Human DNA polymerase α has a strong mutagenic potential at the initial steps of DNA synthesis

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ABSTRACT

DNA polymerase α (Polα) is essential for DNA replication initiation and makes a notable contribution to genome mutagenesis. The activity and fidelity of Polα during the early steps of DNA replication have not been well studied. Here we show that at the beginning of DNA synthesis, when extending the RNA primer received from primase, Polα is more mutagenic than during the later DNA elongation steps. Kinetic and binding studies revealed substantially higher activity and affinity to the template:primer when Polα interacts with ribonucleotides of a chimeric RNA–DNA primer. Polα activity greatly varies during first six steps of DNA synthesis, and the bias in the rates of correct and incorrect dNTP incorporation leads to impaired fidelity, especially upon the second step of RNA primer extension. Furthermore, increased activity and stability of Polα/template:primer complexes containing RNA–DNA primers result in higher efficiency of mismatch extension.

INTRODUCTION

During replication of the eukaryotic genome at each cell division, DNA polymerase α (Polα) initiates DNA synthesis at the thousands of replication origins and on the millions of Okazaki fragments (1,2). In the tightly regulated primosome complex, Polα receives from primase the 9-mer RNA primer (3) for further extension with deoxyribonucleotides, which results in generation of a chimeric RNA–DNA primer. Polα activity greatly varies during first six steps of DNA synthesis, and the bias in the rates of correct and incorrect dNTP incorporation leads to impaired fidelity, especially upon the second step of RNA primer extension. Furthermore, increased activity and stability of Polα/template:primer complexes containing RNA–DNA primers result in higher efficiency of mismatch extension.

MATERIALS AND METHODS

Protein expression and purification

Cloning, expression, and purification to homogeneity of Polα catalytic domain have been described elsewhere (13). Peak fractions obtained from a Heparin HP HiTrap column (Cytiva) were combined and dialyzed to 25 mM Tris–HEPES, pH 7.8, 200 mM NaCl, 1% glycerol and 1 mM tris(2-carboxyethyl)phosphine (TCEP), concentrated

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†The authors wish it to be known that, in their opinion, the first two authors should be regarded as Joint First Authors.

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Figure 1. Structure of a mature RNA–DNA primer synthesized by primase-Polα (primosome) on a parental DNA template.

Figure 2. Experimental design. Schematics of experiments for estimation of misinsertion efficiency (A) and mismatch extension efficiency (B). $k_c$ and $k_w$ describe the rates of primer extension with correct (cognate) and wrong (non-cognate) nucleotide. $k_{obs}$ and $k_{off}$ describe the rate of primer extension and the rate of Polα/T:P complex dissociation, respectively.

Analysis of binding kinetics was done at 23 °C on an Octet K2 (Sartorius AG) as previously described (24). This device uses Bio-Layer Interferometry technology to monitor molecular interactions in real time. This allows for obtaining the rate constants of complex formation ($k_{on}$), dissociation ($k_{off}$), and the dissociation constant ($K_D$). The template with a biotin-TEG at the 5′-overhang was annealed to the primers (Supplemental Table S1) and immobilized on a streptavidin-coated biosensor (SAX, Sartorius AG). Primers were added at two-fold molar excess to the template. To prevent DNA polymerase reaction, a dideoxy-cytidine was placed at the 3′-end of primers. SAX sensors were loaded with oligonucleotide-biotin at 50 nM concentration for 7 min at 500 rpm. Then sensors were blocked by incubating for 2 min in 1000 gg/ml biocytin. In the first row of a 96-well microplate (Greiner Bio-One), the first six wells contained the buffer, consisting of 25 mM Tris-Hepes, pH 7.8, 150 mM KCl, 2 mM TCEP, and 0.002% Tween 20. The next six wells contained the two-fold dilutions of hPolα in the same buffer. When binding studies were performed in presence of an incoming nucleotide, 50 μM dNTP and 5 mM MgCl2 were added to the buffer. All wells in the second row contained only the buffer for reference. Data Analysis HT software (version 11.1, Sartorius AG) was used for calculation of binding constants ($k_{on}$, $k_{off}$ and $K_D$). The average value and standard deviation were calculated from three independent experiments.

Kinetic studies

Pre-steady-state kinetic studies were performed at single-turnover conditions on the QFM-4000 rapid chemical quench apparatus (BioLogic, France) at 35 °C. Reactions contained 3 μM hPolαCDT, 0.25 μM duplex, varying concentrations of dNTP, 25 mM Tris–HEPES, pH 7.8, 0.15 M KCl, 5 mM MgCl2, 2 mM TCEP and 0.2 mg/mL BSA. hPolα was incubated with a fluorescence-labeled 15-mer primer annealed to a 25-mer DNA template (Supplemental Table S1), to allow for the formation of the binary complex, and rapidly mixed with 10 μM dTTP and 5 mM MgCl2 (final concentrations) followed by quenching with 0.3 M EDTA. Mismatch insertion kinetics were conducted in the presence of 50 μM dNTP to avoid prolonged incubation. Products were collected in a tube containing 17 μl 100% formamide and separated by denaturing urea PAGE. The fluorescence-labeled products were visualized by a Typhoon FLA 9500 (GE Healthcare) and quantified by ImageJ, version 1.5.3 (NIH). The extended primer fraction was calculated by dividing the amount of extended primer by the amount of primer added in reaction. The percent of extended primer was plotted against time and the data were fit to a single exponential equation:

$$[\text{product}] = Ax \left(1 - e^{-k_{obs}t}\right)$$
RESULTS AND DISCUSSION

To probe the Polα mutagenic potential during the early and late DNA elongation steps, we compared the rates of misinsertion and extension of mismatched duplexes using a chimeric RNA–DNA primer with two deoxynucleotides at the 3’-end as well as a 15-mer DNA primer, respectively (Supplemental Table S1). Reactions were conducted at single-turnover conditions in the presence of 3 μM Polα and 0.25 μM T: P, which were premixed before the reaction start, initiated by addition of catalytic Mg2+ ions and dNTP. It was found that misinsertion rates for A–C and T–C MMs are 3- to 4-fold higher in the case of a chimeric primer (Supplemental Figure S1). Moreover, the rates of extension of mismatched duplexes are 10-fold higher on a chimeric primer (Supplemental Figure S2). These results indicate that MM insertion and extension efficiencies are

where $A$ is the amplitude, $k_{\text{obs}}$ is the observed rate for dNTP incorporation and $t$ is the time. The mean $k_{\text{obs}}$ values and standard deviations were calculated from three independent experiments.
Figure 4. hPola shows the increased rates of MM extension at the second, third, and fifth steps of DNA synthesis. (A) The rate of A–C MM extension depends on the length of synthesized DNA. The numbers above the bars indicate the ratio of rates obtained for the corresponding primer and a DNA primer. (B) The rates of T–C MM extension show spikes at the same DNA elongation steps as for A–C MM. For DNA synthesis steps one to eighth, the primers P2 to P8 were used, respectively; P1 was used for sixteenth step. Templates T4 and T5 were used in the case of A–C and T–C MM extension, respectively. All primers were extended with cognate dTTP.

highly dependent on primer structure, and Polα has an elevated mutagenic potential at the beginning of DNA primer synthesis.

In order to gather more information about Polα fidelity at each step of RNA primer extension, we analyzed the efficiency of A–C MM incorporation upon addition of the first through the sixth dNMPs. Upon attachment of the first dNMP to an RNA primer, Polα shows the lowest rate of A–C MM generation (Figure 3A). Previously, we observed this phenomenon for all 12 mismatches using a semi-quantitative assay (13). Interestingly, upon attachment of the second dNMP, there is a spike in misinsertion activity with an almost six-fold higher rate of A–C MM generation in comparison to a DNA primer (Figure 3A). Notably, the rate of A–C MM formation is 30-fold higher at the second versus the first step of an RNA primer extension with deoxynucleotides (Figure 3A). Similar to A–C MM, Polα shows 7.4- and 13-fold higher misinsertion rates of A–A and T–C MM generation, respectively, at the second versus the sixteenth DNA synthesis step (Figure 3B). Upon A–A MM formation, human primosome demonstrates comparable with Polα rates resulting in 5.3-fold difference between the same DNA synthesis steps (Supplemental Figure S3). This result indicates that the other primosome subunits do not affect the accuracy of DNA synthesis by Polα.

Next, we measured the rates of dTMP insertion opposite template Ade using the same primers. Surprisingly, the rate of correct nucleotide insertion also significantly varies during RNA extension with the first six deoxynucleotides (Figure 3C), but the activity spikes are distributed differently compared to A–C MM formation (compare panels A and C). The significant variation in the DNA polymerization rate at the early stage of DNA synthesis is likely due to the rigid T:P-binding cleft near the active site and primer bending by Polα at the fourth position from the 3′-end (13). A normalization of misinsertion rates revealed the highest efficiency of A–C MM formation upon the second dNMP insertion, with an almost three-fold bigger value in comparison to the late DNA elongation step (Figure 3D).

Analysis of A–C MM extension showed a more than 10-fold higher activity at the second and third steps of DNA elongation than on a DNA primer (Figure 4A). Interestingly, extension of A–C MM on the RNA primer is 3.4 times more efficient than on DNA. This might be important for efficient extension of MMs introduced by primase during RNA primer synthesis (25). Of note, Polα showed five-fold lower activity upon A–C MM generation on the RNA primer versus DNA (Figure 3A). Experiments with T–C MM extension also showed an activity spike at the second, third, and fifth steps of DNA synthesis (Figure 4B). Notably, upon extension of both MMs, A–A and T–C, Polα activity is at least 10-fold higher at the third step of RNA extension with DNA than on a DNA primer. This seems to be important for efficient extension of MMs introduced at the second step of DNA synthesis when Polα fidelity is compromised at most (Figure 3D).

Stability of the Polα/T:P complex is an important factor for MM extension because it defines the chances of a primer to be extended before the complex dissociates. In this regard, we analyzed hPolα interaction with DNA and RNA–DNA chimeric primers by using Octet K2, which employs the Bio-Layer Interferometry technology to monitor molecular interactions in real time. Pre-annealed template:primers with a biotin at the template 5′-end (Supple-
that RNA-containing primers may stabilize the Pol primer with three dNMPs at the 3′-end of Pol. We analyzed the effect of different MMs on the stability site (26,27) but exhibits no selectivity toward RNA–DNA chimeric primers. Notably, hPol and dipped into Pol mental Table S1) were loaded on streptavidin biosensors and dipped into Polα solutions with different concentrations.

Analysis of Polα affinity to template:primers containing a DNA primer or chimeric RNA–DNA primers with one, three, and seven dNMPs at the 3′-end revealed an ∼10-fold stronger interaction with RNA-containing primers (Figure 5), which is mostly due to reduced $k_{off}$ values (Supplemental Table S2). In line with our recent report about the effect of dNTP on Polα interaction with a DNA duplex (24), Polα/T:P complexes containing chimeric primers are significantly stabilized in the presence of cognate dTTP (Figure 5) and show similar $K_D$ values in a range of 11.3–13.5 nM (Supplemental Table S2). These data revealed that the T:P binding site of hPolα is specific to chimeric primers. Notably, hPol has a similar T:P binding site (26,27) but exhibits no selectivity toward RNA–DNA primers (28).

The results of initial binding studies (Figure 5) indicate that RNA-containing primers may stabilize the Polα/T:P complex in the presence of mismatches. In this regard, we analyzed the effect of different MMs on the stability of Polα/T:P complexes containing a DNA or a chimeric primer with three dNMPs at the 3′-end (Figure 6A and Table 1). To mimic MM at the insertion site of Polα, which must be occupied by a nascent base pair to allow DNA polymerization, we used dCTP or dGTP against template Gua with Thy or Ade. To mimic MM at the post-insertion site, which corresponds to incorrect dNMP insertion during the preceding DNA polymerization step, we used cognate dTTP and template:primers with a 3′-mispair T–C or A–C by replacing template Gua with Thy or Ade, respectively (Supplemental Table S1). These experiments have shown that the chimeric primer stabilizes the Polα/T:P complex in the presence of MMs (Figure 6A and Table 1). Depending on MM, the difference in $K_D$ values for a chimeric and a DNA primer varies from several-fold to >10-fold.

Comparison of Polα interaction with chimeric primers containing seven and eight dNMPs showed a significant change in affinity either for cognate complexes or in the presence of MMs (Figure 6B and Supplemental Table S3). These data indicate that after RNA primer extension with eight dNMPs, Polα affinity to T:P gradually declines. This conclusion is consistent with structural studies showing that hPolα interacts with nine of the duplex bases of the template and with five of the duplex bases of the primer (13). Probably, the T:P-binding site of Polα is optimal for the double helix of a DNA:RNA duplex, which is wider than a DNA:DNA duplex.

Increased efficiency of dNTP insertion and stability of Polα/T:P complexes at the beginning of DNA primer synthesis makes the complexes more productive for MM extension. The probability of primer extension during the existence of the complex Polα/T:P depends on the rates of dNTP insertion and complex dissociation and defines the primer extension efficiency $f_{ext} = k_{obs}/(k_{obs} + k_{off})$. For example, for a Polα/T:P complex with a DNA duplex containing A–C MM, the rate of primer extension ($k_{obs} = 0.00457$ s$^{-1}$; Figure 4A) is 80 times lower in comparison to the rate of complex dissociation ($k_{off} = 0.364$ s$^{-1}$; Table 1), resulting in $f_{ext} = 0.012$. So, on average, ∼80 association/dissociation cycles of the Polα/DNA:DNA complex will take place before extension of A–C MM. This estimate indicates that the Polα complex with a mismatched DNA duplex is highly non-productive for the subsequent extension.

In contrast, the efficiency of A–C MM extension on a chimeric primer with three deoxynucleotides ($f_{ext} = 0.042$; the fourth step of DNA synthesis) is 3.5-fold higher than on a DNA primer, which is due to the increased rate of DNA polymerization ($k_{obs} = 0.0065$ s$^{-1}$; Figure 4A) and the reduced rate of complex dissociation ($k_{off} = 0.149$ s$^{-1}$; Table 1). Furthermore, a DNA polymerization rate is ∼10 times higher at the second and third DNA synthesis steps compared to the fourth step (Figure 4A), thus increasing $f_{ext}$ several-fold.

These studies revealed significant mutagenic potential of hPolα at the first steps of DNA primer synthesis. This effect is dictated by the structure of a chimeric RNA–DNA primer and is based on increased Polα affinity to T:P and its DNA polymerization activity. Efficient MM extension on chimeric primers should prevent Polα and the replisome from stalling at the early DNA elongation stage, when it would be difficult to load the Polβ/PCNA complex on a 11–15 bp duplex (29) to allow Polβ to proofread MM and continue DNA synthesis. Moreover, a very inefficient RNA primer extension by Polβ (30) suggests that its ability to proofread MM on RNA-containing primers is also significantly compromised. Accordingly, a proposed mechanism of correction of Polα-introduced mistakes by Polβ (21) mainly applies to the late DNA elongation stage. Indeed, low efficiency of MM extension on a DNA primer, which results in Polα idling, would facilitate early loading of RFC/PCNA and then Polβ.
Figure 6. hPolα exhibits increased affinity to T:P with chimeric RNA–DNA primers in the presence of MMs. (A) In the presence of different MMs at the insertion and post-insertion sites, hPolα shows higher affinity to a chimeric primer. (B) hPolα interaction with chimeric primers significantly weakens after RNA primer extension with eighth dNMP. For panels A and B, the corresponding $k_{on}$, $k_{off}$, and $K_D$ values are shown in Table 1 and Supplemental Table S3, respectively.

Table 1. Effect of mismatches and primer structure on Pol/H9251 interaction with T:P

| Primer | Template | dNTP | Nascent base/mis-pair | 3'-base/mis-pair | $K_D$ nM | $k_{on}$ mM$^{-1}$ s$^{-1}$ | $k_{off}$ $\times 10^{-3}$ s$^{-1}$ |
|--------|----------|------|-----------------------|------------------|---------|-----------------------------|-----------------------------|
| P12 (R8-D3) | T6 | dTTP | A–T | G–C | 11.3 ± 0.69 | 288 ± 39 | 3.24 ± 0.24 |
| | T9 | dGTP | T–G | G–C | 97.4 ± 1.3 | 255 ± 40 | 24.8 ± 3.6 |
| | T6 | dCTP | T–C | G–C | 125 ± 3.7 | 254 ± 15 | 31.7 ± 0.92 |
| | T6 | dGTP | A–C | G–C | 277 ± 11 | 299 ± 41 | 82.4 ± 7.6 |
| | T7 | dTTP | A–T | A–C | 361 ± 33 | 240 ± 25 | 86.2 ± 1.3 |
| | T8 | dTTP | A–T | A–C | 198 ± 9.2 | 276 ± 23 | 54.7 ± 7.0 |
| | T6 | dCTP | T–C | G–C | 277 ± 11 | 299 ± 41 | 82.4 ± 7.6 |
| | T6 | dGTP | A–C | G–C | 361 ± 33 | 240 ± 25 | 86.2 ± 1.3 |
| | T7 | dTTP | A–T | A–C | 198 ± 9.2 | 276 ± 23 | 54.7 ± 7.0 |
| | T8 | dTTP | A–T | A–C | 198 ± 9.2 | 276 ± 23 | 54.7 ± 7.0 |

*$K_D$ values are obtained by dividing $k_{off}$ by $k_{on}$. Mispairs are in bold. Data are presented as mean ± SD ($n$ = 3).

In the primase-Polα complex, the C-terminal domain of the primase accessory subunit (p58C) is flexibly tethered to the rest of primosome and works as a processivity factor for Polα, by holding the 5′-end of an RNA primer when Polα extends the primer 3′-end with deoxynucleotides (4). Human Polα has low intrinsic processivity (13,31) and the p58C/T:P interaction increases it, providing quick reloading of the Polα catalytic domain on T:P (4). The critical role in p58C/primer interaction plays the triphosphate group located at the 5′-end of an RNA primer (32). This explains why the primosome demonstrates higher processivity of DNA synthesis on RNA polymerase- or primase-synthesized primers compared to the chemically synthesized primers containing no triphosphate (4,33).

Previously it was shown that increased processivity of DNA synthesis on primase-synthesized primers is mediated by a reduced rate of the primosome/T:P complex dissociation and not by changes in DNA polymerization rate (33).

Similar to correct dNMP insertion, catalysis of MM formation and extension should not depend on primase interaction with the primer 5′-end. Accordingly, we propose that the described here effect of primer structure on Polα fidelity plays an important role in vivo upon elongation of primase-synthesized primers. Consistent with our findings, changing the primer from DNA to RNA significantly enhanced the ability of human Polα to bypass abasic lesions (34) and to tolerate arabinofuranosyl nucleotides (35).

The outcome of mutations introduced by Polα at the beginning of DNA synthesis needs further investigation. At the final step of Okazaki fragment synthesis, Polβ and flap endonuclease 1 (FEN1) work sequentially to remove an RNA primer located at the 5′-end of the preceding Okazaki fragment (36). In this iterative process termed nick-translation, Polβ inserts one nucleotide resulting in strand displacement and formation of a 1-nt flap, then Polβ hands off the flapped substrate to FEN1, which removes
5′-rNMP. When all ribonucleotides from the downstream strand are removed, DNA ligase 1 is able to seal the nick. According to structural data, human DNA ligase 1 has a relaxed binding site for the DNA strand synthesized by Polα (37). This suggests that DNA ligase 1 may not discriminate efficiently against MMs downstream of the nick, especially those that do not substantially disturb the DNA duplex, like T–G, T–C and A–C. There is an evidence for existence of a specific MutSα-dependent, MutLa-independent mismatch repair process called Polα-segment error editing (AEE) where Polα generated errors are excised by the FEN1/MSH2 complex (38).

**DATA AVAILABILITY**

The data that support the findings of this study are included in the Supplementary Data file or available from the corresponding author upon request.

**SUPPLEMENTARY DATA**

Supplementary Data are available at NAR Online.

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