Steric and Electrostatic Effects in DNA Synthesis by the SOS-Induced DNA Polymerases II and IV of *Escherichia coli*[†]

Adam P. Silverman,[‡] Qingfei Jiang,[§] Myron F. Goodman,*,[§] and Eric T. Kool*,[‡]

Department of Chemistry, Stanford University, Stanford, California 94305-5080, and Department of Biological Sciences, University of Southern California, Los Angeles, California 90089-2910

ABSTRACT: The SOS-induced DNA polymerases II and IV (pol II and pol IV, respectively) of *Escherichia coli* play important roles in processing lesions that occur in genomic DNA. Here we study how electrostatic and steric effects play different roles in influencing the efficiency and fidelity of DNA synthesis by these two enzymes. These effects were probed by the use of nonpolar shape analogues of thymidine, in which substituted toluenes replace the polar thymine base. We compared thymine with nonpolar analogues to evaluate the importance of hydrogen bonding in the polymerase active sites, while we used comparisons among a set of variably sized thymine analogues to measure the role of steric effects in the two enzymes. Steady-state kinetics measurements were carried out to evaluate activities for nucleotide insertion and extension. The results showed that both enzymes inserted nucleotides opposite nonpolar template bases with moderate to low efficiency, suggesting that both polymerases benefit from hydrogen bonding or other electrostatic effects involving the template base. Surprisingly, however, pol II inserted nonpolar nucleotide (dNTP) analogues into a primer strand with high (wild-type) efficiency, while pol IV handled them with an extremely low efficiency. Base pair extension studies showed that both enzymes bypass non-hydrogen-bonding template bases with moderately low efficiency, suggesting a possible beneficial role of minor groove hydrogen bonding interactions at the N-1 position. Measurement of the two polymerases' sensitivity to steric size changes showed that both enzymes were relatively flexible, yielding only small kinetic differences with increases or decreases in nucleotide size. Comparisons are made to recent data for DNA pol I (Klenow fragment), the archaeal polymerase Dpo4, and human pol κ.

DNA damage from reactive oxygen species, UV light, or chemical carcinogens can lead to structural changes in the bases, resulting in modified bases such as 8-oxoguanine and adducts such as those of *N*-2-acetylaminofluorene and benzo[*a*]pyrene diol epoxide. Structurally altered bases often block the progression of replicative DNA polymerases such as pol III of *Escherichia coli* (*1*). For replication to continue, specialized "translesion synthesis" enzymes that can progress past the damage are required. In response to DNA damage, a biological response (the SOS response) is induced in *E. coli*, leading to upregulation of more than 40 genes, including DNA polymerases II, IV, and V (*2*). Pol II is a high-fidelity polymerase that has a 3′-exonuclease proofreading domain, while pol IV and pol V are more error-prone than pol II, leading to mispairing errors approximately once per 10^3–10^4 bp (*3*, *4*). Specialized roles for these polymerases have been proposed over the past decade. While pol V is responsible for the catalysis of error-prone translesion

synthesis, pol II is used for error-free replication restart, and pol IV is responsible for generating nontargeted frameshift mutations while rescuing stalled replication forks (*5*). The polymerase utilized at a given site may depend on the type of damage; for example, DNA pol IV has been shown to afford translesion synthesis past benzo[*a*]pyrene diol epoxide adducts to guanine, while pol II can replicate past *N*-2-acetylaminofluorene adducts (*6*).

Adaptive mutations, such as *lacZ* adaptive frameshift mutations and other −1 frameshift mutations, are attributed to pol IV, which has constitutive cellular levels 5 times higher than that of pol II (*7*). Pol II is conversely responsible for decreasing the number of adaptive mutations through error-free replication and its 3′-exonuclease domain (*8*, *9*). Thus, pol II and pol IV are believed to carry out a mutational balancing act to maximize cellular fitness.

To improve our understanding of how polymerases deal with damaged DNA, it is important to consider the enzymes' ability to accept noncanonical nucleoside substrates. A number of factors are involved in recognition of substrates, including base and base pair size and shape (steric effects), as well as hydrogen bonding and solvation of the dNTP or template base (electrostatic effects) (*10*). Research in the past decade has demonstrated that Watson−Crick hydrogen bonding is in some cases not required for polymerase activity (*11*−*13*), particularly for family A high-fidelity enzymes. This has led to the hypothesis that steric effects play a
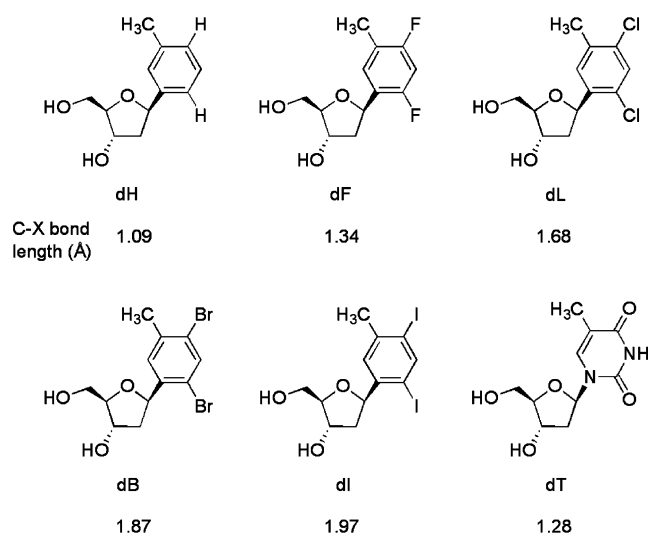
FIGURE 1: Structures of nonpolar thymidine analogues of varied size used in this study.

dominant role in determining the fidelity of such enzymes (*10*, *14*, *15*). Studies on the incorporation efficiencies and fidelities of nonpolar nucleoside isosteres (*16*) as well as variously substituted fluorinated and methylated base mimics have shown that active site size and shape can play critical roles in polymerase activity and fidelity (*17–19*), and practical applications of shape-selective, non-hydrogen-bonded base pairs have begun to be developed recently (*20*).

To test steric effects with DNA polymerases in a systematic way, we recently described a set of nonpolar thymidine analogues (Figure 1) that are nearly identical in shape to thymidine but use structural mimics [toluene (H), difluorotoluene (F), dichlorotoluene (L), dibromotoluene (B), and diiodotoluene (I)] that differ in size by ~1 Å across the entire series (*21*, *22*). Kinetics studies in which natural template bases or nucleoside triphosphates were replaced with the variably sized thymidine analogues using *E. coli* DNA pol I Klenow fragment (Kf *exo−*) (*23*) and T7 DNA polymerase (*24*) demonstrated large differences in fidelity and efficiency as the base size was changed, demonstrating a high degree of steric rigidity. A similar result was also observed for this series in an *E. coli* cellular replication assay (*23*). In contrast to this, a low-fidelity family Y enzyme, Dpo4 DNA polymerase (*25*), was found to be quite flexible in accepting variably sized analogues and also exhibited a strong kinetic preference for hydrogen-bonded base pairs over nonpolar analogues. We ascribed the differences in fidelity between these distinct enzyme classes to differences in active site tightness of high-fidelity enzymes to low-fidelity ones, with the notion that repair enzymes need to process structurally altered (damaged or mispaired) substrates and thus can benefit from steric flexibility.

Since pol II and pol IV offer contrasting replication fidelities and different biological roles, it is important to understand what role sterics and electrostatics play in the ability of these polymerases to replicate unnatural or damaged DNA. In this work, we test the active site flexibilities of pol II (an exonuclease deficient mutant) and pol IV using the variably sized nonpolar thymidine analogues and evaluate their dependence on electrostatic effects. Results of single-nucleotide incorporation and extension suggest that differences in active site tightness play a significant part in the differing fidelities of pol II and pol IV, and the data also reveal the importance of hydrogen bonding and solvation effects for these SOS-induced enzymes.

RESULTS

Single-Nucleotide Insertion Kinetics. Single-nucleotide insertion experiments were performed using a 23-mer−28-mer primer−template duplex. The primer was 5′ end-labeled with ^{32}P, and products were evaluated on denaturing PAGE gels. Steady-state kinetics were measured by use of varied nucleotide concentrations, and the single-nucleotide insertion efficiencies (as V_{max}/K_M) were evaluated for the nonpolar thymidine analogue series. The steady-state method measures overall catalytic efficiency without assumptions about which microscopic step in the catalytic cycle is rate-limiting; note that mispaired bases or nucleotide analogues may cause changes in which step is limiting.

To evaluate steric and electrostatic effects during base pair synthesis by pol IV, we first studied the efficiency of natural dNTP insertion opposite the variably sized nonpolar thymidine analogues. The data are listed in Table 1 and are compared graphically in Figure 2. Results showed that dATP was inserted preferentially opposite these analogues, but only 2−12-fold more efficiently than dTTP. This was in strong contrast to natural nucleotides: dATP was inserted opposite T 4200-fold more efficiently than dTTP was, similar to the reported fidelity of pol IV (*3*). Overall, incorporation of dATP opposite the template base mimics was 10^3−10^4-fold less efficient than incorporation of dATP opposite natural thymidine, suggesting that the polar electrostatics of thymine in the T-A pair are important for base pair synthesis by this enzyme. A small size influence (~8-fold) was observed across the series, with the dichlorotoluene template base analogue (dL) giving the greatest incorporation efficiency.

We next carried out the converse experiment, attempting to insert the nonpolar thymidine analogues as dNTPs opposite natural template bases; however, no incorporation of any of the nonnatural dNTPs was observed even at very high enzyme concentrations and long reaction times. We estimate a maximum efficiency (V_{max}/K_m) of ≤2 for this insertion [compared with 2.8×10^5 for dATP insertion opposite dT (Table 1)]; thus, insertion of dATP opposite dL was more efficient than dLTP opposite dA by a factor of at least 50, demonstrating substantial asymmetry in the pairing efficiency.

The analogous experiments were then carried out with DNA pol II (Figure 3 and Tables 2 and 3). We used an exonuclease-deficient mutant (D155A/E157A) (*27*) to follow insertion in the absence of proofreading activity. As with pol IV, pol II was also inefficient at inserting natural dNTPs opposite the nonpolar template base analogues (Figure 3A). Also like pol IV, there was selectivity for dATP, which was preferred over dTTP by ~40-fold for medium-sized analogues dF and dL, but by less than 3-fold for dH, dB, and dI. Overall, dATP was incorporated 10^3−10^4-fold less efficiently opposite the unnatural bases compared with opposite thymidine. Some size dependence was observed, with dATP being incorporated opposite dL 30-fold more efficiently than opposite the smallest and largest analogues, dH and dI, respectively. Poor incorporation was observed for dCTP and dGTP in a somewhat size-dependent manner,

Table 1: Steady-State Kinetics Data for DNA pol IV Insertion of Natural dNTPs Opposite Variably Sized Nonnatural Template Thymine Analogues[a]

dNTP	template base	V_{max} (% min^{-1})	K_M (μM)	efficiency (V_{max}/K_M)	f_{inc}
dATP	H	0.0075 (0.0023)	580 (370)	1.5×10 (0.4)	5.4×10^{-5}
	F	0.0051 (0.0008)	210 (20)	2.5×10 (0.3)	8.9×10^{-5}
	L	0.017 (0.005)	190 (70)	9.7×10 (3.3)	3.5×10^{-4}
	B	0.013 (0.004)	750 (550)	2.0×10 (0.8)	7.1×10^{-5}
	I	0.0072 (0.0060)	790 (950)	1.3×10 (0.5)	4.6×10^{-5}
	T	2.2 (0.2)	8.2 (2.2)	2.8×10^5 (0.7)	1
dTTP	H	0.0051 (0.0004)	400 (20)	1.2×10 (0.1)	4.3×10^{-5}
	F	0.0057 (0.0002)	1100 (200)	5.4 (0.9)	1.9×10^{-5}
	L	0.0064 (0.0012)	810 (280)	8.4 (2.4)	3.0×10^{-5}
	B	0.0028 (0.0004)	330 (30)	8.4 (1.9)	3.0×10^{-5}
	I	0.0041 (0.0032)	970 (1100)	6.5 (4.1)	2.3×10^{-5}
	T	0.012 (0.005)	250 (220)	6.7×10 (4.1)	2.3×10^{-4}

[a] Values in parentheses indicate standard deviations from multiple experiments. f_{inc} is calculated as the ratio of apparent V_{max}/K_m relative to the A → T case.
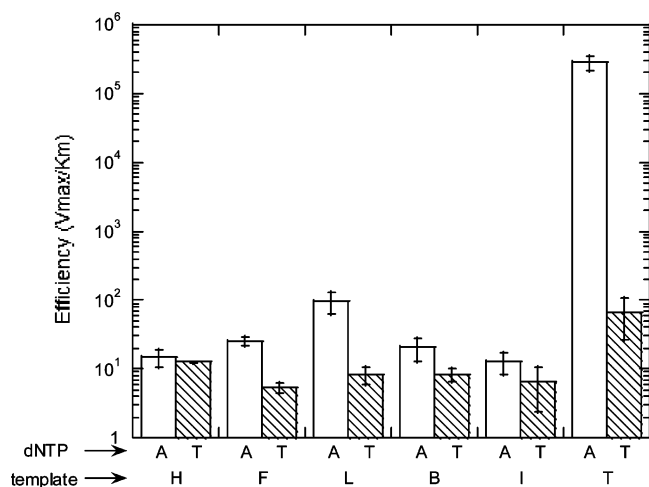

FIGURE 2: Steady-state efficiencies (V_{max}/K_M) for insertion of natural dNTPs opposite template base analogues of increasing size for DNA pol IV. Note the log scale of efficiencies. Primer−template duplexes had the sequence (5′-TAATACGACTCACTATAGGGAGA)·(5′-ACTGXTCTCCCTATAGTGAGTCGTATTA). Kinetics were measured at 37 °C in a buffer containing 20 mM Tris (pH 7.5), 20 mM sodium glutamate, 4% (v/v) glycerol, 8 mM MgCl$_2$, and 5 mM DTT. The primer was 5′-end-labeled with [γ-^{32}P]ATP and was extended by the polymerase in the presence of a single dNTP over various concentrations and times; products of single-nucleotide insertions were resolved from unreacted primer by 20% denaturing polyacrylamide gel electrophoresis and quantified by autoradiography. See Table 1 for numerical data.

with maximum efficiency at dL for dCTP and at dF for dGTP. With natural nucleotides, there was a much higher fidelity, with a 6100-fold enzymatic preference for insertion of dATP rather than dTTP opposite a template T.

Next we looked at the ability of DNA pol II to insert variably sized nonpolar dNTPs opposite natural template bases (Figure 3B and Table 3). The results were strikingly different than those with pol IV (above): the four largest thymidine triphosphate analogues were inserted opposite adenine by pol II with equal or greater efficiency than natural dTTP was. Indeed, the most efficient nucleotide (dLTP) was inserted 3.6-fold more efficiently than dTTP. The efficiency of incorporation opposite A was greatest with dLTP and decreased as the base became larger or smaller, decreasing by 154-fold for the smallest nucleotide triphosphate, dHTP. The unnatural dNTPs were incorporated selectively opposite A, except for dHTP which was inserted opposite T ~8-fold
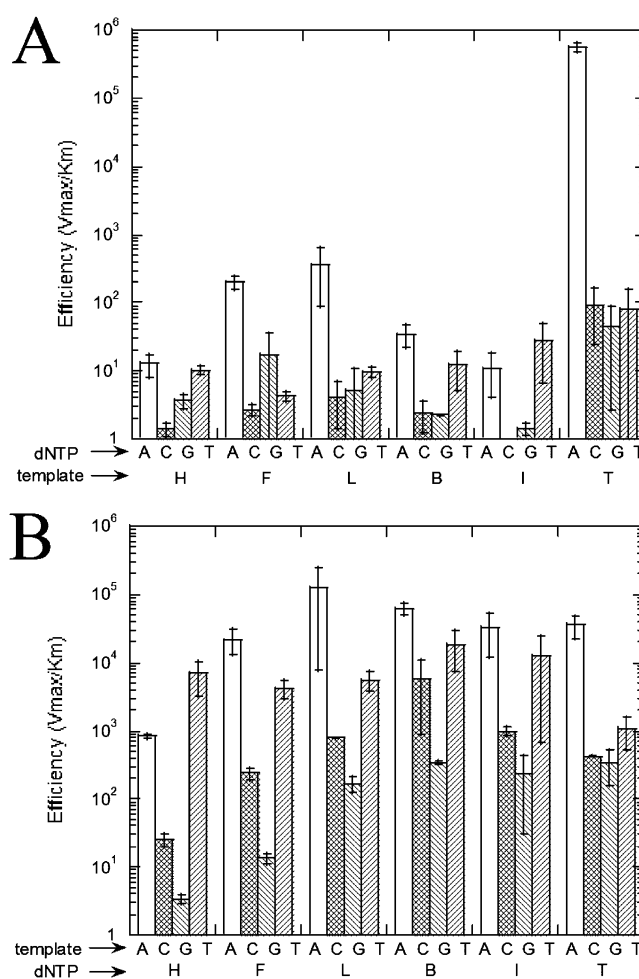
FIGURE 3: Effects of varying base size on DNA pol II. Single-nucleotide insertion efficiencies (V_{max}/K_M) are shown on a log scale. (A) Insertion of natural dNTPs opposite template base analogues of increasing size, with T for comparison. (B) Insertion of nucleoside triphosphate analogues of increasing size opposite natural template bases, with dTTP for comparison. Conditions are the same as those described in the legend of Figure 2. See Tables 2 and 3 for data.

more efficiently. Selectivity for a template A over a T for the most selective case (dLTP) was slightly weaker than that of the natural nucleotide dTTP (24-fold vs 33-fold). Interestingly, incorporation of the analogues opposite the other mismatched bases, C and G, was also highly dependent on

Table 2: Steady-State Kinetics Data for DNA pol II Insertion of Natural dNTPs Opposite Variably Sized Nonnatural Template Thymine Analogues[a]

dNTP	template base	V_{max} (% min^{-1})	K_M (μM)	efficiency (V_{max}/K_M)	f_{inc}
dATP	H	0.0019 (0.0007)	150 (10)	1.2×10 (0.5)	2.1×10^{-5}
	F	0.018 (0.010)	86 (32)	2.0×10^2 (0.5)	3.5×10^{-4}
	L	0.027 (0.019)	74 (3)	3.6×10^2 (2.7)	6.3×10^{-4}
	B	0.0054 (0.0011)	160 (20)	3.4×10 (1.2)	6.0×10^{-5}
	I	0.0046 (0.0030)	430 (10)	1.1×10 (0.7)	1.9×10^{-5}
	T	0.12 (0.06)	0.19 (0.09)	5.7×10^5 (0.8)	1
dCTP	H	0.00078 (0.00004)	580 (160)	1.4 (0.3)	2.5×10^{-6}
	F	0.00045 (0.00025)	170 (60)	2.6 (0.5)	4.6×10^{-6}
	L	0.00042 (0.00024)	100 (10)	4.1 (2.7)	7.2×10^{-6}
	B	0.00040 (0.00009)	180 (50)	2.4 (1.1)	4.2×10^{-6}
	I	*b*	*b*	*b*	−
	T	0.0077 (0.0046)	87 (16)	9.4×10 (7.0)	1.6×10^{-4}
dGTP	H	0.0023 (0.0006)	630 (10)	3.7 (0.8)	6.5×10^{-6}
	F	0.0044 (0.0059)	170 (160)	1.7×10 (1.8)	3.0×10^{-5}
	L	0.0025 (0.0012)	690 (450)	5.2 (5.1)	9.1×10^{-6}
	B	0.00069 (0.00065)	310 (290)	2.2 (0.1)	3.9×10^{-6}
	I	0.00037 (0.00030)	300 (280)	1.4 (0.3)	2.5×10^{-6}
	T	0.012 (0.005)	370 (230)	4.5×10 (4.2)	7.9×10^{-5}
dTTP	H	0.0066 (0.0042)	690 (520)	1.0×10 (0.1)	1.8×10^{-5}
	F	0.0018 (0.0008)	420 (120)	4.2 (0.8)	7.4×10^{-6}
	L	0.0019 (0.0009)	190 (60)	9.7 (1.6)	1.7×10^{-5}
	B	0.0046 (0.0015)	500 (410)	1.2×10 (0.7)	2.1×10^{-5}
	I	0.0076 (0.0010)	370 (240)	2.8×10 (2.1)	4.9×10^{-5}
	T	0.0045 (0.0036)	72 (28)	7.9×10 (8.1)	1.4×10^{-4}

[a] Values in parentheses indicate standard deviations from multiple experiments. [b] No incorporation observed with 29 μg/mL enzyme and a reaction time of 30 min.

Table 3: Steady-State Kinetics Data for DNA pol II Insertion of Nonnatural dNTPs Opposite Natural Template Bases[a]

template base	dNTP	V_{max} (% min^{-1})	K_M (μM)	efficiency (V_{max}/K_M)	f_{inc}
A	dHTP	0.12 (0.009)	140 (20)	8.3×10^2 (0.7)	2.3×10^{-2}
	dFTP	0.43 (0.10)	21 (0.4)	2.2×10^4 (0.9)	6.1×10^{-1}
	dLTP	0.45 (0.06)	5.8 (5.0)	1.3×10^5 (1.2)	3.6
	dBTP	0.39 (0.16)	6.7 (3.9)	6.1×10^4 (1.2)	1.7
	dITP	0.21 (0.01)	8.3 (5.1)	3.2×10^4 (2.1)	8.9×10^{-1}
	dTTP	0.39 (0.20)	11 (2)	3.6×10^4 (1.3)	1
C	dHTP	0.0034 (0.0005)	140 (10)	2.5×10 (0.6)	6.9×10^{-4}
	dFTP	0.024 (0.001)	100 (10)	2.4×10^2 (0.4)	6.7×10^{-3}
	dLTP	0.028 (0.0014)	36 (3)	7.9×10^2 (0.3)	2.2×10^{-2}
	dBTP	0.017 (0.0003)	4.6 (3.9)	5.8×10^3 (5.0)	1.6×10^{-1}
	dITP	0.0055 (0.0014)	5.5 (0.7)	1.0×10^3 (0.1)	2.8×10^{-2}
	dTTP	0.055 (0.0005)	130 (10)	4.2×10^2 (0.2)	1.2×10^{-2}
G	dHTP	0.00021 (0.00008)	66 (33)	3.3 (0.5)	9.2×10^{-5}
	dFTP	0.00066 (0.00012)	50 (1)	1.3×10 (0.2)	3.6×10^{-4}
	dLTP	0.0031 (0.0007)	20 (9)	1.7×10^2 (0.4)	4.7×10^{-3}
	dBTP	0.0057 (0.0028)	17 (9)	3.4×10^2 (0.2)	9.4×10^{-3}
	dITP	0.0024 (0.0001)	17 (15)	2.3×10^2 (2.0)	6.4×10^{-3}
	dTTP	0.048 (0.020)	140 (80)	3.3×10^2 (1.8)	9.2×10^{-3}
T	dHTP	0.0093 (0.0078)	1.9 (2.1)	6.9×10^3 (3.6)	1.9×10^{-1}
	dFTP	0.014 (0.004)	3.5 (2.0)	4.2×10^3 (1.2)	1.2×10^{-1}
	dLTP	0.041 (0.017)	7.2 (0.6)	5.5×10^3 (1.9)	1.5×10^{-1}
	dBTP	0.084 (0.036)	6.3 (5.7)	1.8×10^4 (1.1)	5.0×10^{-1}
	dITP	0.051 (0.018)	6.2 (4.4)	1.3×10^4 (1.2)	3.6×10^{-1}
	dTTP	0.013 (0.005)	12 (2)	1.1×10^3 (0.5)	3.1×10^{-2}

[a] Values in parentheses indicate standard deviations from multiple experiments.

base size. The level of incorporation opposite C increased 240-fold as base size was increased from dHTP to dBTP and then decreased 15-fold for dITP. A similar but less dramatic trend was observed for incorporation opposite G, which also peaked with dBTP and varied by ∼100-fold across the series.

Extension beyond Variably Sized Template Bases. Because an important function of SOS-induced polymerases is extension beyond damaged or mispaired bases, we proceeded to evaluate steric effects in the extension step, separate from

insertion. We measured steady-state kinetics for extension past an A·X base pair, where X is a variably sized thymidine template base analogue, and compared the results with those for extension past natural A·T base pairs.

Results for pol IV showed a moderate size dependence in extension past A·X base pairs (Figure 4B and Table 5). The A·L base pair was extended most efficiently, 240-fold less efficiently than an A·T base pair, and the extension efficiency dropped as base pair size was increased or decreased. The poorest extension occurred with the smallest (A·H) and
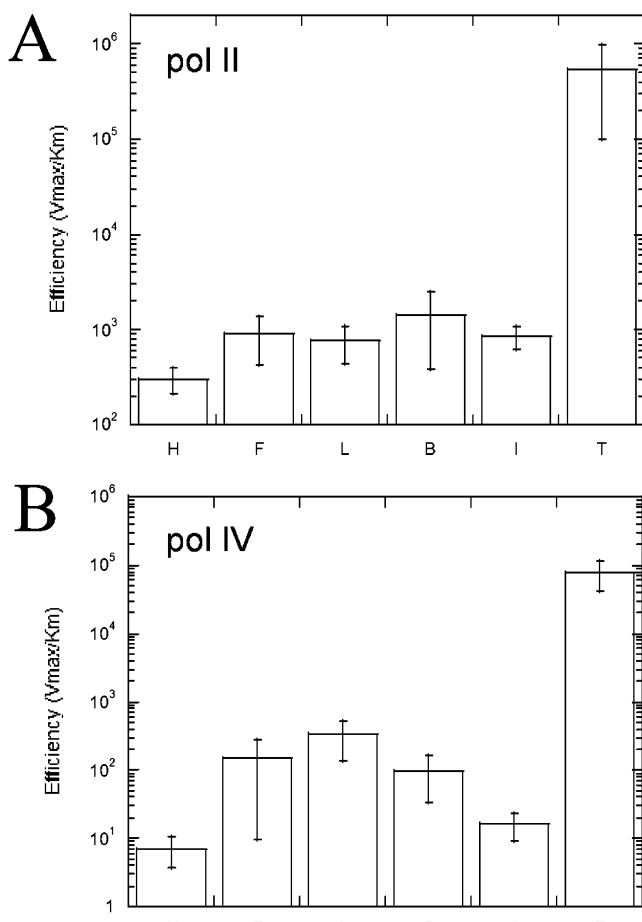
FIGURE 4: Efficiencies (V_{max}/K_M) vs base size for extension past an A·X base pair, where X is a nonpolar thymidine of variable size in the template strand. (A) Extension by DNA pol II. (B) Extension by pol IV. Primer−template duplexes had the sequence (5′-TAATACGACTCACTATAGGGAGAA)·(5′-ACTGXTCTC-CCTATAGTGAGTCGTATTA). Other conditions are described in the legend of Figure 2. See Tables 4 and 5 for data.

Table 4: Steady-State Kinetics Data for Extension beyond Variably Sized Base Pairs by DNA pol II[a]

terminal base pair	V_{max} (% min^{-1})	K_M (μM)	efficiency (V_{max}/K_M)	f_{ext}
A·H	0.013 (0.008)	49 (43)	3.0×10^2 (0.9)	5.6×10^{-4}
A·F	0.013 (0.008)	14 (2)	9.1×10^2 (4.8)	1.7×10^{-3}
A·L	0.022 (0.007)	34 (24)	7.5×10^2 (3.2)	1.4×10^{-3}
A·B	0.029 (0.018)	34 (37)	1.4×10^3 (1.0)	2.6×10^{-3}
A·I	0.025 (0.024)	34 (37)	8.6×10^2 (2.4)	1.6×10^{-3}
A·T	0.13 (0.09)	0.25 (0.04)	5.4×10^5 (4.4)	1

[a] Values in parentheses indicate standard deviations from multiple experiments. f_{ext} is calculated as the ratio of apparent V_{max}/K_m relative to the A → T case.

largest (A·I) base pairs, which were extended ∼4 orders of magnitude less efficiently than an A·T pair. Across the entire series, extension efficiencies varied by 48-fold.

Pol II extension results showed that the enzyme, like pol IV, was also poor at extending past unnatural base pairs (Figure 4A and Table 4). Analogue A·X base pairs were extended with an efficiency 2−3 orders of magnitude lower than those of natural A·T base pairs. Unlike pol IV extension, however, there was very little effect of base pair size on extension efficiency, as the extension efficiencies across the entire series varied by less than 5-fold.

Table 5: Steady-State Kinetics Data for Extension beyond Variably Sized Base Pairs by DNA pol IV[a]

terminal base pair	V_{max} (% min^{-1})	K_M (μM)	efficiency (V_{max}/K_M)	f_{ext}
A·H	0.0051 (0.0032)	830 (610)	7.0 (3.3)	9.0×10^{-5}
A·F	0.024 (0.016)	190 (70)	1.5×10^2 (1.4)	1.9×10^{-3}
A·L	0.071 (0.011)	270 (190)	3.3×10^2 (2.0)	4.2×10^{-3}
A·B	0.022 (0.002)	280 (160)	9.9×10 (6.6)	1.3×10^{-3}
A·I	0.012 (0.005)	930 (660)	1.6×10 (0.7)	2.1×10^{-4}
A·T	1.3 (0.7)	20 (17)	7.8×10^4 (3.6)	1

[a] Values in parentheses indicate standard deviations from multiple experiments.

DISCUSSION

Taken together, our experiments show that both of the SOS-induced polymerases II and IV are only moderately to weakly sensitive to changes in base size and thus should be classified as relatively flexible polymerases. Pol II can be considered more sterically sensitive (more rigid) than pol IV, with a 33-fold drop in efficiency with an increase in template base size from dL to dI, as compared with a 7.5-fold drop for the latter. Interestingly, this 7.5-fold drop in efficiency for pol IV is identical to that measured previously for the related archaeal enzyme Dpo4 (25). This is in marked contrast to the rigidity found for two high-fidelity family A polymerases, where a drop of 89-fold in efficiency was found for T7 DNA polymerase (24) and an even larger 160-fold drop for Klenow fragment of DNA pol I (23) in this same sequence context. Both pol II and IV show the same size preference as other polymerases, with a maximum in efficiency at dichlorotoluene, and a lower efficiency for both smaller and larger analogues, which suggests that the active site size in the closed conformation is similar and that the differences in steric responsiveness arise from a differential ability to flex inward or outward to adjust to varied substrate size or shape (23−25).

The experiments also clearly show that electrostatic and solvation effects are very important for both pols II and IV, in contrast to the insensitivity to these effects found previously for family A polymerases. Comparison of activities with nonpolar analogues difluorotoluene (or dichlorotoluene) to those with thymine in the template DNA show that pol II loses a large fraction of its activity (by a factor of 1600 in V_{max}/K_m) when the polar-to-nonpolar change is made, while pol IV appears to be even more dependent on the electrostatics of thymine (2900-fold effect for the same change). Similar effects have been observed for Dpo4 and the human family Y polymerases η and κ (25, 28, 29). The electrostatic charges in the template are also very important for fidelity: the nonpolar template bases are processed by both enzymes with low fidelity (correct base pair preference of 12-fold for pol IV and 37-fold for pol II), whereas thymine yields a much higher fidelity for both (4200- and 6100-fold, respectively). As a whole, then, we conclude that both enzymes depend on electrostatic interactions to gain the majority of their efficiency and fidelity in synthesis of a new base pair and that their reliance on steric selectivity is considerably weaker (intermediate steric selectivity for pol II and low selectivity for pol IV). This is in strong contrast to the family A DNA polymerases pol I and T7 pol, which have shown near wild-type efficiency with nonpolar template bases, and which
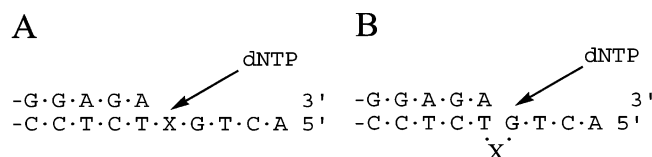
A　　　　　　　　　　　B

FIGURE 5: Sketch showing a potential frameshift caused by DNA slippage. (A) Normal nucleotide incorporation geometry. (B) Possible incorporation geometry with unnatural base slipped out, leading to a −1 frameshift mutation. No evidence for the frameshift mode was seen with nonnatural template bases.

appear to rely nearly entirely on steric rigidity for their fidelity (*23*, *24*, *29*).

One very great difference between pol II and pol IV is that the latter accepts the nonpolar analogues as dNTP derivatives extremely poorly [as does pol κ, a possible ortholog (*30*, *31*)], while pol II accepts them with even better activity than natural dTTP. Thus, for pol II, it is clear that the lack of hydrogen bonds in a L-A or B-A base pair does not affect this enzyme's efficiency. We have not observed this large asymmetry (i.e., nonpolar bases being readily accepted as dNTPs but having poor efficiency as template bases) before in other polymerases, although pol II is the first family B enzyme studied kinetically with nonpolar nucleoside isosteres. One possible explanation for the remarkable electrostatic asymmetry in this enzyme is that desolvation of the incoming dNTP in some way relies on polar groups of the template base; this would explain why nonpolar dNTP is a good substrate while the nonpolar template is an extremely poor substrate. Alternatively, it is possible that asymmetry in the active site causes nonpolar bases in the template strand to be misaligned in some way that prevents dNTP insertion. Structural studies of the related family B RB69 polymerase with DNA and dNTP bound (*32*–*34*) indicate that the template base of the terminal pair undergoes minor groove water-mediated interaction with a tyrosine, while the primer terminal base has no polar interaction. It is possible that this electrostatic asymmetry may be related either to misalignment or to asymmetric desolvation. Similar structural asymmetry might be present in pol II; although this enzyme reportedly has been crystallized (*35*), the structure has not yet been published. Some related polymerases (but lacking DNA and dNTP) have also been studied crystallographically (*36*–*38*).

One mechanism by which pol IV achieves adaptive mutations and bypasses damage is through base slippage, leading to −1 frameshift mutations (*39*). Our current experiments do not show evidence that this mechanism is operating when nonpolar base analogues are in the template. If base slippage were in play for the unnatural template base analogues, we would expect to see dCTP incorporation, as the next template base is G (Figure 5). However, dCTP incorporation was not observed at all, implying that base slippage is not used to bypass these nonpolar bases. This is somewhat unexpected in light of the fact that dATP, the "correct" dNTP, was poorly incorporated.

The experiments described here suggest that hydrogen-bonding groups in the incipient base pair may be highly important for the damage response activity of DNA pol IV. This polymerase is known to replicate past bulky template adducts such as those with benzo[*a*]pyrene diol epoxide. A crystal structure of this adduct with the related Dpo4 enzyme

and an incoming dTTP exhibited two different conformations (*40*). In one, the benzo[*a*]pyrene diol was intercalated and the base pairing between dTTP and the terminal A was distorted such that only a single hydrogen bond was formed. In the other conformation, the benzo[*a*]pyrene diol was in the major groove and the face bearing the diol was packed perpendicular to the plane of the base pairs, while the nonpolar face was fully exposed to solvent. In this positioning, hydrogen bonding between the incipient base pair can occur normally. The authors suggested that the nucleotidyl transfer reaction could proceed in only this latter conformation and not when the adduct intercalates the DNA. Our experiments are in agreement with this hypothesis and suggest that this enzyme in general should be more proficient at processing adducts that can retain normal hydrogen bonding (and solvation) potential than those that cannot (such as ethenocytosine or ethenoadenine). Moreover, the high steric flexibility of the enzyme would allow differently shaped and sized damaged bases to proceed through the active site successfully. Recent studies of effects of temperature on the Dpo4 polymerase also point to flexibility as an important factor in processing a bulky lesion (*41*).

A similar assessment may be made about the ability of DNA pol II to process damaged DNA templates. Pol II can allow replication bypass for certain DNA lesions, including abasic sites, N^4-ethenocytosine, 2-acetylaminofluorene, and some cross-linking lesions (*6*, *42*–*44*). Aside from abasic sites, all of these lesions are polar and may not lead to disruption of electrostatic interactions between water and the template base, which our studies suggest are important.

Cellular studies using a phage replication assay in *E. coli* showed that the bacterium is able to replicate templates containing the variably sized nonpolar thymidine analogue series (*23*). It was found that the replication machinery processed non-hydrogen-bonding thymidine analogues with relatively high efficiency but that the efficiency and fidelity varied strongly with the incremental size changes. Indeed, the most efficient analogue (dL) was processed as thymine with relatively high fidelity of 1 error in 1000. The data presented here suggest that pol II and pol IV are most likely not the polymerases responsible for this activity, unless in vivo activity is very different from what we have observed in vitro. Pol I Klenow fragment was shown to insert dATP opposite dF and dL with high efficiency and fidelity (*11*, *23*), which suggests that pol I is a better candidate than the enzymes described above for the observed activity. It remains to be seen how the other *E. coli* polymerases (III and V) would respond to systematic changes in electrostatics and sterics in their active sites.

EXPERIMENTAL PROCEDURES

Nonpolar thymidine analogues were synthesized as reported previously (*21*, *22*). Pol II (exo−) and pol IV were purified as described previously (*3*, *27*). For steady-state single-nucleotide incorporation experiments, a 28-mer−23-mer template−primer duplex with the sequence (5′-TAATAC-GACTCACTATAGGGAGA)·(5′-ACTGXTCTCCCTAT-AGTGAGTCGTATTA) was used as a polymerase substrate for insertion kinetics. A 24-mer primer with the sequence 5′-TAATACGACTCACTATAGGGAGAA was used for extension kinetics. Primers were end-labeled with ^{32}P at the

5′ terminus with [γ-^{32}P]ATP (Amersham Bioscience) and T4 polynucleotide kinase (Invitrogen). Labeled primers were purified using spin columns (Bio-Rad). Primer−template solutions were annealed by making a 1:1 mixture of primer and template (4 mM each) in buffer containing 20 mM Tris (pH 7.5), 20 mM sodium glutamate, 4% (v/v) glycerol, 8 mM MgCl$_2$, and 5 mM DTT, then heating to 95 °C, and slowly cooling to 4 °C over 90 min. The primer−template solution was then mixed with a 4× enzyme solution to make a 2× primer−template−enzyme solution.

The primer−template−enzyme solution was mixed with a 2× dNTP solution, and the reaction mixtures were incubated at 37 °C. Reactions were quenched by addition of stop buffer (95% formamide containing 10 mM EDTA, 0.05% xylene cyanol, and 0.05% bromophenol blue). Typical reaction conditions used 1.5−29 μg/mL pol II or 2−40 μg/mL pol IV and a reaction time of 0.5−30 min.

Products were resolved from unreacted primer by 20% denaturing polyacrylamide gel electrophoresis and quantified by autoradiography using a phosphorimager (Molecular Dynamics). Spot intensities on the imaged gels were calculated using ImageQuant (Molecular Dynamics). Reaction velocity v for individual reactions was calculated by the equation $v = I_{n+1}/(I_n + I_{n+1})/t$, where I_n is the intensity of the unreacted primer band and I_{n+1} is the intensity of the product band. Velocities were normalized for enzyme concentration, and V_{max} and K_M values were determined from Hanes−Woolf plots.

SUPPORTING INFORMATION AVAILABLE

Representative gel data showing incorporation of unnatural dNTPs opposite an "A" template with pol II and pol IV (Figures S1 and S2) and representative incorporation data and Hanes−Woolf plots for pols II and IV (Tables S1−S13). This material is available free of charge via the Internet at http://pubs.acs.org.

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