DNA Ligation during Excision Repair in Yeast Cell-Free Extracts Is Specifically Catalyzed by the *CDC9* Gene Product†

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ABSTRACT: Excision repair of DNA is an important cellular response to DNA damage induced by radiation and many chemicals. In eukaryotes, base excision repair (BER) and nucleotide excision repair (NER) are two major excision repair pathways which are completed by a DNA ligation step. Using a cell-free system, we have determined the DNA ligase requirement during BER and NER of the yeast *S. cerevisiae*. Under nonpermissive conditions in extracts of the *cdc9-2* temperature-sensitive mutant, DNA ligation in both BER and NER pathways was defective, and the repair patches were enlarged. At the permissive temperature (23 °C), DNA ligation during excision repair was only partially functional in the mutant extracts. In contrast, deleting the DNA ligase IV gene did not affect DNA ligation of BER or NER. Defective DNA ligation of BER and NER in *cdc9-2* mutant extracts was complemented in vitro by purified yeast Cdc9 protein, but not by DNA ligase IV even when overexpressed. These results demonstrate that the ligation step of excision repair in yeast cell-free extracts is catalyzed specifically by the Cdc9 protein, the homologue of mammalian DNA ligase I.

Excision repair is responsible for removing inappropriate or damaged bases in DNA and constitutes a crucial defense system against cytotoxicity, mutagenesis, and carcinogenesis induced by a variety of DNA damaging agents. There are two major excision repair pathways in eukaryotes: base excision repair (BER)[1] and nucleotide excision repair (NER).

BER is initiated by a DNA glycosylase which catalyzes the hydrolysis of the *N*-glycosyl bond between the base and the sugar phosphate backbone. Glycosylase excision results in the release of a free base and the formation of an apurinic/apyrimidinic (AP) site in DNA. The AP site is cleaved by a 5′ AP endonuclease, generating a 3′-hydroxyl group and a 5′-deoxyribose phosphate moiety. Processing of the latter is a rate-limiting step (*1*), which is mediated by two alternative mechanisms (*2*−*6*). The 5′-deoxyribose phosphate can be removed by a deoxyribophosphodiesterase (dRPase) activity such as that associated with DNA polymerase β (*7*), leaving a one-nucleotide gap in DNA. In mammals, DNA polymerase β then fills in the gap, leading to a one-nucleotide repair patch (*5, 6, 8*−*12*). Alternatively, the 5′-deoxyribose phosphate can be displaced from the complementary DNA as part of a flap structure by DNA strand-displacement synthesis which involves DNA polymerase δ/ϵ and PCNA (*3, 4, 6, 12, 13*). The short stretch of single-stranded DNA containing the 5′-deoxyribose phosphate moiety is then cleaved by the flap endonuclease FEN1 in mammals (*6, 14*) or Rad27 in yeast (*15*−*17*). This BER subpathway generates heterogeneous repair patch sizes of 2−7 nucleotides (*1, 4, 6*). Apparently, the 5′ → 3′ exonuclease activity of FEN1 and Rad27 can also function during BER to remove the 5′-deoxyribose phosphate moiety by nucleotide hydrolysis (*17, 18*). Normally, the longer repair patch mechanism constitutes a minor BER subpathway in mammals. In yeast, DNA polymerase β does not play a major role in BER (*19, 20*) (Wu, X., and Wang, Z., unpublished results); instead, DNA polymerase ϵ plays an important role in the repair synthesis of BER (*21*). BER can also be initiated by a DNA glycosylase with associated AP lyase, as in the case of oxidative damage repair. DNA incision is mediated by the AP lyase activity 3′ to the AP site (*22*−*24*). Thus, this BER pathway is mechanistically different, and its precise biochemistry is less clear.

The NER pathway can be conceptually divided into five distinct steps: damage recognition, incision, excision, repair synthesis, and DNA ligation (*25*−*27*). This mode of excision repair is versatile in that it is capable of correcting a large spectrum of very different DNA lesions (*28*). The repair mechanism and required proteins are well conserved from yeast to humans. A minimum set of proteins required for NER has been identified in yeast and humans as indicated by in vitro reconstitution from purified repair components (*29*−*31*). Early steps of NER depend on proteins Rad1, Rad2, Rad4, Rad10, Rad14, Rad23, TFIIH factor, and RPA in yeast. Human homologues corresponding to the Rad proteins are XPF, XPG, XPC, ERCC1, XPA, and HHR23A/HHR23B, respectively. Additionally, the yeast Rad7/Rad16 protein complex also plays a major role in transcription-independent NER (*32*−*34*). Human homologues of these two proteins have not been identified.

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[1] Abbreviations: BER, base excision repair; NER, nucleotide excision repair; AP, apurinic/apyrimidinic; dRPase, deoxyribophosphodiesterase; UV, ultraviolet.

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In both BER and NER pathways, the repair is completed by a DNA ligation step requiring a ligase activity. In mammals, four biochemically distinct DNA ligases have been identified (*35*−*37*). DNA ligase I is required for DNA replication (*38*−*41*) and may also function in BER (*6*, *11*, *42*, *43*). DNA ligase III is involved in mammalian BER (*6*, *10*, *43*) and may also play a role in meiotic recombination (*44*, *45*). DNA ligase IV is required in nonhomologous double-strand end joining (*46*). In the yeast *S. cerevisiae*, only two DNA ligases are found: DNA ligases I and IV encoded by the *CDC9* and *DNL4/LIG4* genes, respectively (*47*−*51*). Cdc9 DNA ligase is required for DNA replication (*52*, *53*). Additionally, *cdc9* temperature-sensitive mutant cells are sensitive to several DNA damaging agents (*54*−*57*). Deletion of the *DNL4* gene does not significantly affect cellular sensitivities to UV, MMS, or ionizing radiation, but causes a defect in nonhomologous double-strand end joining (*49*−*51*). Sensitivity to DNA damaging agents can result from defects in various cellular responses such as excision repair, postreplication repair, recombinational repair, cell cycle checkpoints, or apoptosis. Hence, the DNA ligase requirement for excision repair needs to be biochemically determined.

Using a cell-free system, we have performed biochemical analyses of yeast BER and NER pathways with respect to the DNA ligase requirement. In this report, we show that DNA ligation of both BER and NER is specifically catalyzed by the Cdc9 DNA ligase (DNA ligase I). DNA ligase IV cannot substitute for the excision repair function of DNA ligase I even when overexpressed.

MATERIALS AND METHODS

Strains and Enzymes. The *S. cerevisiae* wild-type strains used were JJ567 (*58*), SF657-2D (*59*), SX46A (*60*), TC102 (*61*), and BY4741 (*MATa his3 leu2 met15 ura3*) (purchased from ATCC). The temperature sensitive *cdc9-2* mutant strain LP2915-9B (*MATa ade2-1 his3-*Δ*200 leu2-3,112 trp1-*Δ *ura3-52*) was obtained from the Yeast Genetic Stock Center (Berkeley, CA), and the *dnl4* deletion mutant (isogenic to BY4741) was purchased from Research Genetics (Huntsville, AL). Purified yeast Cdc9 DNA ligase (fraction VII) (*62*) was provided by Tomkinson (Institute of Biotechnology, The University of Texas Health Science Center at San Antonio). Pfu DNA polymerase was purchased from Stratagene.

DNA Substrates for Excision Repair. DNA substrates used for in vitro BER were osmium tetroxide-damaged plasmid pUC18 and a 30-mer duplex oligonucleotide containing a site-specific uracil residue at position 13. In vitro NER substrate was plasmid pUC18 DNA containing AAF adducts. Osmium tetroxide-damaged DNA was prepared as previously described (*21*, *63*). Nicked plasmid DNA (minor products of osmium tetroxide damage) was removed by sucrose gradient centrifugation (*21*, *63*). The substrate contained approximately five oxidized bases (mostly thymine glycols) per pUC18 DNA on average (*63*). Substrate U-mse1 containing a site-specific uracil residue was prepared as described (*1*). The nucleotide sequence of the uracil-containing strand is 5′-GGATGGCATGCA**U**TAACCG-GAGGCCGCGCG-3′. AAF-pUC18 DNA was prepared by treating the plasmid with *N*-acetoxy-2-acetylaminofluorene (NCI Chemical Carcinogen Reference Standard Repositories, Kansas City, MO) and subsequent purification by sucrose gradient centrifugation as previously described (*63*).

Yeast Extracts. Both nuclear extracts and whole cell extracts were used in this study. Nuclear and whole cell extracts of wild-type cells were prepared according to the methods of Wang et al. (*63*−*65*). Extracts of the temperature-sensitive *cdc9-2* mutant cells were prepared similarly except that the cells were grown at 23 °C and the cell wall digestion with yeast lytic enzyme (70 000 units/g, ICN) was performed at 23 °C for ∼2 h instead of at 30 °C for ∼1 h. Extracts were aliquoted and stored at −80 °C.

Overexpression of Dnl4 and Lif1. Based on YEplac112 and YEplac195 (*66*), we cloned the *GAL1/10* promoter into the *Eco*RI and the *Bam*HI sites of these plasmids to obtain two yeast overexpression vectors, pEGT and pEGU, respectively. Two sets of PCR primers were synthesized (Operon, Inc.): CGCGGATCCATGATATCAGCACTAGATTCTA-TACCC and CCCAAGCTTAAGTTAATTGACTGGCCA-ATC for Dnl4, and GAAGATCTCATATGTCCCAGCT-GACGGAG and CGGGATCCCATATGACCCTAAAC-TACTTCTAAACCCTC for Lif1. *DNL4* and *LIF1* genes were amplified by Pfu DNA polymerase using yeast DNA as the template. *DNL4* gene was cloned into the *Bam*HI and *Hin*dIII sites of pEGU to yield pEGU-Dnl4, and *LIF1* was cloned into the *Bam*HI site of pEGT to yield pEGT-Lif1. To overexpress Dnl4 and Lif1, *cdc9-2* mutant cells containing pEGU-Dnl4, pEGT-Lif1, or both were grown at room temperature in minimum media containing 2% sucrose to stationary phase. Overexpression from the *GAL1* promoter was induced by diluting the culture 10-fold in rich medium (1% yeast extract and 2% Bacto Peptone) containing 0.5% sucrose and 1.5% galactose and growth at room temperature for 16 h. Extracts containing overexpressed Dnl4, Lif1, or both were prepared in a Mini-Beadbeater (Biospec Products) as previously described (*63*).

To confirm the overexpression, Dnl4 and Lif1 were tagged with six histidine residues at their N-termini using vectors pEGUh6 and pEGTh6, respectively, which were derived from pEGU and pEGT, respectively. Overexpression was induced by identical treatments as for the untagged Dnl4 and Lif1 and confirmed by Western blot analysis using antibodies specific to the His tag (Invitrogen).

In Vitro BER. A standard reaction mixture (50 μL) contained 200 ng of osmium tetroxide-damaged 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 of dATP, dGTP, and dCTP, 4 μM dTTP, 1 μCi of [α-^{32}P]dTTP (3000 Ci/mmol), 40 mM phosphocreatine (disodium salt), 2.5 μg of creatine phosphokinase, 4% glycerol, 100 μg/mL bovine serum albumin, and 50−80 μg of yeast extracts. After incubation at various temperatures as indicated for 2 h, the reaction was stopped by adding EDTA and RNase A to 20 mM and 20 μg/mL, respectively, and incubated at 37 °C for 10 min. DNA was then extracted by phenol/chloroform and precipitated in ethanol. Repair products were separated by electrophoresis on a 1% agarose gel in the presence of 0.5 μg/mL ethidium bromide. For BER of the uracil-containing substrate U-mse1, 2 pmol of the 30-mer 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 dried gel (agarose gel) or the wet gel (denaturing polyacrylamide gel). The relative distribution of repair synthesis in ligated versus unligated DNA was determined by estimating the band intensity on the autoradiogram.

In Vitro NER. Standard NER buffer components were mixed in a volume of 50 μL with 200 ng of AAF-pUC18 DNA, 250 μg of yeast whole cell extracts, and 50 μg of yeast extracts containing overexpressed Rad2 in the respective strains as previously described (*63*−*65*). After incubation for 2 h at the temperatures indicated, 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 DNA, respectively, and incubated at 37 °C for 30 min. Plasmid DNA was then purified by phenol/chloroform extraction. Detection and estimation of ligated versus unligated repair products were similarly carried out as in the BER assays described above.

The same yeast extracts were used for both BER and NER assays in vitro. As described in greater detail before (*63*−*65*), these two assay systems mainly differ in: (a) buffer components; (b) extract concentrations; (c) repair substrates; and (d) reaction temperatures. In vitro NER depends on 5% PEG in the reaction buffer, requires 200−300 μg of extract proteins, acts on NER-specific substrates such as AAF−DNA, and is inactive at 37 °C. In contrast, in vitro BER is not affected by PEG, requires only 50−80 μg of extract proteins, acts on BER-specific substrates such as uracil−DNA or osmium tetroxide-damaged DNA, and is active at 37 °C. Additionally, in vitro NER is stimulated by overexpressed Rad2 (supplied as Mini-Bead beater extracts), whereas in vitro BER does not require supplements.

RESULTS

Defective DNA Ligation during BER in cdc9-2 Mutant Extracts. Uracil-containing DNA or osmium tetroxide-damaged DNA can be specifically repaired in vitro by the BER pathway in a yeast cell-free system (*1, 59*). The last step of this repair pathway is a DNA ligation event which is also observed in the yeast cell-free system (*63*). BER in yeast extracts can be monitored by radiolabeling the repair patch at the DNA repair synthesis step (*1*). To define the DNA ligase required during BER, we examined the repair in *cdc9-2* mutant extracts. The yeast *cdc9-2* mutant is a temperature-sensitive strain. To determine an optimal in vitro repair condition in the *cdc9-2* mutant extracts, we first examined its growth properties at various temperatures. As shown in Figure 1, the viability of *cdc9-2* cells was significantly reduced at 28 °C, suggesting that the ligase activity of the mutant Cdc9-2 protein was significantly affected in vivo at this temperature. At 37 °C, no viable cells were observed as expected (Figure 1). Thus, *cdc9-2* mutant extracts were prepared from cells grown at the permissive temperature (23 °C) and used for in vitro repair at various temperatures.

Using osmium tetroxide-damaged plasmid DNA as a BER substrate, we performed repair in *cdc9-2* mutant extracts at various temperatures. Repair products were labeled by [α-^{32}P]dTTP during in vitro BER and separated by electrophoresis on a 1% agarose gel in the presence of ethidium bromide. The ligated repair products existed as closed circular DNA, whereas the unligated ones existed as opened circular DNA and migrated slower on the gel (Figure 2A). In wild-type cell extracts, most BER products were in the ligated form after in vitro repair at 23 or 30 °C (Figure 2A, lanes 4 and 5). This result was observed with various wild-type strains and was not significantly affected by different genetic backgrounds (data not shown). We consistently observed a significant decrease in DNA ligation during BER at 37 °C in wild-type extracts (Figure 2A, lane 6). This may have resulted from a disruption of the coordination between DNA repair synthesis and ligation, and/or thermal inhibition of the DNA ligase activity at 37 °C, which is significantly higher than the physiological growth temperatures for yeast. In contrast, after repair in *cdc9-2* mutant extracts at 30 °C or above, all products were in the unligated form, indicating totally defective DNA ligation during BER in the mutant extracts (Figure 2A, lanes 2 and 3). Even at 23 °C (a permissive temperature for growth), deficient DNA ligation during BER in *cdc9-2* mutant extracts was detected, with only ∼30% repair products being ligated (Figure 2A, lane 1). DNA ligation deficiency at 23 °C was also observed with ethidium bromide-stained DNA (Figure 2A, compare lanes 1 and 4 of the top panel). These results indicate that the *cdc9-2* mutation results in a thermally labile DNA ligase whose activity for BER is completely inactivated above 30 °C.

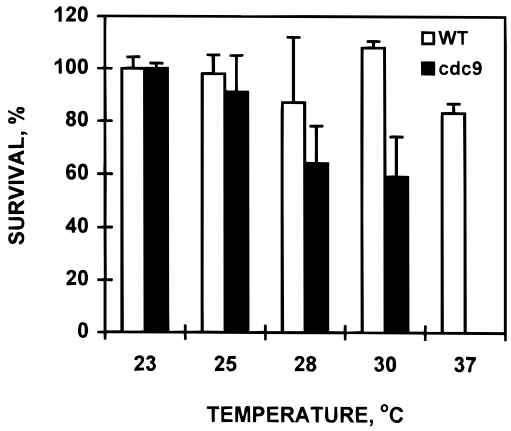

FIGURE 1: Effect of temperature on the growth of *cdc9-2* cells. Cells grown in YPD medium (1% yeast extract, 2% Bacto Peptone, and 2% glucose) at 23 °C to the logarithmic phase of growth were diluted in YPD medium and plated onto YPD plates that had been prewarmed to the indicated temperatures. Plates were incubated at the indicated temperatures, and colonies were counted after incubation for 2−4 days. Survival is expressed relative to the colony formation at 23 °C. Results are the averages of triplicate experiments. WT, wild-type strain JJ567.

To determine if the thermal inactivation of the Cdc9-2 mutant protein is reversible, we incubated the mutant extracts at 37 °C for 15 min prior to in vitro repair. Subsequently, BER was performed at 23 °C in the preincubated mutant extracts. As shown in Figure 2B (lane 7), DNA ligation was not observed. Identical treatment to wild-type extracts did not affect DNA ligation of BER (Figure 2B, compare lanes 1 and 3). Thus, heat inactivation of Cdc9-2 mutant protein leads to permanent loss of activity.

Effect of Defective DNA Ligation on the Repair Patch Size of BER. We consistently observed more repair synthesis in *cdc9-2* mutant extracts (Figure 2). This observation could

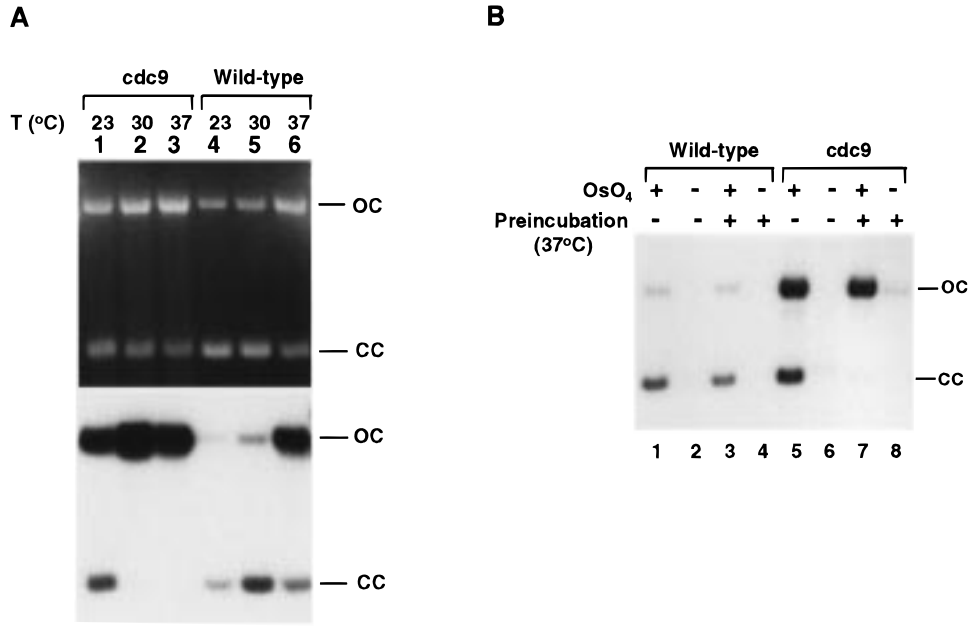

FIGURE 2: DNA ligation during BER in *cdc9-2* mutant extracts. (A) In vitro BER of OsO_4-pUC18 DNA was performed in yeast nuclear extracts (80 μg) of *cdc9-2* mutant or wild-type cells (SF657-2D) for 2 h at the indicated temperatures. Top, ethidium bromide-stained gel; bottom, autoradiograph of the gel. (B) Irreversible thermal inactivation of Cdc9-2 mutant protein for BER. Whole cell extracts (80 μg) of wild-type (SX46A) or *cdc9-2* mutant cells were mixed with BER buffer and preincubated at 37 °C for 15 min without DNA and [α-^{32}P]-dTTP (lanes 3, 4, 7, and 8). In vitro BER was then performed at 23 °C for 2 h after adding DNA and [α-^{32}P]dTTP. Standard in vitro BER without preincubation was performed at 23 °C for 2 h as the control (lanes 1, 2, 5, and 6). BER of OsO_4-pUC18 DNA (OsO_4, +) or undamaged pUC18 control DNA (OsO_4, −) are indicated. Repair products labeled with [α-^{32}P]dTTP were separated into ligated (CC, closed circle) and unligated DNA (OC, opened circle) by electrophoresis on a 1% agarose gel containing 0.5% ethidium bromide, and visualized by autoradiography.

result from increased repair events or enlarged repair patch sizes in *cdc9-2* mutant extracts. To distinguish between these two possibilities, we used a 30-mer duplex oligonucleotide containing a site-specific uracil residue at position 13 to examine the repair patch sizes after BER in the mutant extracts. We have previously shown that repair of uracil residues is specifically mediated by the BER pathway and leads to heterogeneous repair patches of one to five nucleotides with the one-nucleotide patch as a major event (*1*). Consistent with our previous results (*1*), the uracil-containing duplex was fully repaired as ligated 30-mer DNA (labeled with [^{32}P]dTTP) in wild-type extracts (Figure 3, lane 1). At the permissive temperature (23 °C) in *cdc9-2* mutant extracts, ligated repair products (30-mer DNA) were reduced and unligated intermediates of 16−26 nucleotides corresponding to 4−14-nucleotide repair patches were observed (Figure 3, compare lanes 1 and 2). This result confirms the conclusion that *cdc9-2* mutant extracts are partially deficient for DNA ligation of BER at the permissive temperature. After thermal inactivation of Cdc9-2 protein in the mutant extracts by preincubation at 37 °C for 15 min, the DNA ligation defect was clearly indicated by the extensive accumulation of 16−26-nucleotide fragments as the unligated repair products (Figure 3, lane 4). The small amount of the 30-mer repair products probably resulted from repair synthesis extending to the end of the duplex (18-nucleotide repair patch) without ligation (Figure 3, lane 4). In the identically treated wild-type extracts, minor repair intermediates (13−17-mer fragments) representing repair patches of 1−5 nucleotides were observed as previously reported (*1*), and longer repair patch products were not detected (Figure 3, lane 3). These results show that repair patch sizes during BER of uracil-containing DNA are significantly larger in the absence of DNA ligation.

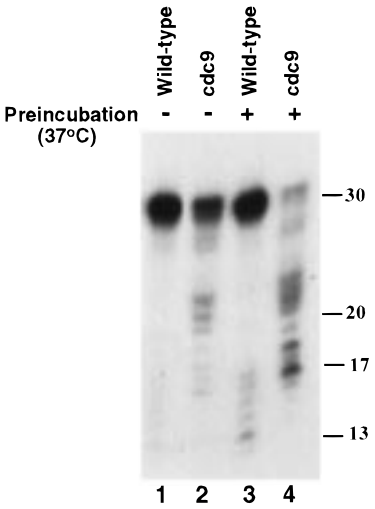

FIGURE 3: Effect of *cdc9-2* mutation on the repair patch size of BER. Whole cell extracts (50 μg) of wild-type (TC102) or *cdc9-2* mutant cells were preincubated in BER buffer at 37 °C for 15 min without DNA and [α-^{32}P]dTTP. In vitro BER was subsequently performed at 23 °C for 2 h after adding the site-specific uracil−DNA (substrate U-mse1) and [α-^{32}P]dTTP (lanes 3 and 4). Standard in vitro BER without preincubation was performed at 23 °C for 2 h as the control (lanes 1 and 2). After purification, repair products were separated by electrophoresis on a 20% denaturing polyacrylamide gel and visualized by autoradiography. DNA markers in nucleotides are indicated on the right.

Complementation of cdc9-2 Mutant Extracts for DNA Ligation of BER. To show that DNA ligation during in vitro BER requires the direct participation of the Cdc9 protein in the repair pathway, we complemented *cdc9-2* mutant extracts with purified yeast Cdc9 protein. As shown in Figure 4 (lanes 2−4), Cdc9 protein complemented the defective DNA

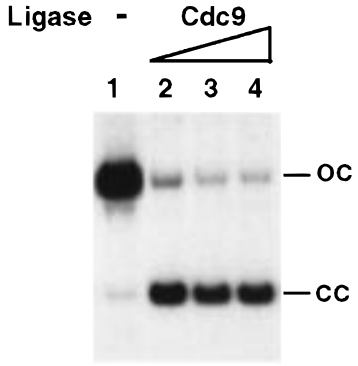

FIGURE 4: In vitro complementation for DNA ligation of BER. To inactivate Cdc9 DNA ligase, *cdc9-2* whole cell extracts (80 μg) were preincubated in BER buffer at 37 °C for 15 min without DNA and [α-^{32}P]dTTP. After adding OsO_4-pUC18 DNA and [α-^{32}P]-dTTP, in vitro BER was performed at 23 °C for 2 h without (lane 1) or with the addition of 30, 90, or 150 ng of purified Cdc9 DNA ligase (lanes 2−4, respectively). Repair products were separated into the ligated (CC) and the unligated form (OC) by electrophoresis on a 1% agarose gel containing 0.5% ethidium bromide and visualized by autoradiography.

ligation during BER in thermal-inactivated *cdc9-2* mutant extracts as evidenced by the conversion of repair products from the opened circular form to the closed circular form. Furthermore, repair synthesis in complemented extracts was reduced (Figure 4). Since the purified CDC9 protein did not significantly affect repair synthesis in wild-type yeast extracts (data not shown), this result suggests that abnormally longer repair patch sizes in *cdc9-2* mutant extracts were concomitantly corrected to the normal sizes by the added DNA ligase. Taken together, these results show that the DNA ligation step of BER in yeast cell-free extracts is catalyzed by the *CDC9* gene product.

Defective DNA Ligation during NER in cdc9-2 Mutant Extracts. Under a different reaction condition, the yeast cell-free system also supports NER in vitro (*64*, *65*). Using DNA containing AAF adducts as the substrate, repair in yeast cell-free extracts is specifically mediated by the NER pathway (*64*, *65*). NER in yeast extracts can be monitored by radiolabeling the repair patch during DNA repair synthesis, and the ligation step can be followed by examining the repair products as closed circular versus opened circular DNA (*63*).

We prepared yeast extracts from *cdc9-2* cells that were grown at the permissive temperature and performed in vitro NER at various temperatures. Since yeast NER is not active at 37 °C in vitro (*63*), we performed the repair assays at temperatures from 26 to 33 °C. Consistent with our earlier results (*63*), repair synthesis of AAF−DNA was gradually reduced with increasing reaction temperatures in wild-type yeast extracts (Figure 5A, lanes 1−4). Nevertheless, NER at each temperature yielded mainly the ligated repair products as indicated by the closed circular DNA (Figure 5A, lanes 1−4). NER in *cdc9-2* mutant extracts, however, yielded significant amounts of unligated repair products (∼40%) at the permissive temperature (23 °C) compared to repair in wild-type extracts (∼20%) (compare lanes 1 in Figure 5A,B), indicating a partial deficiency in DNA ligation during NER at this temperature. With increasing temperatures, ligation during NER in the mutant extracts became increasingly more deficient and reached near total defect at 33 °C (Figure 5B, lanes 1−4). Furthermore, repair synthesis also increased at higher temperatures (Figure 5B, lanes 1−4), which may reflect longer repair patch sizes in the absence of DNA ligation (see below). Like BER, DNA ligation of the NER pathway was irreversibly inactivated by preincubating *cdc9-2* mutant extracts at 37 °C for 10 min, in which the ligated repair products were not detected (Figure 5C). This treatment did not significantly affect NER in wild-type extracts (data not shown). Hence, we conclude that thermal inactivation of the Cdc9-2 mutant protein results in defective DNA ligation during NER.

To show direct participation of the Cdc9 protein in the NER pathway, we complemented *cdc9-2* mutant extracts with purified yeast Cdc9 protein. As shown in Figure 6 (lanes 2−4), addition of Cdc9 protein to thermal-inactivated *cdc9-2* mutant extracts resulted in conversion of significant amounts of unligated repair products to the ligated form. As in ligase complementation for BER (Figure 4), the repair synthesis of NER in complemented extracts was also reduced (Figure 6). In wild-type extracts, the DNA ligase did not significantly affect repair synthesis (data not shown). Thus, similar to BER, repair patch sizes of NER were probably longer in the absence of DNA ligation, and this abnormality in *cdc9-2* mutant extracts was also corrected by purified Cdc9 DNA ligase. Taken together, these results show that the DNA ligation step of the NER pathway in yeast cell-free extracts is catalyzed by the Cdc9 DNA ligase.

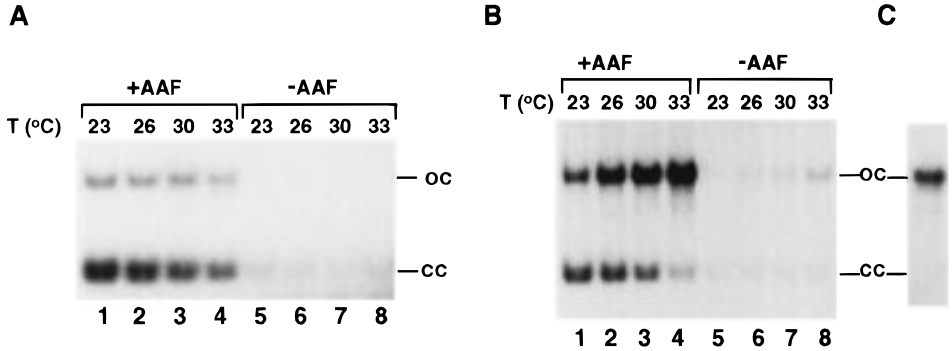

FIGURE 5: DNA ligation during NER in *cdc9-2* mutant extracts. (A) In vitro NER was performed in 300 μg of wild-type SX46A extracts for 2 h at various temperatures as indicated. (B) In vitro NER in *cdc9-2* mutant extracts (300 μg) at various temperatures. +AAF, pUC18 DNA containing AAF adducts; −AAF, undamaged pUC18 DNA. (C) The *cdc9-2* mutant extract (300 μg) was preincubated in NER buffer at 37 °C for 10 min without DNA and [α-^{32}P]dCTP. After adding AAF-pUC18 DNA and [α-^{32}P]dCTP, in vitro NER was then performed at 23 °C for 2 h. Repair products were separated into the ligated (CC) and the unligated form (OC) by electrophoresis on a 1% agarose gel containing 0.5% ethidium bromide and visualized by autoradiography.

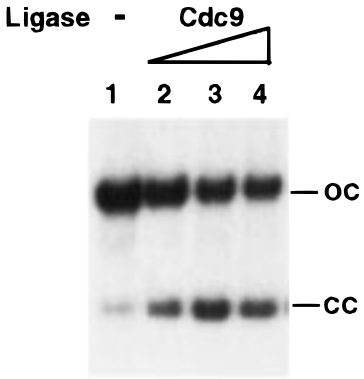

FIGURE 6: In vitro complementation for DNA ligation of NER. To inactivate Cdc9 DNA ligase, *cdc9-2* extracts (300 μg) were preincubated in NER buffer at 37 °C for 7 min without DNA and [α-^{32}P]dCTP. After adding AAF-pUC18 DNA and [α-^{32}P]dCTP, in vitro NER was performed at 23 °C for 2 h without (lane 1) or with the addition of 30, 90, or 150 ng of purified Cdc9 DNA ligase (lanes 2−4, respectively). Repair products were separated into the ligated (CC) and the unligated form (OC) by electrophoresis on a 1% agarose gel containing 0.5% ethidium bromide and visualized by autoradiography.

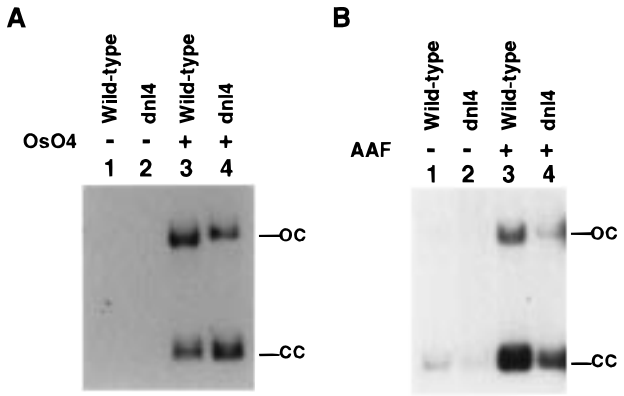

FIGURE 7: DNA ligation during excision repair in *dnl4* mutant extracts. (A) BER in 80 μg of yeast extracts using undamaged (lanes 1 and 2) or OsO4-damaged (lanes 3 and 4) pUC18 DNA. (B) NER in 300 μg of yeast extracts using undamaged (lanes 1 and 2) or AAF-damaged (lanes 3 and 4) pUC18 DNA. In vitro repair was performed at 23 °C for 2 h in extracts of the wild-type BY4741 or its isogenic *dnl4* deletion mutant strains as indicated. Repair products were separated into the ligated (CC) and the unligated form (OC) by electrophoresis on a 1% agarose gel containing 0.5% ethidium bromide and visualized by autoradiography.

DNA Ligase IV Cannot Substitute for Cdc9 Protein in Excision Repair. In contrast to the *CDC9* gene, DNA ligase IV encoded by *DNL4* is not essential for growth (*49*−*51*). To examine if DNA ligase IV plays a role in BER and NER, we performed in vitro repair assays in *dnl4* deletion mutant extracts. DNA ligation in *dnl4* mutant extracts was proficient in both BER (Figure 7A) and NER (Figure 7B) pathways. Lower NER repair synthesis activity in *dnl4* mutant extracts (Figure 7B, lane 4) presumably reflected a less optimal extract preparation, as we occasionally observed with wild-type strains.

Although the *DNL4* gene is intact in *cdc9-2* mutant cells, a total defect of DNA ligation was observed in the mutant extracts for BER and NER (Figures 2, 5B, and 5C). This result can be interpreted as the inability of DNA ligase IV to substitute for Cdc9 protein in excision repair. Alternatively, DNA ligase IV may not be present in sufficient quantity in *cdc9-2* mutant extracts. To definitively determine if DNA

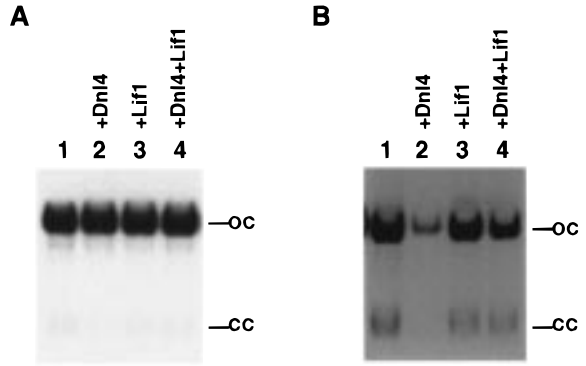

FIGURE 8: Effect of overexpressed DNA ligase IV on excision repair in *cdc9-2* mutant extracts. *DNL4* and *LIF1* were overexpressed in *cdc9-2* mutant cells under the *GAL1* promoter control. Cell extracts were prepared by disruption in a Mini-Beadbeater and subsequent centrifugation (*63*) from cells containing overexpressed Dnl4, Lif1, or both. (A) BER. To inactivate Cdc9 DNA ligase, 80 μg *of cdc9-2* extracts (lane 1) or 60 μg of *cdc9-2* extracts supplemented with 20 μg of cell extracts containing overexpressed Dnl4, Lif1, or both (lanes 2−4, respectively) were preincubated in BER buffer at 37 °C for 15 min without DNA and [α-^{32}P]dTTP. After adding OsO4-pUC18 DNA and [α-^{32}P]dTTP, in vitro BER was performed at 23 °C for 2 h. (B) NER. To inactivate Cdc9 DNA ligase, 300 μg of *cdc9-2* extracts (lane 1) or 250 μg of *cdc9-2* extracts supplemented with 50 μg of cell extracts containing overexpressed Dnl4, Lif1, or both (lanes 2−4, respectively) were preincubated in NER buffer at 37 °C for 7 min without DNA and [α-^{32}P]dTTP. After adding AAF-pUC18 DNA and [α-^{32}P]dTTP, in vitro NER was performed at 23 °C for 2 h. Ligated (CC) and unligated (OC) repair products were separated by electrophoresis and detected by autoradiography.

ligase IV can function in excision repair in the absence of Cdc9 protein, we overexpressed *DNL4* in *cdc9-2* mutant cells. Subsequently, we attempted to complement the defective *cdc9* mutant extracts with the overexpressed DNA ligase IV in vitro. Defective DNA ligation of BER (Figure 8A) and NER (Figure 8B) in *cdc9-2* mutant extracts could not be corrected by overexpression of DNA ligase IV with or without co-overexpression of its interacting partner Lif1 (*67*). Both genes were tagged by six histidine residues at their N-termini to allow for detection by Western blots using a tag-specific monoclonal antibody. Western blot analysis confirmed the expression of Dnl4 and Lif1 proteins in yeast extracts used for the complementation experiments (data not shown). The overexpressed native DNA ligase IV was also unable to complement defective DNA ligation in *cdc9* mutant extracts (data not shown). These results show that the DNA ligation step of excision repair specifically requires Cdc9 DNA ligase and that DNA ligase IV cannot function in excision repair even in the absence of Cdc9 protein.

We consistently observed that repair synthesis of NER was inhibited in *cdc9-2* mutant extracts by DNA ligase IV overexpression (Figure 8B, lane 2). The molecular basis for this effect is not clear. To examine if in vivo NER was interfered by Dnl4 overexpression, we determined the UV sensitivity of wild-type yeast cells containing overexpressed Dnl4. As shown in Figure 9, overexpression of Dnl4 slightly increased the UV sensitivity of wild-type cells. In contrast, overexpression of Dnl4 and Lif1 together slightly enhanced the resistance of wild-type cells to UV radiation, which may reflect stimulated repair by nonhomologous recombination. These observations suggest the possibility that a large excess of DNA ligase IV may interfere with the normal function of

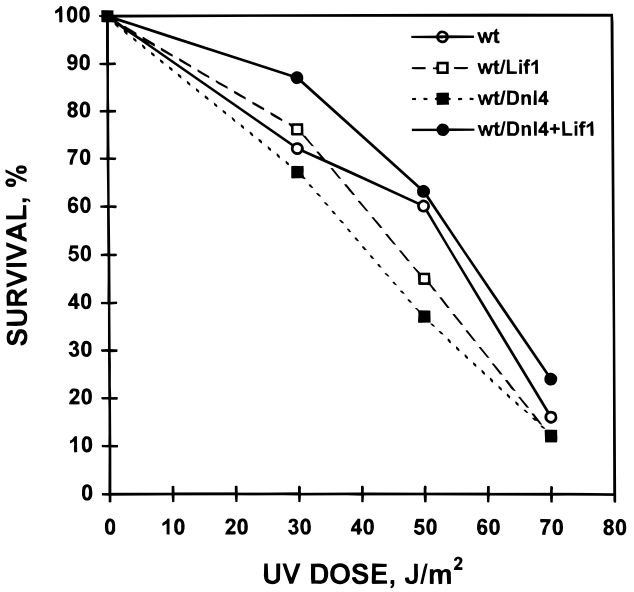

FIGURE 9: Effect of Dnl4 overexpression on UV sensitivity of wild-type yeast cells. Yeast cells were grown at 30 °C to stationary phase in minimum media containing 2% sucrose. The cultures were then diluted 10-fold in YP medium containing 0.5% sucrose and 1.5% galactose (induction medium). Galactose induction of Dnl4 and Lif1 overexpression from the *GAL1* promoter were achieved by growth in the induction medium for 16 h at 30 °C. After dilution, cells were plated onto the induction medium plates. The uncovered plates were irradiated with UV light at the indicated doses. Surviving colonies were counted after incubation at 30 °C for 3 days. Survival rates are expressed relative to those of nonirradiated cells. Yeast strains are the wild-type SX46A (wt) and SX46A containing the overexpression plasmids pEGTh6-Lif1 (wt/Lif1), pEGUh6-Dnl4 (wt/Dnl4), or both plasmids together (wt/Dnl4+Lif1). Results are the averages for duplicate experiments.

NER. Sequestering ligase IV by forming protein complexes with Lif1 may alleviate such an effect of Dnl4.

DISCUSSION

Using in vitro biochemical analyses in yeast cell-free extracts, we have demonstrated that the *CDC9* gene product, the mammalian DNA ligase I homologue (*38*, *62*), is specifically required in both BER and NER pathways. This result provides a biochemical basis for the observed hypersensitivity of *cdc9* mutant cells to DNA damaging agents (*54*−*57*).

Repair of uracil residues and osmium tetroxide-induced damage (mainly thymine glycols) represents two different BER mechanisms. In the former case, DNA incision is catalyzed by the 5′ AP endonuclease (*1*), while in the latter case the incision is mediated by the glycosylase-associated AP lyase 3′ to the AP site (*68*). Thus, regardless of the specific mechanisms involved in the early steps of BER, the ligation step is catalyzed by Cdc9 DNA ligase. Since DNA ligation of NER also depends on Cdc9 protein, it is apparent that the ligase is probably targeted to DNA nicks independent of how the nicks are formed. Nevertheless, we observed that complementation of *cdc9-2* mutant extracts by purified Cdc9 ligase was significantly more effective for BER than for NER (compare Figures 4 and 6, lanes 2−4). Thus, it is plausible that the excision repair mechanisms may influence the ligation reaction to a certain extent. One possibility is that the Cdc9 DNA ligase may function as part of a preformed repair complex during NER. Thus, adding pure Cdc9 protein to *cdc9-2* mutant extracts would not very efficiently complement the defective DNA ligation of NER as we observed. Supporting this possibility, a complex between human DNA ligase I and the replication/repair factor PCNA has been observed (*69*). A much larger "repairosome" complex containing many yeast NER proteins has also been identified and partially purified from normally growing cells in the apparent absence of exogenous DNA damage (*70, 71*). It remains to be determined whether Cdc9 is contained in such a "repairosome".

In the absence of DNA ligation, we showed that repair patch sizes of BER were significantly enlarged. This effect is probably true of NER as suggested by enhanced repair synthesis in *cdc9* mutant extracts which was abrogated by complementation with the purified Cdc9 DNA ligase. Two explanations could be offered. Without DNA ligation, the nicks could be recognized by a DNA polymerse leading to strand displacement or nick translation synthesis. Alternatively, the ligase may be present during DNA repair synthesis through protein−protein interactions such that when a ligatable DNA end is reached repair synthesis is stopped and ligation is initiated. Without the ligase, repair synthesis may continue resulting in enlarged repair patch sizes. Protein interaction between DNA ligase I and the DNA polymerase accessory factor PCNA in humans (*69*) is consistent with such an interpretation.

We show that DNA ligase IV cannot substitute the excision repair function of Cdc9. Additionally, despite its ability to ligate double-stranded DNA breaks in vitro (*62*), Cdc9 cannot substitute the nonhomologous double-strand end-joining function of DNA ligase IV (*49*−*51*). We attribute such in vivo specificity to protein cofactors that may target the ligases to specific sites in DNA. Consistent with this notion, the interacting partner of DNA ligase IV was identified in both yeast (Lif1) and mammals (XRCC4) (*67*, *72*). This factor may efficiently deliver ligase IV to the protein-protected DNA ends, which may not be recognized by DNA ligase I due to the presence of DNA end-binding proteins. Similarly, DNA ligase I may be recruited to DNA replication and excision repair through its interaction with the replication and repair protein PCNA. Thus, multiple DNA ligases in eukaryotes may be a result of functional specificity rather than functional redundancy.

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