Published in final edited form as:

J Mol Biol. 2011 March 4; 406(4): 558–570. doi:10.1016/j.jmb.2010.12.033.

Variation in Mutation Rates Caused by RB69pol Fidelity Mutants Can Be Rationalized on the Basis of Their Kinetic Behavior and Crystal Structures

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Abstract

We have previously observed that stepwise replacement of amino acid residues in the Nascent base-pair Binding Pocket (NBP) of RB69 DNA polymerase (RB69pol) with Ala or Gly expanded the space in this pocket resulting in a progressive increase in misincorporation. However, results *in vivo* with the similar RB69 pol NBP mutants, showed that mutation rates, as determined by the T4 phage *rI* forward and *rII* reversion assays, were significantly lower for the RB69pol S565G/Y567A double mutant than for the Y567A single mutant, the opposite of what we would have predicted. To investigate the reasons for this unexpected result we have determined the pre-steady-state kinetic parameters and crystal structures of the relevant ternary complexes. We found that the S565G/Y567A mutant generally had greater base selectivity than the Y567A mutant and that the kinetic parameters for dNMP insertion, excision of the 3' terminal nucleotide residue as well as primer extension beyond a mispair, differed not only between these two mutants but also between the two highly mutable sequences in the T4 *rI* complementary strand. Comparison of the crystal structures of these two mutants with correct and incorrect incoming dNTPs provides insight into the unexpected increase in fidelity of the S565G/Y567A double mutant. Taken together, the kinetic and structural results provide a basis for integrating and interpreting the *in vivo* and *in vitro* observations.

Keywords

DNA polymerase; kinetics; mutation rates; mutation spectra; crystal structures

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[†]This work was supported by a USPHS grant # (2R01-GM063276-09 and 3R01GM063276-08S1) and by funding from the Steitz Center for Structural Biology, Gwangju Institute of Science and Technology, Republic of Korea to J.W. and T.A. Steitz.

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Mina Wang, Shuangluo Xia, Jimin Wang and Gregor Blaha determined the crystal structures. Shuangluo Xia, Harold Lee, Arjun Sinha, and Thomas Christian performed the kinetic and binding assays. William Konigsberg designed the experiments and wrote the paper.

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Introduction

The fidelity of replicative DNA polymerases depends mainly on their ability to discriminate among the four dNTPs and to excise non-Watson-Crick (non-W-C) base-paired (incorrect) nucleotide residues from a growing primer strand. Ongoing studies are focused on understanding how these DNA polymerases (pols) can copy a genome without making more than one base substitution error in $\sim 10^6$ incorporation events, but a comprehensive solution to this problem has thus far eluded investigators. In pursuing this problem we have chosen the well characterized pols from T4 and RB69 bacteriophages. These are highly homologous replicative DNA pols¹ that make very few errors.² Since they are members of the B family and have extensive sequence as well as structural similarities to human and other eukaryotic replicative pols,³ they have served as good models for studying structure/function relationships and fidelity mechanisms.^{4; 5} RB69 pol in particular has been extensively investigated and there is abundant information about its structure and mechanism of action.^{6; 7; 8; 9; 10; 11; 12; 13; 14; 15; 16; 17; 18; 19; 20; 21; 22; 23}

We had shown previously that incremental expansion of the space in the Nascent base-pair Binding Pocket (NBP) of RB69pol resulted in a progressive increase in misincorporation efficiency of most mispaired dNMPs.^{24; 25} An RB69pol triple mutant, where three residues in the NBP, namely L561, S565 and Y567, were replaced by amino acids with smaller side chains, showed the greatest loss in base selectivity.²⁴ However, we were surprised to learn that the S565G/Y567A double mutant exhibited a lower mutation rate *in vivo* than the Y567A single mutant that we had already studied.²⁶ This unexpected result prompted us to construct and express the RB69 pol S565G/Y567A double mutant. In preliminary experiments we found that the S565G/Y567A mutant was considerably less permissive than the Y567A mutant for incorporating most incorrect dNMPs *in vitro*, a result consistent with *in vivo* observations.²⁶ Because these findings raised questions about the generality of the conclusions in our previous report,²⁴ namely that expanding the space in the NBP would lead to enhanced misincorporation, we decided to investigate the kinetic behavior of the S565G/Y567A double mutant in more detail and to compare it with the more permissive Y567A single mutant. We also wanted to see if we could approximate the differences in the *in vivo* mutation rates between the Y567A single, and the S565G/Y567A double mutant by evaluating several kinetic parameters that should be relevant to the *in vivo* situation. In addition we decided to determine crystal structures of the Y567A and the S565G/Y567A mutant ternary complexes with correct and incorrect dNTPs using primer/template duplexes with one of the relevant hotspot sequences to see if we could rationalize their kinetic behavior in structural terms.

Results and Discussion

One of our goals in this study was to account for the unexpected behavior of the RB69pol S565G/Y567A double mutant with respect to the decrease in mutation rates relative to the Y567A single mutant.²⁶ For this purpose we prepared the S565G/Y567A mutant and investigated various kinetic parameters for both the Y567A and S565G/Y567A mutants with correct and incorrect incoming dNTPs. We also determined crystal structures of the corresponding ternary complexes with the aim of understanding the features that might be responsible for the differences in their behavior with respect to insertion, excision and extension beyond a mispair.

In attempting to correlate the *in vivo* and *in vitro* behavior of these two mutants, we chose P/Ts with sequences that were closely related to the two hotspot sequences present in the complementary strand of the T4 *rI* region.²⁷ Although we realized that it would not be

possible to exactly mimic the *in vivo* conditions *in vitro*, we thought that by comparing the *in vitro* kinetics of the Y567A mutant with the S565G/Y567A variant, we might be able to approximate the differences that were observed in the forward mutation rates between the two mutants in the T4 *rI* tester system.²⁶ Accordingly, we tested the ability of each mutant to: (i) incorporate incorrect dNMPs, particularly those that gave rise to the changes observed in the *rI* mutational spectra;²⁷ (ii) extend primers beyond mispairs that were responsible for the mutations observed at the hotspots and; (iii) excise the mispaired nucleotide residues at the 3' end of the primers. To determine the effect of sequence context, we used P/Ts with the two hotspot related sequences in the insertion, mispair-extension and excision assays (see Fig. 2 for the P/T sequences). We also determined the binding affinities of P/Ts with correct and mismatched primer-termini for each of these mutants using fluorescence anisotropy to see whether we would observe the same variation in K_{ds} between the two mutants that were reported by Trzemecka, et al.²⁶ We want to verify their findings using a different method before including it in our attempt to correlate the *in vivo* mutation rates with our *in vitro* kinetics parameters.

Pre-Steady-State Kinetic Parameters for dNMP Incorporation by the RB69pol NBP Mutants

The pre-steady-state kinetic parameters for dNMP incorporation by the wild type (wt), the S565G, the Y567A and the S565G/Y567A mutants are given in Table 1. The P/T substrates had a G:C base-pair penultimate to the P/T junction. A G:C pair was also present at a similar position in the two hotspot sequences of the T4 *rI* complementary strand (Fig. 1). The templating bases were either G or C opposite the correct dNTPs or the two incorrect dNTPs that gave rise to the sequences found in the mutated hotspot region.²⁷ As shown in Table 1, the k_{pol} values obtained for the wt and all the mutants showed only slight variations for insertion of correct dNMPs. However, the k_{pol} values for incorporation of incorrect dNTPs fell into two groups, one represented by the wt and the S565G mutant, and the other by the Y567A and S565G/Y567A mutants, with a 40–100 fold reduction from the first group to the second group (Table 1). Interestingly, relative to the Y567A mutant, the S565G/Y567A double mutant exhibited lower $K_{d,app}$ values for correct dNTPs but higher $K_{d,app}$ values for insertion of incorrect incoming dNMPs, resulting in 3 to 8 fold increase in the specificity constants ($k_{pol}/K_{d,app}$) for correct dNMP incorporation and up to a 3 fold decrease of the specificity constants for incorrect dNMP incorporation (Table 2).

Extension of Primers Beyond a Mismatch at the Primer-Template Terminus

In view of the ability of the Y567A mutant to insert incorrect dNMPs more efficiently than the S565G/Y567A mutant, we wanted to determine how well each mutant would bury a mismatch, as this could tend to preserve errors as DNA synthesis proceeded and thus be expected to contribute to the observed mutation rate. We found that the Y567A mutant was 8 times more efficient at extending a primer past a T:G mismatch in the HS1B-P/T than the S565G/Y567A mutant. With the HS2B-P/T containing an A:C mismatch, the Y567A mutant was only 3 times more efficient than the S565G/Y567A mutant in burying a mismatch during primer extension (Table 3). These results are consistent with the expectation that the mutation rates with the Y567A mutant would be greater *in vivo* than with the S565G/Y567A mutant, as was observed.²⁶

Excision Rates for *exo*⁺ RB69pol Mutants Acting on P/Ts Containing Hotspot Sequences

In addition to insertion efficiencies and extension past mismatches, the relative editing rates for misincorporated dNMPs would also be expected to influence mutation rates *in vivo*. To investigate this we compared the Y567A and the S565G/Y567A mutants with respect to their editing capability when acting on P/Ts containing hotspot sequences. As shown in Table 4, the S565G/Y567A mutant excised mispaired nucleotide residues at a rate that was, on average, 1.7 times faster than the Y567A mutant. Again, this was in line with the

observations that the S565G/Y567A mutant exhibited a lower mutation rate than the Y567A single mutant *in vivo*.²⁶

Correlating *in vivo* and *in vitro* Discrimination Ratios Between the Single and Double Mutants

The kinetic parameters that we have determined for the Y567A and the S565G/Y567A mutants, with respect to their relative efficiencies for dNMP incorporation, excision, and extension beyond a mispair, are all individually consistent with the finding that the Y567A mutant produced higher mutation rates than the S565G/Y567A double mutant *in vivo*, but we wanted to see whether the *in vitro* kinetic results could approximate the forward mutation rates observed *in vivo* in the *rl* tester system.²⁶ For this purpose we calculated the overall discrimination ratios between the single and double mutants which included all three processes mentioned above. We also made the assumption that these processes were independent of one another. Our objective was to provide a range of values that might encompass the observed forward *in vivo* mutation rates. We first calculated the discrimination factor for the insertion efficiency of correct versus incorrect dNMPs by each mutant with the HS1A and HS2A-P/T sequences as follows.

$$D_{Y567A-HS1A} = \frac{(k_{pol}/K_d)_{dCTP/dG}}{(k_{pol}/K_d)_{dCTP/dG}} = \frac{3.1}{1.4 \times 10^{-3}} = 2.2 \times 10^3 \quad \text{Eq.1}$$

$$D_{S565G/Y567A-HS1A} = \frac{(k_{pol}/K_d)_{dCTP/dG}}{(k_{pol}/K_d)_{dTTP/dG}} = \frac{10}{1.3 \times 10^{-3}} = 7.7 \times 10^3 \quad \text{Eq.2}$$

From equations 1 and 2, the discrimination factor for Y567A mutant acting on the HS1A sequence is 2.2×10^3 , and the discrimination factor for the S565G/Y567A mutant acting on the HS1A sequence is 7.7×10^3 . The relative discrimination ratio for the HS1A sequence, D_{r-HS1A} , is the ratio between $D_{S565G/Y567A-HS1A}$ and $D_{Y567A-HS1A}$, which is $7.7 \times 10^3 / 2.2 \times 10^3 = 3.5$. We then applied the same set of calculations for the HS2A sequence where the incorrect dATP had to compete with the correct dGTP opposite dC with both mutants as follows.

$$D_{Y567A-HS2A} = \frac{(k_{pol}/K_d)_{dGTP/dC}}{(k_{pol}/K_d)_{dATP/dC}} = \frac{4.8}{3.3 \times 10^{-3}} = 1.5 \times 10^3 \quad \text{Eq.3}$$

$$D_{S565G/Y567A-HS2A} = \frac{(k_{pol}/K_d)_{dGTP/dC}}{(k_{pol}/K_d)_{dATP/dC}} = \frac{41}{1.0 \times 10^{-3}} = 41 \times 10^3 \quad \text{Eq.4}$$

The relative discrimination ratio for the HS2A sequence, D_{r-HS2A} , is the ratio of $D_{S565G/Y567A-HS2A}$ divided by $D_{Y567A-HS2A}$, which is $41 \times 10^3 / 1.5 \times 10^3 = 27$.

For extension past a mismatch, we compared the ratios of the extension efficiencies, E_{xt} , for each of the mutants to insert a correct dNMP after a terminal mismatch at the P/T terminus.

The ratio (R) for incorporating dGMP opposite dC with the HS1B sequence, $R_{\text{ext-HS1B}} = E_{\text{xt}}(\text{Y567A-HS1B})/E_{\text{xt}}(\text{S565G/Y567A-HS1B})$, was 8. The ratio for the HS2B sequence where dCMP was inserted opposite dG for the two mutants, $R_{\text{ext-HS2B}} = E_{\text{xt}}(\text{Y567A-HS2B})/E_{\text{xt}}(\text{S565G/Y567A-HS2B})$, was 3.

The ratio of the excision rates, R_{exc} , for each of the mutants acting either on the HS1B or HS2B sequences were nearly equal. The $R_{\text{exc-HS1B}} = E_{\text{xc}}(\text{Y567A-HS1B})/E_{\text{xc}}(\text{S565G/Y567A-HS1B})$ was 0.66, while the $R_{\text{exc-HS2B}} = E_{\text{xc}}(\text{Y567A-HS2B})/E_{\text{xc}}(\text{S565G/Y567A-HS2B})$ was 0.56.

To obtain the overall discrimination ratio for the HS1B sequence, $D_{\text{overall-HS1AB}}$, we multiplied the relative discrimination ratio $D_{\text{r-HS1A}}$, by the extension ratio for the two mutants $R_{\text{ext-HS1B}}$, and then by the ratio of the relative excision rates $R_{\text{exc-HS1B}}$, which we calculated to be 18, as shown in equation 5. When the same set of calculations were performed for HS2A and HS2B, the result was 45, as shown in equation 6.

$$D_{\text{overall-HS1AB}} = D_{\text{r-HS1A}} \times R_{\text{ext-HS1B}} \times R_{\text{exc-HS1B}} = 3.5 \times 8 \times 0.66 = 18 \quad \text{Eq.5}$$

$$D_{\text{overall-HS2AB}} = D_{\text{r-HS2A}} \times R_{\text{ext-HS2B}} \times R_{\text{exc-HS2B}} = 27 \times 3 \times 0.56 = 45 \quad \text{Eq.6}$$

Thus the range from 18 to 45 spans the value of 38, which was the ratio found when the Y567A and the S565G/Y567A mutants were compared with their respect to their forward mutation rates using the T4 *rI* tester system.²⁶

Determination of the Enzyme-P/T Dissociation Constants

Another concern raised by the results reported by Trzemecka et al²⁶ was that P/Ts containing the hotspot sequences might differ in their binding affinities for RB69pol and that the exo^+ versus the exo^- versions of RB69pol might also display different affinities for these P/Ts. If confirmed, this would have a significant impact on the interpretation of our results. Accordingly we tested this possibility using fluorescence anisotropy, an equilibrium method that can accurately determine the relative difference between the enzyme-P/T dissociation constants for both mutants. As shown in Table 5, the K_d s for the exo^+ wt protein showed less than a two-fold difference compared to that of the exo^- wt protein for both hotspot sequences. For the two mutant proteins, the K_d of Y567A mutant binary complex was almost the same as that of the S565G/Y567A mutant binary complex with both complimentary HS1A-P/T and partially complimentary HS1B-P/T sequences (Table 5). Thus, the differences in P/T-RB69pol dissociation constants between the two mutants observed by Trzemecka et al,²⁶ using a different method, is unlikely to be the sole explanation for the differences in mutation rates *in vivo*.

Structures of Ternary Complexes of Y567A and S565G/Y567A Mutants with Correct and Incorrect Incoming dNTPs

As described above, the difference in mutation rates exhibited by the Y567A and the S565G/Y567A mutants in the *in vivo* forward mutation assay can be approximated by the kinetic behavior of these two mutants *in vitro*. To see whether we could rationalize these differences in structural terms, we solved crystal structures of the following ternary complexes: (i) the RB69 pol Y567A mutant with dCTP:dG at 2.1 Å resolution (Fig. 3); (ii) the same Y567A mutant but with dTTP:dG at 1.9 Å resolution (Fig. 4); (iii) the S565G/Y567A double mutant with dCTP:dG at 2.0 Å resolution (Fig. 5) and; (iv) the same double mutant but with

dTTP:dG at 2.5 Å resolution (Fig. 6). The refinement statistics for these structures are summarized in Table 6 and pairwise comparisons among them were carried out by superimposing their C α coordinates so that relevant differences could be discerned. All the structures were nearly identical with C α root mean square deviations that ranged from 0.11–0.18 Å among pairs of these structures except for the region comprising the NBP where the amino acid residues and the nascent base-pairs differed. These comparisons are depicted in Figs. 3 to 6 and features that pertain to their kinetic behavior are described below. The S565G/Y567A double mutant, reported here, differs from the other NBP mutants studied previously where the increase in size of the NBP cavity is mainly contributed by the removal of large hydrophobic side chains. The S565G substitution involves removal of the hydroxymethyl moiety and replacing it with a hydrogen atom. Moreover, the S565 side chain interacts with the aromatic plane of the templating base in a different way from the other single NBP mutants, and the space vacated by the S565G substitution is now filled with the DNA duplex that undergoes a small adjustment when moving into this cavity. The S565G single mutant differs only slightly from the wt RB69pol in terms of its pre-steady-state kinetic parameters, but when it is combined with the Y567A substitution, the kinetic behavior of the resulting S565G/Y567A double mutant (Table 1) is dominated by the structural features of the Y567A single mutant. By superimposing the crystal structures of the relevant ternary complexes we hoped to be able to answer the following questions: (i) Why are the k_{pol} values so much greater for incorporation of incorrect dNMPs by the S565G/Y567A mutant compared to the S565G mutant? (ii) What structural features are responsible for the low $K_{\text{d,app}}$ values for incorporation of the correct dNMPs by the S565G/Y567A double mutant?

Structural Basis for Increased Incorporation Efficiency of Incorrect dTTP Opposite dG by the Y567A and S565G/Y567A Mutants Relative to wt RB69 Pol

Superposition of both the dCTP/dG-containing and the dTTP/dG-containing ternary complexes formed by the Y567A and S565G/Y567A mutants showed that the conformation of the triphosphate tail of both incorrect and correct incoming dNTPs are identical (Figs. 3–6). This suggests that the incorrect dTTP and the correct dCTP are both positioned to allow facile movement of the substrates in the complexes toward the transition state for nucleotidyl transfer. The fact that the reactive centers appear to be optimally aligned even for mispaired complex suggests that the crystal structures represent a state directly on the nucleotidyl transfer reaction pathway, and that this state is highly populated prior to chemistry. Because of this we believe that the structural features of these complexes are relevant in attempting to rationalize the pre-steady-state kinetic parameters for insertion of both correct (dCMP) and incorrect (dTMP) nucleotide residues opposite dG with both mutants. To the best of our knowledge, these are the first pol structures that have an incorrect incoming dNTP in what appears to be a catalytically competent ternary complex in a pre-insertion state. Although the structure of a dTTP/dG-containing ternary complex of Dpo4 pol has been previously determined²⁸ the α -phosphorous atom (P α) of the incoming dTTP opposite dG was about 5 Å away from the corresponding P α of correct incoming dTTP opposite dA, suggesting that this complex was not likely to be catalytically competent. Although this Dpo4 pol structure may adequately explain how an incorrect dTTP opposite to dG is rejected, it does not allow quantitative structure-kinetic correlations. Other mispaired dNTP ternary complexes have also been reported,²⁸ where the binding mode of an incorrect dNTP also differs greatly from that of a correct dNTP. All these structures can account for the rejection of incorrect incoming dNTPs, but they cannot explain how incorrect dNMPs are incorporated at reduced rates. Our structures of the mismatched dTTP/dG ternary complex in the pre-insertion state provides a more accurate picture than any previous pol structures for the incorporation pathway of incorrect dNMPs that were derived from computer simulations.

In ternary complexes of both the Y567A and S565G/Y567A mutants, the pyrimidine base of dTTP is hydrogen bonded to the templating dG with wobble geometry. In these complexes dG is displaced into the cavity created by the Y→A substitution present in both the Y567A and S565G/Y567A mutants, whereas dTTP occupies nearly the same position as dCTP opposite dG (Figs. 4, 6). When the dG position, observed in the dTTP:dG-containing ternary complexes of these mutants was modeled onto the wt complex, dG clashed with the side chain of Y567. Thus, wt RB69 pol cannot bind the dTTP/dG nascent base-pair in the same way as the two NBP mutants. The only way to eliminate the steric clash with the wt RB69 pol, while maintaining wobble geometry, would be to shift the entire nascent base-pair away from the pre-insertion site, which was exactly what has been observed in Dpo4 pol.²⁸ In that case, the bound dTTP was at a considerable distance from the catalytically relevant position. In our structures, we show how an incorrectly paired dTTP/dG is properly aligned allowing incorporation of an incorrect dTMP without additional unknown energetic barriers, and why the incoming dTTP of this same nascent base-pair cannot be incorporated by wt RB69 pol because it would not fit into the NBP with aligned reaction centers.

Comparison of the dTTP/dG-containing ternary complex of the Y567A mutant with that of the S565G/Y567A mutant showed no major differences in the overall orientation of either dTTP or dG in the two structures (Fig. 6B), which could explain why the corresponding kinetic parameters for both mutants are so similar (Table 1 and 2). Superposition of the dTTP/dG-containing ternary complex with the dCTP/dG-containing ternary complex of the Y567A mutant however shows a subtle reorientation of the glycosidic bond of the incoming dNTPs (Fig. 4D). This difference could be one of the contributing factors responsible for the reduction of k_{pol} for incorporation of dTMP, relative to dCMP, opposite dG with this mutant (Table 1).

Structural Basis for the Increased Incorporation Efficiency of Correct dNMPs in the S565G/Y567A Mutant Relative to wt RB69 pol and Other NBP Mutants

The increased incorporation efficiency of *correct* dNMPs catalyzed by the S565G/Y567A mutant relative to wt RB69 pol and other NBP mutants was unexpected based on our previous report.²⁴ In attempt to explain this finding, we decided to compare the corresponding dCTP/dG-containing ternary complexes of wt RB69 pol and the NBP mutants. In all of these structures, the hydrogen bonding network, which includes the OH groups of Y567, Y391 and T587 in the wt RB69 pol,²⁹ has been disrupted in the two NBP mutants that have the Y567A substitution. The space created by the replacement of the phenolic side chain of Y567 with a methyl group is now occupied by two ordered water molecules, W1 and W2. W1 forms two hydrogen bonds, one with the backbone carbonyl oxygen of A583 and the other with W2. W2 in turn forms a hydrogen bond with the OH group of Y391 and with the backbone carbonyl oxygen of Y567A, displacing it slightly toward Y391. Comparison of the dCTP/dG-containing ternary complex of the Y567A mutant with that of the wt RB69 pol shows a similar displacement of G568 (Fig. 3C), suggesting that an inter-residue hydrogen bond between Y567 and Y391 in the wt RB69 pol is responsible for G568 and helix P being in a strained geometry. Once this hydrogen bond is removed in these NBP mutants containing the Y567A substitution, G568 can relax into a new position even in the presence of the correct dCTP/dG nascent base-pair. When G568 is in this relaxed position, the extra space created by the Y567A substitution can now more readily accommodate a nascent non-W-C base-pair. Despite the presence of these ordered water molecules, considerable flexibility has been introduced into the NBP as a result of the disruption of the Y567 hydrogen bonding network mentioned above. Consequently, the Y567A and, to a lesser extent, the S565G/Y567A mutants can now more easily tolerate mispaired bases and utilize incorrect dNTPs for primer extension.²⁴ Since these changes mainly affect the binding of incorrect dNTPs, structural alterations responsible for increased

incorporation efficiency of correct dNMPs relative to wt are situated elsewhere within the NBP, and based on the kinetic results, the S565G replacement is a good candidate.

In the structure of the Y567A single mutant (Fig. 4E,F), the S565 hydroxyl group can alternate between two rotamer orientations, one where the S565 hydroxyl group is hydrogen bonded to the L561 backbone carbonyl oxygen in the presence of the correct dCTP/dG nascent base pair (Fig. 3B,4F), a situation that is also seen in the ternary complex of wt RB69pol with a correct nascent base-pair.²⁹ In the other orientation, the S565 hydroxyl group is hydrogen bonded to the L562 backbone carbonyl oxygen in the presence of dTTP/dG, the incorrect nascent base-pair (Fig. 4E), a situation that is also seen in the apo form of wt RB69pol.¹⁸ In this latter rotamer conformation, the hydroxyl group of S565 points away from the templating base, creating a more hydrophobic environment around dG so that the base can move up against the S565 side chain maintaining a hydrophobic contact with the methylene moiety. Indeed, comparison of the dCTP/dG-containing ternary complex of the Y567A mutant with that of the wt RB69 pol shows that the templating dG follows the displacement of G568 so that the same distance between C α of G568 and N3 of dG is maintained (Fig. 5C). In a hydrophobic environment, with polarizable π -electrons in the aromatic base, the hydrogen on the α -carbon of G568 may be sufficiently polarized to become slightly acidic and thus capable of forming a hydrogen bond with a suitable acceptor to create a fixed geometry between them.^{29–31} Such polar-nonpolar interactions differ from classic hydrophobic interactions where the interacting proteins do not have to have a fixed geometry, i.e., they can slide against each other.²⁹ With a fixed interaction geometry between G568 and the templating base, the downward displacement of dG occurred in both the Y567A and S565G/Y567A mutants (Figs. 3–6). When the hydrogen bond between Y567 and Y391 is present, G568 in the S565G single mutant would not be expected to have a similar displacement as observed in the two Y567A-containing NBP mutants. It is also noteworthy that the interaction pattern of the G568 residue with the templating base is conserved in the ternary complexes of all members of the pol α family.²⁹ An interesting finding here is that enlarging the NBP by substitutions allows other NBP residues to adopt alternative conformations (i.e., reorientation of S565 and movement of G568) so that different shapes of specific nascent base-pair(s) can now be accommodated.

In the dCTP/dG-containing ternary complex of the S565G/Y567A mutant, the side chain of L561 is reoriented toward the space vacated by the replacement of S565 with Gly (Fig. 5A). Both the side chains of L561 and the C α backbone of S565G are now in Van der Waals contact with the templating dG base. This provides the templating base with more rigidity prior to base pairing so that there is likely to be less of an entropic penalty when inter-base hydrogen bonds are formed between dG and an incoming dCTP. This could explain why the S565G/Y567A mutant has markedly lower $K_{d,app}$ values for insertion of correct dNMPs than the wt or the Y567A mutant.

The other significant change in the environment of the templating dG in the S565G/Y567A mutant, is the displacement of the base of dG as it comes into closer contact with the C α of S565G compared to the situation in the Y567A mutant and the wt enzyme. Because this carbon atom is adjacent to electron-withdrawing groups (the peptide bond), the C α H atom could become more acidic especially in a hydrophobic environment^{30, 31} and should be able to form an associative interaction with the π electron cloud of dG (Fig. 4E), which is a specific polar-nonpolar interaction. In comparison, the hydroxyl group of S565, in the wt and in the Y567A mutant, points toward the plane of the aromatic proton of dG at a distance of 3.33 Å, providing a different type of polar-nonpolar interaction with the heterocyclic ring (Fig. 4E). Another consequence of introducing the S565G substitution into the Y567A mutant is that the templating base can interact even more extensively with helix P of the NBP than it does in the case of wt RB69pol. These interactions help to firmly

anchor the templating base, reducing the entropic penalty for binding the correct dCTP and enhancing its apparent affinity, which was observed to be ~6-fold greater than that of the wt or any of the other NBP mutants (Table 1). In the Y567A mutant, the orientations of the L561 and S565 side chains are exactly the same as in the wt, but dG in the Y567A ternary complex is more flexible with the possibility of reorienting the S565 side chain and allowing movement of G568 when dCTP is paired with dG (Figs. 3, 4) compared to the situation when dCTP is part of the S565G/Y567A ternary complex (Figs. 5, 6). Taken together, these comparisons provide a structural basis for rationalizing the differences in kinetic behavior of these two mutants when they incorporate correct dNMPs.

Materials and Methods

Materials and reagents were of the highest quality commercially available. T4 polynucleotide kinase was purchased from New England Biolabs (Boston, MA). [α - 32 P]ATP was from Perkin-Elmer Life Science, Inc (Waltham, MA). The *E. coli* strain BL21(DE3) was obtained from the Stratagene Corp (Santa Clara, CA). DH5 α cells and dNTPs were purchased from Invitrogen (Carlsbad, CA). The Ni-NTA resin was obtained from Qiagen (Valencia, CA). Other chemicals were analytical grade and were purchased from Sigma-Aldrich Inc (St. Louis, MO).

Enzymes and DNA substrates

Wild type RB69pol and the Y567A, S565G, S565G/Y567A mutants were produced in both the exonuclease-proficient and exonuclease-deficient (D222A/D327A) backgrounds in *Escherichia coli*, BL21(DE3) cells, purified, and stored as previously described.²⁴

Oligonucleotides were synthesized by W. M. Keck Foundation Biotechnology Resource Laboratory (Yale University). Primers were labeled at the 5'-end using T4 polynucleotide kinase and [γ - 32 P]ATP for kinetic studies and annealed to unlabeled templates as previously described.²⁵ Oligo nucleotides that were used as templates for fluorescence anisotropy were labeled at the 3' end with TAMRA and then annealed to the complementary primers.

Pre-Steady state kinetic experiments for insertion and extension beyond a mispair

All experiments were performed at 25°C using the KinTek rapid quench flow instrument (model RFQ-3). Reactions were performed in 66 mM Tris at pH 7.4 and 10 mM MgSO₄ and quenched with 0.5 M EDTA. K_d and k_{pol} values were determined using single turnover conditions as previously described,²⁵ where the enzyme was in twenty-fold excess over the [P/T]. The concentration of the enzyme was 1.6 μ M and the [P/T] was 83 nM. For each $K_{d,app}$ and k_{pol} determination, six different [dNTP] were used according to the procedures described previously.²⁵ For extension beyond mispairs, the same methods as used for incorporations were employed²⁵ except that the HS1B and HS2B P/Ts were employed with corresponding correct incoming dGTP and dCTP, respectively.

Nucleotide excision experiments

For reactions too fast to be measured by manual quenching, a rapid quench-flow apparatus (RFQ-3) was used. Excision assays were performed at 25°C in a buffer containing 66 mM Tris-HCl at pH 7.4 and 10 mM MgSO₄. Enzyme at 1.6 μ M was incubated with 83 nM 32 P-labeled duplex DNA in the presence of 6 mM EDTA. Reactions were initiated by the addition of MgSO₄ to a final concentration of 10 mM and then quenched with 0.5 M EDTA. Products were separated on 15% denaturing 8 M urea polyacrylamide sequencing gels, imaged on a Molecular Dynamics Storm 860 instrument, and quantified using ImageQuant software (Amersham Biosciences, Uppsala, Sweden).

Data analysis

Data from single-turnover experiments were fit to equation 7 with a single exponential ($n=1$).

$$Y = \sum_{i=1}^n A_i e^{-k_i t} + C \quad \text{Eq.7}$$

where Y is the concentration of the DNA product formed during the reaction, C is the offset constant, A_i is the observed amplitude of product formation, and k_i is the observed rate constant k_{obs} for i -th exponential term ($i=1$ in all of our cases). The kinetic parameters k_{pol} (the maximum rate of dNMP incorporation) and $K_{\text{d,app}}$ (defined as the [dNTP] that provides an insertion rate that is one half of the maximum rate that can be attained under conditions of saturating substrate) were obtained by fitting plots of k_{obs} versus [dNTP] to Equation 8:

$$k_{\text{obs}} = \frac{k_{\text{pol}} [\text{dNTP}]}{K_{\text{d,app}} + [\text{dNTP}]} \quad \text{Eq.8}$$

Determination of dissociation constants for RB69pol:P/T binary complexes

Fluorescence anisotropy experiments were performed as described previously³² using a two channel QuantaMaster C-61 fluorescence spectrophotometer (Photon Technology International, ON, Canada). Briefly, 560 nm light polarized along the vertical axis with a 5nm slit width was used to excite the TAMRA-labeled DNA duplex. Emission intensities at 580 nm (5 nm slit width) from both the parallel and perpendicular planes were recorded simultaneously using 1s integration for 60 s. The fluorescence anisotropy for each time point was determined using equation 9, where r is the fluorescence anisotropy, $I_{\parallel}$ is the polarized emission intensity parallel to the excitation polarization, and $I_{\perp}$ is the polarized emission intensity perpendicular to the excitation polarization.

$$r = \frac{(I_{\parallel} - I_{\perp})}{(I_{\parallel} + 2I_{\perp})} \quad \text{Eq.9}$$

The average anisotropy for each concentration of polymerase was then determined and plotted vs. concentration. The resulting curve was fitted using the Hill Equation (equation 10), where L_b is the lower bound, U_b is the upper bound, [P] is the protein concentration, K_d is the dissociation constant, and n is the cooperativity (values, typically between 1 and 3 were used to obtain the best fit in our experiments).

$$f([P]) = L_b + (U_b - L_b) \left[\frac{[P]^n}{K_d^n + [P]^n} \right] \quad \text{Eq.10}$$

Crystallization of RB69pol Y567A and S565G/Y567A mutant ternary complexes with dCTP:dG and dTTP:dG

The RB69pol Y567A or S565G/Y567A mutants were mixed in an equimolar ratio with freshly annealed dideoxy-terminated P/T (ddP/T) to give a final protein concentration of 110 μM . dCTP or dTTP was then added to give a 3 mM final concentration. For equilibration,

micro-batch vapor-diffusion methods were used to obtain crystals. For the Y567A:ddP/T:dCTP complex, equal volumes of a solution containing 175 mM CaCl₂, 10% (w/v) PEG350 MME, and 100 mM Na Cacodylate (pH 6.5) was added to the mixture. For the Y567A:ddP/T:dTTP mispaired ternary complex, equal volumes of a solution containing 150 mM CaCl₂, 13% (w/v) PEG350 MME, and 100 mM Na Cacodylate (pH 6.5) was added to the mixture. For the S565G/Y567A:ddP/T:dCTP complex, equal volumes of a solution containing 175 mM CaCl₂, 7% (w/v) PEG 350 MME, and 100 mM Na Cacodylate (pH 6.5) was added to the mixture. Crystals typically grew in 2–3 days at 20°C. The square rod-shaped crystals had typical dimensions of 200 μm × 50 μm × 50 μm. Crystals were transferred from the mother liquor to a cryoprotectant/precipitant stabilization solution containing 20% (w/v) PEG 350 MME, 100 mM CaCl₂, and 100 mM Na Cacodylate (pH 6.5) with 1 mM dCTP or dTTP. The S565G/Y567A: ddP/T:dTTP ternary complex was obtained by soaking the dCTP-containing complex with 1 mM dTTP for ten minutes at a time with four changes. Crystals were then transferred into a cryoprotectant/precipitant solution with the same components but containing 30% (w/v) PEG 350 MME prior to freezing in liquid nitrogen.

Data collection, structure determination, and refinement

X-ray diffraction data were collected using the synchrotron radiation source at the beamline X29A_Q315 at Brookhaven National Laboratory (NSLS) (Upton, NY) for crystals of the S565G/Y567A mutant ternary complexes with dCTP:dG and dTTP:dG as well as the Y567A mutant ternary complexes with dCTP:dG. For the crystals of the Y567A mutant ternary complex with dTTP:dG, we used the synchrotron radiation source at the beamline 24ID-E_NECAT_Q315 at Argonne Advanced Photon Source (APS, Chicago, IL). All crystals belonged to orthorhombic space groups P2₁2₁2₁ with slightly different unit cell dimensions (Table 6).

All four structures were solved by the automated molecular replacement method using AMORE as implemented in CCP4,³³ starting with the wt RB69 pol structure of the ternary complex (1IG9).¹⁸ The primer-template DNA duplex and the incoming dCTP or dTTP were built into the residual electron density maps, which were phased with the partially refined RB69 pol model, using the program COOT.³⁴ The structures were refined using Refmac 5,³⁵ and refinement statistics are summarized in Table 6. All figures were made using the program Ribbons.³⁶

PDB accession numbers

Coordinates and structure factors for the Y567A mutants with dCTP:dG or dTTP:dG ternary complex structures have been deposited in the Protein Data Bank under accession codes 3NDK and 3NGI, respectively, and coordinates and structure factors for the S565G/Y567A with dCTP:dG or dTTP:dG ternary complex structures have been deposited in the Protein Data Bank under accession codes 3NE6 and 3NHG, respectively.

Abbreviations

P/T	primer-template
NBP	nascent base-pair binding pocket
PEG-MME	polyethylene glycol monomethyl ether
pol	DNA polymerase
RB69pol	RB69 DNA polymerase

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Fig. 1. Mutational spectra for the RB69pol Y567A exo^+ mutant. The sequence shown is part of the T4 wt *rI* strand complementary to the coding strand. Transitions are entered above the wt bases and transversions are entered below. This figure was adapted from Fig. 2 in a previous report.² The hotspot sequences are underlined.

HS1A	TAT AAA AAT GAC C ATA TTT TTA CTG GGC ACG AG
HS1B	TAT AAA AAT GAC CT ATA TTT TTA CTG GGC ACG AG
HS2A	AGG CGC GGA GAT G TCC GCG CCT CTA CCG TGT TA
HS2B	AGG CGC GGA GAT GA TCC GCG CCT CTA CCG TGT TA
Std	GC GGA CTG CTT AC GCG CCT GAC GAA TG(C/G)TCT
ddP/T	GC GGA CTG CTT AC ^{dd} GCG CCT GAC GAA TGG ACT

Fig. 2.

Sequences of “Hotspots” (HS1A, HS1B, HS2A, HS2B), standard (Std) and dideoxy-terminated (ddP/T) primer-templates. The HS1A P/T that was used for determining pre-steady-state kinetic parameters had a matched P/T terminus while the HS1B P/T used for primer extension past a mismatch had a T at the 3' terminus of the primer strand opposite dG in the template strand. The HS1B and HS2B 14/20mer sequences had mispaired bases at the 3' ends of the primers. These were used for primer extensions past a mismatch and for determining excision rates of the 3'-terminal residue of the primer. The mismatched bases in the primer strands are ones that produced the majority of the mutations shown above the sequence in Fig. 1.

Fig. 3.

Structures of dCTP:dG-containing ternary complex formed by the wt and the Y567A single mutant. (A) The wt RB69 pol ternary complex containing dCTP:dG.²⁹ (B) The Y567A ternary complex containing dCTP:dG. The two ordered water molecules labeled W1 and W2 are in the space formerly occupied by the side chain of Y567 in the wt enzyme. (C) Superposition of the Y567A mutant (yellow and cyan) with the wt enzyme (golden and green).

Fig. 4.

Structure of the Y567A mutant ternary complex containing dTTP:dG. (A) Final 2Fo-Fc map using omitted model phases calculated at 1.9 Å resolution, contoured at 1.5 σ , superimposed onto the wobble geometry of the refined dTTP:dG mispair in the ternary complex of the Y567A mutant. (B) An additional hydrogen bond is observed for the displaced dG with W2. (C) Superposition of the dTTP:dG-containing ternary complex (yellow and cyan) with the dCTP:dG-containing ternary complex (golden and green) of the Y567A mutant. (D) Superposition of the dTTP:dG-containing ternary complex (yellow and cyan) of the Y567A mutant with the dCTP:dG-containing (golden and green) and with the dCTP:dG-containing wt enzyme (silver). (E) Hydrophobic interactions (highlighted with large spheres) between S565 and the five-membered ring of the dG purine base in the dTTP:dG-containing ternary complex of the Y567A mutant. (F) Polar interactions (highlighted with large spheres) between S565 and the five-membered ring of the templating dG purine base in the dCTP:dG-containing ternary complex of the Y567A mutant.

Fig. 5.
 (A) Structure of the dCTP:dG-containing ternary complex of the S565G/Y567A mutant. (B-C) Two orthogonal views of the superposition of the S565G/Y567A mutant (yellow and cyan) with the corresponding wt enzyme (golden and green).

Fig. 6. (A) Structure of the dTTP:dG-containing ternary complex of the S565G/Y567A mutant. (B-C) Two orthogonal views of the superposition of the S565G/Y567A mutant (yellow and cyan) with the corresponding Y567A mutant structure (golden and green).

Table 1
Pre-Steady-State Kinetic Parameters for exo^- wt RB69 Pol and Three of its exo^- NBP Mutants

	$K_{d,\text{app}}$ (μM)				k_{pol} (sec^{-1})			
	wt	S565G	Y567A	S565G/Y567A	wt	S565G	Y567A	S565G/Y567A
dNTP/dN	70	16	68	12	200	100	213	121
dCTP/dG								
dGTP/dC	66	N.D.	27	3.7	160	N.D.	130	153
dATP/dC	1800	940	803	1080	0.08	0.025	2.61	1.09
dTTP/dG	N.D.	495	1011 ^b	1141	N.D.	0.013	1.37	1.52

^aP/T sequences used for the correct insertion (dCTP/dG, dGTP/dC) were the standard sequences given in Fig. 2. The P/T sequences for dATP/dC and dTTP/dG were the HS1A and HS2A sequences respectively, as shown in Fig. 2. The standard deviation for the $K_{d,\text{app}}$ values was $\pm 15\%$ when the values were less than $100\mu\text{M}$, $\pm 25\%$ when the values were above $100\mu\text{M}$. The standard deviation for k_{pol} values was $\pm 10\%$. N.D. = not determined.

^bThis value differs from the one we reported previously,²⁴ which could be due to the difference in the P/T sequence used for the assays.

Table 2

Incorporation Efficiencies [k_{pol}/K_d ($\mu\text{M}^{-1}\text{sec}^{-1}$)] for dNMPs by wt RB69 Pol and Two of its NBP Mutants Calculated from the Data in Table 1

dNTP/dN	wt	Y567A	S565G/Y567A	Ratio ^a
dCTP/dG	2.9	3.1	10	0.31
dGTP/dC	2.4	4.8	41	0.12
dATP/dC	4.4×10^{-5}	3.3×10^{-3}	1.0×10^{-3}	3.30
dTTP/dG	7×10^{-5}	1.4×10^{-3}	1.3×10^{-3}	1.08

^aThe ratio was calculated by dividing the incorporation efficiency obtained with the Y567A mutant by the incorporation efficiency of the S565G/Y567A mutant.

Table 3

Efficiency for Extension Beyond a Mismatch by the Y567A and Y567A/S565G exo^- Mutants with P/Ts Having Hotspot Related Sequences ($k_{\text{cat}}/K_{\text{m}}$) ($\mu\text{M}^{-1}\text{sec}^{-1}$)^a

Substrate	exo^- RB69pol Mutants			Ratio ^b
	P/T ^a	wt	Y567A S565G/Y567A	
HS1B		5.9×10^{-4}	11×10^{-3}	7.8
HS2B		7×10^{-4}	6.6×10^{-3}	3.3

^aThe P/T sequences are given in Fig 2. The $k_{\text{cat}}/K_{\text{m}}$ values are reported here because the high K_{ds} did not allow saturating [dNTP] to be reached.

^bThe ratio was calculated by dividing the extension efficiency obtained with the Y567A mutant by the extension efficiency observed with the S565G/Y567A mutant

* The standard deviation was $\pm 20\%$.

Table 4Excision Rates (sec^{-1}) for exo^+ RB69pol Mutants Acting on Primer-Templates Containing Hotspot Sequences

Substrate	exo ⁺ RB69pol Mutants			Ratio ^b
P/T ^a	wt	Y567A	S565G/Y567A	
HS1B	32	41	62	0.66
HS2B	37	53	95	0.56

^aThe P/T sequences are given in Fig 2.^bThe ratio was calculated by dividing the excision rates obtained with the Y567A mutant by the excision rate obtained with the S565G/Y567A mutant.* The standard deviation was $\pm 15\%$.

Table 5

Ground-state Dissociation Constants of RB69pol:P/T Binary Complexes

RB69 pol	HS1B (nM)	HS2B (nM)	HS1A (nM)
wt exo^-	70.9 ± 10.8	60.3 ± 7.4	
wt exo^+	114.0 ± 24	74.3 ± 12.8	
Y567A exo^+	86.5 ± 6.7		71.6 ± 5.7
S565G/Y567A exo^+	58.6 ± 4.7		52.6 ± 3.7

Table 6

Crystallographic data collection and refinement statistics

Mutants:	Y567A	Y567A	S565G/Y567A	S565G/Y567A
Ternary complex with:	dCTP:dG	dTTP:dG	dCTP:dG	dTTP:dG
Synchrotron Sources	NSLS	APS	NSLS	NSLS
Beamline	X-29A	24ID-E	X-29A	X-29A
Wavelength (Å)	1.07500	0.97918	1.10000	1.07500
Space group	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁	P2 ₁ 2 ₁ 2 ₁
Unit cell dimensions[a,b,c(Å)]	75.006,120.015,130.781	74.989,120.33,130.76	75.135,119.783,130.652	75.052,119.999,130.382
Resolution range (Å) ^a	50.00–2.00(2.07–2.00)	50.00–1.88(1.95–1.88)	50.00–2.00(2.07–2.00)	50.00–2.50(2.59–2.50)
Number of reflections				
Unique	76738(7648)	95329(9446)	79966(7743)	39761(3861)
Redundancy	3.3(3.2)	4.4(4.4)	4.6(4.3)	4.9(4.7)
Completeness (%)	95.3(96.0)	99.6(99.7)	99.7(97.8)	96.1(95.2)
R _{merge} (%) ^b	14.0(87.9)	11.0(>100)	8.3(78.1)	10.8(>100)
I/s	8.5(1.2)	12.4(1.04)	18.1(1.6)	12.2(1.1)
Refinement statistics				
Reflections	72811	90257	75880	37719
R _{work} (%) ^c	18.7	19.2	18.4	18.6
R _{free} (%) ^d	22.9	22.6	22.6	24.4
Final model				
Non-hydrogen atoms	9139	9213	8993	8537
Amino acid residues	903	903	903	903
Water molecules	932	956	758	354
Ca ²⁺ ions	7	8	5	5
Template nucleotides	18	18	18	18
Primer nucleotides	13	13	13	13
dNTP	1	1	1	1
Average B-factors(Å ²)				
Protein (w/TLS)	28.7	30.2	22.1	58.8
Waters	36.4	39.5	29.6	34.5
r.m.s.d. ^e				
Bond length (Å)	0.0067	0.0066	0.007	0.0086
Bond angles (°)	1.023	1.010	1.055	1.197
PDB accession ID	3NDK	3NGI	3NE6	3NHG

^aThe highest resolution shell statistics are in parenthesis.^bR_{merge} = $\sum_{hkl} \sum_j |I_j(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_j \langle I(hkl) \rangle$, merging statistics for all symmetry-mates and equivalent observations.^cR_{work} = $\sum_{hkl} |F_{obs}(hkl) - F_{calc}(hkl)| / \sum_{hkl} |F_{obs}(hkl)|$, crystallographic R-factor.^dR_{free}: Cross-validation R-factor for a randomly selected ~5% of the total unique reflections.

^e r.m.s.d.: root-mean-squares deviation from ideal values.