Fidelity and Processivity of Saccharomyces cerevisiae DNA Polymerase η*

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The yeast RAD30 gene functions in error-free replication of UV-damaged DNA, and RAD30 encodes a DNA polymerase, pol η, that has the ability to efficiently and correctly replicate past a cis-syn-thymine-thymine dimer in template DNA. To better understand the role of pol η in damage bypass, we examined its fidelity and processivity on nondamaged DNA templates. Steady-state kinetic analyses of deoxynucleotide incorporation indicate that pol η has a low fidelity, misincorporating deoxynucleotides with a frequency of about 10⁻² to 10⁻³. Also pol η has a low processivity, incorporating only a few nucleotides before dissociating. We suggest that pol η’s low fidelity reflects a flexibility in its active site rendering it more tolerant of DNA damage, while its low processivity limits its activity to reduce errors.

Mutations in the RAD30 gene of Saccharomyces cerevisiae confer moderate sensitivity to ultraviolet (UV) radiation, and genetic studies have indicated the involvement of this gene in error-free bypass of UV-damaged DNA (1–3). RAD30 encodes a DNA polymerase, pol η, that can efficiently and correctly bypass a cis-syn-thymine-thymine (T-T) dimer in template DNA by inserting two adenines opposite the two thymines of the dimer (4). The Rad30 DNA polymerase activity is essential for resistance to UV radiation and for its role in error-free bypass (3). Recently, the human homologue of pol η has been identified, and mutational alterations in this protein are responsible for the cancer-prone genetic disorder xeroderma pigmentosum (XP-V) (5, 6).

The oligodeoxynucleotide primer, 5′-CGAGC ATGCT AGGCC ACTCC AGTGT AGGCA, was 5′-32P-end-labeled using polynucleotide kinase (Roche Molecular Biochemicals) and [γ-32P]ATP (Amersham Pharmacia Biotech). Labeled primer (0.5 μM) was then annealed to the various templates (0.8 μM each) in the presence of 50 mM Tris-HCl, pH 7.5, and 100 mM NaCl by heating to 90 °C for 2 min, followed by cooling to 25 °C over several hours.

Analysis of Fidelity—Analysis of the deoxynucleotide incorporation assays was done as described previously (12–14). Gel band intensities of the substrates and products were quantitated using a PhosphorImager and the ImageQuant software (Molecular Dynamics). For each concentration of dNTP, the observed rate of deoxynucleotide incorporation (Vobs) was determined by dividing the relative amount of the extended product by the incubation time. The observed rate of deoxynucleotide incorporation was plotted as a function of dNTP concentration, and the data were fit by nonlinear regression using SigmaPlot 4.0 to the Michaelis-Menton equation describing a hyperbola as follows (Equation 1).

Vobs = (Vmax × [dNTP])/(Km + [dNTP])

(Eq. 1)

Apparent Km and Vmax steady-state parameters for the incorporation of the correct and incorrect deoxynucleotides were obtained from the fit and used to calculate the frequency of deoxynucleotide misincorporation (finc) using the following equation (Equation 2).

finc = (Vmax/Km)correct/(Vmax/Km)incorrect

(Eq. 2)

The following four (53-mer) oligodeoxynucleotides were used as templates, and they differ only in the underlined sequences: Template G, 5′-ATGCC TGCAG GAAGA GTTCC TAGTG CCTAC ACTGT AGTTCG CTATG CCTAC ACTGG AGTAC CGGAG CATCG TCG; Template A, 5′-ATGCC TTCACC AGAAGA GTTCC TAGTG CCTAC ACTGT AGTTCG CTATG CCTAC ACTGG AGTAC CGGAG CATCG TCG; Template T, 5′-ATGCC TGCAG GAAGA GTTCC TAGTG CCTAC ACTGT AGTTCG CTATG CCTAC ACTGG AGTAC CGGAG CATCG TCG; Template C, 5′-ATGCC TTCACC AGAAGA GTTCC TAGTG CCTAC ACTGT AGTTCG CTATG CCTAC ACTGG AGTAC CGGAG CATCG TCG.

Materials and Methods

DNA Substrates—The following four (53-mer) oligodeoxynucleotides were used as templates, and they differ only in the underlined sequences: Template G, 5′-ATGCC TGCAG GAAGA GTTCC TAGTG CCTAC ACTGT AGTTCG CTATG CCTAC ACTGG AGTAC CGGAG CATCG TCG; Template A, 5′-ATGCC TTCACC AGAAGA GTTCC TAGTG CCTAC ACTGT AGTTCG CTATG CCTAC ACTGG AGTAC CGGAG CATCG TCG; Template T, 5′-ATGCC TGCAG GAAGA GTTCC TAGTG CCTAC ACTGT AGTTCG CTATG CCTAC ACTGG AGTAC CGGAG CATCG TCG; Template C, 5′-ATGCC TTCACC AGAAGA GTTCC TAGTG CCTAC ACTGT AGTTCG CTATG CCTAC ACTGG AGTAC CGGAG CATCG TCG.

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substrate before the addition of dNTPs and MgCl₂. After various times, reactions were quenched and run on a 10% polyacrylamide gel as described for the deoxynucleotide incorporation assays.

**Analysis of Processivity**—The processivity, \( P_n \), after each deoxynucleotide incorporation was calculated by a method derived from one previously described (15). Briefly, gel band intensities of the deoxynucleotide incorporation products at the 240 s incubation time were quantitated using the PhosphorImager and ImageQuant software. First, for each deoxynucleotide addition \( n \), the percentage of active polymerases incorporating at least \( n \) deoxynucleotides is given by the following equation (Equation 3),

\[
\% \text{ active polymerases at } n = (I_1 + I_{1+1} + \ldots) \times 100\% / (I_1 + I_2 + \ldots + I_n + \ldots) \quad (\text{Eq. 3})
\]

where \( I_1 \) is the intensity of the band at position 1, \( I_n \) is the intensity of the band at position \( n \), and so on. For each deoxynucleotide incorporation \( n \), processivity, \( P_n \), is the probability that the polymerase will incorporate the next deoxynucleotide rather than dissociating (15) and is given by the following equation (Equation 4).

\[
P_n = \frac{\% \text{ active polymerases at } n + 1/\% \text{ active polymerases at } n \quad (\text{Eq. 4})
\]

Applying Equation 3 to Equation 4 gives an expression for \( P_n \) in terms of gel band intensities, and this equation was used to calculate the processivity as follows (Equation 5).

\[
P_n = \frac{(I_{n+1} + I_{n+2} + \ldots)(I_1 + I_{n+1} + I_{n+2} + \ldots)}{(I_1 + I_{n+1} + I_{n+2} + \ldots)} \quad (\text{Eq. 5})
\]

**RESULTS**

**Fidelity of Pol \( \eta \)—**Fidelity is a measure of the frequency of incorporating a correctly base-paired versus an incorrectly base-paired deoxynucleotide (7, 12, 13, 16). To determine the frequency of misincorporation by pol \( \eta \), we measured the \( V_{\text{max}} \) and \( K_m \) steady-state parameters for the incorporation of correct and incorrect deoxynucleotides opposite each template residue, using a standing-start assay, wherein the target template resi
due immediately follows the end of the primer (13).

Pol \( \eta \) (2 nM) was incubated with the primer-template DNA substrate (50 nM) and various concentrations of one of the four deoxynucleotides, after which reaction products were resolved by polyacrylamide gel electrophoresis and band intensities quantitated. Fig. 1A shows the deoxynucleotide incorporation pattern opposite a template T residue. The concentrations of dGTP, dTTP, and dCTP were varied from 0 to 200 \( \mu \)M, whereas the concentrations of dATP was varied from 0 to 10 \( \mu \)M. Under the reaction conditions, the kinetics of deoxynucleotide incorporation were linear with time.

The kinetics of single deoxynucleotide incorporation opposite the template T residue are shown in Fig. 1B. These data were fit to the Michaelis-Menton equation (Equation 1) and used to determine the apparent \( K_m \) and \( V_{\text{max}} \) values for each deoxynucleotide (Table I). The frequency of misincorporation, \( f_{\text{inc}} \), of G, T, and C opposite the template T was then calculated using Equation 2 (12–14, 17):

\[
f_{\text{inc}}(\text{G})(\text{T})(\text{C}) = \frac{V_{\text{max}}}{K_m + [\text{dNTP}]_{\text{inc}}} \quad (\text{Eq. 2})
\]

For each deoxynucleotide incorporation resulting from a single DNA binding event, the reactions in lanes 7–12 (Fig. 2A) were performed by first preincubating pol \( \eta \) with the DNA substrate for 20 min. Excess herring sperm DNA, MgCl₂, and all four deoxynucleotides were then added to initiate the reaction. The excess herring sperm DNA is included to trap all pol \( \eta \) molecules that dissociated from the substrate ensuring that all DNA synthesis resulted from a single DNA binding event. The reactions in lanes 7–12 (Fig. 2A) were performed by first preincubating pol \( \eta \) with the DNA substrate and the excess herring sperm DNA for 20 min followed by the addition of MgCl₂ and deoxynucleotides. The lack of DNA synthesis in these lanes shows that the excess herring sperm DNA is sufficient to trap all pol \( \eta \) molecules.

**Processivity of Pol \( \eta \)—**Processivity is a measure of how many deoxynucleotides a DNA polymerase incorporates in a single DNA binding event (15, 18). To ensure that we were observing deoxynucleotide incorporation resulting from a single DNA binding event, we monitored DNA synthesis in the presence of an excess of nonradioabeled, sonicated herring sperm DNA as a trap (Fig. 2A). The reactions in lanes 1–6 (Fig. 2A) were performed by first preincubating pol \( \eta \) with the DNA substrate for 20 min. Excess herring sperm DNA, MgCl₂, and all four deoxynucleotides were then added to initiate the reaction. The excess herring sperm DNA is included to trap all pol \( \eta \) molecules that dissociated from the substrate ensuring that all DNA synthesis resulted from a single DNA binding event. The reactions in lanes 7–12 (Fig. 2A) were performed by first preincubating pol \( \eta \) with the DNA substrate and the excess herring sperm DNA for 20 min followed by the addition of MgCl₂ and deoxynucleotides. The lack of DNA synthesis in these lanes shows that the excess herring sperm DNA is sufficient to trap all pol \( \eta \) molecules.

The processivity of a DNA polymerase is quantitatively expressed as the probability, \( P_n \), for each deoxynucleotide incorporation event \( n \) that the polymerase will move ahead to incorporate the next nucleotide \( n + 1 \) rather than dissociate from
The percentage of active polymerases adding at least one deoxynucleotide was set as 100%, and the percentage of active polymerases decreased after each subsequent addition because of the dissociation of some fraction of polymerase molecules. For example, 93% added at least two deoxynucleotides, 88% added at least three deoxynucleotides, 80% added at least four deoxynucleotides, and so on (Fig. 2B). On this DNA substrate, ~50% of the pol η molecules incorporate at least six deoxynucleotides before dissociating from the DNA.

Next, we calculated the processivity $P_n$ after each deoxynucleotide incorporation using the following equation (Equation 5) (15):

$$ P_n = \frac{I_1 + I_{n+1} + I_{n+2} + \ldots}{I_n + I_{n+1} + I_{n+2} + \ldots}, $$

where $I_n$ is the intensity of band $n$, $I_{n+1}$ is the intensity of band $n + 1$, and so on. For example, of the polymerase molecules that incorporated one nucleotide, 93% incorporated at least one additional nucleotide. Thus, $P_1 = 0.93$. The values of $P_n$ ranged from 0.94 in the case of $n = 2$ deoxynucleotide additions to 0.40 in the case of $n = 7$ deoxynucleotide additions with an average value of 0.76 ± 0.20. Thus after each nucleotide incorporation, on average 76% of the bound pol η molecules incorporate at least one additional deoxynucleotide, while 24% dissociate from the DNA substrate. Thus pol η synthesizes DNA with low processivity.

**DISCUSSION**

Replicative DNA polymerases incorporate wrong deoxynucleotides with very low frequencies (7, 12, 13, 16). This high fidelity has been suggested to arise in part because of the intolerance of the active site to geometric distortions in DNA (7). Structures of several DNA polymerases have indicated that a conformational change in the enzyme is critical for the formation of the phosphodiester bond between the primer and the incoming deoxynucleotide, and it plays an important role in fidelity (for a review, see Ref. 19). Furthermore, these structures are consistent with the proposal that the incoming deoxynucleotide initially binds in the active site of the polymerase in a manner independent of the template base, but precise Watson-Crick base pairing geometry between the incoming deoxynucleotide, and the template base is required for this catalytically essential conformational change to occur (19). Thus, if the geometry is not correct, the phosphodiester bond will not be formed efficiently.

Even though the thymine bases of a cyclobutane pyrimidine dimer can properly base pair with adenines (8, 9), the distorted geometry of the dimer (10, 11) presumably blocks polymerases, because they cannot tolerate the distortion (7). The ability of pol η to efficiently and correctly bypass dimers would suggest that relative to other DNA polymerases, the active site of pol η has an increased tolerance of DNA distortions.

The *in vitro* misinsertion frequencies, $f_{\text{inc}}$, vary for different DNA polymerases. Using a steady-state kinetics assay, the error rates for T4 DNA polymerase and *Escherichia coli* DNA polymerase III holoenzyme, both replicative DNA polymerases, were found to vary from $10^{-4}$ to $10^{-7}$ (14, 20). Using an approach involving the synthesis of DNA in a gap across from the α-complementation *lacZ* gene of M13mp2, the eukaryotic replicative DNA polymerase pol δ was found to have an error rate of about $10^{-5}$ (21), whereas pol α, required for lagging strand DNA synthesis, and pol β, involved in short patch base excision repair, are less accurate and have an error rate of about $10^{-3}$ to $10^{-4}$ (21, 22). Here, using the steady-state kinetics assay, we find pol η to have an error rate of $10^{-2}$ to $10^{-3}$. Thus, relative to these other DNA polymerases, pol η has a low fidelity, which may arise from an active site more tolerant of distortions in DNA. It would be of much interest to compare a high resolution structure of pol η with the structures of other DNA polymerases to determine the structural basis of the flexibility of
pol η's active site that gives it a higher tolerance for distortions and a lower fidelity.

Despite its low fidelity, pol η functions in error-free bypass of UV lesions. Both in yeast and humans, inactivation of pol η enhances the frequency of UV-induced mutations (1, 3, 23, 24), and as a consequence, XP-V patients suffer from a high incidence of skin cancers. Additionally, pol η has little effect on spontaneous mutations, as the rate of spontaneous CAN1 to can1 forward mutations is not affected in the rad30Δ strain.2 Thus, there is no evidence that pol η contributes significantly to the generation of mutations in vivo. This likely occurs because the DNA synthesis activity of pol η is limited. Pol η may synthesize just enough DNA to bypass lesions, thus affording little opportunity to make errors. Additionally, the activity of pol η may be regulated by the Rad6-Rad18 complex (25), which may have a role in targeting pol η to DNA damage sites and in ensuring that the action of pol η is limited to damage bypass. Very likely, pol δ takes over soon after the damage has been bypassed by pol η.

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