Activity and fidelity of human DNA polymerase α depend on primer structure

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DNA polymerase α (Polα) plays an important role in genome replication. In a complex with primase, Polα synthesizes chimeric RNA–DNA primers necessary for replication of both chromosomal DNA strands. During RNA primer extension with deoxyribonucleotides, Polα needs to use double-stranded helical substrates having different structures. Here, we provide a detailed structure–function analysis of human Polα’s interaction with dNTPs and DNA templates primed with RNA, chimeric RNA–DNA, or DNA. We report the crystal structures of two ternary complexes of the Polα catalytic domain containing dCTP, a DNA template, and either a DNA or an RNA primer. Unexpectedly, in the ternary complex with a DNA:DNA duplex and dCTP, the “fingers” subdomain of Polα is in the open conformation. Polα induces conformational changes in the DNA and hybrid duplexes to produce the universal double helix form. Pre-steady-state kinetic studies indicated for both duplex types that chemical catalysis rather than product release is the rate-limiting step. Moreover, human Polα extended DNA primers with higher efficiency but lower processivity than it did with RNA and chimeric primers. Polα has a substantial propensity to make errors during DNA synthesis, and we observed that its fidelity depends on the type of sugar at the primer 3′-end. A detailed structural comparison of Polα with other replicative DNA polymerases disclosed common features and some differences, which may reflect the specialization of each polymerase in genome replication.

Three DNA polymerases are required for genome replication in eukaryotes: DNA polymerase α (Polα),5 Polε, and Polδ (1). All of them belong to the B-family and fulfill the different tasks. Polε synthesizes most of the leading strand, Polδ is mainly involved in synthesis of the lagging strand, and Polα generates the primers for both polymerases (2, 3). Polα alone cannot synthesize DNA primers de novo and relies on RNA primers created by primase. Polα works in a tight complex with primase, called the primosome (4, 5). Synthesis of the chimeric RNA–DNA primers by the primosome is highly coordinated by autoregulation through the alternating activation/inhibition of two catalytic centers, which is mediated by the C-terminal domain of the primase accessory subunit (6). Relatively low fidelity of Polα, which does not possess a proofreading activity, results in mutational hot spots predominantly on the lagging strand (7). In addition to the established role of primosome in nuclear replication, it is involved in formation of hybrid DNA:RNA duplexes in the cytosol, which are important for regulation of the type I interferon response (8). Polα is a direct target of an anti-tumor toxin CD437, an attractive anti-cancer lead molecule, which induces apoptosis selectively in cancer cells (9).

Human Polα (hPolα) is composed of two polypeptides: the catalytic subunit (p180) and the accessory B-subunit (p70), with calculated molecular masses of 166 and 66 kDa, respectively. p180 contains two domains, the catalytic (residues 338–1250) and the C-terminal (Polα CTD, residues 1266–1462) domains, which are flexibly connected by a 15-residue-long linker (4). The catalytic domain possesses DNA-polymerizing activity but has no proofreading exonuclease activity, in contrast to other replicative DNA Pols, δ and ε (10). Polα CTD connects the catalytic domain with p70 and primase and contains two conserved zinc-binding modules, where each zinc ion is coordinated by four cysteines (6, 11, 12). The N terminus of p180 (residues 1–337) is predicted to be poorly folded and does not participate in primer synthesis. The structural information for this region is limited to a small peptide in the catalytic subunit of yeast Polα (yPolα; residues 140–147) that interacts with the replisome (13).

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This article contains Figs. S1 and S2.

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5 The abbreviations used are: Pol, DNA polymerase; hPol, human Pol; yPol, yeast Pol; p180core, catalytic domain of hPola; Exo, exonuclease subdomain of Polα; H-bond, hydrogen bond; RMSD, root mean square deviation; TCEP, tris(2-carboxyethyl)phosphine; bp, base pair(s); TEMED, N,N,N′,N′-tetramethylethylenediamine; PDB, Protein Data Bank.
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Table 1

| Data collection and refinement statistics | p180core/DNA:RNA/dCTP (PDB code 4QCL) | p180core/DNA:DNA/dCTP (PDB code 6AS7) |
|-----------------------------------------|--------------------------------------|--------------------------------------|
| Space group                             | P 3,21                               | P 4,2,2                               |
| a = b, c (Å)                            | 140.76, 181.32                       | 151.81, 113.3                        |
| Resolution (Å)                          | 40–2.2 (2.24–2.2)"                  | 50–2.95 (3–2.95)                     |
| Rmerge                                  | 0.055 (0.531)                        | 0.072 (0.47)                         |
| Rfree                                   | 19.76 (1.9)                          | 18.2 (2.2)                           |
| Completeness (%)                        | 91 (81)                              | 99.6 (99.3)                          |
| Unique reflections                      | 95,874 (4196)                        | 28,565 (1406)                        |
| Redundancy                              | 2.7 (1.9)                            | 9.6 (5.5)                            |
| Resolution (Å)                          | 29.51–2.2 (2.34–2.2)                | 46.2–2.95 (3.13–2.95)                |
| No. of reflections                      | 88,726 (12,002)                      | 26,970 (4410)                        |
| Test set                                | 4683 (643)                           | 1380 (209)                           |
| Rmerge / Rfree                          | 0.21/0.239 (0.38/0.404)              | 0.259/0.301 (0.38/0.402)             |
| No. of atoms                            | 6947                                 | 6996                                 |
| Protein                                 | 496                                  | 487                                  |
| DNA and RNA                             | 48/5                                 | 28/4                                 |
| Water                                   | 414                                  | 1                                    |
| Mean B-factors (Å²)                     | 51.1                                 | 57.6                                 |
| RMSDs                                   | 0.007                                | 0.01                                 |
| Bond lengths (Å)                        | 1.3                                  | 1.6                                  |
| Ramachandran plot (%)                   | 89.7                                 | 82.1                                 |
| Core                                    | 97.7                                 | 16.8                                 |
| Allowed                                 | 5.0                                  | 1.0                                  |
| Generously allowed                      | 0.1                                  | 0.1                                  |

| a Numbers in parentheses refer to highest-resolution shell. |

In this work, we use the structural and kinetic approaches to analyze hPolα interaction with the template:primer and dNTP and the effect of the primer structure on hPolα catalysis, processivity, and fidelity.

Results

Overall structure of the catalytic domain of human DNA polymerase α in complex with a DNA template, RNA, or DNA primer, incising dCTP, and divalent metal ions

The structures of the ternary complexes of p180core (the part of hPolα containing the catalytic domain, residues 335–1257) with dCTP and DNA:RNA or DNA:DNA duplexes have been determined at 2.2 and 2.95 Å resolution, respectively (Table 1). The p180core adopts the universal “right-hand” DNA polymerase fold consisting of five subdomains (14), which encircle the active site (upper schematic in Fig. 1A): N-terminal (residues 338–534 and 761–808), exonuclease (Exo; residues 535–760; inactive), palm (residues 834–908 and 968–1076), fingers (residues 909–967), and thumb (residues 1077–1250) domains. The palm is the most conserved subdomain in DNA polymerases and responsible for catalytically proficient positioning in the active site of two substrates to be polymerized: a template:primer and incoming dNTP. The palm harbors two catalytic aspartates (Asp1086 and Asp1088), which coordinate two divalent metal ions important for dNTP binding and nucleophilic attack on its α-phosphate by the primer’s 3’-OH (15). The thumb makes additional contacts with a template:primer, and their pattern significantly differs between B-family Pols (see below). The L-shaped thumb has two parts: the all-helical base adjacent to the palm and the tip with mixed α/β-fold (residues 1135–1207), which packs against the Exo. Polα lost proofreading exonuclease activity by substitution of the catalytic residues in the Exo subdomain during evolution (10). The fingers are composed of two antiparallel α-helices (Fig. 1A), which make additional contacts with the triphosphate moiety of dNTP and oscillate between the open and closed conformations. The thumb encloses the circle of p180core subdomains around the active site by connecting the palm with Exo (Fig. 1A). The interactions at the interfaces of the thumb with the palm and Exo have a lower amount of hydrophobic contacts in Polα compared with Pol6 and Polε, which allows the thumb to rotate relative to the palm. Such rotation of the thumb away from Exo was observed in apo-forms of the human primosome and yPolα (6, 16).

The alignment of hPolα ternary complexes containing DNA:DNA and DNA:RNA duplexes with a root mean square deviation (RMSD) of 0.86 Å for 648 Ca atoms shows good superposition for the first four bp from the primer 3’-end and for p180core subdomains except the thumb and fingers (Fig. 1B). It is likely that the crystal packing affected the conformation of these subdomains in the hPolα/DNA:DNA/dCTP complex. The thumb interacts with the palm and the N-terminal subdomain of one neighboring molecule and with Exo of the other neighboring molecule. The fingers are stabilized in the open conformation due to interaction with the N-terminal and Exo subdomains of the neighboring molecule. Significant difference in the position of the template:primer for bp 5–11 from the primer 3’-end (the bottom part of Fig. 1B) is due to steric hindrance with the thumb tip, which moves closer to the template upon the outward rotation of the thumb. In addition, the blunt end of the DNA:DNA duplex is packed against the N-terminal domain of the other molecule. In support of the idea that crystal packing affected the conformation of the most flexible subdomains in the structure of p180core/DNA:DNA/dCTP, the
thumb of hPol in the binary complex with DNA:DNA has a very similar position as in the hPol/DNA:RNA/dCTP complex (alignment was provided in Ref. 17). Moreover, DNA:DNA from the binary complex superimposes well with DNA:RNA from the ternary complex (the bottom part of Fig. 1B). Accordingly, hPol has similar affinity for DNA and hybrid duplexes.

In the ternary complex containing DNA:DNA and dCTP, the fingers have the open conformation as in the binary complex hPol/DNA:RNA (Fig. 2A) and in the complex hPol/DNA:RNA/aphidicolin (Fig. 2B). In all structures of yeast and human Polα, only two conformations of fingers were observed: open and closed (16–18). This indicates that intermediate positions of fingers are energetically much less favorable. Upon closing, one of the fingers α-helices slides along the N-terminal subdomain toward to Exo (Fig. 2C). Analysis of intersubdomain interfaces in the open and closed forms revealed the switching of Ile944 of the fingers between two adjacent pockets. One pocket is formed by Asn760, Pro763, Leu764, and Gln767 of the N-terminal subdomain. The other is formed by Leu764 of the N-terminal subdomain and by Leu700, Ile701, and Leu759 of Exo. The open and closed conformations are also stabilized by a hydrogen bond between the side chain of Gln941 and the main-chain oxygens of Leu770 and Cys757, respectively.

In the structures of both ternary complexes, all three disordered regions (residues 674–677, 810–833, and 883–895) are situated near the template:primer. The loop 878–903 originates from the same site of the palm as the processivity domain (P-domain), which is found in Polα and provides additional contacts with a template:primer (Fig. 3). The linker 808–837 between the N-terminal and palm subdomains of p180corresponds to the shorter linker in Polδ, which adds two β-strands to the stem of the P-domain. In Polδ, the corresponding region is also shorter than in Polα and well-structured in the crystal (shown in blue in Fig. 3).

**Interaction of hPolα with dNTP**

The dNTP-binding site of B-family Pols is located at the junction between the palm and the fingers (Fig. 4A). Upon dNTP binding, the latter subdomain is able to change its conformation from open to the closed one, which results in stabilization of the precatalytic ternary complex Pol/template:primer/dNTP. Despite the open conformation of fingers in the complex of hPolα with a DNA duplex, dCTP occupies the dNTP-binding site (Fig. 4A). To our knowledge, this is the first structure of the ternary complex of a B-family DNA polymerase with a DNA duplex and dNTP where the fingers are open. This structure catches the transient step of dNTP binding followed by fingers closing and phosphodiester bond formation. It revealed that fingers interact with dNTP even in the open conformation. Asn954, which establishes the water-mediated contact with the β-phosphate in the closed-fingers conformation, directly interacts with this phosphate in the open-fingers state (Table 2). Interaction of Lys950 with dCTP switched from the α-
to γ-phosphate upon fingers opening. Interestingly, in the two reported structures of Polα in the complex with DNA:DNA (in all cases, dNTP was added to the crystallization mix), the fingers are open (this work; also, see Ref. 17, where dCTP was not detected in the active site). This may indicate a weak stabilization of Polα fingers in the closed conformation. Indeed, analysis of dNTP-finger interactions in replicative DNA Pols shows that two conservative residues of hPolα, Lys926 and Asn954, do not directly interact with the triphosphate (Table 2). In yPolβ, Asn930 makes an H-bond with the β-phosphate. In yPolε, both water-mediated contacts observed in the hPolα ternary complex are changed to direct interactions. Thereby, only two residues of Polα fingers (Arg927 and Lys956 in hPolα) directly interact with dNTP in the closed state, whereas yeast Polδ and -ε use three and four residues, respectively.

In hPolα, the sugar and the base of dNTP fit into the hydrophobic pocket formed by Leu864, Tyr865, Pro866, Tyr957, and Thr1003 (Fig. 4B). The conserved Tyr865 defines a steric gate for rNTPs due to steric hindrance with the ribose 2'-OH, which is facilitated by a C3'-endo pucker. Upon fingers closing, the base of dCTP is fixed between Asn954 and the 3'-nucleotide of the primer in the position that is optimal for pairing with guanine from the DNA template. The triphosphate of dCTP is secured by interactions with the side chains of Arg922 and Lys950 and five coordinate bonds with two divalent ions, which in turn are coordinated by Asp860, Asp1004, and the main-chain oxygen of Phe861. hPolα also makes two water-mediated contacts with the phosphates of dCTP, using the side chains of Lys926 and Asn954. The presence of Zn2+ at the metal-binding site A in the hPolα/DNA:RNA/dCTP complex is probably due to the missing hydroxyl at the primer 3'-end, which disrupts the octahedral coordination of Mg2+. In contrast to Mg2+, Zn2+ is flexible with respect to the number of ligands that it can adopt in its first coordination shell (19). Moreover, Zn2+ forms a stronger complex with carboxylates and the triphosphate group of nucleotides than Mg2+ (20, 21). Accordingly, Mg2+ was missing at the site A in the crystal structure of yPolε ternary complex, where the primer 3'-end also contained dideoxycytidine (22). Alignment of the hPolα/DNA:RNA/dCTP complex with the ternary complexes of hPol and yPol containing a DNA primer shows good superposition for incoming dCTP and the residues coordinating dNTP and the catalytic metals (Fig. 4). This indicates a high structural conservation of dNTP-binding pocket in replicative Pols and that the type of the primer does not affect it. Mutation of the conserved fingers residues, Arg922 and Lys950, which interact with dNTP, dramatically reduced hPolα
Interaction of human Polα with DNA:DNA and DNA:RNA duplexes

Figure 4. Interaction of Polα with incoming dNTP. A, hydrophilic interactions between hPolα and the triphosphate moiety of dNTP. In aligned ternary complexes containing DNA:RNA and DNA:DNA duplexes (PDB codes 4QCL and 6A57, respectively), there is a good superposition of dCTP-interacting residues except the fingers. The subdomains of hPolα in the complex with DNA:RNA are colored according to Fig. 1. Polα in the complex with DNA:DNA is colored gray (only α-helices of fingers are shown for clarity); dCTP from this complex is shown as lines and colored dark brown. In both complexes, the side or main chains of the hPolα residues involved in hydrophilic interactions with dCTP and metals are shown as sticks. Potential H-bonds between Polα (in complex with DNA:RNA), dCTP, and catalytic metals are depicted by pink dashed lines. The A and B metal-binding sites of hPolα in complex with DNA:RNA or DNA:DNA contain Zn2+ and Mg2+ or only Mg2+, respectively. Asp24 and Asp204 each interact with both divalent metals; the metal-B interacts with all phosphates of dNTP, whereas the metal-A makes a contact only with the α-phosphate. B, comparison of dNTP-binding pockets in hPolα and yPolα. The ternary complexes hPolα/DNA:RNA/dCTP and yPolα/DNA:DNA/dCTP (PDB codes 4QCL and 3IAY, respectively) were aligned with RMSD of 2.08 Å for 524 Cα atoms. The carbons of hPolα and yPolα are colored according to Fig. 1 and gray, respectively. hPolα Phe651 and yPolα Phe638, as well as two water molecules coordinated by zinc are not shown for clarity. dCTP from Polα complex is shown as lines and colored dark brown. Atoms of magnesium, calcium, and zinc are presented as spheres at reduced scale and colored green, light orange, and slate, respectively. A, different conformation of Asp24 in yPolα is probably due to the coordination of a third calcium ion. Accordingly, Asp204 of hPolα is oriented similarly to Asp204 in yPolα where Mg2+ occupies the site B (22). In both panels, DNA:DNA and DNA:RNA duplexes are not shown for clarity.

Table 2
Amino acids of replicative DNA Pols making hydrophilic contacts with incoming dNTP

| Ternary complex   | Finger conformation | PDB code | Residues of the palm | Residues of the fingers |
|-------------------|---------------------|----------|----------------------|------------------------|
| hPolα/DNA:RNA/dCTP | Closed              | 4QCL     | Ser863*               | Leu864*     Arg922      |
| hPolα/DNA:DNA/dCTP | Open               | 6A57     | Ser863*               | Leu864*     (Arg922)   |
| yPolα/DNA:RNA/dCTP | Closed              | 4FYD     | Asn611*               | Leu612*     Arg917      |
| yPolα/DNA:DNA/dCTP | Closed              | 3IAY     | Ser643*               | Met644*     Arg674      |
| yPolα/DNA:DNA/dATP | Closed              | 4M80     | Ser863*               | Leu864*     Arg781      |

* Structure resolution does not allow a view of water-mediated contacts; open conformation of the fingers affected their interaction with the triphosphate.

Activity in the presence of Mg2+ (Fig. 5). Consistent with that, mutation of Lys944 in yPolα is lethal (23). Substitution of Asn954, which makes a water-mediated contact with the triphosphate in the closed conformation, resulted in a modest effect on DNA polymerase activity. Kinetic studies have demonstrated that changes of Lys950 primarily affect affinity for the incoming dNTP and the processivity of DNA synthesis, whereas their effect on catalysis was much less (24). Interestingly, activity of p180core mutants R922Q and K950S was almost completely restored in the presence of manganese, whereas the activity of the WT protein was weakly affected (Fig. 5). These data are consistent with studies of metal dependence of the activity of entire hPolα and its mutants with different substitutions of Lys950 (24). Manganese provides a tighter complex with dNTP versus magnesium (25), allowing rescue of the compromised interaction of dNTP with the fingers in these mutants. The restoration of DNA-polymerizing activity of the finger mutants in the presence of Mn2+ explains the well-known mutagenic effect of this metal, which reduces Polα fidelity by 2 orders of magnitude (24, 25). Briefly, Mn2+-mediated binding of dNTP becomes less dependent on finger closure, an important selectivity step preventing mispairs.

Interaction of human Polα with DNA:DNA and RNA:DNA double helices

Like other B-family DNA polymerases, Polα makes continuous contact with the minor groove of the double helix of a template:primer (16, 22, 26, 27). The pal makes interactions with phosphates of P1, P2, and T2–T4 (Fig. 6B), which provides the optimal positioning of the primer 3′-end for catalysis. The flexible thumb secures the polymerase grip on the double helix by interacting with phosphates of T7, T9, and P3–P5. Two thumb residues, Arg1082 and Thr1140, make double contacts with primer phosphates, using both the side- and main-chain atoms. Each of the N-terminal and Exo subdomains make only one contact with a template:primer. Interestingly, the position of T1 phophate is not fixed in Polα (Fig. 6, A–D), whereas Polδ and Polε make a number of H-bonds with this phosphate (Fig. 6, E and F). In both ternary complexes of hPolα, the side chain of Arg355 extends into the major groove and packs against the base of T3.
Interaction of human Polα with DNA:DNA and DNA:RNA duplexes

On the other side of the double helix, the side chains of conserved Arg^{1081} and Lys^{1053} protrude into the minor groove and together with Arg^{834} serve as rails for template:primer translocation.

The reduced number of hPolα-DNA contacts in the hPolα/DNA:DNA/dCTP complex (Fig. 6A) is probably due to the crystal packing, which affected the thumb position (see above). This explanation is supported by the crystal structure of the hPolα/DNA:DNA binary complex showing the same network of DNA-protein interactions as in the hPolα/DNA:RNA/25-mer DNA:DNA complex reported in three reported complexes of Polα with a hybrid duplex (16, 18), the conformation of the P1 phosphate is different compared with the complexes of hPolα, yPolδ, and yPolε with a DNA duplex (Fig. 8). The conformation of the P1 phosphate was not affected by the addition of two deoxyribonucleotides to the 3'-end of the RNA primer, as seen in the yPol ternary complex (Fig. 8B), which indicates that it depends on primer bending due to the presence of ribose at P4 (Fig. 7). Thus, accommodation of RNA and chimeric primers is accomplished by dislocation of the P1 phosphorus, which results in disruption of the H-bond between this phosphate and the conserved Tyr^{1055} (Fig. 6B; this H-bond is replaced by the weaker water-mediated interaction in the case of a DNA duplex).

Polα does not exhibit typical polymerase burst kinetics

A pre-steady-state burst experiment was performed to provide insight into the relative rates of DNA polymerization steps in the kinetic mechanism of Polα. It has been frequently observed that under burst conditions (slight excess of template:primer over enzyme), many polymerases exhibit biphasic kinetics (28-30). This biphasic pattern indicates that in the overall mechanism, the release of products is rate-limiting and slow compared with steps governing chemical catalysis. p180core (3 μM) was incubated with either a 15-nucleotide DNA (D15) or RNA primer (R15) annealed to a 25-nucleotide DNA template (Fig. 9A). The preincubated solution was rapidly mixed with dATP (200 μM) using an RQF-3 rapid chemical quench apparatus. When the concentrations of products were plotted versus reaction time, an apparent biphasic curve was not observed but rather a linear formation of product for the elongation of both the DNA (k_{ss} = 104.3 ± 3.1 s^{-1}) and RNA (k_{ss} = 31.7 ± 1.7 s^{-1}) primer substrates (Fig. 9, B and C). Therefore, it appears that the Polα catalytic core does not exhibit typical biphasic kinetics observed in reactions with main replicative polymerases, Polδ and Polε (31, 32). This kinetic behavior indicates that for Polα, the rate of product release is equal to or faster than the rate of chemistry. These data are consistent with structural information showing weaker stabilization of Polα fingers in the closed state compared with Polω δ and ϵ (Table 2), which should increase the rate of finger opening and dissociation of a pyrophosphate/template:primer from a polymerase after catalysis. Conversely, the increased rate of finger opening may slow down

![Figure 5](image-url)

**Figure 5.** The effect of finger mutations on hPolα activity depends on the divalent metal. Extension of dA15 primer annealed to dT15 was carried out in the presence of 30 nM p180core, 1.5 μM template/primer, 0.1 mM dATP, and specified divalent metal ions. The dashed line indicates splicing of the original image.

The side chain of Arg^{1082} is located between the phosphates of P3 and P4 and can interact with any of them. Binding of a hybrid duplex results in potential steric hindrance between Arg^{1081} and the 2'-OH of the P4 ribose (Fig. 7A) and in a local change of RNA conformation (Fig. 7B), which includes the unusual syn-conformation of P4 (torsion angle χ = −70.3°), 2'-endo pucker of its ribose, and unstacking between the bases of P4 and P3. This bending of the RNA primer was also observed in the yPolα/DNA:RNA/dGTP complex (16). It is notable that the orientation of the P3 phosphate in the RNA primer is more favorable for hydrogen bonding with Arg^{1082} and Arg^{702}, compared with the P3 phosphate of the DNA primer. The same position of Arg^{1081} analog in ternary complexes of yeast Pols δ and ϵ indicates a common mechanism of RNA sensing in replicative DNA polymerases.

In three reported complexes of Polα with a hybrid duplex (16, 18), the conformation of the P1 phosphate is different compared with the complexes of hPolα, yPolδ, and yPolε with a DNA duplex (Fig. 8). The conformation of the P1 phosphate was not affected by the addition of two deoxyribonucleotides to the 3'-end of the RNA primer, as seen in the yPol ternary complex (Fig. 8B), which indicates that it depends on primer bending due to the presence of ribose at P4 (Fig. 7). Thus, accommodation of RNA and chimeric primers is accomplished by dislocation of the P1 phosphorus, which results in disruption of the H-bond between this phosphate and the conserved Tyr^{1055} (Fig. 6B; this H-bond is replaced by the weaker water-mediated interaction in the case of a DNA duplex).

![Table 3](image-url)

**Table 3.** Interaction of human Polα with DNA:DNA and DNA:RNA duplexes

| Reaction time (min) | 0.2 mM Mg^{2+} | 0.2 mM Mn^{2+} |
|--------------------|----------------|----------------|
| WT                 | 0.5 0.5 6 6   | 0.5 0.5 6 6    |
| R92Q               | 0.5 0.5 6 6   | 0.5 0.5 6 6    |
| K950S              | 0.5 0.5 6 6   | 0.5 0.5 6 6    |
| N954S              | 0.5 0.5 6 6   | 0.5 0.5 6 6    |

The reduced number of hPolα-DNA contacts in the hPolα/DNA:DNA/dCTP complex (Fig. 6A) is probably due to the crystal packing, which affected the thumb position (see above). This explanation is supported by the crystal structure of the hPolα/DNA:DNA binary complex showing the same network of DNA-protein interactions as in the hPolα/DNA:RNA/dCTP ternary complex (Fig. 6, B and C), with the additional contact between the P1 phosphate and Tyr^{1055}. The binding constants for p180core complexes with both duplexes are comparable (K_d = 58 ± 2 nM for DNA:DNA and K_d = 101 ± 6 nM for DNA:RNA; Table 3); the former value is in good agreement with a K_d of 44 nM for the hPolα/DNA:DNA complex reported by Copeland et al. (25). The discrepancy with data reported by Coloma et al. (17) (Table 3) might be due to different methods and experimental settings. Regardless, in both cases, the DNA and hybrid duplexes were compared at the same conditions, and their binding characteristics are similar.

As was observed in yeast Pols α, δ, and ϵ (Fig. 6, D–F), the invariant arginines 1081 and 1082 of hPol trap the sugar-phosphate backbone of the primer near P4. The side chain of Arg^{1081} extends into the minor groove and packs its guanidinium moiety against the sugar (Figs. 6 A–C and 7A). Such a position of Arg^{1081} is fixed by two H-bonds with Asp^{1083}. The
the overall rate of chemistry because finger closure stabilizes the triphosphate moiety of dNTP and, therefore, should facilitate the formation of a new phosphodiester bond. These results establish a similarity between Pol H9251 and Pol H9261, a polymerase implicated in base excision repair, which does not exhibit significant subdomain movements in response to nucleotide binding (33, 34).

Figure 6. Schematic diagrams of the interactions of eukaryotic replicative Pols with the sugar-phosphate backbone of a template:primer. The crystal structures of the following complexes were analyzed: hPol/DNA:DNA/dCTP (A), hPol/DNA:RNA/dCTP (B), hPol/DNA:DNA (C), yPol/DNA:RNA/dGTP (D), yPol/DNA:DNA/dCTP (E), and yPol/DNA/DNA/dATP (F). Among residues making van der Waals interactions with a template:primer, only the arginines contacting the P4 sugar are shown for clarity. Residues highlighted in italic type can make a hydrogen bond with a template:primer in their alternative conformation. Ribose, deoxyribose, and dideoxyribose rings are colored red, gray, and olive, respectively. The residues interacting with a template:primer are color-coded according to the subdomains they belong to (see Fig. 1 for color-coding). The conservative amino acids are underlined. The residues marked by an asterisk use a main-chain atom to establish H-bond with a sugar-phosphate backbone. The pound sign indicates that the residue makes double contact with a template:primer, using both the side- and main-chain atoms. The type of the double helix (A, B, or intermediate AB) was determined for each dinucleotide step using Web 3DNA software (Rutgers University) (69). This software requires the presence of two consecutive dinucleotide steps of the same type to confidently determine the helix type, so the third dinucleotide step of DNA:RNA was downgraded by the program to unclassified. We assume that in locally distorted double helices, the presence of a single isolated step may take place, which is in line with data on yPol (16). In the p180core/DNA:RNA/aphidicolin complex (PDB code 4Q5V), the pattern of interactions between hPol and the template:primer is the same as shown in B except for the loss of the H-bond between T and Ser786.

Table 3

| Duplex      | $K_d$ This study | $K_d$ Ref. 17 | $K_d$ Ref. 25 |
|-------------|-----------------|---------------|---------------|
| DNA:DNA     | 58 ± 2          | 430           | 44            |
| DNA:RNA     | 101 ± 6         | 320           |               |

Polα incorporates the first and second nucleotides into a DNA primer more efficiently than into an RNA

Single-nucleotide incorporation experiments were done under single-turnover conditions (excess Polα over template:primer) to quantify the maximal rate of catalysis, $k_{pol}$, and the apparent binding constant for the incoming nucleotide, $K_d$. p180core (3 μM) was incubated with a template:primer (100 nM) and rapidly mixed with varying concentrations of dATP (1–300 μM) under rapid chemical quench conditions (Fig. 9D). For the DNA primer substrate, the $k_{pol}$ is 33.8 ± 3.7 s$^{-1}$, the $K_d$ is 9.2 ± 3.4 μM, and the incorporation efficiency ($k_{pol}/K_d$) is 3.7 μM$^{-1}$ s$^{-1}$ (Table 4). For the RNA primer substrate, the $k_{pol}$, $K_d$, and $k_{pol}/K_d$ values are 48.0 ± 2.7 s$^{-1}$, 62.2 ± 10.4 μM, and 0.8 μM$^{-1}$ s$^{-1}$, respectively. It appears that Polα incorporates a
Interaction of human Polε with DNA:DNA and DNA:RNA duplexes

nucleotide more efficiently to a DNA primer by approximately a factor of 4.5. Strikingly, this discrepancy is primarily due to differences in the $k_{pol}$ for the incoming nucleotide. This difference might be explained by reduced stability of the 3′-end of an RNA primer due to lack of interaction between Tyr1055 and the P1 phosphate (Fig. 6, B and D). As a result, increased dynamics of the primer 3′-end may affect dNTP loading to the active site through steric hindrance. In addition, the position of the primer 3′-end and dNTP binding might be affected by the presence of ribose at P1 (see "Discussion").

Experiments were conducted to examine whether subsequent incorporations to a DNA primer were more efficient as compared with an RNA primer after the initial dNTP incorporation event. During the second incorporation, a 3′′′-hydroxyl of deoxyribonucleotide attacks the α-phosphate of incoming dNTP in both the DNA and RNA primer extension assays. Double-incorporation assays were conducted to estimate the kinetic parameters of a subsequent incorporation after the first incorporation. Experiments were designed to observe a dATP followed by a dTTP incorporation under single-turnover conditions. Preincubated solutions of p180core (3 μM) and template-primer (100 nm) were mixed with a solution containing both dATP and dTTP. Because the kinetic parameters of the second incorporation were desired, concentrations of dATP were held at saturating amounts (100 μM for DNA primer and 300 μM for RNA, based on single-incorporation experiments), and concentrations of dTTP were varied from 1 to 300 μM (Fig. 10A).

The appearance of the second incorporation product is complex due to its dependence on the first product being made; product concentration cannot simply be fit to a single-exponential curve like the single-incorporation assays. Thus, the KinTek Global Explorer program (35) was utilized to fit the data and provide an estimate for the $k_{pol}$ and $K_d$ of the dTTP incorporation (Fig. S1). For the second incorporation to a DNA primer, the $k_{pol}$ is estimated to be 65.8 s$^{-1}$, and the $K_d$ is 5.4 μM. The estimates for the RNA second incorporation are $k_{pol} = 23.1$ s$^{-1}$ and $K_d = 13.4$ μM. This leads to incorporation efficiencies of 10.4 and 1.7 μM$^{-1}$ s$^{-1}$ in favor of DNA primer extension (Table 5). This 6-fold difference in incorporation efficiency for DNA versus RNA for sequential dNTPs is comparable with the single-incorporation results. Contrary to the single-incorporation experiments, a major difference in $k_{pol}$ values is observed in the double-incorporation experiments. We hypothesize that the ~3-fold difference in $k_{pol}$ values may stem from the primer bending at P4 in the hybrid duplex. The altered helix structure possibly affects the rate of any step in subsequent incorporations after the initial incorporation, such as translocation or necessary protein conformational changes. The reason for similar $k_{pol}$ values during the first incorporation is the preforma-
Comparison of Pol\textsubscript{H9251}/H9262 processivity on DNA:DNA and DNA:RNA duplexes

A processivity experiment was employed to examine multiple rounds of dNTP incorporation and look at the full extension of the primers and determine whether the rates of each incorporation were different over time between DNA and RNA primers. This analysis observes the $k_{\text{pol}}$ for each incorporation event as well as $k_{\text{off}}$, which characterizes the dissociation rate of the Pol\textsubscript{H9251}/H9262/template:primer complex. The assay was conducted under single-turnover conditions and saturating concentrations (240 $\mu$M) of all dNTPs (Fig. 10, B and C). The products were plotted against time and fit to a single-exponential equation to obtain the observed rates of product formation. The rates were plotted against concentration of dATP and fit to a hyperbolic equation to obtain $K_d$ and $k_{\text{pol}}$ values.

Table 4

Incorporation efficiencies of dATP from single-incorporation assays

| Primer | $k_{\text{pol}}$ | $K_d$ | Efficiency$^a$ |
|--------|-----------------|-------|---------------|
| DNA    | 33.8 $\pm$ 3.7  | $9.2 \pm 3.4$ | 3.7 $\mu$M$^{-1}$ s$^{-1}$ |
| RNA    | 48.0 $\pm 2.7$  | 62.2 $\pm 10.4$ | 0.8 $\mu$M$^{-1}$ s$^{-1}$ |

$^a$ Efficiency values are calculated by dividing $k_{\text{pol}}$ by $K_d$.

to fourth incorporations during DNA primer extension, with an average value of 26.8 s$^{-1}$ compared with 12.0 s$^{-1}$ for RNA. As was shown above, the first incorporation does not show the real $k_{\text{pol}}$ values due to preformation of Pol\textsubscript{H9251}/H9262/template:primer complex; that is why it was omitted from averaging.

Interestingly, we observe an increase in the rate of incorporation after the fourth incorporation into the RNA primer, with an average $k_{\text{pol}}$ for the fifth to seventh incorporations of 24.4 s$^{-1}$, which is similar to the value obtained on a DNA primer (22.2 s$^{-1}$). This is particularly interesting in light of our structural results pertaining to the kink at P4 in the hybrid duplex structure. This observation can be explained in structural terms by the fact that after four incorporations, the rigid and conserved DNA-binding cleft of Pol\textsubscript{H9251}/H9262 no longer makes contacts with the ribonucleotides in the hybrid duplex. This increase in $k_{\text{pol}}$ agrees with our previous experiments demonstrating that DNA primer elongation is more efficient. Furthermore, to confirm that the increase from 12.0 to 24.4 s$^{-1}$ after the fourth incorporation is significant, we simulated how the time course of processive elongation of an RNA primer would change if the fourth incorporation rate was modified to match the rate observed in the DNA experiment (Fig. 10E, dotted lines). Changing 8.3 s$^{-1}$ to 19.3 s$^{-1}$ results in a drastically different product distribution. The most prominent effects are the decreased accumulation of R18 and faster production of R20 because the rate of R19 formation is no longer significantly rate-limiting.
Interaction of human Polα with DNA:DNA and DNA:RNA duplexes

Figure 10. Sequential incorporation modeling of hPolα kinetics. A, a representative gel of the elongation of the DNA primer substrate in the presence of 100 μM dATP and 2 μM dTTP. The n band represents the 15-mer primer: n + 1, the primer elongated by dATP across template dT; n + 2, the primer elongated first by dATP followed by dTTP across template dA. p180core (3 μM) was incubated with radiolabeled DNA template:primer (100 nM) and rapidly mixed with saturating concentrations of dATP (100 μM) and various concentrations of dTTP (1–100 μM). B, the time course of DNA primer elongation. p180core (3 μM) was incubated with radiolabeled DNA template:primer (100 nM) and rapidly mixed with saturating concentrations of dNTPs (240 μM) and various concentrations of dTTP (1–100 μM). C, the time course of RNA primer elongation. D, processivity modeling for DNA primer elongation. Product formation was plotted versus time, and the incorporations were fit to a processive kinetic model using KinTek Global Explorer to provide estimates of the kinetic model using KinTek Global Explorer to provide estimates of the parameters in the kinetic model. The logo for hPolα indicates that on average (for the second to fourth incorporations) hPolα incorporates approximately three or four and six consecutive nucleotides before complex dissociation in the case of DNA and RNA primer, respectively. Using a DNA-trap, it was shown that hPolα incorporates six consecutive nucleotides before dissociation from the DNA duplex (36). Slightly higher processivity observed by Copeland and Wang’s assay may be due to the absence of the monovalent salt in reaction (0.1 M NaCl was used in our experiments). In extension of the DNA primer, yPolδ has the same processivity as hPolα, whereas yPolε catalytic core containing an unique processivity domain incorporates 27 nucleotides in one binding event, in the absence of monovalent salt in reaction (22). Interestingly, k_{pol} values estimated from kinetic modeling do not provide error measurements.

Table 5

| Primer | k_{pol} | K_{d} | Efficiencya |
|--------|---------|-------|-------------|
| DNA    | 65.8 ± 11.6 | 5.4b | 12.2        |
| RNA    | 23.1 ± 3.5  | 13.4b| 1.7         |

a Efficiency values are calculated by dividing k_{pol} by K_{d}
b K_{d} values estimated from kinetic modeling do not provide error measurements.

It was also observed that the average k_{off} value for DNA primer elongation is 7.0 s^{-1} compared to 2.3 s^{-1} for RNA (for the second to fourth incorporations). The higher k_{off} value for the DNA duplex may be due to the tendency for it to adopt the B-form, which is slightly narrower than the template:primer-binding cleft. In contrast, the hybrid duplex is squeezed between Gly^{341} and Gly^{1076} (Fig. 6B; see “Discussion”), which may potentially slow the dissociation and association of the duplex. Indeed, the estimates for k_{on} values (0.12 and 0.02 nm^{-1} s^{-1} for DNA and the hybrid duplex, as a first approximation) calculated by dividing the average k_{off} for the first four incorporations by the K_{d} show a higher rate of complex formation with the DNA duplex. It is noteworthy that the interaction of the thumb with the distant part of a template:primer (T_{5};P_{5};T_{y};P_{y}) can also contribute to slower dissociation of Polα from the hybrid duplex because this DNA-binding region is optimal for recognition of the double helix of mixed AB-form (Fig. 6B). Accordingly, two dinucleotide steps in this region of the DNA duplex have the mixed AB-form (Fig. 6C).

The ratio k_{pol}/k_{off} provides an estimate of processivity and indicates that on average (for the second to fourth incorporations) hPolα incorporates three or four and six consecutive nucleotides before complex dissociation in the case of DNA and RNA primer, respectively. Using a DNA-trap, it was shown that hPolα incorporates six consecutive nucleotides before dissociation from the DNA duplex (36). Slightly higher processivity observed by Copeland and Wang’s assay may be due to the absence of the monovalent salt in reaction (0.1 M NaCl was used in our experiments). In extension of the DNA primer, yPolδ has the same processivity as hPolα, whereas yPolε catalytic core containing an unique processivity domain incorporates 27 nucleotides in one binding event, in the absence of monovalent salt in reaction (22). Interestingly, k_{off} values are comparable for hPolα and yPolε, and the difference in processivity is mainly due to the higher incorporation rate of the latter. Higher processivity of Polα on the hybrid duplex can prevent the enzyme from premature dissociation.
Interaction of human Polα with DNA:DNA and DNA:RNA duplexes

Table 6

Kinetic parameters from processivity assays

| Incorporation | DNA $k_{\text{pol}}$ (s$^{-1}$) | RNA $k_{\text{pol}}$ (s$^{-1}$) | DNA $k_{\text{off}}$ (s$^{-1}$) | RNA $k_{\text{off}}$ (s$^{-1}$) |
|---------------|---------------------------------|----------------|-----------------------------|-----------------------------|
| First         | 14.0 ± 0.9                      | 14.1 ± 0.7     | 4.5 ± 1.2                   | 4.5 ± 0.8                   |
| Second        | 49.9 ± 7.1                      | 16.2 ± 1.1     | 11.0 ± 3.6                  | 4.0 ± 0.8                   |
| Third         | 11.2 ± 1.3                      | 11.4 ± 1.0     | 4.2 ± 1.6                   | 2.1 ± 0.7                   |
| Fourth        | 19.3 ± 4.5                      | 8.3 ± 1.0      | 5.8 ± 3.7                   | 0.9 ± 0.8                   |
| Fifth         | 38.0 ± 16.8                     | 28.1 ± 8.9     | 6.5 ± 7.6                   | 1.8 ± 2.5                   |
| Sixth         | 14.6 ± 4.8                      | 20.6 ± 6.2     | 2.4 ± 3.8                   | 1.8 ± 2.5                   |
| Seventh       | 14.1 ± 7.4                      | 24.6 ± 9.9     | 1.2 ± 5.0                   | 2.0 ± 3.4                   |
| Average (second to fourth) | 26.8                          | 12.0             | 7.0                           | 2.3                          |
| Average (fifth to seventh) | 22.2                          | 24.4             | 3.4                           | 1.9                          |

Additionally, the processivity of a polymerase can be qualitatively assessed by inspection of separated products on a gel. Comparing the processivity gels from Polα (Fig. 10, B and C) and T7 DNA polymerase (30), the striking difference is the presence of primers that have not been fully extended with dNTPs at later points in the experimental time course. Through our experiments, it is observed that there is a significant population of primers that have been only singly elongated ($D_{1e}$) at later time points even when the final product is finally produced ($D_{2e}$). In contrast, once T7 DNA polymerase incorporates a dNTP into a primer, it continues to incorporate nucleotides into the same primer. In the direction of increasing time, the pattern on the gel for the Polα can be described as extending a ladder upward, whereas T7 can be described as shifting a ladder upward. Processivity experiments have also been done with Polɛ (31), and the pattern of processive polymerization is similar to Polα. Although Pol is considered more processive than Polα, the striking difference between T7 and the former polymerases highlights the highly variable kinetic characteristics of polymerases.

The sugar at the primer 3'-end affects Polα fidelity

The potential of Polα to generate 12 possible mismatches during the extension of a DNA or RNA primer was compared. This study revealed that p180core has pronounced difference in nucleotide selectivity during the addition of the first dNMP to the RNA and DNA primers (Fig. 11). It is relatively accurate on RNA but easily generates various mismatches when extending the DNA primer. The most prominent of them are A:dA, T:dG, G:dT, G:dG, and C:dT (the left letter indicates a nucleotide of the template). Moreover, p180core inefficiently extends some mismatches (Fig. 11B, lanes 6, 7, 11, 13, and 16), even with noncognate nucleotides (lanes 6, 7, and particularly lane 16 showing two consecutive A:dC mismatches and their extension). Interestingly, Polα is not able to extend the most prominent mismatch, A:dA, whereas it easily extends C:dA and G:dA by generating the next consecutive mismatch, A:dA (Fig. 11B, compare lanes 6–8). The results are consistent with kinetic studies of human and yeast Polα, showing that they discriminate against noncognate dNTPs and the mismatched primer terminus by a factor of $10^3 \text{ to } 10^4$ and 500, respectively (25, 37, 38). The discrimination mainly depends on the difference in affinity for a correct versus incorrect incoming nucleotide, thus explaining reduced selectivity in the presence of 0.2 mM dNTP in our experiments (this concentration is ~20-fold higher than concentration of dNTPs in mitotic cells (39, 40)).

Intriguingly, Polα becomes less accurate after incorporation of just one dNTP to an RNA primer. For example, no product was detected during the first step of RNA primer extension with dATP opposite the template adenine (Fig. 11C, lane 8), whereas in the same time frame, a significant amount of A:dA mismatch was generated during the second step of RNA primer extension, following incorporation of the first dAMP opposite the template thymine (lane 9). More efficient mismatch generation during the second step is not due to formation of the weak T:dA bp because the same effect was observed using other dNTPs (Fig. 11C, comparison of lanes 2 and 4 (dGTP), 11 and 12 (dTTP), and 15 and 16 (dCTP); appearance of the second and/or the following bands above the product indicates mismatch formation). Dramatic accuracy reduction after the insertion of just one dNMP indicates that Polα fidelity depends on the presence of ribose at P₁, but not on the primer bending at P₄ (Fig. 7).

Initial burst experiments, performed on a broad time scale to establish the relative rate of dNTP incorporation by the p180core, revealed that Polα incorporates an incorrect dNTP at a notable rate (Fig. 12). This is illustrated at later time points by the appearance of a second band that corresponds to a dATP.
Table 2

| Conformational Dynamics | DNA Synthesis by Polα |
|-------------------------|-----------------------|
| Benefits of utilizing a pre-steady-state kinetic approach. | The elongation of the DNA primer in the presence of only dATP. The n + 1 band corresponds to a correct incorporation of dATP across from the templating dA. The sequence shows where the incoming dATP is incorporated onto the substrate. |

**Figure 12. Polα displays misincorporation on a rapid time scale.**

p180core (3 μM) was incubated with radiolabeled DNA template:primer substrate (10 μM) and rapidly mixed with saturating dATP (500 μM). The gel shows the elongation of the DNA primer in the presence of only dATP. The n + 1 band corresponds to a correct incorporation of dATP across from the templating dT. The n + 2 band corresponds to a misincorporation of dATP across from a templating dA. The sequence shows where the incoming dATP is incorporated onto the substrate.

**Discussion**

Currently, the structure of Polα catalytic domain is thoroughly studied. Eight crystal structures have been reported for yeast and human orthologues, which include the apo-form, binary, and ternary complexes, containing DNA or RNA primer, as well as the structures of human primosome and the complex of hPolα with the natural inhibitor aphidicolin (6, 16–18). In addition, the crystal structures of the CTD–B-subunit complexes are known for yeast and human Polα (11, 12). With regard to the catalytic subunits of Polδ and Polε, only the structures of the ternary complexes have been determined for their catalytic domains (22, 26, 42) as well as the structure of the Polε CTD–B-subunit complex (43).

**The role of conformational dynamics in DNA synthesis by Polα**

Accumulated structural information for the catalytic domain of Polα indicates that the N-terminal and exonuclease subdomains are firmly associated, whereas positions of other subdomains relative to the N-terminal part of p180core and each other are quite flexible (this work; also, see Refs. 6 and 16). Such flexibility of the subdomains responsible for substrate binding and catalysis defines the conformational dynamics of the polymerase during DNA synthesis. For example, significant mobility of the thumb might be important for accommodation and translocation of duplexes containing lesions, mismatches, and different types of primers: RNA, DNA, and chimeric RNA–DNA. Accordingly, Polα has notable translesion activity and the ability to extend the mismatched 3’ terminus of a primer (25, 44).

Relatively weak interactions of the mobile part of the fingers with the other subdomains allow them to switch quickly between the open and closed states. High dynamics of the fingers is important for rapid closing after dNTP binding and opening after catalysis to release the products. The binding of dNTP results in the shift of equilibrium to the closed conformation, which in hPolα is primarily due to formation of two H-bonds between Arg⁹²² and the γ-phosphate (Table 2). The dNTP becomes trapped after fingers closing, promoting effective catalysis. Crystal packing may have helped to stabilize the open conformation of the fingers in the hPolα/DNA:DNA/dCTP complex because, in free energy terms, the open state should be less favorable versus the closed one in the presence of bound dNTP. The other known example of a DNA polymerase crystallized in the open ternary complex is Poλβ, which belongs to the X-family (45). That structure was obtained after mutating the conserved Arg²₈₅ important for stabilization of the closed state, which shifted the equilibrium between two conformations of the helix N to the open one. Whereas the helix N fulfills a similar task as the fingers in Polα, it does not interact with a triphosphate of an incoming nucleotide.

This study revealed that Polα fingers sense the triphosphate moiety of dNTP even in the open state (Fig. 4A and Table 2). Interaction of open fingers with dNTP might be important for stabilization of the ternary complex during the first step of its formation and could trigger fingers closing. Currently, there is a lack of information on how the fingers of yeast Polδ and Polε interact with dNTP in the open conformation. Furthermore, the structures of these Pols in the apo-form are not reported, which precludes the possibility to model potential interactions between the residues of open fingers and the triphosphate. The absence of a rate-limiting step for product release revealed from pre-steady-state burst experiments reflects fast dynamics of Polα fingers caused by weak stabilization of the closed conformation. This structural peculiarity of Polα results in higher probability of fingers opening, facilitating mismatch accommodation at the nascent bp-binding pocket (see below). This finding may provide a key insight into the mechanism of the generation of mutation hot spots in the genome (7). Interestingly, all amino acid changes in hPolα conferring resistance to CD437, a potential anticancer molecule recently discovered to target Polα, are located at or near the interface between the fingers and the N-terminal/Exo subdomains (9).

**Comparison of template:primer binding by replicative DNA Pols**

All invariant residues of replicative DNA polymerases contacting the template:primer interact with the first five bp of the duplex (Fig. 6). The most interesting feature of these polymerases is the interaction of T₃ and P₂ phosphates with two conservative glycines of the palm (Gly⁸²¹ and Gly¹₀₇₆ in hPolα). These glycines determine the width of the double helix between the second and third bp from the primer 3’-end, with similar distances between their main-chain nitrogens for hPolα in complex with DNA:RNA (20.2 Å) and yeast Polδ and -ε in complex with DNA:DNA (20.4 and 20.3 Å, respectively). The other common feature of replicative polymerases is the absence of interaction with a T₄ phosphate (Fig. 6), which might be important for adjusting the position of the templating nucleotide to obtain optimal pairing with an incoming dNTP. In contrast to Polε, the thumbs of Polα and Polδ are engaged almost exclusively with the primer and do not interact with T₅ and T₆.
exonuclease subdomains of Polα and Polε interact with the P₃ phosphate, whereas there is no such interaction in Polδ. The catalytic domain of yPolα has the smallest footprint on the double helix (eight bp), and that of yPolε has the largest one (10 bp, with a potential up to 12 bp (22)). The low footprint of Polδ on the template is compensated by the double contact with the P₆ phosphate (Fig. 6E). In contrast, Polα and Polε sense only five continuous nucleotides of the primer. The recently discovered P-domain of Polε allows it to make three additional contacts with a DNA duplex (Fig. 6F), one with a primer (P₆) and two with a template (T₂ and T₃), and two extra contacts are possible (22). These additional contacts and the extensive network of polar contacts between the thumb and the template explain the higher processivity of Polε catalytic domain compared with Polδ and Polα. In both ternary complexes of hPolα, the side chain of Arg⁸³⁴ located on the flexible loop extends into the major groove and packs against the base of T₃. For comparison, yeast Polα and Polε have no contacts with the major groove, whereas the β-hairpin of yPolδ is anchored in it (26).

In solution, the RNA, DNA, and hybrid duplexes (DNA:RNA) prefer to be in the A-, B-, and mixed AB-form, respectively (46–48). The A-form is wider and shorter than the B-form, with a diameter of 23 Å versus 20 Å and a rise per base of 2.6 Å versus 3.4 Å, respectively. DNA-protein interactions can affect the shape of the double helix, locally or globally (49). The conserved character of interaction with the first four bp determines the universal form of the double helix for this region in the ternary complexes of Polα, Polδ, and Polε, independent of the primer nature (Fig. 6). This is also true for the chimeric RNA–DNA primer (Fig. 6D), which reflects the initial steps of RNA primer extension by Polα. Replicative DNA polymerases transform the first two dinucleotide steps of DNA:DNA into the mixed AB-form and the third dinucleotide step of DNA:RNA into the B-like form (the latter is currently shown only for ternary complexes of Polα). Interestingly, in all ternary complexes of eukaryotic replicative DNA polymerases, the sugars of T₀, T₁, P₁, and incoming dNTP have a 3′-endo pucker (or closely C2′-exo and C4′-exo pucks), which might be important for catalysis.

The alignment of hPolα/DNA/RNA/dCTP and yPolδ/DNA:DNA/dCTP complexes (RMSD of 2.08 Å for 524 Ca atoms) shows good superposition for the first four bp of the double helix, which is consistent with the same duplex form in this region as well as with high structural similarity for the palm and the N-terminal part of the thumb (Fig. 13). In contrast to Polα and Polε, Polδ has a short loop in the tip of the thumb (residues 895–900; highlighted in blue), which allows it to interact with the P₂ phosphate using the main-chain nitrogens of Thr⁸⁹⁸ and Asn⁹⁰⁹ (Fig. 13). This loop also contains the conserved Tyr⁸⁸⁷, which interacts with P₆ and, together with Thr⁸⁹⁸ and Asn⁹⁰⁹, would clash with P₆ and P₇ upon DNA:RNA binding by Polδ. The additional steric gate residues for the wider AB-form of the hybrid duplex in yPolδ are Leu⁸³⁵ and Tyr⁹³⁶, which are located on the 310-helix of the thumb tip and correspond to Ala¹¹⁸⁸ and Ser¹¹³⁹ of hPolα, respectively (Fig. 13). This region may have a minor role in hybrid duplex sensing by Polδ due to
Interaction of human Pol\(\alpha\) with DNA:DNA and DNA:RNA duplexes

Potential flexibility of the thumb. Consistent with this structural analysis, Pol\(\delta\) extends the DNA primer with significantly higher efficiency versus RNA (50). Similar to Pol\(\alpha\), Pol\(\epsilon\) does not interact with P\(_e\) (Fig. 6, B and F) and efficiently extends primers containing up to five consecutive ribonucleotides at the 3’-end (51). Pol\(\delta\) also differs from Pol\(\alpha\) and Pol\(\epsilon\) (22) by the presence of a β-hairpin near the unprimed region of the template (Fig. 13).

In summary, all replicative DNA Pols have the same rigid template:primer-binding cleft for the first four bp (from T\(_2\):P\(_1\) to T\(_4\):P\(_3\)), which is mainly formed by the palm residues (Fig. 6). The DNA-binding site distant from the catalytic center (for the duplex region spanning 5–10 bp from the primer 3’-end) significantly differs in B-family Pols and probably reflects the role of each polymerase in DNA replication and repair. For example, the low efficiency of RNA primer extension by Pol\(\delta\) as well as increased processivity of the Pol\(\epsilon\) catalytic domain are mainly due to polymerase-specific structural peculiarities of this part of the DNA-binding cleft.

**Structural basis of Pol\(\alpha\) fidelity and infidelity**

Pol\(\alpha\) demonstrates relatively high fidelity during RNA primer extension with the first dNTP, whereas the incorporation of the following nucleotides is less accurate (Fig. 11). Surprisingly, the bulky purine:purine mismatches are among the most prominent, which is probably due to the ability of Pol\(\alpha\) to easily fit them into the nascent bp-binding pocket. Current structural information suggests that at least four factors may assist in formation of the purine:purine mismatches: fingers opening (Fig. 4A), syn-conformation of T\(_0\) stabilized by Arg\(^{784}\) (17, 18), absence of the β-hairpin (Fig. 13); in Pol\(\delta\), the T\(_{-1}\) sugar and the T\(_0\) phosphate are secured between the β-hairpin and the N-terminal subdomain, and weak interaction of Pol\(\alpha\) with the template 5′-overhang (Fig. 6B), which allows more conformational freedom for T\(_1\)–T\(_{-1}\) during mismatch accommodation. The exact mechanism of Pol\(\alpha\) low fidelity is not known due to the absence of structural information for the WT B-family DNA polymerases in the mismatch-containing ternary complexes.

Intriguingly, the structure of the p180core/DNA:RNA/aphidicolin complex (18) provides a clue for how the most prominent A:dATP mispair fits the active site of Pol\(\alpha\). Indeed, all four factors listed above play a role in the accommodation of the bulky aphidicolin in place of the incoming dCTP, where the local change of template conformation (Fig. 14A) resulted in disruption of interaction between T\(_{-1}\) and Ser\(^{785}\). The modeling shows that Pol\(\alpha\) can easily accommodate all purine:purine mismatches when the T\(_0\) purine is in syn-conformation (Fig. 14B). In addition, A:dATP can fit the nascent bp-binding pocket when the templating adenine is in the anti-conformation. Small steric hindrance between two purines in this case might be relieved by the additional minor adjustments in the active site. This model of A:dATP mispair accommodation is applicable to the other mismatches. Kinetic studies of yeast Pols \(\delta\) and \(\epsilon\) are consistent with the role of the β-hairpin in the fixation of the template position at T\(_0\) which should significantly reduce the chance to generate the bulky purine:purine mismatches. Among replicative Pols of eukaryotes, only Pol\(\delta\) has the β-hairpin and generates the purine:purine mismatches with relatively low efficiency (32). Accordingly, exonuclease-deficient yPol\(\delta\) and yPol\(\epsilon\) discriminate against the G:dG mismatch by a factor of 6 × 10\(^4\) (32) and 5 × 10\(^3\) (52), respectively.

The apparent low fidelity of Pol\(\alpha\) during incorporation of the second and following dNMPs allows us to compare the enzyme with other well-studied polymerases. HIV-1 reverse transcriptase has been established as a low-fidelity polymerase (53). Its activity is beneficial for the virus, allowing for rapid mutation and development of resistance against antivirals. Although poor discrimination between nucleotides may be desirable for viruses, this trait is not particularly advantageous for host polymerases, which are responsible for perfectly replicating the genome. The low accuracy of Pol\(\alpha\) might be important for the evolution of regulatory elements in eukaryotic genomes (7).
Polα has a significant contribution to the mutation rate in vivo, despite the fact that only 1.5% of Polα-synthesized DNA has been shown to be retained in the mature genome (7). Poor fidelity in host polymerases is not limited to Polo and translesion polymerases, including the other member of the B-family, Polθ. Recently, PrimPol, a primase-polymerase that is implicated to reinitiate replication after mitochondrial DNA damage, has been characterized to have low fidelity (54, 55). It is interesting that we observe a prominent purine:purine mismatch in our experiments with Polα (A:dA) as well as with PrimPol (G:dA).

High accuracy during the addition of the first dNMP to the RNA primer, which Polα receives from primase during intra-molecular transfer (6), might be important for a smooth switch from the RNA- to DNA-polymerizing mode of the primosome. The structure of the p180core/DNA:RNA/dCTP complex can provide a key to the effect of the 3'-ribose on Polα fidelity. The 2’-OH modeled at the primer 3'-end has steric hindrance with the conserved Thr1003 (Fig. 14C). The P1 ribose shall move from Thr1003 by ~0.4 Å to avoid steric clashing, which will affect the position of the T1−T2, and can alter their ability to change the conformation upon mispair binding.

The structures of the catalytic domain of replicative DNA Pols provide a rationale for the mutagenic effect of substitutions of the conservative leucine (37, 44, 56, 57), whose main chain interacts with the dNTP β-phosphate (Leu864 in hPolα; Fig. 4). This leucine is located in the hydrophobic pocket formed in hPolα by Phe861, Tyr865, Ile868, Ile983, and Leu1036. Bulky side chains of methionine and phenylalanine in place of Leu864 cannot fit into this pocket, which would result in the distortion of the upper part of the α-helix (Fig. 4), affecting the position of Ser863, Leu864, and Tyr865. Among these residues, Tyr865 is the most important for Pol selectivity. Such distortion of the dNTP-binding pocket can result in dNTP wobbling and reduced discrimination against mismatches.

**The overall mechanism of RNA primer extension by Polα**

Accumulated information from structural and functional studies allows us to understand the mechanism of chimeric RNA–DNA primer synthesis by the primosome, including the ability of Polα to efficiently extend primers with varying DNA/RNA ratio and bind double helices with different helical parameters. The following structural peculiarities of the Polo catalytic domain are important to perform this task: small primer-binding interface, thumb flexibility, and the universal template:primer binding site, which is able to accommodate the substrates with different structures. Perhaps during evolution, Polo developed the ability to fit the template:primer into a universal form of the double helix by deforming both DNA:RNA and DNA:DNA duplexes.

Structural studies of the human primosome point out that Polα receives a 9-mer RNA primer from primase (6). The data presented here establish the four main steps of RNA primer extension by Polα: incorporation of the first dNMP (first step), second to fourth (second step), fifth to ninth (third step), and tenth and further (fourth step). The main features of the first step are the enhanced accuracy (Fig. 11) and the relatively low affinity for dNTP (Table 3). The increased Polα selectivity for a cognate dNTP is probably due to the presence of ribose at P3 (Fig. 14C), whereas dNTP binding is also affected by the primer bending at P4 (Fig. 7). The second step of RNA primer extension is characterized by reduced polymerization rate and increased processivity compared with Polα activity on a DNA primer (Tables 5 and 6). For this step, primer binding at P4 and the squeezing of a hybrid duplex between Gly441 and Gly1076 may affect template:primer translocation along the DNA binding cleft and dissociation from the polymerase. The third step of extension is similar to the fourth step, which corresponds to extension of a DNA primer (Table 6). The hybrid part of the template:primer leaves the rigid and relatively narrow section of the DNA binding cleft and interacts only with the thumb, which is slightly more selective for a mixed AB-form of the double helix versus a B-form (Fig. 6, B and C). During the fourth step, the Polα catalytic domain does not sense the hybrid part of a template:primer, and the characteristics of DNA synthesis should be identical to that on a DNA:DNA duplex.

The size of DNA tracks synthesized by Polα on the leading and lagging strands has not been determined yet. It is possible that the length of chimeric RNA–DNA primers is precisely regulated, as was recently shown for the RNA primer (6). Only the structural properties of the primosome define the mechanism of RNA primer switching between primase and Polα, whereas additional factors should play a role in chimeric primer switching between Polα and Polθ on the leading or lagging strand, respectively. In 2013, the mechanism of primer synthesis termination by Polα, based on its low activity in extension of homopolymeric DNA primers, had been proposed (16). As was shown later, the low activity of Polα in primer synthesis on poly(dT) template is due to formation of unconventional DNA structures, which depends on the type of the divalent ion and its concentration (58). Moreover, comparable activity was observed during extension of DNA and RNA heteroprimer (59, 60). The data presented here indicate that Polα works on the DNA duplex with higher efficiency than on the hybrid one (Tables 4 and 5). On the other hand, Polα has low processivity on the DNA duplex, which can favor its displacement from the template:primer by Polθ on the leading strand and by the complex of the replication factor C and proliferating cell nuclear antigen on the lagging strand (replication factor C is then exchanged for Polθ) (61).

It is possible that additional factors play a role in determination of the size of DNA track synthesized by Polo. For example, the primase accessory subunit (ps8 in humans) has higher affinity for the template:primer (Kd is 33 nM (62)) than Polo (Kd is 58 nM; Table 3) and can hold the 5'-end of RNA primer during its extension with dNTPs and promote Polo reloading on the DNA primer (6). In this case, the linkers in primosome would allow Polα to generate a ~20-mer DNA track. The primosome structure also predicts significant steric hindrance after synthesis of a ~10-mer DNA track due to ~360° rotation of the catalytic domain of Polα relative to the C-terminal domain of p58; this would require a temporary dissociation of Polα from the template:primer to untwist the linkers. In addition, it might be the signal from the replisome, leading to primosome dissociation from the template:primer. For example, as was shown in yeast, Polα is tethered to the CMG helicase through the interaction between the N-terminal domain of the catalytic subunit and the replication factor Ctf4 (13). In humans, however, the
N-terminal domain of the accessory B-subunit connects Pol\(\alpha\) to a replisome (63), similar to the mode of Pol\(\varepsilon\) recruitment to a replication fork (64). In both cases the CMG-interacting domain of Pol\(\alpha\) is connected with the rest of the primosome by a long flexible linker (4). During Okazaki fragment synthesis, Pol\(\alpha\) moves along the parental DNA strand in the direction opposite to the replication fork, and at some point, the tension in the linker, connecting the catalytic core with the replisome, will result in Pol\(\alpha\) displacement from the primer 3'-end. Conducting structural and functional studies with the reconstituted eukaryotic replisome will shed more light on the mechanism of the template:primer switch from Pol\(\alpha\) to Pol\(\delta\) and Pol\(\varepsilon\).

**Experimental procedures**

**Oligonucleotides and reagents**

Oligonucleotides were manufactured by IDT Inc. Deoxyribonucleotides used to extend the primers were obtained from Roche Life Sciences. Reagents for crystallization were obtained from Hampton Research. [\(\gamma\)-\(^{32}\)P]ATP was obtained from PerkinElmer Life Sciences.

**Protein expression and purification**

Cloning, expression, and purification to homogeneity of p180core (residues 335–1257), which contains the catalytic domain of hPol\(\alpha\), have been described elsewhere (18). Peak fractions obtained from Heparin HP HiTrap column (GE Healthcare) were combined and dialyzed to the buffer specific for each application (see below).

**Analysis of DNA-polymerizing activity in extension of fluorophore-labeled primers**

Purified p180core was dialyzed to 10 mM Tris-HCl, pH 7.7, 100 mM KCl, 1% glycerol, and 1 mM DTT, and flash-frozen in aliquots. The primers had a Cy5 label at the 5'-end. Reactions were started by mixing two solutions; one of them contained p180core and the template:primer, and the other one contained dNTP and the divalent metal (their concentrations are indicated in the figure legends; other components were the same in both stocks).

**Crystallization**

DNA:RNA and DNA:DNA duplexes were obtained at 0.2 mM concentration by annealing at 43 °C for 30 min (after heating at 70 °C for 1 min) in buffer containing 10 mM Tris HCl, pH 7.9, and 70 mM KCl. In the case of DNA:RNA duplex, the sequences for DNA template and RNA primer were 5'-ATTACATTAGGCTCCAGGCG (the region complementary to a primer is underlined) and 5'-rGrCrUrGrGrArGrCrG/ddC/, respectively (/ddC/is a dideoxycytidine, which prevents polymerization during crystallization). In the case of DNA:DNA duplex, the template and primer were 5'-ATAGGCGGCTCCAGGC and 5'-GCCGTTGAGGC/ddC/, respectively. Dialyzed protein sample (15 \(\mu\)M p180core) was diluted 1.5-fold with dialysis buffer (10 mM Tris-HCl, pH 7.7, 100 mM KCl, 1% glycerol, and 1 mM DTT) containing 36 \(\mu\)M template:primer, 3.6 mM MgCl\(_2\), and 12 mM dCTP. The ternary complex was concentrated 10-fold and flash-frozen in liquid nitrogen. Before crystallization experiments, the aliquots of protein/template:primer/dCTP solutions were defrosted and centrifuged to remove the precipitate, and the sample monodispersity was verified with the dynamic light scattering. The screening of crystallization conditions was performed with the sitting-drop vapor diffusion method at 295 K by mixing 1 \(\mu\)L of ternary complex solution with 1 \(\mu\)L of reservoir solution. Initial screen solutions producing tiny crystals were optimized to produce well-shaped crystals at 295 K with reservoir solutions containing 0.8 mM zinc sulfate, 8.8% (v/v) PEG MME 550, and 50 mM MES, pH 6.5, for p180core/DNA:RNA/dCTP and 0.8 mM cobalt chloride, 2 mM tris(2-carboxyethyl) phosphine (TCEP), pH 7.5, 250 mM 1,6-hexanediol, and 50 mM sodium acetate, pH 4.6, for p180core/DNA:DNA/dCTP.

**Data collection**

For data collection, each crystal was soaked in cryoprotectant solution (100 mM potassium chloride, 0.8 mM zinc sulfate, 1.1 mM magnesium chloride, 15% (v/v) PEG MME 550, 15% (v/v) ethylene glycol, and 50 mM MES, pH 6.5, for p180core/DNA:RNA/dCTP crystals, and 0.8 mM cobalt chloride, 2 mM TCEP, pH 7.5, 230 mM 1,6-hexanediol, 26% (v/v) ethylene glycol, and 50 mM sodium acetate, pH 4.6, for p180core/DNA:DNA-dCTP crystals) for a few seconds, scooped in a nylon-fiber loop, and flash-cooled in a dry nitrogen stream at 100 K. All initial diffraction data were obtained on a Rigaku R-AXIS IV imaging plate using Osnic Varimax HR mirror-focused CuK\(\alpha\) radiation from a Rigaku FR-E rotating anode operated at 45 kV and 45 mA. Complete diffraction data sets were collected using synchrotron X-rays on the Argonne National Laboratory Advanced Photon source beamline 24-ID-E using ADSC Quantum 315 detector. All intensity data were indexed, integrated, and scaled with DENZO and SCALEPACK from the HKL-2000 program package (65). The crystals of p180core/DNA:RNA/dCTP belong to trigonal space group P3\(_2\)1 and diffract up to 2.2 Å resolution, and the crystals of p180core/DNA:DNA/dCTP belong to tetragonal space group P4\(_3\)2\(_1\)2\(_1\) and diffract up to 2.95 Å resolution. Both crystals contained only one copy of the ternary complex in the asymmetric unit. The crystal parameters and data-processing statistics are summarized in Table 1.

**Crystal structure determination**

Initial phases for p180core/DNA:RNA/dCTP structure were determined by the molecular replacement method using the coordinates of backbone atoms of the yeast Pol\(\delta\) catalytic core derived from its ternary complex with template:primer and dCTP (PDB code 3AY) as a search model. The positions of magnesium and zinc ions were determined using an anomalous difference Fourier map. Molecular replacement and initial automated model rebuilding with Phenix (66) revealed over 70% of correctly built protein structure. The model building was continued and completed manually with Turbo-Frodo, and the structure was refined using standard protocols of CNS version 1.1 (67). After the addition of solvent molecules, the model was refined at 2.2 Å resolution to an R\(_{\text{cryst}}\) of 21% and an R\(_{\text{free}}\) of 23.9%. The structured region of protein starts with Glu\(^{138}\) and ends with Thr\(^{1224}\). The electron density maps were poor or were missing also for the inter-
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Processivity assays

Processive polymerization assays were performed under similar single-turnover conditions as the single- and double-nucleotide incorporation assays with excess Polα (3 μM) to template:primer (100 nM). The Polα/template:primer complex was mixed with saturating concentrations of all dNTPs (240 μM each) to allow for complete extension of the primer substrate. The processivity data were modeled in KinTek Global Explorer to determine the $k_{\text{pol}}$ and $k_{\text{off}}$, the rate of dissociation of the template:primer substrate from Polα, for each incorporation event.

In fitting the data to the models, the following assumptions were made: 1) all incorporation events were irreversible, 2) the $K_d$ and $k_{\text{pol}}$ values obtained from the single-incorporation experiments were manually fixed for the first incorporation in the double-incorporation modeling, 3) the $k_{\text{on}}$ values for template:primer binding to Polα were assumed to be the same, disregarding primer length differences.

Data analysis and kinetic models

Analysis of the radiolabeled products through the phosphor imager provides raw data in count values. For each time point, the counts of each band present were summed to calculate the total amount of counts and subsequently the percentage of each product length present. These values were then normalized to add up to 100% and then converted into concentration amounts based on the total amount of nucleic acid substrate used in the assay.

The KinTek Global Explorer program requires user-inputted models to fit kinetic data for the estimation of rates (Fig. S2) (35, 68). Briefly, the data from double-incorporation assays were fit to a two-step mechanism of dNTP binding followed by incorporation that was modified to account for two incorporations. The processivity data were fit using a model that allows for binding or dissociation of each DNA product and combines the dNTP binding and incorporation event into one rate.

Author contributions—A. G. B. expressed and purified the catalytic domain of hPolα and prepared the ternary complexes. N. D. B. and T. H. T. crystallized the ternary complexes and collected the diffraction data. V. N. D. performed kinetic studies under the supervision of K. S. A. Y. Z. analyzed polymerase activity under the supervision of A. G. B. and Y. I. P. T. H. T. initiated the project and determined the crystal structures. A. G. B. and V. N. D. wrote the manuscript, with contributions and critical comments from the other authors.

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