The Enhanced DNA Replication Fidelity of a Mutant Herpes Simplex Virus Type 1 DNA Polymerase Is Mediated by an Improved Nucleotide Selectivity and Reduced Mismatch Extension Ability[▽]

Wang Tian, Ying T. Hwang, and Charles B. C. Hwang*

Department of Microbiology and Immunology, State University of New York, Upstate Medical University, Syracuse, New York 13210

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We previously demonstrated that a recombinant herpes simplex virus containing a mutation within the finger domain of DNA polymerase replicated DNA with increased fidelity. In this study, we demonstrate that, compared with wild-type polymerase, the mutant enzyme exhibited improved nucleotide selectivity and a reduced ability to extend from mismatched primer termini, which would contribute to the increased DNA replication fidelity.

Herpes simplex virus (HSV) DNA polymerase (Pol) is a member of the B family of Pols. Like other replicative Pols, HSV Pol contains several conserved regions that fold into the thumb, finger, and palm domains. These domains, along with the exonuclease domain, form the Pol and exonuclease active sites (12), which function in the polymerization reaction and the removal of misinserted nucleotides, respectively. Studies of HSV *pol* mutants that are resistant to antiviral drugs demonstrate that the majority of drug resistance is due to mutations within the conserved regions. This supports the notion that the conserved regions of Pol are critical for interaction with the incoming nucleotides and DNA as well as the catalytic reaction (reviewed in references 2 [and references therein] and 10).

We previously engineered a recombinant virus containing a point mutation (L774F) within conserved region VI. The mutant virus replicates viral DNA with increased fidelity compared with the control virus expressing the wild-type Pol (10). Since amino acid L774 is located at the beginning of the finger domain and interacts with the incoming deoxynucleoside triphosphate (dNTP), we hypothesized that the L774F mutation may slightly alter the structure of the finger domain and lead to an altered catalytic activity, dNTP binding affinity, and polymerization rate, which may lead to an improved DNA replication fidelity. In this study, we examined the enzyme kinetics of the L774F mutant Pol to illustrate the biochemical mechanisms leading to the enhanced fidelity of DNA replication.

The mutant Pol exhibits a different nucleotide incorporation pattern and decreased proofreading activity. Wild-type (BBP3) and L774F mutant (B5) Pols were expressed and purified from the corresponding recombinant baculovirus-infected Sf9 insect cells as described previously (9). The purified proteins were more than 99% pure, and the Coomassie blue-stained sodium

dodecyl sulfate-polyacrylamide gels showed the lack of any contaminated protein (Fig. 1). We performed a Pol activity assay to measure the ability of these Pols to incorporate [α -³²P]dCTP into activated salmon sperm DNA as described previously (9). The results demonstrate that both BBP3 and B5 had no significant difference in Pol activity (data not shown). In order to further examine the relative Pol and exonuclease activities of these Pols, we performed experiments using a defined primer/template consisting of a 17-mer primer annealed to a 34-mer template (Fig. 2A), in which the 17-mer oligonucleotide was 5' ³²P labeled. In these experiments, the reaction mixture contained 5 nM primer/template, 100 nM Pol, and 50 μ M dNTPs in a total of 10 μ l of reaction buffer A [20 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 40 μ g/ml of bovine serum albumin, 4% glycerol, 3 mM MgCl₂, 5 mM dithiothreitol, 150 mM (NH₄)₂SO₄]. The reaction mixture was incubated at 37°C for 30 min, and the reaction was stopped by quenching with loading buffer. The reaction products were analyzed on 15% denaturing acrylamide gels containing 8 M urea. The Pol activity was demonstrated by the formation of a ladder of bands (P+1, P+2, . . . , and P+17) above the labeled primer band, and the 3'-5' exonuclease activity was assayed by determining the ability to degrade the labeled primers and form a ladder of shorter bands (P-1, P-2, and P-3). The integrated band intensity was measured using a PhosphorImager (Molecular Dynamics) and quantified using the program ImageQuant. The relative Pol activity was determined from the ratio of gel band intensity, $R = I_{P+1}^{\Sigma} / I_{P}^{\Sigma}$.

In the presence of 50 μ M dNTP, both the wild-type (BBP3) and mutant (B5) Pols were able to incorporate dNTPs from the primer terminus with similar overall Pol activities, extending ~90% of the primers (Fig. 2B). However, the B5 Pol exhibited a different pattern of polymerization, having stronger pauses at several positions than wild-type Pol. For example, B5 Pol exhibited the strongest pause after the insertion of one nucleotide (P+1), compared with the wild-type Pol (30% versus 2% for B5 and wild-type Pol, respectively). B5 Pol also formed stronger pauses at several other sites, including P+2, P+3, and P+4, and those near the end of the template. Inter-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, SUNY, Upstate Medical University, 750 E. Adams St., Syracuse, NY 13210. Phone: (315) 464-8739. Fax: (315) 464-4417. E-mail: hwangc@upstate.edu.

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FIG. 1. Coomassie blue-stained gel of purified Pols. Wild-type (BBP3) and B5 (L774F) mutant Pols expressed in Sf9 cells infected with recombinant baculovirus were purified as previously described (9). For each Pol, 10 μ l of the peak fraction from the single-stranded DNA agarose column was analyzed separately on sodium dodecyl sulfate-10% polyacrylamide gels, which were stained with Coomassie blue. Positions of molecular mass markers (lane M) are shown on the left. The arrow indicates the purified Pols.

estingly, it appeared that the B5 Pol had a weaker activity in extending nucleotide incorporation to the last template base than did the wild-type Pol (1% versus 44% for B5 and wild-type Pol at the position P+17, respectively). It seems that the sequence context could influence the Pol activity of B5 Pol. The insertion of dGMP from the dCMP:G primer terminus led to the strongest pause at P+1 (Fig. 2B), which was not seen when B5 Pol inserted dGMP from the dAMP:T primer terminus (Fig. 3, lane 11). Furthermore, it can be proposed that the stronger the pause, the more frequent the dissociation of the Pol, although we cannot rule out the possibility that the Pol may stall after the incorporation. Thus, it will be interesting to examine whether the processivity factor UL42, the accessory subunit of Pol (6), can alter the pattern of polymerization.

Although we did not use 3'-labeled primer to examine whether these Pols could excise the primer terminal base, the experiments described above clearly demonstrated that both Pols were able to degrade approximately 9% of the primers to form smaller oligonucleotides, which were marked as P-1, P-2, etc., despite the matched-primer termini and the presence of dNTPs, demonstrating that both Pols were exonuclease proficient (Fig. 2B). However, the B5 Pol produced more P-1 and fewer P-2 bands than the wild-type Pol under the assay conditions. The exonuclease activity was further demonstrated by the ability of the Pols to degrade the primer and form shorter ladders from the matched-primer terminus in the absence of dNTPs (Fig. 2C). Under this condition the B5 Pol had a relatively weaker exonuclease activity than did the wild-type Pol in that 38% of the primers were degraded by the B5 Pol compared with 59% by the wild-type Pol. Therefore, these Pols exhibit different Pol and exonuclease activities.

The mutant Pol exhibits a reduced nucleotide misincorporation activity. While the experiments described above demonstrated that B5 Pol exhibited a weaker exonuclease activity than did wild-type Pol for matched primers, we sought to examine whether these Pols have the same activity as proofreading mismatched primers. In these experiments, we annealed the 5'-³²P-labeled 17-mer primer to the 34-mer oligo-

FIG. 2. (A) Sequence of the primer and template used in these experiments. (B) The Pol activity (%), shown at the bottom of the gel images, was calculated as the ratio of the sum of the integrated band intensity of the ladders (P+1, P+2, ..., P+17) above the primer band (P) over the sum of the integrated band intensity of all bands (I_P). Lane 1, primer alone; lane 2, wild-type BBP3 Pol; lane 3, mutant B5 Pol. (C) The exonuclease activity (%), shown at the bottom of the gel image, was calculated as the ratio of the sum of the integrated band intensity of the shorter ladder (P-1, P-2, P-3) over the sum of the intensity of all the bands. The bands labeled with P+ indicate that the products resulted from the incorporation of different numbers of nucleotides by the Pol activity, and those labeled with P- indicate the products from exonucleolytic degradation.

nucleotide (Fig. 3) to create a dCMP:T mismatched-primer terminus, which was then incubated in the absence of dNTPs or in the presence of dATP, dGTP, dATP plus dGTP, or all four dNTPs. The reaction mixture lacking any dNTPs is a control for the proofreading activity in removing the mismatched base. The results show that, compared with the wild-type Pol, B5 appeared to have a slightly weaker exonuclease activity in removing the mismatched dCMP:T (Fig. 3, 30% [lane 7, B5] versus 42% [lane 2, BBP3]).

The results from the reaction containing dATP (Fig. 3, lanes 3 and 8) demonstrated the ability of Pol to reinsert the correct nucleotide after excision of the mismatched base. In the reactions including dGTP, which is the nucleotide to be incorporated opposite the template C base (lanes 5 and 10), or all four dNTPs (lanes 6 and 11) will allow the Pol to excise the wrong base, reincorporate the correct dATP, and extend synthesis from the primer dAMP:T terminus. Under both of these conditions, the wild-type and B5 Pols were each able to proofread and reinsert the correct dATP, based on the reduced intensity of the shorter ladders and increased intensity of the band migrating at the primer position, compared to the control reaction with no dNTPs (compare lanes 3 and 8 with lanes 2

FIG. 3. Exonucleolytic proofreading, resynthesis, extension, rescued insertion, and misinsertion by wild-type and mutant Pols. The activities of the Pols were analyzed using a primer/template containing a dCMP:T mismatched-primer terminus shown at the top of the figure. The representative gel image shows the results of the reaction, including the primer alone (lane 1), the exonuclease activity (lanes 2 and 7), the resynthesis activity of the Pol after proofreading and extension, and misinsertion and/or rescued insertion of the nucleotide(s) provided in the reaction mixture (lanes 3 to 6 and 8 to 11). The relative activity (%), which was the average of two experiments, was calculated as described for Fig. 1 and is shown below the gel image. Lanes 2 to 6 and 7 to 11 are the results from wild-type and B5 Pol, respectively. Symbols * and # indicate the bands resulting from misinsertion. -, not applicable; A, dATP; G, dGTP; AG, dATP plus dGTP; All, all four dNTPs; Exo, exonuclease activity; ReSyn, resynthesis; Ext, extension; MisIn/Resc, misinsertion and/or rescued insertion.

and 7, respectively). The relative percentage of exonuclease activity in reaction mixtures containing the indicated dNTP refers to the relative amount of the remaining exonucleolytic products after the resynthesis, extension, and/or misinsertion (Fig. 3, lanes 3, 5, 8, and 10). Thus, most proofread products were repaired by the reincorporation of the correct nucleotides by both Pols. Furthermore, the wild-type Pol could extend beyond the primer band after the resynthesis, which accounts for 2% of the total DNA substrates (Fig. 3, lane 3). In addition, it seems that the faster-migrating band produced by the wild-type Pol was the result of misinsertion of the dATP paired with the template C base, since the reaction mixture did not contain dGTP and the mobility of this band was slightly higher than that of the band with the correct insertion of dGTP (shown in lanes 5, 6, 10, and 11). Also, it should be noted that the lack of any detectable misincorporation of dAMP by the B5 Pol could be due to the limited sensitivity of the PhosphorImager (Fig. 3, compare lane 3 with lane 8 for the band marked with the asterisk). This notion, together with the data demonstrating

that the B5 Pol was able to misincorporate in the presence of both dATP and dGTP (Fig. 3, lane 10), indicated that the B5 Pol had a weaker misinsertion capability than did the wild-type Pol.

In the reaction mixture containing both dATP and dGTP, both Pols were able to remove the mismatched nucleotide and reinsert the correct nucleotide, as well as insert the next correct nucleotide (dGTP, lanes 5 and 10). It was also noted that both Pols were able to misinsert either dATP or dGTP and formed the band migrating in between the P+2 and P+3 bands (marked by the symbol #) after the formation of a dGMP:C primer terminus. Interestingly, the wild-type Pol exhibited a stronger misinsertion activity than did B5 Pol (5% versus 1%, respectively). Therefore, the wild-type Pol exhibited a greater ability to polymerize the misinsertion of either dGTP:G or dATP:G (which cannot be differentiated by this experiment) than did the B5 Pol.

In the reaction mixtures containing all four dNTPs, the two Pols were able to proofread, resynthesize, and extend the primers with similar activities (lanes 6 and 11). In these reactions, we did not detect any misinsertion. Similar to the results observed for the reactions with the matched-primer terminus, the B5 Pol failed to extend the polymerization to the last template base compared to the results with the wild-type Pol (Fig. 2B). However, the stop sites generated by the two Pols were quite similar. This is in contrast to the reactions containing the matched-primer terminus dCMP:G (Fig. 2B). The differences may be due to the difference of the sequence context as described above.

In the reaction containing only dGTP (Fig. 3, lanes 4 and 9), both Pols exhibited efficiency in removing the mismatched primer and the wild-type Pol showed a stronger ability to misinsert dGMP. However, the misinsertion of dGMP could also be the result of a rescued incorporation. Although we could not distinguish whether the insertion of dGTP was a misinsertion or a rescued incorporation, the B5 Pol's mutation impaired the ability of the Pol to misincorporate nucleotides (compare 1%, lane 9, versus 6%, lane 4, for B5 and wild-type Pol, respectively). This also suggests that the B5 Pol has a weaker capability to synthesize premutagenic products than does the wild-type Pol.

We also examined the proofreading and polymerization activities on mismatched-primer termini of dCMP:C and dCMP:A. These Pols exhibited different activities similar to those observed on the dCMP:T mismatched primer (data not shown). These included the following: (i) the B5 Pol showed a lower capability to remove the mismatched bases than did the wild-type Pol, (ii) the wild-type Pol exhibited stronger abilities to insert incorrect nucleotides than did the B5 Pol, and (iii) while the wild-type Pol was able to extend from the mismatched-primer terminus, the mutant Pol was impaired in such activity.

The mutant Pol has increased nucleotide selectivity. The weaker activity of B5 Pol than of the wild type in extension from the primer termini suggests that there may be a difference between these two Pols in discriminating nucleotide incorporation. To test this hypothesis, we performed gel kinetics assays according to the method described by Creighton and Goodman (3) to measure the kinetic parameters K_m and V_{max} for the correct nucleotide insertions. Briefly, the kinetics of the Pols

FIG. 4. Gel kinetic assays of the parameters K_m and k_{cat} . The primer/template used for each gel kinetic experiment is shown above the graphic plot. The template base for the insertion of the corresponding nucleotide is bold and underlined. The gel kinetic assay was performed as described in the text. The dashes shown in the primer indicate the positions that require the corresponding nucleotides for the running-start reactions. The rectangular hyperbola plot derived from nonlinear regression using the GradFit software program (11) and the representative gel images are shown. The values for the kinetic parameters, including the standard deviations, are indicated below each graph. P, primer; T, T+1, and T+2, target of nucleotide incorporations; T-1, T-2, and T-3, running-start bases.

were analyzed using 10 μl of reaction buffer A containing 5 nM of Pol, 5 nM of primer/template, 50 μM dNTP (corresponding to the nucleotide required for the running-start reaction), and various concentrations (0 to 50 μM) of dNTP (corresponding to the target nucleotide). The reaction mixture was incubated for 1 min at 37°C for correct incorporation to create a single complete hit condition, in which less than 10% of the primers were utilized. Reactions were quenched by adding stop solution, and reaction mixtures were analyzed on a 15% denaturing acrylamide gel. A PhosphorImager was used for the detection of the reaction products. The intensity of each band was quantified using the program ImageQuant and analyzed using the method previously described (3). The ratio of integrated gel band intensities, $R = I_{\text{T}}^{\Sigma}/I_{\text{T-1}}$, was plotted as a function of the target dNTP concentrations, where T refers to the target band(s), including those above the target band, and T-1 is the running-start primer band. K_m and V_{max} values were calculated by fitting the data to a rectangular hyperbola using nonlinear regression with GradFit software version 3.0 (11). k_{cat} values were determined by dividing V_{max} by the enzyme concentration.

The primer/templates used in the gel kinetic assays are displayed above each graphic plot shown in Fig. 4. The graphic plot, gel image of the representative experiment for each correct nucleotide insertion, and the corresponding V_{max} and k_{cat}

values are shown in Fig. 4 and were the averages of at least two independent experiments. Although the two Pols did not have any significant difference in their k_{cat} values for the correct insertion of each nucleotide, B5 exhibited significantly higher K_m values ($P < 0.05$; Student's t test) for the insertion of the correct dCTP, dGTP, and dATP opposite the G, C, and A bases, respectively, than did the wild-type Pol. Therefore, the B5 Pol exhibited a lower affinity and thus had a better selectivity for the correct insertion of dCTP, dGTP, and dATP during the polymerization step than did the wild-type Pol.

Possible role of the finger domain of HSV Pol in enzymatic activity and DNA replication fidelity. Both the nucleotide discrimination during incorporation and the 3'-5' exonuclease activity are the key steps in maintaining DNA replication fidelity (reviewed in reference 4). Our previous study demonstrated that the HSV type 1 L774F Pol mutant replicated DNA with increased fidelity (10). Since the L774 residue is highly conserved among Pols and is located at the beginning of one helical structure of the finger domain, which together with the palm and thumb domains forms the Pol active site (12), the substitution of a phenylalanine for the leucine residue may induce a local structure change in the finger domain. The change may directly affect its interaction with incoming dNTPs or alter its interaction with other residues involved in the formation of the Pol active site. This may subsequently impact

the interaction of Pol with incoming dNTPs, thereby influencing the fidelity of DNA replication. Alternatively, the mutation may result in a stiffer finger domain structure and influence the catalytic step during polymerization. In this study, the biochemical data demonstrate that the mutant Pol exhibits a higher K_m or a better selectivity for the correct insertion of three nucleoside triphosphates with an unchanged k_{cat} , supporting the hypothesis that the L774F mutation affects the interaction of Pol with the incoming nucleotide. This also is consistent with the idea that K_m is the major factor contributing to fidelity (7, 13). Furthermore, despite the fact that the mismatch has a significant kinetic barrier to extension (1, 13), the wild-type Pol is able to extend from the mismatched-primer terminus. Compared with the wild-type Pol, this mutation impairs the Pol activity of misinsertion and extension from a mismatched primer (Fig. 3), which may contribute in part to the increased fidelity.

The finding that this mutant Pol exhibits a modest effect on the exonuclease activity is intriguing. Since the exonuclease activity requires movement of the primer terminus from the Pol active site into the exonuclease active center and involves rotation of the thumb and finger domains toward or away from the palm, as demonstrated for other B family Pols (5, 8), it is possible that this mutation affects the conformational change required for switching of the active sites. Alternatively, the mutation may slightly alter the conformation of the exonuclease active center and affect the proofreading activity. Indeed the HSV type 1 Pol crystal structure shows two asymmetry units, one of which has a structural conformation consisting of the pre-N-terminal domain, the N-terminal domain, the exonuclease domain, and the finger domain (12). Thus, the mutation in the finger domain may affect the structure of the exonuclease active center and/or the motion required for active-site switching. Further studies are necessary to explore the detailed mechanisms leading to the altered activity. It also is

important to examine whether the processivity factor has any effects on the altered properties of the mutant Pol.

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