**Drosophila** DNA Polymerase ζ Interacts with Recombination Repair Protein 1, the Drosophila Homologue of Human Abasic Endonuclease 1*

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Abasic (AP) sites are a threat to cellular viability and genomic integrity, since they impede transcription and DNA replication. In mammalian cells, DNA polymerase (pol) β plays an important role in the repair of AP sites. However, it is known that many organisms, including *Drosophila melanogaster*, do not have a pol β homologue, and it is unclear how they repair AP sites. Here, we screened for DNA polymerases that interact with the *Drosophila* AP endonuclease 1 homologue, Rrp1 (recombination repair protein ζ), and found that *Drosophila* pol ζ (Dmpol ζ), DmREV3 and DmREV7 bound to Rrp1 in a protein affinity column. Rrp1 directly interacted with DmREV7 *in vitro* and *in vivo* but not with DmREV3. These findings suggest that the DNA polymerase partner for Rrp1 is Dmpol ζ and that this interaction occurs through DmREV7. Interestingly, DmREV7 bound to the N-terminal region of Rrp1, which has no known protein homologue, suggesting that this binding is a species-specific event. Moreover, DmREV7 could stimulate the AP endonuclease activity of Rrp1, but not the 3'-exonuclease activity, and form a homomultimer. DmREV3 could not incorporate nucleotides at the 5’-incised tetrahydrofran sites but did show strand displacement activity for one-nucleotide-gapped DNA, which was not influenced by either DmREV7 or Rrp1. Methyl methanesulfonate and hydrogen peroxide treatments increased mRNA levels of *DmREV3* and *DmREV7*. On the basis of the direct interaction between DmREV7 and Rrp1, we suggest that Dmpol ζ may be involved in the repair pathway of AP sites in DNA.

In short patch BER, APE1 recruits DNA polymerase (pol) β to the nick with a 5’-deoxyribose phosphate group and a 3’-hydroxyl group. There, pol β catalyzes DNA synthesis and 5’-deoxyribose phosphate excision. DNA ligase then completes the repair of the AP sites by sealing pol β nicks. In long patch BER, pol β, δ, and ε carry out the strand displacement synthesis (2–10 nucleotides), and flap endonuclease 1 cleaves the 5’-protruding flaps. Finally, the nicks are sealed by DNA ligase in a fashion similar to short patch BER (4).

Most of the genes that code for mammalian BER proteins show conservation across a wide range of organisms. Pol β is an exception in that no homologue has been identified in any other organism (5). Yeast and higher plants contain pol β-like gene products, pol IV and pol Λ, respectively, which are probably involved in BER (6, 7). However, a pol β homologue is absent in *Drosophila melanogaster*. Trf4, the only known member of the pol X family of proteins in *Drosophila*, is thought to be involved in the establishment of sister chromatid cohesion and RNA quality control (5, 8–11). We therefore sought to determine which DNA polymerase is required for the repair of AP sites in *Drosophila*.

*Drosophila* Rrp1 (recombination repair protein ζ) belongs to the *Escherichia coli* exonuclease III family (12). The C-terminal 252-amino acid region of Rrp1 shows high homology with exonuclease III and human APE1 and possesses AP endonuclease, 3'-exonuclease, 3'-phosphatase, and 3'-phosphodiesterase activities. In contrast, the N-terminal 421-amino acid region is not homologous to any known protein and shows the unique *in vitro* characteristics of DNA strand transfer and single-stranded DNA renaturation (13). A previous study suggested that Rrp1 was involved in the repair of oxidative DNA damage *in vivo* (14), but little is known of its possible role in this type of repair. Therefore, we hypothesized that Rrp1 recruits DNA polymerases, such as APE1 (15), that participate in the repair of incised AP sites.

We sought to identify DNA polymerases that bind to Rrp1 using a GST-tagged Rrp1-conjugated affinity (Rrp1 protein affinity) column and found that *Drosophila* DNA polymerase ζ (Dmpol ζ) can interact with Rrp1. Pol ζ consists of the catalytic subunit REV3, which belongs to the B-family of DNA polymerases, and REV7 (16). Yeast pol ζ has the biochemical ability to extend from mismatched termini opposite various DNA lesions and plays major roles in most of the spontaneous and DNA damage-induced mutations (16, 17). Human and mouse pol ζ are not only thought to participate in mutagenesis and somatic hypermutation but also are essential for early embryonic development (18–24). However, *Drosophila* REV3 (DmREV3) does not appear to participate in mutagenesis and cannot extend mismatched termini in (6–4)-photoproducts *in vitro* (16, 25, 26). Here, we report that DmREV7, the accessory subunit of Dmpol ζ, directly interacts with Rrp1 and stimulates the AP endonuclease activity of Rrp1. We also discuss the potential role of Dmpol ζ in repair of AP sites in association with Rrp1.
Drosophila DNA Polymerase $\zeta$ and Rrp1

**EXPERIMENTAL PROCEDURES**

**Materials**—[$\alpha$-$^32$P]dTTP (~3000 Ci/mmol) and [$\gamma$-$^32$P]ATP (~3000 Ci/mmol) were purchased from Amersham Biosciences. Ni$^{2+}$-nitrilotriacetic acid-agarose was purchased from Qiagen (Hilden, Germany). Glutathione-Sepharose 4B, HiTrap Heparin HP, and Resource S were purchased from Amersham Biosciences. Bradford reagents and molecular mass markers for SDS-PAGE were from Bio-Rad. DmREV3 and recombinant *Rattus norvegicus* pol $\beta$ were purified as described in our previous report (26). All other reagents were analytical grade and were purchased from Sigma, Amersham Biosciences, or Wako Chemicals (Osaka, Japan).

**Collection of *D. melanogaster* Embryos**—The basic procedures for the collection of *Drosophila* embryos have been described previously (27). Embryos of *D. melanogaster* (Oregon R and M316) from 0 to 24 h of age were collected from large population cages.

**Expression and Purification of Rrp1—Rrp1** cDNA fragments were isolated by reverse transcription (RT)-PCR from cultured cells using primers based on sequences obtained from the *Drosophila* Genome database (available on the World Wide Web at flybase.net/). The full-length Rrp1 coding region was cloned into the pGEX 6P-1 expression vector (Amersham Biosciences) and transformed into *E. coli* BL21(DE3) (Novagen). One colony was incubated in 10 ml of LB medium containing 1% (w/v) glucose and 50 $\mu$g/ml ampicillin. The culture was grown overnight at 30 °C and transferred into 1 liter of fresh LB medium containing 1% (w/v) glucose and 50 $\mu$g/ml ampicillin. The cells were grown at this temperature until the A$_{600}$ value reached 0.4, at which point they were incubated on ice for 1 h. Isopropyl-$\beta$-d-thiogalactopyranoside was added to 0.1 mM, and the culture continued at 30 °C for 3 h. The cells were then harvested by centrifugation at 4 °C, frozen in liquid nitrogen, and stored at −80 °C. Frozen cells were resuspended in 50 ml of TEMG buffer (50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 5 mM 2-mercaptoethanol, 10% (v/v) glycerol, 0.01% (v/v) Nonidet P-40, 1 $\mu$g/ml each pepstatin A and leupeptin, and 1 mM phenylmethylsulfonyl fluoride (ph 8.0)) supplemented with 10 mM imidazole, sonicated 5 times for 30 s, and centrifuged for 30 min at 30,000 × g at 4 °C. The supernatant was loaded onto a Ni$^{2+}$-nitrilotriacetic acid-agarose column (Qiagen) pre-equilibrated with His buffer containing 10 mM imidazole, washed thoroughly with His buffer containing 30 mM imidazole, and eluted with elution buffer (50 mM Na$_2$HPO$_4$, 100 mM NaCl, 200 mM imidazole, 5 mM 2-mercaptoethanol, 10% (v/v) glycerol, 0.01% (v/v) Nonidet P-40, 1 $\mu$g/ml each pepstatin A and leupeptin, and 1 mM phenylmethylsulfonyl fluoride (pH 7.7)). The fractions containing T7-DmREV7 were loaded onto a HiTrap Heparin HP column (Amersham Biosciences) pre-equilibrated with TEMG buffer containing 0.1 mM NaCl, and washed with the same buffer. T7-DmREV7 was eluted with a linear gradient of 0.1–1.0 M NaCl in TEMG buffer, dialyzed against TEMG buffer supplemented with 50 mM NaCl, and loaded onto a Resource S column (Amersham Biosciences) pre-equilibrated with TEMG buffer containing 50 mM NaCl. After washing with the same buffer, T7-DmREV7 was eluted with a linear gradient of 50–800 mM NaCl in TEMG buffer. The purified T7-DmREV7 was dialyzed against TEMG buffer supplemented with 150 mM NaCl, frozen in liquid nitrogen, and stored at −80 °C until use.

**Polyclonal Antibodies**—Polyclonal antibodies against *Drosophila* pol $\delta$, pol $\epsilon$, and REV3 were obtained as described previously (26). A polyclonal antibody against DmREV7 was obtained by expressing and purifying T7-DmREV7 as described above and using this protein to immunize mice.

**Rrp1 Protein Affinity Chromatography**—Eight nmol of GST or GST-Rrp1 was loaded onto 300 $\mu$l of glutathione-Sepharose 4B and washed with 6 ml of TEMG buffer containing 150 mM NaCl. Frozen *Drosophila* embryos (2 g) were crushed in a Waring-type blender running at maximum speed, and the material was suspended in 10 ml of TEMG buffer supplemented with 150 mM NaCl. The embryo suspension was then filtered through two layers of nylon mesh, and sonicated and then centrifuged for 60 min at 50,000 × g at 4 °C. Twenty-five mg of the supernatant was loaded onto each affinity column and washed with TEMG buffer containing 150 mM NaCl. After equilibration with digestion buffer, the columns were incubated with 20 units of PreScission Protease for 4 h at 4 °C. Material dissociated from the affinity columns was collected and analyzed by SDS-PAGE. To confirm the amount of GST protein conjugated to each column, GST proteins were eluted with vectors. We induced expression of the vectors and then purified the GST-Rrp1N and GST-Rrp1C proteins as well as GST-Rrp1 protein.

**Expression and Purification of DmREV7—Recovery of the T7-tagged DmREV7 (T7-DmREV7) from the soluble fraction was carried out by first digesting the expression plasmid containing DmREV7 (26) with NdeI and XhoI and then inserting the NdeI/XhoI DNA fragment into the pCold vector (TaKaRa Bio Inc.). BL21 (DE3), harboring the pColdl plasmid containing T7-DmREV7, was incubated in 20 ml of LB medium containing 1% (w/v) glucose and 50 $\mu$g/ml ampicillin. The culture was grown overnight at 37 °C and transferred into 2 liters of fresh LB medium containing 1% (w/v) glucose and 50 $\mu$g/ml ampicillin. The cells were further grown at the same temperature until the A$_{600}$ value reached 0.3 and incubated at 15 °C for 1 h. Isopropyl-$\beta$-d-thiogalactopyranoside was added to 0.1 mM, and the incubation was continued at 15 °C for 20 h. The cells were then harvested by centrifugation at 4 °C, frozen in liquid nitrogen, and stored at −80 °C. Frozen cells were resuspended in 50 ml of His buffer (50 mM Na$_2$HPO$_4$, 300 mM NaCl, 5 mM 2-mercaptoethanol, 10% (v/v) glycerol, 0.01% (v/v) Nonidet P-40, 1 $\mu$g/ml each pepstatin A and leupeptin, and 1 mM phenylmethylsulfonyl fluoride (pH 8.0)) supplemented with 10 mM imidazole, sonicated 5 times for 30 s, and centrifuged for 30 min at 30,000 × g at 4 °C. The supernatant was loaded onto a Ni$^{2+}$-nitrilotriacetic acid-agarose column (Qiagen) pre-equilibrated with His buffer containing 10 mM imidazole, washed thoroughly with His buffer containing 30 mM imidazole, and eluted with elution buffer (50 mM Na$_2$HPO$_4$, 100 mM NaCl, 200 mM imidazole, 5 mM 2-mercaptoethanol, 10% (v/v) glycerol, 0.01% (v/v) Nonidet P-40, 1 $\mu$g/ml each pepstatin A and leupeptin, and 1 mM phenylmethylsulfonyl fluoride (pH 7.7)). The fractions containing T7-DmREV7 were loaded onto a HiTrap Heparin HP column (Amersham Biosciences) pre-equilibrated with TEMG buffer containing 0.1 mM NaCl, and washed with the same buffer. T7-DmREV7 was eluted with a linear gradient of 0.1–1.0 M NaCl in TEMG buffer, dialyzed against TEMG buffer supplemented with 50 mM NaCl, and loaded onto a Resource S column (Amersham Biosciences) pre-equilibrated with TEMG buffer containing 50 mM NaCl. After washing with the same buffer, T7-DmREV7 was eluted with a linear gradient of 50–800 mM NaCl in TEMG buffer. The purified T7-DmREV7 was dialyzed against TEMG buffer supplemented with 150 mM NaCl, frozen in liquid nitrogen, and stored at −80 °C until use.
TEMG buffer (adjusted to pH 8.0) containing 3 mg/ml glutathione and analyzed by Western blotting with anti-GST tag polyclonal antibody.

In Vitro Protein-Protein Interaction Assay—GST-Rp1, GST-Rp1N, GST-Rp1C, and GST (0.5 nmol of each) were separately incubated with 0.5 nmol of T7-DmREV7 on ice for 30 min in TEGN buffer (50 mM Tris-HCl (pH 7.5); 50 mM NaCl; 1 mM EDTA; 10% (v/v) glycerol; and 0.01% Nonidet P-40), and mixed with 50 μl of glutathione-SEpharose 4B in 100 μl of the same buffer. After incubation at 4 °C for 1 h, the beads were washed with the same buffer and mixed with 4 units of PreScission protease in digestion buffer. After incubation for 4 h at 4 °C, the bound proteins were eluted by boiling in 1× SDS sample buffer and analyzed by Western blotting with a monoclonal antibody against the T7 epitope tag (Novagen). To confirm the amount of GST conjugated to the beads, the eluted fractions were analyzed by Western blotting with an anti-GST tag polyclonal antibody. Conversely, 0.2 nmol of GST-Rp1, 2.5 μl of anti-T7 tag monoclonal antibody, and 20 μl of bovine γ-globulin supplemented with 10% fetal bovine serum at 27 °C and transfected with Schneider 2 (S2) cells were grown in Schneider’s medium supplemented with 10% fetal bovine serum at 27 °C and transfected with pAc5.1 vectors containing both FLAG and anti-HA monoclonal antibodies. 3F10; Roche Applied Science), or T7 epitope tag at 4 °C for 4 h. After incubation with a monoclonal antibody against FLAG (M2; Sigma), HA-Rrp1 conjugated to the beads was purified to near homogeneity (data not shown), the presence of several bands in the elution product from GST-Rp1, 5'-AAAGAGGAAGGCGAGGC-3' and 5'-CTGATGATGGCAT-TATCTTG-3'; DmREV3, 5'-TTCGATCCGGCAGGAGATC-3' and 5'-TTCAAGGGGGATATTGACG-3'; DmREV7, 5'-ATGGAC-GCGGAGATTAAGGC-3' and 5'-CTGAAAGCCAGGAAACTCTCT-3'; Actin5C (as an internal control), 5'-TGTGGATCTCCTCCGACA-3' and 5'-ATCCGGATCCTGACCTTCT-3'. For Rp1, DmREV3, and DmREV7, the PCR conditions were 95 °C for 15 s, 50 °C for 30 s, and 72 °C for 35 s, after a predenaturation step of 2 min 30 s at 95 °C. Twenty-one amplification cycles were used for Rp1, 25 cycles for DmREV3, and 29 or 24 cycles for DmREV7. For the Actin5C control, amplification was carried out, after a predenaturation step of 2 min 30 s at 95 °C, using 17 cycles of 95 °C for 30 s, 50 °C for 30 s, and 72 °C for 1 min 5 s. PCR products were visualized by staining with SYBR Safe (Invitrogen).

RESULTS

Drosophila DNA Polymerase ζ Binds to Rp1 Protein Affinity Beads—Drosophila cells contain eight types of DNA polymerase, pol α, β, δ, ε, ζ, η, θ, and λ. However, they do not have a mammalian pol β-like gene product (5). To investigate how AP sites in Drosophila DNA are repaired without pol β, we used protein-protein affinity chromatography to identify DNA polymerases that interact with Rp1, the Drosophila homologue of APE1. As described under “Experimental Procedures,” the open reading frame region of Rp1 was isolated by RT-PCR from a cDNA library of cultured Kc cells and cloned into the pGEX 6P-1 vector. GST-tagged protein (GST-Rp1) was produced by overexpression of the vector in transformed bacteria. Next, we conjugated equal amounts of purified GST-Rp1 or GST alone to glutathione-Sepharose beads. Crude extracts of Drosophila embryos were loaded onto these beads, and proteins were eluted by digestion with PreScission Protease after extensive washing (see “Experimental Procedures”). The eluted fractions were analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue (Fig. 1A). Several bands were observed in the fraction eluted from Rp1 protein affinity beads (lane 2), whereas there was no band in the elution product from GST-conjugated beads (lane 1). Since the GST-Rp1 conjugated to the beads was purified to near homogeneity (data not shown), the presence of several bands in the elution product from Rp1 protein affinity beads suggests that other proteins may physically interact with Rp1. Therefore, we carried out Western blotting with antibodies against several Drosophila DNA polymerases in order to...
Drosophila DNA Polymerase ζ and Rrp1

FIGURE 1. Dmpol ζ binds to GST-Rrp1 protein affinity column. A, SDS-PAGE analysis of the fractions eluted from GST and GST-Rrp1 protein affinity columns (lanes 1 and 2, respectively). The 12.5% polyacrylamide gel was stained with Coomassie Brilliant Blue. The band with Rrp1 digested by PreScission Protease is marked with an asterisk. B, Western blotting analysis of Dmpol ζ. Lanes are the same as those for A. The indicated proteins were detected with polyclonal antibodies against DmREV3 and DmREV7. To confirm the amount of GST protein conjugated to each column, GST proteins were eluted with glutathione and analyzed by Western blotting with anti-GST tag polyclonal antibody.

FIGURE 2. DmREV7 interacts with Rrp1 in vitro and in vivo. A, 0.5 nmol of T7-DmREV7 and glutathione-Sepharose beads were incubated with 0.5 nmol of GST alone or GST-Rrp1. The bound proteins were eluted by incubation with PreScission protease, followed by boiling in SDS sample buffer. The bound proteins were detected by Western blotting with anti-T7 tag and GST tag antibodies. B, 0.2 nmol of T7-REV7 and GST-Rrp1 was immunoprecipitated with anti-T7 tag monoclonal antibody. The bound proteins were detected by Western blotting with anti-GST tag and T7 tag antibodies. C, 0.5 nmol of T7-DmREV7 and glutathione-Sepharose beads was mixed with 0.5 nmol of GST fusion proteins containing the N-terminal 400-amino acid or the C-terminal 273-amino acid regions of Rrp1 (GST-Rrp1 N or GST-Rrp1 C, respectively). The bound proteins were eluted and detected in the same way as in A. D and E, GST and FLAG epitope-tagged constructs containing Rrp1 and DmREV7, respectively (HA-Rrp1 and FLAG-DmREV7) were co-transfected into S2 cells. The cell lysate was immunoprecipitated with a monoclonal antibody against HA (D) or FLAG (E) and analyzed by Western blotting with monoclonal antibodies against HA and FLAG epitope tags. The asterisks indicate the nonspecific protein included in the immunoprecipitation with anti-T7 monoclonal antibody.

identify those bound to the beads. A sharp signal was found with anti-DmREV3 polyclonal antibody (Fig. 1B). Since we previously demonstrated that DmREV3 and DmREV7 interact in vitro (26), we applied anti-DmREV7 polyclonal antibody to the Western blot to investigate whether DmREV7 also bound to Rrp1 protein affinity beads. We found that both DmREV7 and DmREV3 were present (Fig. 1B). These results indicate that Dmpol ζ is involved in the complex with Rrp1. Additionally, no bands were detected with antibodies against Drosophila pol δ and ε (data not shown), whereas mammalian pol δ and ε are implicated in the long patch BER pathway (1).

DmREV7 Directly Interacts with Rrp1 both in Vitro and in Vivo—To examine how Dmpol ζ interacts with Rrp1, we first performed a GST pull-down assay. We conjugated GST-Rrp1 or GST alone to glutathione-Sepharose beads and incubated the beads with Dmpol ζ subunit, T7-tagged DmREV7 (T7-DmREV7), or DmREV3. T7-DmREV7 bound to GST-Rrp1 (Fig. 2A), but DmREV3 did not (data not shown). Conversely, GST-Rrp1 was precipitated with anti-T7 monoclonal antibody and protein G-Sepharose beads in the presence of T7-DmREV7 but not in its absence (Fig. 2B).

To examine the region of GST-Rrp1 required for the interaction with T7-DmREV7 in vitro, we prepared GST fusion proteins containing the N-terminal 400-amino acid region and the C-terminal 273-amino acid region of Rrp1 (GST-Rrp1N and GST-Rrp1C, respectively). T7-DmREV7 bound to GST-Rrp1N but not to GST alone or to GST-Rrp1C (Fig. 2C).

To confirm this interaction in vivo, we next performed an in vivo co-immunoprecipitation assay. HA and FLAG epitope-tagged constructs, containing either Rrp1 or DmREV7 (HA-Rrp1 and FLAG-DmREV7), were co-transfected into Drosophila S2 cells. Cell lysates were incubated with anti-HA or FLAG monoclonal antibody and protein G-Sepharose beads. We found that FLAG-DmREV7 and HA-Rrp1 were

FIGURE 3. Effect of T7-DmREV7 on AP endonuclease activity of Rrp1. A, purification of Rrp1 by Sephacryl S-300 gel filtration. AP endonuclease activity in the fractions from Sephacryl S-300 gel filtration was assayed at 37 °C for 5 min using the 21F substrate (top). The mobilities of substrates (S) and products (P) are indicated. B, T7-DmREV7 stimulates AP endonuclease activity of Rrp1. A 5 μM concentration of the 21F substrate and 15 fmol of Rrp1 were incubated with four different amounts of T7-DmREV7 (15, 75, 300, and 600 fmol in lanes 2–5, respectively) at 37 °C for 5 min. Lane 6 shows that AP endonuclease activity is absent in the fraction of T7-DmREV7. Control reaction with 600 fmol of BSA is shown in lane 7. C, T7-DmREV7 can form a homomultimer. The purified T7-DmREV7 was dialyzed against the buffer containing 200 mM NaCl and loaded onto a HiPrep 16/60 Sephacryl S-300 column. T7-DmREV7 in the elution was detected by Western blotting with anti-T7 monoclonal antibody. C, control fraction with no protein. The same fractions were separated on a 12.5% SDS-polyacrylamide gel, and the gel was stained with Coomassie Brilliant Blue (bottom). The molecular mass was estimated from a calibration curve with ribonuclease A (13.7 kDa), BSA (67 kDa), adolase (158 kDa), ferritin (440 kDa), and thyroglobulin (669 kDa).
precipitated by the anti-FLAG monoclonal antibody but not by the control anti-T7 monoclonal antibody (Fig. 2D). Moreover, these proteins were also precipitated by the anti-HA monoclonal antibody (Fig. 2E). These results indicate that DmREV7 directly interacted with Rrp1 both in vitro and in vivo and that the unique N-terminal region of Rrp1 was essential for this interaction.

**DmREV7 Stimulates AP Endonuclease Activity of Rrp1 and Forms a Homomultimer in Vitro**—We examined the influence of T7-DmREV7 on the AP endonuclease activity of Rrp1. GST-Rrp1 was overexpressed in bacteria, eluted from glutathione-Sepharose beads with PreScission Protease, and analyzed by Sephacryl S-300 gel filtration. A single peak of AP endonuclease activity appeared at a molecular mass of ~410 kDa (Fig. 3A, upper panel); this was consistent with the SDS-PAGE pattern of the purified Rrp1 (Fig. 3A, lower panel). A previous study reported that Rrp1 is eluted at about 590 kDa by gel filtration but is a monomer of 78 kDa with an extremely asymmetric structure (28). Therefore, we conclude that purified Rrp1 was responsible for AP endonuclease activity and not an E. coli protein.

The purified Rrp1 was used to examine the influence of DmREV7 on AP endonuclease activity. T7-DmREV7 caused an ~8-fold stimulation of Rrp1 activity (Fig. 3B). By itself, T7-DmREV7 had no AP endonuclease activity, and BSA did not influence the activity of Rrp1. However, an excess of T7-DmREV7 was required to produce a significant stimulation of Rrp1 AP endonuclease activity. This finding led us to hypothesize that most of the T7-DmREV7 binds nonspecifically to DNA sequences other than Rrp1 or that T7-DmREV7 forms a homomultimer in vitro. To investigate the first of these possibilities, we carried out an electrophoretic mobility shift assay (29) and found that no DNA binding activity could be detected for T7-DmREV7 (data not shown). To determine whether T7-DmREV7 formed a homomultimer, we carried out Sephacryl S-300 gel filtration in the presence of 0.2 M NaCl and also Western blotting with an anti-T7 tag monoclonal antibody. In the gel filtration assay, one major peak and a second minor peak of T7-DmREV7 were also precipitated by the anti-FLAG monoclonal antibody but not by the anti-HA monoclonal antibody (Fig. 3C). This suggests that T7-DmREV7 can form a homomultimer. If it is a globular complex, it could be a homodimer, because the calculated molecular mass of the former peak was 10 times larger than that of the latter. Although we tried to test whether a T7-DmREV7 homomultimer could stimulate Rrp1 AP endonuclease activity, its tendency to aggregate in the presence of 0.2 M NaCl in the buffer hampered the collection of sufficient material to assay the activity by gel filtration.

**Rrp1 Exonuclease Prefers 3′-Mismatched DNA Substrates**—Recently, several studies showed that APE1 also has a 3′-mismatched exonuclease activity and might be a proofreading enzyme in the BER pathway (30, 31). Sander and Benhaim (32) previously reported that the rate of cleavage of 3′-terminal nucleotides by Rrp1 varied by up to 2 orders of magnitude, depending on the specific nucleotide and its sequence context, but it remained unclear whether Rrp1 3′-exonuclease was more active for 3′-mismatched DNA substrates than 3′-matched ones. Therefore, we sought to examine the effect of base-base hydrogen bonding on 3′-exonuclease activity. We first confirmed the absence of contaminating E. coli exonucleases in the Rrp1 fraction from the Sephacryl S-300 gel filtration (Fig. 3A). The 3′-exonuclease activity for a recessed DNA substrate with 3′ G/T coincided not only with the elution pattern of AP-endonuclease activity but also with the SDS-PAGE pattern of the purified Rrp1 (data not shown), indicating that 3′-exonuclease activity in this fraction depended on Rrp1.

We carried out a time course experiment using the recessed DNA substrates containing the 3′-terminal nucleotides, 3′ G/C and 3′ G/T (see Table 1). Like APE1, Rrp1 exonuclease showed an ~10-fold preference for the 3′-mismatched DNA substrate over the 3′-matched substrate (Fig. 4A). We next tested the influence of DmREV7 using these recessed DNA substrates and found that it did not alter the 3′-exonuclease activity of Rrp1 (Fig. 4B). Since APE1 exonuclease is known to be more active on nicked and gapped DNA substrates than on recessed substrates (30), we examined the efficiency of Rrp1 in the removal of 3′-terminal nucleotides in these DNA substrates. Rrp1 exonuclease activity was dramatically influenced by the DNA substrate species: for 3′ G/T nucleotides, Rrp1 showed a slightly higher rate of activity for the nicked DNA substrate than for the recessed one but a much lower efficiency for mismatched DNA containing a one-nucleotide gap; for 3′ G/C nucleotides, Rrp1 showed much reduced activities for both nicked and gapped DNA compared with the recessed substrate (Fig. 4C). We also found that DmREV7 did not influence Rrp1 exonuclease activity on any nicked or gapped DNA substrates (data not shown).

**Strand Displacement Synthesis of DmREV3 for Gapped DNA Templates**—In the mammalian BER pathway, pol β, δ, and ε incorporate nucleotides at 5′-incised and reduced AP sites (33, 34). The present results suggest that Dmpol δ might be involved in the repair of AP sites. Therefore, we examined whether DmREV3 could incorporate nucleotides at 5′-incised tetrahydrofran (F) sites. We prepared a 21-bp-long, double-stranded oligonucleotide that contained an incised F residue

### TABLE 1
Sequences of DNA substrates used in this study

| Template | Sequences |
|----------|-----------|
| F1 | 5′-CTCCGCTTGGCAGCCTGTCG-3′ (21-nt) |
| F2 | 5′-CCACAGCTGCACCGGGAGG-3′ (21-nt) |
| F3 | 5′-CTCCGCTTGGCAGCCTGTCG-3′ (9-nt) |
| U1 | 5′-GATACCCGGAATTCGATCCATGGCTG-3′ (36-nt) |
| T1 | 5′-ATGCCTGCAGTCACAGGCGTCTGACCAATAGCAACCTGCAATGTCATGGCTG-3′ (67-nt) |
| T2 | 5′-ATGCCTGCAGTCACAGGCGTCTGACCAATAGCAACCTGCAATGTCATGGCTG-3′ (67-nt) |
| D1 | 5′-ATGGTGACAGCATGATCCGACCATAT-3′ (31-nt) |
| D2 | 5′-TTGTGAGGCAATGGTGCATCCGCAAT-3′ (38-nt) |

**E. coli** 5′-phosphate.
incision (21F). This incision was the result of pretreatment with Rrp1, which cleaved more than 95% of F residues under our experimental conditions (data not shown). DmREV3 was unable to catalyze DNA synthesis across the incised 21F (Fig. 5A, lanes 5–8), whereas recombinant R. norvegicus pol β did so to a significant extent (Fig. 5A, lane 2) as anticipated (33). We previously reported that DmREV3 is a highly processive enzyme (26), but the reaction products for the recessed DNA template (9:21-nt), 15 or 18 nt, were shorter than the 21 nt expected of complete replication of the template (Fig. 5A, lane 4). Since yeast REV3 behaves in a similar manner (35), this biochemical property of REV3 may be universal.

Strand displacement synthesis by DmREV3 was tested using nicked, one-nucleotide-gapped, and recessed 3′/G/C DNA templates (see Table 1). Although reaction products were observed for all DNA templates (Fig. 5B), it is possible that those for nicked DNA were the result of extension on the DNA template by an incorrectly annealed downstream primer. However, the DNA synthesis activity of DmREV3 for a one-nucleotide-gapped DNA was ~2-fold higher than for nicked DNA (Fig. 5B). Therefore, we conclude that DmREV3 can catalyze strand displacement synthesis for one-nucleotide-gapped DNA. We next examined the effects of DmREV7 and Rrp1 on strand displacement synthesis by DmREV3 for one-nucleotide-gapped DNA with a 3′/G/C. Neither T7-DmREV7 nor Rrp1 influenced the activity of DmREV3 (Fig. 5C).

Expression of Rrp1, DmREV3, and DmREV7 during Developmental Stages and in Drosophila Cultured Cells Treated with DNA-damaging Agents—Mouse REV3 is highly expressed around the gastrulation stage of embryogenesis and in adult brain, ovaries, and testes, whereas human REV7 is ubiquitously transcribed (36–38). Therefore, we performed RT-PCR to check the expression patterns of Rrp1, DmREV3, and DmREV7 during Drosophila development. First strand cDNA was synthesized using total RNA extracted from a range of developmental stages of Drosophila: unfertilized eggs, 0–2, 2–4, 4–8, 8–12, 12–16, and 16–20 h embryos, larvae, pupae, and adult males and females. Expression levels were compared in the linear range of RT-PCR amplification. All three genes were highly expressed in early embryos and in adult females (Fig. 6A). It appeared that expression of these genes was particularly required prior to gastrulation, which is known to begin after about 3 h after the eggs are laid in Drosophila.

To examine the DNA damage responses of Rrp1, DmREV3, and DmREV7, we next exposed S2 cells to DNA-damaging agents and carried out RT-PCR. The expression patterns of DmREV3 and DmREV7 were mostly similar after exposure to MMS, H2O2, or UV (Fig. 6B). The transcripts of these genes were increased by MMS and H2O2 and notably stimulated by UV. In contrast, the expression of Rrp1 was less influenced by any DNA-damaging agents than that of DmREV3 and DmREV7.

**DISCUSSION**

In the present study, we screened DNA polymerases to determine whether any of these could bind to Rrp1 and found that Dmpol ζ interacted with Rrp1 (Fig. 1B). Additionally, neither Dmpol δ nor Dmpol ε could be detected in the elution products from GST-Rrp1 protein affinity beads under our experimental conditions, although pol β, δ, and ε are involved in BER pathways in mammalian cells (1). These results suggest that the interaction between Dmpol ζ and Rrp1 may have a role in the repair of oxidative DNA damage and that Dmpol δ and ε are involved in an Rrp1-independent repair pathway for AP sites. We previously reported that DmREV7 directly interacts in vitro with the central, 360-amino acid region of DmREV3 (26). As shown in Fig. 2, DmREV7, but not DmREV3, directly interacted with Rrp1 in vitro and in vivo. Therefore, DmREV3 may bind to GST-Rrp1 protein affinity beads through interaction with DmREV7 (Fig. 1B). We also found that interaction between Rrp1 and DmREV7 required the N-terminal 400-amino acid region of Rrp1, which has a unique sequence (12), suggesting that this interaction may be a species-specific event in the repair of oxidative DNA damage.

We showed that DmREV7 significantly stimulated Rrp1 AP endonuclease activity (Fig. 3B). We sought to examine the mechanism of this effect in detail via an electrophoretic mobility shift assay (29). However,
FIGURE 5. Strand displacement activity of DmREV3 for one-nucleotide-gapped DNA templates. A, DmREV3 cannot incorporate nucleotides at the 5'-incised tetrahydrofuran sites by Rrp1. Incised 21F (lanes 1, 2, and 5–8) and 9:21-nt (lanes 3 and 4) were used as DNA templates (see Table 1). 100 fmol of R. norvegicus pol β (lanes 1 and 2) or 1000 fmol of DmREV3 (lanes 3–8) were incubated with [α-32P]dTTP. The reaction products were separated on a 20% polyacrylamide, 8M urea gel. B, DNA synthesis of DmREV3 for nicked, one-nucleotide-gapped, and recessed DNA templates. 400 fmol of DmREV3 was incubated with the nicked, one-nucleotide-gapped, or recessed DNA template with 3' G/C (lanes 1–4, 5–8, or 9–12, respectively). The reaction products were separated on a 10% polyacrylamide, 8M urea gel. The left panel demonstrates that the DNA synthesis activity of DmREV3 for the one-nucleotide-gapped DNA (closed squares) was about 2-fold higher than for the nicked DNA (open circles). C, strand displacement activity of DmREV3 for the one-nucleotide-gapped DNA template was not influenced by T7-DmREV7 and Rrp1. Left, 400 fmol of DmREV3 were incubated with three different amounts of T7-DmREV7 (400, 800, and 2000 fmol in lanes 3–5) at 37 °C for 60 min. Lane 6 shows that DNA synthesis activity was absent in the T7-DmREV7 fraction. Control reactions with no protein and 2000 fmol of BSA are shown in lanes 1 and 7, respectively. The reaction products were separated on a 10% polyacrylamide, 8M urea gel. Right, 400 fmol of DmREV3 and T7-DmREV7 were incubated with three different amounts of Rrp1 (4, 10, and 40 fmol in lanes 3–5) at 37 °C for 60 min. Lane 6 shows that DNA synthesis activity was absent in the Rrp1 fraction. Control reactions with no protein and 40 fmol of BSA are shown in lanes 1 and 7, respectively.
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(A) Untreated eggs Embryos Adults 
Rrp1 DmREV3 DmREV7 Actin 5C

(B) 

FIGURE 6. RT-PCR analysis of Rrp1, DmREV3, and DmREV7. PCR conditions are described under “Experimental Procedures.” A, expression patterns during developmental stages. B, DNA-damaging agents increased the levels of DmREV3 and DmREV7 transcripts. S2 cells were exposed to 0.005% (v/v) MMS, 1.5 mM H2O2, or 20 J m−2 UV, respectively. The relative amounts of transcript were normalized using that of Actin 5C. Each data point represents the mean of three independent measurements. Error bars, ± S.D.

we unfortunately failed, because the complexes between Rrp1 and DNA containing AP sites were unstable (data not shown). An earlier study, using an electrophoretic mobility shift assay, showed that APE1 has a high affinity for AP sites in DNA. Therefore, the unique N-terminal region of Rrp1 may cause the instability of these complexes. Although the mechanism of stimulation of AP endonuclease activity by DmREV7 remains unclear, it is known that mammalian pol β enhances the dissociation of APE1 from incised AP sites in DNA (15). Thus, one possibility is that DmREV7 may promote Rrp1 to dissociate from incised AP sites. Another possibility is that DmREV7 may change the structure of Rrp1 to a more active one. Human REV7 forms a homodimer and is thought to act as a regulator of DNA repair (39). We found that DmREV7 could also form a homodimer (Fig. 3C), suggesting that the homomultimerization of DmREV7 may play an important role in the regulation of Rrp1 and DmREV3 functions.

Reardon et al. (28) showed that the 3′-exonuclease activity of Rrp1 is dramatically decreased (210-fold lower) by removal of its N-terminal region, whereas its AP endonuclease activity is not altered. In the present study, Rrp1 exonuclease, like APE1, preferred a 3′-mispaired DNA substrate, although its preference for gapped and nicked DNA was apparently different from that of APE1 (Fig. 4). These findings suggest that the additional N-terminal region of Rrp1 causes the differences between the exonuclease properties of Rrp1 and APE1.

Although human pol δ can incorporate nucleotides at 5′-incised and reduced AP sites (33, 34), DmREV3 could not do so (Fig. 5A). The N-terminal 300-amino acid and C-terminal 850-amino acid regions of DmREV3 are similar to human pol δ, whereas the central 950-amino acid region is unique (25), suggesting that the central region of DmREV3 may suppress DNA synthesis for nicks after Rrp1 cleavage. Drosophila has a homologue of the enzyme flap endonuclease 1 that, reportedly, can excise the 5′-deoxyribose-5-phosphate as a part of the dinucleotide from the 5′-end in vitro (40). Therefore, we speculate that Rrp1 and DmREV7 may, in association with flap endonuclease 1, remove AP sites in the Drosophila genome and recruit DmREV3 in order to fill in the dinucleotide gap. Alternatively, AP sites may be excised in combination with bifunctional DNA glycosylases. Rrp1 and DmREV7 may cleave the 3′-terminal α,β-unsaturated aldehydes produced by AP lyase of bifunctional glycosylases.

Transcription levels of Rrp1, DmREV3, and DmREV7 varied between developmental stages, with the highest levels of expression in early cleavage stages (before gastrulation) (Fig. 6A). Our data suggest that AP sites may often be produced in early embryos and that expression of all three genes is important in blastogenesis.

The expression patterns of DmREV3 and DmREV7 showed similar responses to DNA-damaging agents (Fig. 6B). DmREV3 and DmREV7 transcripts were increased after MMS and H2O2 treatment. These DNA-damaging agents do not specifically induce AP sites in DNA, but they are believed to promote the release of bases from DNA (1). Therefore, these results suggest that an increase in the levels of transcription of these genes may be necessary for the repair of AP sites, although it is difficult to determine the biological roles only from gene expression patterns. In contrast, the transcripts of Rrp1 were less influenced by DNA-damaging agents than those of DmREV3 and DmREV7, possibly because Rrp1 may be expressed constitutively at a relatively high level in nuclei in a similar fashion to APE1 (3, 41).

In this study, we analyzed the relationship between Dmpol ζ and Rrp1 using biochemical approaches. Therefore, the in vivo function of each gene remains unclear. The detailed analysis of transgenic flies in which each gene is repressed or overexpressed will help us understand the function of each gene in Drosophila. In summary, we identified a DNA polymerase that bound to AP endonuclease (Rrp1) in Drosophila, which has no pol β-like gene. DmREV7, the accessory subunit of Dmpol ζ, directly interacted with Rrp1 and stimulated its AP endonuclease activity. DmREV3 could not incorporate nucleotides at incised tetrahydrofuran sites but catalyzed strand displacement synthesis in one-nucleotide-gapped DNA. From these results, we suggest that Dmpol ζ may be involved in the repair pathway of AP sites in DNA.

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