Supporting Information

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SI Materials and Methods

Reagents. Deoxyribonucleoside triphosphates were from Sigma. Labeled γ[32P] ATP was from Perkin-Elmer. All other reagents were of analytical grade and from Fluka, Sigma, or Merck. Gel-purified oligonucleotides were from Oligos Etc. Nonhydrolyzable dUMPNPP was from Jena Bioscience.

DNA Polymerases. WT yeast DNA polymerase (Pol) δ and Pol ε were purified as described previously (1, 2). RB69 Pol and its L415F variant were expressed and purified as described previously (3). In these studies, the RB69 Pol used were exonuclease deficient (D222A/D327A).

Bypass Assays. For Pol δ, the reaction mixture contained 20 mM Tris·HCl (pH 7.8), 200 μg/mL BSA, 1 mM DTT, 90 mM NaCl, 8 mM Mg acetate, 400 nM proliferating cell nuclear antigen (PCNA), 5 nM Pol δ, 100 nM primer template 1–5 (Table S1), 16 μM dATP, 30 μM dTTP, 12 μM dGTP, and 14 μM dCTP. For four-subunit Pol ε, the reaction mixture contained 40 mM Tris·HCl (pH 7.8), 200 μg/mL BSA, 1 mM DTT, 100 mM NaCl, 8 mM Mg acetate, 1 nM Pol ε, 100 nM primer template 1–5 (Table S1), 16 μM dATP, 30 μM dTTP, 12 μM dGTP, and 14 μM dCTP. For RB69 Pol or L415F RB69 Pol, the reaction mixture contained 25 mM Tris·HCl (pH 7.5), 2 mM DTT, 150 mM potassium acetate, 5 mM $MgCl_2$, 100 pM of either RB69 Pol or L415F RB69 Pol, 100 nM primer template 6–10 (Table S1), and 10 μM dNTPs. All components except the polymerase were mixed on ice and incubated at 37 °C for 2 min. The polymerase was added to initiate reactions, which were terminated after 0, 4, 8, and 12 min. These mixtures were heated at 95 °C for 3 min, and the DNA products were separated by electrophoresis through a 12% (wt/vol) denaturing polyacrylamide gel. A PhosphorImager was used to visualize the DNA products, which were quantified using Image Quant software from Molecular Dynamics. Termination and bypass probabilities were calculated as described previously (4). Termination probability at any template position is defined as the band intensity at that position divided by the intensity at that position plus all longer products. Bypass probability is defined as the band intensity at the +1 position plus all longer products divided by the intensity at the −1 position plus all longer products. In this study, −1 precedes the first ribonucleotide encountered. Relative bypass efficiency is the bypass probability with the ribonucleotide-containing substrate divided with the bypass probability for the all-DNA substrate.

Protein Crystallization. Crystals of ternary complexes were formed using the vapor diffusion sitting drop method. The crystal for structure (all-DNA) in Table S2 was formed by mixing 2 μL of the protein solution containing 74 μM polymerase, 74 μM primer template 11, and 1.3 mM dUMPNPP with 2 μL of the reservoir solution containing 50 mM Tris·HCl, pH 7.5, 10% (vol/vol) PEG-350 monomethyl ether (MME), and 180 mM $CaCl_2$. The crystal for structure (0) in Table S2 was formed by mixing 2 μL of the protein solution containing 74 μM polymerase, 74 μM primer template 12, and 1.6 mM dTTP with 2 μL of the reservoir solution containing 50 mM Tris·HCl, pH 7.5, 10% (vol/vol) PEG-350 MME, and 220 mM $CaCl_2$. The crystal for structure (−1) in Table S2 was formed by mixing 2 μL of the protein solution containing 74 μM polymerase, 74 μM primer template 13, and 1.6 mM dTTP with 2 μL of the reservoir solution containing 50 mM Tris·HCl, pH 7.0, 180 mM $CaCl_2$, and 13% (vol/vol) PEG-350 MME. The crystal for structure (−2) in Table S2 was formed by mixing 2 μL of the protein solution containing 74 μM polymerase, 74 μM primer template 14, and 1.6 mM dTTP with 2 μL of the reservoir solution containing 50 mM Tris·HCl, pH 7.0, 180 mM $CaCl_2$, and 19% (vol/vol) PEG-350 MME. The crystal for structure (−3) in Table S2 was formed by mixing 2 μL of the protein solution containing 74 μM polymerase, 74 μM primer template 15, and 1.6 mM dTTP with 2 μL of the reservoir solution containing 50 mM Tris·HCl, pH 7.5, 180 mM $CaCl_2$, and 17.5% (vol/vol) PEG-350 MME. The crystal for structure (−1,0) in Table S2 was formed by mixing 2 μL of the protein solution containing 74 μM polymerase, 74 μM primer template 16, and 1.6 mM dTTP with 2 μL of the reservoir solution containing 50 mM Tris·HCl, pH 7.5, 180 mM $CaCl_2$, and 16% (vol/vol) PEG-350 MME. The crystal for structure (−2,−1) in Table S2 was formed by mixing 2 μL of the protein solution containing 74 μM polymerase, 74 μM primer template 17, and 1.6 mM dTTP with 2 μL of the reservoir solution containing 50 mM Tris·HCl, pH 8.0, 180 mM $CaCl_2$, and 16% (vol/vol) PEG-350 MME. For data collection, crystals were transferred into a cryo-solution containing 100 mM Tris·HCl, pH 7.0, 7.5, or 8.0, dependent on the crystallization conditions, 100 mM NaCl, 5% (vol/vol) glycerol, 220 mM $CaCl_2$, and 30% (vol/vol) PEG-350 MME. All crystals were frozen in liquid nitrogen and then mounted on a goniometer in a cold stream of nitrogen gas at 95 K.

Data Collection and Processing. Data for structures all-DNA, (0), (−1), (−2), (−1,0), and (−2,−1) (designations in Table S1), were collected on a Saturn 92 charge-coupled device (CCD) area detector system mounted on a 007HF rotating anode generator equipped with VarimaxHF mirrors. Structure (−3) was collected at the Advanced Photon Source, Argonne National Laboratory, on the Southeast Regional Collaborative Access Team BM beam line, on a MAR225 CCD detector. All data were processed using the HKL2000 data processing software (5).

Molecular Replacement and Refinement. For all structures, the 3CQ8 coordinates were used as a starting model for refinement maintaining the same *R*free reflection test set (3). Model building was performed using iterative cycles of manual model building using the program COOT (6) and refinement with Phenix (7) with dihedral restraints turned off for the DNA and incoming nucleotide. The electron density maps were of sufficient quality to build most of the side chains, backbone, and DNA. The quality of the models was assessed using Molprobity (8), and all models were found to have good geometry.

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Table S1. Primer-templates used in this study

Primer-templates	Sequence	Figure
1	5′-TCAGGTCTAACACTACCGGCGGACTGCT-3′ 3′-AGTCCAGATTGTGATGGCCGCCTGACGAATGGACACGGACATGGGATCTT-5′	1 *A* and *B*
2	5′-TCAGGTCTAACACTACCGGCGGACTGCT-3′ 3′-AGTCCAGATTGTGATGGCCGCCTGACGAATGG**A**CACGGACATGGGATCTT-5′	1 *A* and *B*
3	5′-TCAGGTCTAACACTACCGGCGGACTGCT-3′ 3′-AGTCCAGATTGTGATGGCCGCCTGACGAATG**GA**CACGGACATGGGATCTT-5′	1 *A* and *B*
4	5′-TCAGGTCTAACACTACCGGCGGACTGCT-3′ 3′-AGTCCAGATTGTGATGGCCGCCTGACGAAT**GGA**CACGGACATGGGATCTT-5′	1 *A* and *B*
5	5′-TCAGGTCTAACACTACCGGCGGACTGCT-3′ 3′-AGTCCAGATTGTGATGGCCGCCTGACGAA**UGGA**CACGGACATGGGATCTT-5′	1 *A* and *B*
6	5′-TCAGGTCTAACACTACCGGCGGACT-3′ 3′-AGTCCAGATTGTGATGGCCGCCTGACGAATGGACACGGACATGGGATCTT-5′	1 *C* and *D*
7	5′-TCAGGTCTAACACTACCGGCGGACT-3′ 3′-AGTCCAGATTGTGATGGCCGCCTGACGAATGG**A**CACGGACATGGGATCTT-5′	1 *C* and *D*
8	5′-TCAGGTCTAACACTACCGGCGGACT-3′ 3′-AGTCCAGATTGTGATGGCCGCCTGACGAATG**GA**CACGGACATGGGATCTT-5′	1 *C* and *D*
9	5′-TCAGGTCTAACACTACCGGCGGACT-3′ 3′-AGTCCAGATTGTGATGGCCGCCTGACGAAT**GGA**CACGGACATGGGATCTT-5′	1 *C* and *D*
10	5′-TCAGGTCTAACACTACCGGCGGACT-3′ 3′-AGTCCAGATTGTGATGGCCGCCTGACGAA**UGGA**CACGGACATGGGATCTT-5′	1 *C* and *D*
11 (all-DNA)	5′-GCGGACTGCTTACC-3′ 3′-GCGCCTGACGAATGGACA-5′	2 *B, E, H,* and *K*
12 (0)	5′-GCGGACTGCTTACC-3′ 3′-GCGCCTGACGAATGG**A**CA-5′	2 *A–C*
13 (−1)	5′-GCGGACTGCTTACC-3′ 3′-GCGCCTGACGAATG**G**ACA-5′	2 *D–F*
14 (−2)	5′-GCGGACTGCTTACC-3′ 3′-GCGCCTGACGAAT**GG**ACA-5′	2 *G–I*
15 (−3)	5′-GCGGACTGCTTACC-3′ 3′-GCGCCTGACGAA**U**GGACA-5′	2 *J–L*
16 (−1,0)	5′-GCGGACTGCTTACC-3′ 3′-GCGCCTGACGAATG**GA**CA-5′	3 *A–E*
17 (−2,−1)	5′-GCGGACTGCTTACC-3′ 3′-GCGCCTGACGAAT**GG**ACA-5′	4 *A–F*

The number in parentheses refers to the position of the ribonucleotide in the template. Characters in bold face indicate the position of the ribonucleotide.

Table S2. Crystallographic data collection and refinement statistics

PDB ID code	4KHQ	4KHS	4KHU	4KHW	4KHY	4KI4	4KI6
Structure*	all-DNA	0	−1	−2	−3	−1,0	−2,−1
Primer template†	11	12	13	14	15	16	17
Data collection							
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions							
a (Å)	80.66	80.69	80.98	80.36	80.80	80.73	80.85
b (Å)	119.36	119.57	119.35	118.63	118.24	118.84	118.40
c (Å)	128.07	127.80	127.86	126.31	127.61	127.52	127.97
α, β, γ (°)	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90
Resolution (Å)	50.0–2.2	50.0–2.1	50.0–2.1	50.0–2.4	50.0–2.2	50.0–2.4	50.0–2.6
R_{merge} (%)‡	7.4 (52.0)	7.6 (46.8)	8.9 (50.3)	7.0 (45.3)	7.7 (58.2)	7.9(52.3)	6.2 (42.3)
$I/\sigma I$‡	11.9 (3.1)	13.3 (2.1)	8.8 (2.6)	9.7 (3.0)	10.4 (2.9)	8.8(2.5)	14.5 (2.4)
Completeness (%)‡	99.9 (99.0)	99.9 (99.8)	97.9 (84.0)	98.7 (95.3)	98.4 (88.9)	99.8(97.3)	97.4 (86.2)
Redundancy‡	6.0 (5.2)	7.2 (6.0)	5.8 (3.9)	7.0 (4.4)	7.1 (5.8)	6.0(4.8)	4.9 (2.9)
Refinement							
Resolution (Å)	2.2	2.1	2.1	2.4	2.2	2.4	2.6
No. reflections	64,171	69,508	73,809	48,447	57,059	45,787	39,756
R_{work}/R_{free} (%)	17.9/21.3	17.6/21.6	20.5/24.0	17.2/22.4	19.7/23.3	19.2/22.1	19.5/23.3
No. atoms							
Protein	7,345	7,234	7,296	7,274	7,337	7,247	7,106
DNA	649	647	651	651	649	643	652
Nucleotide	28	29	29	29	29	29	29
Solvent	589	672	596	544	574	426	182
Overall Wilson B factor (Å^2)	40.5	36.7	33.6	37.5	40.4	42.7	54.2
B factors (Å^2)							
Protein	44.8	42.4	38.0	42.8	45.9	43.3	62.0
DNA	49.4	47.7	46.3	49.5	53.6	49.6	70.4
Nucleotide	30.2	27.9	23.8	28.2	30.5	28.6	45.6
Solvent	47.4	46.2	39.1	41.7	47.2	40.4	55.1
Root mean square deviations							
Bond length (Å)	0.010	0.009	0.009	0.007	0.007	0.002	0.002
Bond angles (°)	0.724	0.815	0.890	0.764	0.731	0.685	0.652
Ramachandran statistics§							
Favored regions (%)	97.4	97.4	97.7	96.9	96.9	96.7	95.9
Allowed regions (%)	99.9	100	100	100	100	100	99.8

*Ribonucleotide position in template.
†See Table S1.
‡Highest resolution shell is shown in parentheses.
§Values obtained using MolProbity (8).