pol ι , a remarkably error-prone human DNA polymerase

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The *Saccharomyces cerevisiae* RAD30 gene encodes DNA polymerase η . Humans possess two Rad30 homologs. One (RAD30A/POLH) has previously been characterized and shown to be defective in humans with the Xeroderma pigmentosum variant phenotype. Here, we report experiments demonstrating that the second human homolog (RAD30B), also encodes a novel DNA polymerase that we designate pol ι . pol ι is a distributive enzyme that is highly error-prone when replicating undamaged DNA. At template G or C, the average error frequency was $\sim 1 \times 10^{-2}$. Our studies revealed, however, a striking asymmetry in misincorporation frequency at template A and T. For example, template A was replicated with the greatest accuracy, with misincorporation of G, A, or C occurring with a frequency of $\sim 1 \times 10^{-4}$ to 2×10^{-4} . In dramatic contrast, most errors occurred at template T, where the misincorporation of G was, in fact, favored $\sim 3:1$ over the correct nucleotide, A, and misincorporation of T occurred at a frequency of $\sim 6.7 \times 10^{-1}$. These findings demonstrate that pol ι is one of the most error-prone eukaryotic polymerases reported to date and exhibits an unusual misincorporation spectrum *in vitro*.

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All organisms duplicate their genomic DNA using a highly processive and accurate DNA polymerase (Kornberg and Baker 1992; Kelman and O'Donnell 1995; Hub-scher et al. 2000). In addition to their main replicase, many organisms possess additional DNA polymerases that fulfill vital roles in DNA synthesis and/or in DNA repair. Recently, a new class of DNA polymerase has been reported that appears to be used to replicate genomic DNA when the cell's replicase is unable to do so (Tang et al. 1998, 1999, 2000; Reuven et al. 1999; Johnson et al. 1999c; Masutani et al. 1999a; Wagner et al. 1999; Woodgate 1999). Indeed, it is thought that the primary role of this so-called UmuC/DinB/Rev1/Rad30 super-family of DNA polymerases is to facilitate translesion DNA synthesis of otherwise replication-blocking lesions. However, it is very likely that they are also capable of acting on undamaged DNA (Fijalkowska et al. 1997; Tang et al. 1998, 2000; Wagner et al. 1999; Maor-Shoshani et al. 2000). Whether this mode of replication is considered error-free or error-prone depends on the polymerase used and/or the particular DNA lesion encountered. Some, like the UmuD'₂C complex (*Escherichia coli* pol V) are intrinsically error-prone whereas others, like *Saccharomyces cerevisiae* and human pol η , are considered to be error-free when they perform translesion

replication of a *cis-syn* thymine-thymine dimer or an acetylaminofluorene guanine (AAF-G) adduct (McDonald et al. 1997; Johnson et al. 1999c; Masutani et al. 1999b; Yuan et al. 2000; Washington et al. 2000).

The importance of this class of enzymes is obvious at both ends of the evolutionary spectrum. In *E. coli*, cells carrying a deletion of the *umuDC* operon are unable to bypass many types of lesions in single-stranded DNA (Szekeres et al. 1996; Lawrence et al. 1996; Koffel-Schwartz et al. 1996; Napolitano et al. 1997) and, as a consequence, do not exhibit the large increase in mutagenesis normally seen in wild-type bacteria exposed to a variety of mutagens (Kato and Shinoura 1977; Steinborn 1978; Woodgate 1992). In addition, the spontaneous mutation rate in *umuDC* cells is also three- to fivefold lower than wild-type cells (Sargentini and Smith 1981; Woodgate 1992) indicating that *E. coli* polV also plays an important role in spontaneous mutagenesis. In humans, individuals expressing a severely truncated (and apparently inactive) pol η are said to have the Xeroderma pigmentosum variant (XP-V) phenotype (Masutani et al. 1999b; Johnson et al. 1999a, 2000b). These patients exhibit sunlight sensitivity and a concomitant increased incidence of cancers (predominantly of the skin), and their cultured cells exhibit altered mutation spectra (Cleaver and Carter 1973; Wang et al. 1993; Raha et al. 1996; McGregor et al. 1999; Kuwamoto et al. 1999).

Recent phylogenetic studies suggest that many organisms possess multiple homologs of the UmuC/DinB/

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Rev1/Rad30 family of DNA polymerases (McDonald et al. 1999; Gerlach et al. 1999; Ogi et al. 1999). For example, humans have at least four. These include a homolog of Rev1 (Lin et al. 1999); one is the aforementioned pol η (encoded by the human *POLH* gene and also known as *XPV* and *RAD30A*); another is a second homolog of *S. cerevisiae* *RAD30*, termed *RAD30B* (McDonald et al. 1999); finally, the last homolog is more closely related to the *E. coli* *dinB* (pol IV) gene and has been called *DINB1* (Gerlach et al. 1999; Ogi et al. 1999).

Given the relationship of the last two homologs to pol η and pol IV, respectively, it seemed reasonable to predict that both the human *RAD30B* and *DINB1* genes encoded novel DNA polymerases (Woodgate 1999). Indeed, such an activity has very recently been shown for *DINB1* (Johnson et al. 2000a; Ohashi et al. 2000). In this manuscript, we present data demonstrating that the Rad30B protein is also a DNA polymerase. After consultation with the Human Genome Organization (HUGO) nomenclature committee (<http://www.gene.ucl.ac.uk/nomenclature/>), it has been agreed that the *RAD30B* gene should be renamed *POLI* (for DNA polymerase iota). We report here, an initial characterization of pol ι and demonstrate that the polymerase is highly error-prone when replicating undamaged DNA. Surprisingly, the fidelity of the enzyme was strongly influenced by the template nucleotide being replicated. Possible roles for this novel error-prone DNA polymerase within the cell are discussed.

Results

Overproduction and purification of wild-type and mutant pol ι

To test the hypothesis that Rad30B might possess intrinsic DNA polymerase activity, we fused the human *RAD30B* gene in-frame with the *glutathione S-transferase* (GST) gene. The chimeric GST-Rad30B protein (~108 kD) was expressed in insect cells and subsequently purified by GST-affinity and hydroxylapatite ion-exchange chromatography. We estimate that the purity of the GST-Rad30B fusion protein after these columns to be >90% (Fig. 1A). However, it should be noted that we also observed small quantities of two lower molecular weight proteins in our preparation. We believe that these are amino-terminal proteolytic fragments of the fusion protein, as they were much more intense in earlier preparations that lacked protease inhibitors. Furthermore, they did not cross-react with antibodies raised to the carboxyl terminus of the Rad30B protein. Such truncations are apparently quite common in the Rad30-like polymerases as they were also observed in preparations of the related pol η and pol κ enzymes (Masutani et al. 1999a; Ohashi et al. 2000). Fractions from the hydroxylapatite column were assayed for DNA polymerase activity and the fraction with the highest purity and greatest activity was used for subsequent studies (Fig. 1B). Replication was refractory to inhibition by aphidicolin (100 μ M), low levels of dideoxy nucleotides and low levels of N-ethylma-

Figure 1. (A) Purity of wild-type and mutant forms of GST-tagged pol. Purified wild-type and mutant GST-tagged pol fusion proteins (~250 ng) was run on a 4%–20% polyacrylamide-SDS gel and visualized after staining with Coomassie brilliant blue R-250. (S) 10 kD molecular weight protein ladder (Life Technologies, MD); the migration of the 20, 40, 60, and 100 kD markers are indicated at left; (wt) wild-type GST-pol preparation used in the replication experiments; (m) D126A-E127A double alanine substituted mutant GST-pol protein. (B) DNA polymerase activity of the purified proteins. The position of the unextended radiolabeled primer (*P) as well as the products (P + 1, P + 2, etc.) are noted at the side of the figure. Reaction mixes were incubated at 37°C for 30 min and assays were performed in the absence of dNTPs (0) or in the presence of all four dNTPs (4). The lack of any discernable primer degradation in the absence of dNTPs suggests that pol does not possess any 3'–5' exonucleolytic activity.

leimide (NEM; 100 μ M) but was completely abolished in the presence of 1 mM NEM. To confirm that the polymerase activity is, in fact, intrinsic to the purified fusion protein, we were interested in analyzing a mutant variant that might lack the observed polymerase activity. Conserved motifs can be found in all of the UmuC/DinB/Rev1/Rad30 superfamily of proteins (Kulaeva et al. 1996; McDonald et al. 1999; Masutani et al. 1999b; Johnson et al. 1999b) and, in particular, two adjacent invariant aspartate and glutamate residues within motif III are believed to be critical for the catalytic activity of these proteins, as they are most likely involved in the chelation of divalent Mg^{2+} ions necessary for polymerase activity (Kulaeva et al. 1996). In support of this hypothesis, mutations at these residues have been shown to result in a complete loss of polymerase activity in *E. coli* UmuC (Tang et al. 1999; Reuven et al. 1999), *E. coli* DinB (Wagner et al. 1999), *S. cerevisiae* Rad30 (Johnson et al. 1999b,c) and human pol κ (Ohashi et al. 2000). As a consequence, we changed the appropriate Asp-126 and Glu-127 residues of *RAD30B* to alanine. The mutant Rad30B-GST fusion protein had similar physical properties to the wild-type protein and was purified to approximately the same degree after the GST- and hydroxylapatite columns (Fig. 1A). Unlike the wild-type protein, however, the mutant variant exhibited no DNA polymerase activity (Fig. 1B). Our observations suggest,

therefore, that the polymerase activity observed with the wild-type protein is intrinsic to the chimeric GST-Rad30 protein. We propose to rename Rad30B as DNA polymerase iota (*poli*) and the gene will henceforth be referred to as *POLI*.

Poli is a highly error-prone DNA polymerase

As noted above, in our replication assays, we found that in the presence of all four dNTPs *poli* extended the radiolabeled primer by an average of 2–3 nucleotides (Fig. 1B). In the absence of dNTPs there was no significant degradation of the annealed primer, suggesting that *poli* lacks any associated 3′–5′ exonucleolytic activity (Fig. 1B). Interestingly, closer inspection of the replication products (P + 1, P + 2, etc.) revealed that each contained multiple gel bands corresponding to the slightly different electrophoretic mobility of each nucleotide added to the primer. This observation suggests that in addition to the correct nucleotide, *poli* is able to misincorporate incorrect nucleotides with relatively high efficiency. We investigated this further by performing reactions in the presence of individual dNTPs (Fig. 2). In these reactions, the respective oligonucleotide templates are virtually identical, differing only at the template nucleotide immediately downstream from the annealed primer (Fig. 2). This allows us to measure the incorporation of each nucleotide from the same “standing-start” primer. In most cases, it appeared that *poli* preferentially inserted the correct nucleotide opposite the complementary template base as was evident by the increased amount of primer utilization. However, there was also significant misincorporation of incorrect nucleotides at all template sites (Fig. 2). In particular, misincorporation of G at template T appeared to be better than, the correct nucleotide, A. *poli* also appears able to extend some of these mispairs. For example, in addition to correctly inserting G opposite C, *poli* appeared to efficiently misincorporate

G opposite T and then extend this mispair by adding the correct next nucleotide of G opposite C (see Fig. 2; dGTP track with template C). This observation is quite remarkable as it means that *poli* can not only misinsert an incorrect base but is able to elongate from a mismatched base efficiently, a property not usually associated with a high fidelity enzyme.

To accurately determine the fidelity of *poli*, we used a steady-state gel assay that allows one to calculate the apparent $V_{\max}$ and K_m of the enzyme with respect to the enzyme’s usage of the correct and incorrect nucleotide and thereby determine the frequency at which a misincorporation is likely to occur (Boosalis et al. 1987; Creighton et al. 1995) (Table 1). In these experiments, the same oligonucleotide templates used in Figure 2 were used and the ability of a limiting amount of *poli* to extend the primer in the presence of varying concentrations of each correct or incorrect dNTP was measured. Under these conditions, we found that for template A, G, and C, incorporation of the correct nucleotide was favored over the incorrect nucleotide (Table 1). The exception was, quite strikingly, misinsertion of G opposite template T, where the misinsertion was favored ~3:1 over the correct nucleotide, A (Fig. 3; Table 1). Of all the mispairs, G·T is most readily formed (Mendelman et al. 1989). However, misincorporation frequencies of G opposite T for most replicative polymerases are usually in the range of 10^{-3} to 10^{-5} (Mendelman et al. 1989) and it is certainly not favored over the correct nucleotide. Although as noted above, the correct nucleotide was generally favored over the incorrect dNTP at the remaining template sites, discrimination was particularly poor, with error frequencies generally ranging from 6.7×10^{-1} for the T·T mispair to 6.3×10^{-3} for the A·C mispair (Table 1). The exception was at template A, where *poli* appears to be relatively accurate, incorporating dTTP efficiently and misincorporating G, A, or C at a frequency of 1×10^{-4} to 2×10^{-4} (Fig. 3; Table 1). The reason for this

Figure 2. Ability of *poli* to incorporate nucleotides at various template sites. The extent of *poli*-dependent (mis)incorporation was measured at each template site in the absence (0) or presence of all four dNTPs (4) or presence of each individual dNTP (100 μ M) (G, A, T, C). Reactions were for 30 min at 37°C. The sequence context of each template is given above each panel. In particular, note the very efficient misincorporation of G opposite T.

Table 1. Fidelity of human pol λ as determined by steady state kinetics^a

dNTP · Template	$V_{\max}/K_m$ ($M^{-1} \min^{-1}$)	f_{inc}^b
dGTP·G	0.005	6.5×10^{-3}
dATP·G	0.02	2.2×10^{-2}
dTTP·G	0.100	1.3×10^{-1}
dCTP·G	0.76	1
dGTP·A	0.008	1.0×10^{-4}
dATP·A	0.02	2.0×10^{-4}
dTTP·A	73.5	1
dCTP·A	0.009	1.0×10^{-4}
dGTP·T	0.33	3
dATP·T	0.11	1
dTTP·T	0.078	6.7×10^{-1}
dCTP·T	nd ^c	$<1.0 \times 10^{-5}$
dGTP·C	3.0	1
dATP·C	0.019	6.3×10^{-3}
dTTP·C	0.023	7.7×10^{-3}
dCTP·C	0.022	7.4×10^{-3}

^aThe data were derived from experiments similar to that depicted in Fig. 3. However, in these experiments the length of the reaction time was varied considerably, to ensure that primer utilization was measurable in the inefficient reactions, yet was kept to <20% in those that were more efficient. The reaction times were as follows: C, incorporation of dGTP was 2 min and of dATP, dTTP, or dCTP was 10 min; T, incorporation of dGTP, dATP, or dTTP was 2 min and of dCTP was 10 min; A, incorporation of dTTP was 2 min of dGTP or dATP was 10 min, and of dCTP was 15 min; G, the reaction time for the incorporation of dCTP was 2 min and misincorporation of dGTP, dATP, and dTTP was 10 min. Note that the $V_{\max}/K_m$ is given in $M^{-1} \min^{-1}$, and therefore takes into account the differing reaction times.

^bThe nucleotide misincorporation ratio, $f_{inc} = (V_{\max}/K_m)_{\text{incorrect}} / (V_{\max}/K_m)_{\text{correct}}$.

^c(nd) Not detected.

accuracy is the especially high $V_{\max}$ and low K_m of dTTP incorporation opposite template A. Interestingly, the fact that pol λ prefers to use dTTP is also apparent at other template sites. With the exception of the G·T mispair noted above, the most frequent mispairs were T·T (6.7×10^{-1}) and T·G (1.3×10^{-1}).

Thus, pol λ is highly error-prone compared to most replicative enzymes and even appears to have slightly lower fidelity than that recently reported for the related human pol η (Matsuda et al. 2000). Perhaps the most striking aspect of these studies is the observation that the fidelity of pol λ is unusually template dependent, being relatively accurate at template A and highly inaccurate at the complementary template T. Nearest-neighbor effects on polymerase insertion fidelity have been observed with all enzymes studied to date (e.g., see Mendelman et al. 1989). It is likely, therefore, that the same will be true for pol λ . In an attempt to address this issue, we are presently analyzing the fidelity of pol λ at a variety of different template sites. It should be stressed, however, that any such nearest-neighbor effects are unlikely to change the major conclusion of this present work, namely that pol λ is a remarkably error-prone DNA polymerase.

pol λ is a distributive enzyme

Based on our initial observations (Fig. 2) pol λ appears to be a distributive polymerase as even in the presence of relatively high levels of the enzyme and all 4 nucleotides, the primer was, on average, only extended 2–3 nucleotides. Although such properties would be consistent with the reported activities of the related pol η and pol κ enzymes (Masutani et al. 1999a; Washington et al. 1999; Ohashi et al. 2000), we considered the possibility that this distributive mode of synthesis occurred as a result of the use of the relatively short synthetic oligonucleotide template (40-mer) in the replication assays. To test this hypothesis, we compared the ability of pol λ to extend the same radiolabeled primer when annealed to a larger (7.2 kb), circular single-stranded template, M13mp19, or the linear 40-mer oligonucleotide. However, both the shorter oligonucleotide template and the larger M13 DNA gave qualitatively similar results with the primer only extended 2–3 nucleotides.

The ability of pol λ to accurately incorporate T opposite template A (Fig. 3; Table 1) prompted us to investigate whether this propensity might affect the distributive nature of pol λ . To examine this possibility, we synthesized a new 40-mer oligonucleotide template that contained a short run of six As immediately downstream from the primer and assayed the ability of decreasing amounts of pol λ to extend a fixed amount of primer–template [either the wild-type or poly(A) template] (Bambara et al. 1995; Fig. 4). Under conditions where we had a twofold molar excess of enzyme to primer/template, we only observed

Figure 3. Kinetic analysis of pol λ -dependent (mis)incorporation at template T and template A. The ability of pol λ to incorporate the correct or incorrect nucleotide at both template sites was assayed at varying concentrations of dNTPs in a 5-min reaction, to allow a direct visual comparison of (mis)insertion fidelity. In most cases, the dNTP concentration varied from 0.1 to 100 μM . The exception was the incorporation of dTTP opposite template A, where the range was 1 nM to 1 μM . The sequence context of each template is given above each respective panel.

Figure 4. *pol*₁ is a distributive enzyme. The ability of *pol*₁ to extend the primer annealed to the wild-type template or the poly(A) template was assayed over a range of primer/template to enzyme concentrations. The primer/template was kept fixed at 20 nM and the enzyme varied from 40 nM to 0.4 nM as indicated. Reactions contained all four dNTPs (100 μ M each) and were performed for 30 min at 37°C.

the addition of three nucleotides with the wild-type template but eight with the poly(A) template. Interestingly, the replication pattern observed on the poly(A) track in the presence of all four dNTPs or dTTP alone was virtually identical, suggesting that *pol*₁ preferentially used dTTP even when given a pool of dNTPs. Despite the fact that synthesis was clearly much more efficient than on the wild-type template, it still appeared distributive as is evident by the appearance of pause sites at each template A replicated. At lower enzyme to primer/template ratios, we only observed the addition of a single nucleotide on the wild-type template, but faint bands were visible at P + 2 and P + 3 with the poly(A) template (Fig. 4). Thus, *pol*₁ is a distributive enzyme, but the number of nucleotides replicated in one binding/extension event appears to be template dependent, varying from 1 to 3 nucleotides.

Discussion

*Preliminary characterization of human pol*₁

Based on the homology of the deduced primary amino acid sequence of the *POL1* (*RAD30B*) gene to *S. cerevisiae* *RAD30* and human *POLH* (*RAD30A/XPV*) we previously hypothesized that it would encode a novel DNA polymerase (McDonald et al. 1999). The experiments outlined in this paper support such an hypothesis and detail the preliminary characterization of human DNA polymerase *pol*₁. Based on these findings, we believe that *pol*₁ is unlike any other human DNA polymerase reported to date, in that it is an extraordinarily low-fidelity enzyme. Remarkably, at template T, *pol*₁ prefers to misinsert G rather than the “Watson-Crick” complementary base, A. At template G, T, or C, most mispairs occur

with a frequency ranging from 6.7×10^{-1} to 6.3×10^{-3} . One interesting property of *pol*₁ is its preferred utilization of dTTP. As a consequence, misincorporations at template A are relatively rare compared to other sites, occurring in the range of 1×10^{-4} – 2×10^{-4} which is similar to other DNA polymerases such as *pol* α (Mendelman et al. 1989, 1990) or *pol* β (Boosalis et al. 1989; Chagovetz et al. 1997).

*pol*₁ is a highly distributive enzyme, meaning that the activity of this error-prone polymerase is likely to be limited to just 1–2 base pairs. However, the apparent enzyme activity also appears to be strongly stimulated by the template sequence, with the greatest activity on short homopolymeric runs of template A (Fig. 4). By comparison, we have also noticed that when replicating templates with a random DNA sequence, *pol*₁ tends to pause/dissociate more frequently at template G than at any other base (Fig. 2, cf. templates with all 4 dNTPs).

*Speculations as to a cellular role for error-prone pol*₁

In general, cells prefer to use a highly accurate DNA polymerase to replicate their DNA, so they presumably must possess a variety of mechanisms to limit the activity of *pol*₁ in vivo. Conceivably, such regulation might occur through additional protein–protein interactions (that are lacking in our in vitro assay) that could increase its in vivo fidelity. Alternatively, the in vivo activity of *pol*₁ may be strictly limited by targeting the enzyme for rapid proteolytic degradation. Such regulation may, in fact, be common to Rad30-like enzymes, as in addition to *pol*₁ (Fig. 1), the related *pol* η and *pol* κ also appear susceptible to proteolysis (Masutani et al. 1999a; Ohashi et al. 2000). Given the fact that homologs of human *POL1* have been identified in many higher eukaryotes including mice (McDonald et al. 1999) and the recently sequenced *Drosophila melanogaster* genome (Adams et al. 2000), the presence of error-prone *POL1* must presumably provide some evolutionary advantage to these organisms. Indeed, there are special cases where a low-fidelity polymerase might be useful. A good example is the generation of mutations in the variable region of human immunoglobulin (IgV) genes, as it would provide the necessary genetic diversity required for an efficient immune response. The mechanism of this somatic hypermutability is still poorly understood (for recent reviews, see Harris et al. 1999; Spencer and Dunn-Walters 1999). However, it does not seem to require nucleotide excision repair (Jacobs et al. 1998) and mismatch repair appears to operate after the mutational event rather than cause it (Winter et al. 1998; Kim et al. 1999). The prevailing model is that the hypermutability occurs as a result of the action of an error-prone DNA polymerase (Diaz and Flajnik 1998; Diaz et al. 1999). Interestingly, the error-rate over the 1.5–2 kb variable region in rearranged IgV gene is estimated to be between 10^{-2} and 10^{-3} and is certainly within the range of that described here for *pol*₁. In general, transitions are favored 2:1 over transversions, but perhaps more importantly, there is a strand bias in that mutations from T occur with a much lower

frequency than the complementary A (Yelamos et al. 1995; Smith et al. 1996). Such findings have previously been thought to indicate a requirement for transcription in the hypermutability process (Yelamos et al. 1995). We would like to suggest, however, that an involvement of error-prone pol η in the generation of somatic hypermutations might help explain this strand bias. For example, our observation that pol η is relatively accurate when replicating template A means that fewer mutations from T will occur, whereas the fact that pol η is much more error-prone when replicating template T, means that there are likely to be many more mutations from A. Furthermore, the fact that under certain conditions pol η is also able to extend one or more mispaired nucleotides (Fig. 2), suggests that it might also contribute to the tandem mutations observed in the IgV gene that are revealed in mismatch-repair deficient mice (Winter et al. 1998).

Another hypothesis is that somatic hypermutability requires separate AT/GC mutators (Spencer et al. 1999). Based on a recent literature search, humans now appear to possess at least 12 DNA polymerases. Many of these are considered to be error-prone and might also be candidates for one of the aforementioned mutators. The best studied is pol β , which although required for base-excision repair does not appear to be involved in somatic hypermutation (Esposito et al. 2000). Although *S. cerevisiae* pol ζ is error-prone when replicating damaged DNA (Nelson et al. 1996), the human equivalent has yet to be characterized. Human pol η is also apparently error-prone on undamaged DNA (Johnson et al. 2000b; Matsuda et al. 2000), however humans with defects in pol η (XP-V patients), apparently do not exhibit any major immunological defect, suggesting that like pol β , pol η is not involved in somatic mutagenesis. Other potential candidates for the presumed error-prone polymerase appear to be the recently described pol κ (Ohashi et al. 2000), the pol β -like enzyme, pol λ (Genbank accession no. AJ131890) and the terminal-transferase-like, pol μ (Dominguez et al. 2000). Although in vitro experiments provide clues as to the involvement of error-prone DNA polymerases in this fascinating process, a more definitive answer will clearly be found in transgenic mice that lack pol η or one of the aforementioned error-prone DNA polymerases and such experiments are currently in progress in a number of laboratories.

Another possible role for pol η is that of a back-up to pol η . The primary role for human pol η is to accurately replicate the major UV-induced DNA lesion, a *cis-syn* T-T dimer (Masutani et al. 1999a,b; Washington et al. 2000). Humans lacking pol η show a greatly reduced capacity to replicate past the T-T dimer (Masutani et al. 1999a), but the little replication that does traverse the lesion is apparently error-prone, as XP-V patients exhibit an increased rate of cancers and cultured cells exhibit a very different mutation spectrum from wild-type cells. The majority of the mutations in XP-V cells are C-A and T-A transversions (Wang et al. 1993; McGregor et al. 1999). This would be consistent with incorporation of dTTP opposite the C or T of a pyrimidine dimer. pol η appears to use dTTP efficiently and it will be interesting

to determine if such a preference is observed at *cis-syn* pyrimidine dimers, if so, it is possible that pol η is the DNA polymerase that generates the mutations that ultimately lead to a higher rate of cancers in XP-V patients.

Materials and methods

Overproduction of wild-type and mutant forms of GST-tagged pol η

The baculovirus expression vector used in these studies was pAcG2T (PharMingen, La Jolla, CA). A chimeric *GST-POL* gene was constructed by first synthesizing two oligonucleotides pAcG2T5, 5'-CGGGATCCCAGGCCTGG GCCATGGACCC-GGGCGTACGTTCGACCTGCAGAAATTCGG-3' and pAcG2T6, 5'-CGGAATTCTGCAGGTTCGACGTACGCCCCGGTCCAT-GGCCCAGGCCTGGGATCCCG-3' that when annealed form a 55-mer with unique *Bam*HI, *Eco*RI, and *Nco*I restriction enzymes sites (*Nco*I underlined). The annealed oligonucleotides were digested with *Bam*HI and *Eco*RI and ligated into the similarly digested pAcG2T vector to create pJM296. An in-frame *GST-POL* fusion was generated by cloning an ~2.4-kb *Nco*I to *Pst*I fragment of human *POL* (formerly *RAD30B*) from clone 6-1 (McDonald et al. 1999) (Genbank accession no. AF140501) into *Nco*I-*Pst*I-digested pJM296 to create pJM299. A mutant form of the *GST-POL* gene was constructed by using the Quick Change mutagenesis kit (Stratagene, La Jolla, CA). Adjacent residues, D126 and E127 in *POL* where changed to A126-A127 in clone 6-1 using primers R30AAA, 5'-CTTGGATTTCG-TGCCAATTTTGTGGATCTA-3' and R30AAB, 5'-CACAAA-ATTGGCAGCAAATCCAAGTCTCTC-3' to create plasmid pJM300. For the convenience of subsequent identification, these primers also contain silent changes that result in a novel *Bst*XI restriction enzyme site (underlined). An ~0.28-kb *Bst*EII to *Bst*Z17I fragment from pJM300 was sequenced to verify that the region only contained the desired mutation and then subcloned into the similarly digested wild-type *GST-POL* expression vector, pJM299, to create pJM302, which expresses the nonfunctional mutant *POL*-*GST* protein.

Purification of wild-type and mutant forms of GST-tagged pol η

SE9 insect cells (~1 liter) (PharMingen) were infected at a multiplicity of infection (M.O.I) of 3 with a high-titer stock of either pJM299- or pJM302-derived baculovirus. After 60 hr incubation, the cell pellet was recovered by centrifugation and lysed on ice for 45 min in 50 ml of Insect lysis buffer (PharMingen) containing 1× Protease inhibitor cocktail (PharMingen). After cell lysis, the mixture was centrifuged at 45,000 rpm in a Beckman 50.2 Ti rotor for 1 hr. The supernatant was incubated with 4 ml of Glutathione-Agarose (PharMingen), equilibrated with 1× PBS containing 0.01% NP-40, by rotating at 4°C for 2 hr. The matrix was subsequently packed into a disposable column, washed with 10 column volumes of A-buffer (20 mM Tris-HCl at pH 8.0, 1 M NaCl, 10 mM 2-mercaptoethanol, 0.01% NP-40) and 10 column volumes of B-buffer (1× PBS, 10 mM 2-mercaptoethanol, 0.01% NP-40) at 4°C. The GST-pol η fusion protein was eluted from the GST column in a stepwise fashion by washing with 4 column volumes of E-buffer (50 mM Tris-HCl at pH 8.0, 20 mM reduced Glutathione (Sigma, MO), 100 mM NaCl, 10 mM 2-mercaptoethanol, 0.01% NP-40). The GST-pol η containing eluate was applied to a hydroxylapatite (BioRad, CA) column, equilibrated with P-buffer (10 mM Na-phosphate at pH 7.7, 100 mM NaCl, 10 mM 2-mercaptoethanol). The column was subse-

quently washed with 10 column volumes of P-buffer and the GST-pol fusion protein eluted with a 10–200 mM linear gradient of Na-phosphate in P-buffer. Finally, glycerol was added to the GST-pol containing fractions (10% vol/vol) prior to storing at -80°C .

DNA templates

All synthetic oligonucleotides were synthesized by Loftstrand Laboratories (Gaithersburg, MD) using standard techniques and were gel purified prior to use. The templates for the replication reactions were designed such that a universal 16-mer primer, 5'-CTTGAAAACATAGCGA-3', could be used in all of the replication reactions. The primer was 5'-labeled with [γ - ^{32}P]ATP (5000 Ci/mmol; 1 Ci = 37 GBq) (Amersham Pharmacia Biotech, Piscataway, NJ) using T4 polynucleotide kinase (Life Technologies, Gaithersburg, MD). All synthetic oligonucleotide templates were 40mers in length. The sequence of the wild-type template was 5'-AGCGTCTTAATCTAAGCTATCGCTATG-TTTTCAAGGATTC-3'. This sequence corresponds to nucleotides 4072–4121 of M13mp18. The nucleotides underlined indicate the location of the annealed universal primer (Fig. 1B). This template was also used to measure the fidelity of nucleotide insertion at template A (Figs. 2 and 3). To measure the fidelity of nucleotide insertion at template G, C, or T, similar oligonucleotides were synthesized but which contained G or C at nucleotide 19 of the 40mer template or AT at nucleotides 18–19 of the template (Figs. 2 and 3). Similarly, a template with a run of six As immediately downstream of the primer was synthesized by replacing nucleotides 16–18 of the wild-type template with A (Fig. 4).

Replication reactions

Radiolabeled primer–template DNAs were prepared by annealing the 5'- ^{32}P -labeled 16-mer universal primer to the unlabeled template DNA at a molar ratio of 1:1.5. Standard reactions of 10 μl contained 40 mM Tris-HCl at pH 8.0, 5 mM MgCl_2 , 100 μM of each ultrapure dNTP (Amersham Pharmacia Biotech, NJ), 10 mM DTT, 250 $\mu\text{g/ml}$ BSA, 60 mM KCl, 2.5% glycerol, 20–30 nM 5'-[^{32}P] primer–template DNA and 3 nM GST-Pol. After incubation at 37°C for 30 min, reactions were terminated by the addition of 10 μl of 95% formamide/10 mM EDTA and the samples heated to 100°C for 5 min. Reaction mixtures (5 μl) were subjected to 20% polyacrylamide/7 M Urea gel electrophoresis and replication products visualized by autoradiography or PhosphorImager analysis.

Kinetic analysis of replication products

Time course reactions using the standard replication conditions were initially performed so as to ensure that the reaction was in the linear range (usually <20% of primer utilization) (Boosalis et al. 1987; Creighton et al. 1995). Subsequent reactions measuring the incorporation of the correct nucleotide were performed for 2–5 min, whereas those measuring the incorrect nucleotide were for 5, 10, or 15 min. In most cases, the concentration of the single correct or incorrect nucleotide in the reaction mixture varied from 0.1 μM to 100 μM . The exceptions were in the analysis of the incorporation of G opposite template C, where the concentration of dGTP was varied from 10 nM to 10 μM and also in the analysis of the correct incorporation of dTTP at template A, where the concentration of dTTP was varied from 1 nM to 1 μM . All of the reactions use the same standing-start primer and reactions were initiated by the addition of the appropriate dNTP. Reaction products were separated in a 20% polyacryl-

amide gel containing 7 M urea and gels dried prior to quantitative PhosphorImager analysis using the ImageQuant software (Molecular Dynamics, CA). Saturation plots of velocity as a function of dNTP concentration were determined by dividing the percent of product generated by the respective reaction time of the reaction. The apparent V_{max} and K_m values were derived from nonlinear least squares fits to a rectangular hyperbola using the Sigma Plot software (SPSS, Chicago, IL). Nucleotide misincorporation frequencies were subsequently calculated using the formula: $f_{\text{inc}} = (V_{\text{max}}/K_m)_{\text{incorrect}} / (V_{\text{max}}/K_m)_{\text{correct}}$ as described previously (Boosalis et al. 1987; Creighton et al. 1995).

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