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DNA Repair Synthesis during Base Excision Repair In Vitro Is Catalyzed by DNA Polymerase ε and Is Influenced by DNA Polymerases α and δ in *Saccharomyces cerevisiae*

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Base excision repair is an important mechanism for correcting DNA damage produced by many physical and chemical agents. We have examined the effects of the *REV3* gene and the DNA polymerase genes *POL1*, *POL2*, and *POL3* of *Saccharomyces cerevisiae* on DNA repair synthesis in nuclear extracts. Deletional inactivation of *REV3* did not affect repair synthesis in the base excision repair pathway. Repair synthesis in nuclear extracts of *pol1*, *pol2*, and *pol3* temperature-sensitive mutants was normal at permissive temperatures. However, repair synthesis in *pol2* nuclear extracts was defective at the restrictive temperature of 37°C and could be complemented by the addition of purified yeast DNA polymerase ε. Repair synthesis in *pol1* nuclear extracts was proficient at the restrictive temperature unless DNA polymerase α was inactivated prior to the initiation of DNA repair. Thermal inactivation of DNA polymerase δ in *pol3* nuclear extracts enhanced DNA repair synthesis ~2-fold, an effect which could be specifically reversed by the addition of purified yeast DNA polymerase δ to the extract. These results demonstrate that DNA repair synthesis in the yeast base excision repair pathway is catalyzed by DNA polymerase ε but is apparently modulated by the presence of DNA polymerases α and δ.

Both prokaryotic and eukaryotic cells effect the repair of damaged or inappropriate bases in DNA by enzyme-catalyzed excision processes. These processes are designated base excision repair or nucleotide excision repair, depending on whether the damaged bases are excised as free entities (base excision repair) or as components of oligonucleotide fragments (nucleotide excision repair) (10). Both processes include nonsemiconservative repair synthesis of DNA during which gaps generated by the excision events are filled. These gaps vary in size, depending on the particular excision repair mode in operation (8, 12, 15).

In the yeast *Saccharomyces cerevisiae*, multiple genes for DNA polymerases have been identified, and there is considerable information on the biochemistry of the proteins encoded by a number of these genes. Three DNA polymerases that are known to be required for DNA replication in *S. cerevisiae* are encoded by the *POL1* (*CDC17*), *POL2*, and *POL3* (*CDC2*) genes (3, 17, 20, 24). Consistent with their requirement for DNA replication, all three genes are essential (7, 17, 20, 24), and temperature-sensitive mutants have been isolated for each (1, 14). These three genes encode the catalytic subunits of DNA polymerases I, II, and III, respectively, which are the mammalian homologs of DNA polymerases α, ε, and δ, respectively (3, 17, 20). In the interests of uniformity, the Greek letter nomenclature has recently been adopted for the yeast enzymes (6).

Yeast DNA polymerase α has an associated primase activity (4) and is believed to be responsible for initiating DNA replication on both strands (20). Purified yeast DNA polymerases δ and ε are multiprotein complexes consisting of two and five subunits, respectively (2, 11). They are believed to be involved in DNA synthesis by extending primers formed by DNA polymerase α-primase complexes (5, 20). There is a requirement for additional protein cofactors during DNA replication in yeast cells. These include proliferating cell nuclear antigen, single-strand binding protein, and replication factor C (2, 5, 9, 28).

In addition to the three enzymes mentioned above, there are indications of a fourth DNA polymerase in *S. cerevisiae*. Examination of the translated nucleotide sequence of the *REV3* gene reveals homology with yeast and Epstein-Barr virus DNA polymerases (19). This gene is not essential, but it is required for DNA damage-induced mutagenesis in *S. cerevisiae* (18, 19). Hence, it has been suggested that Rev3 protein may be a DNA polymerase that participates in an error-prone cellular response(s) to DNA damage (19).

There is considerable uncertainty as to which DNA polymerase(s) is involved in repair synthesis during excision repair of DNA and whether the same polymerase(s) is utilized during the base and nucleotide excision repair modes. In mammalian cells, the lack of appropriate mutants has prompted the extensive use of polymerase inhibitors to investigate these questions (13, 22, 23, 25). Such studies have suffered from the limited specificity of the inhibitors used and the implicit assumption that their effects on purified DNA polymerases in vitro accurately mimic those in living cells. Additionally, experimental conditions have varied considerably, and little attention has been paid to which particular excision repair mode was being measured.

The availability of mutants for all four known DNA polymerase genes suggests that *S. cerevisiae* might be particularly suited for studies on the role of the individual enzymes during excision repair in eukaryotic cells. Here we report the results of an experimental approach that utilized a recently developed in vitro assay for specifically measuring repair synthesis during base excision repair of DNA (27). We show that in yeast nuclear extracts, repair synthesis during base excision repair is catalyzed exclusively by DNA polymerase ε. In addition, our experiments suggest that DNA

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TABLE 1. Yeast strains used

Strain	Genotype	Source
BJ2168	*MATa RAD leu2 trp1 ura3-52 pep4-3 prb1-1122 prc1-407*	Yeast Genetic Stock Center
CL1265-7C	*MATα arg4-17 leu2-3,112 his3-Δ1 trp1 ura3-52*	C. W. Lawrence, Rochester, N.Y.
AMY32	*MATα rev3⁰ arg4-17 leu2-3,112 his3/4 trp1 ura3-52*	C. W. Lawrence, Rochester, N.Y.
488	*MATα trp1 leu2 ura3-52 his1-7 can1 pol1-17*	A. Sugino, NIEHS[a]
YHA302	*MATa ade5-1 leu2-3,112 ura3-52 trp1-289 pol2-3::LEU2* [YCp*pol2-18*]	A. Sugino, NIEHS
ts370	*MATa cdc2-1 ade1 ade2 his7 gal1*	Yeast Genetic Stock Center

[a] NIEHS, National Institute of Environmental Health Sciences.

polymerases α and δ indirectly influence repair synthesis during base excision repair. The *REV3* gene product has no effect on repair synthesis.

MATERIALS AND METHODS

Materials. Plasmid pGEM-3Zf(+) was purchased from Promega. Osmium tetroxide, protease inhibitors, phosphocreatine (disodium salt), creatine phosphokinase, ATP, and deoxyribonucleoside triphosphates were purchased from Sigma Chemical Co. Ultrapure sucrose and acetylated bovine serum albumin were from Bethesda Research Laboratories. Zymolyase 100-T was purchased from ICN Biomedicals, Inc.

Strains. *Escherichia coli* DH5α was used to propagate plasmids and was purchased from Bethesda Research Laboratories. *E. coli* CJ236 was obtained from Bio-Rad. The yeast strains used are listed in Table 1.

Enzymes. Yeast DNA polymerase α with associated primase activity was a gift from David C. Hinkle, Department of Biology, University of Rochester. Yeast DNA polymerase δ was generously provided by Akio Sugino, National Institute of Environmental Health Sciences, and Peter M. J. Burgers, Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine. Yeast DNA polymerase ε was generously provided by Akio Sugino. One unit of DNA polymerase α incorporates 1 nmol of total nucleotide per h at 30°C, using activated salmon sperm DNA as the substrate. One unit of DNA polymerase δ or ε incorporates 1 nmol of total nucleotide per 30 min at 30°C, using poly(dA) · oligo(dT) as the substrate (11).

Preparation of yeast nuclear extracts. Yeast nuclear extracts from strains BJ2168, CL1265-7C, and AMY32 were prepared as previously described (27). Nuclear extracts from DNA polymerase temperature-sensitive mutants were similarly prepared with minor modifications. Cells grown at 23°C in YPD medium (1% yeast extract, 2% Bacto Peptone, 2% glucose) to late logarithmic phase were harvested by centrifugation, washed once in water, and resuspended to 0.1 g/ml in 0.1 M EDTA-KOH (pH 8.0)–10 mM dithiothreitol. Cell suspensions were incubated at 23°C for 10 min with shaking (50 rpm) and centrifuged for 10 min at 6,000 rpm in a JA-14 rotor (Beckman) at 4°C. Cell pellets were resuspended to 1 g/ml in YPD medium containing 1 M sorbitol, and zymolyase 100-T was added to 1 mg/g of cells. Following incubation at 23°C for ~1.5 h with shaking (50 rpm), ice-cold YPD medium containing 1 M sorbitol was added (10 ml/g of cells). Cells were harvested by centrifugation as before, washed twice in the same medium, resuspended to 0.1 g/ml in YPD-sorbitol medium, and incubated at 23°C for 30 min with shaking.

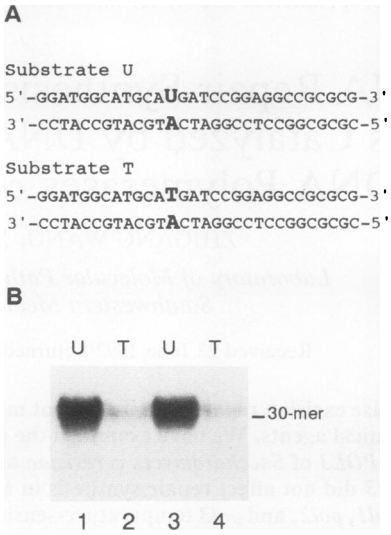

FIG. 1. (A) Sequences of 30-mer duplex oligonucleotide substrates U and T containing a dUMP or TMP residue, respectively, at nucleotide 13. (B) DNA repair synthesis in uracil-containing duplex oligonucleotides. Repair synthesis was performed with 2 pmol (40 ng) of substrate U (U) or substrate T (T) in 50 μg of nuclear extracts from yeast *pol2* (lanes 1 and 2) or *pol3* (lanes 3 and 4) cells in the presence of [^{32}P]TTP as described in Materials and Methods. After incubation at 23°C for 2 h, 120 ng of oligonucleotide was added, and the DNA was purified by phenol-chloroform extractions. Duplex oligonucleotides were recovered by ethanol precipitation, dissolved in water, and electrophoresed on a 15% nondenaturing polyacrylamide gel. The gel was then dried and autoradiographed.

After the addition of phenylmethylsulfonyl fluoride (0.5 mM, final concentration), cells were pelleted by centrifugation, washed once in 1 M sorbitol, and resuspended to 0.25 g/ml in 5 mM Tris-HCl (pH 7.4)–20 mM KCl–2 mM EDTA-KOH (pH 7.4)–0.125 mM spermidine–0.05 mM spermine–18% Ficoll–1% thiodiglycol–protease inhibitors (pepstatin, leupeptin, chymostatin, and antipain at 1 μg/ml each, benzamidine at 300 μg/ml, and 1 mM phenylmethylsulfonyl fluoride). Spheroplasts were lysed in a motor-driven homogenizer with 10 strokes. Cell debris and unlysed spheroplasts were removed by centrifugation twice at 7,500 rpm in a JA-20 rotor (Beckman) for 10 min at 4°C and then subjected to repeated 5-min centrifugation at 6,000 rpm to obtain a clear supernatant. Nuclei were pelleted by centrifugation at 13,000 rpm for 30 min at 4°C. Extracts were then prepared from the nuclei as previously described (27).

Preparation of damaged DNA for repair synthesis. Single-stranded oligonucleotides for substrates containing thymine or uracil (Fig. 1A) were synthesized in a DNA synthesizer (Integrated DNA Technologies, Inc.). To anneal complementary strands, equimolar amounts of both oligonucleotides were incubated for 5 min at 85°C in 10 mM Tris-HCl (pH 7.5)–1 mM EDTA–100 mM NaCl (STE buffer) and allowed to cool slowly to 23°C. To prepare uracil-containing plasmid pUC18 DNA, the plasmid was propagated in *E. coli* CJ236 (*ung dut*). Plasmid DNA was then isolated and purified by alkaline lysis and CsCl-ethidium bromide equilibrium centrifugation. UV-irradiated plasmid pUC18 DNA was obtained by exposure to UV radiation of the DNA (50 μg/ml)

on ice in a thin layer under a germicidal lamp at a dose of 450 J/m^2. To prepare osmium tetroxide-damaged DNA, plasmid pUC18 (100 μg) was treated with the agent (300 μg/ml) at 70°C for 90 min in STE buffer (300 μl). The preparation was then purified by centrifugation in a linear 5 to 20% sucrose gradient to remove nicked plasmid DNA as previously described (27).

DNA repair synthesis. Standard DNA repair synthesis was assayed in yeast nuclear extracts essentially as described by Wang et al. (27). Standard reaction mixtures (50 μl) contained 300 ng each of damaged plasmid pUC18 and undamaged plasmid pGEM-3Zf(+) DNA, 45 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-KOH (pH 7.8), 7.4 mM MgCl, 0.9 mM dithiothreitol, 0.4 mM EDTA, 2 mM ATP, 20 μM each dATP, dGTP, and TTP, 8 μM dCTP, 1 μCi of [α-^{32}P]dCTP (3,000 Ci/mmol), 40 mM phosphocreatine (disodium salt), 2.5 μg of creatine phosphokinase, 4% glycerol, 100 μg of bovine serum albumin per ml, and yeast nuclear extract. When [α-^{32}P]TTP (3,000 Ci/mmol) was used instead of [α-^{32}P]dCTP, deoxyribonucleoside triphosphate concentrations were readjusted to 20 μM each dATP, dGTP, and dCTP and 8 μM TTP. We observed that [^{32}P]TTP yielded more extensive radiolabeling for DNA repair synthesis of UV- or osmium tetroxide-damaged DNA than did [^{32}P]dCTP in yeast nuclear extracts. The assay measures incorporation of [^{32}P]dCMP or [^{32}P]TMP into DNA during repair synthesis reactions. Undamaged DNA was used as an internal control for nonspecific background DNA synthesis. After incubation for 2 h at 23, 30, or 37°C as indicated, plasmid DNA was purified, linearized with restriction endonuclease *Hin*dIII, and separated by 1% agarose gel electrophoresis. Gels were stained with ethidium bromide, dried, and autoradiographed. To quantitate isotope incorporation, DNA bands were sliced from dried gels and radioactivity was measured by liquid scintillation counting.

RESULTS

Repair synthesis in uracil-containing DNA. In previous studies (27), we showed that yeast extracts prepared under the conditions described here do not support repair synthesis associated with nucleotide excision repair but do support base excision repair. Specifically, repair synthesis observed in UV-irradiated plasmid DNA was not dependent on multiple *RAD* genes tested, all of which are absolutely required for nucleotide excision repair. Additionally, no repair synthesis was observed in plasmid DNA treated with cisplatin, the repair of which is exclusively by nucleotide excision. Repair synthesis was also observed in DNA treated with osmium tetroxide, which produces oxidative base damage known to be removed by base excision repair, and was accompanied by the excision of free damaged bases (27).

To confirm that the repair synthesis observed in UV-irradiated and osmium tetroxide-treated DNA does indeed reflect bona fide base excision repair in yeast nuclear extracts, we carried out preliminary experiments using defined substrates in which thymine residues were replaced with uracil. The repair of uracil in DNA has been extensively characterized and is known to be mediated by base excision repair initiated by uracil-DNA glycosylase, a repair-specific enzyme (10).

When a 30-mer duplex oligonucleotide containing a single uracil residue in a defined position was incubated with yeast nuclear extract at 23°C, [^{32}P]TMP incorporation was confined to a single band at the position of the intact 30-mer duplex oligonucleotide (Fig. 1B). Essentially no synthesis was observed in the control substrate without uracil (Fig. 1B). Hence, the demonstration of repair synthesis in yeast nuclear extracts is not restricted by nonspecific nuclease activity, and the incorporation of radioactive nucleotides faithfully reflects the enzymatic processing of modified bases in DNA. Qualitatively identical results were obtained with use of plasmid DNA in which uracil instead of thymine was randomly incorporated by propagating the plasmid in a *dut ung* strain of *E. coli* (Fig. 2, lanes 1, 3, and 5). Figure 2 also shows that the uncontrolled excision of uracil by endogenous uracil-DNA glycosylase in yeast nuclear extracts results in significant degradation of the plasmid substrate, as evidenced by the presence of lower-molecular-weight bands. Hence, in subsequent experiments, we mainly used osmium tetroxide-treated DNA, in which the extent of oxidative base damage can be more rigorously controlled.

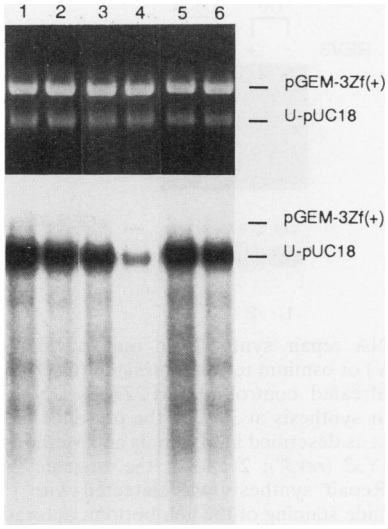

FIG. 2. DNA repair synthesis in uracil-containing plasmid DNA. Yeast nuclear extracts (50 μg) were preincubated for 30 min at 30°C (lanes 1, 3, and 5) or 37°C (lanes 2, 4, and 6) in standard repair synthesis buffer in the absence of DNA and [^{32}P]TTP. Uracil (5 mM) was included in the repair synthesis buffer to reduce the extent of DNA degradation initiated by uracil-DNA glycosylase. Uracil-containing plasmid DNA (U-pUC18), unmodified control DNA [pGEM-3Zf(+)], and [^{32}P]TTP were then added, and the repair synthesis reactions proceeded at 30°C for 30 min. Reactions were stopped by phenol-chloroform extractions, and the DNA was treated as described in Materials and Methods. Nuclear extracts were from *pol1* (lanes 1 and 2), *pol2* (lanes 3 and 4), and *pol3* (lanes 5 and 6) strains.

DNA repair synthesis in *rev3* nuclear extracts. The *REV3* gene is believed to encode a DNA polymerase (19). We therefore examined the role of this putative enzyme in base excision repair in vitro. As shown in Fig. 3 and confirmed by quantitative analysis (data not shown), comparable levels of damage-specific repair synthesis were observed in UV-irradiated or osmium tetroxide-treated plasmid pUC18 DNA after incubation with yeast nuclear extracts from either a *rev3* deletion mutant or its otherwise isogenic wild-type parent. Identical results were obtained with use of a *rev3-1* point mutant (data not shown). These results suggest that Rev3 protein is not required for repair synthesis during base excision repair.

Repair synthesis in nuclear extracts from various polymerase mutant strains. *S. cerevisiae* encodes three essential

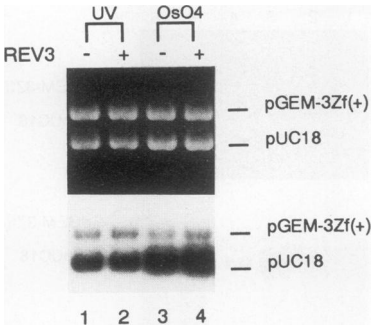

FIG. 3. DNA repair synthesis in *rev3* nuclear extracts. UV-irradiated (UV) or osmium tetroxide-treated (OsO_4) plasmid pUC18 DNA and untreated control pGEM-3Zf(+) DNA were used to measure repair synthesis at 30°C in the presence of 80 μg of yeast nuclear extract as described in Materials and Methods. Lanes: 1 and 3, strain AMY32 (*rev3*^{0}); 2 and 4, the isogenic wild-type strain CL1265-7C. Repair synthesis was detected with [^{32}P]TTP. Top, ethidium bromide staining of the gel; bottom, autoradiograph of the gel.

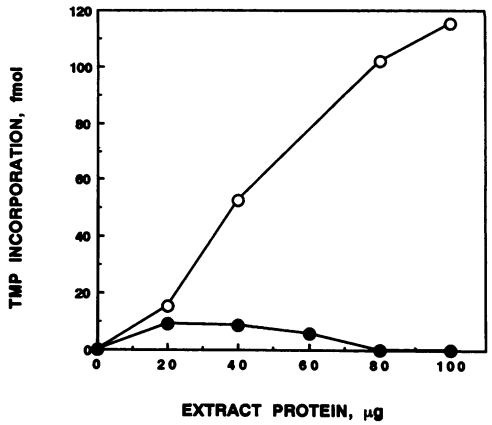

FIG. 5. Repair synthesis as a function of protein concentration. Various amounts of yeast *pol2* or wild-type nuclear extract were preincubated at 37°C for 30 min in the standard repair synthesis buffer in the absence of DNA and [^{32}P]TTP. Subsequently, repair synthesis was initiated by adding UV-irradiated pUC18 DNA, unirradiated control [pGEM-3Zf(+)] DNA, and [^{32}P]TTP. Repair reactions were at 37°C for 2 h. Incorporation of [^{32}P]TMP into DNA was determined as described in Materials and Methods. Background incorporation was subtracted. Open circles, wild-type repair synthesis; closed circles, *pol2* repair synthesis.

DNA polymerases designated DNA polymerases α, δ, and ε (3, 16, 17, 20). Temperature-sensitive mutants for all three *POL* genes have been isolated and characterized (1, 14). We examined the effect of thermally inactivating each of these DNA polymerases on repair synthesis in vitro. We prepared nuclear extracts from yeast *pol1*, *pol2*, and *pol3* mutant strains grown at the permissive temperature (23°C). Repair reactions were carried out at both permissive (23 or 30°C) and restrictive (37°C) temperatures. UV radiation damage-dependent repair synthesis was observed at 23°C with all three nuclear extracts, at levels comparable to that observed with extracts of wild-type cells (Fig. 4, lanes 1 to 4). When the reaction temperature was increased to 30°C, the extent of the repair synthesis increased slightly in each of the extracts (Fig. 4, lanes 5 to 8). However, when reactions were carried out at 37°C, repair synthesis was dramatically reduced uniquely in *pol2* nuclear extracts (Fig. 4; compare lane 11 with lanes 7, 9, 10, and 12). A significant reduction in the amount of repair synthesis was also observed when nuclear extracts from the *pol2* mutant that were preincubated at 37°C were subsequently incubated with uracil-containing plasmid DNA (Fig. 2, lane 4). This reduction was not observed with extracts of *pol1* and *pol3* mutants (Fig. 2, lanes 2 and 6). Increasing the amount of *pol2* extract protein during reactions at 37°C did not increase the amount of DNA repair synthesis in UV-irradiated DNA (Fig. 5). In contrast, repair synthesis in wild-type nuclear extracts increased linearly with increasing amounts of protein (Fig. 5). These results suggest that repair synthesis during base excision repair in vitro is catalyzed by DNA polymerase ε.

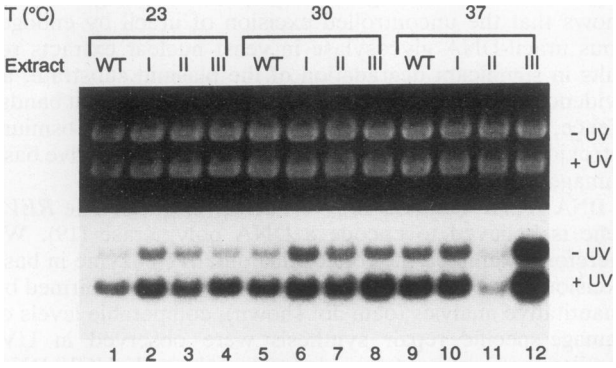

FIG. 4. DNA repair synthesis in nuclear extracts of yeast DNA polymerase temperature-sensitive mutants. Plasmid DNA exposed to UV radiation (+UV) and unirradiated (−UV) control DNA were used to measure repair synthesis in 80 μg of yeast nuclear extract from yeast *pol1* (I), *pol2* (II), *pol3* (III), or wild-type (WT) strains. Standard repair synthesis assays were performed at 23°C (lanes 1 to 4), 30°C (lanes 5 to 8), or 37°C (lanes 9 to 12) as described in Materials and Methods, using [^{32}P]dCTP. Top, ethidium bromide staining of the gel; bottom, autoradiograph of the gel.

Complementation of defective DNA repair synthesis in *pol2* extracts with purified DNA polymerases. The amount of repair synthesis was also dramatically reduced in osmium tetroxide-treated plasmid DNA when *pol2* nuclear extracts were incubated at 37°C. A typical experimental result is shown in Fig. 6A, in which an ~13-fold reduction was observed relative to the level of repair synthesis at 30°C (compare lanes 1 and 2). When this extract was supplemented with increasing amounts of purified yeast DNA polymerase ε, repair synthesis was progressively restored (Fig. 6A, lanes 3 to 5). As little as 0.01 U of the purified enzyme detectably increased damage-specific repair synthesis, and when the extract was supplemented with 0.05 U of the enzyme, repair synthesis was increased ~9-fold.

The addition of purified yeast DNA polymerase δ also resulted in moderately increased repair synthesis in *pol2* nuclear extracts. However, it required 25 times more DNA polymerase δ to achieve levels of repair synthesis comparable to those observed with DNA polymerase ε (Fig. 6A, lanes 6 to 8), and repair synthesis never achieved wild-type levels. Purified yeast DNA polymerase α failed to complement defective repair synthesis in *pol2* nuclear extracts, even when as much as 1.5 U of the enzyme was added (Fig. 6B).

Similar results were obtained with plasmid DNA exposed to UV radiation (Fig. 6C). The addition of 0.05 U of purified

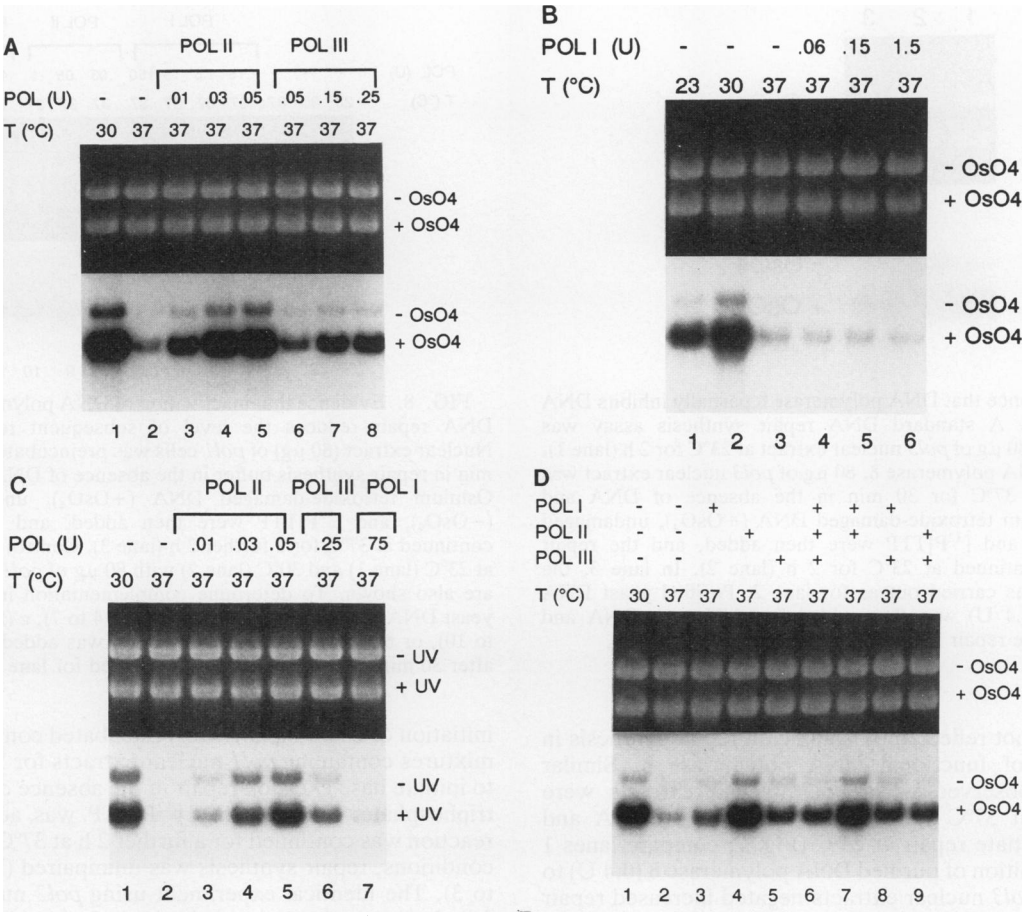

FIG. 6. Complementation of DNA repair synthesis in *pol2* nuclear extracts with purified yeast DNA polymerases. (A) A standard repair synthesis reaction was carried out with 80 μg of yeast *pol2* nuclear extract at 30°C for 2 h (lane 1). Additionally, 80 μg of *pol2* nuclear extract was preincubated at 37°C for 30 min in standard repair synthesis buffer in the absence of DNA and [^{32}P]TTP. Osmium tetroxide-damaged DNA (+OsO_4), undamaged DNA (−OsO_4), and [^{32}P]TTP were then added, and the reaction was continued at 37°C for 2 h (lane 2). For complementation studies, preincubation was performed as in lane 2. Subsequently, 0.01 to 0.05 U of purified yeast DNA polymerase ε (POL II) (lanes 3 to 5) or 0.05 to 0.25 U of purified yeast DNA polymerase δ (POL III) (lanes 6 to 8) was added to the preincubated *pol2* nuclear extract together with DNA and [^{32}P]TTP. Reactions proceeded at 37°C for 2 h. (B) Standard repair synthesis reactions were carried out with *pol2* nuclear extract for 2 h at 23°C (lane 1) or 30°C (lane 2). Repair synthesis of osmium tetroxide-damaged DNA with preincubated *pol2* nuclear extract was performed as described for panel A at 37°C for 2 h without (lane 3) or with (lanes 4 to 6) 0.05 to 1.5 U of purified yeast DNA polymerase α (POL I). (C) Repair synthesis reactions with yeast *pol2* nuclear extract at 30°C (lane 1) and preincubated *pol2* nuclear extract at 37°C (lane 2) were performed as described for panel A. Complementation experiments were performed as for lane 2, with the addition of purified yeast DNA polymerases ε (POL II) (lanes 3 to 5), δ (POL II) (lane 6), or α (POL I) (lane 7) to the preincubated *pol2* nuclear extract. UV-irradiated and unirradiated DNA are indicated by +UV and −UV, respectively. (D) Repair synthesis was performed with 80 μg of *pol2* nuclear extract in the standard reaction either at 30°C for 2 h (lane 1) or with 80 μg of preincubated *pol2* nuclear extract at 37°C for 2 h (lane 2, as described for panel A). For complementation experiments, purified yeast DNA polymerases α (POL I) (lane 3), ε (POL II) (lane 4), δ (POL III) (lane 5), α, ε, and δ (lane 6), α plus ε (lane 7), α plus δ (lane 8), or ε plus δ (lane 9) were added to the preincubated *pol2* nuclear extract (80 μg of each). DNA polymerases α, ε, and δ were added in the amounts of 150, 0.05, and 0.25 U, respectively. Repair synthesis reactions were at 37°C for 2 h. Osmium tetroxide-damaged and undamaged DNA are indicated by +OsO_4 and −OsO_4, respectively.

yeast DNA polymerase ε fully restored defective repair synthesis in *pol2* nuclear extracts (Fig. 6C; compare lanes 1 and 5). However, 0.25 U of yeast DNA polymerase δ had only a partial effect (Fig. 6C, lane 6), and the addition of 75 U of DNA polymerase α (Fig. 6C, lane 7) had no detectable effect on the defective repair synthesis.

We considered the possibility that DNA polymerases α and δ might contribute more substantially to repair synthesis if all three functional polymerases were present at the same time. To examine this possibility, we added various combinations of the three purified enzymes to heat-inactivated *pol2* nuclear extracts. As shown in Fig. 6D, no combinations led to a cooperative increase in repair synthesis compared with the levels observed in the presence of DNA polymerase ε alone.

Yeast DNA polymerase δ limits DNA repair synthesis. Several experimental results demonstrated that repair synthesis catalyzed by DNA polymerase ε is limited by the presence of DNA polymerase δ. We reproducibly detected increased levels of repair synthesis in both damaged and control plasmid DNA when *pol3* extracts were incubated at temperatures that are restrictive for DNA polymerase δ activity (Fig. 4; compare lanes 4 and 12). The increased DNA synthesis in the control DNA was observed uniquely under these experimental conditions, suggesting that very low levels of spontaneous damage and or nicks in these DNA

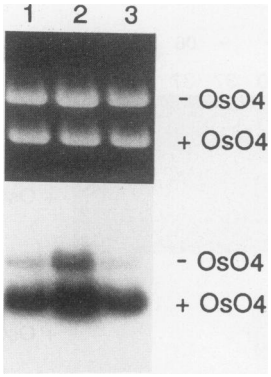

FIG. 7. Evidence that DNA polymerase δ partially inhibits DNA repair synthesis. A standard DNA repair synthesis assay was carried out with 80 μg of *pol3* nuclear extract at 23°C for 2 h (lane 1). To inactivate DNA polymerase δ, 80 μg of *pol3* nuclear extract was preincubated at 37°C for 30 min in the absence of DNA and [^{32}P]TTP. Osmium tetroxide-damaged DNA (+OsO_4), undamaged DNA (−OsO_4), and [^{32}P]TTP were then added, and the repair reaction was continued at 23°C for 2 h (lane 2). In lane 3, the preincubation was carried out as for lane 2. Purified yeast DNA polymerase δ (0.4 U) was then added in addition to DNA and [^{32}P]TTP, and the repair reaction proceeded at 23°C for 2 h.

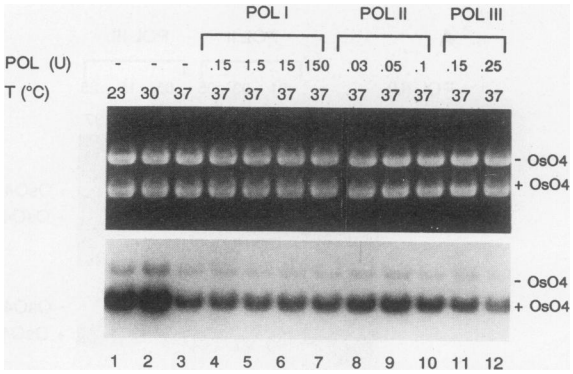

FIG. 8. Evidence that inactivation of DNA polymerase α prior to DNA repair reduces the level of subsequent repair synthesis. Nuclear extract (80 μg) of *pol1* cells was preincubated at 37°C for 30 min in repair synthesis buffer in the absence of DNA and [^{32}P]TTP. Osmium tetroxide-damaged DNA (+OsO_4), undamaged DNA (−OsO_4), and [^{32}P]TTP were then added, and incubation was continued at 37°C for a further 2 h (lane 3). Control repair reactions at 23°C (lane 1) and 30°C (lane 2) with 80 μg of *pol1* nuclear extract are also shown. To determine complementation in vitro, purified yeast DNA polymerase α (POL I) (lanes 4 to 7), ε (POL II) (lanes 8 to 10), or δ (POL III) (lanes 10 and 12) was added to the extracts after 30 min of preincubation as described for lane 3.

molecules are not reflected by significant repair synthesis in the presence of functional DNA polymerase δ. Similar results were observed when *pol3* nuclear extracts were preincubated at 37°C prior to the addition of DNA and [^{32}P]TTP to initiate repair at 23°C (Fig. 7; compare lanes 1 and 2). The addition of purified DNA polymerase δ (0.4 U) to preincubated *pol3* nuclear extracts negated increased repair synthesis in both the damaged and control substrates (Fig. 7, lane 3). Quantitative determinations of the amount of damage-dependent repair synthesis in lane 2 of Fig. 7, i.e., after subtraction of the synthesis in control DNA, showed an ~3-fold increase relative to that measured in lanes 1 and 3. Finally, we observed that the correction of defective repair synthesis in *pol2* extracts by purified DNA polymerase ε was reduced ~70% by the simultaneous addition of purified DNA polymerase δ (Fig. 6D; compare lane 4 with lanes 6 and 9). The addition of purified DNA polymerase α did not produce this effect (Fig. 6D; compare lanes 4 and 7).

Thermal inactivation of DNA polymerase α prior to DNA repair reduces repair synthesis. In the experiments shown in Fig. 4, incubation mixtures were assembled at room temperature prior to reaction at various temperatures. As indicated previously, the levels of repair synthesis in *pol1* nuclear extracts were unchanged when reactions were shifted to 37°C. However, when *pol1* nuclear extracts were preincubated at 37°C in the absence of the DNA substrate, i.e., prior to the initiation of excision repair, the amount of repair synthesis of osmium tetroxide-treated DNA during continued incubation at 37°C was reduced relative to that observed during extended incubation at 23 or 30°C (Fig. 8; compare lane 3 with lanes 1 and 2). This result was not observed when extracts of wild-type cells were preincubated at 37°C prior to initiating repair (data not shown). The reduction in repair synthesis was not corrected by the addition of purified yeast DNA polymerase α, ε, or δ following the preincubation (Fig. 8, lanes 4 to 12).

To further demonstrate that repair synthesis in *pol1* nuclear extracts was proficient at the restrictive temperature unless DNA polymerase α was inactivated prior to the initiation of DNA repair, we preincubated complete reaction mixtures containing *pol1* nuclear extracts for 15 min at 23°C to initiate base excision repair in the absence of radiolabeled triphosphate. Subsequently [^{32}P]TTP was added, and the reaction was continued for a further 2 h at 37°C. Under these conditions, repair synthesis was unimpaired (Fig. 9, lanes 1 to 3). The identical experiment using *pol2* nuclear extracts failed to restore repair synthesis at the restrictive temperature (Fig. 9, lanes 4 to 6).

DISCUSSION

Utilizing an assay that supports base excision repair of plasmid DNA in vitro, we have demonstrated that the *REV3*

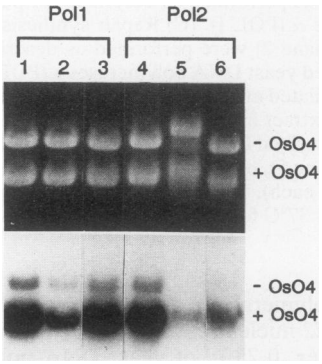

FIG. 9. Evidence that initiating DNA repair at the permissive temperature results in proficient repair synthesis at the restrictive temperature in *pol1* extracts. Repair reactions were carried out at 30°C with 80 μg of *pol1* (lane 1) or *pol2* (lane 4) nuclear extracts. Preincubation of 80 μg of *pol1* (lane 2) or *pol2* (lane 5) nuclear extract at 37°C prior to DNA repair was also carried out. To initiate DNA repair before shifting reactions to 37°C, 80 μg of *pol1* (lane 3) or *pol2* (lane 6) nuclear extract was incubated with osmium tetroxide-damaged DNA (+OsO_4) and undamaged DNA (−OsO_4) in the standard reaction buffer at 23°C for 15 min. [^{32}P]TTP was added, and reactions were continued at 37°C for an additional 2 h.

gene of the yeast *S. cerevisiae* is not required for repair synthesis during this repair mode. Hence, if as has been suggested (19), the *REV3* gene encodes a nonessential DNA polymerase, this polymerase is not responsible for repair synthesis during base excision repair of DNA. On the other hand, the observations that (i) repair synthesis of DNA damaged by UV radiation or osmium tetroxide was totally defective in *pol2* nuclear extracts under nonpermissive conditions and (ii) the addition of purified yeast DNA polymerase ε to *pol2* nuclear extracts uniquely complemented this defect indicate that DNA polymerase ε is required for repair synthesis during base excision repair. These results are consistent with those of Nishida et al. (21), who observed that repair synthesis of DNA in permeabilized human fibroblasts exposed to UV radiation requires the human DNA polymerase ε and that DNA polymerases α or β cannot substitute for polymerase ε. Though not explicitly stated, it is likely that the studies of Nishida et al. (21) measured repair synthesis primarily, if not exclusively, during nucleotide excision repair of DNA. Hence, our results suggest that the polymerase requirements for gap filling associated with excision repair in eukaryotes are similar, if not identical, during base and nucleotide excision repair.

We demonstrated that under normal conditions, repair synthesis is proficient at the restrictive temperature in *pol1* nuclear extracts. However, when DNA polymerase α was thermally inactivated prior to the initiation of base excision repair, the extent of repair synthesis was reduced ~3-fold. Neither purified DNA polymerases α, δ, nor ε corrected this reduction. Additionally, we showed that DNA polymerase δ has an inhibitory effect on DNA polymerase ε-dependent repair synthesis. Hence, while DNA polymerases α and δ do not play a role in directly catalyzing repair synthesis during base excision repair, both enzymes can apparently influence repair synthesis catalyzed by DNA polymerase ε.

The mechanism by which DNA polymerase δ inhibits repair synthesis is not known. It is interesting to note that DNA polymerases δ and ε have similar properties (26). For example, both enzymes are able to associate with proliferating cell nuclear antigen and replication factor C proteins on primed single-stranded DNA to form an initiation complex (5). Both enzymes might therefore recognize and bind to gaps in DNA, but DNA polymerase ε might utilize such substrate sites more efficiently. Hence, DNA polymerase δ might compete with DNA polymerase ε for repair synthesis substrate. Alternatively, DNA polymerases ε and δ might compete for one or more accessory proteins that are required for repair synthesis of DNA during base excision repair. The observation of the partial correction of repair synthesis in nuclear extracts defective in DNA polymerase ε following the addition of a vast excess of purified DNA polymerase δ is consistent with this notion.

From the observed effect of DNA polymerase α on repair synthesis, we postulate that this enzyme normally interacts with a factor(s) that is mobilized following the initiation of DNA repair and is required for repair synthesis catalyzed by DNA polymerase ε. Thermal inactivation of polymerase α may somehow result in inactivation of this factor(s), perhaps by trapping with denatured polymerase α, thereby preventing its participation in DNA polymerase ε-dependent repair synthesis.

In conclusion, our studies reveal a complex involvement of DNA polymerases α, δ, and ε in DNA repair synthesis during base excision repair in yeast cells. All three enzymes affect the level of DNA repair synthesis under certain conditions, either directly or indirectly. The yeast DNA polymerases are biochemically very similar to their mammalian counterparts. Hence, our results may be reasonably extrapolable to mammalian DNA repair and may provide at least a partial explanation for the contradictory conclusions obtained in earlier studies with mammalian systems which relied mainly on the use of polymerase inhibitors (13, 22, 23, 25).

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REFERENCES

1. **Araki, H., P. A. Ropp, A. L. Johnson, L. H. Johnson, A. Morrison, and A. Sugino.** 1992. DNA polymerase II, the probable homolog of mammalian DNA polymerase ε, replicates chromosomal DNA in the yeast *Saccharomyces cerevisiae*. EMBO J. **11**:733–740.
2. **Bauer, G. A., and P. M. J. Burgers.** 1988. The yeast analog of mammalian cyclin/proliferating-cell nuclear antigen interacts with mammalian DNA polymerase δ. Proc. Natl. Acad. Sci. USA **85**:7506–7510.
3. **Boulet, A., L. M. Simon, G. Faye, G. A. Bauer, and P. M. J. Burgers.** 1989. Structure and function of the *Saccharomyces cerevisiae CDC2* gene encoding the large subunit of DNA polymerase III. EMBO J. **8**:1849–1854.
4. **Brooke, R. G., R. Singhal, D. C. Hinkle, and L. B. Dumas.** 1991. Purification and characterization of the 180- and 86-kilodalton subunits of the *Saccharomyces cerevisiae* DNA primase-DNA polymerase protein complex: the 180-kilodalton subunit has both DNA polymerase and 3′→5′-exonuclease activities. J. Biol. Chem. **266**:3005–3015.
5. **Burgers, P. M. J.** 1991. *Saccharomyces cerevisiae* replication factor C. J. Biol. Chem. **266**:22698–22706.
6. **Burgers, P. M. J., R. A. Bambara, J. L. Campbell, L. M. S. Chang, K. M. Downey, U. Hubscher, M. Y. W. T. Lee, S. M. Linn, A. G. So, and S. Spadari.** 1990. Revised nomenclature for eukaryotic DNA polymerases. Eur. J. Biochem. **191**:617–618.
7. **Conrad, M. N., and C. S. Newlon.** 1983. *Saccharomyces cerevisiae cdc2* mutants fail to replicate approximately one-third of their nuclear genome. Mol. Cell. Biol. **3**:1000–1012.
8. **Dianov, G., A. Price, and T. Lindahl.** 1992. Generation of single-nucleotide repair patches following excision of uracil residues from DNA. Mol. Cell. Biol. **12**:1605–1612.
9. **Fien, K., and B. Stillman.** 1992. Identification of replication factor C from *Saccharomyces cerevisiae*: a component of the leading-strand DNA replication complex. Mol. Cell. Biol. **12**: 155–163.
10. **Friedberg, E. C.** 1985. DNA repair. W. H. Freeman, New York.
11. **Hamatake, R. K., H. Hasegawa, A. B. Clark, K. Bebenek, T. A. Kunkel, and A. Sugino.** 1990. Purification and characterization of DNA polymerase II from the yeast *Saccharomyces cerevisiae*. J. Biol. Chem. **265**:4072–4083.
12. **Hansson, J., M. Munn, W. D. Rupp, R. Kahn, and R. D. Wood.** 1989. Localization of DNA repair synthesis by human cell extracts to a short region at the site of a lesion. J. Biol. Chem. **264**:21788–21792.
13. **Hardt, N., G. Pedrali-Noy, F. Focher, and S. Spadari.** 1981. Aphidicolin does not inhibit DNA repair synthesis in ultraviolet-irradiated HeLa cells. A radioautographic study. Biochem. J. **199**:453–455.
14. **Hartwell, L. H., R. K. Mortimer, J. Culotti, and M. Culotti.** 1973. Genetic control of the cell division cycle in yeast. V.

genetic analysis of *cdc* mutants. Genetics **74:**267–286.
15. **Huang, J.-H., D. L. Svoboda, J. T. Reardon, and A. Sancar.** 1992. Human nucleotide excision nuclease removes thymine dimers from DNA by incising the 22nd phosphodiester bond 5′ and the 6th phosphodiester bond 3′ to the photodimer. Proc. Natl. Acad. Sci. USA **89:**3664–3668.
16. **Hubscher, U., and P. Thommes.** 1992. DNA polymerase ε: in search of a function. Trends Biochem. Sci. **17:**55–58.
17. **Johnson, L. M., M. Snyder, L. M. S. Chang, R. W. Davis, and J. L. Campbell.** 1985. Isolation of the gene encoding yeast DNA polymerase I. Cell **43:**369–377.
18. **Lemontt, J. F.** 1971. Mutations of yeast defective in mutation induced by ultraviolet light. Genetics **68:**21–33.
19. **Morrison, A., R. B. Christersen, J. Alley, A. K. Beck, E. G. Bernstine, J. F. Lemontt, and C. W. Lawrence.** 1989. *REV3*, a *Saccharomyces cerevisiae* gene whose function is required for induced mutagenesis, is predicted to encode a nonessential DNA polymerase. J. Bacteriol. **171:**5659–5667.
20. **Morrison, A., A. Hiroyuki, A. B. Clark, R. K. Hamatake, and A. Sugino.** 1990. A third essential DNA polymerase in S. cerevisiae. Cell **62:**1143–1151.
21. **Nishida, C., P. Reinhard, and S. Linn.** 1988. DNA repair synthesis in human fibroblasts requires DNA polymerase δ. J. Biol. Chem. **263:**501–510.
22. **Perrino, F. W., and L. A. Loeb.** 1990. Animal cell DNA polymerases in DNA repair. Mutat. Res. **236:**289–300.
23. **Popanda, O., and H. W. Thielmann.** 1992. The function of DNA polymerases in DNA repair synthesis of ultraviolet-irradiated human fibroblasts. Biochim. Biophys. Acta **1129:**155–160.
24. **Sitney, K. C., M. E. Budd, and J. L. Cambell.** 1989. DNA polymerase III, a second essential DNA polymerase, is encoded by the S. cerevisiae *CDC2* gene. Cell **56:**599–605.
25. **Smith, C. A., and D. S. Okumoto.** 1984. Nature of DNA repair synthesis resistant to inhibition of polymerase α in human cells. Biochemistry **23:**1383–1391.
26. **Syvaoja, J. E.** 1990. DNA polymerase epsilon: the latest member in the family of mammalian DNA polymerases. BioEssays **12:**533–536.
27. **Wang, Z., X. Wu, and E. C. Friedberg.** 1992. Excision repair of DNA in nuclear extracts from the yeast *Saccharomyces cerevisiae*. Biochemistry **31:**3694–3702.
28. **Yoder, B. L., and P. M. J. Burgers.** 1991. *Saccharomyces cerevisiae* replication factor C. I. purification and characterization of its ATPase activity. J. Biol. Chem. **266:**22689–22697.