Fidelity of hepatitis B virus polymerase

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Although efficient vaccines are available, chronic hepatitis B (HBV) infection poses a major health problem worldwide, and prolonged treatment of chronically infected HBV patients with nucleoside analogs often results in drug-resistant HBV variants. Therefore, it is critical to evaluate the contribution of the HBV polymerase to mutations. FLAG-tagged wild-type (FPoIE) and mutant (FPoIE/D551A) HBV polymerases have been expressed in insect cells and purified. The purified FPoIE showed DNA polymerase activity, but FPoIE/D551A did not, implying that the activity was derived from FPoIE. No 3' to 5' exonuclease activity was detected in FPoIE. The fidelity of FPoIE was investigated and compared with that of HIV-1 RT, which is highly error-prone. The fidelity of HBV polymerase seems to be achieved by increasing the $K_m$ for the dNTP being misinserted.

The hepatitis B virus (HBV) is a member of the hepadnaviridae, a family of enveloped hepatotropic DNA viruses. The virus can cause severe liver disease with eventual progression to cirrhosis and primary hepatocellular carcinoma. Nevertheless, the number of chronic HBV carriers is estimated to exceed 350 million [1] and HBV chronic infection remains among the 10 most common causes of death worldwide according to the 1997 World Health Organization report [2]. Moreover, deaths from liver cancer caused by HBV infection probably exceed one million per year worldwide [3,4].

The mature virus consists of a partially duplex, relaxed circular genome of 3.2 kb [5,6]. The HBV genome contains four open reading frames (ORFs) coding for the viral core antigen, the viral surface antigen, the viral DNA polymerase, and the transactivator protein X. Unlike most DNA viruses, it replicates via reverse transcription of an RNA genome in the presence of the reverse transcriptase and a C-terminal RNase H domain [9–11].

Retroviruses exhibit a relatively high rate of mutation attributed to the inaccuracy of the replication machinery that is unique to the retroviral life cycle [12]. The generation of HIV variants is facilitated by the overall low polymerase fidelity of viral reverse transcriptase [13–15]. As RT is a preferred target for the development of viral inhibitors as antiviral drugs, researches have focused on the structural and catalytic properties of RTs, including three-dimensional crystal studies [16–18]. Because HBV RT is functionally and structurally related to HIV RT, some of the nucleoside analogs (such as lamivudine) developed to treat HIV infection are highly potent against HBV infection [1,19] at concentrations below cytotoxic thresholds [20]. However, short-term monotherapy with lamivudine is insufficient to clear viral infection and prolonged use has caused the increased emergence of lamivudine-resistant HBV [21]. HBV polymerase mutants may occur due to the fast viral turnover rate [22], which may lead to the heterogeneity of HBV viral genomes. Mutations of viral genomes also result in the existence of quasispecies in infected individuals that evolve during the course of infection depending on the host-selective pressure [23]. The existence of HBV as quasispecies may be favored by the infidelity of HBV polymerase.

Keywords: HBV polymerase; HBV; fidelity; misinsertion; mispair; exonuclease.

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Abbreviations: AMV, avian myeloblastosis virus; HBV, hepatitis B virus; FPoIE, FLAG-fused HBV polymerase; MLV, murine leukemia virus; ORF, open reading frame; PVDF, poly(vinylidene difluoride); RT, reverse transcriptase.

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polymerase, which would account for the emergence of the many natural mutants with point substitutions and the hypermutation phenomenon [24]. Naturally occurring mutations of HBV have been identified in all the HBV viral genes and regulatory elements, indicating that HBV mutation may affect infection, viral clearance, and response to antiviral therapy [25]. While research toward understanding the extent and source of HBV variation is lacking, fidelity of HIV-1 RT has been investigated by many researchers in in vitro conditions using Pol expressed in heterologous systems. Thus, it is important to evaluate the contribution of the HBV polymerase to mutations.

In this study, FLAG-tagged wild-type and catalytic mutant HBV polymerases have been expressed in insect cells and purified using immunoaffinity column chromatography. We show that the purified HBV polymerase exhibits DNA-dependent DNA polymerase activity, but the purified mutant HBV polymerase does not show the activity. This result indicates that polymerase activity is not caused by host polymerase contamination. In addition, the purified wild-type polymerase does not have 3′ → 5′ exonucleolytic proofreading activity, like other RTs. The nucleotide insertion fidelity of the HBV polymerase was examined and compared with that of HIV-1 RT, and the result shows that HBV polymerase may have similar mutation rates to HIV-1 RT. This is the first study on the fidelity of HBV polymerase.

### Experimental procedures

#### Materials

T4 polynucleotide kinase was purchased from New England Biolabs. Unlabeled nucleotides were purchased from Pharmacia. Oligonucleotides were synthesized by Integrated DNA Technology Inc. Homopolymer template poly(dA)–oligo(dT)12–18 was obtained from Amersham Pharmacia, and [α-32P]dTPP (3000 Ci mmol⁻¹) was purchased from NEN Life Science Products. HIV-1 RT (specific activity, > 5000 U mg⁻¹) was purchased from Roche Molecular Biochemicals. Poly(vinylidene difluoride) (PVDF) blotting membrane was from Millipore, and M2 monoclonal antibody was from Sigma.

#### Methods

**Construction and purification of the wild-type and mutant HBV polymerase.** Two recombinant plasmids, pFPoE and pFPoE/D551A, containing entire HBV polymerase gene (subtype adw [26]), and catalytic mutant HBV polymerase gene, respectively, with FLAG sequences at the N-terminal region were used. The FLAG tag was used to isolate the wild-type and mutant HBV polymerase. pFPoE/D551A has a mutation in nucleotide 1654 changing A to C, altering amino acid residue 551 from aspartic acid to alanine. Each recombinant baculovirus was expressed in Sf-9 cells, and the proteins were purified as described previously [27].

**SDS/PAGE and immunoblot analysis.** The partially purified proteins were separated by 7.5% SDS/PAGE. For immunoblot analysis, proteins were electrophoretically transferred to a PVDF blotting membrane. The membranes were probed with M2 monoclonal antibody and resuspended in NaCl/P₄, containing 0.5% skimmed milk and 0.3% Tween-20. The immunoblots were then incubated with horseradish peroxidase-conjugated antimouse antiserum. The immunoreactive bands were visualized using the ECL system (Amersham Pharmacia).

**DNA polymerase activity.** The DNA polymerization reaction (total reaction volume of 50 µL) contained 50 ng of homopolymer template poly(dA)–oligo(dT)12–18, 2 µCi of [α-32P]dTPP (3000 Ci mmol⁻¹), 50 mM Tris/HCl pH 7.4, 0.01% NP-40, 10 mM MgCl₂, 1 mM dithiothreitol, 10 mM KCl and 50 µM unlabeled dTTP. The reaction was started by adding 90 ng (1 pmol) of either the purified proteins of wild type (FPoE) or mutant (FPoE/D551A), and incubated at 37 °C for 30 min. The reaction was quenched by the addition of 2 µL 0.5 M EDTA, and the reaction products were phenol-extracted and ethanol-precipitated. Two microliters of 95% formamide were added and the proteins were immediately denatured by incubating at 95 °C for 3 min and analyzed by electrophoresis in 7 M urea/16% polyacrylamide gels. The gel was then dried and exposed to a phosphoimager system (BAS FLA2000, Japan).

**Template-primer.** Different template-primer substrates were used for measuring exonuclease activity and site-specific nucleotide misinsertion (Table 1). Each primer was end-labeled with [γ-32P]ATP (3000 Ci mmol⁻¹), using T4 polynucleotide kinase (20 U). Reaction was started by adding 100 nm oligonucleotide and 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′ → 5′ exonuclease activity, hybrid molecules between 16-mer oligonucleotide and 24-mer template were made. To measure site-specific nucleotide misinsertion, four different primers (1510G, 2226A, 5385T and 1212C) were hybridized to the M13mp18 single-stranded template. Partially double-stranded template-primer structures were created by

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<tr>
<th>Table 1. Template/primers used in exonuclease activity assay and site-specific misinsertion assay.</th>
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<tr>
<td><strong>Exonuclease activity assay</strong></td>
</tr>
<tr>
<td>Primer 5′-CCC CTA GAA GAA GAA G-- ---- ---- 3′</td>
</tr>
<tr>
<td><strong>Template 3′-GGG GAT CTT CTT CTT AGG ATA GCG-5′</strong></td>
</tr>
<tr>
<td><strong>Site-specific misinsertion assay</strong></td>
</tr>
<tr>
<td>Primer 1510 G: 5′-GTT TAT CAG CTT GCT TT-</td>
</tr>
<tr>
<td>M13mp18: -CAA ATA GTC GAA GCA GAG-</td>
</tr>
<tr>
<td>Primer 2226 A: 5′-TGA TAT TCA CAA AGC AA-</td>
</tr>
<tr>
<td>M13mp18: -ACT ATA AGT GTT TGC TTA-</td>
</tr>
<tr>
<td>Primer 5385 T: 5′-TTT TAG ACA GGA AGC GT-</td>
</tr>
<tr>
<td>M13mp18: -AAA ATT GTC CTT TGC CAT-</td>
</tr>
<tr>
<td>Primer 1212 C: 5′-GTT TTC CCA GTC AGC AC-</td>
</tr>
<tr>
<td>M13mp18: -CAA AAG GGT CAG TGC TGC-</td>
</tr>
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</table>
combining 740 nm $^{32}$P-end-labeled primer with 4 µm template in 50 mM Tris/HCl pH 7.4, 5 mM MgCl$_2$, 2 mM 2-mercaptoethanol, and 17 µg bovine serum albumin. The mixture was heated at 95 °C and allowed to cool down slowly to room temperature.

Exonuclease activity. The 3′ → 5′ exonuclease activity was measured by the removal rate of mismatched 3′-terminal nucleotides from the 5′-[γ-$^{32}$P] end-labeled oligonucleotide. The reactions were carried out 25 µL reaction mixture containing 300 ng mismatched template-primer, 50 mM Tris/HCl pH 7.4, 10 mM KCl, 10 mM MgCl$_2$, 1 mM dithiothreitol, and 0.01% NP-40. The reaction was started by adding 1 pmol FPoE, 100 mU Klenow fragment of *Escherichia 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 formamide dye mix. The reaction mixtures were electrophoresed in 7 m urea/16% polyacrylamide sequencing gels, and dried. The dried gel was exposed to the phosphorimager system.

Site-specific nucleotide misinsertion. The template-primer substrates for measuring the rates of dNTP incorporation opposite the G, A, T and C are shown in Table 1. Before measuring the kinetic constants of correct and wrong nucleotide incorporation, a time course study was carried out to decide the time frame during which products accumulated linearly with time and less than 30% of the original primer was extended [28]. Reaction times were chosen to be 30 min for FPoE, 1 min for correct insertion and 4 min for misinsertion for HIV-1 RT according to the results of time course experiments (data not shown). The specific activity of the partially purified enzyme was 40 units µg$^{-1}$. 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)$_{12-18}$-directed reaction in 30 min at 37 °C. Reactions were started by combining 4 µL enzyme-primer-template solution (12.5 nM FPoE or 5.3 nM HIV-1 RT, 25 µg bovine serum albumin and 2 µL of the original annealed primer-template solution), 4 µL dNTP-salts solution (52 mM Tris/HCl pH 7.8, 20 mM KCl, 10 mM MgCl$_2$, 5 mM dithiothreitol, 150 µg bovine serum albumin and increasing concentrations of single dNTP), and were incubated at 37 °C. The reaction was terminated by the addition of EDTA to a final concentration of 50 mM in 95% formamide buffer. Reaction products were denatured by incubating at 95 °C for 3 min and analyzed by electrophoresis in 16% polyacrylamide/7 m urea gels. Analysis of deoxynucleotide incorporation assays was carried out using a gel based steady-state kinetic assay [28,29] to determine misinsertion efficiency for all the mispairs. Gel band intensities of the substrates and products were quantitated using the phosphorimager system within the linear response range. For each concentration of dNTP, 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 dNTP concentration, and the data were fitted to the Michaelis–Menten equation using nonlinear least-squares methods. 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 nucleotide misinsertion ($f_{mis}$) [30].

Results

Expression and purification of FLAG-fused wild-type (FPoE) and mutant (FPoE/D551A) HBV polymerase in insect cells

The reverse transcriptase domain of HBV polymerase has several conserved motifs, and the YMDD motif is involved in nucleotide binding in the catalytic site of the polymerase [31,32]. In this study, to determine whether polymerase activity of purified wild-type HBV polymerase due to contamination by host polymerase or not, single amino acid change was made in this YMDD motif and the mutant is called FPoE/D551A. FLAG-fused wild-type and mutant HBV polymerases were expressed in insect cells using the recombinant baculovirus expression system. SF-9 cells were infected with the FPoE or FPoE/D551A baculovirus and were harvested 48 h postinfection. From the infected cells, the polymerases were purified as described in the Materials and methods. In the purified fractions of FPoE (lane 1) and FPoE/D551A (lane 2), five prominent proteins with a molecular weight of approximately 110, 84, 70 and 60 kDa were eluted as shown in Fig. 1A. The purified 84 kDa protein was immuno-stained with the M2 monoclonal antibody that is specific to the FLAG epitope, representing the band was recombinant FPoE or FPoE/D551A proteins as shown in Fig. 1B. In addition through MALDI-TOF analysis, it has been reconfirmed that the 84 kDa
protein is the two HBV polymerases (FPoIE and FPoIE/D551A). Possibly, 110 kDa protein is endogenous protein that binds both tightly and nonspecifically to M2 agarose resin. The other two proteins (70 kDa and 60 kDa proteins) were Hsp70 and Hsp60, respectively. The function of the Hsps for HBV polymerase is described in previous studies [27,33].

**DNA polymerase activity**

The purified HBV polymerase showed DNA-dependent DNA polymerase activity (Fig. 2, lane 1). To confirm that the polymerase activity is from HBV polymerase, polymerase assays were performed with the purified FPoIE/D551A which lacks polymerase activity due to a point mutation. Under the standard reaction conditions described in Experimental procedures, polymerase reactions were conducted with the purified fractions of wild-type or mutant HBV polymerase. Reaction products from FPoIE or FPoIE/D551A were subjected to electrophoresis in a 7 M urea/16% polyacrylamide sequencing gel as shown in Fig. 2. Polymerization products were detected from FPoIE (lane 1), but not from FPoIE/D551A (lane 2), indicating that the polymerization activity was clearly derived from the FPoIE.

**Analysis of the 3' → 5' exonuclease activity**

All the RTs studied thus far lack 3' → 5' exonuclease activity [34]. Therefore, it was interesting to check whether the polymerase of this small DNA virus, HBV, displaying similarities to retroviral transcriptases, has any exonuclease activity. Terminal mismatched template-primer pairs (Table 1) were incubated with the partially purified FPoIE proteins (Fig. 3). Terminal nucleotide excision capability was analyzed in the presence of no proteins (lane 1), HIV-1 RT (lane 2), FPoIE proteins (lane 3) and the Klenow fragment of *E. coli* 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 the FPoIE proteins were used (lane 3), in the same way as with HIV-1. However, efficient excision of the terminal nucleotide was found to occur 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 RTs.

**Site-specific nucleotide misincorporation**

The ratio of the insertion efficiency for wrong (W) vs. right (R) base pairs indicates frequency of nucleotide misinsertion, \( f_{\text{ins}} \). The nucleotide insertion fidelity is defined as the reciprocal of \( f_{\text{ins}} \): \( f_{\text{ins}} = V_{\text{max}}/K_{m}\text{R}/(V_{\text{max}}/K_{m}\text{R}) \) [17]. The lack of proofreading activity permits the sole analysis of the fidelity of DNA polymerization activity. To determine the nucleotide misinsertion frequency of FPoIE and HIV-1 RT, we measured the \( V_{\text{max}} \) and \( K_{m} \) steady-state parameters for the incorporation of correct and incorrect deoxynucleotides (G, A, T and C) opposite the G, A, T and C residues on native M13mp18 template-strand primed with \( 5'\text{P}3'\text{P} \) end-labeled oligonucleotide primers 1510G, 2226A, 5385T and 1212C (Table 1). Four separate reactions were carried out and
each reaction included a single dNTP to measure the rate of synthesis of the correct pair and three possible mispairs. From the quantitation of unextended and extended primers from each reaction set, the initial velocities of product formation were plotted against the dNTP concentrations, and the double-reciprocal plots for the initial velocities vs. the substrate concentrations were made. Only the data of primer 5385T-template are shown to avoid overlapping of data (Fig. 4). The \( f_{\text{max}} \) values for each of the 16 possible insertion events, i.e. four correct

Fig. 4. Kinetic assay for site-specific nucleotide misinsertion. The 5'-\([^{32}\text{P}]\) labeled primer 1510G, 2226 A, 5385T or 1212C was annealed to M13mp18 template strand to produce the 3'-terminal mispairs in the presence of increasing concentrations of single dNTP as indicated, with FPolE or HIV-1 RT. The data pertaining only to the primer 5385T-template are shown.
Table 2. The apparent $V_{\text{max}}$, $K_m$ values and misinsertion frequency ($f_{\text{ins}}$) for wild-type HBV polymerase (FPolE) and HIV-1 RT. Data shown are the mean values ± standard deviation. Standard deviations presented are derived from three (FPolE) or two (HIV-1 RT) independent measurements and the variations were mostly < 20%. Misinsertion frequency, $f_{\text{ins}}$ were evaluated from ratio of relative $V_{\text{max}}$ to $K_m$ as using the equation $f_{\text{ins}} = (V_{\text{max}}/K_m)_{\text{correct}}/(V_{\text{max}}/K_m)_{\text{incorrect}}$. Base pairs are shown with the template (T) first.

<table>
<thead>
<tr>
<th>Base pair (T:dNTP)</th>
<th>$K_m$ (μM)</th>
<th>$V_{\text{max}}$ (% min$^{-1}$)</th>
<th>$f_{\text{ins}}$</th>
<th>$1/f_{\text{ins}}$</th>
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<tbody>
<tr>
<td></td>
<td>FPolE</td>
<td>HIV-1 RT</td>
<td>FPolE</td>
<td>HIV-1 RT</td>
</tr>
<tr>
<td>G : G</td>
<td>61.9 ± 4.5</td>
<td>28.4 ± 2.4</td>
<td>0.161 ± 0.011</td>
<td>4.40 ± 0.81</td>
</tr>
<tr>
<td>G : A</td>
<td>49.3 ± 3.7</td>
<td>46.6 ± 5.1</td>
<td>0.195 ± 0.001</td>
<td>5.91 ± 1.35</td>
</tr>
<tr>
<td>G : T</td>
<td>43.8 ± 5.4</td>
<td>42.0 ± 4.5</td>
<td>0.244 ± 0.036</td>
<td>6.53 ± 0.33</td>
</tr>
<tr>
<td>G : C</td>
<td>0.0909 ± 0.0002</td>
<td>0.0936 ± 0.0003</td>
<td>0.332 ± 0.035</td>
<td>8.97 ± 0.88</td>
</tr>
<tr>
<td>A : G</td>
<td>31.3 ± 3.3</td>
<td>31.4 ± 0.1</td>
<td>0.290 ± 0.006</td>
<td>8.67 ± 1.77</td>
</tr>
<tr>
<td>A : A</td>
<td>45.0 ± 5.2</td>
<td>25.2 ± 2.3</td>
<td>0.192 ± 0.023</td>
<td>7.85 ± 1.88</td>
</tr>
<tr>
<td>A : T</td>
<td>0.0404 ± 0.0088</td>
<td>0.0486 ± 0.0012</td>
<td>0.316 ± 0.036</td>
<td>11.3 ± 3.8</td>
</tr>
<tr>
<td>A : C</td>
<td>33.1 ± 1.4</td>
<td>20.0 ± 0.2</td>
<td>0.269 ± 0.021</td>
<td>6.26 ± 0.11</td>
</tr>
<tr>
<td>T : G</td>
<td>202 ± 20</td>
<td>32.9 ± 1.3</td>
<td>0.606 ± 0.095</td>
<td>20.1 ± 0.7</td>
</tr>
<tr>
<td>T : A</td>
<td>0.122 ± 0.011</td>
<td>0.0674 ± 0.0033</td>
<td>0.749 ± 0.033</td>
<td>26.1 ± 4.1</td>
</tr>
<tr>
<td>T : T</td>
<td>98.4 ± 7.6</td>
<td>71.8 ± 9.8</td>
<td>0.357 ± 0.054</td>
<td>15.7 ± 0.4</td>
</tr>
<tr>
<td>T : C</td>
<td>96.5 ± 10.9</td>
<td>39.9 ± 3.0</td>
<td>0.391 ± 0.039</td>
<td>9.58 ± 0.14</td>
</tr>
<tr>
<td>C : G</td>
<td>0.0470 ± 0.0035</td>
<td>0.0676 ± 0.0012</td>
<td>0.655 ± 0.086</td>
<td>46.4 ± 6.0</td>
</tr>
<tr>
<td>C : A</td>
<td>94.7 ± 4.1</td>
<td>76.9 ± 5.5</td>
<td>0.541 ± 0.059</td>
<td>23.3 ± 4.1</td>
</tr>
<tr>
<td>C : T</td>
<td>58.1 ± 3.5</td>
<td>108 ± 2</td>
<td>0.291 ± 0.014</td>
<td>13.0 ± 2.0</td>
</tr>
<tr>
<td>C : C</td>
<td>36.8 ± 3.4</td>
<td>45.2 ± 5.7</td>
<td>0.260 ± 0.041</td>
<td>8.64 ± 0.95</td>
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Fig. 5. Relative misinsertion efficiencies ($f_{\text{ins}}$) by HBV polymerase (FPolE) and HIV-1 RT. A comparative plot of misinsertion efficiencies, $f_{\text{ins}}$, for individual mismatches from Table 2 are given in bar graph form.

Discussion

HBV has the smallest genome of all known human DNA viruses (3.2 kb) and a unique replication strategy with a reverse transcription step. In retroviruses, reverse transcription is error-prone, which contributes to the high genetic variability of retroviruses with the mutation rates of $10^{-3}$–$10^{-5}$ misincorporation per base [13,35]. In wild-type isolates of HBV, the sequence of the genome may vary up to 10% despite conservation of open reading frame and function [36]. Published HBV genomes showed high nucleotide sequence variability in S, C and P genes, region X, the precore region, and the pre-S2/pre-S1 regions ranked in the order of increasing variability [37]. HBV mutants affecting all known reading frames of the viral genome have been demonstrated in patients with fulminant or chronic HBV infection. Moreover, novel variants of HBV genomic sequences from patients with unusual serological profiles are continually discovered. The exact contribution of the mutations to the natural course of HBV infection remains to be elucidated, but the genetic variations of HBV are possibly related to the infidelity of the HBV polymerase and reverse transcription strategy of HBV.

Fidelity of DNA synthesis is a major determinant in generating spontaneous mutation. However, the molecular mechanisms governing fidelity of DNA synthesis are largely unknown. Judging from the spontaneous mutation rates, the frequency of errors during DNA replication in prokaryotic and eukaryotic cells are between $10^{-7}$ and $10^{-10}$ substitutions per base pair in each cell generation [38]. These low mutation rates are achieved by multiple steps in error discrimination including base selection by DNA polymerase, $3' \rightarrow 5'$ exonucleolytic proofreading, and post-replicative repair [39]. In the present study, HBV polymerase was found to lack $3' \rightarrow 5'$ proofreading exonuclease activity like all RTs studied so far, suggesting that it
has high mutation rate, at least during DNA replication. Especially for RT lacking proofreading activity, nucleotide misinsertion rates are important parameters contributing to the overall polymerase fidelity [39], but it is not the only factor because retroviral RTs lacking a proofreading exonuclease, such as avian myeloblastosis virus (AMV) and murine leukemia virus (MLV) RT have 10-fold and 18-fold higher fidelity than HIV-1 RT, respectively [35].

Because HIV-1 RT is a well-studied enzyme, there are many reports about the error rate of the enzyme. Misinsertion efficiency of HIV-1 RT for all the possible mispairs were between 5.60 × 10^{-2} (C : T) and 1.55 × 10^{-2} (G : T) [40], and misinsertion efficiency for some mismatches were 4.4 × 10^{-5} (C : T), 1.2 × 10^{-4} (T : T) and 1.6 × 10^{-4} (G : T) on oligonucleotide DNA template, and 1.2 × 10^{-4} (C : T), 1.8 × 10^{-4} (T : T) and 4.4 × 10^{-4} (G : T) on M13 DNA template [15]. Another report shows that $f_{\text{ins}}$ values of HIV-1 RT for some mismatches were 1/6000 (1.7 × 10^{-3}) (A : C), 1/32 550 (3.1 × 10^{-5}) (A : G), and 1/75 000 (1.5 × 10^{-5}) (A : A) [41]. In contrast, our $f_{\text{ins}}$ data for HIV-1 RT were from 1.75 × 10^{-4} (1/5714) to 1.62 × 10^{-3} (1/6172) with the overall $f_{\text{ins}}$ value for all the possible mismatches of 6.03 × 10^{-3} (1/1658). Although reported $f_{\text{ins}}$ values for HIV-1 RT are variable depending on template and assay system, our data are within the reported range, representing the reliability of our data. In this report, overall misinsertion efficiency for HBV polymerase was 6.28 × 10^{-4} (1/1591), and the error rates for each G : T, A : C, and A : A mismatch were 1.51 × 10^{-3}, 1.03 × 10^{-3}, and 5.45 × 10^{-4}, respectively (Table 2). Conclusively, HBV polymerase is fairly error-prone, compared to other reported RTs for misincorporation of nucleotides on the DNA templates.

The fidelity of DNA synthesis by HIV-1 RT is several-fold higher with an RNA template than with a DNA template. Misaligned intermediates are formed less frequently with an RNA template than with a DNA template [42]. However, there are some reports that the parameters for fidelity of DNA synthesis in vitro depend primarily on the sequences of nucleic acids copied, rather than DNA or RNA templates [34]. In addition to efficient misinsertion, efficient extension of mismatched 3′-termini of the nascent DNA was found to be a major factor for the infidelity of HIV-1 and HIV-2 RTs [43,44]. Therefore, it is important to examine insertion and extension efficiency on both RNA- and DNA-templated DNA synthesis reactions by HBV DNA polymerase, given the possible role of replication infidelity in generating mutant viruses. As the present study only focuses on the fidelity of misincorporation of nucleotides on the DNA template, a more extensive study remains to be performed to reveal the relationships between fidelity of HBV polymerase and genetic variability.

In this report, HBV polymerase is shown to be highly error-prone, compared to other reported RTs, in contrast to a previous report that HBV and the related animal hepadnaviruses are known to have a mutation rate which is intermediate between DNA and RNA viruses [45]. Although HBV polymerase shows a high error rate similar to HIV-1 reverse transcriptase in vitro conditions, mutation rate of HBV is lower compared to that of HIV-1 in vivo conditions. The reason may be due to the fact that mutations in HBV are not well tolerated because of more overlapping reading frames in HBV than other retroviruses genomes. Therefore from this data it can be inferred that the rate of mutation by the HBV polymerase is higher than mutation rates in in vivo HBV replication.

Acknowledgments

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References

human immunodeficiency virus type 1 reverse transcriptase com-


Supplementary material

The following material is available from: http://www.blackwellpublishing.com/products/journals/suppmat/EJB/EJB3650/EJB3650sm.htm

**Figure S1.** Kinetic assay for site-specific nucleotide misinsertion with 5385T primer. The 5′-[32P]-labeled primer 5385T was annealed to M13mp18 template strand to produce the 3′-terminal mispairs in the presence of increasing concentrations of single dNTP as indicated, with FPoIE or HIV-1 RT.

**Figure S2.** Kinetic assay for site-specific nucleotide misinsertion with 2226A primer. The 5′-[32P]-labeled primer 2226A was annealed to M13mp18 template strand to produce the 3′-terminal mispairs in the presence of increasing concentrations of single dNTP as indicated, with FPoIE or HIV-1 RT.

**Figure S3.** Kinetic assay for site-specific nucleotide misinsertion with 1510G primer. The 5′-[32P]-labeled primer 1510G was annealed to M13mp18 template strand to produce the 3′-terminal mispairs in the presence of increasing concentrations of single dNTP as indicated, with FPoIE or HIV-1 RT.

**Figure S4.** Kinetic assay for site-specific nucleotide misinsertion with 1212C primer. The 5′-[32P]-labeled primer 1212C was annealed to M13mp18 template strand to produce the 3′-terminal mispairs in the presence of increasing concentrations of single dNTP as indicated, with FPoIE or HIV-1 RT.