Repair of DNA double strand breaks by nonhomologous end joining (NHEJ) requires enzymatic processing beyond simple ligation when the terminal bases are damaged or not fully compatible. We transformed yeast with a series of linearized plasmids to examine the role of Pol4 (Pol IV, DNA polymerase β) in repair at a variety of end configurations. Mutation of POL4 did not impair DNA polymerase-independent religation of fully compatible ends and led to at most a 2-fold reduction in the frequency of joins that require only DNA polymerization. In contrast, the frequency of joins that also required removal of a 5′- or 3′-terminal mismatch was markedly reduced in pol4 (but not rev3, exo1, apn1, or rad1) yeast. In a chromosomal double strand break assay, pol4 mutation conferred a marked increase in sensitivity to HO endonuclease in a rad52 background, due primarily to loss of an NHEJ event that anneals with a 3′-terminal mismatch. The NHEJ activity of Pol4 was dependent on its nucleotidyl transferase function, as well as its unique amino terminus. Paradoxically, in vitro analyses with oligonucleotide substrates demonstrated that although Pol4 fills gaps with displacement polymerases have been described in eukaryotic cells (27–30). DNA polymerase (Pol) α (designated Pol I in yeast, where POL1 is the gene for the polymerase subunit), Pol ε (Pol II, POL2), and Pol δ (Pol III, POL3) together catalyze the essential functions of DNA replication. Pol δ and Pol ε are also involved in certain DNA repair events, notably nucleotide excision repair. Pol ζ (REV3) and Pol η (RAD30, 31) mediate different forms of translesion bypass synthesis in yeast. Pol β (Pol IV, POL4) is a 39-kDa monomeric polymerase in vertebrates that mediates base excision repair (BER) (32–34). Of these polymerases, Pol β has features that might suggest its involvement in NHEJ as well as BER, including low processivity and a preference for short strand gaps. Yeast Pol4 shares these biochemical properties, but has an additional 30 kDa of sequence of underdetermined function and is not required for BER (35–37). Rather, pol4 yeast exhibit reduced spore viability with abnormally high levels of intragenic meiotic recombination and persistent meiotic DSBs (36, 38). These phenotypes might be explained by impaired NHEJ.

We have previously used a color-based plasmid transformation assay to document that the yeast NHEJ pathway can create processed joins at compatible restriction site ends (17).
Here, we extend the versatility of our assay to examine in detail the handling of more complex end configurations, with an emphasis on defining the role of Pol4 in NHEJ, if any. We find that like mammalian cells, yeast can efficiently execute remarkably complex NHEJ reactions, making extensive use of even very limited base pairing (microhomologies) between protruding single-stranded ends. Pol4 is indeed involved in this, a finding confirmed using chromosomal cutting assays. Surprisingly, although nucleotide transfer is essential to Pol4’s NHEJ function, the pol4 phenotype cannot be explained by this activity alone. Rather, Pol4 is stringently required only for joins that necessitate removal of 5’- or 3’-terminal mismatches. Despite this, in vitro studies using gapped oligonucleotide substrates confirm the absence of nuclease activities in Pol4. We discuss the implications of these potentially paradoxical findings in the context of the multiple pathways of NHEJ.

**EXPERIMENTAL PROCEDURES**

**Reagents—**Except as noted, components for yeast media were from United States Biologicals, chemical reagents were from Sigma, nucleotides were from Amersham Pharmacia Biotech, DNA modifying enzymes were from New England Biolabs, and oligonucleotides were synthesized by either the Protein and Nucleic Acid Chemistry Laboratory or Jeffrey Milbrandt laboratory at the Washington University School of Medicine.

**Construction and Maintenance of Yeast Strains—**The genotypes of all strains are shown in Table I. All are haploid and closely related to the strain YW112, a derivative of YPH499 (17, 39). Complete deletions of the POL4, REV3, APN1, and RAD1 coding sequences were made by PCR-mediated HIS3 gene replacement as described (40). apn1::HIS3 and rad1::HIS3 alleles were additionally verified by documenting increased sensitivity to methylenemethane sulfonate and ultraviolet radiation, respectively. The rad52/p04 double mutant strain YW153 was made from YW144 and YW150 by mating and sporulation. Yeast meiotic DNA was either YPD, or minimal medium (41) supplemented with the carbon source (2% glucose or 2% raffinose plus 2% galactose). Yeast were grown at 30 °C for all experiments.

**Construction of Plasmids—**pES16 and pES26 were described previously (17). New derivatives were constructed using a pES16 derivative in which the polylinker sites had been fused and destroyed (pTW288). The sequences inserted between the 2nd and 3rd codons of the ADE2 gene were (restriction sites are in bold, excisable stop codons are underlined): pM1, 5′-AAGCGGTATCGACGGAGGCGGCCGCGGATTCAT-3′. Downstream PCR products were made with specifically tailed reverse primers that spanned the 5′-end of the ADE2 gene (restriction sites are in bold, excisable stop codons are underlined): pm1, 5′-AAGCGGTATCGACGGAGGCGGCCGCGGATTCAT-3′. This study

| Strain | Genotype | Source |
|--------|----------|--------|
| YW112  | MATa, ade2Δ·TRP1, his3Δ·200, leu2Δ·1, lys2Δ·801, trp1Δ·63, ura3Δ·52 | Ref. 11 |
| YW114  | YW112 + dnl4Δ·HIS3 | Ref. 11 |
| YW130  | MATa, ade2Δ·TRP1, his3Δ·200, leu2Δ·2, rad52Δ·LEU2, trp1Δ·63, ura3Δ·52 | Ref. 11 |
| YW132  | MATa, ade2Δ·TRP1, dnl4Δ·HIS3, his3Δ·200, leu2Δ·2, rad52Δ·LEU2, trp1Δ·63, ura3Δ·52 | Ref. 11 |
| YW247  | YW112 + exol1Δ·HIS3 | Ref. 54 |
| YW144  | YW112 + pol4Δ·HIS3 | This study |
| YW145  | YW112 + rad1Δ·HIS3 | This study |
| YW290  | YW112 + apn1Δ·HIS3 | This study |
| YW291  | YW112 + rad1Δ·HIS3 | This study |
| YW153  | MATa, ade2Δ·TRP1, his3Δ·200, leu2Δ·2, lys2Δ·4, HIS3, rad52Δ·LEU2, trp1Δ·63, ura3Δ·52 | YW130 × YW144 |

Plasmid expression plasmids were derived from the URA3/CEN/ARS plasmid pTW268, which contains the CDC9 coding sequence fused to glutathione S-transferase under the control of the ADH1 promoter (17). The first, the glutathione S-transferase coding sequence was replaced with a HindIII-BamHI-tailed PCR product made from mPS127b (42) that encoded a His9-Myc epitope tag, to create pTW283. Next, the CDC9 coding sequence was replaced with a BamHI-SalI-tailed PCR fragment made from total Saccharomyces cerevisiae genomic DNA (Novagen) that encoded the Pol4 protein from the second to the last amino acid, to create pTW284. Unlike the published POL4 sequence, our POL4 PCR fragment did not contain an internal BamHI site (38), but rather had a silent mutation of codon 303 from GAT to GAC. To create the LEU2 selectable derivative pTW285, the ADH1-HM-POL4-containing Asp718-NotI fragment of pTW284 was ligated into Smal-NotI-digested pRS315 (39) after blunting of the Asp718 5′ overhang. To create the LEU2-selectable derivative pTW286, the XhoI-NotI fragment of pTW285 was ligated into SacI-NotI-digested pRS317.

The plasmid expressing the Δ1–61 amino-terminal deletion of Pol4 (ptVW301) was created by replacing the BamHI-SalI fragment of pTW300 (pTW285 with the polylinker SalI destroyed) with a PCR fragment corresponding to the truncated Pol4 sequence. The Δ676E and K247R/K248R point mutations were created by a gap repair strategy. First, a three-way BamHI-XhoI-SalI ligation of PCR products was used to create an internal 2477–369 Pol4 deletion in pTW300 with XhoI and SmalI sites at the deletion junction (ptTW304). YW144 was then transformed with SmalI-digested pTW304 and PCR products that spanned the 2477–369 deletion and that included degenerate bases in this region. Plasmids were recovered from transformants by the gap repair region sequenced to verify the presence of targeted but not unexpected mutations (D367E, pTW305; K247R/K248R, pTW306), and retransformed into YW144 for functional analysis. Wild-type isolates expressed functional Pol4, ensuring that pTW304 was not cryptically mutated.

pGAL-HO was constructed by ligating a HindIII-SalI-tailed PCR fragment from YCP50-HO (43) that encoded HO into HindIII-SalI-digested pBM272 (44). This places HO expression under control of the galactose-regulated GAL1 promoter on a URA3/CEN/ARS vector.

Plasmid Transformation Assay—Plasmids were prepared byseveral-fofold digestion with the appropriate combination of restriction enzymes, followed by phenol/chloroform extraction and ethanol precipitation. DNA were examined by agarose gel electrophoresis with ethidium bromide staining to ensure complete digestion and equivalent concentrations in parallel preparations. More than 95% of Min1, BamHI-, and MinI-BamHI-digested pMB0 and Sphl-, KpnI-, and Spfl-KpnI-digested pSK3 could be religated in vitro, as determined by treatment with T4 DNA ligase followed by agarose gel electrophoresis. Partially end filled pm1 was prepared by an additional incubation of 5 μg of plasmid with 0.1 μl of Taq polymerase (a kind gift of Wayne Barnes, unit definition not available) for 20 min at 65 °C in 10 μl of KLA buffer (50 mM Tris-HCl, pH 9.2, 16 mM ammonium sulfate; 2.5 mM MgCl2; 0.1% Tween 20) with 100 μM dCTP, followed by a second phenol/ chloroform extraction and ethanol precipitation. Yeast were grown to exponential phase (A600 0.5 to 0.8) in a total volume of 25 ml, harvested, washed, incubated at 30 °C for 30 min in 10 ml of 0.1 M LiAc, and washed again in 1 ml of 0.1 M LiAc per A600 unit of the original culture. 100 μl of yeast suspension, 0.5 μg of plasmid in 10 μl of TRe (10 mM Tris,
of frameshifting nucleotides, for example pBX2 contains Plasmid names reflect the combination of inserted sites and the number larly by a mechanism that (re)establishes the correct reading frame. information only when the digested restriction site(s) are joined intracellu-

ADE2 given linearized plasmid will yield pES16 by engineering novel restriction site(s) and frameshifting nucleotide(s) just after the first two codons of the pES26 is pES16 with an excisable polytermina- tor inserted at the ADE2 gene. In this way, a given linearized plasmid will yield ADE2 (white) yeast after transformation only when the digested restriction site(s) are joined intracellularly by a mechanism that (re)establishes the correct reading frame. Plasmid names reflect the combination of inserted sites and the number of frameshifting nucleotides, for example pBX2 contains BamHI and XhoI sites with a reading frame of +2 relative to ADE2. B, pES26 transformation data are plotted as a ratio of the colony count obtained with BamHI-digested plasmid over the colony count obtained in a parallel transformation with undigested plasmid, a measure of the relative rate of simple religation at the BamHI ends. Each point represents an independent transformation experiment.

Characterization of Join Types by Sequencing and PCR—Plasmids contained within independent yeast transformants were recovered into Escherichia coli by glass bead lysis (41) and sequenced with primer OW563 (5'-GCCGAGAAGATTTTCAAGCATC, a reverse primer 114-base pairs downstream of the ADE2 start codon). Colony PCR was performed by touching a plastic pipette tip to a fresh yeast streak (<24 h) and inoculating 40 μl of PCR buffer (10 mM Tris, pH 9.2, 50 mM KCl, 2.5 mM MgCl2, 400 μM each dNTP) containing 0.625 units Taq polymerase (Promega) and 50 ngml of each primer. After amplification (1 cycle of 94 °C for 4 min, 40 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, and 1 cycle of 72 °C for 5 min) products were electrophoresed on a 1.25% agarose gel with ethidium bromide visualization. Negative and positive controls were included that had been verified by sequencing, because faint false bands were occasionally observed but readily distinguished from true positives as exemplified by the controls. Join rates were calculated as the fraction of colonies positive for the join multiplied by the normalized transformation rate for the relevant colony color. Join- 

pH 7.5, 1 mM EDTA), and 5 μg of single-stranded carrier DNA in 10 μl of TE (41) were mixed and incubated at 30 °C for 30 min, followed by the addition of 1 ml of 40% polyethylene glycol 3350 and incubation at 30 °C for 30 min. Cells were heat-shocked at 42 °C for 15 min, washed in water, and plated to appropriate minimal medium. Colonies were counted after 3 days growth and further incubation at 4 °C as needed to enhance the red/white color difference.

Characterization of Join Types by Sequencing and PCR—Plasmids contained within independent yeast transformants were recovered into Escherichia coli by glass bead lysis (41) and sequenced with primer OW563 (5'-GCCGAGAAGATTTTCAAGCATC, a reverse primer 114-base pairs downstream of the ADE2 start codon). Colony PCR was performed by touching a plastic pipette tip to a fresh yeast streak (<24 h) and inoculating 40 μl of PCR buffer (10 mM Tris, pH 9.2, 50 mM KCl, 2.5 mM MgCl2, 400 μM each dNTP) containing 0.625 units Taq polymerase (Promega) and 50 ng/ml of each primer. After amplification (1 cycle of 94 °C for 4 min, 40 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s, and 1 cycle of 72 °C for 5 min) products were electrophoresed on a 1.25% agarose gel with ethidium bromide visualization. Negative and positive controls were included that had been verified by sequencing, because faint false bands were occasionally observed but readily distinguished from true positives as exemplified by the controls. Join rates were calculated as the fraction of colonies positive for the join multiplied by the normalized transformation rate for the relevant colony color. Join-
specific primer sets coupled OW620 (5'-CTTGACTAGGCGACTCAGC, a forward primer just near the 5'-end of the ADE2 fragment) with the following reverse primers: XB(5'-ATACCAACTGTTCTAGAG); MB(5'-GATCCTAGTTCTAGAGGATCGC); SK(5'-GATCCTAGTTCTAGAGGATCGC); pMB0(5'-CTTGACTAGGCGACTCAGC), not determined.

Structure-based Sequence Alignment—First, the entire Pol4 sequence was submitted to the web-based Swiss Model utility (45) for mapping onto the hPol β polypeptide contained in a gapped DNA substrate ends of Mu1-BamHI-digested pMB0, as in Fig. 2A. B, pMB0 transformation data are plotted as a ratio of the colony count obtained with Mu1-BamHI-digested plasmid over the colony count obtained in a parallel transformation with BamHI-digested plasmid (open circles, white colonies; filled circles, red colonies). C, joins predicted to occur at Mu1-BamHI-digested ends, as in Fig. 2D. Undigested pMB0 yields red colonies. ND, not determined.

HO Endonuclease Sensitivity Assay—Saturated overnight cultures in pGAL-HO-selective glucose minimal medium were washed and raffinose-galactose plates 5 days. PCR diagnosis of MAT join types was performed essentially as described (11).

Structure-based Sequence Alignment—First, the entire Pol4 sequence was submitted to the web-based Swiss Model utility (45) for mapping onto the hPol β polypeptide contained in a gapped DNA substrate in 50 mM HEPES, pH 7.9, 100 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol, 10% glycerol, 100 μM each dNTP. Klenow and exonuclease I. 2 μl of Ni-NTA eluate or buffer B and mixing by pipetting up and down, then the maximum join rate for SK(+) and SK(−) joins in the pol4 strain was calculated using the upper limit of the 95% confidence interval from the binomial distribution for 0 of 29 events. Undigested pSK3 yields red colonies. ND, not determined.

For activity assays, labeled strands (5 pmol) were phosphorylated using 5 units of T4 polynucleotide kinase in 20 μl of the supplied buffer with 50 μCi [γ-32P]ATP (NEN Life Science Products) for 15 min at 37 °C. The slurry was poured into a disposable mini-column (Bio-Rad), the resin washed with 1 ml of buffer A and proteins eluted with 2 ml of Ni-NTA eluate or buffer B and mixing by pipetting up and down, then the maximum join rate for SK(+) and SK(−) joins in the pol4 strain was calculated using the upper limit of the 95% confidence interval from the binomial distribution for 0 of 29 events. Undigested pSK3 yields red colonies. ND, not determined.

2 μg/ml apronin, 1 μg/ml pepstatin A, 1 μg/ml leupeptin. 3-pellet volumes of glass beads were added, and the cells disrupted by vortexing. Buffer A was added to 9 ml, the lysate brought to 0.1% Nonidet P-40, and proteins extracted by rocking 30 min at 4 °C. After clearing by centrifugation at 12,000 × g for 15 min, 0.35 ml of packed volume Ni-NTA beads (Qiagen) were added, and batch binding allowed to proceed with rocking for 1 h at 4 °C. The slurry was poured into a disposable mini-column (Bio-Rad), the resin washed with 10 × 1 ml of buffer A and proteins eluted with 2 × 250 μl of buffer A, 500 mM imidazole, 50 mM KCl. Eluates were dialyzed against 2 × 1 liter of buffer B (10 mM Tris, pH 7.5, 50 mM KCl, 5 mM MgCl₂, 1 mM dithiothreitol, 10% glycerol, 0.1 mM phenylmethylsulfonyl fluoride), flash frozen in 50-μl aliquots, and stored at −70 °C.

For activity assays, labeled strands (5 pmol) were phosphorylated using 5 units of T4 polynucleotide kinase in 20 μl of the supplied buffer with 50 μCi [γ-32P]ATP (NEN Life Science Products) for 15 min at 37 °C. For activity assays, labeled strands (5 pmol) were phosphorylated using 5 units of T4 polynucleotide kinase in 20 μl of the supplied buffer with 50 μCi [γ-32P]ATP (NEN Life Science Products) for 15 min at 37 °C. For activity assays, labeled strands (5 pmol) were phosphorylated using 5 units of T4 polynucleotide kinase in 20 μl of the supplied buffer with 50 μCi [γ-32P]ATP (NEN Life Science Products) for 15 min at 37 °C. For activity assays, labeled strands (5 pmol) were phosphorylated using 5 units of T4 polynucleotide kinase in 20 μl of the supplied buffer with 50 μCi [γ-32P]ATP (NEN Life Science Products) for 15 min at 37 °C. For activity assays, labeled strands (5 pmol) were phosphorylated using 5 units of T4 polynucleotide kinase in 20 μl of the supplied buffer with 50 μCi [γ-32P]ATP (NEN Life Science Products) for 15 min at 37 °C. For activity assays, labeled strands (5 pmol) were phosphorylated using 5 units of T4 polynucleotide kinase in 20 μl of the supplied buffer with 50 μCi [γ-32P]ATP (NEN Life Science Products) for 15 min at 37 °C. For activity assays, labeled strands (5 pmol) were phosphorylated using 5 units of T4 polynucleotide kinase in 20 μl of the supplied buffer with 50 μCi [γ-32P]ATP (NEN Life Science Products) for 15 min at 37 °C. For activity assays, labeled strands (5 pmol) were phosphorylated using 5 units of T4 polynucleotide kinase in 20 μl of the supplied buffer with 50 μCi [γ-32P]ATP (NEN Life Science Products) for 15 min at 37 °C.
in 2 μl of buffer B) served as controls. After 60 min at 30 °C, the reactions were stopped by addition of 40 μl of sequencing dye and heating to 90 °C for 5 min. 5 μl (~5,000 rpm) was then electrophoresed on a 20% sequencing gel, which was exposed wet to a PhosphorImager screen for 3 h before imaging and quantitation.

RESULTS

The Plasmid Transformation Assay—In this assay, test plasmids are linearized in vitro with restriction enzymes and transformed into yeast, where recircularization is required for plasmid maintenance. pES16 (Fig. 1A) contains the URA3 gene to select for transformants and the CEN1ARS sequence for plasmid maintenance, which also prevents recovery of integrated plasmids, because this results in a lethal dicentric chromosome. DSBs are introduced into the color indicator gene ADE2 at a variety of restriction sites. First, we have previously used BglII-digested pES16 and pES26 to show that yeast DNA ligase IV (Dn14, also called Lig4) catalyzes NHEJ ligation (17). Second, we have created a series of new pES16 variants in which unique restriction sites have been inserted after the second codon of the ADE2 gene. Essentially any combination of restriction sites (and therefore encoded amino acids) can be introduced at this location without affecting Ade2 function. Different NHEJ events yield different reading frames of the ADE2 gene, however, and therefore different color colonies (specifically, ADE2 yeast are white, ade2 yeast are red). Joins are illustrated as the intermediate alignment structures inferred from the sequences of the final products, assuming no prior end degradation (Figs. 2-5, see Discussion). Fully compatible overhangs can be joined by simple religation, i.e., requiring only DNA ligase. Repair events that proceed via partial end annealing (i.e. microhomology usage) are of three types. Gap joins anneal in a fashion that requires fill-in synthesis by a DNA polymerase. Flap joins anneal in a fashion that extrudes excess bases that must be removed. Mixed joins show characteristics of both gap and flap joins. Because all yeast used in this study bear a complete chromosomal deletion of ADE2 (17), non-NHEJ transformation events arise only from the small fraction of undigested plasmid and rare macrodeletions.

Pol4 Is Not Required for Simple Religation—In the plasmid transformation assay, the relative rate of plasmid DSBR is typically revealed by normalizing the transformation rate obtained with linearized plasmid to that obtained in parallel with uncut plasmid. A subset of colonies are next analyzed to determine the mechanism of plasmid recircularization, either by sequencing of recovered plasmids or colony PCR, with the relative rate of a given repair event calculated by multiplying the normalized transformation rate by the fraction of colonies positive for the event. In the experiments below, we wanted to specifically examine the effects of polymerase gene deletions on end processing by normalizing instead to parallel transformations with linearized plasmids that are joined by simple religation. To validate this approach, we first transformed all the wild-type strain with pRS317 (indicated as ADH-POL4 white colonies with pM1. Mutation of POL4 did not significantly affect M(0) simple religation but did slightly and reproducibly reduce the rate of polymerization-dependent M(+) gap join, i.e., white colonies with pM1. Mutation of POL4 again did not significantly affect M(0) simple religation but did slightly and reproducibly reduce the rate of polymerization-dependent M(+) gap join, i.e., white colonies with pM1. Mutation of POL4 again did not significantly affect M(0) simple religation but did slightly and reproducibly reduce the rate of polymerization-dependent M(+) gap join, i.e., white colonies with pM1. Mutation of POL4 again did not significantly affect M(0) simple religation but di stantly and}
A Pol4-dependent Pathway of Nonhomologous End Joining

TABLE II
Survival, mating type, and join rates among HO-expressing rad52 strains

| Strain       | Survivala | a | o | Sterile | Join type among sterile survivorsc | Join rates |
|--------------|-----------|---|---|---------|-----------------------------------|-----------|
|              |           | HO (+2) | HO (+3) | Other  | HO (+2) | HO (+3) |
| rad52        | (7.0 ± 1.5) × 10⁻⁴ | 0/86 (0%) | 19/86 (22%) | 67/86 (78%) | 12/20 (60%) | 5/20 (25%) | 3/20 (15%) | 3.3 × 10⁻⁴ | 1.4 × 10⁻⁴ |
| rad52/pol4   | (7.9 ± 3.5) × 10⁻⁵ | 4/88 (5%) | 54/88 (61%) | 30/88 (34%) | 0/19 (0%)  | 12/19 (63%) | 7/19 (37%) | <4.7 × 10⁻⁴ (>70) | 1.7 × 10⁻⁴ |
| rad52/hdf1   | (3.7 ± 4.9) × 10⁻⁵ | 0/21 (0%) | 21/21 (100%) | 0/21 (0%) | NA       | NA       | NA       | NA       | NA       |

a p values for the survival of rad52/pol4 and rad52/hdf1 strains are 3.1 × 10⁻⁷ and 1.3 × 10⁻⁴, respectively, as compared with the rad52 strain (Student's t test).

b p values for the 2 × 3 table of strain vs. mating type are 2.7 × 10⁻⁸ and 7.3 × 10⁻¹¹ for the rad52/pol4 and rad52/hdf1 strains, respectively, as compared with the rad52 strain (χ² distribution).

c The maximal HO (+2) rate was calculated using the upper limit of the 95% confidence interval from the binomial distribution for 0 of 19 events.

* NA, not applicable.

to the wild type strain, verifying the specificity of the pol4 phenotype (data not shown). Thus, although Mlu1-digested pM1 did have a high frequency of gap joining that revealed a reproducible pol4 effect, the magnitude of this effect was small, suggesting a redundancy of polymerase action.

We next sought to eliminate competition with simple religation in an attempt to increase the dependence on Pol4. Mlu1-digested pM1 was partially end filled with dCTP, yielding a plasmid with a 3-base 5’ overhang that can anneal using the same base pairing as M (+2) but now with only single nucleotide gaps. Fig. 2B shows that this treatment largely blocked red colony formation (i.e. M (0) joining) as expected, and correspondingly increased the rate of white colony formation (i.e. M (+2) joining) about 2-fold. The Pol4 dependence was similar to that seen with untreated ends, however. BamHI-XhoI-digested pXB2 presents “incompatible” ends that can only anneal as 2-base gap and flap joins (Fig. 2). The XB (+2) gap join formed at 60% of the rate of BamHI simple religation, demonstrating that joining via partially annealed intermediates can be quite efficient. Despite this, mutation of pol4 caused only a very slight reduction in the frequency of XB (+2) gap joining (1.4-fold, p = 0.01).

pol4 Mutants Are Markedly Deficient in Mixed Joining—We next tested substrates with only single nucleotide micro-homologies in an attempt to reveal a greater Pol4 dependence by destabilizing the annealed intermediate. Figs. 3 and 4 present two such substrates: pMB0 juxtaposes the incompatible 5’ overhangs Mlu1-BamHI, and pSK3 the incompatible 3’ overhangs Sphi-KpnI. As illustrated, four alignments are predicted for each: a 3-base gap, a 3-base flap (5’ versus 3’), and two mixed joins. The 3-base gap joins, i.e. MB (+3) and SK (+3), were initially of greatest interest, because they require only DNA polymerization. With pMB0, white colonies bearing MB (+3) did form in the wild type strain at 1.5% of the rate of BamHI simple religation, but this join showed only the same 2-fold decrease in the pol4 mutant as the 2-base gap joins (p = 0.003, Fig. 3). It became clear that the red colonies revealed a more striking result, however. MB (+1) formed even more efficiently than MB (+3) at 6.5% of the rate of simple religation, even though the MB (+1) annealed intermediate includes a 2-base 5’-terminal mismatch. Further, MB (+1) joining was markedly impaired in the pol4 mutant (28-fold). We did not detect macrodeletion, MB (-3), or MB (-1) events in the wild type strain. The remaining red colonies contained intact pMB0, arising from either single or undigested plasmid (see “Experimental Procedures” for details regarding stop codons that are excised during double digestion).

pSK3 revealed a different pattern from pMB0 in that the SK (+3) 3-base gap join was not detected. Rather, white colonies resulted from SK (-3) flap joining, at 2.6% of the rate of KpnI simple religation (Fig. 4). pSK3 was similar to pMB0, however, in that mixed joins (red colonies) predominated, despite the presence of 3’-terminal mismatches. SK (-1) was favored, but both SK (-1) and SK (+1) were detected and together formed at 10% of the rate of simple religation. Most dramatically, SK (-1) joining was more than 95-fold reduced in the pol4 mutant, and the combination of the mixed joins was more than 120-fold reduced. The few red colonies obtained with the pol4 mutant were a mixture of intact plasmid (44%) and rare macrodeletion (44%) and microdeletion (9%) events. For both pMB0 and pSK3, mixed joining was REV3-independent and restored by plasmid-expressed His₆-Myc₃-Pol4 (HM-Pol4), demonstrating the specificity of the phenomenon for the pol4Δ allele (Figs. 3 and 4).

pol4 Yeast Are Deficient in Chromosomal Mixed Joining—We next sought to verify the role of Pol4 in mixed joining at a chromosomal rather than a plasmid DSB. Expression of HO endonuclease (HO) in yeast that bear a rad52 mutation is largely lethal, because this prevents homologous repair of the DSB created by HO at MAT (11, 47). Rare cells (~0.1%) escape the effects of HO, however, predominantly by repair events that create HO-resistant (i.e. nonreconcilable) MAT alleles. The two most common HO-resistant MAT alleles, called +CA and =ACA by Moore and Haber (11), are readily modeled as a mixed join with a 3’-terminal mismatch (HO (+2)) and a 3-base flap (HO (-3)), respectively (Fig. 5, A and C). It seemed likely that the same mechanism was responsible for both these and plasmid NHEJ, because each is Rad50/Mre11-dependent (8, 11). Indeed, hdf1 rad52 and dnl4 rad52 yeast each showed a more than 10-fold lower survival than rad52 yeast after induction of HO expression from the GAL1 promoter (Fig. 5B and not shown) (8). MATα/hdf1 rad52 HO survivors all retained the a mating type, indicative of escape by inactivation of HO with an intact MATa allele, implying a complete absence of NHEJ at MAT DSBs in hdf1 yeast (Table II). A pol4 rad52 strain also showed a 33-fold reduction in HO survival compared with rad52 yeast with essentially complete loss of HO (+2) joining (>70-fold), although a substantial fraction of MATα/pol4 rad52 HO survivors became sterile as a result of HO (-3) joins and lower frequency events. Overexpression of HM-Pol4 from a plasmid corrected the pol4 but not the hdf1 mutant phenotypes (Fig. 5B). This pattern is similar to the plasmid results, except for the greater effect of pol4 mutation on 3’ flap joining in the chromosomal assay (8-fold for HO (-3) versus 2-fold for SK (-3)).
Mutational Analysis of Pol4—The simplest interpretation of
the strong Pol4 dependence of MB(1), SK(2), SK(1), and
HO(1) mixed joins is that Pol4 itself removes terminal mis-
matches. To examine the Pol4 protein in detail to see if such a
gap-dependent nuclease activity could be discovered, we per-
formed a structure-based sequence alignment of Pol4 with the
hPol beta polypeptide resolved in a co-crystal with gapped DNA (Fig. 6
A, and Ref. 46). Pol beta binds to both the 3' and 5' termini
of short nucleotide gaps as a critical part of its function (34, 46,
48). At the 3' terminus, the highly conserved “fingers,” “palm,”
and “thumb” polymerase subdomains cooperate to bind and
catalyze nucleotidyl transfer, with the three universal aspartic
acid residues coordinating the incoming Mg2+-dNTP. At the 5' terminus, the “8 kDa” lyase domain binds and catalyzes re-
moval of a 5'-deoxyribose phosphate moiety by beta-elimination,
with Lys-72 forming the critical Schiff base intermediate.
These regions and amino acids are well conserved in Pol4. The
nonaligned regions of the much larger Pol4 protein encompass
primarily a 23.4-kDa NH2-terminal extension in place of the
2-kDa NH2-terminal helix of hPol beta, with an additional 8.5-kDa
Pol4 sequence inserted in the palm subdomain.

Guided by this analysis, we made a series of focused HM-
Pol4 mutations and tested them for their ability to complement
joining of Mlu I-Bam HI-digested pMB0 and Sph I-Kpn I-di-
gested pSK3 by pol4 yeast (Fig. 6, B-D). First, the D367E palm
mutation behaved similarly to the null allele with loss of mixed
joining, consistent with loss of nucleotidyl transfer via dis-
rupted geometry of Mg2+-dNTP coordination. Second, we mu-
tated the lysine residues in the pocket where Schiff base formation should occur, to test whether Pol4 cleaves 5' mismatches by a β-elimination reaction similar to removal of a 5’-deoxyribose phosphate. The protein, K247R/K248R, did not demonstrate the specific loss of MB(+1) joining that would be expected with loss of a 5’ nuclease, but rather showed a slight reduction in all joins, consistent with weakened binding to the 5’ terminus (K247A and K248A mutations gave similar results, not shown). Third, removal of even a small part of the unique Pol4 amino terminus in Δ1–61 led to complete inactivation of Pol4 (similar results were obtained with Δ1–145 and Δ1–205 mutants, not shown). Although this result demonstrates the importance of the unique sequence of Pol4, it unfortunately does not provide any information regarding the function of this domain.

**Pol4 Lacks Nuclease Activity in Vitro on Gapped Substrates**—We next used Ni-NTA-agarose to partially purify HM-Pol4 and its mutant derivatives and examined their biochemical activities on a series of gapped oligonucleotide substrates (Fig. 7). Ni-NTA fractionation was not sufficient to yield pure protein, but no contaminating polymerase activities were detected in the vector-only control fraction (Fig. 7C, lane 7). Also, no Pol4-dependent bands were copurified in this single step that might be candidates for a Pol4-associated nuclease (Fig. 7B). HM-Pol4 (but not D367E) filled a 2-nt gap and stopped (Fig. 7C, lane 6), unlike the displacement synthesis seen with Klenow (lane 5), similar to published results (35). Interestingly, Δ1–61 activity on gapped substrate (lane 9) was similar to the reduced HM-Pol4 activity in the absence of distal strand (lane 11), which suggests that this mutant may have impaired gap recognition, explaining its *in vivo* phenotype. When a 5’-terminal mismatch was present on the distal strand, the resulting 3-nt gap was filled efficiently, but again with no continued displacement into base paired positions (lane 12). The 5’-terminal mismatch was not cleaved during this reaction, which would be evident as a specific reduction of probe counts when the mismatched distal strand was labeled, although this was complicated by a low level phosphatase activity in all Ni-NTA fractions (Table III). A 3’-terminal mismatch on the proximal strand significantly impaired extension by HM-Pol4 and exo-Klenow, but not by Klenow (Fig. 7C, lanes 13–15). The limited remaining extension by HM-Pol4 is best explained by simple incorporation of the mismatch as opposed to removal and resynthesis, because it did not depend on dGTP (lane 17). Further, prevention of polymerization by the D367E mutation or by removal of dNTPs revealed no proximal strand shortening (lanes 16 and 20). Collectively, then, we find Pol4 to be a gap-filling polymerase, but with no detectable gap-dependent nuclease activity.

**Exo1, Apn1, and Rad1 Do not Provide Pol4-associated Nuclease Activity**—Finally, we have tested three genes as candidates for providing the essential Pol4-associated nuclease activities. Exo1, a major mitotic 5’ nuclease in yeast cells (49), was not required for MB(+1) joining (Fig. 3B). The apurinic-apyrimidinic endonuclease interacts functionally with Pol β during BER, cleaving on the 5’ side of an abasic site (33). It was possible that a similar interaction is utilized in NHEJ, with the yeast homologue Apn1 providing 3’ nuclease function (50). Alternatively, this might be provided by the Rad1-Rad10 complex, similar to cleavage of nonhomology tracts, i.e. 3’ flaps, during rDSBR (51). *apo1* and *rad1* mutants made SK(-1)/SK(+1) joins at wild-type rates, however (Fig. 4B).

**DISCUSSION**

**A Pol4-dependent Pathway of NHEJ**—In this study, we demonstrate that yeast are capable of efficient NHEJ at incompatible ends via intermediates that make extensive use of terminal microhomology, as required at DSBs that are created by genotoxic agents and believed to lead to chromosomal translocation. Further, both plasmid and chromosomal assays show conclusively that Pol4 is one of the processing enzymes recruited for this purpose. This was established only by the subset of mixed joins that required resolution of terminal mismatches, however, especially at 3’ overhangs (see Fig. 8 for summary of join results). Thus, MB(+1), SK(-1), SK(+1), and HO(+2) mixed joins were 28-, >95-, >24-, and >70-fold reduced in *pol4* mu-
TABLE III

| Expressed protein | Labeled strand | Fraction of probe extended | Substrate 1 | Fraction of counts remaining in lane | Substrate 4 | Fraction of probe extended | Fraction of counts remaining in lane |
|-------------------|----------------|---------------------------|-------------|-------------------------------------|-------------|---------------------------|-------------------------------------|
| Vector            | Proximal       | <0.03                     | 0.76        | <0.01                               | 0.69        |
|                   | Distal         | NA                        | 0.79        | NA                                  | 0.66        |
| HM-Pol4           | Proximal       | 0.59                      | 0.70        | 0.69                                | 0.67        |
|                   | Distal         | NA                        | 0.68        | NA                                  | 0.72        |

* NA, not applicable.

A Pol4-dependent Pathway of Nonhomologous End Joining

Labeled oligonucleotides and Ni-NTA fractions were incubated and electrophoresed similar to Fig. 7C and quantitated using a PhosphorImager. The fraction of lane counts extended to longer distances was calculated as a measure of the nucleotidyl transferase activity. The fraction of total input counts remaining in each lane (in comparison with probe-only control lanes) reflects 5'-phosphatase or 5'-exonuclease activity.

| Substrate 1 | Substrate 4 |
|-------------|-------------|
| Vector      | 0.76        | 0.69        |
| HM-Pol4     | 0.70        | 0.67        |

A unifying model that incorporates interaction with a nuclease with the concept of polymerase redundancy states that Pol4 is required for only one of the potentially several NHEJ pathways in yeast cells. A first more limited pathway can join ends only via simple religation or gapped intermediates, but is more promiscuous with regards to polymerase usage, explaining the limited effect of the pol4 mutation on gap joining. A second more versatile pathway can join a greater variety of end configurations, such as mixed joins, presumably by recruitment of additional processing activities. This pathway is also more selective, however, and will only utilize Pol4. There is precedent for the presence of multiple NHEJ pathways in yeast. Moore and Haber (11) examined NHEJ events at HO-cut MAT DNA, similar to Fig. 5, and showed that HO(+2) joins predominate in S/G2 and are dependent on RAD50/MRE11. HO(-3) joins are less cell cycle dependent, and less impaired by loss of RAD50/MRE11, suggesting that they proceed through a different NHEJ pathway. Our pol4 data are very similar to the rad50 pattern observed by Moore and Haber, suggesting Pol4 recruitment as part of the molecular basis for the Rad50/Mre11-dependent NHEJ pathway dominant in S/G2, which in turn further implicates Mre11 as the missing 3' nuclease activity.

End Processing in Higher Eukaryotes—Unlike initial observations regarding DNA ligase IV (17, 21), it is uncertain whether our Pol4 results indicate a role for Pol β in NHEJ in higher eukaryotes. hPol β mediates BER, whereas Pol4 is dispensable for this function (54). Further, the yeast-specific portions of Pol4 may be required for specific recruitment to the NHEJ active site. Finally, Sobol et al. (92) have examined Pol β-deficient fibroblasts and found no increase in IR sensitivity, indicative of an intact NHEJ mechanism. Despite this, the end alignment and processing we observed in yeast is very similar to that observed for vertebrate NHEJ both in vivo (55-57) and in vitro (58, 59). Indeed, NHEJ in Xenopus egg extracts is inhibited by ddNTPs but not aphidicolin (58), a pattern characteristic of Pol β.
Toward Understanding the Concerted NHEJ Mechanism—

In the model suggested by this study and many others, DSB recognition leads to formation of a complex on each DNA end that presumably involves at least the Ku proteins and probably the Sir and Rad50-Mre11 complexes. Ends are brought together, and base pairing drives formation of a stable alignment structure that recruits enzymes for resolution of strand discontinuities and ultimately nick ligation. Importantly, our data indicate that the yeast NHEJ apparatus does not attempt to judge the correctness of an alignment register by biasing against discontinuous alignments by a means other than their relative thermodynamic stability. For example, the alignment gaps in \( M(+) \) are not frankly inhibitory, because this join formed once for every five \( M(0) \) joins (Fig. 2). Indeed, when only gap joining is possible it can be nearly as efficient as simple religation, as seen with \( X(B)(+) \) (Fig. 2).

The most tenuous aspect of the above model is the ordering of events. The alignments diagrammed in the figures are inferred from the join sequences based on the assumption that alignment occurs before processing. From a technical standpoint, we have verified that plasmid ends are ligation-competent \textit{in vitro}, and therefore base loss must occur \textit{in vivo} (see “Experimental Procedures”). Limited \textit{in vivo} degradation of ends prior to joining could explain the observed join types but would provide no explanation for the differential effects of \( pol4 \) mutation. Further evidence that base removal occurs after alignment is provided by our finding that FEN-1 is involved in NHEJ, as discussed elsewhere (52). A final issue is the exten-

\begin{table}
\begin{tabular}{|c|c|c|c|}
\hline
Join & Interred alignment & Polarity & \textit{pol4} Told Reduction \\
\hline
SK(-1) & AGCA CT & 3' & >95 \\
& TC TGGA CA & & \\
HO(+2) & GCAACA GT & 3' & >70 \\
& CG TGTCAT & & \\
MB(+1) & TA GATCCT & 5' & 28 \\
& ATGC G & & \\
& AGCAT CT & 3' & >24 \\
& TC ATGGA C & & \\
HO(-3) & GCAAGT & 3' & 8 \\
& CGTCA TG & & \\
MB(+3) & TA GATCCT & 5' & 2.3 \\
& ATGC GA & & \\
SK(-3) & AGCCT & 3' & 1.7 \\
& TGGA TCA G & & \\
M(+2) & AA CGCGTT & 5' & 1.7 \\
& TTGCCA AA & & \\
XB(+2) & AC GATCCT & 5' & 1.4 \\
& TGAGCT GA & & \\
\hline
\end{tabular}
\end{table}

FIG. 8. Summary of findings. Processed joins that formed at a significant rate are displayed as in Fig. 2D and sorted according to the magnitude of the decrease in join rate caused by \textit{pol4} mutation. Lines stratify the results into strong, intermediate, and weak \textit{pol4} effects.
sive and efficient 5' degradation seen in yeast and known to precede rDSBR events. The nature of the balance between 5' degradation and end preservation is uncertain, because Rad50 and Ku are both required for NHEJ and yet the former promotes end degradation, whereas the latter stabilizes ends (60).

We cannot rule out the possibility that extensive 5' degradation occurs before NHEJ of 3' overhangs, because the 3'-terminal bases would still be available for pairing. Even limited 5' strand degradation would prevent occurrence of all joins observed at 5' overhangs, however. It will be of great interest to examine this dynamic relationship between NHEJ, rDSBR, and the degradation and polarity of ends in more detail.

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