A Physical and Functional Interaction between Yeast Pol4 and Dnl4-Lif1 Links DNA Synthesis and Ligation in Nonhomologous End Joining*

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Genetic studies have implicated the Saccharomyces cerevisiae POL4 gene product in the repair of DNA double-strand breaks by nonhomologous end joining. Here we show that Pol4 preferentially catalyzes DNA synthesis on small gaps formed by the alignment of linear duplex DNA molecules with complementary ends, a DNA substrate specificity that is compatible with its predicted role in the repair of DNA double-strand breaks. Pol4 also interacts directly with the Dnl4 subunit of the Dnl4-Lif1 complex via its N-terminal BRCT domain. This interaction stimulates the DNA synthesis activity of Pol4 and, to a lesser extent, the DNA joining activity of Dnl4-Lif1. Notably, the joining of DNA substrates that require the combined action of Pol4 and Dnl4-Lif1 is much more efficient than the joining of similar DNA substrates that require only ligation. Thus, the physical and functional interactions between Pol4 and Dnl4-Lif1 provide a molecular mechanism for both the recruitment of Pol4 to in vivo DNA double-strand breaks and the coupling of the gap filling DNA synthesis and DNA joining reactions that complete the microhomology-mediated pathway of nonhomologous end joining.

Mechanisms for the repair of DNA double-strand breaks (DSBs) can be divided into two classes based on the requirement for DNA sequence homology. In the major homology-dependent pathway, repair involves an intact duplex that is homologous to the broken molecule. This is the major DSB repair pathway in the yeast Saccharomyces cerevisiae and is mediated by members of the RAD52 epistasis group that includes RAD50, RAD51, RAD52, RAD54, RAD55, RAD57, RAD59, MRE11, XRS2, and RDH54/TID1 (1). Alternatively, broken DNA ends are simply brought together, processed, and then ligated by repair mechanisms, known collectively as nonhomologous end joining (NHEJ) (2). Unlike the major recombinational repair pathway that faithfully restores the genetic information, nonhomologous end joining frequently causes genetic alterations that range from the loss or addition of a few nucleotides at the break site to gross rearrangements such as chromosomal translocations (2).

Genetic studies in S. cerevisiae have identified the products of the HDF1, HDF2, RAD50, MRE11, XRS2, DNL4, and LIF1 genes as key players in the major NHEJ pathway (3–14). HDF1 and HDF2 encode subunits of a heterodimeric DNA end-binding complex that is functionally homologous to the mammalian Ku70-Ku80 complex (3–6). Similarly, the Rad50-Mre11-Xrs2 and Dnl4-Lif1 complexes appear to be functional homologs of the hRad50-hMre11-NBS1 (7, 8, 15–21) and DNA ligase IV-XRCC4 complexes (9–13, 22, 23), respectively. Congruent with genetic analysis in yeast, a recent biochemical study has reconstituted DNA end joining with the purified NHEJ factors Hdf1-Hdf2, Rad50-Mre11-Xrs2, and Dnl4-Lif1 and demonstrated functional interactions among these complexes (24). Recently, a novel yeast NHEJ gene, NEJ1, has been identified, but the exact role of this gene product in NHEJ remains to be determined (25–28).

Many of the genetic studies and the biochemical study with purified NHEJ factors have focused on the joining of linear DNA molecules with short complementary single strands at their termini (3, 4, 7–10, 12, 13, 24). However, the majority of DSBs generated by agents such as ionizing radiation will have ends that are neither complementary nor ligatable, indicating that end processing reactions will be critical for the repair of in vivo DSBs by NHEJ. In fact, analysis of DNA molecules repaired by NHEJ has revealed that a favored mode of end processing involves short tracts of DNA sequence homology, so-called microhomologies, close to the break site that presumably facilitate alignment of the DNA ends (14, 29, 30). Following alignment, the processing of DNA ends by nucleases and DNA polymerases to remove noncomplementary nucleotides and fill-in gaps is likely to be required to generate ligatable termini (31, 32).

Interestingly, biochemical studies with purified human Mre11 have shown that this nuclease can act on DNA ends to expose and align microhomologies that can then be ligated (33, 34). However, the efficiency of recircularization of linear plasmid DNA molecules with complementary single-strand ends in vivo is not affected by inactivation of yeast Mre11 nuclease activity (8, 35). This observation suggests that, although the nuclease activity of Mre11 may not be required for nonhomologous end joining, the Rad50-Mre11-Xrs2 complex has another critical role in this repair pathway. Indeed, recent biochemical studies have shown that the Rad50-Mre11-Xrs2 complex has end bridging activity and functionally interacts with the Dnl4-Lif1 complex (24).

Recent genetic studies have shown that pol4 and rad27 strains have no defect in the recircularization of linear plasmid DNA double-strand breaks by nonhomologous end joining. Here we show that Pol4 preferentially catalyzes DNA synthesis on small gaps formed by the alignment of linear duplex DNA molecules with complementary ends, a DNA substrate specificity that is compatible with its predicted role in the repair of DNA double-strand breaks. Pol4 also interacts directly with the Dnl4 subunit of the Dnl4-Lif1 complex via its N-terminal BRCT domain. This interaction stimulates the DNA synthesis activity of Pol4 and, to a lesser extent, the DNA joining activity of Dnl4-Lif1. Notably, the joining of DNA substrates that require the combined action of Pol4 and Dnl4-Lif1 is much more efficient than the joining of similar DNA substrates that require only ligation. Thus, the physical and functional interactions between Pol4 and Dnl4-Lif1 provide a molecular mechanism for both the recruitment of Pol4 to in vivo DNA double-strand breaks and the coupling of the gap filling DNA synthesis and DNA joining reactions that complete the microhomology-mediated pathway of nonhomologous end joining.
DNA molecules with complementary single-strand ends but exhibit reduced joining of linearized plasmid DNA molecules with noncomplementary termini (31, 32). These observations, together with enzymatic properties of the DNA polymerase, Pol4 (32, 36, 37), and the flap endonuclease, Fen-1 (Rad27) (38, 39), suggest that Pol4 and Fen-1 participate in microhomology-mediated NHEJ events requiring gap-filling and nucleolytic processing. In our hands, Pol4 purified to near homogeneity from either pGST-BRCT or pGSTag by glutathione-Sepharose 4B affinity chromatography and gel filtration through a Superdex 75 column. (Fig. 1) (36, 37, 40–43). Within this family, mammalian Pol β catalyzes gap filling DNA synthesis in base excision repair (43, 44), whereas terminal transferase adds nucleotides in a template-independent manner during V(D)J recombination (45). The cellular functions of Pol μ and Pol λ are less well understood (40–42). In this study we describe a functional interaction between Pol4 and Dnl4-Lif1 that links the gap-filling and ligation steps of NHEJ.

MATERIALS AND METHODS

Plasmid Construction—The S. cerevisiae POL4 open reading frame was amplified from BJ5464 genomic DNA by the polymerase chain reaction. After the DNA sequence of the amplified product was verified, it was ligated into the Escherichia coli expression plasmids pGStag (46) and pET28b (Novagen) to generate the plasmids pGST-Pol4 and pET28b-Pol4, which express Pol4 as a glutathione S-transferase (GST) fusion and His-tagged polypeptide, respectively. Using a similar strategy we constructed the plasmids pGST-Pol4BRCT and pET28b-Pol4BRCT, which encode tagged versions of Pol4 lacking the N-terminal 112 amino acids encompassing the breast cancer susceptibility gene 1 C terminus (BRCT) domain (see Fig. 1) that was originally identified in the breast cancer susceptibility gene BRCA1 (47, 48) and the plasmid pGSt-Pol4BRCT that encodes the N-terminal 112-amino acid BRCT domain as a GST fusion protein.

Purification of His-tagged Pol4 and Pol4BRCT—Overnight cultures (100 ml) of E. coli BL21(DE3) cells harboring either pET28b-Pol4 or pET28b-Pol4BRCT were inoculated into 10 liters of LB medium containing kanamycin (0.025 mg/ml) and chloramphenicol (0.034 mg/ml) and grown at 37 °C. At an absorbance of 600 nm of 0.5, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM, and growth was continued at 25 °C for 4 h. The cells were harvested by centrifugation, flash frozen, and stored at −80 °C. Frozen cells were resuspended in 40 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 300 mM NaCl, 10% glycerol, 10 mM mercaptoethanol, 0.1% Nonidet P-40, 1 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine HCl, 1 mM leupeptin, 2 µM aprotinin, and 1 µg/ml pepstatin) and lysed by sonication. After centrifugation at 15,000 rpm for 20 min at 4 °C, the cleared lysate was supplemented with imidazole to a final concentration of 20 mM prior to incubation with 1 ml of nickel-nitrioltriacetic acid-agarose beads (Qiagen) for 2 h at 4 °C. The beads were collected by centrifugation and then washed extensively with lysis buffer containing 40 mM imidazole. His-tagged Pol4 polypeptides were eluted with lysis buffer containing 250 mM imidazole and then further purified to near homogeneity by Resource Q and Resource S column chromatography. Approximately 40 µg of Pol4 and 30 µg of Pol4BRCT were obtained from 2-liter cultures. Protein concentrations were measured by the Bradford assay (49) using bovine serum albumin as the standard.

Purification of GST Fusion Proteins—An overnight culture (1 liter) of E. coli BL21 (DE3) cells harboring either pET28b-Pol4 or pGST-Pol4BRCT were inoculated into 10 liters of LB medium containing ampicillin (0.1 mg/ml) and chloramphenicol (0.034 mg/ml) and grown at 37 °C. When the absorbance at 600 nm reached 0.6, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.5 mM, and growth was continued at 25 °C for 2 h. The cells were harvested by centrifugation, flash frozen, and stored at −80 °C. The frozen cells were resuspended in 40 ml of lysis buffer and lysed by sonication. After centrifugation, GST fusion proteins were purified from the clarified lysate by glutathione-Sepharose 4B affinity chromatography, gel filtration through a Superdex 75 column, and Resource S ion exchange chromatography. Approximately 30 µg of nearly homoogenous GST-Pol4 was obtained from the 1-liter culture. A similar quantity of GST-Pol4BRCT was obtained, but this preparation contained two proteolytic fragments of the fusion protein. GST-BRCT and GST were purified to near homogeneity from E. coli BL21 (DE3) cells harboring either pGST-BRCT or pGSTtag by glutathione-Sepharose 4B affinity chromatography and gel filtration through a Superdex 75 column.

DNA Polymerase Assays—Three oligonucleotides, 34M (5′-TCCCTCA-AGAGTGACCTGAGCATGCAAGTGTG-3′, 34-mer), 5′-labelled 34M (5′-CACCTGCTGATGCTGCTGG-3′, 17-mer), and 34M (5′-CTACGAGAAGAC-3′, 18-mer) were annealed by incubation at 70 °C for 10 min, 50 °C for 10 min, 40 °C for 10 min, 18 °C for 10 min, and then on ice for 5 min to generate a linear duplex with a single nucleotide gap.

Linear duplexes with complementary single-strand ends were constructed by annealing the following oligonucleotides: 50M (5′-GTACAAAGTTGAGCTTACTACGGCGCTGG-3′, 50-mer) annealed to 41M (5′-GGACGCAGACCCAGCGATGAG-3′, 43-mer) and 34M (5′-TCCCTCAAGAGTGACCTGAGCATGCAAGTGTG-3′, 34-mer) assembled to 5′-labelled 34M (5′-CACCTGCTGATGCTGCTGG-3′, 17-mer) and 34M (5′-GTCAGAGAAGAC-3′, 18-mer) annealed by incubation at 70 °C for 10 min, 50 °C for 10 min, 40 °C for 10 min, 18 °C for 10 min, and then on ice for 5 min to generate an RNA ligase I complex with a single nucleotide gap.

To detect a direct interaction between purified Pol4-Lif1 complex and Pol4, beads (10 µl) with GST-Pol4, GST-Pol4BRCT, or GST as the ligand were incubated with 0.5 µg of purified Dnl4-Lif1 complex (24) in Buffer A containing 2% bovine serum albumin in a final volume of 20 µl at 4 °C for 4 h. After collection by centrifugation, the beads were washed extensively with Buffer A and then incubated for 15 min at 25 °C in 20-µl reaction mixtures containing 60 mM Tris-HCl, pH 8.0, 10 mM MgCl2, 5 mM dithiothreitol, 50 µg/ml bovine serum albumin, and 0.5 µCi of [35S]ATP (3000 Ci/mmol; Amersham Biosciences). The reactions were stopped by the addition of SDS sample buffer (51). To detect a direct interaction between purified Dnl4-Lif1 complex and Pol4 and Dnl4-Lif1, GST-Pol4 or GST as the ligand were incubated with 0.5 µg of purified Dnl4-Lif1 complex (24) in Buffer A containing 2% bovine serum albumin in a final volume of 20 µl at 4 °C for 4 h. After collection by centrifugation, the beads were washed extensively with Buffer A containing 150 mM NaCl and then incubated with 1 µCi of [35S]ATP. To further characterize the interaction between Dnl4 and Pol4, beads (10 µl) with the indicated ligand were incubated with labeled ATP in vitro translated Dnl4 supplemented with 2% bovine serum albumin (22 µl) for 4 °C for 4 h. After centrifugation, the supernatant was removed, and the beads were washed with Buffer A. The beads were resuspended in 20 µl of SDS sample buffer to yield the eluate (E). After electrophoresis through a 7.5% SDS-polyacrylamide gel (51), labeled Dnl4 was detected using a PhosphorImager screen.

DNA Pull-down Assays—Oligodeoxynucleotides, 34M (5′-TCCCTCAAGAGTGACCTGAGCATGCAAGTGTG-3′, 34-mer), 5′-labelled 34M (5′-CACCTGCTGATGCTGCTGG-3′, 17-mer), and 16M (5′-CTACGAGAAGAC-3′, 18-mer) were annealed to 41M (5′-GGACGCAGACCCAGCGATGAG-3′, 43-mer) annealed to 5′-labelled 30M (5′-GTCAGAGAAGAC-3′, 18-mer) and 34M (5′-GTCAGAGAAGAC-3′, 18-mer) annealed to 41M (5′-GGACGCAGACCCAGCGATGAG-3′, 43-mer). Alignment of the complementary single strands generates a nonillustrable gap in the DNA duplex (see Fig. 2C). A similar strategy was used to construct pairs of duplexes with single-strand extensions that, when aligned, give differently sized gaps with and without single-strand flaps (see Fig. 3). Unless indicated, DNA concentrations are expressed as DNA molecules.

Equal amounts of the labeled and unlabeled duplexes (100 nM) were incubated by sonication for 30 s at 25 °C. The resulting duplexes were resuspended in 35 mM Tris-HCl, pH 7.5, 10 mM MgCl2, 0.05 mM of each of the four dNTPs, and 1 mM ATP at 25 °C. The reactions were stopped by the addition of gel loading buffer (95% (v/v) formamide, 0.09% (w/v) bromophenol blue, and 0.09% (w/v) xylene cyanol). After separation by denaturing gel electrophoresis, labeled DNA was visualized. The nucleotide composition in the dried gel were detected and quantitated by PhosphorImager analysis.

Ligation Assay—Linear duplexes with complementary single-strand ends were constructed by annealing the following pairs of oligonucleotides; 50M annealed to 5′-phosphorylated 41M and 5′-phosphorylated
Completion of DNA End Joining by Pol4 and Dnl4-Lif1

RESULTS

DNA Synthesis Activity of Pol4—Studies on the efficiency of in vivo recircularization of linear plasmid DNA molecules with noncomplementary single-strand ends have implicated Pol4 in a subset of DNA joining events that involve end processing (32). Full-length and truncated versions of Pol4 lacking the N-terminal BRCT domain (Fig. 1) were expressed as His-tagged polypeptides in E. coli and then purified to near homogeneity (Fig. 2A, lanes 1 and 2). The DNA polymerase activity of Pol4 was compared with that of Pol β. As shown in Fig. 2B, Pol4 and Pol β have similar gap filling activity on a DNA substrate containing a single-nucleotide gap. In contrast, Pol4 was at least 4-fold more effective at filling in a single-nucleotide gap generated by the alignment of linear duplex DNA molecules with complementary ends than Pol β (Fig. 2C, compare lanes 4 and 5 with lanes 6 and 7). Moreover, it appears that this substrate specificity is an intrinsic property of the Pol4 catalytic domain because deletion of the BRCT domain had no significant effect on DNA synthesis activity (Fig. 2C, compare lanes 2 and 3 with lanes 4 and 5).

Next we examined the DNA synthesis activity of Pol4 on differently sized gaps generated by the alignment of linear duplex DNA molecules with complementary ends (Fig. 3). There was an inverse relationship between gap size and the amount of fully filled-in product (Fig. 3). The presence or absence of a phosphate group at the 5′ end of the gaps did not affect Pol4 DNA synthesis activity (data not shown). DNA synthesis by Pol4 was distributive, with the enzyme preferentially adding a single nucleotide, even with larger gaps. Although a ladder of products corresponding to incompletely filled gaps were detectable, the fully filled-in product was the most abundant species. With gaps sizes greater than a single nucleotide, strand displacement synthesis of one to two nucleotides occurred (Fig. 3, compare lanes 2 and 3). Interestingly, the presence of a short 5′ flap increased the amount of strand displacement DNA synthesis about 2-fold (Fig. 3, compare lane 3 with lanes 5 and 6). In summary, the DNA substrate specificity of Pol4 is compatible with its in vivo role in DSB repair because small gaps, possibly with noncomplementary flaps, are predicted intermediates in the subpathway of NHEJ that involves microhomology-mediated alignment of DNA ends (31, 32).

Pol4 Interacts with the Dnl4-Lif1 Complex via the Dnl4 Subunit—To detect associations between Pol4 and NHEJ factors, we expressed and purified Pol4 as a GST fusion protein (Fig. 4A, lane 2). In affinity chromatography experiments with extracts from a wild type yeast strain, no specific binding of the core NHEJ factors, Hdf1-Hdf2, Rad50-Mre11-Xrs2, and Dnl4-Lif1, to GST-Pol4 beads was observed (data not shown). We
suspected that associations were not detected because of the low endogenous levels of NHEJ factors. Therefore, we performed a similar experiment with an extract from a strain overexpressing Dnl4-Lif1 (24) and observed the specific binding of Dnl4 to the GST-Pol4 resin (Fig. 4B). To determine whether there is a direct interaction between Pol4 and Dnl4-Lif1, glutathione beads with GST-tagged full-length Pol4 (Fig. 4A, lane 2), GST-tagged Pol4 lacking the N-terminal BRCT domain (Fig. 4A, lane 4), or GST (Fig. 4A, lane 1) as the ligand were incubated with purified Dnl4-Lif1 that was then labeled by adenylation (24). The binding of Dnl4-Lif1 to the GST-Pol4 beads but not to either the GST-Pol4ABRCT or the GST beads (Fig. 4C) demonstrates that these protein factors interact directly and suggests that this interaction is mediated by the N-terminal BRCT domain of Pol4. In similar experiments, we did not observe specific binding of purified Lif1 to GST-Pol4 beads (data not shown), suggesting that the interaction is either mediated by Dnl4 or requires complex formation between Dnl4-Lif1. Because Lif1 is required for Dnl4 stability in yeast cells (9, 24), we examined the interaction of labeled in vitro translated Dnl4 with Pol4. Dnl4 bound to glutathione beads with either GST-Pol4 (Fig. 4A, lane 2) or GST fusion protein with only the N-terminal BRCT domain of Pol4 (Fig. 4A, lane 3) as the ligand but did not bind to glutathione beads with GST (Fig. 4A, lane 1) as the ligand (Fig. 4D). In similar experiments, in vitro translated Lif1 did not bind specifically to Pol4 beads (data not shown). Thus, we conclude that Pol4 interacts directly with the Dnl4 subunit of the Dnl4-Lif1 complex via its N-terminal BRCT motif.

**Dnl4-Lif1 Specifically Stimulates the DNA Polymerase Activity of Pol4**—To elucidate the functional consequences of the interaction between Pol4 and Dnl4-Lif1, we examined the effect of Dnl4-Lif1 on gap filling DNA synthesis catalyzed by Pol4. As shown in Fig. 5A, Dnl4-Lif1 greatly stimulates DNA synthesis
FIG. 5. The effect of Dnl4-Lif1 on DNA synthesis by Pol4 at a one-nucleotide gap. A, effect of Dnl4-Lif1 concentration on DNA synthesis by Pol4 at a one-nucleotide gap generated by the alignment of linear duplex DNA molecules with complementary ends. Lanes 1 and 8, DNA substrate alone. Pol4 (7 nM) and Pol4ΔBRCT (7 nM) and the indicated DNA duplexes (100 nM of each) that, when aligned, form a one-nucleotide gap in the labeled bottom strand were incubated at 25 °C for 2 h with increasing amounts of Dnl4-Lif1 where indicated. Lanes 2 and 9, no Dnl4-Lif1; lanes 3 and 10, 1.25 nM; lanes 4 and 11, 2.5 nM; lanes 5 and 12, 5 nM; lanes 6 and 13, 10 nM; lanes 7 and 14, 20 nM. After separation by denaturing gel electrophoresis, labeled oligonucleotides in dried gels were detected and quantitated by PhosphorImager analysis. The arrows indicate the positions of the one-nucleotide fill-in product (F, 51-mer) and substrate (S, 50-mer). The results of three independent experiments are shown graphically.

B, time course of DNA synthesis by Pol4 and Pol4ΔBRCT in the presence of Dnl4-Lif1. Dnl4-Lif1 (40 nM) and the DNA duplexes (100 nM of each) described above were incubated at 25 °C with either Pol4 (7 nM, lanes 1–4) or Pol4ΔBRCT (7 nM, lanes 6–9) for the indicated times. Lane 5, DNA substrate alone. After separation by denaturing gel electrophoresis, labeled oligonucleotides in dried gels were detected and quantitated by PhosphorImager analysis. The arrows indicate the positions of the one-nucleotide fill-in product (F, 51-mer) and substrate (S, 50-mer). The results of the experiment are also shown graphically.

C, effect of Dnl4-Lif1 concentration on DNA synthesis by Pol4 at a one-nucleotide gap within a linear duplex DNA molecule. Lane 1, DNA substrate alone. Pol4 (7 nM) and Pol4ΔBRCT...
Completion of DNA End Joining by Pol4 and Dnl4-Lif1

by Pol4 on nonligatable gaps formed by the alignment of partial duplex oligonucleotides in a concentration-dependent manner. Notably, Dnl4-Lif1 is much more effective at stimulating full-length Pol4 compared with either a truncated version of Pol4 lacking the BRCT motif (Fig. 5A) or Pol β (data not shown). At a ratio of about 1:1, Dnl4-Lif1 increased Pol4-catalyzed DNA synthesis by 5–6-fold. When measured as a function of time, Dnl4-Lif1 increased both the rate and extent of Pol4-mediated DNA synthesis (Fig. 5B). Again the effect on DNA synthesis was dependent on the BRCT domain of Pol4. These results demonstrate that the stimulation of Pol4 DNA synthesis activity is mediated, at least in part, by the protein-protein interaction between Dnl4-Lif1 and Pol4. Dnl4-Lif1 also stimulated gap filling DNA synthesis by Pol4 on a linear duplex containing a single nucleotide nonligatable gap in a BRCT domain-dependent manner (Fig. 5C). Because the stimulatory effect of Dnl4-Lif1 on DNA synthesis by Pol4 was similar whether the gap was within a linear DNA duplex or formed by the alignment of linear duplex DNA molecules with complementary ends (Fig. 5, A and C), it appears that the stimulation of Pol4 activity by Dnl4-Lif1 is mediated by mechanisms other than DNA end alignment. Finally, we examined whether the effect of Dnl4-Lif1 on the DNA synthesis activity of Pol4 was influenced by either increased gap size or the presence of a 5′ flap. Dnl4-Lif1 stimulated the DNA synthesis activity of Pol4 on cohesive ended DNA molecules that when aligned form a three-nucleotide gap either without (Fig. 6A) or with a three-nucleotide flap (Fig. 6B) but did not significantly alter the distribution of reaction products.

Pol4 Specifically Stimulates the DNA Joining Activity of Dnl4-Lif1—In assays with partial duplex oligonucleotides that when aligned form a ligatable structure, Dnl4-Lif1 exhibited a low activity that was stimulated by intact Pol4 (Fig. 7A) but not by the truncated version of Pol4 lacking the N-terminal BRCT domain. At a ratio of about 1:1, Pol4 increased Dnl4-Lif1-catalyzed DNA joining by about 2-fold. These results demonstrate that the stimulation of DNA joining by Dnl4-Lif1 is mediated, at least in part, by the protein-protein interaction between Dnl4-Lif1 and Pol4, but the magnitude of this effect is less than that of Dnl4-Lif1 on Pol4 DNA synthesis activity.

These observations suggest that the interaction between Pol4 and Dnl4-Lif1 co-ordinates the gap filling DNA synthesis and ligation reactions that complete NHEJ. To provide support for this model, we compared the amount of ligated product produced by Pol4 and Dnl4-Lif1 in assays with oligonucleotide duplexes that when aligned either form a ligatable nick (Fig. 7A) or a single nucleotide gap (Fig. 7B). As shown in Fig. 7C, joining of the DNA substrate that requires both DNA synthesis and ligation (15 fmol) was significantly higher than the joining of the DNA substrate requiring only ligation (0.5–1 fmol). This synergistic effect indicates that the interaction between Pol4 and Dnl4-Lif1 not only enhances the catalytic activity of both these enzymes but co-ordinates their action, ensuring the efficient hand-over of pathway intermediates.

DISCUSSION

The preferred pathway for the repair of in vivo DSBs by NHEJ appears to involve microhomologies that are presumably revealed by nucleolytic digestion (14, 29, 30, 34). After the alignment of short complementary sequences, it is likely that single-strand flaps are removed and gaps are filled in to generate ligatable structures (31, 32). Our biochemical studies have revealed differences in the DNA substrate specificity of the catalytic domain of Pol4 compared with Pol β, the prototypic member of the Pol X DNA polymerase family (Fig. 1) (36, 37, 40–43) that are compatible with the predicted role of the POLA gene product in end joining events that involve gap filling DNA synthesis (32). Specifically, Pol4 preferentially acts upon short gaps formed by the alignment of linear duplexes with complementary single-strand ends.

Hdf1-Hdf2, Rad50-Mre11-Xrs2, and Dnl4-Lif1 complexes have been identified as critical factors in the major NHEJ pathway in yeast (3–14). Rad50-Mre11-Xrs2 stimulates DNA joining both by aligning DNA ends and by recruiting Dnl4-Lif1 via a direct interaction between Xrs2 and Lif1 (24). Furthermore, DNA end joining mediated by these factors is dependent on Hdf1-Hdf2 at physiological salt concentrations, suggesting
DNA duplexes (100 nM of each) that, when aligned, form a ligatable nick concentration on Dnl4-Lif1 activity. Dnl4-Lif1 (40 nM) and the indicated DNA synthesis and ligation by Pol4 and Dnl4-Lif1.

**FIG. 7.** Effect of Pol4 on DNA joining by Dnl4-Lif1; coupled DNA synthesis and ligation by Pol4 and Dnl4-Lif1. A, effect of Pol4 concentration on Dnl4-Lif1 activity. Dnl4-Lif1 (40 nM) and the indicated DNA duplexes (100 nM of each) that, when aligned, form a ligatable nick in the labeled bottom strand were incubated at 25 °C for 2 h with increasing amounts of Pol4, where indicated as described under "Materials and Methods." Lane 1, no protein; lane 2, no Pol4; lane 3, 11 nM Pol4; lane 4, 22 nM Pol4; lane 5, 44 nM Pol4; lane 6, 88 nM Pol4. After separation by denaturing gel electrophoresis, labeled oligonucleotides in dried gels were detected and quantitated by PhosphorImager analysis. The results of the experiment are shown graphically. B, effect of DNA synthesis on DNA joining. Pol4 (14 nM) and Dnl4-Lif1 (40 nM) were incubated at 25 °C for 2 h with the one-nucleotide gap substrate described for 4 and the ligatable substrate described for A as indicated. After separation by denaturing gel electrophoresis, labeled oligonucleotides in dried gels were detected and quantitated by PhosphorImager analysis. The results of the experiment are shown graphically. C, effect of DNA synthesis on DNA joining by Dnl4-Lif1. Pol4 and Dnl4-Lif1 (40 nM) were incubated at 25 °C for 2 h with the one-nucleotide gap substrate described for B and the ligatable substrate described for A as indicated. After separation by denaturing gel electrophoresis, labeled oligonucleotides in dried gels were detected and quantitated by PhosphorImager analysis. The results of the experiment are shown graphically.

Analysis of the assembly of NHEJ factors at in vivo DSBs by chromatin immunoprecipitation has shown that the recruitment of Dnl4 to DSBs is dependent upon Hdf1-Hdf2 and Lif1 (55). Because inactivation of POL4 has no effect on the repair of DNA breaks with cohesive ends (32), it appears that the Hdf1-Hdf2, Rad50-Mre11-Xrs2, and Dnl4-Lif1 factors can form a functional nucleoprotein complex in the absence of Pol4. Thus, the recruitment of Pol4 to in vivo DSBs may be mediated via its interaction with Dnl4. Our biochemical studies demonstrating that the BRCT domain of Pol4 is critical for the interaction with Dnl4-Lif1 but not for Pol4 DNA synthesis activity provide a molecular explanation for genetic studies showing that N-terminal deletions inactivate Pol4 function in vivo (32). Interestingly, several members of the Pol X family have an N-terminal BRCT domain (Fig. 1) (36, 37, 40–42, 45, 47, 48), suggesting that this may be a common mechanism for recruiting these enzymes to their in vivo substrates. This idea is supported by a recent report describing interactions between the human Pol X family members, Pol µ and terminal transferase, and DNA ligase IV-XRCC4 (56).

In summary, Pol4 efficiently fills in short gaps formed by the alignment of complementary single strands at the ends of duplex DNA and is specifically stimulated by Dnl4-Lif1. It will be interesting to determine whether end bridging by Rad50-Mre11-Xrs2 (24) will further stimulate the coupled DNA synthesis and ligation reaction mediated by Pol4 and Dnl4-Lif1.
Moreover, genetic studies indicate that Pol4 participates in end joining events that require nucleolytic processing (32). Future studies are necessary to determine whether Pol4 specifically associates with and/or modulates the activity of nucleases such as Mre11 (33, 34) and Fen-1 (31) within the nucleoprotein structure formed by NHEJ factors.

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