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Kinetics of DNA Polymerase I (Klenow Fragment Exo$^-$) Activity on Damaged DNA Templates: Effect of Proximal and Distal Template Damage on DNA Synthesis†

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ABSTRACT: Mutagenic DNA adducts have been analyzed with respect to the rate of nucleotide insertion opposite the modified base, extension from that "mispair", and nucleotide insertion preference. To complement and extend these studies we have investigated the long-range effects of DNA adducts on DNA polymerase activity. To address this question, primer extension reactions were performed using DNA polymerase I, Klenow fragment exo$^-$. Templates containing 7,8-dihydro-8-oxoguanine, dG-C8-aminofluorene, dG-C8-(acetylamino)fluorene, and the model abasic site, tetrahydrofuran, were used for these studies, and the steady-state kinetics of correct nucleotide insertion were determined at positions (−2), (−1), (+1), (+2), (+3), and (+5) with respect to the template lesion. The kinetics of primer extension by Klenow fragment exo$^-$ at template positions 3′ to the lesion showed only a small inhibitory effect, <3-fold, even for the strongly blocking lesion, dG-C8-(acetylamino)fluorene, indicating that Klenow fragment exo$^-$ activity is not greatly affected by lesions in the single-stranded portion of the template-primer. In contrast, a dramatic decrease in the frequency of primer extension was observed at template sites 5′ to the site of adduction. Inhibition of polymerase activity decreased as the distance from the lesion increased; however, a relatively large effect was seen at the (+2) and (+3) positions for dG-C8-(acetylamino)fluorene and tetrahydrofuran. For these blocking lesions, the effect on extension 5 bases from the lesion was greatly reduced. We conclude from these studies that DNA damage at positions remote from the site of the lesion affects DNA polymerase function.

DNA damage can be an initiating event in mutagenesis and carcinogenesis; a wide variety of endogenously- and exogenously-produced lesions have been detected in genomic DNA (*1*). Some lesions block DNA replication and are potentially lethal; others generate mutations that are associated with carcinogenesis and cellular aging (*2*). As an aid toward understanding molecular mechanisms that promote mutagenesis, it is useful to characterize the processing of DNA lesions by the different enzymes involved in DNA metabolism. Much of what we know about the effects of DNA adducts on translesion synthesis has been gained from the analysis of primer extension reactions catalyzed by DNA polymerases on site-specifically modified templates.

DNA adducts have been analyzed with respect to the rate of nucleotide insertion opposite the modified base, extension of the primer from the "mispair", and nucleotide insertion preference. Translesion synthesis also is dependent on subtleties of neighboring sequence and properties of the DNA polymerase involved. For instance, there are several examples where the surrounding sequence context has been shown to have a significant effect on the mutagenicity of a DNA lesion (*3*−*7*). In addition, DNA polymerases differ in their miscoding specificity and the rate of translesion synthesis (*8*, *9*). The molecular basis for sequence context effects and differences in DNA polymerase specificity is not understood even though structures of several DNA polymerases and many DNA lesions have been determined. In addition, active sites of DNA polymerases are known to interact with bases flanking the 3′ primer terminus in duplex and single-stranded DNA. Therefore, in addition to sequence (nearest neighbor) effects and differences in individual polymerases, it is important to study the effect of lesions on DNA polymerase synthesis at positions distant from the primer terminus.

To address the long-range effects of DNA adducts on DNA polymerase activity, primer extension reactions were performed using the exo$^-$ Klenow fragment of DNA polymerase I (KF exo$^-$).[1] Four different DNA lesions were used for this study: 7,8-dihydro-8-oxoguanine (8-oxo-dG), dG-C8-aminofluorene (dG-AF), dG-C8-(acetylamino)fluorene (dG-AAF), and tetrahydrofuran, a model abasic site. These lesions differ in their abilities to block DNA replication *in vitro*. 8-Oxo-dG (*9*) and dG-AF (*3*, *6*, *10*, *11*) have relatively little effect on DNA synthesis *in vitro*, the tetrahydrofuran moiety has a moderate inhibiting effect on the process (*12*−*14*), and dG-AAF (*3*, *10*, *11*) strongly inhibits chain extension from the 3′ primer terminus. Using templates containing these lesions, kinetic parameters (k_{cat} and K_m) of nucleotide insertion were determined in the absence of proofreading at positions proximal (−2) and (−1) and distal (+1), (+2), (+3), and (+5) to the template lesion.

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[1] Abbreviations: dNTP, deoxynucleoside triphosphate; dNMP, deoxynucleoside monophosphate; 8-oxo-dG, 7,8-dihydro-8-oxoguanine; dG-AF, *N*-(deoxyguanosin-8-yl)-2-aminofluorene; dG-AAF, *N*-(deoxyguanosin-8-yl)-2-(acetylamino)fluorene; tetrahydrofuran, 3-hydroxy-2-(hydroxymethyl)tetrahydrofuran; KF exo$^-$, DNA polymerase I, Klenow fragment, 3′-5′ exonuclease free.

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EXPERIMENTAL PROCEDURES

Materials. [γ-^{32}P]ATP was obtained from Amersham Corp. The plasmid vector used for expression of Klenow fragment exo⁻ was a gift from Dr. C. Joyce (Yale University). Cloned Klenow fragment exo⁻ was overexpressed and purified as described (*15*−*17*). T4 polynucleotide kinase was purchased from U.S. Biochemical Corp.

Synthesis and Purification of Oligonucleotides. Oligodeoxynucleotides were synthesized by solid-state methods using an automated DNA synthesizer. DNA templates (18-mer) containing a single dG-AAF or a single dG-AF were prepared as described (*3*, *18*, *19*). Published methods were used for the synthesis and purification of oligodeoxynucleotides containing 8-oxoguanine (*20*, *21*) and the modified tetrahydrofuran moiety (*13*). Oligonucleotides were purified by electrophoresis on a 20% polyacrylamide gel in the presence of 7 M urea. DNA was eluted from gel slices passively and desalted using a SEP−PAK C18 cartridge. Oligonucleotides were quantified by A_{260} using molar extinction coefficients. Oligonucleotides were 5′-radiolabeled with T4 polynucleotide kinase and [γ-^{32}P]ATP according to the manufacturer's protocol. Primer−template substrates were prepared by hybridizing 5′-radiolabeled primer to the complementary 18-mer at a 1:1.2 molar ratio and heating to 80 °C followed by slow cooling.

Primer Extension Reactions. An 18-mer template containing a single dG, 8-oxo-dG, dG-AF, dG-AAF, or tetrahydrofuran was annealed to a 5′-^{32}P - labeled primer. Oligonucleotide sequences used for these experiments are listed in Figure 1. The standard reaction (10 μL) contained 50 mM Tris−HCl pH 7.5, 5 mM $MgCl_2$, 5′-^{32}P -primer/template at 100 nM, and 0−500 μM of the next correct dNTP. KF exo⁻ (0.1−310 nM) was diluted in a solution containing 50 mM Tris−HCl, pH 7.5, 0.5 mg/mL BSA, and 10% glycerol. Reactions were performed at 25 ± 1 °C for 1−60 min and quenched by addition of 20 μL of 100% ethanol. Samples were dried *in vacuo* and resuspended in 4 μL of 95% formamide dye mixture (90% formamide, 0.001% xylene cyanol, 0.001% bromphenol blue). Samples were heated at 100 °C for 3 min, and aliquots (2 μL) were subjected to electrophoresis on a 20% polyacrylamide denaturing gel. The amounts of primer and product were quantified using a Molecular Dynamics PhosphoImager.

Data Analysis. Kinetic parameters were determined from primer extension reactions. Values for the Michaelis−Menten constant (K_m) and V_{max} were obtained by least−squares nonlinear regression to a rectangular hyperbola. Less than 20% of the primer is extended under the steady state conditions used in our kinetic studies, ensuring single hit kinetics (*22*, *23*).

RESULTS

Steady-state kinetic parameters (K_m and k_{cat}) for correct nucleotide insertion were measured for the six template−primers shown in Figure 1. To facilitate comparison of K_m and k_{cat} values for different positions on the template and for different DNA lesions, the relative extension frequency, F_{ext}, also is reported.

$$F_{ext} = (k_{cat}/K_m)_{lesion}/(k_{cat}/K_m)_{dG\ control} \quad (1)$$

The numbers (−2), (−1), (+1), (+2), (+3), and (+5) designate the position of nucleotide incorporation with respect to the DNA lesion, X. Positions −1 and −2 are located proximal to the lesion; positions +1, +2, +3, and +5 are distal to the lesion. The structures of the DNA lesions used in these studies, 8-oxo-dG, dG-AF, dG-AAF, and tetrahydrofuran, are shown in Figure 1. The unmodified template has dG at position X. In experiments that measure incorporation distal to the lesion (positions (+1), (+2), and

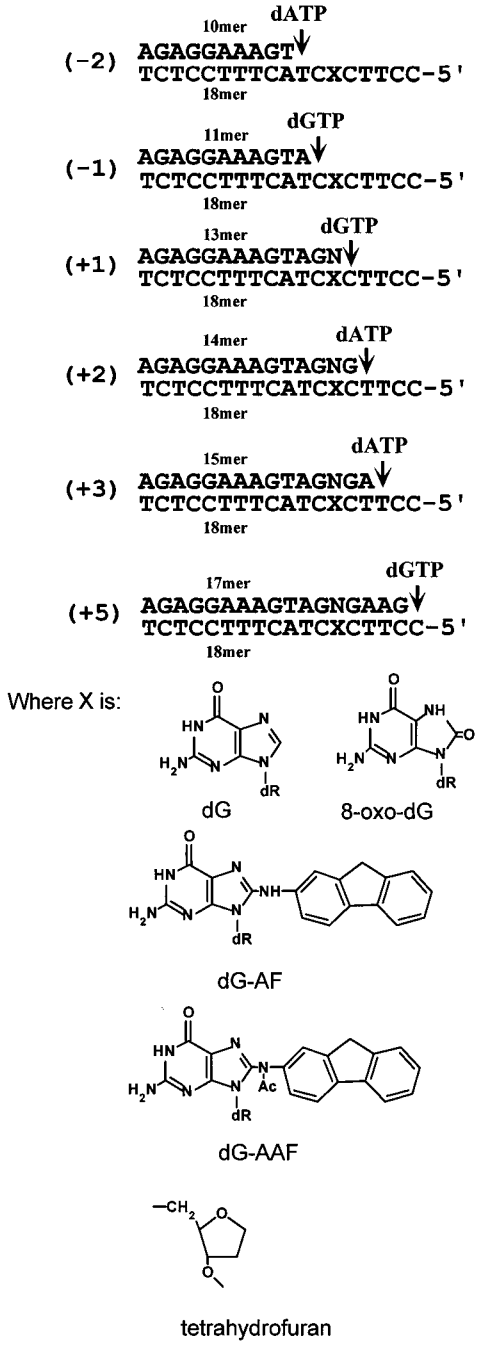

FIGURE 1: Oligonucleotide substrates used for the single nucleotide extension experiments. The oligonucleotides used for these experiments are shown at the top of the figure. The template oligonucleotides (18mer) contain a lesion or dG at position X (for the 17/18 template X = dG, tetrahydrofuran, or dG-AAF only). The chemical structures of 8-oxo-dG, (7,8-dihydro-8-oxoguanosine), dG-AF, (*N*-(deoxyguanosin-8-yl)-2-amino fluorene), dG-AAF, (*N*-(deoxyguanosin-8-yl)-2-acetylamino)fluorene, and tetrahydrofuran (3-hydroxy-2-(hydroxymethyl)tetrahydrofuran) used for these studies are diagrammed. dR represents deoxyribose. The primer oligonucleotides varied from 10 to 17 nucleotides and differed at position N. All possible template/primers were analyzed in this study (for the 17/18 template N = dA, dC only).

Table 1: Kinetic Parameters for Nucleotide Insertion Catalyzed by KF Exo$^-$ at Positions Proximal to the Lesion

	position of primer terminus with respect to lesion site					
	(−2)			(−1)		
lesion	K_m (μM)	k_{cat} (min^{-1})	F_{ext}[a]	K_m (μM)	k_{cat} (min^{-1})	F_{ext}
dG	0.124 ± 0.068[b]	11.6 ± 1.3	1	0.118 ± 0.009	40.6 ± 0.91	1
8-oxo-dG	0.212 ± 0.082	9.4 ± 0.9	0.47	0.0475 ± 0.005	29.8 ± 2.4	1.8
dG-AF	0.094 ±0.04	13.3 ± 1.4	1.5	0.103 ± 0.04	13 ± 1.1	0.37
dG-AAF	0.0587 ± 0.03	6.7 ± 0.66	1.2	0.059 ± 0.012	19.7 ± 1.0	0.97
tetrahydrofuran	0.263 ± 0.045	9.12 ± 0.48	0.37	0.045 ± 0.005	20.8 ± 0.45	1.3

[a] $F_{ext} = (k_{cat}/K_m)_{lesion\ template}/(k_{cat}/K_m)_{dG\ control\ template}$. [b] Standard error calculated from nonlinear regression.

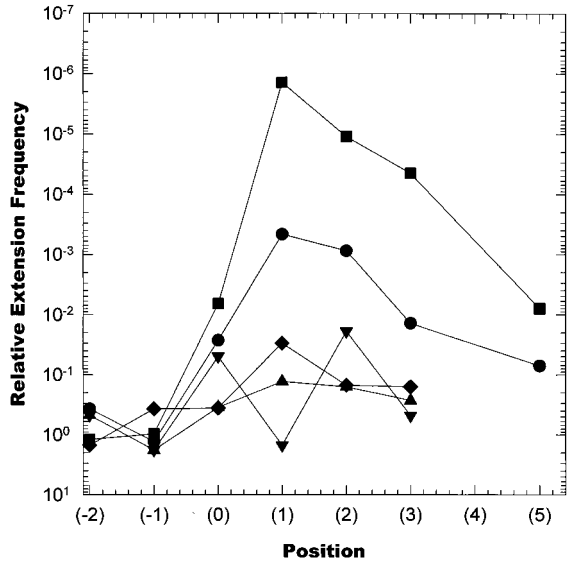

FIGURE 2: Relative extension frequencies for DNA lesions along the template. Some of the data from from Tables 1−5 are summarized in this figure. The relative frequency of extension, $F_{ext} = (k_{cat}/K_m)_{lesion\ template}/(k_{cat}/K_m)_{dG\ control\ template}$, is plotted versus the position of incorporation with respect to the template lesion: (●) dA opposite tetrahydrofuran; (■) dA opposite dG-AAF; (▲) dC opposite 8-oxo-dG; (▼) dA opposite 8-oxo-dG; (◆) dC opposite dG-AF. All values are relative to the control dG template with the correctly paired primer. The data for primer extension at position (+5) are as follows: dG:dC $k_{cat}/K_m = 64.4$ min^{-1} M^{-1}; tetrahydrofuran:dA $k_{cat}/K_m = 0.071$; $F_{ext} = 1.1 \times 10^{-3}$ min^{-1} M^{-1}; dG-AAF:dC $k_{cat}/K_m = 0.014$ min^{-1} M^{-1}, $F_{ext} = 2.1 \times 10^{-4}$.

(+3)), four different primers were used for each template. This strategy allowed determination of the kinetic constants for each of all four possible nucleotides (N = A, C, G, T) opposite the DNA lesion. For incorporation at position (+5) only primers with N = A or C were used.

Effect of DNA Lesions on DNA Synthesis Proximal to the Lesion. Initially, we measured the kinetic parameters for DNA synthesis 3′ to the lesion. In general, this effect was small (Table 1, Figure 2). Two bases proximal to the lesion (−2), we see little effect on k_{cat} and less than a 3-fold decrease on the relative incorporation efficiency, F_{ext}, for any of the DNA lesions. Even one base before dG-AAF, a bulky lesion, k_{cat} is diminished only 2-fold. The maximum change in F_{ext} at this position (less than 3-fold) was observed with the dG-AF modified template. These data support a model for KF exo$^-$ translesion synthesis whereby the polymerase is not affected by DNA lesions in the region of the template 5′ to the primer terminus and suggest that the single-stranded template is not bound in the active site in a precise conformation.

DNA Synthesis Opposite the Lesion. For the four lesions used in this study, the relative insertion frequency, F_{ext}, at position (0) and the preferred base inserted in the presence of all four nucleotides has been established (*3*, *9*, *14*). These data, summarized in Table 2 and Figure 2, show that 8-oxo-dG and dG-AF are not serious impediments to nucleotide insertion compared to the tetrahydrofuran and dG-AAF where F_{ext} is decreased several orders of magnitude.

Table 2: Relative Nucleotide Insertion Frequencies, F_{ext}, for KF Exo$^-$ at Lesion Site

lesion	F_{ext} C:X[e]	F_{ext} A:X	nucleotide preference[f]
8-oxo-dG[a]	0.35	0.05	dC > dA
dG-AF[b]	0.36	1.1×10^{-5}	dC
dG-AAF[b]	6.6×10^{-3}	3.8×10^{-4}	dC > dA
tetrahydrofuran[c]	N.D.[d]	2.72×10^{-2}	dA

[a] Data from ref 9. [b] Data from ref 3. [c] Data from ref 14. [d] N.D.: not determined. [e] $F_{ext} = (k_{cat}/K_m)_{lesion\ template}/(k_{cat}/K_m)_{dG\ control\ template}$. [f] Preferred nucleotide inserted under conditions where all four nucleotides are provided.

DNA Synthesis Distal to the Lesion. We tested the hypothesis that not only insertion opposite the lesion and extension from the primer terminus determines the amount of translesion synthesis but also synthesis more distal to the lesion plays a significant role in determining the impact of DNA adducts on DNA synthesis *in vivo*. To address this proposal, we performed a systematic analysis of the kinetics of nucleotide incorporation at positions (+1) (Table 3), (+2) (Table 4), and (+3) (Table 5) (see also Figure 2). For nucleotide incorporation using the unmodified template (dG:dC) the K_m, k_{cat}, and specificity constants (k_{cat}/K_m) were comparable. This result indicates a lack of sequence-specific effects of the template or end effects due to the primer template itself even though synthesis at (+5) occurs one base from the template terminus. Data recorded for the unmodified template reflect the kinetic parameters for correct as well as mispair extension. A decrease in k_{cat} is the major effect seen for synthesis on the damaged template and is largely responsible for the observed differences in extension frequencies. K_m for dNTP is not greatly altered by the lesions. The data reported here were obtained using 100 nM primer−template concentrations. To confirm that this concentration of primer−template was saturating, several experiments were performed at 200 nM, 500 nM, and 1 μM primer−template concentrations (data not shown). From these experiments we conclude that the enzyme is saturated with DNA substrate even for the dG-AAF-modified templates.

The largest effect of the template lesion is seen at the (+1) position. This observation is in agreement with previously published reports that extension at (+1) is more difficult than base insertion opposite a lesion (*3*, *9*, *14*). The single exception to this rule is extension from the 8-oxo-dG:dA primer−template, which is approximately as efficient as

Table 3: Kinetic Parameters Determined for Chain Extension by KF Exo⁻ One Base Distal to the Lesion (+1)

lesion	base opposite	K_m	k_{cat}, min^{-1}	k_{cat}/K_m	F_{ext}
dG	A	12.1 ± 0.88	0.14 ± 0.0032	0.011	3.15×10^{-4}
dG	C	5.6 ± 2.5	203 ± 23	36.34	1
dG	G	17.2 ± 4.6	0.63 ± 0.06	0.036	1×10^{-3}
dG	T	5.7 ± 1.5	82.7 ± 5.6	14.5	0.4
8-oxo-dG	A	1.9 ± 0.43	103 ± 4.5	53.74	1.5
8-oxo-dG	C	7.0 ± 1.7	33.9 ± 2.2	4.83	0.13
8-oxo-dG	G	17.7 ± 1.8	0.47 ± 0.017	0.025	7.03×10^{-4}
8-oxo-dG	T	28.0 ± 18	0.38 ± 0.099	0.014	3.8×10^{-4}
dG-AF	A	7.4 ± 1.5	0.019 ± 0.001	0.0027	7.46×10^{-5}
dG-AF	C	6.8 ± 0.2	8.1 ± 0.08	1.18	0.032
dG-AF	G	35.2 ± 37.4	$1.4 \pm 0.63 \times 10^{-4}$	4×10^{-6}	1.1×10^{-7}
dG-AF	T	23.7 ± 13.9	$8.8 \pm 2 \times 10^{-4}$	3.71×10^{-5}	1.02×10^{-6}
dG-AAF	A	19.7 ± 11.7	$14 \pm 2.8 \times 10^{-4}$	7.05×10^{-5}	1.9×10^{-6}
dG-AAF	C	18.2 ± 13.8	$9.1 \pm 2.4 \times 10^{-4}$	4.97×10^{-5}	1.4×10^{-6}
dG-AAF	G	N.D.[a]	N.D.	N.D.	N.D.
dG-AAF	T	N.D.	N.D.	N.D.	N.D.
tetrahydrofuran	A	27.9 ± 2.62	0.46 ± 0.020	0.017	4.6×10^{-4}
tetrahydrofuran	C	25.7 ± 5.4	$69 \pm 5 \times 10^{-4}$	2.3×10^{-4}	6.4×10^{-6}
tetrahydrofuran	G	14.9 ± 3.6	$14 \pm 1.1 \times 10^{-4}$	9.5×10^{-5}	2.6×10^{-6}
tetrahydrofuran	T	23 ± 5.9	$52 \pm 5.2 \times 10^{-4}$	2.3×10^{-4}	6.3×10^{-6}

[a] N.D.: no detectable extension.

Table 4: Kinetic Parameters Determined for Chain Extension by KF Exo⁻ Two Bases Distal to the Lesion (+2)

lesion	base opposite	K_m	k_{cat}, min^{-1}	k_{cat}/K_m	F_{ext}
dG	A	48.9 ± 13.4	18.9 ± 1.9	0.38	0.0048
dG	C	2.2 ± 1.2	174 ± 19	79.9	1
dG	G	66.7 ± 24	26 ± 3.8	0.39	0.0049
dG	T	17.2 ± 7.9	148 ± 14	8.60	0.11
8-oxo-dG	A	197 ± 53	129 ± 25	1.59	0.02
8-oxo-dG	C	18.4 ± 7.7	236 ± 25	12.87	0.16
8-oxo-dG	G	44.1 ± 8.4	71.9 ± 3.8	1.63	0.02
8-oxo-dG	T	49.2 ± 18.4	27.7 ± 3.7	0.56	7×10^{-3}
dG-AF	A	83 ± 10	$18 \pm 1.3 \times 10^{-3}$	2.3×10^{-4}	2.9×10^{-6}
dG-AF	C	1.9 ± 0.3	23.4 ± 0.7	12.33	0.15
dG-AF	G	72.5 ± 12.2	8.8 ± 1.3	0.13	1.6×10^{-3}
dG-AF	T	42.3 ± 4.1	0.68 ± 0.018	0.016	2×10^{-4}
dG-AAF	A	99.8 ± 21.8	$32 \pm 4 \times 10^{-3}$	3.0×10^{-4}	3.81×10^{-6}
dG-AAF	C	48.6 ± 7.9	$38 \pm 2.6 \times 10^{-3}$	8.9×10^{-4}	1.12×10^{-5}
dG-AAF	G	66.8 ± 14.8	0.30 ± 0.02	4.5×10^{-3}	5.66×10^{-5}
dG-AAF	T	298 ± 15.6	$61 \pm 1.3 \times 10^{-4}$	2.0×10^{-5}	2.56×10^{-7}
tetrahydrofuran	A	35.8 ± 5.5	2.5 ± 0.16	0.070	8.7×10^{-4}
tetrahydrofuran	C	30.8 ± 0.8	1.04 ± 0.009	0.034	4.2×10^{-4}
tetrahydrofuran	G	37.7 ± 26.1	$32 \pm 6.8 \times 10^{-3}$	9.1×10^{-4}	1.13×10^{-5}
tetrahydrofuran	T	46.1 ± 4.9	0.92 ± 0.027	0.020	2.5×10^{-4}

extension from the unmodified template and 20 times more efficient than insertion of dA opposite 8-oxo-dG. The least efficient synthesis at (+1) occurs on the dG-AAF template. For primer−templates containing dG-AAF:dG and dG-AAF:dT, there was no detectable primer extension indicating values for F_{ext} of less than 1×10^{-7}. At this position in the template, it is clear that certain lesion−base pairs are more easily extended by KF exo⁻. Discrimination between extension of dG:dC and a mispair is as high as 10^4; a similar range of discrimination is seen for the dG-AF lesion, where dG-AF:dC extension is favored 10^3−10^5-fold over extension of dG-AF positioned opposite the other bases (dG, dA, dT). For the tetrahydrofuran:dA there is a 100-fold preference above primer extension for tetrahydrofuran positioned opposite the other bases; both dG-AAF:dA and dG-AAF:dC are extended equally poorly but at least 10-fold more efficiently that dG-AAF:dG or dG-AAF:dT. The base found opposite dG-AAF after translesion synthesis with all four nucleotides present in the reaction is C or A, with only a 3-fold preference for C (*3*). This preference probably reflects the 10-fold difference in insertion frequency of dCMP opposite template dG-AAF (Table 2, Figure 2) because there are only minor differences in the extension efficiencies from either dC or dA opposite dG-AAF until position (+3).

Two bases beyond the template lesion (+2; Table 4, Figure 2), the barriers to mispairing and lesion extension are still high, except in the case of 8-oxo-dG. However, even purine/purine mispairs, the most difficult mispairs to extend, are several orders of magnitude more efficiently extended than a primer in which dA, dC, dG, or dT is positioned opposite dG-AAF. The barrier to primer extension on dG-AAF-modified templates is seen at (+3) and, although lower, is also seen at (+5) (Figure 2). dG-AAF and tetrahydrofuran have been described as lesions that effectively block replication based on F_{ins} and F_{ext} values 6−9 orders of magnitude lower than a normal DNA base pair (*3*, *14*). From the data presented here, it is clear that the barrier to extension from dG-AAF:dN persists for five bases. This kinetic barrier allows ample opportunity for proofreading or polymerase/DNA disassembly, mechanisms that help to maintain the fidelity of DNA synthesis. The tetrahydrofuran lesion is most efficiently extended when dA is opposite the lesion. This preference persists three bases away from the site of the lesion.

Table 5: Kinetic Parameters Determined for Chain Extension by KF Exo$^-$ Three Bases Distal to the Lesion (+3)

lesion	base opposite	K_m	k_{cat}, min^{-1}	k_{cat}/K_m	F_{ext}
dG	A	9.8 ± 4.6	4.5 ± 0.43	0.46	0.0035
dG	C	1.7 ± 0.32	224 ± 9.6	132	1
dG	G	6 ± 1.3	85.8 ± 4.7	14.	0.11
dG	T	10.8 ± 0.82	20.4 ± 0.43	1.9	0.014
8-oxo-dG	A	2.6 ± 0.72	159 ± 11	63	0.48
8-oxo-dG	C	4.6 ± 0.57	160 ± 5.2	35	0.27
8-oxo-dG	G	13.5 ± 1.4	35.3 ± 1.1	2.6	0.02
8-oxo-dG	T	22 ± 6.7	18 ± 1.4	0.92	0.007
dG-AF	A	33.9 ± 12.4	2.6 ± 0.38	0.076	5.8×10^{-4}
dG-AF	C	3.1 ± 0.52	66.8 ± 2.8	21.5	0.16
dG-AF	G	9.5 ± 3.74	0.24 ± 0.022	0.025	1.9×10^{-4}
dG-AF	T	38.2 ± 1.0	0.94 ± 0.0080	0.024	1.9×10^{-4}
dG-AAF	A	27.3 ± 4.0	0.68 ± 0.029	0.025	1.9×10^{-4}
dG-AAF	C	14.3 ± 5.056	0.061 ± 0.0064	59×10^{-4}	4.49×10^{-5}
dG-AAF	G	36.1 ± 3.8	0.13 ± 0.0059	37×10^{-4}	2.85×10^{-5}
dG-AAF	T	97.2 ± 12.8	0.040 ± 0.0030	4.4×10^{-4}	3.35×10^{-6}
tetrahydrofuran	A	30.3 ± 4.3	54.4 ± 2.5	1.8	0.014
tetrahydrofuran	C	23.6 ± 6.5	0.85 ± 0.079	0.036	2.7×10^{-4}
tetrahydrofuran	G	190.2 ± 22.8	18.1 ± 1.5	0.095	7.2×10^{-4}
tetrahydrofuran	T	30.9 ± .028	1.21 ± 0.082	0.041	3.1×10^{-4}

Effect of Lesions on Overall Translesion Synthesis. To illustrate the long-range effects of DNA lesions on polymerase efficiency, selected data from our experiments are presented graphically in Figure 2. For each lesion, the relative extension frequency, F_{ext}, is plotted versus the position with respect to the lesion:dNMP on the DNA template. There is a very small effect on F_{ext} proximal to the template lesion and a relatively moderate effect opposite the adduct. In all but one case (8-oxo-dG:dA), the largest effect is at position (+1). It is clear from this analysis that dG-AAF has the largest inhibitory effect on polymerase activity and that the decrease in activity is propagated along template DNA at least five bases from the lesion. The effect of the tetrahydrofuran is qualitatively similar, but the decrease in extension frequency is 2−3 orders of magnitude less for this lesion than for dG-AAF. However, by position (+5) the effect of the tetrahydrofuran lesion is small. The increased activity for the 8-oxo-dG:A primer template at (+1) is in agreement with data obtained by pre-steady-state methods (*24*) where there is a dramatic difference in the rate of extension of a 8-oxo-dG:A primer template compared to the 8-oxo-dG:C primer template.

DISCUSSION

The data presented in this paper combined with results of previous studies provide a quantitative analysis of translesion synthesis by KF exo$^-$. Several conclusions can be drawn from this study. First, KF exo$^-$ is not highly discriminatory with respect to the single-stranded region of the template. This is evidenced by the small effect that even bulky lesions have on DNA synthesis preceding the DNA adduct. A similar finding has been reported for T7 DNA polymerase (*25*). Translesion synthesis by T7 DNA polymerase on templates containing dG-AF and dG-AAF, investigated using pre-steady-state methods, showed there was little effect of these lesions on DNA synthesis proximal to the lesion. DNA synthesis proximal to the tetrahydrofuran or the exocyclic adduct, propanodeoxyguanosine (propanodG), by DNA polymerase α is stimulated 3−4-fold relative to the unmodified DNA primer template at positions (−4) and (−3), respectively (*26, 27*). In the case of propanodG, results obtained with KF at position (−3) were similar (*27*). However, these effects are small compared to those on DNA synthesis distal to the lesion.

Second, strongly-blocking lesions in DNA templates affect DNA polymerase function at least five bases away from the lesion. Even lesions that are easily bypassed (i.e., 8-oxo-dG) have as much as a 50-fold effect two bases from the lesion and 2- to 3-fold effect three bases from the lesion. These experiments were performed with KF exo$^-$, a polymerase lacking proofreading 3′→5′ exonuclease activity. The decrease in the rate of polymerization (k_{cat}) observed at template sites three or more nucleotides away from the lesion would allow for "correction" by a proofreading competent DNA polymerase. Since most replicative polymerases have 3′→5′ exonuclease activity, proofreading may have a large effect on translesion synthesis *in vivo*. The data presented in Table 2 show that the effect of several template lesions is several orders of magnitude greater than a mispair. This long-range effect of template lesions has been observed for other DNA polymerases. In pre-steady-state experiments using T7 DNA polymerase (exo$^-$) and dG-AAF, it was observed that the rate of incorporation even two nucleotides past the adduct was decreased in comparison to templates without lesions (*25*). In an analysis performed using tetrahydrofuran or propanodG template lesions, DNA polymerase α showed decreased activity four or fewer bases from the lesion site (*26, 27*). We conclude that lesions that disrupt Watson−Crick hydrogen bonding, for example, an abasic site, or DNA adducts, such as dG-AAF and propanodG, inhibit DNA polymerization at least three or more bases after the initial translesion synthesis event. This effect has been observed for all DNA polymerases examined to date; therefore, synthesis at these distal positions should be considered when analyzing translesion synthesis *in vitro*.

A body of evidence obtained from thermodynamic studies indicates that DNA duplexes with and without a lesion may show different thermodynamic stabilities even though they are structurally similar (*28, 29*). This may explain the observed long-range effects of DNA lesions on polymerase activity since crystallographic and NMR studies of DNA adducts do not show structural differences five base pairs away from the lesion. The only lesion that results in a dramatic alteration of the DNA structure is the (acetylamino)-

fluorene modification of guanine, which induces a rotation from *anti* to *syn*. There may also be local denaturation of the dG-AAF:dC base pair and insertion of the AAF moiety into the helix (*11*, *30*). Aminofluorene modification of guanine (dG-AF) paired opposite dC results in two interconvertable conformations (*31*, *32*). In one conformation, dG-AF lies in the major groove of the helix and results in only minor distortions. In the other, the AF moiety is stacked within the duplex, disrupting base pairing between dG-AF and dC and displacing the modified guanine base into the minor groove and the cytosine into the major groove (*31*, *32*). A tetrahydrofuran moiety positioned opposite dA does not disrupt the B-DNA structure (*33*). Both the unpaired adenine base and the tetrahydrofuran residue reside within the helix (*33*). 8-Oxo-dG opposite dC also causes little disruption to the overall DNA structure (*34*). 8-Oxo-dG opposite dA in the duplex does not perturb significantly the helical structure, although the glycosidic torsion angle of the modified dG is *syn* (*35*). The 8-oxo-dG:dA base pair is stabilized by hydrogen bonding and both bases are within the helix (*35*). Therefore, the long-range effects these template lesions have on polymerase activity is not only due to major disruptions of the DNA helix; more subtle structural or thermodynamic factors must be involved.

Many crystallographic, kinetic, and site-specific mutagenesis studies have been performed on KF exo⁻, and these data offer insight into the mechanism of polymerase fidelity from both a structural and a kinetic perspective. The barrier to mispair extension is an important aspect of DNA replication fidelity. Our data indicate there is also a barrier for lesion base pair extension as demonstrated by the strong effect at distal positions. The structural model for KF exo⁻ primer−template binding shows the primer terminus bound in the large cleft of the polymerase domain with the template strand in contact with residues of the *O*-helix (*36*). Site-directed mutation experiments support this conclusion (*37*). This region of the active site may be important for determining the rate of mispair or lesion base pair extension. Pre-steady-state kinetic experiments led to the proposal of two important conformational change steps, one before and one after the chemical step of phosphoryl transfer. Slow extension of mispairs may be governed by the chemical step and by the conformational changes both preceding and after the phosphoryl transfer (*38*, *39*). Structurally, this may be the result of steric interference of the conformation change when the base pair is not precisely Watson−Crick in form. For example, recent structural studies of DNA polymerase β indicate that a conformational change may be important for fidelity (*40*). An induced fit model has also been proposed for T7 DNA polymerase where misincorporation is limited by a slow conformational change step preceding phosphoryl transfer and extension of the mispair is inhibited by a rate-limiting chemistry step (*41*). Another mechanism for preventing mispair extension has been proposed by Freemont *et al*. in which there is diffusion between the polymerase and exonuclease active sites after each polymerization step with these two activities in balanced competition (*42*). Although KF exo⁻ cannot excise the 3′ terminus, perhaps transient DNA binding in the exonuclease site is facilitated by a lesion base pair as it would be by a mispair. Certain residues are thought to be important for stabilizing the single stranded region in the exonuclease binding site and the tendency for the mispaired primer terminus to become single stranded leads to higher residence time in this binding site (*43*). Perhaps, lesion base pairs also exhibit this tendency to become single stranded. It would therefore be of interest to study, in detail, the effect of 3′→5′ exonuclease activity on translesion synthesis.

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