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Single-nucleotide base excision repair DNA polymerase activity in *C. elegans* in the absence of DNA polymerase β

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ABSTRACT

The base excision DNA repair (BER) pathway known to occur in *Caenorhabditis elegans* has not been well characterized. Even less is known about the DNA polymerase (pol) requirement for the gap-filling step during BER. We now report on characterization of *in vitro* uracil-DNA initiated BER in *C. elegans*. The results revealed single-nucleotide (SN) gap-filling DNA polymerase activity and complete BER. The gap-filling polymerase activity was not due to a DNA polymerase β (pol β) homolog, or to another X-family polymerase, since computer-based sequence analyses of the *C. elegans* genome failed to show a match for a pol β-like gene or other X-family polymerases. Activity gel analysis confirmed the absence of pol β in the *C. elegans* extract. BER gap-filling polymerase activity was partially inhibited by both dideoxynucleotide and aphidicolin. The results are consistent with a combination of both replicative polymerase(s) and lesion bypass/BER polymerase pol θ contributing to the BER gap-filling synthesis. Involvement of pol θ was confirmed in experiments with extract from pol θ null animals. The presence of the SN BER in *C. elegans* is supported by these results, despite the absence of a pol β-like enzyme or other X-family polymerase.

INTRODUCTION

Throughout the life cycle of an organism, damage in genomic DNA is produced from endogenous and exogenous sources, ultimately threatening genomic integrity. For example, uracil is generated from deamination of cytosine during normal cellular processes and, if unrepaired, will be mutagenic by pairing with adenine during the next cycle of DNA replication. Base excision repair (BER) purifies the genome of DNA base damage such as uracil. In the canonical mammalian single-nucleotide (SN) BER pathway for uracil-DNA, uracil-DNA glycosylase (UDG) recognizes and removes the base, leaving an apurinic/apyrimidinic (AP) site. AP endonuclease (APE) incises the DNA strand 5′ to the AP site to create a SN gap with 3′-OH and 5′-deoxyribose phosphate (dRP) at the margins. DNA polymerase (pol) β removes the 5′-dRP group from the gap and fills the SN gap with a correct nucleotide. Finally, DNA ligase seals the resulting nick to complete the repair pathway (1–4).

Caenorhabditis elegans is a popular model organism for investigating development, neurological maturation, aging and genome instability in developmental biology research (5–10). Although *C. elegans* is known to possess repair homologs to some of the proteins in mammalian DNA repair pathways (11–14), BER has not been well elucidated. Several *C. elegans* BER repair enzymes have been identified and characterized, including: UDG for uracil removal (15); the nth1 glycosylase for oxidized pyrimidine base removal (16) and two APEs, APN-1 and

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EXO-3, for AP site incision and processing (17). In addition to these enzymes, *C. elegans* also is known to express pol θ, a lesion bypass polymerase and back-up BER polymerase in vertebrate systems (18–21). Even though a complete BER system has not been reported in *C. elegans*, the existence of the above noted enzymes strongly suggested the presence of BER in the organism.

In this study, we further evaluated BER in *C. elegans*. To identify gap-filling DNA polymerases in *C. elegans,* we first conducted computer-based analyses of the genomic sequence. Computer-based sequence alignment analyses identified several DNA polymerases, including a pol θ homolog, but failed to identify a pol β homolog. In spite of the absence of a pol β homolog, we found robust *in vitro* BER in the extract prepared from L1 stage *C. elegans* and then examined the DNA polymerase requirement for the BER reaction using uracil-DNA as substrate. The DNA polymerase activity in the extract was characterized using inhibitors, and the activity was not consistent with a mammalian pol β-like enzyme, but was consistent with a combination of replicative polymerase and pol θ-like enzyme. The results illustrate the presence of complete BER in *C. elegans* and are discussed in the context of enzymes that fulfill the DNA polymerase requirement in the absence of a pol β homolog.

MATERIALS AND METHODS

Materials

Radioactive [γ-^{32}P]ATP (7000 Ci/mmol) was from MP Biomedicals (Irvine, CA, USA). [α-^{32}P]dCTP (3000 Ci/mmol), [α-^{32}P] dTTP (3000 Ci/mmol), [α-^{32}P] Cordycepin (3000 Ci/mmol) from PerkinElmer Life Sciences (Waltham, MA, USA), dCTP, ddCTP and MicroSpin G-25 columns were from GE Healthcare (Piscataway, NJ, USA). OptiKinase and terminal deoxynucleotidyl transferase were obtained from USB Corporation (Cleveland, OH, USA). Protease inhibitor cocktail was from Roche Diagnostic Corp. (Indianapolis, IN, USA). Human pol β (22,23), UDG (24), APE (25), DNA ligase I (26) and human pol θ domain (27) were purified as described. Activated calf thymus DNA, dimethyl sulfoxide (DMSO) and aphidicolin were obtained from Sigma-Aldrich (St Louis, MO, USA).

DNA substrates

The primer-template duplex DNA substrate for primer extension assays was constructed by annealing two synthetic oligodeoxynucleotides (Midland Certified Reagent Company, Inc., Midland, TX, USA): 15PRM, 5′-CTG CAGCTGATGCGC-3′ and 37COM, 5′-GCCGTA CCCGGGGATCCGTACCGCGCATCAGCTGCAG-3′. The uracil-containing DNA substrate for BER was prepared by annealing two synthetic oligonucleotides: 35U, 5′-GCCCTGCAGGTCGAUTCTAGAGGATCCC CGGGTAC-3′ and 35COM, 5′-GTACCCGGGGATCC TCTAGAGTCGACCTGCAGGGC-3′.

Preparation of L1 stage *C. elegans* extract

Caenorhabditis elegans strains Bristol N2 (wild-type) and tm2572 (*polq-1* mutant) were obtained from the *Caenorhabditis* Genetic Center (Minneapolis, MN, USA) and the National Bioresource Project (Tokyo, Japan), respectively. The tm2572 insertion–deletion strain was back-crossed three times into the Bristol N2 wild-type strain and screened by nested PCR using the following primer sets: external primers (forward 5′-GGT GCA CCA TGA TAG GTA TT-3′ and reverse 5′-TGT ACC ATC GAA AAA GCA GC) internal primers (forward 5′-TTA CGA CAG TGA CAC CAC AA-3′ and reverse 5′-CGA TTC GTC TCG TGG TGC AC-3′). Wild-type nematodes yielded an amplification product of 1776 bp, whereas, the mutant allele produced a 1078 bp product.

Synchronized populations of wild-type nematodes were prepared by sodium hydroxide/hypochlorite treatment, as previously described (28). Adult animals were cultured on 150 mm nematode growth medium agar plates and eggs were obtained by established methods using a sodium hypochlorite and sodium hydroxide mixture (29). The resulting eggs were washed in sterile K-medium (30), resuspended in complete K media (31) and cultured at 20°C overnight in a vented tissue culture flask to allow the eggs to hatch and for growth arrest at the L1 stage. After the overnight incubation, a portion of the complete K media was streaked onto an LB-agar plate to test for bacterial contamination. No bacterial colony formation was identified post-egg preparation.

L1 nematodes were washed with K media and pelleted by centrifugation to remove the majority of culture medium. The compressed nematode pellet was resuspended in two volumes of sonication buffer and then sonicated three times for 30 s each, using a Bioruptor at 4°C. Initially, three sonication buffers were tested in preparation of whole-cell extracts: Buffer A {50 mM Tris–HCl, pH 7.4, 14 mM β-mercaptoethanol, 100 mM NaCl, 1 mM EDTA, 10% glycerol and two mini-EDTA-free protease inhibitor tablets [modified from Morinaga *et al.* (16)]}, Buffer B {25 mM Tris–HCl, pH 7.4, 1 mM DTT, 5 mM KCl, 1.5 mM $MgCl_2$ and two mini-EDTA-free protease inhibitor tablets [modified from Nakamura *et al.* (15)]} or Buffer C (50 mM Tris–HCl, pH 7.4, 500 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5% Triton X-100, 20% glycerol and two mini-EDTA-free protease inhibitor tablets). The resulting extract was clarified by centrifugation at 14 000 rpm at 4°C for 30 min. The clear supernatant fraction was divided into aliquots, quick-frozen in liquid nitrogen and stored at −80°C until use. The protein concentration of the extract was determined by Bio-Rad protein assay using BSA as standard.

Activity gel analysis of DNA polymerase in *C. elegans* extract

The activity gel assay was performed essentially as described previously by Karawya *et al.* (32). Briefly, the protein samples [*C. elegans* extract (50 μg) and purified pol β (0.5 μg) as control] were electrophoresed in 10% discontinuous SDS–PAGE gels containing 100 μg/ml of activated calf thymus DNA as template-primer. Note that

the control sample (pol β) also contained 40 μg of heat-inactivated fetal calf serum to facilitate renaturation process. Before electrophoresis, both *C. elegans* extract and purified pol β samples were heated for 5 min at 37°C in SDS–PAGE sample buffer. After electrophoresis, the gel was washed with four 1-l changes of 50 mM Tris–HCl, pH 7.5, over a 1 h period with gentle agitation on a platform shaker. Then, the gel was placed in 1 l of renaturation buffer (50 mM Tris–HCl, pH 7.5, 6 mM Mg acetate, 40 mM KCl, 400 μg/ml BSA, 16% glycerol, 0.1 mM EDTA, and 1 mM DTT) and incubated overnight at 4°C with gentle agitation. The next morning, the buffer was replaced with 800 ml of fresh renaturation buffer and the incubation continued for 3 h at 4°C and then for 1 h at room temperature. After this renaturation protocol, the gel was rinsed and incubated in a 50 ml reaction mixture containing renaturation buffer and 12.5 μM each dATP, dCTP, dGTP and 1 μM [α-^{32}P]dTTP for the *in situ* DNA polymerase activity. The incubation was in a 37°C water bath with shaking for 20 h. Then, removing the mixture containing unincorporated dNTPs stopped the polymerization reaction. The gel was washed first with three 1-l changes of chilled 5% trichloroacetic acid containing 1% NaPPi for a 1-h period and then with one additional change overnight. The washed gel was then dried and subjected to imaging and data analysis using Typhoon PhosphorImager and ImageQuant™ software (GE Healthcare).

In vitro DNA polymerase assay for primer extension

The primer termed 15PRM was 5′-end labeled by incubation with OptiKinase in the presence of [γ-^{32}P]ATP for 30 min at 37°C according to the manufacturer's protocol. The labeled oligonucleotide was then annealed with the complementary oligonucleotide 37COM, and the DNA was purified with a MicroSpin G-25 column. The ^{32}P-labeled duplex oligonucleotide (50 nM) was incubated with 10 μg of *C. elegans* extract at 25°C for the indicated period in a final reaction mixture volume of 10 μl. The reaction mixture contained 50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 20 mM KCl, 0.5 mM DTT and 20 μM 4dNTPs. The reaction was terminated by the addition of an equal volume (10 μl) of DNA gel-loading buffer and heating for 5 min at 95°C. The reaction products were separated by 15% denaturing PAGE and subjected to imaging and data analysis using Typhoon PhosphorImager and ImageQuant™ software (GE Healthcare).

In vitro uracil-DNA initiated BER assays

The oligonucleotide termed 35U was 5′-end labeled and annealed with 35COM. The duplex DNA was then purified as described above. For verification of this substrate, the ^{32}P-labeled duplex oligonucleotide (50 nM) was incubated with 20 nM UDG and 20 nM APE for 30 min at 37°C in BER reaction buffer containing 50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 20 mM KCl and 0.5 mM DTT. The reaction was terminated by the addition of an equal volume (10 μl) of DNA gel-loading buffer and heating for 5 min at 95°C. The reaction products were separated by 15% denaturing PAGE and analyzed as above.

To conduct the uracil-initiated BER assay, the duplex oligonucleotide (250 nM) was pre-treated with UDG (20 nM) and APE (20 nM) for 30 min at 37°C in the BER reaction buffer to ensure complete incision of the uracil-containing DNA strand. Then, the BER reaction mixture (10 μl final volume) that contained 50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 20 mM KCl, 0.5 mM DTT, 2.3 μM [α-^{32}P]dCTP, 4 mM ATP, 5 mM phosphocreatine, 100 μg/ml phosphocreatine kinase, 0.5 mM NAD and pre-treated duplex DNA (250 nM) was assembled on ice. The repair reaction was initiated by adding 10 μg *C. elegans* extract, followed by incubation at 25°C for the indicated periods. Note that, some reaction mixtures in Figure 3B also contained 250 nM purified human DNA ligase I. Reactions were terminated by the addition of an equal volume of DNA gel-loading buffer and heating for 5 min at 95°C. The reaction products were separated by 15% denaturing PAGE and analyzed as above.

Inhibition of SN BER gap-filling activity in the *C. elegans* extract

To examine the inhibition of SN gap-filling activity in a *C. elegans* extract by ddCTP, uracil-initiated BER reaction was performed under similar reaction conditions as described above, except 1000 μM ddCTP was added along with 2.3 μM [α-^{32}P]dCTP. The control reaction, without ddCTP, was conducted with 2.3 μM [α-^{32}P]dCTP. The reaction mixtures were incubated at 25°C and aliquots were withdrawn at the indicated periods.

For testing SN gap-filling activity inhibition by aphidicolin in a *C. elegans* extract, DMSO was used to dissolve aphidicolin. Uracil-initated BER reaction was performed as above with or without 0.2 mM aphidicolin. The final concentration of DMSO in each reaction mixture was 10%. The reaction mixtures were incubated at 25°C, aliquots were withdrawn at the indicated periods and the reaction products were analyzed as above.

In an alternate method of detecting the incorporation of dCTP or ddCTP by the BER gap-filling DNA synthesis activity in *C. elegans,* extracts was used. In this case, the reaction conditions were the same as those described above, except unlabeled duplex DNA and 2.6 μM [α-^{32}P]dCTP were replaced with a 5′-end ^{32}P-labeled duplex DNA substrate (100 nM) and unlabeled 20 μM dCTP or 20 μM ddCTP, respectively (Figure 7).

RESULTS

Computer-based genomic sequence analyses of DNA polymerase open reading frames in *C. elegans*

The genome of *C. elegans* has been sequenced to completion (18), and we initially used this information to conduct homology search analyses for DNA polymerases by comparison with the human DNA polymerases. This search involved several different approaches as follows:

BLAST analysis in search of DNA polymerase β. To evaluate the presence of pol β or a similar enzyme, a search in KEGG (Kyoto Encyclopedia of Genes and Genomes: http://www.kegg.jp) for ortholog groups was

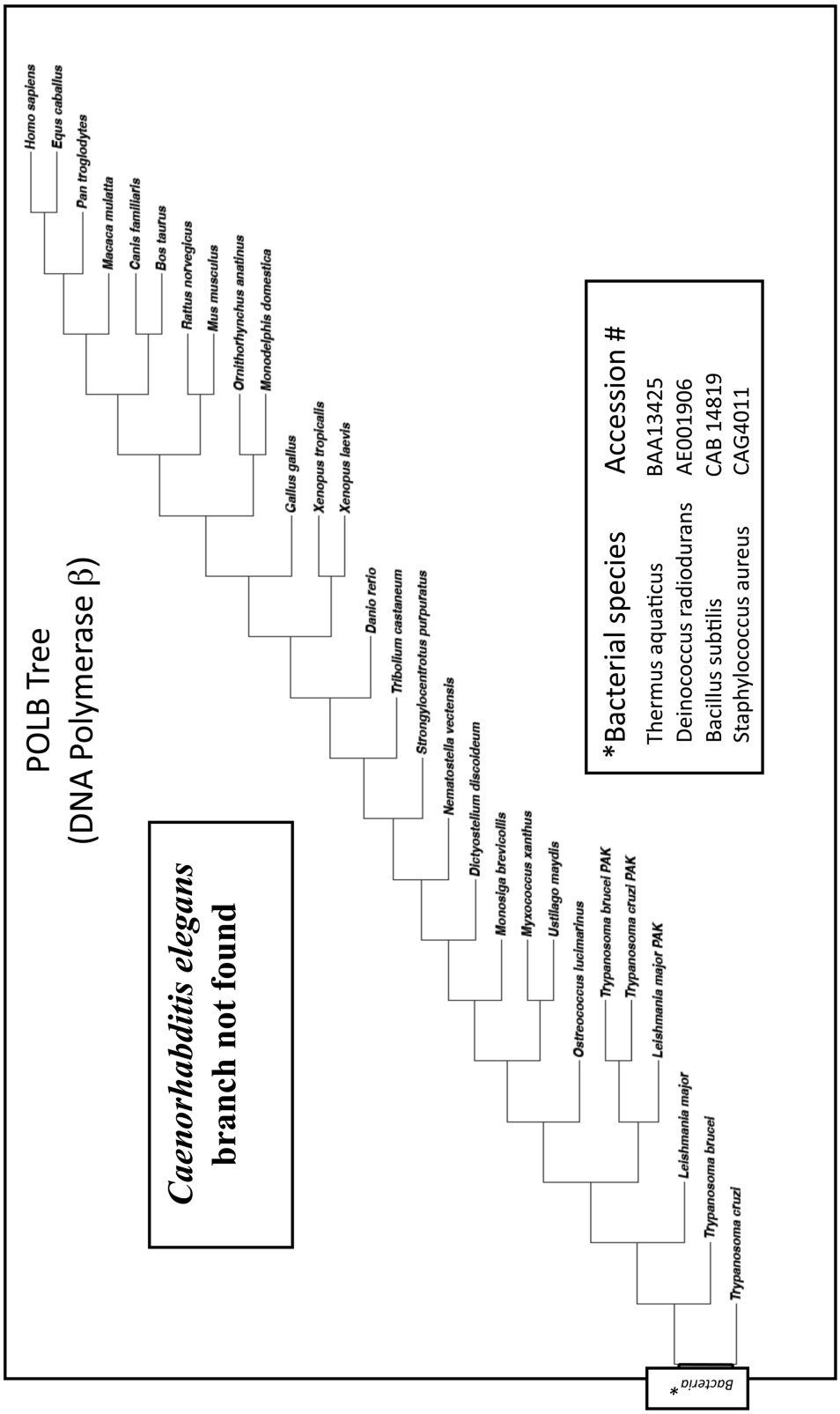

Figure 1. Pol B unrooted phylogenetic tree. Dendrogram was built using all 28 sequences from KEGG orthology cluster K02230. As expected, sequences from more related organisms are found to be closer on the tree. Branch lengths do not correspond to evolutionary distances. A pol β branch was not detected. Pol β homologs are found in multiple bacterial species (33–36); this is not included in the phylogenetic analysis shown.

Table 1. Putative DNA polymerases from *C. elegans*[a]

Human homolog	Uniprot ID	Worm homolog	Uniprot ID	Number of amino acids	kDa
MAD2L2 (REV7)	Q9UI95	Y69A2AR.30a	Q9NGT3	203	23.53
POLA	P09884	Y47D3A.29	Q9NAH1	1428	161.45
POLD1	P28340	F10C2.4	P90829	1081	120.83
POLE	Q07864	F33H2.5	O62218	2144	244.71
POLG	P54098	Y57A10A.15	Q9U208	1072	121.89
POLH	Q9Y253	F53A3.2	O17219	584	66.37
POLK (DINB1)	Q9UBT6	F22B7.6	P34409	503	57.31
POLQ	Q7Z5Q5	W03A3.2	A0FlQ6	1538	174.64
POLS	Q5XG87	ZK858.1	Q94419	845	94.31
REV1L (REV1)	Q9UBZ9	ZK675.2	Q09615	1027	115.85
REV3L (POLZ)	O60673	Y37B11A.2	Q9BKQ3	1133	129.07

[a]In this search, pol β, pol λ and pol ι were not found in *C. elegans*.

performed. The cluster K02330 is a Pol β cluster with 28 distinct sequences from 25 organisms. The trypanosomatids, *Leishimania major*, *Trypanosoma cruzi* and *Trypanosoma brucei* have 2 polymerases (PolB and PolB-PAK). Pol β was found in a variety of clades, including mammals, birds, amphibians, fishes, insects, plants, fungi, algae and cnidarians, but not in nematodes. An unrooted tree of pol β was constructed using the Phylip package and as expected, the sequences for the more related organisms were found to be closer in the tree (Figure 1), yet nematodes were not represented. In addition, pol β is found in multiple species of bacteria that are more closely related to protists (33–36). Thus, this analysis revealed that pol β homologs could be found in a wide variety of organisms; however, pol β was not found in nematodes (including *C. elegans*).

In order to further evaluate open reading frame sequences in *C. elegans* that would encode orthologs of pol β, two methods were used. The first was to align all the sequences and BLAST them against the *C. elegans* proteome. This strategy did not work as expected, due to the fact that mammalian sequences are not similar to trypanosomatids sequences. Therefore, this rendered only small fragments of conserved sequences, which were not long enough for BLAST searches. The second method consisted of mapping conserved domains in the pol β sequences. One domain, called polXc (Conserved domains id: cdd00141, SMART id: smart00483), is conserved in all pol β from cluster K02230. A Position Specific Matrix (a score for each amino acid at that position) for this domain was then used as a subject for a Reversed Position Specific-BLAST (RPS-BLAST). Two RPS-BLAST searches were performed, one using all *C. elegans* transcriptomes (24 143 sequences) as query and another using all *C. elegans* expression sequence tag (EST) sequences (40 5514) as query. The RPS-BLAST search using the complete *C. elegans* as query revealed no significant hits with polXc domains. Also, no significant hits were found when *C. elegans* EST sequences were used as query.

The final step was to use a Psi-T BLAST N, which is a method to find distant orthologs. The focus was on pol β fragments (>15 amino acid contiguous blocks) that are most highly conserved. This method also failed to reveal any pol β orthologs. Taken together, we conclude that the *C. elegans* genome does not contain an open reading frame capable of encoding a pol β homolog. This conclusion is consistent with other computer-based analyses conducted independently (37).

Search for other DNA polymerases. A similar analysis was performed for all known human DNA polymerases, and 12 matches of putative DNA polymerases or related enzymes were found (Table 1). As summarized in Table 1, this analysis failed to identify any X-family polymerases like pol β or pol λ homologs in *C. elegans*, but a number of other polymerase homologs were found. Two of these corresponded in size to the polymerases observed in activity gel analysis (described below) of the extract, including a *C. elegans* open reading frame encoding a protein of 66 kDa corresponding to polH or DNA polymerase *η*. Interestingly, no matches with Pol ι were found.

In the experiments described below, we found BER in extracts, and we emphasized identification of the DNA polymerase responsible for the gap-filling step. In spite of the failure of the computer-based analyses to find a pol β homolog, we conducted experiments to confirm the absence of this enzyme.

Preparation of *C. elegans* L1 stage extracts

Our first challenge toward the study of BER DNA polymerase activity in *C. elegans* was to establish an appropriate experimental protocol for preparation of the extract. This proved to be demanding for several reasons: a major concern was elimination of bacterial contamination, since *C. elegans* use *Escherichia coli* as a food source. To avoid this complication, we used egg preparations as the starting material instead of adult *C. elegans*, along with an extensive egg washing protocol. This washing eliminated bacterial contamination, whereas the *C. elegans* eggs remained viable and grew to the L1 stage before extract preparation. A second concern was disruption of the L1 nematodes during sonication without denaturing the proteins. We used three buffer systems for preparation of extracts as indicated under 'Materials and Methods' section, and tested these extracts for recovery of DNA

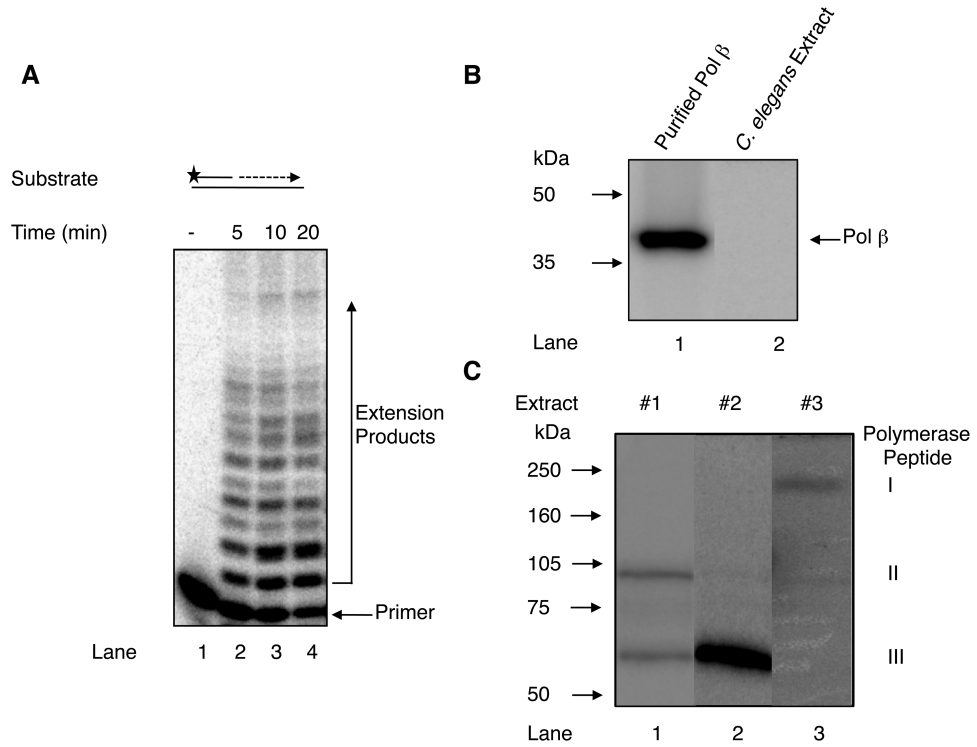

Figure 2. *In vitro* DNA polymerase assay in the *C. elegans* extract. (**A**) Primer extension analysis using *C. elegans* extract. A 5′-end labeled primer-open template duplex was used (lane 1) along with *C. elegans* extract. Incubation was for 5 min (lane 2), 10 min (lane 3) and 20 min (lane 4). The gel migration positions of the primer and the primer extension products are shown on the 'right' side of the image, and a schematic of the substrate and product is shown at the top. The results shown are representative of three similar experiments. The position of ^{32}P-label on DNA is indicated by the '*' sign. (**B**) Example of a typical result from activity gel analysis of DNA polymerases in the *C. elegans* extract. Experiments were conducted as described under 'Materials and Methods' section. Purified human pol β (used as a positive control; lane 1) and *C. elegans* extract (50 μg) (lane 2) were subjected to the activity gel assay, involving SDS–PAGE, renaturing steps and then a DNA polymerase reaction mixture containing [α-^{32}P]dTTP. The phosphorimage of the gel is shown illustrating the incorporation of [α-^{32}P]dTMP into DNA. The relative positions of the protein markers and pol β are indicated. The region of the gel shown corresponds only to the migrating positions human DNA pol β and any potential corresponding enzyme in the *C. elegans* extract. The results shown are representative of three repeat experiments. (**C**) In other activity gel experiments using three independently prepared extracts, higher molecular mass signals were observed. These polymerase peptides, designated as I, II and III, corresponded to ~185 kDa, ~100 kDa and ~65 kDa, respectively.

polymerase activity. Although the concern about loss of enzymatic activities may not have been fully accommodated, the recovery of BER activity in the extract designated as Buffer C was slightly higher than that of the other two extracts (data not shown). Therefore, the procedure for this extract preparation was used in the following experiments.

Initial characterization of DNA polymerase activity in the *C. elegans* extract

To verify the presence of DNA polymerase activity in our preparation of *C. elegans* extract, we conducted routine primer extension assays using an 'open template' DNA substrate. The primer strand of this substrate DNA was ^{32}P-labeled at the 5′-end. After incubation, the reaction products were displayed on a DNA sequencing gel (38). The results revealed that DNA polymerase activity in the extract was capable of extending the primer (Figure 2A).

To verify the absence of a mammalian pol β-like DNA polymerase in the *C. elegans* extract, we first employed activity gel analysis. This assay involves gel-embedded activated DNA as the DNA polymerase reaction substrate (Figure 2B and C). Extract from *C. elegans* and purified human pol β, used as a reference, were separated by SDS–PAGE. The proteins in the gel were renatured as described in 'Materials and Methods' section and subjected to the *in situ* DNA polymerase 'activity gel' assay (32). The results consistently failed to reveal the presence of an enzyme in the size range corresponding to the main mammalian BER DNA polymerase, pol β (Figure 2B, lane 2), an enzyme that is known to be unusually active in this in-gel assay compared with other DNA polymerases. In these experiments, signals corresponding to larger polypeptides were often observed. However, these signals were of variable strength from extract to extract (Figure 2C); the most prominent of these polymerase peptides, designated as I, II and III, corresponded to ~185 kDa, ~100 kDa and ~65 kDa, respectively. The variation in detecting these higher molecular mass DNA polymerase polypeptides from extract to extract was presumably due to uncontrolled proteolysis in the extracts.

Evaluation of BER in the *C. elegans* extract

To investigate uracil-mediated BER activity in the *C. elegans* extract, a 35-bp uracil-containing duplex

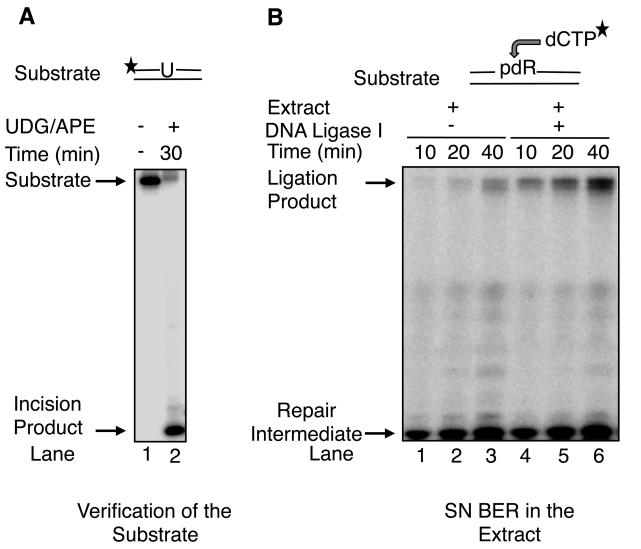

Figure 3. Characterization of BER activities in the *C. elegans* extract. (**A**) 5′-end labeled uracil-containing duplex DNA was incubated with either buffer (lane 1) or UDG and APE at 37 C for 30 min (lane 2) and the products were analyzed as described in 'Materials and Methods' section. The position of ^{32}P-label on DNA is indicated by the 'asterisk' sign. The positions of the ^{32}P-labeled substrate (35 nt) and the UDG/APE-incised product (14 nt) are indicated. (B) Unlabeled uracil-containing duplex DNA substrate was pre-treated with UDG and APE at 37 C for 30 min and then incubated with *C. elegans* extract in the presence of [α-^{32}P]dCTP at 25 C, either without DNA ligase I (lanes 1 and 3) or with DNA ligase I (lanes 4–6). The incubation was for 10 min (lanes 1 and 4), 20 min (lanes 2 and 5), or 40 min (lanes 3 and 6), respectively. The products were analyzed as in (A). The positions of the repair BER intermediate (15 nt) and the ligation product (35 nt) representing complete BER are indicated. The schematic at the top illustrates the DNA and dNTP substrates for the repair assay. The 'asterisk' sign depicts radiolabeled [α-^{32}P]dCTP in the reaction. Note that the substrates in (**B**) was pre-treated with UDG and APE to generate a SN gapped substrate that contained 3′-OH and 5′-dRP groups at the margins. The results shown are representative of three similar experiments.

oligonucleotide substrate was employed. The uracil-containing strand was 5′-end labeled and annealed with the complementary strand containing a guanine base opposite a solitary uracil residue at position 15. The duplex oligonucleotide substrate was then subjected to uracil base removal and AP site incision assay by incubation with UDG and APE (Figure 3A) for verification of the substrate. As expected, the combined actions of purified UDG and APE on this DNA substrate generated a 14 nt BER intermediate (Figure 3A, lane 2). To examine the BER activity after the UDG and APE steps, uracil-containing unlabeled duplex oligonucleotide substrate was pre-incubated with purified UDG and APE to ensure complete processing at the site of the uracil lesion. The resulting SN-gapped DNA substrate was then incubated with the *C. elegans* extract and [α-^{32}P]dCTP as the labeled nucleotide substrate. After this incubation, we observed the SN gap-filling product (15 nt) (within 10 min, Figure 3B, lane 1), representing the repair intermediate stalled at the step immediately before ligation. A relatively small amount of the fully ligated BER product also was observed (Figure 3B, lanes 1–3). However, when the reaction mixtures were supplemented with purified DNA ligase I, a portion of the SN gap-filled BER intermediate was converted to fully ligated product (Figure 3B, lanes 4–6). The results in Figure 3B also indicated that the gap-filled BER intermediate produced during the incubation was a substrate for ligation by the DNA ligase added, in spite of the apparent lack of pol β and its dRP lyase activity. This suggested that a 5′-dRP lyase activity in the *C. elegans* extract was capable of processing the BER intermediate so that ligation could occur.

Characterization of SN BER gap-filling DNA synthesis activity in the *C. elegans* extract

Test for ddNTP inhibition of the SN BER gap-filling activity. The presence of the SN gap-filling polymerase activity described above is consistent with BER in *C. elegans*. In mammalian BER, the SN gap-filling reaction is conducted primarily by pol β and in this case, the pol β active site can accommodate ddNTPs and incorporate them about as well as the normal dNTPs (39), but subsequent ligation, or further dNMP incorporation, is blocked due to the absence of the 3′-OH group on the sugar ring of the newly incorporated nucleotide (40). On the other hand, replicative polymerases are resistant to ddNTP inhibition because they fail to bind the nucleotide analog. Therefore, we decided to characterize the SN gap-filling activity for ddNTP inhibition. We observed that the SN gap-filling reaction in the *C. elegans* extract was partially inhibited when the reaction mixture was supplemented with an excess of ddCTP (1 mM) (Figure 4A, compare lanes 1 and 2 with lanes 3 and 4). In this ddNTP inhibition analysis, the reaction mixture contained the normal level of ^{32}P-labeled dCTP in addition to the large excess of ddCTP. These results suggested the SN gap-filling reaction in the *C. elegans* extract involved a mixture of ddNTP-resistant replicative-type DNA polymerase activity and ddNTP-sensitive DNA polymerase activity, such as those of pol θ and pol γ (19).

Test for aphidicolin inhibition. To further evaluate the enzyme(s) responsible for the BER SN gap-filling, the replicative-type DNA polymerase inhibitor aphidicolin was used. Aphidicolin inhibition of the activity was strong (Figure 4B, lanes 1 and 2 versus lanes 3 and 4), indicating that aphidicolin-sensitive DNA polymerase activity was a contributor of the gap-filling activity. Nevertheless, a portion of the activity was resistant to aphidicolin, again consistent with the interpretation that a mixture of polymerase activities accounted for the gap-filling activity of the wild-type extract.

Characterization of BER gap-filling DNA synthesis activity in the *C. elegans* extract prepared from a *polq-1* gene deletion strain

Taken together, the results described above using inhibitors suggested that the BER gap-filling activity of the wild-type extract was due to a mixture of DNA polymerases, and this included ddNTP-inhibited and aphidicolin-resistant polymerase(s). Pol θ has these inhibition properties, was found in our computer-based analysis of the genome (Table 1), and is also known to be present in

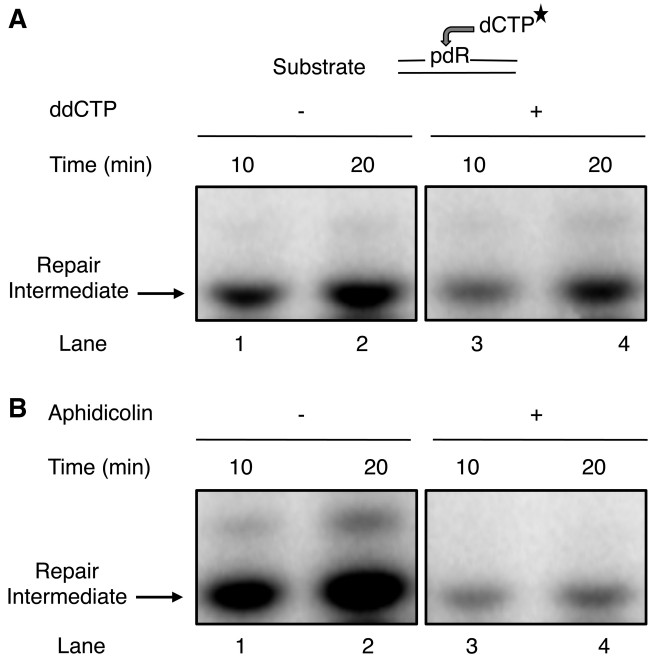

Figure 4. Inhibition of the SN gap-filling activity with ddCTP/aphidicolin in *C. elegans* extract-mediated BER. The schematic at the top illustrates the DNA and dNTP substrates for the repair assay. The 'asterisk' sign depicts radiolabeled [α-^{32}P]dCTP in the reaction. (**A**) To examine the inhibition of SN gap-filling activity in the *C. elegans* extract by ddCTP, uracil-initiated BER reaction was performed under similar reaction conditions as described in Figure 3B, except that the reaction mixtures also contained 1000 μM ddCTP along with 2.3 μM [α-^{32}P]dCTP (lanes 3 and 4). The control reaction (lanes 1 and 2), without ddCTP, was conducted with 2.3 μM [α-^{32}P]dCTP only. The reaction mixtures were incubated at 25 C and aliquots were withdrawn at 10 and 20 min, as indicated. The absence or presence of ddCTP is illustrated by (−) or (+) symbols, respectively, at the top. (**B**) To evaluate the contribution of replicative DNA polymerases in *C. elegans* extract-mediated BER, the repair reaction was performed under similar reaction conditions as described in Figure 3B. The reaction mixture was supplemented either with DMSO (lanes 1 and 2), or with 200 μM aphidicolin (lanes 3 and 4). The incubation was for 10 and 20 min, as indicated at the top of each gel image. The absence or presence of aphidicolin is illustrated by (−) or (+) symbols, respectively, at the top. The position of the repair intermediate is indicated. The experiment shown is a representative of three experiments. Note that the strong activity observed in Figure 4B may be due to the effect of the solvent DMSO, as previously observed with mammalian extracts (41).

C. elegans (21). In addition, vertebrate pol θ is known to function as a BER enzyme in DT40 cells and has BER enzymatic properties (20,27). Therefore, we decided to obtain a *polq-1* gene deletion strain and test the extract for BER gap-filling activity. The gene deletion was confirmed in the experiments shown in Figure 5A. Characterization of the BER gap-filling activity in the extract from the mutant strain revealed that the BER activity was lower, compared with the activity of wild-type extract prepared in parallel (Figure 5B and C). Furthermore, the activity of the null extract was not inhibited by ddNTP (Figure 6A), whereas the activity of the null extract was completely inhibited by aphidicolin (Figure 6B). These results confirm that pol θ was responsible for a portion of the activity of the wild-type extract (Figure 4A). The aphidicolin sensitivity of the null extract was striking and consistent with inhibition of replicative DNA polymerase(s). These results also confirm that pol θ was responsible for a portion of the activity of the wild-type extract (Figure 4B). The strong activity observed in Figures 4B and 6B, lane 1 may be due to the effect of the solvent DMSO, as observed previously with mammalian extracts (41).

Test for ddNTP incorporation by the BER gap-filling DNA synthesis activity in *C. elegans* extracts

The ability to incorporate ddNTP into a primer terminus is a characteristic property of pols β and γ, as well as, many other DNA polymerases used in DNA sequencing, of course (42). On the other hand, pol θ is unable to insert ddNTP, but is inhibited by ddNTPs (21). Therefore, we decided to test the *C. elegans* extracts for this property (Figure 7). Both wild-type and pol θ null extracts were unable to insert ddCTP (Figure 7, lanes 4 and 6), as was the case for the reference pol θ polymerase domain enzyme (Figure 7, lane 8). As expected, pol β was able to insert ddCTP about as well as the normal dCTP (Figure 7, lanes 9 and 10).

DISCUSSION

Caenorhabditis elegans extract DNA polymerase activity capable of supporting SN BER of uracil-DNA was detected here, and then the activity was characterized in the *in vitro* BER assay systems described. Based on our extract preparation procedure and the results of activity gel and primer extension analyses (Figure 2), we conclude that the DNA polymerase activity was intrinsic to the *C. elegans* proteins, rather than a contaminant of the food source, *E. coli*. Nevertheless, none of the observed DNA polymerase activity-positive *C. elegans* proteins in the activity gel analysis matched the size of the main mammalian BER DNA polymerase, pol β (39 kDa), suggesting the absence of this BER enzyme in *C. elegans*. Since mammalian pol β is the most readily detectable DNA polymerase in the activity gel analysis (Figure 2B, lane 1), it seemed likely that a 39 kDa homolog was not present in our *C. elegans* extract. In concert with this interpretation, the computer-based homology searches of DNA polymerases in the *C. elegans* genome failed to reveal any suggestion of a pol β homolog (Table 1). In addition, the *C. elegans* BER gap-filling DNA polymerase activity was not inhibited by anti-pol β antibody, and did not exhibit pol β-like catalytic properties since it did not utilize ddNTP as substrate (data not shown; Figure 7). Finally, a phylogenetic analysis for the presence of pol β-like enzymes [Figure 1 and (37)] failed to reveal the existence of this enzyme in nematodes, including *C. elegans*, confirming the sequence alignment analysis summarized in Table 1. Thus, we concluded that *C. elegans* does not have a human pol β homolog and that the BER gap-filling activity observed is due to other enzymes.

From the activity gel analysis, several polypeptides with DNA polymerase activity were observed, at ~65 kDa, ~100 kDa and ~185 kDa, respectively. Regarding the ~65 kDa polypeptide, one *C. elegans* open reading frame

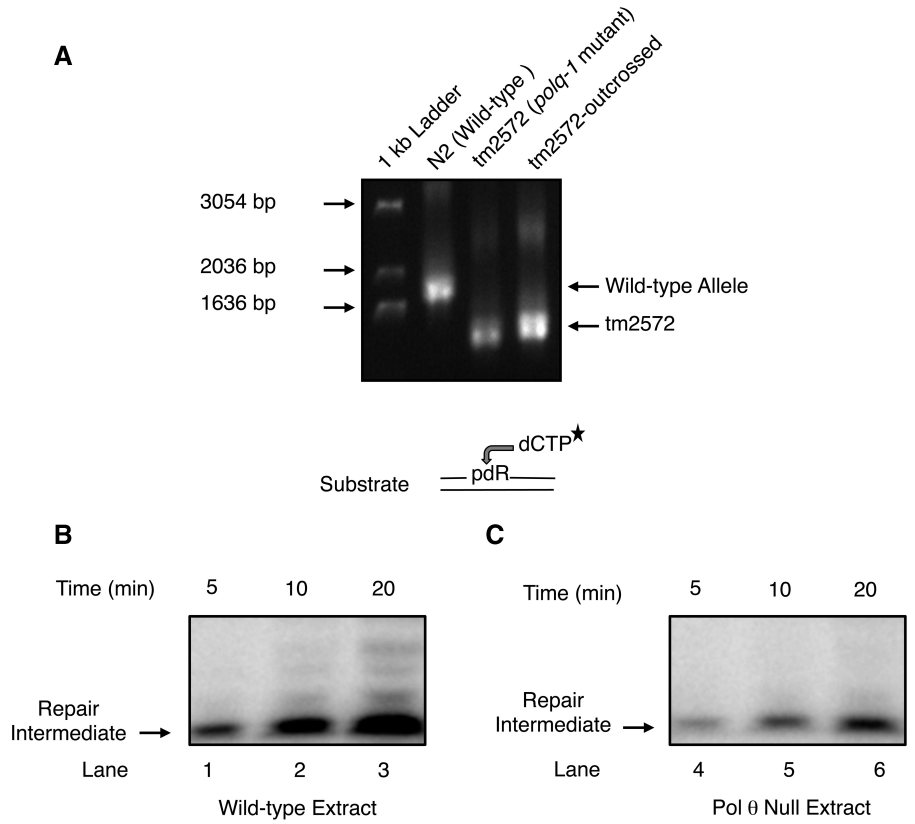

Figure 5. BER gap-filling DNA synthesis activity in the *C. elegans* extract from a *polq-1* gene deletion strain. (**A**) PCR products using N2 (wild-type) and tm2572 (*polq-1* mutant), allele-specific primers on approximately 20 nematodes isolated from a population of N2, tm2572 and tm2572-outcrossed animals before extract preparation. Arrows indicate the PCR product obtained after amplification of the wild-type (1776 bp) or pol θ knockout tm2572 (1078 bp) allele. To evaluate the contribution of pol θ in *C. elegans* extract-mediated BER, the repair reaction was performed under similar reaction conditions as described in Figure 3B. The repair reaction was initiated either with the extract prepared from wild-type animals (**B**) or with the extract prepared from pol θ null animals (**C**). The reaction mixtures were incubated at 25 C and aliquots were withdrawn at 5, 10 and 20 min, as indicated. The reaction conditions and products analysis were as in Figure 3B. The position of the repair intermediate is indicated. The results shown are representative of multiple repeat experiments. The 'asterisk' sign depicts radiolabeled [α-^{32}P] dCTP in the reaction.

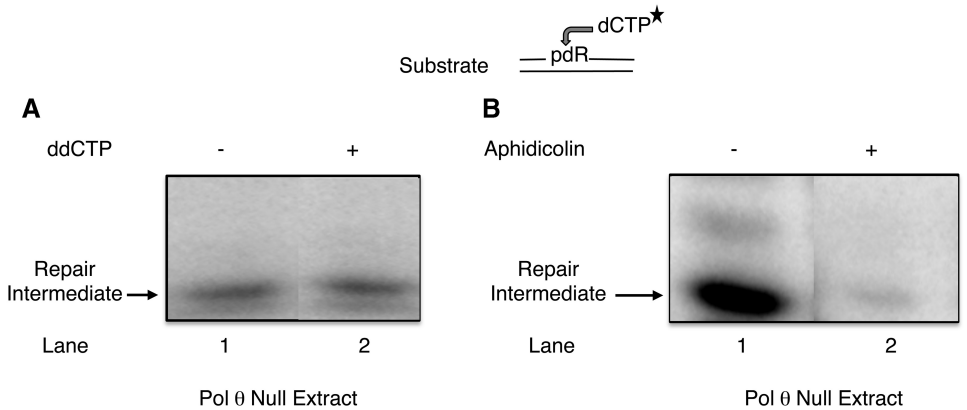

Figure 6. Inhibition of the SN gap-filling activity with ddCTP/aphidicolin in the pol θ null *C. elegans* extract. The schematic at the top illustrates the DNA and dNTP substrates for the repair assay. The 'asterisk' sign depicts radiolabeled [α-^{32}P]dCTP in the reaction. (**A**) To evaluate the contribution of pol θ in *C. elegans* extract-mediated BER, the repair reaction was performed under similar reaction conditions as described in Figure 3B, except that the reaction mixtures were supplemented with 1000 μM ddCTP (+) along with 2.3 μM [α-^{32}P]dCTP. The control reaction, without ddCTP (−), was conducted with 2.3 μM [α-^{32}P]dCTP. The repair reaction was initiated by the addition of extract (10 μg) prepared from pol θ null animals and subsequent incubation at 25 C for 10 min. (**B**) The reaction mixture was supplemented either with DMSO (lane 1), or with 200 μM aphidicolin (lane 2). The repair reaction was initiated as in (A). The absence or presence of aphidicolin is illustrated by (−) or (+) symbol, respectively, at the top of the phosphorimmage of the gel. The reaction products were analyzed as in Figure 3B. The results shown are representative of three repeat experiments.

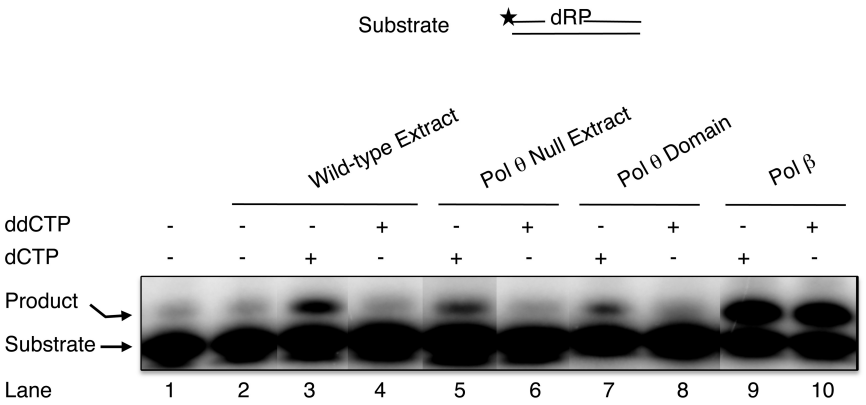

Figure 7. SN gap-filling activity in the presence of dCTP or ddCTP in the wild-type and pol θ null extracts of *C. elegans*. A schematic representation of the ^{32}P-labeled DNA pre-treated with UDG and APE is shown. The 'asterisk' depicts the ^{32}P-label at the 5′ end of DNA. The reaction conditions and product analysis were as described under 'Materials and Methods' section. UDG/APE pre-treated DNA substrate (100 nM) was mixed either with the extract (10 μg) prepared from wild-type (lanes 2–4) or pol θ null (lanes 5 and 6) animals. Reference repair reactions were incubated either with 400 nM purified human pol θ (lanes 7 and 8) or with 20 nM purified human pol β (lanes 9 and 10), respectively. Lane 1 represents DNA substrate alone. The repair reaction was initiated by the addition of dCTP (20 μM) or ddCTP (20 μM) and subsequent incubation at 25 C for 20 min. The reaction products were analyzed as in Figure 3B. The positions of the substrate and product are indicated. The results shown are representative of two similar experiments.

encoding a protein of this size was identified by computer-based sequence analysis (Table 1). This enzyme, termed F53A3.2, has homology to human POLH or polymerase *η*. Yeast and mammalian pol *η* are known to be involved in lesion bypass damage tolerance against UV irradiation (43), but a role in BER has not been suggested and is unlikely. Regarding the ∼100 kDa polypeptide, the sequence analysis in Table 1 failed to identify a matching polymerase. In the case of the ∼185 kDa polypeptide, *C. elegans* pol θ is of similar size (Table 1). Interestingly, human pol θ has a polymerase domain of ∼100 kDa that is capable of conducting BER gap-filling synthesis and dRP lyase activity (19,27). In addition to the absence of a pol β homolog, the analysis failed to identify a pol λ homolog, the other X-family BER polymerase, or a pol ι homolog, the Y-family DNA polymerase implicated in BER (41,44).

In further considering the DNA polymerase(s) involved in the *C. elegans* extract-mediated uracil-DNA BER activity, we examined the effects of ddNTP and aphidicolin. First, ddNTP partially inhibited the gap-filling activity (Figure 4). On the other hand, the extract failed to support ddNTP incorporation at the gap-filling step (Figure 7). This property is known for some B-family DNA polymerases, e.g. RB69 polymerase (45) and Vent DNA polymerase (46), and may depend on the loss of O3′ hydrogen bonding involved in stabilizing the position of the triphosphate moiety of the incoming ddNTP substrate (47). In contrast to this, many DNA polymerases are able to both bind and incorporate ddNTP, resulting in a synthesis block at the following extension step; this feature, of course, is characteristic of the enzymes used for Sanger DNA sequencing and is also characteristic of pol β, pol λ (48), pol γ (49) and pol μ (50). Since the use of ddNTP as substrate was not supported by the *C. elegans*-mediated gap-filling activity, the involvement of pol γ and the X-family DNA polymerases appeared to be unlikely, and these DNA polymerases were not considered as candidates for the *C. elegans* BER DNA synthesis activity. Like the two polymerases mentioned above, human pol θ is inhibited by ddNTP, but does not incorporate it (19), as we observed here for a portion of the *C. elegans*-mediated gap-filling activity. Next, aphidicolin had an inhibitory effect on the BER gap-filling activity (Figure 4B). Aphidicolin is used as an inhibitor for the replicative-type or B-family DNA polymerases, such as pol α, pol *δ* and pol ε, but does not inhibit pol β and only weakly inhibits pol θ (19). The aphidicolin-mediated inhibition of the *C. elegans* gap-filling activity suggested that the B-family DNA polymerases were responsible for a significant portion of the BER activity observed. Yet, the aphidicolin-resistant activity observed was consistent with a pol θ homolog. A stimulatory effect on the extract-based BER activity was observed with the presence of the DMSO solvent alone (data not shown); similar observations with this solvent had been made earlier (41).

In light of these results with polymerase inhibitors, we obtained a strain of *C. elegans* with a deletion in the *polq-1* gene, i.e. pol θ. The extract from these animals had lower BER gap-filling activity; use of the polymerase inhibitors suggested that this extract relied on the replicative-type enzymes for its gap-filling activity (Figures 4–7). Concerning observations of BER gap-filling activity in the absence of pol β expression, our results with *C. elegans* can be compared with results reported for early stage and developing zebrafish (51). In this case, the pol β gene is present, but expression is not observed, and aphidicolin-sensitive polymerase(s) accounted for most of the extract-based BER activity (51). Thus, pol β gene expression can vary as a function of developmental stage in this model organism. The phenomenon of developmental stage-specific DNA polymerase expression has not been observed as yet in *C. elegans*. However, we note that the larval stage extract-based BER characterized here may differ in extract from adult stage *C. elegans*.

In the BER assay in *C. elegans* extract, we failed to observe robust processing of the SN repair intermediate into the final ligated product of BER (Figure 3B). However, upon addition of purified DNA ligase I to the

Table 2. Summary of DNA polymerase inhibitors and *C. elegans* extract-mediated SN BER

	Inhibition of SN-BER	
Inhibitor	Wild-type extract	Pol θ null extract
ddCTP	Partial inhibition	No inhibition
Aphidicolin	Strong inhibition	Complete inhibition

reaction mixture, a substantial amount of ligated product was observed (Figure 3B), indicating that the BER intermediate was a substrate for DNA ligase and that 5′-dRP lyase activity was present in the extract.

The inhibitor profiles of *C. elegans* pol θ and other DNA polymerases have not been reported, although the profiles can be inferred from results with mammalian systems. In the absence of pol θ, the extract failed to exhibit any ddNTP sensitivity and there was no aphidicolin-resistance activity (Figure 6 and Table 2). Yet, on the other hand, the wild-type extract was (partially) sensitive to ddNTP and had some aphidicolin-resistant activity (Table 2 and Figure 4B). These results were consistent with a pol θ-like enzyme playing a role in the BER activity in the wild-type extract along with other polymerases, most likely a replicative enzyme(s). Pol γ has a similar profile of inhibitor sensitivity as that of pol θ, except pol γ, like pol β, is able to incorporate ddNTPs. Both the wild-type and pol θ null extract-mediated BER activities were unable to incorporate ddNTP (Figure 7). Thus, our results with polymerase inhibitors are consistent with the pol θ homolog and replicative DNA polymerases as contributing the extract-mediated BER gap-filling activity. These observations suggesting pol θ involvement in *C. elegans* BER are reminiscent of an earlier report on interstrand cross-link repair in the organism. Expression of a pol θ homolog in *C. elegans* was confirmed and a role in interstrand cross-link repair was revealed (21).

In summary, the results described here suggested that a mixture of DNA polymerases contributed to the BER gap-filling activity in the *C. elegans* extract. The observation of reduced BER activity in the extract from a pol θ deletion strain, clearly pointed to a pol θ homolog as contributing a portion of the BER gap-filling activity. Nevertheless, a significant amount of BER activity was observed with the pol θ null extract. It appeared that, the replicative polymerases also contribute to the BER activity, and the ddNTP and aphidicolin inhibition results (Table 2) are consistent with this interpretation. That a deficiency in BER gap-filling activity was observed in the absence of pol θ suggests the option of future biological experiments in pol θ-deficient strain of *C. elegans* to investigate the phenotypes of a BER deficiency. Finally, the purification and characterization of the DNA polymerases responsible for repair synthesis steps in *C. elegans* BER remains to be conducted.

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