ASFV DNA Polymerase X Is Extremely Error-Prone under Diverse Assay Conditions and within Multiple DNA Sequence Contexts†

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Received June 30, 2006; Revised Manuscript Received October 11, 2006

ABSTRACT: We previously demonstrated that the DNA repair system encoded by the African swine fever virus (ASFV) is both extremely error-prone during the single-nucleotide gap-filling step (catalyzed by ASFV DNA polymerase X) and extremely error-tolerant during the nick-sealing step (catalyzed by ASFV DNA ligase). On the basis of these findings we have suggested that at least some of the diversity known to exist among ASFV isolates may be a consequence of mutagenic DNA repair, wherein damaged nucleotides are replaced with undamaged but incorrect nucleotides by Pol X and the resultant mismatched nicks are sealed by ASFV DNA ligase. Recently, this hypothesis appeared to be discredited by Salas and co-workers [(2003) *J. Mol. Biol. 326*, 1403−1412], who reported the fidelity of Pol X to be, on average, 2 orders of magnitude higher than what we previously published. In an effort to address this discrepancy and provide a definitive conclusion about the fidelity of Pol X, herein we examine the fidelity of Pol X-catalyzed single-nucleotide gap-filling in both the steady state and the pre-steady state under a diverse array of assay conditions (varying pH and ionic strength) and within different DNA sequence contexts. These studies corroborate our previously published data (demonstrating the low fidelity of Pol X to be independent of assay condition/sequence context), do not reproduce the data of Salas et al., and therefore confirm Pol X to be one of the most error-prone polymerases known. These results are discussed in light of ASFV biology and the mutagenic DNA repair hypothesis described above.

ASFV[1] causes a disease of varying mortality [depending on the particular isolate (*1*)] in domestic pigs in Africa, the Iberian Peninsula, and the Caribbean (*2*). Possessing a large [168−189 kb (*3*)] double-stranded DNA genome encoding 151 proteins (*4*), ASFV is one of the most complex viruses known. In its target cells, marcrophages and monocytes (*5*), ASFV utilizes the host cell nucleus during an early phase of viral DNA synthesis but appears to complete the replication/assembly of its genome in cytoplasmic/perinuclear viral factories (*6*−*8*). Consistent with the latter intracellular location, ASFV encodes its own replicative polymerase in addition to a minimalist DNA repair system consisting of an AP endonuclease (APE), a repair polymerase (Pol X), and an ATP-dependent DNA ligase (*4*). While this tripartite repair system would appear to have been retained for the purpose of processing spontaneously[2] generated apurinic/apyrimidinic (AP) sites and/or reactive oxygen species (ROS)-induced single-strand breaks in the viral genome (*9*), the unique substrate specificities of *both* Pol X and ASFV DNA ligase have led us to hypothesize that this "repair" system may have subsequently evolved a secondary function, that of viral genome mutagenesis (*9*−*12*).

In an initial communication on Pol X we demonstrated it to be extremely error-prone during single-nucleotide gap-filling; in particular, Pol X catalyzes formation of the G:G[3] mismatch with an efficiency comparable to that of the correct, Watson−Crick G:C base pair (*10*). Though we noted a potential mutagenic role for Pol X at that time, it was clear that, in order for this error-proneness to be biologically relevant, the downstream DNA ligase would need to have the unusual property of sealing mismatched nicks (the products of aberrant gap-filling by Pol X) with high efficiency (*11*). This is indeed the case: we recently demonstrated that the ASFV DNA ligase is more tolerant of mismatched nicks than any other ligase characterized to date (*12*).

The presence, in the same virus, of an extremely error-prone gap-filling DNA polymerase and the most error-tolerant DNA ligase known seems unlikely to be coincidental and raises questions about what purpose these relaxed substrate specificities have been selected for. We have hypothesized that by replacing damaged nucleotides with undamaged but incorrect nucleotides the ASFV DNA repair

† This work was supported by NIH Grant GM43268. B.J.L. was supported in part by a predoctoral NIH CBIP fellowship (2T32 GM08512).

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[1] Abbreviations: AP, apurinic/apyrimidinic; APE, AP endonucleoase; ASFV, African swine fever virus; BSA, bovine serum albumin; DTT, dithiothreitol; f_{inc}, misincorporation ratio; PNK, polynucleotide kinase; Pol, DNA polymerase; ROS, reactive oxygen species.

[2] ASFV does not encode a DNA glycosylase.

[3] In the base pair notation X:Y, X refers to the templating position and Y denotes the incoming dNTP.

10.1021/bi0613325 CCC: $33.50
Published on Web 11/16/2006

Table 1: Comparison of Previously Reported Pol X Fidelity[a] Values

base pair[b]	Tsai[c] fidelity	Salas[d] fidelity	(Salas fidelity)/(Tsai fidelity)
G:A	140	16132	115
G:G	1.9	1399	736
G:T	16	4038	252
C:A	65	16547	254
C:C	7700	300416	39
C:T	95	23485	247

[a] When examined in the pre-steady state (Tsai data), fidelity is defined as $[(k_{pol}/K_{d,app})_{cor} + (k_{pol}/K_{d,app})_{inc}]/(k_{pol}/K_{d,app})_{inc}$, where the subscripts "cor" and "inc" refer to correct and incorrect nucleotide incorporation, respectively. When examined in the steady state (Salas data), fidelity is defined as $[(k_{cat}/K_M)_{cor} + (k_{cat}/K_M)_{inc}]/(k_{cat}/K_M)_{inc}$. [b] In the base pair notation X:Y, X refers to the templating position and Y denotes the incoming nucleotide. [c] Data taken from ref *10*. [d] Data taken from ref *14*; this study only analyzed the six mismatched base pairs shown here (i.e., mismatches containing templating A or T were not studied).

system could contribute to viral genomic drift (*9*−*12*). Importantly, this hypothesis is consistent with a restriction fragment length polymorphism analysis which suggested that genetic diversity in ASFV may arise from point mutations or small insertions and/or deletions (*13*).

The work described here was undertaken as part of an ongoing effort to understand whether the in vitro error-proneness/error-tolerance of ASFV Pol X and DNA ligase actually correlate with an in vivo enhancement of base substitutions in the viral genome. Importantly, the extreme error-proneness of Pol X was recently called into question by Salas and co-workers who reported the fidelity[4] of this polymerase to be, on average, 2 orders of magnitude higher than what we previously published (*10*, *14*) (Table 1). The specific objective of the work described here is to address this discrepancy and provide a definitive conclusion about the magnitude of Pol X's error-proneness. Toward this end, we have reevaluated the fidelity of Pol X-catalyzed pre-steady state (and steady state) single-nucleotide gap-filling (focusing on the G:C and G:G base pairs) as a function of both assay condition (varying pH and ionic strength) and DNA substrate sequence context. These analyses corroborate our previous findings, do not reproduce the data of Salas and co-workers, and therefore confirm Pol X to be one of the most error-prone DNA polymerases known. These results are discussed in light of ASFV biology and the mutagenic DNA repair hypothesis described above.

MATERIALS AND EXPERIMENTAL PROCEDURES

Materials. Bovine serum albumin (BSA) was from Roche. Oligonucleotides were from Integrated DNA Technologies. [γ-^{32}P]ATP and Microspin G-25 columns were from Amersham Biosciences. T4 polynucleotide kinase (PNK) was from New England Biolabs. Sep-Pak C18 columns were from Waters. Materials and reagents not listed here were of standard molecular biology grade.

[4] When analyzed in the pre-steady state, fidelity is defined as $[(k_{pol}/K_{d,app})_{cor} + (k_{pol}/K_{d,app})_{inc}]/(k_{pol}/K_{d,app})_{inc}$, where the subscripts "cor" and "inc" denote correct and incorrect base pair synthesis, respectively. When analyzed in the steady state, fidelity is defined as $[(k_{cat}/K_M)_{cor} + (k_{cat}/K_M)_{inc}]/(k_{cat}/K_M)_{inc}$.

Preparation of DNA Substrates. All oligonucleotides were purified by polyacrylamide gel electrophoresis, extracted into a buffer consisting of 500 mM ammonium acetate with 1 mM EDTA (pH 7.5), desalted on Sep-Pak C18 columns, dried in a CentriVap, and then resuspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) prior to storage at −20 °C. The concentration of purified oligonucleotides was determined by UV absorbance at 260 nm using extinction coefficients provided by the vendor. Upstream primers were ^{32}P labeled at their 5′ termini using [γ-^{32}P]ATP and PNK. After heat inactivating PNK at 65 °C for 20 min, free [γ-^{32}P]-ATP was removed on Microspin G-25 columns. All DNA substrates (Table 2) were assembled at room temperature by mixing primer:template:downstream oligos in a 1:1.1:1.2 molar ratio. Typically, only 5% of the substrate molecules were ^{32}P labeled. Once assembled, substrates were stored at 4 °C prior to use.

Protein Expression, Purification, and Quantitation. Pol X was expressed and purified as described (*10*) except that the protein was supplemented with glycerol to a final concentration of 40% (v/v) prior to storage at −80 °C. The concentration of active protein was determined by burst assays using a KinTek RQF-3 rapid chemical quench apparatus at 37 °C. Syringe A contained Pol X (at different dilutions) and 200 nM TSAI single-nucleotide gapped substrate (containing templating G; see Table 2 for details) in buffer consisting of 50 mM Tris-borate, 50 mM KCl, 3% glycerol (v/v), pH → 9.0 with KOH while at 25 °C, 2 mM DTT, and 200 μg/mL BSA. Syringe B contained 12 mM dCTP and 20 mM *free*[5] $MgCl_2$ in the same buffer, but lacking DTT and BSA. Reactions were quenched with 600 mM Na_2EDTA (pH 8.0), followed by resolution on 19% denaturing polyacrylamide gels. Gel visualization was by phosphor screen autoradiography using a STORM840 scanner from Molecular Dynamics. Band intensity quantitation and data plotting were performed with ImageQuant (GE Healthcare) and SigmaPlot 9.0 (Systat Software Inc.), respectively. Data were fit to the burst equation [product] = $A[1 - \exp(-k_{obs}t)] + ct$, where A is the burst amplitude, k_{obs} is the apparent first-order rate constant, and c is the steady state rate. Multiplying the burst amplitude by the protein dilution factor for a given assay yielded the concentration of active enzyme.

Pre-Steady State Fidelity Analysis under Different Buffer Conditions. Assays were performed at 37 °C in a buffer whose postmixing composition was 50 mM Tris-borate, *X* mM KCl, 3% glycerol (v/v), pH = *Y*, 1 mM DTT, and 100 μg/mL BSA, where *X* was either 0, 50, or 140 and *Y* was either 7.5 (adjusted at 37 °C) or 9.0 [adjusted at room temperature to facilitate comparison with previously published data (*10*)]. Assays were initiated by mixing a protein/DNA solution (consisting of 450 nM Pol X, 200 nM TSAI single-nucleotide gapped DNA substrate, 2 mM DTT, and 200 μg/mL BSA) with a dNTP/$MgCl_2$ solution (consisting of varying dNTP concentration and sufficient $MgCl_2$ to achieve a postmixing *free* Mg^{2+} concentration of 10 mM[5]) in a 1:1 ratio. While incorporation of dCTP was monitored by rapid chemical quench [using 600 mM Na_2EDTA (pH 8.0) to quench], dGTP incorporation was monitored by

[5] To achieve a final $[Mg^{2+}]_{free}$ of 10 mM, $[Mg^{2+}]_{total}$ was adjusted assuming that the Mg^{2+}·dNTP complex has a K_d of 333 μM.

Table 2: DNA Substrates

substrate	sequence[a]
TSAI[b]	5′-^{32}P-GCCTCGCAGCCGTCCAACCAACTCA GCTCGATCCAATGCCGTCC 3′-CGGAGCGTCGGCAGGTTGGTTGAGT**G**CGAGCTAGGTTACGGCAGG
SALAS[b]	5′-^{32}P-GATCACAGTGAGTAC ATAGAACGACGGCCAGT 3′-CTAGTGTCACTCATG**G**TATCTTGCTGCCGGTCA

[a] In these gapped substrates the downstream oligonucleotide is 5′-phosphorylated. [b] "TSAI" indicates that this is the same substrate used in our previous analysis of Pol X (*10*); "SALAS" indicates that this is the same substrate used by Salas and co-workers in their previous analysis of Pol X (*14*).

manually quenching aliquots with formamide. Reaction products were resolved and visualized as described above, and data were fit to the single exponential equation [product] $= A[1 - \exp(-k_{obs}t)]$. The apparent rate constants (k_{obs}) thus obtained were plotted as a function of [dNTP] and fit to the hyperbolic equation $k_{obs} = k_{pol}[\text{dNTP}]/(K_{d,app} + [\text{dNTP}])$ to obtain the equilibrium dissociation constant ($K_{d,app}$) and the catalytic rate constant (k_{pol}). The error values reported for the k_{obs} and $K_{d,app}$ parameters are the asymptotic standard errors generated by the fitting software. The fidelity of nucleotide incorporation was calculated as fidelity $= [(k_{pol}/K_{d,app})_{cor} + (k_{pol}/K_{d,app})_{inc}]/(k_{pol}/K_{d,app})_{inc}$, where the subscripts "cor" and "inc" refer to correct and incorrect incorporation, respectively. Saturation curves were typically repeated at least twice; the reported data represent the best (i.e., highest R^2, coefficient of determination, value) independent trial.

Steady State Fidelity Analysis. Assays were performed at 37 °C in a buffer consisting of 50 mM Tris-borate, 3% glycerol, pH 7.5 (adjusted at 37 °C). Reactions were initiated by mixing a protein/DNA solution (consisting of either 2 or 50 nM Pol X, 24 nM SALAS single-nucleotide gapped DNA substrate, 2 mM DTT, and 200 μg/mL BSA) with a dNTP/$MgCl_2$ solution (consisting of varying dNTP concentration and sufficient $MgCl_2$ to achieve a postmixing *free* Mg^{2+} concentration of 10 mM[5]) in a 1:1 ratio; for synthesis of G:C and G:G, the premixing Pol X concentrations were 2 and 50 nM, respectively. Time points, representing less than 30% substrate consumption, were acquired by manually quenching 10 μL aliquots in 10 μL of formamide. Product resolution and data analysis were performed as described above. Saturation curves were generated by plotting initial velocity (v_0) as a function of [dNTP] and fitting the data to the hyperbolic equation $v_0 = V_{max}[\text{dNTP}]/(K_M + [\text{dNTP}])$. k_{cat} was obtained by dividing V_{max} by the active enzyme concentration. Steady state fidelity was calculated as $[(k_{cat}/K_M)_{cor} + (k_{cat}/K_M)_{inc}]/(k_{cat}/K_M)_{inc}$, where "cor" and "inc" denote correct and incorrect nucleotide incorporation, respectively.

RESULTS

Rationale behind Fidelity Analyses. Critical to the ASFV mutagenic DNA repair hypothesis described above is the high frequency at which Pol X synthesizes mismatched base pairs. The report of Salas and co-workers that Pol X has a fidelity ~740-fold higher than what we had previously published (Table 1) appeared to discredit the mutagenic DNA repair hypothesis (*14*) and prompted us to investigate this discrepancy exhaustively. Though confident in our published data, we reassayed Pol X under the same conditions that we had used previously and found the fidelity of G:G synthesis to be essentially invariant (1.6 vs 1.9; see Table 3). Though it seemed unlikely, we asked whether our data might differ from the Salas data because of differences in experimental protocol. Three differences were evident. *(i) Buffer conditions:* While we had assayed Pol X in 50 mM Tris-borate (pH 9.0), 50 mM KCl, 3% glycerol, 1 mM DTT, 100 μg/mL BSA, and 10 mM *free* Mg^{2+} (*10*), Salas et al. used 50 mM Tris (pH 7.5), 0 mM KCl, 4% glycerol, 1 mM DTT, 100 μg/mL BSA, and 10 mM *total* Mg^{2+} (*14*). pH and [KCl] seemed to be the most important differences between these two buffer/cosolvent systems and were therefore singled out for analysis. Rather than focusing solely on the buffer condition used by Salas (pH 7.5/0 mM KCl), we examined multiple combinations of pH and [KCl] in order to determine the extent to which the fidelity parameter is dependent upon these; this seemed important because polymerases are often assayed under nonphysiological conditions of pH and ionic strength (*15*−*19*) despite the fact that the condition dependence of polymerase fidelity has not hitherto been rigorously analyzed. *(ii) DNA substrate sequence:* Since DNA sequence context can influence the catalytic properties (including fidelity) of a polymerase (*20*−*25*), Pol X was reassayed using the same substrate employed by Salas. *(iii) Pre-steady state vs steady state:* Our previous analyses of Pol X were conducted in the pre-steady state (*10*), while those of Salas and co-workers were done in the steady state (*14*). Though the fidelities determined under these two conditions ought to be similar, a subset of the assays reported here were

Table 3: Kinetic Parameters for Pol X-Catalyzed Pre-Steady State Single-Nucleotide Gap-Filling Using the TSAI Substrate at Varying pH and [KCl]

pH	[KCl] (mM)	base pair[a]	k_{pol} (s^{-1})	$K_{d,app}$ (μM)	$k_{pol}/K_{d,app}$ (M^{-1} s^{-1})	fidelity[b]
7.5	0	G:C[c]				
7.5	0	G:G	0.013 ± 0.0007	130 ± 20	100	
7.5	50	G:C[c]				
7.5	50	G:G	0.0089 ± 0.0003	60 ± 5	150	
7.5	140	G:C	0.074 ± 0.005	200 ± 30	370	
7.5	140	G:G	0.0028 ± 0.0001	21 ± 1	130	3.8
9.0	50	G:C	0.23 ± 0.02	930 ± 270	250	
9.0	50	G:G	0.035 ± 0.002	87 ± 16	400	1.6
9.0	140	G:C	0.11 ± 0.01	84 ± 20	1300	
9.0	140	G:G	0.022 ± 0.001	28 ± 4	790	2.6

[a] In the base pair notation X:Y, X refers to the templating position and Y denotes the incoming nucleotide. [b] Fidelity is defined as $[(k_{pol}/K_{d,app})_{cor} + (k_{pol}/K_{d,app})_{inc}]/(k_{pol}/K_{d,app})_{inc}$, where the subscripts "cor" and "inc" refer to correct and incorrect nucleotide incorporation, respectively. [c] Under these conditions Pol X was unstable and catalytic parameters could not be obtained.

conducted in the steady state to enable direct comparison with the kinetic parameters reported by Salas et al., thereby elucidating specific differences between the enzyme preps being used by each group.

Because both our previously published data and that of the Salas group show the G:G mismatch to be the lowest fidelity base pair synthesized by Pol X, and because the discrepancy in the fidelity value being reported by the two groups is largest for G:G (Table 1), all fidelity analyses described in this work focused on this particular mismatch and its corresponding Watson−Crick base pair, G:C. By accounting for the discrepancy in G:G fidelity, we hoped to arrive at a general explanation that would reconcile the conflicting fidelities of the other mismatches as well.

Pre-Steady State Fidelity Assays Using the TSAI Substrate under Diverse Buffer Conditions. Table 3 lists kinetic parameters for Pol X-catalyzed pre-steady state single-nucleotide gap-filling using the TSAI substrate at different combinations of pH (7.5 or 9.0) and [KCl] (0, 50, or 140 mM); note that pH 9.0/0 mM KCl was the only permutation of these conditions that was not examined. When using the same buffer condition as Salas (pH 7.5/0 mM KCl) we were unable to generate usable data for G:C synthesis; this assay could not be conducted by the manual quench method because at moderate to high dCTP concentrations primer extension was nearly complete by the time the first aliquot could be quenched (6 s); however, assaying this base pair by rapid quench yielded saturation curves of very poor quality. One explanation for the latter finding is that at neutral pH and low ionic strength Pol X may be less stable, and therefore not able to withstand the pressure/turbulent flow associated with the rapid quench technique; note that we have observed a similar phenomenon with ASFV APE (*9*). Similar difficulties were encountered when assaying the G:C base pair at pH 7.5/50 mM KCl. We were, however, able to obtain data under all other conditions examined. Important findings are summarized below.

(i) The Fidelity of Pol X Is Largely Independent of Assay Conditions. The data in Table 3 indicate that the more closely the assay buffer resembles physiological conditions (pH 7.5/140 mM KCl), the higher the fidelity of Pol X. However, the magnitude of the fidelity changes is quite small: at pH 9.0, increasing [KCl] from 50 to 140 mM enhances fidelity just 1.6-fold (1.6 vs 2.6); at 140 mM KCl, decreasing pH from 9.0 to 7.5 enhances fidelity just 1.5-fold (2.6 vs 3.8). To our knowledge, this represents the first quantitative analysis of polymerase fidelity as a function of buffer ionic strength (i.e., monovalent salt concentration), so it is unclear whether the behavior of Pol X is typical in this regard. Fidelity as a function of pH has been examined previously, but since these studies employed base substitution reversion assays (involving the *lacZ*α gene) (*26*−*28*), their results cannot be directly compared to the data in Table 3.[6] The one exception to this, that we are aware of, is Eckert and Kunkel's steady state analysis of mismatch synthesis by *Escherichia coli* Pol I *exo*$^-$ (*28*), where it was found that decreasing pH from 7.6 to 6.2 enhanced the fidelity of T:G synthesis 23-fold, reduced the fidelity of G:A synthesis 1.4-fold, and had negligible influence on the fidelity of A:C synthesis.

Though not expansive enough to provide an authoritative guideline for the condition dependence of polymerase fidelity, collectively the above data suggest that while a given change in assay conditions can alter the catalytic efficiencies of match and mismatch synthesis in opposite directions, the magnitude of these changes is typically small (less than 10-fold), and consequently the fidelity parameter displays only weak dependence on assay conditions. However, as in the above case of T:G synthesis by *E. coli* Pol I *exo*$^-$ (*28*), the fidelity of some mismatches may be considerably more condition sensitive than others; the identity of these condition sensitive mismatches is expected to vary from one polymerase to the next.

Though when using the TSAI substrate we were unable to determine the fidelity of Pol X under buffer conditions identical to those used by Salas, the fact that the fidelity of G:G synthesis varies less than 3-fold over a wide range of pH and [KCl] strongly suggests that differences in buffer conditions alone cannot reconcile our data with that of the Salas group.

(ii) Pol X Is Extremely Error-Prone. Under the physiological condition of pH 7.5/140 mM KCl Pol X synthesizes the G:G mismatch with a fidelity of 3.8 (i.e., G:G is synthesized ∼35% as efficiently as the Watson−Crick G:C base pair) (Figure 1 and Table 3). For comparison, when using the exact same DNA substrate, eukaryotic Pol β (Pol X's closest homologue) synthesizes G:G with a fidelity of 59 000, and it synthesizes T:C (its preferred mismatch) with a fidelity of 1700 (*29*). When compared with other low-fidelity polymerases (Table 4), it can be concluded that Pol X is indeed one of the most error-prone polymerases known.

(iii) Influence of Buffer Conditions on k_{pol} and $K_{d,app}$. It is interesting to note that, at a fixed pH, as the [KCl] is increased both the G:C and G:G base pairs display—without exception—a decrease in k_{pol} and $K_{d,app}$ (Table 3). The enhancement in nucleotide affinity with increasing ionic strength is fairly dramatic: at pH 7.5 the $K_{d,app}$ for G:G improves from 130 μM (at 0 mM KCl) to 60 μM (at 50 mM KCl) to 21 μM (at 140 mM KCl); at pH 9.0, upon increasing [KCl] from 50 to 140 mM the $K_{d,app}$ for G:G improves from 87 to 28 μM while the $K_{d,app}$ for G:C improves from 930 to 84 μM. While increasing ionic strength is expected to decrease the component of substrate affinity resulting from charge−charge interactions, it has the capacity to enhance substrate affinity resulting from dispersion forces. Significant enhancement of the latter, however, seems unlikely considering the relatively low salt concentrations used here. An alternative explanation for this phenomenon is not immediately apparent.

Pre-Steady State Fidelity Assays Using the SALAS Substrate under Different Buffer Conditions. Since, when using the TSAI DNA substrate, we were unable to reproduce the higher fidelity observed by Salas and co-workers, assays were repeated using the SALAS substrate (see Table 2 for substrate details). Importantly, since Pol X uses this substrate less efficiently than the TSAI substrate, we were able to acquire data for G:C synthesis at pH 7.5/0 mM KCl (the same condition used by Salas) by the manual quench method (whereas this had not been possible with the TSAI substrate).

[6] Since the fidelity monitored by these reversion assays is a complex function of mismatch synthesis, mismatch extension, and other fidelity-influencing phenomena such as polymerase processivity (*28*), the data cannot be directly compared with the data in Table 3.

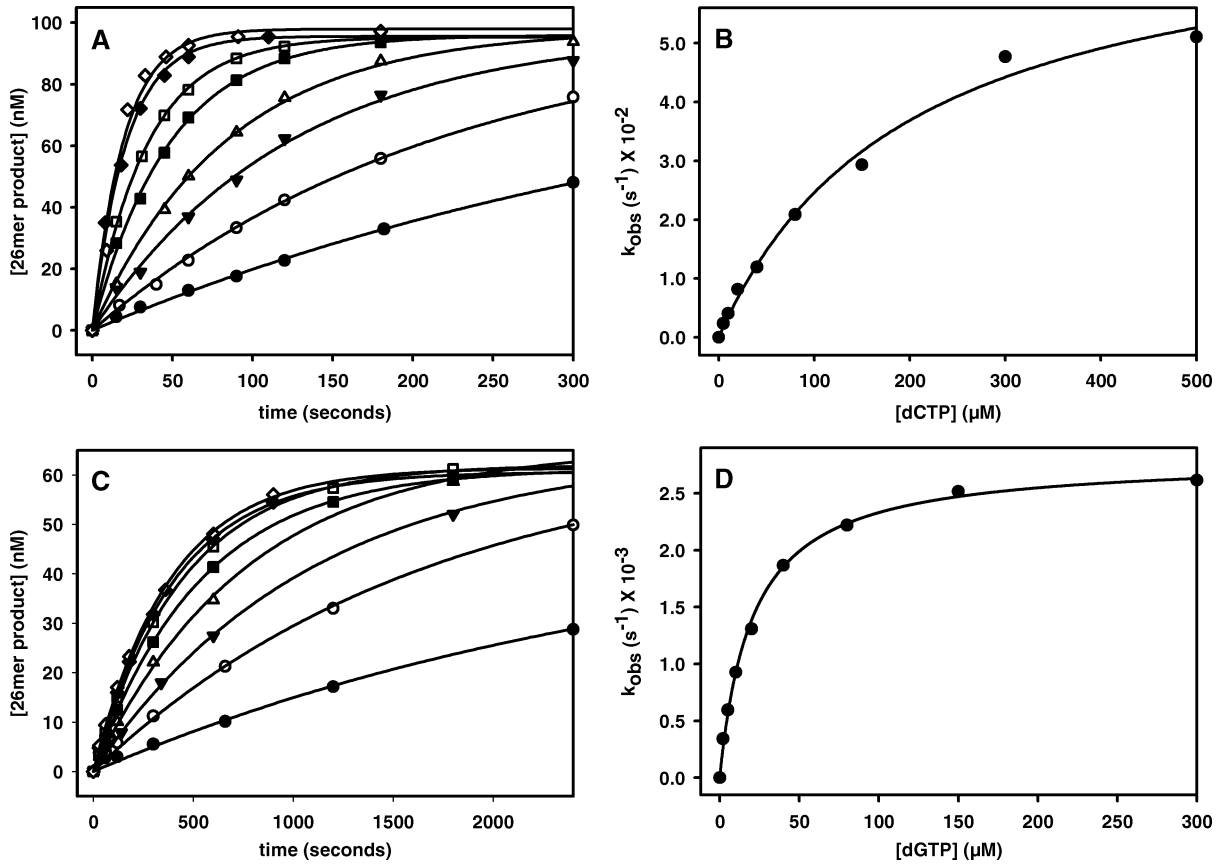

FIGURE 1: Pre-steady state analyses of Pol X-catalyzed single-nucleotide gap-filling using the TSAI substrate at pH 7.5 and 140 mM KCl. (A) Time courses for incorporation of C opposite G at varying nucleotide concentration. dCTP concentrations are 5 μM (●), 10 μM (○), 20 μM (▼), 40 μM (△), 80 μM (■), 150 μM (□), 300 μM (◆), and 500 μM (◇). Data are fit to a single exponential. (B) Observed rate constants from (A) as a function of dCTP concentration. Data points (●) are fit to a hyperbola. (C) Time courses for incorporation of G opposite G at varying nucleotide concentration. dGTP concentrations are 2 μM (●), 5 μM (○), 10 μM (▼), 20 μM (△), 40 μM (■), 80 μM (□), 150 μM (◆), and 300 μM (◇). Data are fit to a single exponential. (D) Observed rate constants from (C) as a function of dGTP concentration. Data points (●) are fit to a hyperbola. Kinetic parameters are reported in Table 3 (along with those obtained under different assay conditions).

Table 4: Comparison of the Fidelity of Error-Prone DNA Polymerases When Copying Undamaged DNA

enzyme	substrate type	range of f_{inc}[a]	average f_{inc}[b]	reference
human Pol ι	template-primer	6.7×10^{-1} to 1.0×10^{-4}	3.5×10^{-1}	*47*
ASFV Pol X	single-nucleotide gap	3.5×10^{-1} to 1.3×10^{-4}	4.8×10^{-2}	*10*, this study[c]
human Pol κ	template-primer	5.8×10^{-2} to 5.2×10^{-4}	1.3×10^{-2}	*19*
human Pol η	template-primer	1.1×10^{-2} to 1.1×10^{-3}	5.6×10^{-3}	*48*
E. coli Pol V	template-primer	4.8×10^{-3} to $<1.0 \times 10^{-5}$	1.6×10^{-3}	*49*
S. solfataricus[d] Dpo4	template-primer	3.2×10^{-3} to 1.5×10^{-4}	6.8×10^{-4}	*50*
E. coli Pol IV	template-primer	1.7×10^{-3} to 3.6×10^{-5}	3.7×10^{-4}	*49*

[a] For data obtained in the steady state (Pols ι, κ, η, V, and IV), the misincorporation ratio (f_{inc}) is defined as $(V_{max}/K_M)_{incorrect}/(V_{max}/K_M)_{correct}$. For data obtained in the pre-steady state (Pol X and Dpo4), f_{inc} is defined as $(k_{pol}/K_{d,app})_{incorrect}/(k_{pol}/K_{d,app})_{correct}$. [b] Enzymes are ordered by error-proneness as determined by the *average* misincorporation ratio (for all 12 mismatches). [c] The f_{inc} values for Pol X are derived both from ref *10* (all mismatched base pairs other than G:G) and from this study (the G:G base pair; using the kinetic parameters obtained with the TSAI substrate at pH 7.5 and 140 mM KCl). [d] *Sulfolobus solfataricus*.

There are two significant findings. First, the ionic strength dependence of k_{pol} and $K_{d,app}$ described above for the TSAI substrate is recapitulated here with the SALAS substrate: at the fixed pH of 7.5, increasing [KCl] from 0 to 140 mM reduces k_{pol} and $K_{d,app}$ for both the G:C and G:G base pairs (Table 5). Second, when using the same substrate and buffer condition employed by Salas, we find Pol X to have a fidelity of 12 (vs 1399 reported by Salas). Upon adjusting the buffer condition to pH 7.5/140 mM KCl the fidelity increases to 20 (Table 5), which is a ~5-fold enhancement relative to the TSAI substrate under this same buffer condition.

Fidelity has been shown to vary with DNA sequence context for other polymerases (*20*, *21*, *23*−*25*), but the magnitude of change is usually modest (less than 15-fold); in the most extreme case that we are aware of, WT eukaryotic Pol β was shown to synthesize the A:G mismatch with fidelities of 140 000 and 5000 (a 28-fold difference) in two different sequence contexts (*21*). Clearly Pol X does not deviate from this trend, necessitating an alternative explanation for the higher fidelities reported by Salas and co-workers.

Steady State Fidelity Assays Using the SALAS Substrate at pH 7.5 and 0 mM KCl. By assaying Pol X in the steady

Table 5: Kinetic Parameters for Pol X-Catalyzed Pre-Steady State Single-Nucleotide Gap-Filling Using the SALAS Substrate at pH 7.5 and Varying [KCl]

pH	[KCl] (mM)	base pair[a]	k_{pol} (s^{-1})	$K_{d,app}$ (μM)	$k_{pol}/K_{d,app}$ (M^{-1} s^{-1})	fidelity[b]
7.5	0	G:C	0.11 ± 0.007	1020 ± 230	110	
7.5	0	G:G	0.0096 ± 0.0004	990 ± 150	9.7	12
7.5	140	G:C	0.054 ± 0.002	320 ± 53	170	
7.5	140	G:G	0.00073 ± 0.00002	81 ± 8.3	9.0	20

[a] In the base pair notation X:Y, X refers to the templating position and Y denotes the incoming nucleotide. [b] Fidelity is defined as $[(k_{pol}/K_{d,app})_{cor} + (k_{pol}/K_{d,app})_{inc}]/(k_{pol}/K_{d,app})_{inc}$, where the subscripts "cor" and "inc" refer to correct and incorrect nucleotide incorporation, respectively.

Table 6: Kinetic Parameters for Pol X-Catalyzed Steady State Single-Nucleotide Gap-Filling Using the SALAS Substrate at pH 7.5 and 0 mM KCl

base pair[a]	k_{cat} (min^{-1})	K_M (μM)	k_{cat}/K_M (M^{-1} min^{-1})	fidelity[b]	reference
G:C	0.0301 ± 0.0013	86 ± 16.9	356.5		*14*
G:G	0.0001964 ± 0.0000092	777 ± 50	0.2550	1399	*14*
G:C	0.34 ± 0.007	87 ± 7.1	3900		this study
G:G	0.13 ± 0.007	620 ± 87	210	20	this study

[a] In the base pair notation X:Y, X refers to the templating position and Y denotes the incoming nucleotide. [b] Fidelity is defined as $[(k_{cat}/K_M)_{cor} + (k_{cat}/K_M)_{inc}]/(k_{cat}/K_M)_{inc}$, where the subscripts "cor" and "inc" refer to correct and incorrect nucleotide incorporation, respectively.

state, we obtained kinetic parameters that could be directly compared with those reported by Salas et al. Table 6 shows that when using the same substrate, same buffer condition, and same assay method as the Salas group, we still observe a fidelity that is 70-fold lower than what they have reported (20 vs 1399). Our consistent observation of low fidelity values under an array of assay/substrate conditions, and our inability to reproduce the higher fidelity value of Salas, strongly suggest that Pol X is in fact very error-prone when incorporating opposite to undamaged templates within the context of a single-nucleotide gap.

DISCUSSION

Condition Dependence of Polymerase Fidelity. Collectively, our data and that in the literature (*28*) suggest that the *relative* efficiencies at which different base pairs are synthesized by a given polymerase are likely to display little variability over a range of assay conditions. It is worthwhile to note, however, that for some polymerases one or more base pairs may be especially sensitive to assay conditions. As an example, consider human Pol ι, which has recently been suggested to protonate N3 of incoming dCTP to facilitate Hoogsteen pairing with templating G (*30*). This protonation event is not requisite for incoming dTTP. Thus, all else being equal, for Pol ι the ratio $[(k_{pol}/K_{d,app})_{G:C} + (k_{pol}/K_{d,app})_{G:T}]/(k_{pol}/K_{d,app})_{G:T}$ should show considerable pH dependence. While this caveat appears pedantic, it seems justified considering the fact that in vitro base pair specificities/fidelities are relied upon heavily to predict the in vivo functions of a polymerase (*31*−*33*) as well as to correlate in vivo mutation profiles with a particular enzyme (*34*, *35*). In a situation where in vitro and in vivo fidelities/base pair specificities appear to conflict with one another, in vitro assay conditions ought to be considered as a potential source of artifacts.

Fidelity of Pol X. Though the Pol X fidelities reported by Salas and co-workers are higher than what we originally published (*10*, *14*), they are still quite low relative to most polymerases; the average misincorporation ratio (f_{inc})[7] for the six base pairs analyzed by Salas is 1.9×10^{-4}, placing the fidelity of Pol X at an intermediate position among the representative error-prone enzymes listed in Table 4. Importantly, however, this average f_{inc} of 1.9×10^{-4} (1 error per ~5300 incorporations) is dramatically lower than the average of 1.1×10^{-1} (1 error per 9 incorporations) that our previously published data for all 12 possible mismatches suggested (*10*). The biological implication of these different error frequencies is especially important considering the fact that Pol X is a repair enzyme (*14*, *36*), presumably only responsible for synthesizing a small number of the ~180 000 base pairs in each ASFV genome. Thus, if the Salas data is accurate, Pol X is expected to contribute very little to ASFV genomic diversity; if our previously published data is accurate, then Pol X *at the very least* has the capacity to contribute greatly to ASFV genomic diversity.

Herein, focusing on the G:C and G:G base pairs, we have reproduced our previously published data, have demonstrated very low Pol X fidelity values over a range of assay conditions and DNA sequence contexts, and have not been able to reproduce the higher fidelity values of Salas and co-workers. On the basis of these findings for G:C and G:G, we conclude that (i) our previously reported data for the 14 other base pairs (*10*) is also likely to be accurate/reproducible, and (ii) Pol X is one of the most error-prone polymerases known. Though further in vitro and in vivo analyses may demonstrate mutagenic DNA repair to be inconsequential in ASFV, on the basis of the results presented here we contend that such a conclusion will not derive from a lack of mutagenic potential on the part of Pol X (i.e., though as of yet uncharacterized or unquantified fidelity enhancement mechanisms—such as mismatched nick editing by ASFV APE (*9*)—may render ASFV DNA repair a high fidelity process, it is clear that Pol X is itself of sufficiently low fidelity to drive viral mutagenesis).

Conflicting Pol X Fidelity Data. How can the higher fidelities reported by the Salas group be accounted for? Both our group and theirs are using the Pol X gene from the BA71V isolate of the virus, so amino acid substitutions[8] cannot account for the different catalytic efficiencies and fidelities. Comparison of the catalytic efficiencies listed in Table 6 indicates that, for G:C and G:G synthesis, our enzyme is 11-fold and 824-fold more efficient, respectively. This disproportionate enhancement in the efficiency of mismatch (G:G) synthesis is clearly the source of the discrepancy in the fidelity value being reported by the two groups, but how can it be explained? Note that the difference

[7] For data obtained in the steady state, the misincorporation ratio (f_{inc}) is defined as $(k_{cat}/K_M)_{incorrect}/(k_{cat}/K_M)_{correct}$. For data obtained in the pre-steady state, f_{inc} is defined as $(k_{pol}/K_{d,app})_{incorrect}/(k_{pol}/K_{d,app})_{correct}$.

[8] Comparison of the Pol X gene from eight different ASFV isolates (NCBI accession numbers NC_001659, AY261360, AY261361, AY261362, AY261363, AY261364, AY261365, and AY261366) reveals that mutations are present at 13 different positions of the 174 amino acid polypeptide.

in catalytic efficiency between the two enzyme preps is due almost entirely to differences in the k_{cat} parameter: though K_M shows little variation (86 vs 87 μM for G:C, and 777 vs 620 μM for G:G), relative to the Salas k_{cat} our k_{cat} is enhanced 11-fold for G:C synthesis and 660-fold for G:G synthesis. This raises two important and related questions: (i) How can we account for the enhanced k_{cat} of our enzyme? (ii) Why is the ratio $(k_{cat})_{tsai\ data}/(k_{cat})_{salas\ data}$ so much larger for G:G than it is for G:C?

Since k_{cat} is obtained by dividing V_{max} by the enzyme concentration, a potential explanation for the lower k_{cat} values reported by the Salas group is that they overestimated their enzyme concentrations. This might have occurred by (i) the UV-determined protein concentration exceeding the actual concentration of active Pol X, and/or (ii) excessive "dilution death."[9] Though a combination of the above mechanisms could *possibly* account for the fact that $(k_{cat})_{tsai\ data}/(k_{cat})_{salas\ data} = 11$ for the G:C base pair, in our experience the combined effect of both mechanisms is not large enough in magnitude to give rise to the 660-fold difference in $(k_{cat})_{tsai\ data}/(k_{cat})_{salas\ data}$ that is observed for the G:G base pair. Moreover, an overestimation of active enzyme concentration (regardless of the mechanism responsible) cannot explain why the ratio $(k_{cat})_{tsai\ data}/(k_{cat})_{salas\ data}$ is so different for the G:G and G:C base pairs, since the k_{cat} parameter should be affected similarly for all base pairs. We conclude that a different explanation for the discrepancies in k_{cat} and fidelity between our data and that of the Salas group is needed; however, alternative rationales for these disparities are not immediately apparent.

The Big Picture. Important features of our ASFV mutagenic DNA repair hypothesis are efficiently illustrated by comparison with recent data for another important pathogen, *Mycobacterium tuberculosis*. *DNA damage is critical:* Merely overexpressing the *M. tuberculosis* error-prone DNA polymerase, DnaE2, in the absence of DNA damage is insufficient for increasing the rate of spontaneous mutagenesis; though the precise details await elucidation, this implies that it is only through DNA damage that this mutator polymerase gains access to the pathogen's genome (*37*). Since our initial characterization of Pol X in 2001 we have analogously suggested that damage to the ASFV genome serves as the gateway for Pol X-induced mutagenesis (*9*−*12*). This is an important point because it has been suggested, on the basis of sequencing a dispensable genomic segment from 17 clones of the Vero cell-adapted ASFV isolate BA71V, that the ASFV genome shows little diversity as a function of time (*38*). Since this finding contradicts what has been observed in numerous other studies of ASFV isolates passaged in pigs or in porcine macrophages (*39*, *40*), and since it has been shown that the ASFV APE is essential for viral viability in macrophages but not in Vero cells (*41*), we suggest that damage to the ASFV genome is more extensive in macrophage cells than it is in Vero cells, and that this in turn affords the ASFV DNA "repair" system greater opportunity to introduce point mutations. Importantly, this is consistent with the fact that ASFV field isolates passaged in porcine macrophages display greater antigenic diversification than does the Vero cell-adapted BA71V isolate after passage in Vero cells (*40*). *Controlling the extent of mutagenesis:* In many organisms the capacity of error-prone polymerases for rampant mutagenesis is mitigated by control of both *when*, such as during the SOS responses of *M. tuberculosis* and *E. coli* (*37*, *42*−*45*), and *where*, such as within immunoglobulin loci (*46*), these enzymes are allowed access to DNA. The potent mutagenic potential of Pol X is presumably tolerated because, being a repair polymerase (*10*, *14*, *36*), it only gains access to the viral genome via a relatively small number of single-nucleotide gaps. This scenario is advantageous because, without the need for regulatory mechanisms, the capacity for generating sequence diversity is directly dependent on the extent of environmental challenge (i.e., DNA damage). *Type of DNA substrates:* A critical difference between the hypothesized ASFV mutagenic DNA repair pathway and the mutagenesis induced by other error-prone polymerases (including *M. tuberculosis* DnaE2) is that the latter are thought to employ, if not exclusively then at least predominantly, lesion-containing templates during mutagenesis. In contrast, Pol X is envisioned to predominantly employ single-nucleotide gaps containing *undamaged* templating nucleotides.

Characterization of a large number of DNA polymerases from all three kingdoms of life has demonstrated a very strong correlation between error-proneness when copying undamaged templates and the ability to efficiently copy damaged templates. This begs the question of whether the error-proneness of Pol X is a consequence of it having been selected for the ability to catalyze translesion synthesis. Note, however, that regardless of what specialized activities Pol X might conceivably possess, or what sort of selective pressures have guided its evolution, the fact remains that at this point in time Pol X is error-prone when copying undamaged templates. Whether this property actually contributes to ASFV genomic diversity and viral fitness remains to be seen.

[9] We have found that under some buffer conditions the serial dilution of Pol X (which is required for setting up most assays) inactivates a small percentage of the enzyme (during *each* dilution step), and the extent of activity loss increases if DTT and BSA are not included as stabilizing agents in the dilution buffer.

ACKNOWLEDGMENT

S.K. thanks M. Su for help with purification of Pol X and for helpful discussions.

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BI0613325