The repair of DNA double-strand breaks is critical for maintaining genetic stability. In the non-homologous end-joining pathway, DNA ends are brought together by end-bridging factors. However, most in vivo DNA double-strand breaks have terminal structures that cannot be directly ligated. Thus, the DNA ends are aligned using short regions of sequence microhomology followed by processing of the aligned DNA ends by DNA polymerases and nuclease to generate ligatable termini. Genetic studies in Saccharomyces cerevisiae have implicated the DNA polymerase Pol4 and the DNA structure-specific endonuclease FEN-1(Rad27) in the processing of DNA ends to be joined by Dnl4/Lif1. In this study, we demonstrated that FEN-1(Rad27) physically and functionally interacted with both Pol4 and Dnl4/Lif1 and that together these proteins coordinate processed and joined DNA molecules with incompatible 5' ends. Because Pol4 also interacts with Dnl4/Lif1, our results have revealed a series of pair-wise interactions among the factors that complete the repair of DNA double-strand breaks by non-homologous end-joining and provide a conceptual framework for delineating the end-processing reactions in higher eukaryotes.

The repair of DNA double-strand breaks (DSBs) is critical for the maintenance of genomic integrity and stability. There are two main DSB repair pathways in eukaryotes: homologous recombination and non-homologous end joining (NHEJ) (1). In the error-free homologous recombination pathway, an intact homologous DNA duplex acts as a template for repair, resulting in the accurate restoration of the broken DNA molecule. By contrast, in NHEJ, the two broken ends are simply rejoined to each other in a process that frequently causes loss and/or gain of nucleotides at the break site and occasionally results in the joining of previously unlinked DNA molecules. Notably, defects in the repair of DSBs by either homologous recombination or NHEJ have been linked with cancer predisposition (2, 3).

Studies with mammalian cells identified the DNA-dependent protein kinase (DNA-PK), which is composed of the DNA end binding Ku70/Ku80 heterodimer and the DNA-PK catalytic subunit, and the DNA ligase IV/XRCC4 complex as key NHEJ factors (4). In Saccharomyces cerevisiae, Hdf1/Hdf2 and Dnl4/Lif1 are the functional homologs of Ku70/Ku80 and DNA ligase IV/XRCC4, respectively (4). Although yeast lacks a DNA-PK catalytic subunit homolog, genetic studies have revealed that the Rad50/Mre11/Xrs2 complex is a key player in NHEJ (4–7). Using purified protein complexes, it has been shown that the Rad50/Mre11/Xrs2 complex has robust end-bridging activity and specifically stimulates intermolecular DNA joining by Dnl4/Lif1 (8). Furthermore, efficient intermolecular DNA joining mediated by the Rad50/Mre11/Xrs2 and Dnl4/Lif1 complexes was dependent upon Hdf1/Hdf2 at physiological salt concentrations (8). Based on these results, it seems likely that Hdf1/Hdf2 enhances the recruitment of the Rad50/Mre11/Xrs2 end-bridging complex to DNA ends and that Dnl4/Lif1 joins the resultant juxtaposed DNA ends.

The in vitro reconstitution studies described above used linear DNA molecules with cohesive single-strand ends generated by restriction endonucleases. However, the termini of the majority of DSBs generated in vivo by agents such as ionizing radiation will be noncomplementary and may also have damaged termini. Analysis of DNA molecules repaired by NHEJ has shown that short complementary sequences, so-called microhomologies, are preferentially used for alignment of broken DNA ends (7, 9, 10). This suggests that, after DNA ends are brought together, the DNA regions next to the ends are compared in some manner for short complementary sequences. A prediction of this mechanism is that the aligned DNA molecules will have noncomplementary terminal nucleotides that will need to be removed and the resultant gaps filled in before ligation.

The conserved eukaryotic DNA structure-specific endonuclease FEN-1(Rad27), is involved in DNA replication, DNA repair, and the maintenance of genome stability (11–16). FEN-1 is a member of the growing family of proteins that bind to the interdomain connector loop of proliferating cell nuclear antigen, which functions as a ring-shaped homotrimer (17–20). This interaction with proliferating cell nuclear antigen, which involves residues in addition to the conserved proliferating cell nuclear antigen binding motif, stimulates the DNA structure-specific endonuclease activity of FEN-1 and presumably contributes to the participation of FEN-1 in Okazaki fragment maturation and long patch base excision repair (13, 17, 21–23). Although genetic studies in yeast indicate that FEN-1(Rad27) is involved in the processing of aligned NHEJ intermediates with 5' flaps (16, 24), the protein-protein interactions that specifically recruit FEN-1 to the NHEJ intermediates have not been identified.

Yeast Pol4, a member of the Pol X family of DNA polymerases has been implicated in NHEJ events that involve gap-filling DNA synthesis (24). In accordance with these observations, we recently characterized a physical interaction between Pol4 and Dnl4/Lif1 that coordinates the DNA synthesis
and ligation reactions that complete NHEJ (25). Furthermore, there is evidence linking the mammalian Pol X family members, Pol μ, Pol λ, and terminal deoxynucleotidyl transferase with DNA ligase IV-mediated end-joining, indicating that the functional interaction between the gap-filling and DNA-joining enzymes in NHEJ is conserved among eukaryotes (26, 27). An interesting feature of the Pol4-mediated NHEJ events is that, although they also required removal of 5’ or 3’ terminal mismatches, purified preparations of Pol4 did not have nuclease activity (24).

In this study, we examined the role of FEN-1(Rad27) in the processing of DNA substrates that mimic NHEJ intermediates with non-complementary 5’ ends. We demonstrate that FEN-1(Rad27) physically and functionally interacts with both Pol4 and the Dnl4 subunit of the Dnl4/Lif1 complex. Furthermore, we show that these factors act in a coordinated manner to

**Fig. 1.** FEN-1(Rad27) interacts with the Dnl4 subunit of Dnl4/Lif1. **A**, left, after separation by SDS-PAGE, His-tagged FEN-1(Rad27) (1 μg) was stained with Coomassie blue (lane 1). Lane 2, labeled in vitro-translated FEN-1(Rad27) detected by PhosphorImager analysis (Molecular Dynamics). The positions of molecular mass standards are indicated at left. Right, extracts from yeast strain BJ5464 harboring the plasmids pADH-Dnl4 and pYES-Lif1 were incubated with in vitro-translated FEN-1(Rad27). These mixtures were then incubated with either pre-immune serum (lane 3) or polyclonal antiserum against Lif1 (lane 4) as described under “Experimental Procedures.” **B**, labeled in vitro-translated Dnl4 (top) and Lif1 (bottom) were incubated with nickel-nitrilotriacetic acid agarose beads in either the presence or absence of His-tagged FEN-1(Rad27). After centrifugation, the supernatants (S) were removed. The beads were washed and then treated with 250 mM imidazole to elute bound proteins (E). The eluate (E) and supernatant (S, 1/10 of supernatant) fractions were separated by SDS-PAGE. Labeled polypeptides were detected by PhosphorImager analysis (Molecular Dynamics).

**Fig. 2.** FEN-1(Rad27) interacts with Pol4. **A**, purified His-tagged FEN-1(Rad27) (7 μg) and glutathione 4B-Sepharose beads were incubated with either GST or GST-Pol4 (10 μg of each). After centrifugation, the supernatants (S) were removed. The beads were washed and then treated with 250 mM imidazole to elute bound proteins (E). The eluate (E) and supernatant (S, 1/10 of supernatant) fractions were separated by SDS-PAGE. Recombinant His-tagged FEN-1(Rad27) was detected by immunoblotting with an antibody specific for the oligo-histidine tag. **B**, labeled in vitro-translated Pol4, Pol4ΔBRCT, and the BRCT domain of Pol4 were incubated with nickel nitrilotriacetic acid agarose beads either in the presence (FEN-1(Rad27)) or absence (C) of His-tagged FEN-1(Rad27). After centrifugation, the supernatants (S) were removed. The beads were washed and then treated with 250 mM imidazole to elute bound proteins (E). The eluate (E) and supernatant (S, 1/10 of supernatant) fractions were separated by SDS-PAGE. Labeled polypeptides were detected by PhosphorImager analysis (Molecular Dynamics). The positions of molecular mass standards are indicated at left.
process and join DNA molecules with non-complementary 5′ ends. Together these results provide novel mechanistic insights into the end processing reactions that complete the repair of DSBs by NHEJ.

EXPERIMENTAL PROCEDURES

Coupled in Vitro Transcription/Translation—Labeled FEN-1(Rad27), Pol4, Pol4ΔBRCT, the Pol4 BRCT domain, Dnl4, and Lif1 were synthesized from pET-FEN-1(Rad27), pGADT7-Pol4, pGADT7-

Pol4ΔBRCT, pGADT7-BRCT, pBKS-Dnl4, and pBKS-Lif1 templates using T7 RNA polymerase and [35S]methionine (Amersham Biosciences) in the TNT quick-coupled transcription/translation system (Promega). Labeled proteins were partially purified by ammonium sulfate precipitation (28) and then resuspended in 50 μl of Buffer A (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine HCl, 1 μg/ml leupeptin, 2 μg/ml aprotinin, and 1 μg/ml pepstatin).

Co-immunoprecipitation—Yeast cell extracts were prepared from the yeast strain B45464 harboring the plasmids pADH-Dnl4 and pYES-Lif1.
amphenicol (0.034 mg/ml) and grown at 37 °C. At an absorbance of 0.5
Luria-Bertani medium containing kanamycin (0.025 mg/ml) and chlor-
(BDE3) cells harboring pET-FEN-1(Rad27) were inoculated into 1 liter of
incubated with FEN-1(Rad27) and, where indicated, either Dnl4/Lif1 or
eluted with lysis buffer containing 250 mM imidazole and then further
purified from a 1-liter culture. His-tagged Pol4 and the Dnl4/Lif1 complex were
purified to near homogeneity by Resource Q and Resource S column
chromatography. Approximately 0.9 mg of FEN-1(Rad27) was obtained
raised against Lif1 (8) or pre-immune serum at 4 °C overnight. Protein
A-Sepharose beads (10 μl) were added and the incubation continued for 1 h.
After collection by centrifugation, the beads were washed extensively
with Buffer A and then were resuspended in 20 μl of SDS sample buffer.
After electrophoresis through a 7.5% SDS-polyacrylamide gel
(DE3) cells harboring pET-FEN-1(Rad27) was detected in the dried gel by Phosphor-
Imager analysis (Molecular Dynamics).

**Purification of Recombinant His-tagged Proteins**—The FEN-
1(Rad27) expression plasmid pET-FEN-1(Rad27) was a gift from Dr.
Binghui Shen. Overnight cultures (100 ml) of *Escherichia coli* BL21 (DE3) cells harboring pET-FEN-1(Rad27) were inoculated into 1 liter of
Luria-Bertani medium containing kanamycin (0.025 mg/ml) and chlor-
amphenicol (0.034 mg/ml) and grown at 37 °C. At an absorbance of 0.5
at 37 °C. An absorbance of 0.5
600 nm, isopropyl β-D-thiogalactoside (IPTG) was added to a final
centration of 0.05 mM, and growth continued at 25 °C for 4 h. Cells were
harvested by centrifugation, flash-frozen, and stored at −80 °C.
Frozen cells were resuspended in 20 ml of lysis buffer (50 mM Tris-HCl,
pH 7.5, 300 mM NaCl, 10% glycerol, 10 mM 2-mercaptoethanol, 0.1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine
HCl, 1 μg/ml leupeptin, 2 μg/ml 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 15 mM before incubation with 1 ml of nickel-nitri-
lotropic 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 FEN-1(Rad27) was
eluted with lysis buffer containing 250 mM imidazole and then further
purified to near homogeneity by Resource Q and Resource S column
chromatography. Approximately 0.9 mg of FEN-1(Rad27) was obtained
from a 1-liter culture. His-tagged Pol4 and the Dnl4/Lif1 complex were
purified from *Escherichia coli* and yeast, respectively, as described
previously (8, 25).

**Nicking-Nitrilotriacetic Acid Agarose Pull-down Assays**—To detect
interactions between FEN-1(Rad27) and the NHEJ components, Dnl4,
Lif1, and Pol4, labeled in *vitro* translated polypeptides (20 μl) and
bovine serum albumin (50 μg) were incubated either with or without
His-tagged FEN-1(Rad27) (30 μl, 6 μg) at 4 °C for 4 h. The mixtures
were supplemented with imidazole to a final concentration of 40 mM
before addition with nickel-nitritotriacetic acid agarose beads (10 μl
of a 80% slurry in Buffer A containing 40 mM imidazole) for 1 h at 4 °C.
After centrifugation, the supernatant was removed and the beads were
washed extensively with Buffer A containing 40 mM imidazole. An aliquot
of the supernatant (2 μl) was diluted by the addition of Buffer A
(10 μl) before the addition of SDS sample buffer (2 μl). Bound proteins
were eluted from the beads with 15 μl of Buffer A containing 250 mM
imidazole. After electrophoresis through a 10% SDS-polyacrylamide
gel, labeled proteins were detected in the dried gel by PhosphorImager
analysis (Molecular Dynamics).

**Nuclease Assays**—Linear duplexes with short partially complemen-
tary single-strand overhangs were constructed by annealing the follow-
ing pairs of oligonucleotides; 34-mer (5’-ACA AAG TTT GGA TGG CTA
CTG ACC GCC GCT CTC GTC C-3’) annealed to 5’-32P-labeled 39 + 3’flap
(5’-GGT GAC GAG CAC GAG AGC GGT CAG TAG CAA TCC AAA CTT
GTC-3’), 42-mer); 54-mer (5’-TGG TCG GCT TCG TGC GCG CAT
GAC TCT AAA GGG TTC TAA TAG TGA GAC AGA-3’); 50-mer
(5’-GTC TCT GTC TCT GTC GCG CAT GAC TCT AAA GGG TTC TAA
TAG TGA GAC GAC-3’); and 50-mer (5’-GTC TCT GTC TCT GTC
GCG CAT GAC TCT AAA GGG TTC TAA TAG TGA GAC GAC-3’;
50-mer). Alignment of the complementary single strands generates a three-nucleotide gap in the unlabeled strand and a three-nucleotide flap
in the unlabeled strand.

Equal amounts of the labeled and unlabeled duplexes (100 nM) were
incubated with Pol4 and FEN-1(Rad27) in reaction mixture (10 μl
containing 35 mM Tris-HCl, pH 7.5, 10 mM MgCl2, and 1 mM ATP at
25 °C for 2 h. Reactions were stopped by the addition of gel loading
buffer (95% (v/v) formamide, 0.09% (w/v) bromphenol blue, and 0.09%
(w/v) xylene cyanol). After separation by gel electrophoresis, labeled
dNA molecules in the dried gel were detected by PhosphorImager
analysis (Molecular Dynamics).

**RESULTS**

**FEN-1(Rad27) Interacted with Both Dnl4/Lif1 and
Pol4**—We have previously characterized a physical and functional
interaction between two NHEJ factors, Pol4 and Dnl4/Lif1, that coordinates the gap-filling DNA synthesis and liga-
tion steps of NHEJ (25). Because genetic studies have implicated FEN-1(Rad27) in the repair of a subset of NHEJ
events that involve removal of 5’ mismatched nucleotides (16, 24), we examined whether FEN-1(Rad27) interacted with Dnl4/
Lif1 and/or Pol4. To detect an association between FEN-
1(Rad27) and Dnl4/Lif1, labeled in *vitro* translated FEN-
1(Rad27) (Fig. 1A, lane 2) was incubated with a lysate from a yeast strain overexpressing Dnl4/Lif1 (25). Labeled
FEN-1(Rad27) was specifically co-immunoprecipitated by a Lif1 an-
tiserum (Fig. 1A, lane 4). To determine the contribution of the subunits of the Dnl4/Lif1 complex to the interaction with FEN-
1(Rad27), we performed pull-down assays with purified His-
tagged FEN-1(Rad27) (Fig. 1A, lane 1) and labeled in *vitro*
translated Dnl4 and Lif1. Dnl4 was specifically retained on
nickel beads liganded by His-tagged FEN-1(Rad27), whereas
no significant binding of Lif1 was detected (Fig. 1B). Together,
these results provide strong evidence that FEN-1(Rad27) interacts directly with the Dnl4 subunit of the Dnl4/Lif1 complex.

To determine whether there is a direct interaction between Pol4 and FEN-1(Rad27), we performed pull-down assays with purified FEN-1(Rad27) (Fig. 2A and lanes 1, 3, 5, and 7) and glutathione-Sepharose beads liganded by either GST-Pol4 or GST. As shown in Fig. 2A, FEN-1(Rad27) bound specifically to the GST-Pol4 beads. To map the region of Pol4 that interacted with FEN-1(Rad27), we performed pull-down assays with GST-Pol4 beads (Fig. 2A). Thus, FEN-1(Rad27) bound to a different region of Pol4 than Dnl4/Lif1, raising the possibility that these proteins form a ternary end-processing complex.

Activity of FEN-1(Rad27) on DNA Substrates that Mimic NHEJ Intermediates—Although the biochemical properties of FEN-1 have been extensively characterized, these studies have primarily focused on the flap-cleaving activity of FEN-1 using linear DNA substrates containing flaps of varying length (12, 30). To examine the activity of FEN-1(Rad27) on DNA substrates that more closely mimic intermediates generated by microhomology-mediated end alignment during NHEJ, we constructed linear duplexes that, when annealed by their complementary single-strand ends, generated a linear DNA molecule with a 5' flap of three nucleotides (Fig. 3A, middle). With this DSB substrate, FEN-1 acts primarily as an endonuclease, with the major incision site located 1 residue into the duplex region adjacent to the duplex/single-strand junction (Fig. 3A, lanes 5–7). In the absence of the unlabeled duplex (Fig. 3A, right), FEN-1(Rad27) exhibited weak exonuclease activity and also cleaved the duplex/single strand junction, generating labeled 7- and 8-nucleotide fragments (Fig. 3A, lanes 10–12). It is noteworthy that both of these activities were suppressed by the presence of the unlabeled duplex with the partially complementary single strand overhang (Fig. 3A, compare lanes 5–7 with lanes 10–12). Thus, FEN-1(Rad27) cleaved the flap structure formed by the annealing of the partially complementary single strand, releasing a four-nucleotide fragment and leaving a one-nucleotide gap within the annealed region holding the duplexes together. This pattern of cleavage was essentially identical to that generated with the same-sized flap within a contiguous linear duplex (Fig. 3A, compare lane 2 with lanes 5–7).

As expected, FEN-1(Rad27) was significantly less active when the flap was formed by the annealing of two DNA duplexes compared with a flap in a contiguous linear duplex (data not shown) because of the relative instability of the flap structures
formed by the annealing of the complementary four-nucleotide single-strand regions. Together, these results demonstrated that FEN-1(Rad27) cleaved flap structures generated by the annealing of short regions of microhomology. Although these structures are unstable in vitro, we anticipate that they will be stabilized in vivo by the presence of end-binding and -bridging factors, such as Hdf1/Hdf2 and Rad50/Mre11/Xrs2 (8).

Because there may be gaps adjacent to mismatched nucleotides generated during NHEJ, we next examined the effect on FEN-1(Rad27) activity of the size of the gap adjacent to the 5′ flap. We were surprised to find that, in the presence of a one-nucleotide gap, FEN-1(Rad27) acted as an exonuclease on about 50% of the DNA substrate (Fig. 3B, left). In addition, the endonuclease activity generated a ladder of products, ranging from two to five nucleotides in length. When the gap size was increased to two nucleotides, FEN-1(Rad27) acted as an endonuclease but, in this case, the major incision site was located two residues into the duplex region adjacent to the duplex/single-strand junction (Fig. 3B, middle). Finally, with a three-nucleotide gap, FEN-1(Rad27) acted as an endonuclease with the major incision sites at the duplex/single-strand junction and two residues into the duplex region adjacent to the duplex/single-strand junction (Fig. 3B, right). Thus, the length of the gap 3′ to the 5′ mismatched flap dramatically affected the mode of action and cleavage site of FEN-1(Rad27).

Dnl4/Lif1 Specifically Stimulated FEN-1(Rad27) Endonuclease Activity—To determine whether the interaction with Dnl4/Lif1 modulates the nuclease activity of FEN-1(Rad27), we co-incubated these proteins with DNA duplexes that, when aligned, have no gap adjacent to a three-nucleotide 5′ flap. The addition of increasing amounts of Dnl4/Lif1 stimulated the endonuclease activity of FEN-1(Rad27) (Fig. 4, lanes 2–6) with a 3–4-fold increase in cleavage produced at a 3:1 ratio of Dnl4/Lif1 to FEN-1(Rad27). In contrast, DNA ligase IV/XRCC4, the human homolog of Dnl4/Lif1, did not significantly increase FEN-1(Rad27) activity in similar experiments (Fig. 4, compare lanes 3–6 with lanes 7–10).

Pol4 Specifically Stimulated FEN-1(Rad27) Endonuclease Activity—Although Dnl4/Lif1 specifically stimulated FEN-1(Rad27), it did not alter the preferred cleavage site (Fig. 4, compare lanes 2 and 6). Thus, the majority of products generated by FEN-1(Rad27) action would contain a one-nucleotide gap and so would not be ligatable by Dnl4/Lif1. This prompted us to examine whether Pol4, which has been implicated in gap-filling during NHEJ (24, 25), modulated the nuclease activity of FEN-1(Rad27). In assays with the DNA duplexes that, when aligned, have no gap adjacent to the flap, the addition of Pol4 without deoxynucleotide triphosphates markedly stimulated the endonuclease activity but did not change the major incision site of FEN-1(Rad27) (Fig. 5A, lanes 4–7). At a ratio of 2:1, Pol4 increased the extent of FEN-1 cleavage by greater than 30-fold. In contrast, another Pol X family member Pol β only weakly stimulated FEN-1 activity in a similar experiment (Fig. 5A, lanes 8–11). A similar specific stimulation of FEN-1(Rad27) activity by Pol4 was observed when the flap was located within a contiguous duplex (data not shown), indicating that the increase in endonuclease activity did not involve the annealing and/or stabilization of the short complementary sequences. However, when there was a three-nucleotide gap adjacent to the mismatched nucleotides, Pol4 neither markedly increased flap removal nor changed the pattern of incisions by FEN-1(Rad27) (Fig. 5B, lanes 2–5). Similar results were obtained with one- and two-nucleotide gaps adjacent to the mismatched flap (data not shown). Together, these results demonstrate that Pol4 specifically stimulates flap removal by FEN-1(Rad27) when there is no gap adjacent to the flap.

**FIG. 6.** FEN-1(Rad27) inhibits strand displacement DNA synthesis by Pol4 and FEN-1(Rad27).—Next, we examined the processing of DNA duplexes that, when aligned, have a three-nucleotide gap adjacent to the flap by FEN-1(Rad27) and Pol4 in the presence of the deoxynucleotide triphosphates required to fill in the gap. Under these conditions, the addition of Pol4 not only greatly increased the extent of FEN-1 cleavage but also changed the pattern of FEN-1 incisions (Fig. 5B, compare lanes 4 and 5 with lanes 10 and 11). At a ratio of 2:1, Pol4 stimulated cleavage by FEN-1(Rad27) by about 10-fold. It is noteworthy that the major incision site was now located 1 residue into the duplex region adjacent to the duplex/single-strand junction. Although Pol β has gap-filling activity on gapped DSB substrates (25), Pol β did not stimulate FEN-1(Rad27) activity (data not shown). Taken together with our other results, this suggests that Pol4 and FEN-1(Rad27) act in a concerted reaction, with Pol4 stimulating FEN-1(Rad27) endonuclease by two distinct mechanisms. First, Pol4 rapidly filled in the short gap adjacent to the mismatched flap generating the DNA structure preferred by FEN-1(Rad27) endonuclease. Second, the protein-protein interaction between Pol4 and FEN-1(Rad27) specifically stimulated the cleavage of the DNA substrate with no gap adjacent to the flap by FEN-1(Rad27), releasing a four-nucleotide fragment (Fig. 5B).

**FEN-1(Rad27) Inhibited Strand-displacement Synthesis by Pol4—**Although gap-filling synthesis generates the substrate for FEN-1(Rad27) cleavage, the preferred site of incision generates a one-nucleotide gap. This prompted us to examine the effect of FEN-1(Rad27) on DNA synthesis by Pol4. We have shown previously that Pol4 catalyzes strand displacement synthesis to a much greater extent on gapped DNA substrates with mismatched 5′ nucleotides (25). As shown in Fig. 6, the addi-
tion of increasing amounts of FEN-1(Rad27) progressively inhibited strand displacement DNA synthesis by Pol4 on a DNA substrate with a three-nucleotide 5’ flap adjacent to a three-nucleotide gap. Because the majority of DNA synthesis tracts were four nucleotides long even when the concentration of FEN-1(Rad27) was about 20-fold lower than that of Pol4 (Fig. 6, lane 6), it seemed that Pol4 not only filled in the three-nucleotide gap adjacent to the flap to generate the preferred substrate of FEN-1(Rad27) but also then efficiently filled in the one-nucleotide gap generated by FEN-1(Rad27) cleavage. On the other hand, it is possible that DNA synthesis by Pol4 generated a double-flap structure, in which there is a one-nucleotide 3’ tail next to the 5’ flap, because this structure is cleaved even more efficiently by FEN-1(Rad27) (30).

**DISCUSSION**

The repair of DSBs by NHEJ is critical for maintaining genomic integrity. In the initial steps of this repair pathway, protein complexes are assembled on the DNA ends and the ends are then brought together (8, 34–36). Genetic and biochemical studies have identified the key factors and provided insights into the molecular mechanisms involved in these reactions in yeast and mammalian cells (1, 4). In most instances, the juxtaposed DNA ends will have to be processed to generate ligatable termini. Given the diverse nature of the DNA termini generated by agents such as ionizing radiation, it is likely that end processing can occur by several different mechanisms. This complexity and possible redundancy has hindered genetic and biochemical analysis of the end-processing reactions in NHEJ. Many of the end-processing events seem to involve an alignment step in which a short tract of sequence homology at or near the DNA ends is used to align the DNA ends (7, 9, 10). Whether the short microhomologies are exposed by unwinding or by nucleolytic degradation remains unknown. Irrespective of the exact mechanism, the alignment is likely to generate mismatched terminal flaps and gaps adjacent to the aligned, base-paired region.
In elegant genetic studies, Wu et al. (16) provided evidence that the DNA structure endonuclease FEN-1(Rad27) is involved in nucleolytic processing events that occur after end alignment in NHEJ. The participation of FEN-1(Rad27), a multifunctional enzyme, in DNA replication and the long patch subpathway of base excision repair is mediated by an interaction with proliferating cell nuclear antigen (13, 17, 21–23). In this study, we identified and characterized physical and functional interactions between FEN-1(Rad27) and both Pol4 and Dnl4/Lif1, the enzymes that catalyze the gap-filling and ligation steps of NHEJ, respectively. We have shown previously that Dnl4/Lif1 interacts with and stimulates gap-filling DNA synthesis by Pol4 (25). Thus, the pair-wise interactions among Pol4, FEN-1(Rad27), and Dnl4/Lif1 generate a versatile protein machine that can efficiently process and join aligned DNA ends with 5′ mismatches. In the scheme shown in Fig. 8, we have not included Hdf1/Hdf2 and Rad50/Mre11/Xrs2, factors that are likely to mediate end-bridging and -alignment (8). Nonetheless, we anticipate that these factors will play a critical role in stabilizing the joint structures formed between DNA ends aligned by regions of microhomology and will act as a platform for the subsequent actions of the end-processing enzymes. In particular, we propose that, if end alignment has resulted in a gap, Dnl4/Lif1 stimulates the filling in of the gaps by Pol4. If there are mismatched bases at the 5′ end of the gap, gap-filling generates the preferred substrate for FEN-1(Rad27), which also inhibits strand displacement DNA synthesis by Pol4. In the model shown in Fig. 8, Pol4 stimulates the removal of the 5′ mismatched bases by FEN-1(Rad27), generating a one-nucleotide gap that is in turn filled in Pol4 to yield a ligatable nick. Our studies do not exclude the possibility that gap-filling DNA synthesis generates a one-nucleotide 3′ flap adjacent to the 5′ flap. In this case, cleavage of the double-flap structure (which has been proposed as an intermediate generated during Okazaki fragment processing (30)) by Fen1(Rad27) directly generates a ligatable nick.

In the reconstituted end processing and ligation reaction, the DNA joining step was relatively inefficient. It should be noted, however, that DNA joining by Dnl4/Lif1 is greatly stimulated by the Rad50/Mre11/Xrs2 complex (8). Although it is evident that the Rad50/Mre11/Xrs2 complex is the end-bridging factor in yeast NHEJ (8), the role of the Mre11 nuclease in NHEJ is less well defined. It is not required for the joining of DNA molecules with complementary ends (37) but may be involved in the removal of 3′ flaps. The identity of the nuclease(s) that remove 3′ flaps remains unknown. Given its function as an end-bridging factor (8), it is possible that the Rad50/Mre11/Xrs2 complex also mediates end alignment via microhomologies. Indeed, biochemical studies with human Mre11 have provided evidence supporting the involvement of the 3′ to 5′ exonuclease activity of human Mre11 in exposing and aligning microhomologies (38). It is intriguing that this type of degradation and alignment will generate 5′ mismatched nucleotides, the substrate for FEN-1(Rad27).

In mammals, compelling genetic and biochemical evidence links the nuclease activity of Artemis with end processing during the repair of DSBs by NHEJ (39, 40). Moreover, the similar hypersensitivity to killing by ionizing radiation of Artemis-deficient and DNA-PK catalytic subunit-deficient cell lines (41) suggests that Artemis plays a key role in end-bridging and alignment. The interaction between Ku70/Ku80 and the Werner syndrome protein (WRN) that stimulates WRN nuclease activity implicates WRN in some aspect of end processing (42, 43). In contrast to yeast, the evidence linking FEN-1 with mammalian NHEJ is circumstantial. Although WRN protein interacts with and stimulates FEN-1 cleavage activity, this association may reflect the participation of these proteins in DNA replication (41). A functional interaction between FEN-1 and DNA Pol β seems to coordinate gap-filling synthesis and flap removal during long path BER in mammalian cells (45). This raises the possibility that the interaction between FEN-1 and the Pol X family DNA polymerases, μ and λ, implicated in NHEJ may be conserved in mammals (26, 27).

In summary, the studies described here, together with our previous work (25), have characterized a series of pair-wise interactions between FEN-1(Rad27), Pol4, and Dnl4/Lif1 that coordinate the processing and joining of DNA molecules with mismatched 5′ ends. It is noteworthy that the results of our biochemical studies are concordant with genetic analyses implicating these factors in the repair of DSBs with incompatible
ends by NHEJ in vivo (16, 24, 31–33, 46). Given the conservation of the mechanisms of NHEJ between yeast and mammals, our studies with yeast proteins will provide a conceptual framework for elucidating the mechanisms of end processing in mammalian NHEJ.

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