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Low fidelity DNA synthesis by human DNA polymerase-η

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A superfamily of DNA polymerases that bypass lesions in DNA has been described[1–4]. Some family members are described as error-prone because mutations that inactivate the polymerase reduce damage-induced mutagenesis. In contrast, mutations in the skin cancer susceptibility gene *XPV*[5,6], which encodes DNA polymerase (pol)-η, lead to increased ultraviolet-induced mutagenesis[7–11]. This, and the fact that pol-η primarily inserts adenines during efficient bypass of thymine–thymine dimers *in vitro*[8,12,13], has led to the description of pol-η as error-free. However, here we show that human pol-η copies undamaged DNA with much lower fidelity than any other template-dependent DNA polymerase studied. Pol-η lacks an intrinsic proofreading exonuclease activity and, depending on the mismatch, makes one base substitution error for every 18 to 380 nucleotides synthesized. This very low fidelity indicates a relaxed requirement for correct base pairing geometry and indicates that the function of pol-η may be tightly controlled to prevent potentially mutagenic DNA synthesis.

The ability of pol-η to bypass efficiently a DNA adduct that usually blocks synthesis by other polymerases indicates that pol-η may have relaxed discrimination ability. To test this hypothesis, we examined the fidelity of human pol-η during copying of undamaged DNA. Recombinant pol-η containing a carboxy-terminal hexahistidine tag[5] was first examined for the presence of exonuclease activities using a 5′-end labelled template primer containing a 3′-terminal A·dGMP mismatch[14]. Neither 5′ → 3′ nor 3′ → 5′ exonuclease activities were detected (Fig. 1a, lane 2). Thus, pol-η lacks an intrinsic exonuclease activity that can proofread replication errors. We then investigated the fidelity of pol-η during synthesis to fill a five-nucleotide gap with an undamaged DNA template containing a TGA nonsense codon in the *LacZ* gene in M13mp2 DNA[15]. This substrate encodes a colourless M13 plaque phenotype, and errors are scored as blue revertant plaques when the DNA products are introduced into an α-complementation strain of *Escherichia coli* and plated on indicator plates.

Synthesis by pol-η generated gap-filled products (not shown, but see ref. 15 and below) whose revertant frequency was 8,400-fold higher than that of control DNA that had not been copied *in vitro* (Table 1). DNA sequence analysis revealed that 8 of 12 revertants contained a single base substitution and that the other 4 contained 2 substitutions among the 5 nucleotides copied. Both the high reversion frequency and the number of revertants containing multiple substitutions indicate that pol-η has low fidelity. The base substitution error rate calculated from these data is $3{,}100 \times 10^{-5}$ (1 error in 32 bases, 1/32). This rate is 24-fold higher than for human DNA polymerase-β (Table 1), the least accurate among wild-type DNA polymerases that have previously been studied.

To determine whether such low fidelity is site-specific or a general feature of synthesis by pol-η, we used a 407-nucleotide gap that permits detection of hundreds of different substitution, addition, deletion and complex errors generated when copying a 275-nucleotide sequence of the wild-type *LacZ* α-complementation gene[16]. Here, errors are scored as light-blue or colourless mutant plaques and the error specificity is defined by sequence analysis of independent *lacZ* mutants. Analysis of the DNA products of gap-filling synthesis revealed that pol-η filled the 407-nucleotide gap (Fig. 1b, lane 2). These products yielded a *lacZ* mutant frequency of 32%, a value that is unprecedented in comparison with the 0.1 to 3% values obtained with other wild-type, template-dependent DNA polymerases (Table 1). Similar values were observed for synthesis using 10- and 100-fold lower dNTP concentrations or for reactions containing 2- or 4-fold less pol-η. The average mutant frequency for six independent determinations was 34% (Table 1).

In contrast to the predominantly light-blue phenotypes of *lacZ* mutants generated by most other DNA polymerases using this assay, more than 95% of the pol-η-dependent *lacZ* mutants had a colourless rather than light-blue plaque phenotype. Sequence analysis of 24 *lacZ* mutants revealed that they contained from 3 to 22 changes per mutant. A total of 232 single base substitutions, 10 tandem double-base substitutions, 13 additions of 1–3 nucleotides and 32 deletions of 1–56 nucleotides were observed. The recovery of 232 single base substitutions among only 6,600 total nucleotides analysed (24 mutants × 275 nucleotides) yields an average base

Table 1 Fidelity of human pol-η

DNA polymerase	Polymerase family	3′-exo activity	Mutant frequency* (×10^{-4})	Average base substitution rate (×10^{-5})†
TGA codon reversion assay			0.05 (control‡)	
Pol-η	Rad30	no	420	3,100
Pol-β	Pol X	no	26	130§
Forward mutation assay			6 (control‡)	
Pol-η	Rad30	no	3,400	3,500
Pol-β	Pol X	no		67
Pol-α	Pol α	no		16
HIV-1 RT	RT	no		6
E. coli Kf pol	Pol I	no‖		2
Pol-δ	Pol α	yes		~1
Pol-ε	Pol α	yes		≤1
Pol-γ	Pol I	yes		≤1

* The reversion frequency is the average of two determinations; the forward mutant frequency is the average for six experiments where reaction conditions were varied and yielded the following mutant frequencies: 5 mM $MgCl_2$, 10 μM dNTPs: 32%; 5 mM $MgCl_2$, 100 μM dNTPs: 37%; 10 mM $MgCl_2$, 1 mM dNTPs: (18 nM pol-η) 39%, (36 nM pol-η) 37%, (72 nM pol-η) 28 and 32%. In each case, about 1,000 total plaques were scored.
† Error rates for the other DNA polymerases[14,15,27,28] are averages, which vary depending on the mismatch and its location.
‡ Control values are for DNA substrates not subjected to copying *in vitro*.
§ From ref. 16.
‖ The value for exonuclease-deficient Klenow fragment[14].

substitution error rate of 3.5% (Table 1), which is one error for every 28 nucleotides polymerized. We recovered errors consistent with formation of 11 of the 12 possible mispairs (Table 2). Among the 12 mispairs, error rates ranged from 1/18 for a T·dGMP mispair to greater than 1/1900 for a C·dCMP mispair. These values represent the average rates for 68, 67, 61 and 79 template positions for A, T, G and C, respectively (Table 2). The overall average rate of one in 28 is more than 3,000-fold higher than that of eukaryotic replicative DNA polymerases-δ, -ε, and -γ, for results obtained using the same assay. The lower fidelity of pol-η is not merely due to the lack of a proofreading exonuclease activity, as pol-η is 50- to 1,700-fold less accurate than four other exonuclease-deficient DNA polymerases measured with the same assay (Table 1). Further evidence that pol-η has unusually low fidelity when copying undamaged DNA is the recovery of 10 tandem double base substitutions among only 24 mutants. Such mutations require three consecutive rare events—an initial misinsertion, a second misinsertion using a mismatched template primer, and correct extension from a doubly mismatched template primer. The data in Table 1 reveal that pol-η is much less accurate than are representatives of each of the four other DNA polymerase families.

We next examined the infidelity of pol-η with an independent experimental approach, steady-state kinetic analysis (Table 3). Pol-η inserted correct dAMP opposite template T and correct dCMP opposite template G with catalytic efficiencies (k_{cat}/K_m) that are similar to that observed with HIV-1 reverse transcriptase (RT) (Table 3). Thus, pol-η has robust catalytic efficiency for formation of correct T·A and G·C base pairs. However, it also has low nucleotide selectivity, as shown by the high rate of misinsertion of incorrect dGMP or dCMP opposite template T, or of incorrect dGMP opposite template G (Table 3). Note that discrimination is low because of efficient insertion of incorrect nucleotides, rather than inefficient insertion of correct nucleotides. The misinsertion rates ($1/f_{ins}$) for these three mispairs at these two template locations are remarkably similar to the average error rates for the same mispairs observed during gap-filling synthesis (Table 2). Moreover, the pol-η misinsertion rate for the T·dGMP mispair is much higher than that of HIV-1 RT (Table 3), again consistent with the results of gap-filling reactions (Table 1). Thus, an independent experimental approach confirms the low fidelity of pol-η. This may be a common property of pol-η regardless of source, as yeast pol-η also appears to have low nucleotide insertion fidelity with undamaged templates[17].

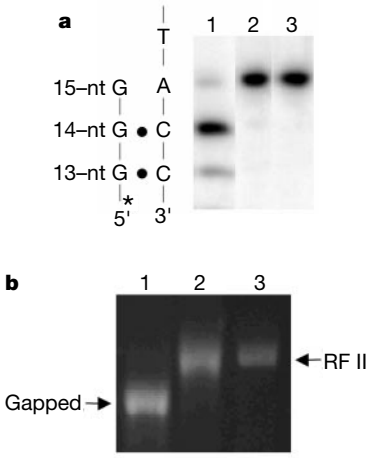

Figure 1 Exonuclease activities and gap-filling synthesis by pol-η. **a**, Exonuclease assay using Klenow fragment (lane 1), pol-η (lane 2) or no enzyme (lane 3). The total pixels in the lanes were similar, indicating no detectable 5′-exonuclease. The n − 1 and n − 2 products generated by pol-η indicate a specific exonuclease activity (% digestion per pmol per min) 80-fold lower than for Klenow fragment, which has modest proofreading capacity[14]. **b**, Products of gap-filling synthesis examined by agarose gel electrophoresis[16]. The reaction included no enzyme (lane 1), pol-η (lane 2) or nicked, double-stranded (RFII) DNA standard (lane 3). nt, nucleotide. Asterisk, ^{32}P labelling.

Table 2 Base substitution error rates of pol-η

Base	Number* (total)†	Mutation from → to	Mispair template·dNMP	Observed substitutions	Error rate
A	68	A → G	A·dCMP	17	1/96
	(1,632)	A → T	A·dAMP	26	1/63
		A → C	A·dGMP	21	1/78
T	67	T → C	T·dGMP	91	1/18
	(1,608)	T → A	T·dTMP	13	1/120
		T → G	T·dCMP	8	1/200
G	61	G → A	G·dTMP	21	1/70
	(1,464)	G → C	G·dGMP	4	1/370
		G → T	G·dAMP	7	1/210
C	79	C → T	C·dAMP	19	1/100
	(1,896)	C → G	C·dCMP	0	≤1/1,900
		C → A	C·dTMP	5	1/380

* The number of template bases within the 275-base *LacZ* target sequence.
† The total number of template bases in 24 sequenced mutants.

Table 3 Kinetic analysis of misinsertion by pol-η

Enzyme	Template nucleotide	dNTP	K_m (μM)	k_{cat} (min^{-1})	(k_{cat}/K_m) (μM min^{-1} × 10^3)	f_{ins} × 10^{3}*
Pol-η	T	dATP	31 ± 6	13 ± 3.2	420 ± 130	—
	T	dGTP	250 ± 84	5.5 ± 2.2	22 ± 10	52 (1/19)
	T	dCTP	2,900 ± 1,600	4.8 ± 0.7	1.6 ± 0.9	3.9 (1/260)
	G	dCTP	11 ± 1.9	8.5 ± 1.9	760 ± 210	—
	G	dGTP	450 ± 111	3.9 ± 0.4	8.7	11† (1/90)
HIV-1 RT	T	dATP	0.20 ± 0.05	0.16 ± 0.01	800	
	T	dGTP	140 ± 24	0.01 ± 0.0005	0.07	0.1 (1/10,000)

Reactions were performed as described in Methods and kinetic constants were derived as described[26].
* f_{ins} is defined as $(k_{cat}/K_m)_{incorrect}/(k_{cat}/K_m)_{correct}$. For ease of comparison with the error rates in Table 2, values for $1/f_{ins}$ are shown in parentheses. The results for HIV-1 RT are from ref. 26.
† Some of this insertion could be templated by the adjacent 5′-template C, through misalignment.

Several possibilities exist to reconcile the observation that pol-η has low fidelity but is also involved in a process that reduces ultraviolet-induced mutagenesis. Pol-η synthesis past lesions could be more accurate than copying of undamaged DNA, or fidelity may be enhanced by replication accessory proteins. Alternatively, pol-η misinsertions could be proofread by a separate exonuclease[18] or removed by DNA mismatch repair[19]. The low fidelity of pol-η during copying of undamaged DNA and its ability to fill gaps typical of those generated during base and nucleotide excision repair suggest that controls may have evolved to limit synthesis by pol-η. The opportunity to generate errors *in vivo* may be limited by the need to form partnerships with specific replication accessory proteins[1–3], and pol-η may only synthesize enough nucleotides to generate a duplex template primer that can be elongated by other, more accurate DNA polymerases. Pol-η function may also be controlled at the level of messenger RNA synthesis[20] or stability, post-translational modification or protein stability. The latter possibility is consistent with the mutator phenotype observed upon disruption of the yeast *RAD6* gene[21]. RAD6 is a ubiquitin conjugase and its inactivation could result in inefficient turnover of yeast pol-η, resulting in enhanced mutagenesis owing to inaccurate DNA synthesis. Indeed, precedents exist for enhanced mutagenesis resulting from an excess of a polymerase. Elevated spontaneous mutation rates have been observed upon overexpression of a mutator mutant of pol-β[22], the *E. coli* DinB polymerase[23] or the murine DINB1 polymerase[4]. *E. coli* DinB polymerase and DINB1 are members of the superfamily of DNA polymerases to which human pol-η belongs. Structural and functional studies of the other four DNA polymerase families reveal a strict requirement for correct Watson–Crick base pair geometry (reviewed in ref. 24). Those enzymes copy undamaged templates with higher fidelity than pol-η, and they have limited ability to bypass bulky adducts. The lower fidelity of pol-η (Table 2) implies that it may incorporate nucleotides that are primarily stabilized by base–base hydrogen bonding and/or stacking interactions and that it may not require strict shape complementarity to bypass bulk lesions efficiently. In fact, there may be an inverse relationship between lesion bypass efficiency and nucleotide selectivity. This hypothesis can be tested by examining the fidelity of other enzymes that bypass lesions, such as pol-ζ, which also bypasses thymine–thymine dimers but at a lower efficiency[25]. □

Methods

Assay for exonuclease activities

Reactions (25 μl) contained 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 10 mM DTT, 6.25 μg BSA, 60 mM KCl, 2.5% glycerol, 2.8 pM 5′-^{32}P-labelled template primer DNA containing a 3′-terminal A·G mismatch[14], and either 96 nM pol-η or 150 nM of exonuclease-proficient Klenow polymerase (Kf). Reactions were incubated at 37 °C for 5 min (Kf) or 30 min (pol-η) and examined by gel electrophoresis. The products were quantified by phosphoimagery. A reduction in total signal intensity indicates the presence of 5′-nuclease activity. Products shorter than 15 nucleotides indicate the presence of 3′-exonuclease activity, with a product of 14 bp indicating excision of the terminal mismatch.

Gap-filling synthesis and fidelity measurements

The sources and descriptions of strains and reagents, and preparation of substrates were as described[15,16]. Reactions (25 μl) contained 40 mM Tris-HCl (pH 8.0), 10 mM DTT, 6.25 μg BSA, 60 mM KCl, 2.5% glycerol, 10 mM $MgCl_2$, 1 mM dNTPs (forward assay) or 0.5 mM dNTPs (reversion assay), 1.4 nM M13mp2 DNA containing a 407-nucleotide gap (from nucleotides, –216 to +191 of the *LacZ* gene), and 72 nM pol-η (about 50:1 ratio of enzyme to DNA). Reactions were incubated at 37 °C for 1 h and terminated by adding EDTA to 15 mM. DNA products were processed for mutant frequencies as described[15,16]. As a control, when pure heteroduplex DNA containing a single mismatch is introduced into the *E. coli* strain used to score polymerase errors, the percentage of M13 plaques having the phenotype of the (newly-synthesized) minus strand is typically about 60% (ref. 16). However, we have previously shown that minus-strand expression values decrease as the number of unpaired nucleotides in the minus strand increases. The high frequency of forward mutants observed for the pol-η products (Table 1) and the multiple mutations in each *lacZ* mutant indicate that all minus strand products of pol-η gap-filling that survived and were expressed in *E. coli* containing errors made by pol-η. Consistent with this, when the DNA from six dark blue M13 clones obtained from pol-η reactions were sequenced, no mutations were found.

Kinetic analysis of misinsertion

Reactions (25 μl) were as for gap-filling, except for use of 200 nM 30-nucleotide template primed at a 1.2 to 1 molar ratio with a 5′-^{32}P-labelled 20 bp oligonucleotide complementary to nucleotides 88–107 or 89–108 of the *lacZ* gene, 2 nM pol-η, and correct dATP or dCTP at concentrations of 1 to 100 μM or incorrect dGTP or dCTP at concentrations of 5 to 3,000 μM. With one exception (one determination with the G·G mismatch), duplicate determinations were performed for each template·dNTP combination at seven different dNTP concentrations. Aliquots were removed at 2, 4, 6 and 8 min and the products separated by gel electrophoresis and quantified by phosphoimagery. Kinetic constants were derived as described[26].

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