45×10^{-4}	1.91
	T : T	503.76 + 37.44	0.286 + 0.021	2.67×10^{-4}	2.21
M550I	T : A	0.04 + 0.01	0.114 + 0.026	1	
	T : G	45.51 + 4.17	0.068 + 0.019	4.81×10^{-4}	2.72
	T : C	189.79 + 21.52	0.061 + 0.014	1.04×10^{-4}	6.36
	T : T	79.93 + 9.47	0.031 + 0.007	1.34×10^{-4}	4.39

Data shown above are the mean values + standard deviation. Standard deviations presented are derived from three independent measurements. The variations were mostly <15%. Mis-insertion frequency, f_{ins}, was evaluated from the *ratio* of relative V_{max} to K_m, using the equation $f_{ins} = (V_{max}/K_m)_{correct}/(V_{max}/K_m)_{incorrect}$. Fold decrease is the *ratio* relative to the corresponding template: primer pair for wild-type.

Lamivudine was developed as a potent treatment for HIV infection and has recently been used for HBV-infected patients. Although it has a strong antiviral effect, the emergence of a resistant strain of the virus has been a serious problem (Schuurman *et al*., 1995). For HIV, resistance to lamivudine in the cell culture and *in vivo* correlated with amino acid substitutions in the methionine residue of the YMDD motif of reverse transcriptase. Similar substitutions have also been reported in HBV (Ling *et al*., 1996; Jardi *et al*., 1999). The YMDD motif is a highly-conserved domain in the RNA-dependent DNA polymerases, and it is involved in nucleotide binding in the catalytic site of the polymerase (Jacobo-Molina *et al*., 1993). Reportedly, the two aspartic acid residues in this motif, which are highly conserved, are constituted as two of the three in a catalytic triad (Jacobo-Molina *et al*., 1993). In general, two aspartic acid residues of the YMDD motif are generally flanked by hydrophobic residues. In the HIV-1 and HBV mutants, there are valine, isoleucine, leucine, and the alanine substitution of methionine, and these are related to reverse transcriptase fidelity in HIV-1. Valine has a higher fidelity than methionine in HIV-1 and MuLV reverse transcriptase (Pandey *et al*., 1996; Oude Essink *et al*., 1997; Hamburg *et al*., 1998; Kaushik *et al*., 2000). The fidelity of the Valine substitution is 1.5 times higher than methionine in the MuLV reverse transcriptase and 3-4.5 times higher than the wild-type methionine in the HIV-1 reverse transcriptase. In the present study, it has 1.46-2.21 times higher fidelity than the wild-type DNA polymerase of HBV. Isoleucine also has a higher fidelity than methionine in the HIV-1 reverse transcriptase. The fidelity of the Isoleucine substitution is 1.5-6 times higher than methionine in the HIV-1 reverse transcriptase (Pandey *et al*., 1996; Hamburg *et al*., 1998). In the present study, it has 2.72-6.36 times higher fidelity than the wild-type DNA polymerase of HBV. Alanine in the MuLV reverse transcriptase has 1.2 times higher fidelity (Kaushik *et al*., 2000).

The Inhibitions of HBV DNA polymerase activity using 5 nucleotide analogs (3TC, FTCTP, ADVDP, PCVTP, LPVTP) show that M550V is 20 times and 15 times more resistant to 3TC and FTCTP, respectively. Also, M550I is 8 times and 20 times more resistant to 3TC and FTCTP, respectively (Das *et al*., 2001). In this report, only valine and isoleucine have effective resistance to the nucleotide analog. The other variants (methionine, alanine, lysine, leucine, arginine, and threonine) have little effect. This means that the lamivudine resistance comes from the stringency to dNTP binding and discrimination of dCTP and lamivudine in M550V and M550I. According to Pandey *et al*. (1996), the nucleotide selection is related to the structure of the side chain of amino acids. Alanine has the smallest side chain and methionine has the biggest side chain among these four amino acids; therefore, valine and isoleucine may be the proper size. The side chain of a substituent amino acid may prevent or increase the correct alignment of the 3'-O of the primer terminus with the a-phosphate moiety of dNTP. In the case of valine, it has a favorable distance, rather than methionine from the sugar

moiety of the primer terminus (Pandey *et al*., 1996). An optimal hydrophobic interaction between valine and the sugar moiety may stabilize the template-primer and the interrelated-dNTP binding site. Consequently, this would result in a higher fidelity, decrease in affinity, and/or the high K_m values for the incorrect nucleotide (Table 1).

Our studies, therefore, indicate that valine and isoleucine at this position play an important role in the fidelity of the DNA synthesis of the enzyme. Lamivudine resistance may also come from this stringency to nucleotide selection in these Met550 variants. Since the present studies only focus on the fidelity of the mis-insertion of the nucleotides on the DNA template, more extensive studies are required to reveal the relationships between the fidelity of the HBV polymerase and genetic variability.

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References

Allen, M. I., Deslauriers, M., Andrew, C. W., Tipple, G. A., Walters, K. A., Tyrrell, D. L., Brown, N. and Condreay, L. D. (1998) Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Lamivudine Clinical Investigation Group. *Hepatology* **27**, 1670-1677.

Bakhanashvili, M., Avidan, O. and Hizi, A. (1996) Mutational studies of human immunodeficiency virus type 1 reverse transcriptase: the involvement of residues 183 and 184 in the fidelity of DNA synthesis. *FEBS Lett.* **391**, 257-262.

Bartholomew, M. M., Jansen, R. W., Jeffers, L. J., Reddy, K. R., Johnson, L. C., Bunzendahl, H., Condreay, L. D., Tzakis, A. G., Schiff, E. R. and Brown, N. A. (1997) Hepatitis-B-virus resistance to lamivudine given for recurrent infection after orthotopic liver transplantation. *Lancet* **349**, 20-22.

Beasley, R. P., Hwang, L. Y., Lin, C. C. and Chien, C. S. (1981) Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22,707 men in Taiwan. *Lancet* **2**, 1129-1133.

Boosalis, M. S., Petruska, J. and Goodman, M. F. (1987) DNA polymerase insertion fidelity. Gel assay for site-specific kinetics. *J. Biol. Chem.* **262**, 14689-14696.

Buti, M., Jardi, R., Cotrina, M., Rodirquez-Frias, F., Esteban, R. and Guardia, J. (1998) Transient emergence of hepatitis B variants in a patient with chronic hepatitis B resistant to lamivudine. *J. Hepatol.* **28**, 510-513.

Coates, J. A., Cammack, N., Jenkinson, H. J., Jowett, A. J., Jowett, M. I., Pearson, B. A., Penn, C. R., Rouse, P. L., Viner, K. C. and Cameron, J. M. (1992) (-)-2'-deoxy-3'-thiacytinine is a potent, highly selective inhibitor of human immunodeficiency virus type 1 and type 2 replication *in vitro*. *Antimicrob. Agents Chemother.* **36**, 733-739.

Das, K., Xiong, X., Yang, H., Westland, C. E., Gibbs, C. S., Sarafianos, S. G. and Arnold, E. (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.

De Clercq, E. (1999) Perspectives for the treatment of hepatitis B virus infections. *Int. J. Antimicrob. Agents* **12**, 81-95.

Dienstag, J. L., Schiff, R., Wright, T. L., Perrillo, R. P., Hann, H. W., Goodman, Z., Crowther, L., Condreay, L.D., Woessner, M., Rubin, M. and Brown, N. A. (1999) Lamivudine as initial treatment for chronic hepatitis B in the United States. *N. Engl. J. Med.* **341**, 1256-1263.

Echols, H. and Goodman, M. F. (1991) Fidelity mechanisms in DNA replication. *Annu. Rev. Biochem.* **60**, 477-511.

Hamburgh, M. E., Drosopoulos, W. C. and Prasad, V. R. (1998) The influence of 3TC resistance mutations E89G and M184V in the human immunodeficiency virus reverse transcriptase on mispair extension efficiency. *Nucleic Acid Res.* **26**, 4389-4394.

Harrison, T. J. (1996) Genetic variation in hepatitis B virus. *Eur. J. Gastroenterol. Hepatol.* **8**, 306-311.

Hubner, A., Kruhoffer, M., Grosse, F. and Krauss, G. (1992) Fidelity of human immunodeficiency virus type I reverse transcriptase in copying natural RNA. *J. Mol. Biol.* **223**, 595-600.

Jacobo-Molina, A., Ding, J., Nanni, R. G., Clark, A. D., Lu, X. Jr., Tantillo, C., Williams, R. L., Kamer, G., Ferris, A. L., Clark, P., Hizi, A., Hughes, S. H. and Arnold, E. (1993) Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0Å resolution shows bent DNA. *Proc. Natl. Acad. Sci. USA* **90**, 6320-6324.

Jardi, R., Buti, M., Rodriguez-Frias, F., Cotrina, M., Costa, X., Pascual, C., Esteban, R. and Guardia, J. (1999) Rapid detection of lamivudine-resistant hepatitis B virus polymerase gene variants. *J. Virol. Methods* **83**, 181-187.

Jarvis, B. and Faulds, D. (1999) Lamivudine. A review of its therapeutic potential in chronic hepatitis B. *Drugs* **58**, 101-141.

Kaushik, N., Chowdhury, K., Pandey, V. N. and Modak, M. J. (2000) Valine of the YVDD motif of moloney murine leukemia virus reverse transcriptase: role in the fidelity of DNA synthesis. *Biochemistry* **39**, 5155-5165.

Kim, S. I., Kim, S. -J., Leem, S. -H., Oh, K. -H., Kim, S. and Park, Y. -M. (2001) Site-directed mutagenesis of two cysteines (155, 202) in catechol 1,2-dioxygenase I_1 of *Acinetobacter lwoffii* K24. *J. Biochem. Mol. Biol.* **34**, 172-175.

Kunkel, T. A. (1992) DNA replication fidelity. *J. Biol. Chem.* **267**, 18251-18254.

Lai, C. L., Chien, R. N., Leung, N. W., Chang, T. T., Guan, R., Tai, D. I., Ng, K. Y., Wu, P. C., Dent, J. C., Barber, J., Stephenson, S. L. and Gray, D. F. (1998) A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. *N. Engl. J. Med*. **339**, 61-69.

Ling, R., Mutimer, D., Ahmed, M., Boxall, E. H., Elias, E., Dusheiko, G. M. and Harrison, T. J. (1996) Selection of mutations in the hepatitis B virus polymerase during therapy of transplant recipients with lamivudine. *Hepatology* **24**, 711-713.

Lok, A. S. (2000) Hepatitis B infection: pathogenesis and management. *J. Hepatol.* **32**, 89-97.

Mendelman, L. V., Boosalis, M. S., Petruska, J. and Goodman, M. F. (1989) Nearest neighbor influences on DNA polymerase insertion fidelity. *J. Biol. Chem.* **264**, 14415-14423.

Mendelman, L. V., Petruska, J. and Goodman, M. F. (1990) Base mispair extension kinetics. Comparison of DNA polymerase alpha and reverse transcriptase. *J. Biol. Chem.* **265**, 2338-2346.

Ono, S. K., Kato, N., Shiratori, Y., Kato, J., Goto, T., Schinazi, R.

F., Carrilho, F. J. and Omata, M. (2001) The polymerase L528M mutation cooperates with nucleotide binding-site mutations, increasing hepatitis B virus replication and drug resistance. *J. Clin. Invest.* **107**, 449-455.

Oude Essink, B. B., Back, N. K. and Berkhout, B. (1997) Increased polymerase fidelity of the 3TC-resistant variants of HIV-1 reverse transcriptase. *Nucleic Acids Res*. **25**, 3212-3217.

Pandey, V. N., Kaushik, N., Rege, N., Sarafianos, S. G., Yandav, P. N. and Modak, M. J. (1996) Role of methionine 184 of human immunodeficiency virus type-1 reverse transcriptase in the polymerase function and fidelity of DNA synthesis. *Biochemistry* **35**, 2168-2179.

Park, S. G. and Jung, G. (2001) Human hepatitis B virus polymerase interacts with the molecular chaperonin Hsp60. *J. Virol*. **75**, 6962-6968.

Park, I. and David, J. I. (2002) Mutations within the putative active site of heterodimeric deoxyguanosine kinase block the allosteric activation of the deoxyadenosine kinase subunit. *J. Biochem. Mol. Biol*. **35**, 244-247.

Preston, B. D., Poiesz, B. J. and Loeb, L. A. (1988) Fidelity of HIV-1 reverse transcriptase. *Science* **242**, 1168-1171.

Randall, S. K., Eritja, R., Kaplan, B. E., Petruska, J. and Goodman, M. F. (1987) Nucleotide insertion kinetics opposite abasic lesions in DNA. *J. Biol. Chem.* **262**, 6864-6870.

Rho, H. M., Kim, K., Hyun, S. W. and Kim, Y. S. (1989) The nucleotide sequence and reading frames of a mutant hepatitis B virus subtype adr. *Nucleic Acids Res*. **17**, 21-24.

Santantonio, T., Mazzola, M., Iacovazzi, T., Miglietta, A., Guastadisegni, A. and Pastore, G. (2000) Long-term follow-up of patients with anti-HBe/HBV DNA-positive chronic hepatitis B treated for 12 months with lamivudine. *J. Hepatol*. **32**, 300-306.

Schuurman, R., Nijhuis, M., van Leeuwen, R., Schipper, P., de Jong, D., Collis, P., Danner, S. A., Mulder, J., Loveday, C. and Christopherson, C., *et al.* (1995) Rapid changes in human immunodeficiency virus type 1 RNA load and appearance of drug-resistant virus populations in persons treated with lamivudine (3TC). *J. Infect. Dis*. **171**, 1411-1419.

Williams, K. J. and Loeb, L. A. (1992) Retroviral reverse transcriptases: error frequencies and mutagenesis. *Curr. Top. Microbiol. Immunol.* **176**, 165-180.

Yu, H. and Goodman, M. F. (1992) Comparison of HIV-1 and avian myeloblastosis virus reverse transcriptase fidelity on RNA and DNA templates. *J. Biol. Chem.* **267**, 10888-10896.

Zoulim, F. (1999) Therapy of chronic hepatitis B virus infection: inhibition of the viral polymerase and other antiviral strategies. *Antiviral. Res.* **44**, 1-30.