© 2001 Oxford University Press Nucleic Acids Research, 2001, Vol. 29, No. 14 **3123–3130**

Accessibility of DNA polymerases to repair synthesis during nucleotide excision repair in yeast cell-free extracts

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Received December 8, 2000; Revised and Accepted May 23, 2001

ABSTRACT

Nucleotide excision repair (NER) removes a variety of DNA lesions. Using a yeast cell-free repair system, we have analyzed the repair synthesis step of NER. NER was proficient in yeast mutant cell-free extracts lacking DNA polymerases (Pol) β, ζ or η. Base excision repair was also proficient without Polβ. Repair synthesis of NER was not affected by thermal inactivation of the temperature-sensitive mutant Polα (*pol1-17*), but was reduced after thermal inactivation of the temperature-sensitive mutant Polδ (*pol3-1*) or Polε (*pol2-18*). Residual repair synthesis was observed in *pol3-1* and *pol2-18* mutant extracts, suggesting a repair deficiency rather than a complete repair defect. Deficient NER in *pol3-1* and *pol2-18* mutant extracts was specifically complemented by purified yeast Polδ and Polε, respectively. Deleting the polymerase catalytic domain of Polε (*pol2-16*) also led to a deficient repair synthesis during NER, which was complemented by purified yeast Polε, but not by purified yeast Polη. These results suggest that efficient repair synthesis of yeast NER requires both Polδ and Polε *in vitro*, and that the low fidelity Polη is not accessible to repair synthesis during NER.

INTRODUCTION

Nucleotide excision repair (NER) is an important mechanism for removing a wide spectrum of different base lesions in DNA. In humans, defects in NER can lead to the hereditary disease xeroderma pigmentosum (XP) (1). XP patients exhibit photosensitivity and a highly increased incidence of skin cancers (1). Therefore, NER plays a crucial defensive role against cytotoxicity, mutagenesis and carcinogenesis induced by a variety of DNA damaging agents.

Conceptually, NER can be divided into five biochemical steps: damage recognition, incision, excision, repair synthesis and DNA ligation. Depending on whether the repaired DNA strand is transcribed by RNA polymerase II or not, NER is further differentiated by two subpathways: global genome repair and transcription-coupled repair (2,3). In the yeast *Saccharomyces cerevisiae*, the first three steps of global genome repair require at least the following proteins and protein complexes: Rad7, Rad16, Rad14, replication protein A (RPA), Rad4, Rad23, TFIIH, Rad2, Rad1 and Rad10 (4–7). In the transcription-coupled repair in yeast, Rad7 and Rad16 are replaced by the presumptive coupling factor Rad26 (8,9). The last step of yeast NER requires DNA ligase I encoded by the *CDC9* gene (10). The presence of DNA ligase IV cannot substitute for the NER function of ligase I (10).

Based on an *in vitro* system reconstituted from purified mammalian NER proteins, the repair synthesis step of NER involves RPA, replication factor C and proliferating cell nuclear antigen (PCNA) (11,12). Either purified DNA polymerase (Pol) δ or Polε can fill the DNA gap in this reconstituted system (11,12). Using permeabilized human fibroblasts exposed to UV radiation, Nishida *et al*. (13) found that Polε is required for repair synthesis. In a separate study, Zeng *et al*. (14) reported that repair of UV-irradiated DNA in HeLa nuclear extract was inhibited by antibodies against human Polδ. In contrast, repair synthesis of NER in oocyte extracts of *Xenopus laevis* was reported to require both Polα and Polβ (15).

In yeast, the requirement for RPA and PCNA in NER has been demonstrated in a cell-free system (16). Yeast cells contain DNA Polα, β, γ, δ, ε, ζ and η. Most recently, the eighth *S.cerevisiae* DNA polymerase was identified as the *TRF4* gene product and was named Polκ (17), which is required for sister chromatid cohesion. It should be noted that this polymerase bears no relation to the recently identified human Polκ encoded by the *DINB1* gene (18,19). By analyzing molecular weight changes in cellular DNA after UV radiation, Budd and Campbell (20) concluded that Polδ and Polε are involved in repair of UV-induced damage in yeast. However, this study did not differentiate between NER and base excision repair (BER) (20). BER has been detected with UV-irradiated DNA in yeast (21), and a requirement for Polε in yeast BER has been reported (22). Therefore, further biochemical analysis would shed more light on the DNA polymerase requirement for yeast NER.

Most recently, it was found that DNA Polη is an extraordinarily low fidelity polymerase (23–25). Furthermore, the expression of yeast Polη is induced by UV radiation (26,27). These observations raised the question of whether Polη is accessible to repair synthesis during NER. Participation or interference of

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Polη in repair synthesis of NER would significantly affect the repair fidelity. Using a cell-free system, we have performed biochemical analyses of yeast NER with respect to the DNA polymerase requirement for and Polη accessibility to the repair synthesis step. In this report, we show that efficient repair synthesis in yeast cell-free extracts requires both Polδ and Polε, and present evidence suggesting that the low fidelity DNA Polη is not accessible to repair synthesis during yeast NER *in vitro*.

MATERIALS AND METHODS

Materials

Osmium tetroxide and *cis*-diamminedichloroplatinum (II) (cisplatin) were purchased from Sigma (St Louis, MO). *N*-acetoxy-*N*-2-acetylaminofluorene (AAAF) was obtained from the Midwest Research Institute (Kansas City, MO). The *S.cerevisiae* wild-type strains used were CL1265-7C (28), SX46A (29), TC102 (30), BY4741 (*MATa his3 leu2 met15 ura3*) and CWY231 (*MATa ade1 his2 leu2-3,112 trp1-1 bar1Δ ura3Δns*) (31). The *S.cerevisiae* mutant strains used were AMY32 (*rev3Δ*) (28), BY4741rad30Δ (*rad30Δ*) SK-2-1β (*pol4Δ*) (32), TAY237 (*pol2-16*) (31), and the temperature-sensitive mutants 488 (*pol1-17*) (22), YHA302 (*pol2-18*) (33) and ts370 (*cdc2-1/pol3-1*) (22).

Purified yeast DNA Polα containing associated primase activity was provided by David C. Hinkle (University of Rochester, Rochester, NY). Purified yeast Polδ and Polε were obtained from Akio Sugino (Osaka University, Osaka, Japan). One unit of DNA Polα incorporates 1 nmol of total nucleotide per hour at 30°C, using activated salmon sperm DNA as the substrate. One unit of DNA Polδ or Polε incorporates 1 nmol of total nucleotide per 30 min at 30°C, using poly(dA).oligo(dT) as the substrate (34). Yeast DNA Polη (Rad30 protein) was purified to near homogeneity as previously described (35).

Damaged DNA substrates

Single-stranded oligonucleotide U-mse1 (30mer) containing a uracil residue at position 13 and its complementary strand (30mer) were synthesized by Operon (Alameda, CA). The nucleotide sequence of the uracil-containing strand is 5′-GGATGGCATGCA**U**TAACCGGAGGCCGCGCG-3′. Equal molar amounts of the two oligonucleotides were mixed and annealed by incubating for 5 min at 85°C in TES (10 mM Tris–HCl pH 7.5, 1 mM EDTA and 100 mM NaCl) buffer followed by cooling slowly to room temperature.

To prepare cisplatin-damaged DNA, plasmid pUC18 (100 µg/ml) in TE (10 mM Tris–HCl pH 7.5 and 1 mM EDTA) buffer was incubated with *cis*-dichlorodiamineplatinum (II) at a drug/nucleotide ratio of 0.005 at 37°C for 20 h in the dark. After adding NaCl to 0.5 M, the DNA was purified by centrifugation in a linear 5–20% sucrose gradient as described by Wang *et al.* (21). Purified DNA was precipitated in ethanol, dissolved in TE buffer, and stored at –20°C. To prepare osmium tetroxide-damaged DNA, plasmid pUC18 (100 µg) was treated with the agent at 70°C for 90 min in TES buffer (300 µl). The DNA was then purified by centrifugation in a linear 5–20% sucrose gradient to remove nicked plasmids (21). To prepare DNA containing *N*-acetyl-2-aminofluorene (AAF) adducts, pUC18 (100 µg) was incubated at 37°C for 3 h in 1 ml of TE buffer containing 3 µM AAAF (the activated form of AAF) and 20% ethanol. The DNA was then purified by centrifugation in a linear 5–20% sucrose gradient (21).

In vitro DNA repair

Yeast cell-free extracts were prepared according to our previously reported methods (36,37). The same extracts were used for both *in vitro* NER and BER. However, NER and BER were carried out under different reaction conditions with different DNA lesions. *In vitro* NER and BER were performed as described by Wang *et al.* (21,36,37) and are described briefly below.

A standard NER reaction mixture (50 µl) contained 200 ng each of damaged pUC18 DNA and undamaged pGEM3Zf DNA, 45 mM HEPES–KOH pH 7.8, 7.4 mM $MgCl_2$, 0.9 mM dithiothreitol, 0.4 mM EDTA, 2 mM ATP, 20 µM each dATP, dGTP and dTTP, 4 µM dCTP, 1 µCi of [α-^{32}P]dCTP (3000 Ci/mmol), 40 mM phosphocreatine (disodium salt), 2.5 µg of creatine phosphokinase, 4% glycerol, 100 µg/ml bovine serum albumin, 5% polyethylene glycol 8000 and 250–300 µg of yeast cell-free extracts. After incubation at 26°C for 2 h, EDTA and RNase A were added to 20 mM and 20 µg/ml, respectively, and incubated at 37°C for 10 min. SDS and proteinase K were added to 0.5% and 200 µg/ml, respectively, and incubated at 37°C for 30 min. Plasmid DNA was purified by phenol/chloroform extraction, and linearized with *Hin*dIII restriction endonuclease. DNA was separated by electrophoresis on a 1% agarose gel and repair synthesis was visualized by autoradiography.

A standard BER reaction mixture (50 µl) contained 200 ng each of osmium tetroxide-damaged pUC18 and undamaged pGEM3Zf DNA, 45 mM HEPES–KOH pH 7.8, 7.4 mM $MgCl_2$, 0.9 mM dithiothreitol, 0.4 mM EDTA, 2 mM ATP, 20 µM each dATP, dGTP and dCTP, 4 µM dTTP, 1 µCi of [α-^{32}P]dTTP (3000 Ci/mmol), 40 mM phosphocreatine, 2.5 µg of creatine phosphokinase, 4% glycerol, 100 µg/ml bovine serum albumin and 50 µg of yeast cell-free extracts. After incubation at 30°C for 2 h, the DNA was purified by phenol/chloroform extraction, separated by electrophoresis, and visualized by autoradiography of the gel as described above for NER assays. For BER of the uracil-containing substrate U-mse1, 2 pmol of the 30mer duplex DNA was used in place of the damaged pUC18 DNA in the standard BER assay described above, and incubated at 23°C for 2 h. Reactions were stopped by phenol/chloroform extraction and the DNA was recovered by precipitation in ethanol. Repair products were separated by electrophoresis on a 20% denaturing polyacrylamide gel. Repair synthesis was visualized by autoradiography of the wet gel.

RESULTS

NER in yeast cell-free extracts lacking Polζ or Polη

Yeast Polζ and Polη are two lesion bypass DNA polymerases encoded by the non-essential genes *REV3* and *RAD30*, respectively (26–28,35,38,39). To examine whether these two DNA polymerases affect repair synthesis of yeast NER, we performed *in vitro* NER in *rev3* and *rad30* deletion mutant extracts, using plasmid DNA containing cisplatin or AAF

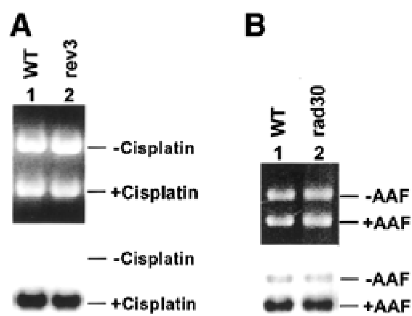

Figure 1. NER in Polζ and Polη mutant extracts. (**A**) *In vitro* NER of cisplatin-damaged DNA was performed in yeast cell-free extracts of the wild-type (WT) strain CL1265-7C (lane 1) and its isogenic Polζ (*rev3*) deletion mutant strain AMY32 (lane 2). (**B**) *In vitro* NER of AAF-adducted DNA was performed in yeast cell-free extracts of the wild-type (WT) strain BY4741 (lane 1) and its isogenic Polη (*rad30*) deletion mutant strain BY4741rad30Δ (lane 2). Top, ethidium bromide-stained gel; bottom, autoradiograph of the gel. +Cisplatin and +AAF, damaged pUC18 DNA; –Cisplatin and –AAF, undamaged pGEM3Zf DNA as the internal control.

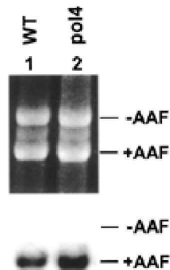

Figure 2. NER in Polβ mutant extracts. *In vitro* NER of AAF-adducted DNA was performed in yeast cell-free extracts of the wild-type (WT) strain TC102 (lane 1) and the Polβ (*pol4*) deletion mutant strain SK-2-1β (lane 2). Top, ethidium bromide-stained gel; bottom, autoradiograph of the gel. +AAF, damaged pUC18 DNA; –AAF, undamaged pGEM3Zf DNA as the internal control.

adducts. We have shown previously that under the conditions used cisplatin and AAF DNA adducts are repaired specifically by the NER pathway in yeast cell-free extracts (6,36,37,40,41). NER was monitored by radiolabeling the repair patch during DNA repair synthesis (36,37). As shown in Figure 1A, repair synthesis of NER in cisplatin-damaged DNA was not affected by deleting the *REV3* gene. Repair synthesis of NER in AAF-adducted DNA was also not affected by deleting the *RAD30* gene (Fig. 1B). These results indicate that yeast Polζ and Polη are not required for NER *in vitro*.

Yeast Polβ is not required for NER or BER *in vitro*

DNA Polβ is an important repair polymerase for BER in mammalian cells (42). Yeast Polβ encoded by the *POL4* gene is not essential for growth (32,43,44). Thus, *pol4* deletion mutants have been isolated (32,43,44). To examine whether Polβ plays an important role in yeast NER or BER, we performed repair in *pol4* deletion mutant extracts. *In vitro* NER assays were carried out using AAF-damaged plasmid DNA as the repair substrate. As shown in Figure 2, deleting the *POL4* gene did not affect repair synthesis of NER in yeast extracts.

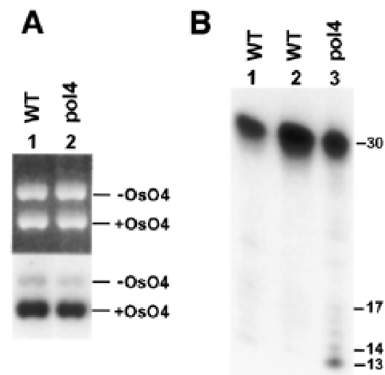

Figure 3. BER in Polβ mutant extracts. (**A**) *In vitro* BER of OsO_4-damaged DNA was performed in yeast cell-free extracts of the wild-type (WT) strain TC102 (lane 1) and the Polβ deletion mutant (*pol4*) strain SK-2-1β (lane 2). +OsO_4, damaged pUC18 DNA; –OsO_4, undamaged pGEM3Zf DNA as the internal control. Top, ethidium bromide-stained gel; bottom, autoradiograph of the gel. (**B**) *In vitro* BER of the uracil-containing 30mer duplex DNA was performed in yeast cell-free extracts of the wild-type (WT) strains SX46A (lane 1) and TC102 (lane 2), or in yeast cell-free extracts of the Polβ deletion mutant (*pol4*) strain SK-2-1β (lane 3). Repair products were separated by electrophoresis on a 20% denaturing polyacrylamide gel and visualized by autoradiography. DNA size markers in nucleotides are indicated on the right.

These results suggest that Polβ is not required for NER in yeast.

Depending on whether the initiating DNA glycosylase is with or without an associated AP lyase, two modes of BER are known. For example, repair of oxidative base damage in DNA is initiated by a glycosylase with associated AP lyase, while repair of uracil residues in DNA is initiated by a glycosylase without associated AP lyase. To examine both modes of BER in yeast *pol4* deletion mutant extracts we used osmium tetroxide-damaged plasmid DNA that contained thymine glycol as the major damage and uracil-containing short duplex DNA as the repair substrates. Under the conditions used, BER was specifically measured without interference by NER (45). As shown in Figure 3A, BER of osmium tetroxide-damaged DNA was not affected by deleting the *POL4* gene. BER of uracil residues in DNA in yeast *pol4* mutant extracts was then compared with that in two different wild-type yeast extracts. As shown in Figure 3B, uracil repair was not significantly affected without yeast Polβ. These results suggest that yeast Polβ is not required for BER *in vitro*.

Deficient NER in *pol2-18* and *pol3-1* mutant extracts

To identify the DNA polymerase(s) required for yeast NER, we examined the repair synthesis step in *pol1-17*, *pol2-18* and *pol3-1* mutant extracts. The *pol1-17*, *pol2-18* and *pol3-1* mutant cells are temperature-sensitive for growth. Previously, Boulet *et al.* (46) showed that the Polα activity in *pol1-17* cells and the Polδ activity in *pol3-1* cells were reduced to undetectable levels after shifting the growth temperature from 24 to 36°C for 2 h. Araki *et al.* (33) showed that partially purified mutant Polε (*pol2-18*) was very sensitive to temperature shift with a half-life of <1 min at 45°C. Therefore, the *pol1-17*, *pol2-18* and *pol3-1* mutant cells were grown at 23°C to late

logarithmic phase and then at 37°C for 2 h before preparation of cell-free extracts. We expected that this growth condition should lead to inactivation of the mutant DNA polymerases in the corresponding mutant cells. To validate this expectation, we determined the effect of the temperature shift on the growth of these mutant cells. One hour after shifting to 37°C, all three mutants stopped growing. From 1 to 2 h at 37°C, viable cells of *pol1-17*, *pol2-18* and *pol3-1* were slightly reduced from 8.4 × 10^7 to 8.2 × 10^7, 2.2 × 10^7 to 1.7 × 10^7, and 2.3 × 10^7 to 1.9 × 10^7 per ml, respectively. In contrast, from 1 to 2 h at 37°C, wild-type cells continued to grow from 3.2 × 10^7 to 4.2 × 10^7 per ml. These results suggest that the temperature-sensitive Polα, δ and ε were inactivated in the mutant cells after incubation for 2 h at 37°C.

NER of AAF adducts in plasmid DNA was performed in the mutant cell extracts at 23°C. As shown in Figure 4 (lanes 1 and 2), repair synthesis was readily detected in wild-type or *pol1-17* mutant extracts. In contrast, repair synthesis activities in *pol2-18* (Fig. 4, lane 3) and *pol3-1* (Fig. 4, lane 4) mutant extracts were significantly reduced. However, residual repair synthesis was observed in both *pol2-18* and *pol3-1* mutant extracts when compared with the undamaged internal control (Figs 4, lanes 3 and 4, 5A, lanes 2 and 5, and 5B, lanes 2 and 7). To show direct participation of DNA Polδ and ε in yeast NER, we complemented *pol2-18* and *pol3-1* mutant extracts with the purified yeast polymerases. Deficient repair synthesis in *pol2-18* mutant extracts was partially complemented by 0.05 U of purified yeast Polε (Fig. 5A, lane 3). In contrast, purified yeast Polα (80 U) had no effect (Fig. 5A, lane 4). Similarly, deficient repair synthesis in *pol3-1* mutant extracts was partially complemented by 0.25 U of purified yeast Polδ (Fig. 5A, lane 6), but not by purified yeast Polα (80 U) (Fig. 5A, lane 7). Furthermore, the catalytic subunit of Polε alone was also able to partially complement *pol2-18* mutant extracts (Fig. 5B, lanes 3 and 4), but not *pol3-1* mutant extracts (Fig. 5B, lanes 5 and 6). The catalytic subunit of Polδ alone was able to partially complement *pol3-1* mutant extracts (Fig. 5B, lanes 10 and 11), but not *pol2-18* mutant extracts (Fig. 5B, lanes 8 and 9). These results suggest that both Polδ and Polε are required for yeast NER *in vitro*.

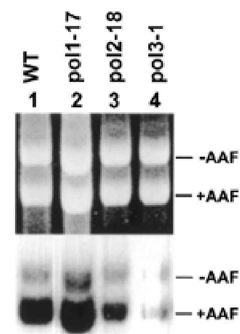

Figure 4. NER in temperature-sensitive mutant extracts of Polα, Polδ and Polε. The mutant yeast cells were grown at the permissive temperature (23°C) to late logarithmic phase of growth. The cultures were then shifted to the restrictive temperature (37°C) for 2 h. Cell-free extracts were prepared for *in vitro* NER. The wild-type extracts were prepared from cells grown at 37°C. *In vitro* NER assays were performed at 23°C for 2 h. WT, the wild-type SX46A; pol1-17, the Polα temperature-sensitive mutant 488; pol2-18, the Polε temperature-sensitive mutant YHA302; and pol3-1, the Polδ temperature-sensitive mutant ts370. +AAF, damaged pUC18 DNA; –AAF, undamaged pGEM3Zf DNA as the internal control. Top, ethidium bromide-stained gel; bottom, autoradiograph of the gel.

Participation of both Polδ and Polε in yeast NER raised the question of whether complete filling of each DNA gap requires both polymerases. To address this question, we performed NER reaction in *pol2-18* and *pol3-1* mutant extracts. After repair, the plasmid DNA was purified and loaded directly onto a 1% agarose gel without prior digestion with the *Hin*dIII restriction endonuclease. This modification of the standard *in vitro* NER assay allowed examination of the ligation step of the repair (10). If the DNA gap was not completely filled, subsequent DNA ligation would not occur. As expected for NER in the wild-type extracts, the vast majority of the repair

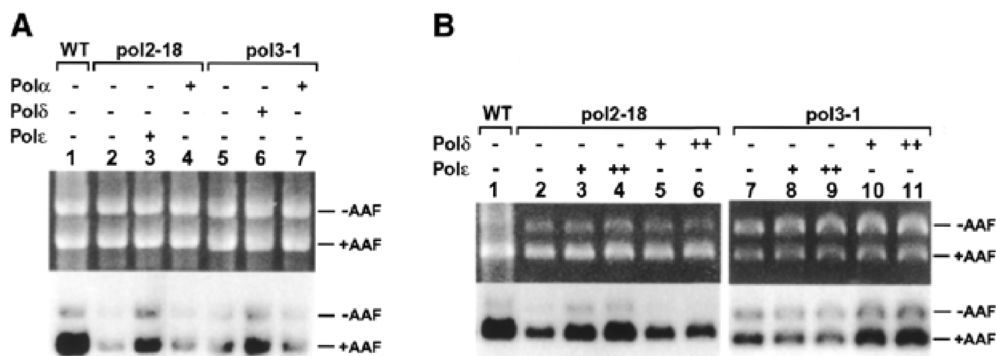

Figure 5. Complementation of deficient NER in *pol2-18* and *pol3-1* mutant cell extracts. (**A**) Deficient repair synthesis of NER in the *pol2-18* and *pol3-1* mutant extracts was complemented by adding purified yeast DNA Polα, δ or ε to the repair reactions as indicated. The amounts of DNA polymerases added were: Polα, 80 U; Polδ, 0.25 U; and Polε, 0.05 U. (**B**) Deficient repair synthesis of NER in the *pol2-18* and *pol3-1* mutant extracts was complemented by adding the purified catalytic subunit of yeast Polδ or Polε as indicated. The His_6-tagged catalytic subunits of Polδ (125 kDa) and Polε (256 kDa) were overexpressed in yeast cells and purified to near homogeneity. Both proteins were active in the polymerase activity. The amounts of proteins added were: Polδ catalytic subunit, 12 ng (+) and 20 ng (++); Polε catalytic subunit, 30 ng (+) and 50 ng (++). WT, the wild-type SX46A. +AAF, damaged pUC18 DNA; –AAF, undamaged pGEM3Zf DNA as the internal control. Top, ethidium bromide-stained gel; bottom, autoradiograph of the gel.

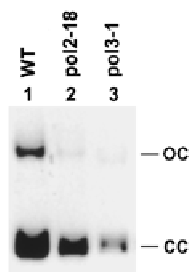

Figure 6. DNA ligation during residual NER in Polδ and Polε mutant extracts. *In vitro* NER reactions were performed at 26°C for 2 h using AAF-damaged pUC18 plasmid DNA. The DNA was then purified and loaded directly onto a 1% agarose gel without prior digestion with *Hin*dIII restriction endonuclease. Electrophoresis was performed in the presence of 0.5 µg/ml ethidium bromide to separate the ligated form (CC, closed circle) of plasmid pUC18 from the unligated form (OC, opened circle). Autoradiograph of the gel is shown. WT, wild-type SX46A.

patches were in the ligated form of the plasmid DNA (Fig. 6, lane 1). Similarly, the repair patches synthesized by the residual repair activities in *pol2-18* and *pol3-1* mutant extracts were mostly contained in the ligated form of the plasmid (Fig. 6, lanes 2 and 3). These results indicate that some DNA gaps were completely filled during residual NER in the mutant extracts. Therefore, we conclude that complete synthesis of a DNA gap during yeast NER can be performed by either Polδ or Polε, although repair synthesis is more efficient when both polymerases are present.

The catalytic domain of yeast Polε is required for NER *in vitro*

Recently, Kesti *et al.* (31) showed that the catalytic domain of Polε is dispensable for viability of yeast cells. To determine whether the catalytic domain of Polε is also dispensable for NER, we performed repair assays in *pol2-16* mutant extracts. The sequence encoding amino acids 176–1134 of yeast Polε that contains the polymerase and the 3′→5′ exonuclease functions has been deleted in *pol2-16* mutant cells (31). As shown in Figure 7 (compare lanes 1 and 2), repair synthesis in *pol2-16* mutant extracts was deficient. Again, residual repair synthesis activity in the mutant extracts was observed (Fig. 7, lane 2). Furthermore, the repair patches synthesized by the residual repair activity in *pol2-16* mutant extracts were mostly contained in the ligated form of the plasmid DNA (Fig. 7, lane 2). The deficient repair synthesis in *pol2-16* mutant extracts was complemented by 0.08 U of purified yeast Polε (Fig. 7, lane 4). In contrast, 0.4 U of purified yeast Polδ was unable to complement the deficient repair synthesis in *pol2-16* mutant extracts (Fig. 7, lane 3). These results show that the catalytic domain of yeast Polε is required for NER *in vitro*.

The low fidelity Polη is not accessible to repair synthesis of NER in yeast extracts

Polη synthesizes DNA with an extraordinarily low fidelity (23,25,47). In yeast, Polη expression is induced by UV radiation (26,27). Thus, it is of particular interest to determine whether Polη is accessible to the DNA gap for repair synthesis during NER, especially when the amount of Polη is increased upon DNA damage. To do this, we performed repair synthesis assays in yeast *pol2-16* mutant extracts. As presented above,

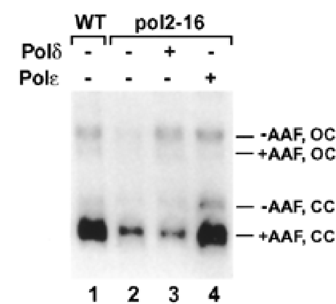

Figure 7. Deficient repair synthesis of NER in *pol2-16* mutant extracts. *In vitro* NER was performed in yeast cell-free extracts of the wild-type strain CWY231 (lane 1) and its isogenic *pol2-16* mutant strain TAY 237 (lane 2). Separately, purified yeast Polδ (0.4 U) or Polε (0.08 U) was added to the *in vitro* NER reaction for complementation, as indicated in lanes 3 and 4, respectively. After reaction, the DNA was purified and loaded directly onto a 1% agarose gel without prior digestion with *Hin*dIII restriction endonuclease. Electrophoresis was performed in the presence of 0.5 µg/ml ethidium bromide to separate the ligated forms (CC, closed circle) of the damaged pUC18 (+AAF) and the undamaged pGEM3Zf (–AAF) from the unligated forms (OC, opened circle) of the plasmids. Autoradiograph of the gel is shown.

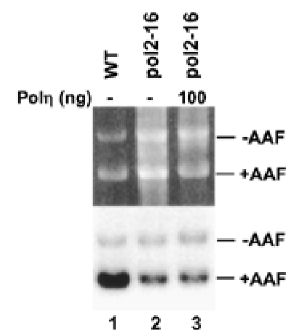

Figure 8. Effect of purified yeast Polη on NER in *pol2-16* mutant extracts. Standard *in vitro* NER assays were performed in the *pol2-16* mutant extracts without (lane 2) or with (lane 3) 100 ng of purified yeast Polη. Lane 1, *in vitro* NER in yeast cell-free extract of the isogenic wild-type strain CWY231. +AAF, damaged pUC18 DNA; –AAF, undamaged pGEM3Zf DNA as the internal control. Top, ethidium bromide-stained gel; bottom, autoradiograph of the gel.

deficient repair synthesis in this mutant extract could be complemented by purified yeast Polε (Fig. 7), suggesting that some DNA gaps were accumulated during NER in *pol2-16* extracts. When we added excess amounts of purified yeast Polη (14-fold molar excess over DNA) during NER in *pol2-16* mutant extracts, deficient repair synthesis could not be complemented (Fig. 8). These results suggest that Polη is not accessible to repair synthesis of NER in yeast cell-free extracts even when the polymerase is present in excess amount.

DISCUSSION

Using biochemical analyses of *in vitro* repair in cell-free extracts, we show that the repair synthesis step of the yeast NER pathway requires both Polδ and Polε. Loss of either Polδ or Polε activity reduced repair synthesis during NER *in vitro*. Nevertheless, residual repair synthesis was present, probably due to functional Polε in the Polδ mutant extract or functional

Polδ in the Polε mutant extract. It is unlikely that the residual repair synthesis activity was derived from partial inactivation of Polδ or Polε in the mutant extracts, because such residual activity was also observed even when the catalytic domain of Polε was deleted. Therefore, efficient NER in yeast cell-free extracts requires the participation of both Polδ and Polε at the repair synthesis step. It is not clear mechanistically how the presence of both polymerases would mediate efficient repair synthesis of NER. One possibility is that both polymerases may be parts of a large protein complex for repair synthesis during NER. Loss of one polymerase may destabilize and/or reduce the activity of the repair synthesis complex. Alternatively, each repair synthesis complex may contain only one of the two polymerases. The presence of mutant Polδ or Polε may form an inactive complex that competes for the functional complex at the repair synthesis step.

Our results indicate that during NER repair synthesis can occur without Polδ or Polε, although less efficiently. These results are consistent with earlier reports that either Polδ or Polε alone can carry out repair synthesis in an *in vitro* system reconstituted from purified human NER proteins (11,12). Overlapping or partially overlapping functions of Polδ and Polε in repair synthesis of NER provide an explanation that the *pol3-1* (*cdc2-1*), *pol2-18* and *pol2-16* mutant cells are not significantly sensitive to UV radiation (20,31) (X.Wu and Z.Wang, unpublished results). With significant repair synthesis activity still present in the mutant cells, it is expected that UV damage can be repaired by NER, even if it may take somewhat longer to do so. It is noted that some *pol3* mutant alleles lead to cellular sensitivity to a DNA damaging agent, such as methyl methanesulfonate (MMS) (48). Since post-replication repair requires Polδ (49), it is likely that the sensitivity is a consequence of defective post-replication repair rather than defective NER or BER.

Mammalian Polβ plays a major role in BER (42). However, our *in vitro* results indicate that yeast Polβ is not required for BER, regardless of whether the repair is initiated by a glycosylase with or without associated AP lyase. Polβ is also not required for NER. Consistent with this conclusion, yeast cells without Polβ are not sensitive to UV radiation or MMS (32,44). In fact, yeast Polβ is expressed at a very low level during vegetative growth (32). Its expression, however, is stimulated during meiosis (32). Recently, Wilson and Lieber (50) showed that yeast Polβ is involved in non-homologous DNA end joining, a special mode of DNA recombination. Therefore, the BER function of Polβ appears to be acquired later during the evolution of higher eukaryotes. The repair synthesis step of BER in yeast is catalyzed by Polε (22). During yeast BER of uracil-containing DNA, the 5′ deoxyribose phosphate moiety resulted from Apn1 incision is removed by the nuclease activities of Rad27 (51). The resulting gapped DNA can then be filled in by Polε and the nick is ligated by DNA ligase I (Cdc9 protein) (10). In higher eukaryotes, the Rad27 homolog is known as FEN1 and DNase IV (52,53), and Polδ–Fen1 combination for BER is also reported (54,55). The Polε–Fen1 combination for BER may also occur in higher eukaryotes (42,56). Unlike yeast, however, this repair mechanism is thought to play only a minor role in human BER (42,54,56).

Recently, it was demonstrated that the yeast *RAD30* gene codes for DNA Polη (26,27,39,57). Originally, Polη was identified as an error-free lesion bypass polymerase opposite TT dimers (39,58). Later, *in vitro* bypass of other DNA lesions by Polη was also demonstrated (35,59,60). Surprisingly, Polη copies DNA from undamaged templates with an extraordinarily low fidelity (23,25). For cells to maintain genomic stability, mechanisms must exist to exclude Polη from DNA replication. One mechanism is to regulate its expression. Indeed, transcription of yeast Polη is UV-inducible (26,27). In this study, we asked whether Polη is also excluded from NER in yeast. We found that excess amounts of purified yeast Polη were unable to complement deficient repair synthesis in *pol2-16* mutant extracts. This result suggests that Polη cannot participate in repair synthesis during NER. Even purified Polδ could not complement deficient repair synthesis in *pol2-18* and *pol2-16* mutant extracts, and purified Polε could not complement deficient repair synthesis in *pol3-1* mutant extracts. Thus, it appears that following damage excision the resulting DNA gap is not exposed. Participation of Polδ and Polε in repair synthesis of NER is likely to involve a recruitment mechanism, which may be coordinated with an earlier reaction in the NER pathway. This process is probably carried out through protein–protein interactions. Possible interactions include Rad2–PCNA, PCNA–Polδ and PCNA–DNA ligase I, which have been reported (29,30,61). Thus, NER is most likely operated by protein complexes from damage recognition to DNA ligation in a coordinated manner.

ACKNOWLEDGEMENTS

We thank Akio Sugino for providing yeast strains YHA302 and 488 and purified yeast DNA Polδ and Polε, David Hinkle for purified yeast DNA Polα, Christopher Lawrence for yeast strains CL1265-7C and AMY32, Judith Campbell for yeast strains TC102 and SK-2-1β, and Curt Wittenberg for yeast strains CWY231 and TAY237. These studies were supported by a research grant CA67978 from NIH. D.G. is a recipient of the Kentucky Opportunity Fellowship.

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