Loss of DNA Minor Groove Interactions by Exonuclease-Deficient Klenow Polymerase Inhibits O^6-Methylguanine and Abasic Site Translesion Synthesis†

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ABSTRACT: The importance of DNA polymerase−DNA minor groove interactions on translesion synthesis (TLS) was examined in vitro using variants of exonuclease-deficient Klenow polymerase and site-specifically modified DNA oligonucleotides. Polymerase variant R668A lacks primer strand interactions, while variant Q849A lacks template strand interactions. O^6-Methylguanine (m6G) and abasic site TLS was examined in three stages: dNTP insertion opposite the lesion, extension from a terminal lesion-containing base pair, and the dissociation equilibrium of the polymerase from the lesion-containing template. Less than 5% TLS was observed at the insertion step for either variant on the lesion-containing templates. While extensive TLS was observed for WT polymerase on the m6G template, only incorporation opposite the lesion was observed for the R668A variant. Loss of the template strand interaction, Q849A, resulted in the inability to insert dNTPs opposite either the m6G or abasic lesion. For both variants, extension of purine-containing m6G primer-templates was increased relative to WT polymerase. We observed similar extension efficiencies for all variants, relative to WT, using abasic template-primers. Polymerase dissociation/reassociation was studied through the use of a competitor primer/template complex. Dissociation for WT polymerase increased 2-fold and 3-fold, respectively, for m6G and abasic lesion-containing templates, relative to the natural template. Variants lacking DNA minor groove interactions displayed increased dissociation from DNA templates, relative to WT polymerase, but do not display an increased level of lesion-induced polymerase dissociation. Our results indicate that the primer and template strand interactions of the Klenow polymerase with the DNA minor groove are critical for maintaining the DNA−polymerase complex during translesion synthesis.

The effect of lesions on DNA synthesis by purified polymerases can be either inhibitory, leading to chain termination, or noninhibitory, leading to translesion synthesis (TLS).[1] The degree of inhibition versus TLS observed in vitro depends on the type of lesion, the DNA sequence context, and the DNA polymerase. Although much information has been catalogued over the past decade regarding the TLS efficiencies of various lesions and polymerases, the salient structural features of the DNA polymerase−DNA substrate interaction that are required to accomplish TLS have not been described. The inability of a polymerase to stably incorporate a base opposite the lesion may reflect the polymerase's inability to position a lesion-containing base pair in a geometric conformation that allows active site assembly and catalysis. Inhibition of synthesis may also be due to the inability of the polymerase to extend the 3′ lesion-containing terminus or due to dissociation of the polymerase from the lesion containing DNA substrate.

Crystal structures of several DNA polymerases have greatly enhanced our understanding of the mechanism of DNA synthesis and polymerase error discrimination (*1*, *2*). Importantly for TLS, the active site is comprised of both protein and nucleic acid elements (*3*−*5*). In A, B, and X family polymerases, the penultimate base pair creates one wall of the dNTP-binding pocket, while a conformational change of the protein moves α-helices to create the other walls. Thus, the binding pocket is believed to provide discrimination for Watson−Crick base pairs by steric complementarity during base incorporation, as premutational intermediates disrupt the geometry of the polymerase active site (*6*, *7*). Accurate nucleotide selection by a DNA polymerase in the absence of hydrogen-bonding potential demonstrates that the DNA polymerase can exhibit base selectivity exclusively on a steric basis (*8*). Specific minor groove recognition (MGR) of the DNA by polymerases is also observed in the physical structures. The polymerase protein hydrogen bonds directly with the O^2-pyrimidine and N3-purine positions of the penultimate base pair. Since the formation of base mispairs alters the pattern of H-bonding positions in the minor groove, this structural interaction is also relevant to error discrimination (*7*).

To elucidate the importance of DNA minor groove interactions during polymerase TLS, we have examined amino acid variants of the Klenow fragment (KF) of *Escherichia coli* DNA polymerase I involved in MGR. High-resolution crystal structures of the binary complex of KF

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[1] Abbreviations: KF, Klenow fragment of *Escherichia coli* DNA polymerase I; m6G, O^6-methylguanine; MGR, minor groove recognition; P/T, primer-template; TLS, translesion synthesis.

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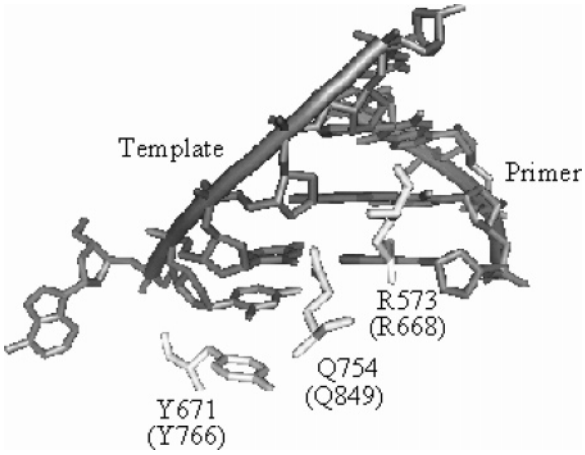

FIGURE 1: Klenow fragment of DNA polymerase I from *T. aquaticus* (PDB ID 1QSS) in the closed conformation. The highlighted residues include R573, Y671, and Q754, corresponding to residues R668, Y766, and Q849 of the *E. coli* Klenow fragment shown in parentheses. The diagram was generated using the PYMOL program.

bound to primer/template (P/T) DNA in the open conformation are available (*9*). Physical structures are also available for the closed ternary complex of the large fragment of DNA polymerase I from *Thermus aquaticus* (*10*, *11*) and *Bacillus stearothermophilus* (BF) (*7*, *12*). These characteristics along with the abundance of information available, due to previous genetic and biochemical studies, make the KF of *E. coli* DNA polymerase I an ideal model for structure−function studies of a DNA polymerase and the interactions involved with TLS (*1*, *13*−*16*). Amino acid residue Arg668 is involved in direct hydrogen bonding with the terminal base of the DNA primer, while Gln849 is involved in hydrogen bonding to the corresponding base of the DNA template (Figure 1) (*17*, *18*). Previous biochemical studies have demonstrated that loss of R668 interactions severely reduces KF catalytic rate and weakens both dNTP and DNA substrate binding (*19*, *20*). The R668A variant polymerase exhibits altered fidelity: specifically, an increased rate of base substitution errors and an increased efficiency of mispair extension (*21*). The mutator phenotype has been postulated to result from loss of polymerase interactions with both the incoming dNTP and the primer terminus which in turn alters the geometry of the polymerase active site and steric discrimination. The Q849A variant polymerase exhibits a decreased catalytic rate, relative to wild-type KF, and weakened DNA substrate binding (*19*, *20*). In contrast to R668A, however, the Q849A variant displays an increased fidelity: specifically, a decreased efficiency of mispair extension (*21*). Residue Tyr766 is involved in the stacking interactions with bases of the template strand, and loss of this interaction increases base miscoding and strand misalignment errors (*22*, *23*). The Y766A variant was used as a control in this study, as it represents an amino acid variant important for error discrimination but not involved in MGR.

We chose to examine two lesions with differing degrees of polymerase inhibition during TLS. Abasic sites are generally very inhibitory, resulting in little detectable TLS products (*24*−*27*). A preference to insert dAMP opposite apurinic sites has been observed for many prokaryotic, eukaryotic, and viral polymerases (*28*, *29*). Alkylation lesions vary in the extent to which they inhibit synthesis. Several DNA alkyl adducts severely inhibit synthesis, including 1-alkylpurines, 3-alkylpyrimidines, and O^2-alkylthymine (*30*). Other adducts, particularly O^6-alkylguanine, generally result in less inhibition and concomitantly higher levels of TLS (*30*, *31*). However, the absolute TLS efficiency for O^6-methylguanine (m6G) has been shown to vary with template sequence (*32*, *33*). In this study, TLS biochemistry was examined at the steps of insertion opposite a lesion, extension from a terminal, lesion-containing base pair, and a novel assay to determine the dissociation equilibrium of KF variants from a lesion-containing complex.

EXPERIMENTAL PROCEDURES

DNA Polymerases. The recombinant Klenow polymerase variants, D424A, R668A, Y766A, and Q849A, were a gift from Dr. Catherine Joyce (Yale University). All of the DNA polymerases contain the D424A mutation, making the polymerases 3′ to 5′ exonuclease deficient. Polymerases were purified to homogeneity as previously described (*34*). Biochemical properties of the variants have been previously reported (*19*, *20*).

DNA Template-Primer Preparation. The sequences and description of all of the oligonucleotides used in this study are given in Table 1. Unmodified DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Abasic (tetrahydrofuran) and m6G template oligonucleotides were purchased from Midland Certified Reagent Co., and the presence of modifications was confirmed by mass spectroscopy by the manufacturer. The oligonucleotides serving as primers were 5′ end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Unincorporated nucleotides were removed by G-25 Sephadex columns (Boehringer Mannheim) prior to use in the polymerase reactions. The templates and primers were hybridized at equal molar ratios by heating to 90°C and cooling slowly to room temperature.

Nucleotide Insertion Experiments. The G primer (13mer) was hybridized to each of the 33mer templates, yielding three P/T substrates. The P/T concentration in the polymerase reactions was 6.67 nM. The DNA:polymerase ratios were determined empirically to ensure that the enzyme was in limiting concentrations to give 10−40% total synthesis: 100:1 for D424A, 20:1 for R668A, 25:1 for Y766A, and 25:1 for Q849A. The reaction conditions were 20 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM DTT, and 10−1000 μM dNTPs. After addition of the polymerase, the reactions were incubated at 37 °C for the indicated times and quenched by the addition of an equal volume of formamide stop dye. Each reaction for the single nucleotide insertion experiments contained 50 μM dATP, corresponding to the running start substrate, and 200 μM dNTP as noted. The remaining reaction conditions were identical to those stated above.

Primer Extension Experiments. The 14mer primers (ExA, ExC, ExG, ExT) were hybridized to the 33mer templates (G, m6G, abasic) to yield 12 different P/T substrates. The reaction conditions were the same as those of the insertion experiments. The DNA:polymerase ratios used were 100:1 for D424A, 3:1 for R668A, 50:1 for Y766A, and 10:1 for Q849A.

Polymerase Dissociation Experiments. The reaction conditions and DNA:polymerase ratios were the same as those

Table 1: Description of Primer and Template Oligonucleotides

name	description	length	sequence (5′−3′)
	templates		
G	normal template	33	CTACTGCGGGTTTAGATC**G**TCGGTCCGCACGGC
m6G	template containing O^6-methylguanine at position 15	33	CTACTGCGGGTTTAGATC**m6G**TCGGTCCGCACGGC
abasic	template containing an abasic site at position 15	33	CTACTGCGGGTTTAGATC**X**TCGGTCCGCACGGC
long T	long (trap) template for dissociation experiments	60	CTACTGCGGGTTTACATCGTCGGTCCGCACGGCATGGGG-AAAACCACCACCACGCAACTC
	primers		
short	primer for insertion experiments	13	GCCGTGCGGACCG
ExA	extension primer for G-A mispair	15	GCCGTGCGGACCGAA
ExC	extension primer for G-C pair	15	GCCGTGCGGACCGAC
ExG	extension primer for G-G mispair	15	GCCGTGCGGACCGAG
ExT	extension primer for G-T mispair	15	GCCGTGCGGACCGAT
long P	long (trap) primer for dissociation experiments	40	GAGTTGCGTGGTGGTGGTTTTCCCCATGCCGTGCGGACCG

described above for the insertion experiments. In the experiment to determine polymerase preference for either short or long P/T complexes, the total P/T concentration remained constant, 73.3 nM, with 13.3 nM being ^{32}P 5′ end labeled. The ratios of short:long complexes tested were 10:1, 5:1, 2:1, 1:1, 1:2, 1:5, 1:10, 1:20, 1:50, and 1:100. When the polymerase variants were tested for dissociation from the short templates, the polymerase was prebound to the short complexes (6.67 nM). Reactions were initiated by addition of dNTPs together with the long complex in 10-fold molar excess (66.7 nM with 10% end labeled). The reactions were incubated at 37 °C for the indicated times and quenched by the addition of an equal volume of stop dye.

Analysis of Polymerase Reactions. The reaction products were separated by 12% or 16% denaturing polyacrylamide gel electrophoresis (PAGE). The completed gels were exposed to a phosphor screen (Molecular Dynamics), and the screen was then scanned by a Storm860 (Amersham Pharmacia). The resulting bands were quantitated using ImageQuant 5.1 software, correcting for gel and primer-specific backgrounds. The total synthesis was calculated as the amount of synthesis of the first incorporation event (14th base or position −1) and beyond divided by the total radioactivity in the lane. The amount of TLS was defined as the amount of incorporation across from the second incorporation event (15th base or position 0) and beyond divided by the total in the lane. The percent of TLS is the amount of TLS divided by total synthesis.

RESULTS

Nucleotide Insertion Experiments. The ability of the Klenow variants to insert dNTPs across from a lesion was examined under enzyme limiting conditions. The DNA substrate was composed of a 13 base primer hybridized to a lesion-containing or control template of 33 bases in length (Table 1). The lesion, if present, was the 15th base (marked Lesion), so that the polymerase has a "running start" before encountering the lesion. Translesion synthesis was defined as stable incorporation of a nucleotide(s) opposite the lesion. Representative gels of the insertion assay are shown in Figure 2. Synthesis by the three Klenow polymerase variants and D424A (referred to as "wild type" from this point onward) was examined using three templates differing at the 15th position: a natural guanine (G), an O^6-methylguanine (m6G) lesion, and an abasic residue. The DNA:polymerase ratio of each variant was determined empirically to yield similar amounts of total synthesis on the G template (Figure 3A).

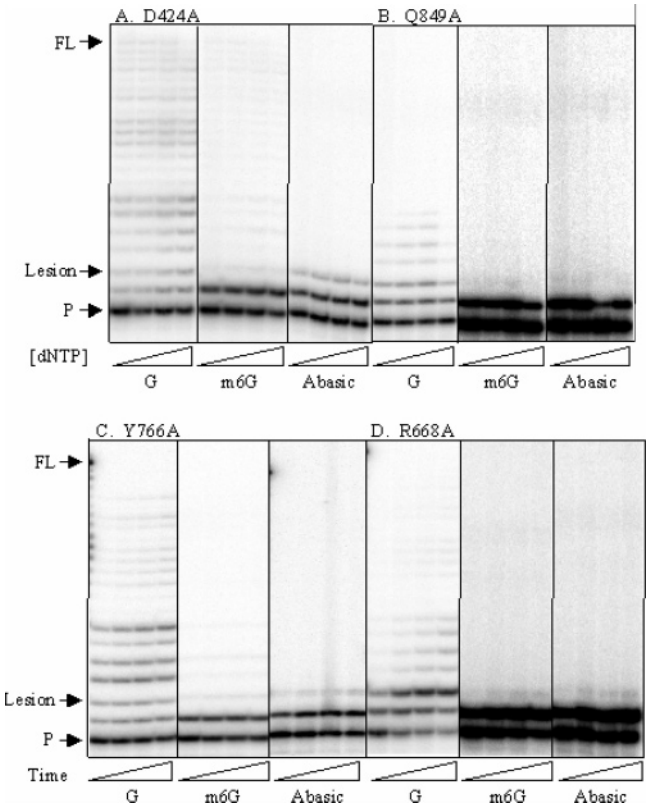

FIGURE 2: Representative gels of nucleotide insertion opposite DNA lesions by KF variants. DNA substrates were created by hybridization of the short primer to the G, m6G, or abasic template. (A, B) D424A and Q849A synthesis with increasing dNTP concentration, 10, 50, 200, 1000 μM, with a reaction time of 180 s. The DNA:polymerase ratios were 100:1 and 25:1, respectively. (C, D) Y766A and R668A synthesis with increasing time, 30, 60, 90, 180 s, with a dNTP concentration of 50 μM. The DNA:polymerase ratios were 25:1 and 20:1, respectively. The reaction products were separated by 16% PAGE. P = primer band, Lesion = G, m6G, or abasic residues, and FL = full-length product.

For the D424A and Q849A variants, TLS products are shown with increasing dNTP concentrations (Figure 2A,B), while for the R668A and Y766A variants, TLS products are shown as a function of reaction time (Figure 2C,D). Varying time for wild type (WT) and Q849A or dNTP concentration for R668A and Y766A resulted in the same gel patterns in relation to TLS. The Klenow polymerase variant Y766A acted similarly to wild type on the m6G template, in that the percentage of TLS decreased from 73% on the normal template to 10% on the m6G-containing template (Figure 3B). On the abasic template, Y766A displayed lower TLS

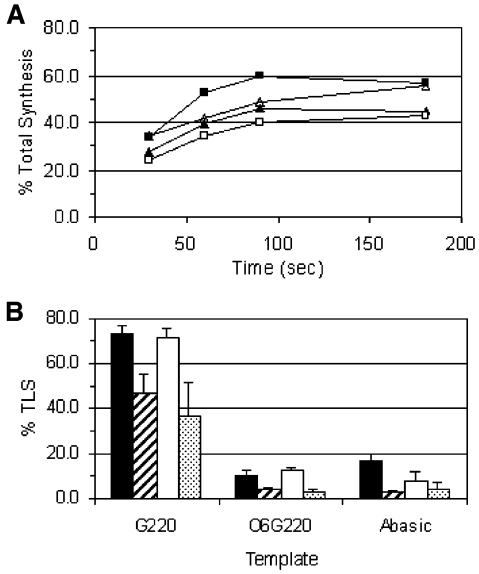

FIGURE 3: Quantitative analysis of insertion experiments. (A) Percentage of total synthesis on the control (G) template. Comparison of percent total synthesis of Klenow variants at 50 μM dNTPs with DNA:polymerase ratios of 100:1 for D424A, 20:1 for R668A, 25:1 for Y766A, and 50:1 for Q849A. Key: (■) D424A; (▲) R668A; (□) Y766A; (△) Q849A. (B) Percent of TLS for the G, m6G, and abasic template measured at 180 s. Key: filled, D424A; hatched, R668A; open, Y766A; stippled, Q849A. The reaction conditions are the same as in panel A. Data are the mean of two or three independent experiments with standard deviations indicated as error bars.

Table 2: Translesion Synthesis by Klenow Polymerase Variants[a]

	template		
variant	G	m6G	abasic
D424A (WT)	+18[b]	+18	0[c]
R668A	+4	0	0
Y766A	+16	+16	0
Q849A	+4	−1[d]	−1

[a] Qualitative analysis of polymerase insertion reactions was performed as described in Figure 2. [b] $+X$ = translesion synthesis (bases beyond the indicated template base). [c] 0 = synthesis opposite the lesion. [d] −1 = prelesion synthesis.

(8%) than wild type (17%). The minor groove recognition variants, R668A and Q849A, lacking either primer or template strand interactions, respectively, were active on the natural G template, but less than 5% TLS was observed for either variant polymerase on the lesion-containing templates (Figure 3B).

The TLS patterns of the variant polymerases also have unique qualitative differences with regard to the longest products observed in relation to the position of the lesion (noted as 0 in Table 2). For instance, while WT and Y766A completed synthesis of the entire G template, R668A and Q849A incorporate only up to six nucleotides even though the amount of total synthesis was similar. The variants can be grouped on the basis of insertion profiles using the lesion-containing templates. While WT and Y766A insert and extend opposite an m6G lesion, R668A only can insert a nucleotide opposite the lesion, while Q849A only can incorporate to the prelesion position. Using abasic site-containing templates, both Y766A and R668A variants can incorporate a nucleotide opposite the abasic site, similar to WT. However, Q849A is unable to accomplish this incorporation.

Single nucleotide insertion experiments were performed to determine dNTP incorporation preferences across from the lesions for each KF variant (Figure 4). Quantitatively, the TLS results for WT polymerase are consistent with previous reports in the literature, in that dTMP incorporation across from a m6G lesion was preferred over dCMP, and dAMP incorporation across from an abasic site is favored over a combination of 50 μM dATP plus 200 μM dGTP, dCTP, or TTP. The synthesis reactions of variants R668A and Q849A with both lesion-containing templates were inhibitory in nature for each dNTP substrate. While Y766A had a preference for dTMP incorporation opposite the m6G lesion similar to WT, indiscriminate insertion of nucleotides was observed opposite the abasic site. This equality of dNTP insertion by Y766A was also seen in the presence of all four dNTPs (Figures 1C and 6C), as doublet products were observed opposite an abasic site, indicating a mixed population of purines and pyrimidines.

Primer Extension Experiments. Primer extension reactions were used to measure the ability of the polymerases to bind and synthesize nascent DNA from preformed, lesion-containing P/T complexes. All possible P/T combinations were examined for the three templates and four possible 3′-terminal nucleotides, totaling 12 different P/T complexes. The amount of extension by the variants of the P/T complexes was compared (Figure 5). Extension of the correct Watson−Crick base pair (C/G) of the polymerase ranged from 8% to 15% for the four variants. For the m6G templates, WT polymerase efficiently extended the expected m6G·T mispair over the over P/T complexes. A pronounced effect on the specificity of extending m6G lesion base pairs was observed when MGR interactions were lost. For the R668A variant, the percent extension of the purine-containing P/T complexes m6G·A (4.1%) and m6G·G (5.7%) was increased over that seen for WT polymerase (m6G·A, 2.2%; m6G·G, 1.1%). A similar trend was also observed for extension of the m6G·A (6.8%) and m6G·G (3.9%) P/T complexes by the Q849A variant. This preference for extension of 3′-terminal guanine by the MGR variants was specific for the m6G·G template and was not observed for the unmodified (G) P/T complex. The Y766A variant was notable in that the highest extension efficiency (3.7%) was observed using the mutagenic m6G·A template-primer mispair. For the abasic templates, WT polymerase displayed the expected preference for extension from a 3′-terminal dAMP. A similar 2-fold preference for extension from dAMP was observed for the other variants as well. Generally, loss of the MGR interaction in Q849A and R668A resulted in an increased extension of primers on the m6G template but had extension patterns similar to those of WT when an abasic site was involved.

Polymerase Dissociation Experiments. A dissociation assay was designed such that the dissociation/reassociation equilibrium of the polymerase during DNA synthesis may be visualized by both the loss and gain of a signal. The KF variants were prebound to the same substrates used in the insertion experiments, which will be referred to as the short (13/33mer) complexes. Synthesis reactions were initiated by the simultaneous addition of dNTPs and a 10-fold molar excess of the long (40/60mer) complex, to act as a competitor

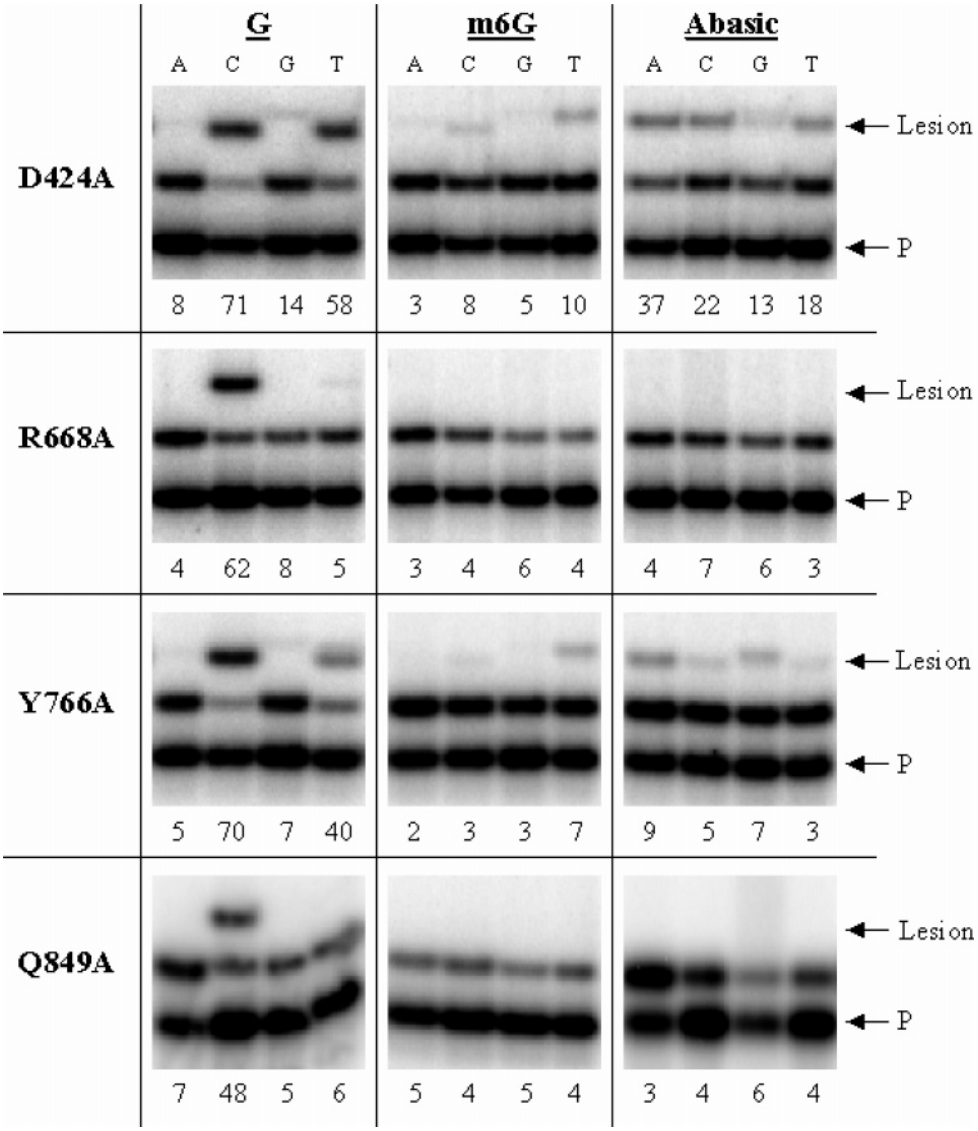

FIGURE 4: Representative gels of single nucleotide insertion opposite G, m6G, and abasic residues. Each reaction contained 50 μM dATP, corresponding to the running start substrate, and 200 μM dNTP as indicated. The DNA:polymerase ratios are the same as in the legend to Figure 3. The percentage of TLS is indicated below each lane.

for free polymerase (Table 1). The sequence surrounding the 3′OH is identical on both complexes. If the polymerase dissociates from the short complex, it will likely reassociate with the long template (in excess) and begin synthesis. Therefore, the amount of synthesis on the long complex with time is a measure of the dissociation equilibrium from the short complex. The ability of the polymerase to equally recognize and bind both the short and long complexes is crucial to this experimental design and was experimentally tested by mixing defined ratios of short and long complexes with unbound WT polymerase and quantitating the amount of product formed. At time points ranging from 1.0 to 15 min, the observed values were nearly identical to the expected values, demonstrating that the short and long complexes are equal substrates for the WT polymerase (data not shown).

Differences among the variant polymerases were observed in the amount of long complex synthesis completed after prebinding to the short complexes (Figure 6), indicating differences in the dissociation equilibrium of the variants. Qualitative examination of the gels demonstrated that complete extension of the long complex was greatest for WT, followed by Y766A, Q849A, and R668A. The pattern of long synthesis for each variant polymerase is similar, regardless of the short complex template (G, m6G, abasic) examined. On the short complex, TLS beyond the m6G lesion by the R668A and Q849A variants was observed when the reactions were extended to 30 min, as compared to the insertion assays in which these variants were unable to bypass the lesion in 3 min. Note that by 30 min, however, synthesis on the competitor long complex was nearly complete, and rebinding of the polymerase to the lesion-containing templates would be likely to have occurred.

Quantitative analyses of the gels in Figure 6 prior to exhaustion of the competitor complex were performed to determine the ratio of TLS on the short complex to total synthesis on the long complex, which is a measure of polymerase dissociation. On the control (G) template, this ratio increases with reaction time for all polymerases (Figure 7), which by definition means that synthesis on the short complex is greater than synthesis on the long complex, consistent with low dissociation from the normal short

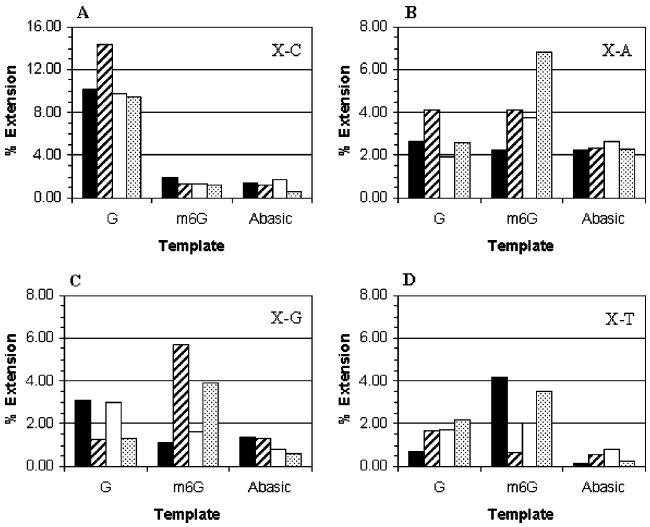

FIGURE 5: Polymerase extension from terminal G, m6G, or abasic residues. The primers (A) ExC, (B) ExA, (C) ExG, or (D) ExT were hybridized to the indicated template. Reactions contained 200 μM dNTPs, 6.67 nM hybridized DNA, and DNA:polymerase ratios of 100:1 for D424A, 3.6:1 for R668A, 50:1 for Y766A, and 10:1 for Q849A. The reactions were quenched at 60 s and the products separated by 16% PAGE. The products were quantitated by phosphorimager to determine the percent extension above background. Key: filled, D424A; hatched, R668A; open, Y766A; stippled, Q849A. The data are the mean of two or three independent experiments. The range of errors for the m6G·A and m6G·G P/T experiments was 7.9−37%.

complex. The higher ratios observed for the WT and Y766A variants, relative to R668A and Q849A, at the earlier time points (prior to exhausting the long complex) likely reflect decreased affinity of the MGR variants for the short DNA template. On lesion-containing complexes, the TLS/long ratio decreased with time for all polymerases, consistent with polymerase dissociation from the short complex prior to TLS, followed by reassociation and synthesis on the long complex. The average TLS/long ratio for WT polymerase decreased 2-fold and 3-fold for m6G and abasic templates, respectively, relative to the natural template (Figure 8). The degree of dissociation by R668A and Q849A variants from lesion-containing templates, relative to the natural template, was similar to WT. Therefore, the two polymerase variants lacking DNA minor groove interactions appear to dissociate from DNA templates more readily than WT but do not display an increased level of lesion-induced polymerase dissociation.

DISCUSSION

The molecular interactions between a DNA polymerase protein and its substrates that mediate TLS are poorly understood. In this study of TLS, we used Klenow polymerase in a structure−function approach with two types of mutagenic DNA lesions: the slightly inhibitory m6G adduct and the highly inhibitory abasic site. We observed that the minor groove recognition residues Gln849 and Arg668 of KF are crucial for successful TLS past both types of lesion. The Q849A variant causes a pronounced inhibition of insertion across from a lesion position. Thus, the direct polymerase interaction with the template DNA in the minor groove is important for stable incorporation of a nucleotide opposite either the m6G or abasic lesion. Loss of the primer strand interaction (R668A) results in a decreased TLS ability. Polymerase minor groove interactions with both the primer and template strands are also important to stabilize the lesion-containing terminal base pair as a substrate for continued DNA synthesis.

Previous models to explain the effects of lesions on DNA polymerase biochemistry have been based on crystallographic and NMR studies of lesion-containing duplex DNA. For example, physical structures of m6G-containing DNA duplexes have demonstrated that the mutagenic m6G·T base pair closely resembles a Watson−Crick base pair geometry with mild perturbations to the sugar−phosphate backbone (*35*). The nonmutagenic m6G·C base pair can exist in two conformations, one of which is a wobble base pair geometry and may be a less efficient substrate for DNA polymerases than the m6G·T base pair (*35*, *36*). The physical structures of abasic site-containing templates have shown that the structure of the abasic DNA strand is collapsed to maximize stacking interactions of flanking base pairs. The polymerase preference for insertion of purines opposite abasic sites may be due to the ability of purine dNTPs to wedge flanking bases apart and stabilize the template structure surrounding the lesion (*29*). The DNA sequence context around an abasic site does alter the stability of the duplex and can therefore influence the dNTP chosen (*37*, *38*). While these studies have led to important conceptualizations of TLS mechanisms, they must be considered with the caveat that the relevant DNA structure for TLS is the one within a DNA polymerase active site. For all DNA polymerases for which ternary complexes have been determined, such a DNA structure is not B-form duplex DNA (*1*, *2*, *12*).

Currently, we have the opportunity to examine our TLS results using BF polymerase structures obtained during DNA synthesis (*7*, *12*). In the open conformation, this A family polymerase sequesters the acceptor template base (which can be a lesion-containing base in our studies) within a pocket formed by helices O and O1 of the fingers region. Movement of the template base between this sequestered preinsertion position and the catalytically competent insertion position (polymerase closed conformation) is proposed to function as a checkpoint for fidelity (*12*). DNA synthesis may be halted when base mispairings impose altered geometries of the primer-template DNA. Upon stalling, rapid formation of the preinsertion site sequesters the acceptor template base. A second structural checkpoint for fidelity is related to the direct hydrogen bonds formed between the polymerase and minor groove positions with the terminal DNA base pair. Structural defects in MGR may interfere with DNA synthesis by disrupting proper formation of the preinsertion site and/or movement of the template base into the insertion site. This model is supported by physical structures of base mispairs within the BF polymerase active site (*7*). For the wobble mispairs (G·T and T·G), the template strand is displaced, and the minor groove interaction with the T-containing strand is lost. Similarly, in the A·G mispair, the disrupted minor groove interaction between template and polymerase is replaced with water-mediated interactions.

Our data are consistent with the hypothesis that MGR residues are required to maintain functional polymerase−DNA interactions during TLS. The Q849A variant polymerase is unable to stably incorporate a dNMP opposite lesions. Loss of the Q849 interaction may weaken the

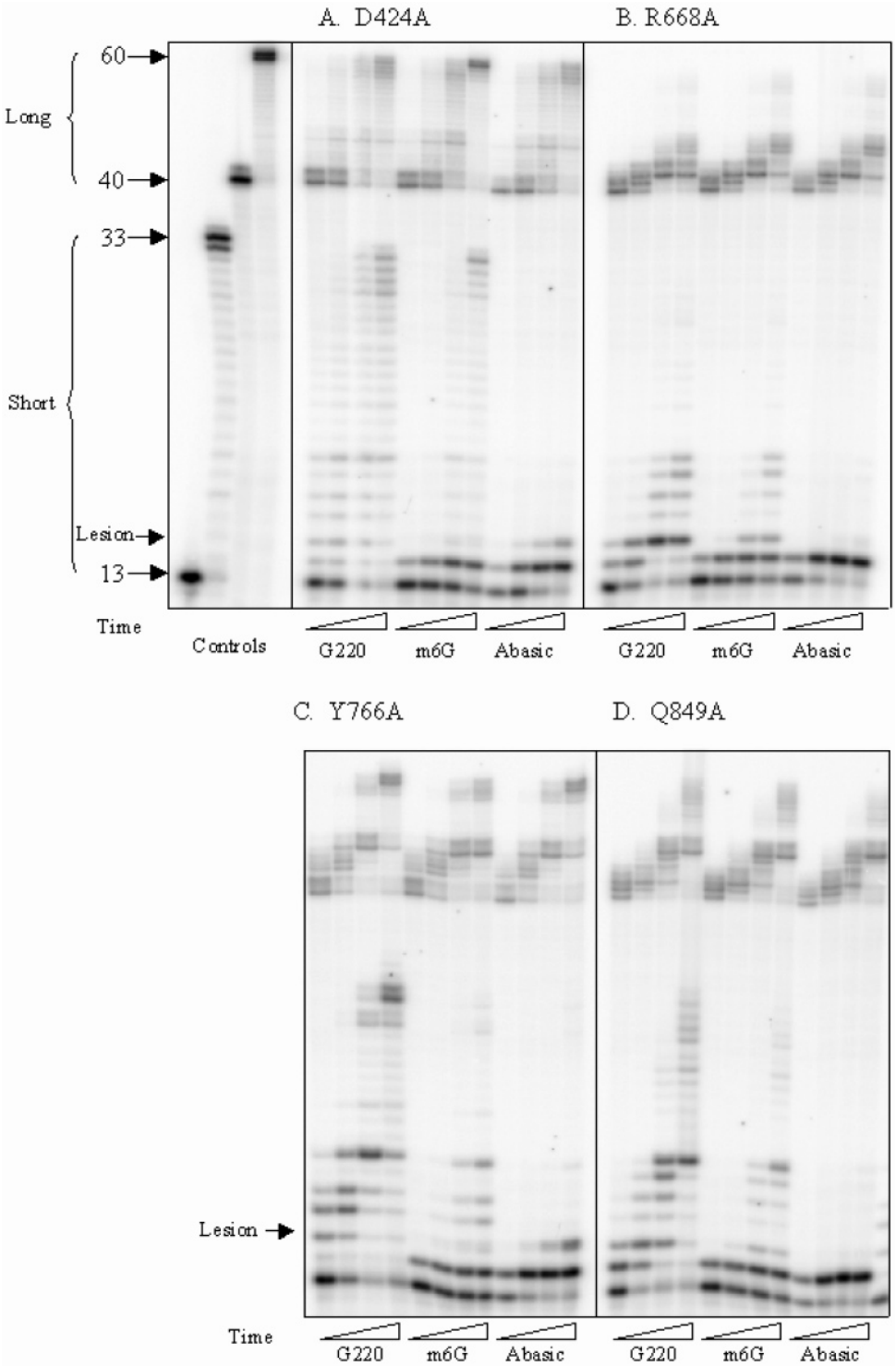

FIGURE 6: Representative gels of the polymerase dissociation assay. Time points of 1, 3, 10, and 30 min are represented for (A) D424A, (B) R668A, (C) Y766A, and (D) Q849A. All of the lanes contained the long P/T complex as well as one of the short P/T complexes (short primer with G, m6G, or abasic template) except for the control lanes. The polymerase was prebound to 6.67 nM of the short complex, and the reactions were initiated by the addition of the 66.7 nM long complex (6.67 nM labeled) and 50 μM dNTPs. The total DNA:polymerase ratios were the same as in the insertion experiments. The reaction products were separated by 12% PAGE. The product length of both short and long complexes is shown to the left of the gels. Lesion = G, m6G, or abasic residues.

stability of the dNMP·lesion base pair at the insertion site and/or alter the equilibrium to favor residence of the lesion-containing acceptor base in the preinsertion site, resulting in the inability to insert across from a lesion. When an abasic site was examined, the variant not only lacks the interaction with the DNA caused by the Q849A mutation but also the interaction the template base would have with the polymerase. In total, two protein−template interactions are lost in a localized area, potentially causing a greater effect, as seen in the inability of Q849A to insert opposite an abasic site.

We observed that loss of polymerase−substrate interactions in variant R668A resulted in a reduced ability of the polymerase to insert opposite a lesion, as well as an altered

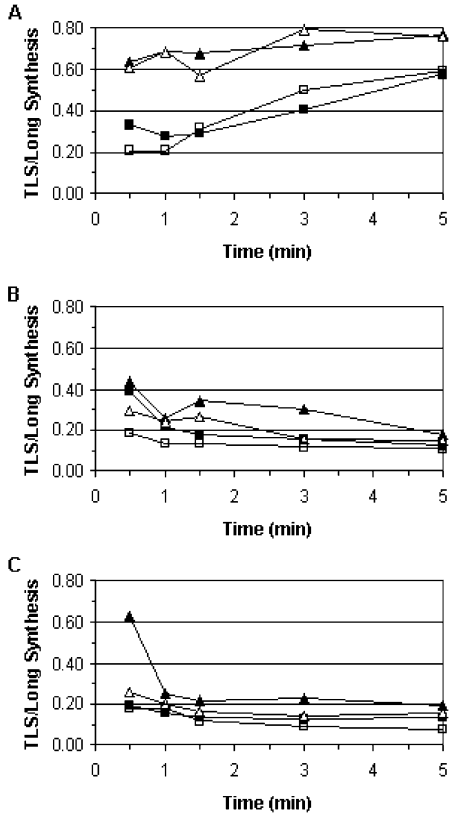

FIGURE 7: Quantitation of the polymerase dissociation assay. Ratio of TLS of the short complex to total synthesis of the long complex in the polymerase dissociation assay with the (A) G, (B) m6G, and (C) abasic short complexes. The products were quantitated by phosphorimager, and the amount of TLS was divided by the amount of synthesis on the long complex. Key: (▲) D424A; (■) R668A; (△) Y766A; (□) Q849A. Data are the mean of three independent experiments.

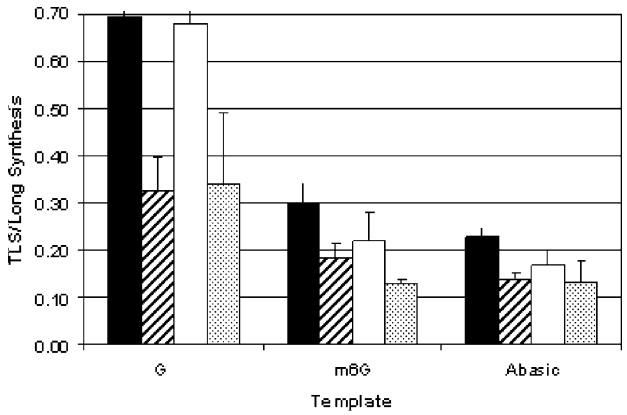

FIGURE 8: Average ratios of TLS of the short complex to total synthesis of the long complex in the polymerase dissociation assay. The ratios from time points (0.5, 1.0, 1.5, and 3.0 min) in which the unextended long complex was in great excess were averaged from three independent experiments. Error bars indicate standard deviation. Key: filled, D424A; hatched, R668A; open, Y766A; stippled, Q849A.

specificity of extension from m6G lesion-containing terminal base pairs. These results were somewhat unexpected, given the mutator phenotype of the R668A variant. On the basis of studies using natural DNA templates, Minnick et al. proposed that loss of Arg668 contacts between the polymerase and the substrates allowed the polymerase more flexibility in positioning the 3′OH and the nascent base pair in a catalytically favorable conformation within the active site (*21*). Because the m6G lesion alters base pair configurations (*35*, *36*), we predicted that the R668A variant polymerase would exhibit an overall increased efficiency of m6G TLS. One explanation for our observed results is that protein−DNA interactions other than discrimination of nascent base pair geometry within the polymerase active site are required for complete TLS.

The Klenow polymerase Y766A variant was used as a control for an amino acid alteration distinct from MGR. In the KF polymerase, the corresponding tyrosine physically blocks the insertion site in the open conformation. During nucleotide selection the polymerase adopts the closed conformation, and conformational changes of the fingers helices remove the tyrosine block. The Y766A variant lacks the ability to interact with bases on the template strand through hydrophobic ring interactions. Effects of this missing interaction were most observable during abasic but not m6G TLS. These results are consistent with the importance of stacking interactions for abasic site TLS and demonstrate the potentially important contribution of protein as well as nucleic acid residues to this process. All three amino acids examined in this study, Q849, R668, and Y766, are predicted to interact with the DNA primer-template (*10*, *11*). The variants display different TLS defects, depending on the lesion studied. Therefore, other lesions, such as 8-oxoguanine (8-oxoG), should be studied to further determine the generality of KF DNA minor groove interactions on TLS.

The polymerase dissociation assay performed here allowed the simultaneous detection of loss of a signal (dissociation from prebound template) as well as gain of a signal (reassociation to the long trap) during TLS. Reported K_D(DNA) values for the KF variants on a natural template agree with our observations (*17*, *19*, *20*, *23*). Our results clearly show that Q849A and R668A dissociate more frequently from unmodified DNA than WT. Therefore, loss of either MGR interaction is sufficient to disrupt polymerase−DNA association. On both lesion-containing templates, WT polymerase displayed an increased dissociation, as the ratio of TLS:long decreased 2−3-fold, relative to the natural template. A quantitatively similar change in dissociation was observed for each variant on lesion-containing templates, indicating that loss of these template interactions does not precipitate lesion-induced dissociation. Our data demonstrate that Klenow polymerase displays an altered dissociation/reassociation equilibrium on lesion-containing substrates. Therefore, DNA polymerase dissociation contributes to the DNA synthesis inhibition observed at m6G lesions and abasic sites.

DNA polymerases vary with respect to the nature of the protein−DNA structural interactions during DNA synthesis, and TLS efficiency is well documented to be dependent upon the DNA polymerase examined. A physical study of polymerase β (X family) TLS using an 8-oxoguanine DNA lesion demonstrated that the flexibility of the template backbone within the polymerase active site is an important parameter influencing TLS (*39*). In this structure, the phosphate backbone of the template lesion base is shifted, and the amino acid residues around the active site are repositioned, relative to the unmodified template structure. Physical studies of a Y family polymerase, Dpo4, with an abasic lesion demonstrated that this protein also alters the template DNA structure

(*40*). In this case, the lesion is looped out of the template strand, and the 5′ base is used to instruct nucleotide incorporation. The unique rigid movement of this polymerase may facilitate accommodation of a distorted DNA template within the polymerase active site during TLS. Our study has utilized an A family DNA polymerase. We demonstrated that KF polymerase residues R668 and Q849, predicted by structural analyses to interact with the DNA substrate in the minor groove, are functionally critical for the process of translesion synthesis.

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