DNA Joint Dependence of Pol X Family Polymerase Action in Nonhomologous End Joining

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DNA double-strand breaks (DSBs) can be directly rejoined by the nonhomologous end-joining (NHEJ) pathway of repair. Nucleases and polymerases are required to promote accurate NHEJ when the terminal bases of the DSB are damaged. The same enzymes also participate in imprecise rejoining and joining of incompatible ends, important mutagenic events. Previous work has shown that the Pol X family polymerase Pol4 is required for some, but not all, NHEJ events that require gap filling in Saccharomyces cerevisiae. Here, we systematically analyzed DSB end configurations and found that gaps on both strands and overhang polarity are the principal factors that determine whether a joint requires Pol4. DSBs with 3’ overhangs and a gap on each strand strongly depended on Pol4 for repair, whereas DSBs with 5’ overhangs of the same sequence did not. Pol4 was not required when 3’ overhangs contained a gap on only one strand, however. Pol4 was equally required at 3’ overhangs of all lengths within the NHEJ-dependent range, but was dispensable outside of this range, indicating that Pol4 is specific to NHEJ. Loss of Pol4 did not affect rejoining of DSBs that utilized a recessed microhomology or DSBs bearing 5’ hydroxyls but no gap. Finally, mammalian Pol X polymerases were able to differentially complement a pol4 mutation depending on the joint structure, demonstrating that these polymerases can participate in yeast NHEJ but with distinct properties.

Double-strand breaks (DSBs¹) can be repaired by two mechanisms: homologous recombination and nonhomologous end-joining (NHEJ) [reviewed in (1)]. Whereas recombination utilizes an intact homologous template to promote accurate repair, NHEJ relies on base-pairing potential in the terminal overhangs, termed microhomologies, to guide religation of the ends. NHEJ is initiated when the Ku heterodimer (Yku70/Yku80 in yeast) binds to the DSB ends. The Mre11/Rad50/Xrs2 complex is thought to tether the two termini together, along with DNA-PK in mammalian cells (2-4). The DNA ligase IV complex, in yeast consisting of the catalytic subunit Dnl4 and its cofactor Lif1, ligates the break to restore the duplex (5).

Agents that create DSBs often leave damaged bases at the break termini (6,7). Processing enzymes, at minimum including a nuclease and a polymerase, must remove and resynthesize these damaged bases before ligation can occur, but such repair is ultimately accurate. The nuclease Artemis is involved in mammalian NHEJ (8), but in yeast the NHEJ nuclease(s) are unknown. The Pol X family of DNA polymerases has been implicated in NHEJ. Pol4, the only Pol X family member in yeast, is required for gap filling in some end configurations but not others (9). Mammals have four Pol X family members: Polβ, Polλ, Polμ, and terminal deoxynucleotidyl transferase (TdT) [reviewed in (10)]. Polβ functions in base excision repair (11), while TdT is specifically associated with V(D)J recombination, a specialized form of NHEJ that occurs only in B- and T-cell maturation (12,13). In contrast, recent reports have implicated Polλ and Polμ in general NHEJ. Immunodepletion of Polλ, but not Polμ, inhibits in vitro NHEJ (14). Additionally, Polμ has been show to interact with Ku and the XRCC4/ligase IV complex, and is
recruited to DNA damage foci (15). Both Polλ and Polµ can act in NHEJ in a reconstituted system (16). In all cases, Pol X polymerases are only required for NHEJ when gaps must be filled, indicating that they are not part of the core NHEJ complex.

While the ability of NHEJ to trim and fill overhangs is of great importance to restorative repair, it also enables imprecise rejoining. NHEJ faithfully repairs DSBs with compatible overhangs the majority of the time, but Pol4-dependent inaccurate repair involving overhang mispairing and gain or loss of nucleotides can occur (17-19). NHEJ can also join incompatible termini by utilizing microhomologies either in the DSB overhangs or exposed by resection into the duplex DNA (9,20). Since incompatible termini necessarily originate from separate or resected DSBs, such imprecise NHEJ events lead to chromosomal rearrangements or deletions. Together, terminal base damage, overhang mispairing and incompatible termini lead to a large variety of processed NHEJ joints and outcomes in vivo. Thus, understanding the mechanistic basis of processed NHEJ, including the differential contributions of processing enzymes to various joint types, is of great importance.

We previously identified some Pol4-dependent NHEJ events, but due to limited ability to create specific DSB termini could not identify the precise characteristics of a joint structure that necessitated Pol4 action (9). Here, we sought to determine why only some joints that require gap filling are Pol4-dependent by using oligonucleotide-modified plasmids (OMPs) to perform a systematic analysis of DSB end configurations (21). We found that only NHEJ events that involve 3’ overhangs and require gap filling on both strands are strongly Pol4-dependent, with Pol4 promoting accurate repair. Repair of Pol4-independent gap-containing DSBs does not require the translesion polymerases Polη or Polζ, implicating the replicative polymerases in these joints. Pol4 appears to be exclusively associated with the NHEJ pathway of DSB repair, as it is not required for Ku-independent DSB rejoining that occurs via long overhangs or recessed microhomologies (i.e. microhomologies within the duplex portion of a DSB). Finally, we extend these findings to mammalian NHEJ by showing that Polβ, Polλ, and Polµ can each partially replace Pol4 when expressed in yeast. Interestingly, these mammalian polymerases reconstituted NHEJ to different extents depending on the joint structure. These data suggest that specialized NHEJ polymerases are recruited to DSBs with termini that are too unstable to utilize the replicative polymerases.

Materials and Methods

Yeast strains – S. cerevisiae strains used for plasmid transformation were isogenic derivatives of the previously described wild-type strain YW389 (MATa ade2D0 his3D200 leu2 lys2-801 trp1D63 ura3D0) (22). YW438 (pol4Δ::MET15), YW459 (yku70Δ::HIS3), YW507 (dnl4Δ::MET15), YW1567 (pol4Δ::MET15 rad30Δ::kanMX4), YW1568 (pol4Δ::MET15 rev3Δ::HIS3), YW1569 (pol4Δ::MET15 rad30Δ::kanMX4 rev3Δ::HIS3), and YW1654 (pol4Δ::MET15 yku70Δ::kanMX4) were created using a PCR-mediated one-step gene replacement technique (23). All disruptions were confirmed by PCR. The suicide deletion strains YW1276 (ADE2::HOSD[+1]) and YW1407 (ADE2::HOSD[+1] pol4Δ::URA3) were described previously (18). When indicated, yeast were transformed with the appropriate polymerase expression plasmids (see below) with continued selection for the plasmid using growth medium lacking uracil.

Polymerase expression plasmids – Polymerase expression plasmids were created by gap repair with tailed PCR fragments into the CEN/LEU2 plasmid pTW435, which uses the ADH1 promoter to drive expression of a nuclear localization signal (NLS)-Myc tagged protein. The POL4 coding sequence was amplified from yeast genomic DNA while the Polβ, Polλ, and Polµ sequences were amplified from human placental cDNA (Clontech) using the Advantage HF PCR Kit (BD Biosciences). Plasmids were sequenced to verify the correct coding sequence.

Oligonucleotide-modified plasmids – OMPs were created as previously described (21). Briefly, two pairs of annealed oligonucleotides designed to restore the ADE2 coding sequence in plasmid pTW423 were ligated onto the ends of pTW423
digested with BglII and XhoI (Supplemental Figure 1A). Plasmids were purified from free oligonucleotides by agarose gel electrophoresis, and product size, linearity, and integrity were verified. DNA concentration was quantified by UV spectrometry. Because the DSB side of the oligonucleotides initially bore 5’ hydroxyls to prevent ligation of the DSB in vitro, purified OMPs were typically treated with T4 polynucleotide kinase (NEB) followed by a second round of purification. The extent of oligonucleotide ligation was then monitored by primer extension (Supplemental Figure 1B). Because the ligation extent is not constant, small differences in NHEJ efficiency between OMPs cannot be considered significant, although large differences likely reflect real changes in the repair rate. Inter-strain differences with a given OMP are independent of this effect.

SfiI digest of plasmids pTW502-507 – Plasmids pTW502-507 are derivatives of the CEN plasmid pES16, which was previously described (5). Two PCR products amplified from pES16 were prepared to insert an SphI site and stop codons in all reading frames flanked by two SfiI sites after the second codon of ADE2. The first product was made using primers OW2003 (5’ ACACCCGCGCCGGTCTAAATG) and OW2041 (5’ TACTAGCATGCGGCC)

GAGTGGGCCATCCATCTTGATGTGGTTTG T). The second product was made with OW2068-2073, depending on the plasmid (5’ TACTAGCATGCGXTAACTGACTAGGCGCCXXCGGCCCATCTGACAAGTTGTATATT AGGA) and OW2008 (5’ TTAACAGATCTCACAATCATGACTGC). The SphI site is indicated in italics, the SfiI sites are in bold, stop codons are underlined, and variable nucleotides that place ADE2 in frame or make up the desired overhang are indicated by X. The products were digested with NotI and SphI or BglII and SphI, respectively, and simultaneously ligated into NotI- and BglII-digested pES16. Plasmids were then digested with SfiI to excise the polyterminator and gel purified as above.

Yeast transformation – Plasmids were transformed into yeast using a high efficiency lithium acetate method as previously described (21). 100 ng of linear plasmid (marked with URA3) was co-transformed with 10 ng supercoiled pRS315 as a transformation control (marked with LEU2). Cells were plated in parallel to glucose medium lacking either uracil or leucine and grown at 30°C for 3 days. The relative repair efficiency for a strain-plasmid combination is expressed as the ratio of Ade+ (white) colonies on plates lacking uracil, which represent in-frame joining of DSBs, to colonies on plates lacking leucine. Note that while in-frame joining effectively selected for the target joint in most cases, alternative Ade+ joints can also occur and will be represented in the graphs. To ensure adequate colony counts, more cells were plated for less efficient joints and/or mutant strains. A minimum of 150, and more typically 500-1200, Ade+ colonies were counted for each joint in wild-type yeast, except where noted. Similar colony numbers were counted for mutant strains when possible, but in instances where the mutant strain is highly deficient at forming a particular joint counts are of the few colonies recovered at the highest plating density. Individual data panels always represent results collected in parallel with a single preparation of the plasmids and carrier DNA to allow valid comparisons. Small variances between graphs are the result of different reagent sets and are not significant as compared to the large changes observed when comparing mutant and wild-type strains within a graph.

Joint analysis – To determine whether Ade+ colonies had formed the target joint in wild-type and/or residual colonies of mutant strains, a sampling of independent colonies for relevant plasmid-strain combinations were subjected to joint analysis. For this, PCR was used to amplify the region of ADE2 containing the break, followed by sequencing. A complete understanding of a particular joint efficiency can only be determined by considering both the transformation efficiency (Ade+/Leu+ ratio) and pattern of joints among sequenced colonies.

Suicide deletion – The HO(+) suicide deletion allele was used to measure the percentage of cells that undergo NHEJ and the percentage of imprecise +2 NHEJ events as previously described (18).
RESULTS AND DISCUSSION

Pol4 is required for gap filling independently of nonhomologous flaps. Based on previous data (9), we considered several joint features, in addition to the presence of gaps, as possible explanations for Pol4 dependence in NHEJ: formation of nonhomologous flaps (i.e. unpaired terminal nucleotides) upon overhang annealing, the stability of the annealed overhangs, and overhang polarity. To test whether nonhomologous flaps rendered gap-filling Pol4-dependent, a series of plasmids were created that bore the four possible combinations of 1-nt flaps and gaps (Figure 1). The fully compatible and gap-only DSBs are the most physiologically relevant of these as they correlate with precise repair of complementary overhangs at a single DSB, where gaps would result from removal of damaged terminal nucleotides. More generally, this panel mimics end structures that might arise when incompatible termini of different DSBs are joined. To make these substrates we took advantage of the fact that the 3-base SfiI 3' overhangs can be comprised of any combination of nucleotides. SfiI sites were placed after the start codon of ADE2 such that the target joint placed ADE2 in-frame. SfiI-digested plasmids were transformed into wild-type, pol4, yku70, and dnl4 yeast to monitor repair, along with a supercoiled LEU2-marked plasmid to control for transformation efficiency. As expected, DSBs with fully compatible overhangs did not require Pol4 for joining (Figure 1, first joint). When a 1-nt gap was introduced, joining became strongly Pol4-dependent (Figure 1, second joint). This joint was also highly dependent on Dnl4 and Yku70, indicating that repair occurred by NHEJ. The few Ade+ colonies that did arise in pol4 yeast utilized other short recessed microhomologies. A joint with a 1-nt gap and a 1-nt nonhomologous flap also showed substantial Pol4-dependence, with the target overhang-to-overhang joint not recovered among 5 sequenced residual events from the pol4 strain (Figure 1, fourth joint). Thus, Pol4 is required for gap-filling in 3’ overhang NHEJ events regardless of the presence or absence of nonhomologous flaps. A joint with 1-nt flaps but no gap showed only a ~2-fold reduction in the pol4 strain (Figure 1, third joint), similar to previous results (9).

Pol4 is not required for MMEJ. Surprisingly, substantially more Ade+ colonies were recovered in yku70 yeast relative to the dnl4 strain with two of the above SfiI-digested plasmids. Sequencing revealed that the joints recovered in yku70 and dnl4 strains utilized a 6-nt recessed microhomology that contained part of the SfiI recognition sequences and placed ADE2 in-frame in these plasmids (Figure 1, first and fourth joints). This joint bears the characteristics of microhomology-mediated end joining (MMEJ) events that occur in NHEJ mutant strains when no homology donor is available for recombination (24). MMEJ shows partial DNA ligase IV-dependence but does not require, and is in fact inhibited by, Ku, consistent with our data (24). These plasmids thus provided a means of assessing the role of Pol4 in MMEJ. For the fourth SfiI joint, 5 of 5 residual repair events formed by the pol4 strain corresponded to this MMEJ event (Figure 1). More strikingly, the increased MMEJ efficiency seen upon yku70 mutation was still observed upon further pol4 mutation. Therefore, Pol4 is not required for MMEJ.

Pol4 is required at all NHEJ-dependent microhomology lengths. We next asked whether the pattern of Pol4-dependence seen in Figure 1 would be recapitulated if the joint contained a more stable (but still NHEJ-dependent) microhomology. The OMP approach was used to create DSBs with the desired overhang configurations. In this system, annealed oligonucleotides are ligated onto restriction site ends within the ADE2 gene of the plasmid pTW423 (Supplemental Figure 1A). Ade positivity largely, but not completely, selects for the target joint by requiring ligation of both oligonucleotide pairs and subsequent rejoining of the DSB (21). We again created a panel of DSBs containing 3’ overhangs and each possible combination of 1-base gaps and 1-base flaps, but now in the context of a 4-base microhomology. The overhang sequence was kept constant to ensure that differences in repair were due to the presence of flaps and gaps. Such ends are again within the spectrum expected to occur at naturally occurring DSBs, with flaps relevant only to mutagenic events. These DSBs showed a pattern of Pol4-dependence similar to the shorter
overhangs. Each gap-containing joint was again highly Pol4-dependent regardless of whether flaps were present (Figure 2A). The deficiency in the pol4 strain was comparable to that of the dnl4 strain, indicating that Pol4 is strongly required for NHEJ of gap-containing DSBs with 3’ overhangs. Residual Ade+ colonies generated by the pol4 strain corresponded to the designed joint, however, indicating that this event can occur in the absence of Pol4 at a low frequency (Figure 2A). A joint with flaps but no gaps again showed slightly decreased joining in the absence of Pol4 (Figure 2A, third joint). Thus, Pol4 is required for efficient gap filling at 3’ overhangs regardless of whether the overhang contains 1, 2, or 4 homologous bases, and in all cases tends to promote accurate rejoining of the microhomology present in the overhangs.

Overhang polarity determines Pol4 dependence. We next generated a comparable panel of OMPs with 5’ overhangs to test the importance of overhang polarity, given that each polarity is expected to occur naturally. These DSBs were markedly less Pol4-dependent than the corresponding DSBs with 3’ overhangs (Figure 2B). When the DSB contained 1-nt gaps, a 2.1-fold decrease in joining efficiency was observed in the pol4 strain as compared to a 39-fold deficiency with 3’ overhangs. Similarly, a 5’ overhang joint with 1-nt flaps and 1-nt gaps was only 1.6-fold reduced in a pol4 mutant versus 31-fold with 3’ overhangs. Sequencing revealed that Ade+ colonies indeed represented the designed joint in both wild-type and pol4 strains for 5’ overhangs (Figure 2B). Dnl4 was still required for 5’ overhang joining, however, indicating that repair still occurred by bona fide NHEJ (Figure 2B). Thus, the NHEJ requirement for Pol4 is greatly relaxed at 5’ overhangs.

Why might 3’ overhang joints depend more strongly on a specialized NHEJ polymerase? The 3’ primer terminus that must be extended during gap filling has a different stability and orientation when comparing 3’ and 5’ overhangs. With 5’ overhangs, the 3’ end is on the stable duplex adjacent to the gap, and synthesis proceeds centrally. With 3’ overhangs, the polymerase must synthesize away from the break center using the overhang-to-overhang base pairing as the primer-template pair. This duplex consists of only one to at most six bases (21), and is therefore much less stable than the primer used for 5’ overhang filling. Thus, Pol4 is likely a specialized polymerase that evolved to function in circumstances where an unstable duplex is the only available primer for DNA synthesis. The recently solved crystal structure of Pol λ, the mammalian Pol X polymerase most related to Pol4, supports this idea (25). Interactions between the polymerase and the 3’ primer terminus are much less extensive in Pol λ than in the replicative polymerases, while the lyase domain of Pol λ makes extensive contacts with the base on the 5’ side of the gap. The unique biochemical properties of Pol X polymerases, including TdT activity and frequent primer-template slippage, appear to reflect this reduced dependence on a stably annealed primer (26-28). It is also possible that accessibility of the primer terminus within the bound NHEJ complex differs with joint polarity, and that only Pol4 is capable of gaining access to internal 3’ ends via interactions with other NHEJ proteins. Indeed, 5’ overhangs could even be filled prior to association of the DSB ends, while 3’ overhangs can only be filled during NHEJ.

Although Pol4 is not strictly required at gapped 5’ overhangs, our results do not rule out a role for Pol4 in these events in wild-type cells. In fact, a modest decrease was observed in pol4 mutants (Figure 2B). We therefore favor a model in which other polymerases can compensate for Pol4 loss at more permissive 5’ overhangs. To attempt to identify these redundant polymerases, we constructed double and triple mutants of pol4 with the two remaining nonessential polymerases in budding yeast, Polη and Polζ. Polη, encoded by the RAD30 gene, and Polζ, consisting of two subunits Rev3 and Rev7, are involved in error-free and error-prone lesion bypass pathways, respectively [reviewed in (29,30)]. Neither of these polymerases was required for gap-filling of 5’ overhangs, as even pol4 rad30 rev3 yeast repaired the gapped 5’ overhang DSB as efficiently as pol4 yeast (Figure 2C). Therefore, one of the essential polymerases or an unknown polymerase must be utilized. Polζ, Polη and Polε are required for DNA replication. Intriguingly, a recent study using mammalian cell extracts identified Polo as providing NHEJ polymerase activity (31). The involvement of Polζ and Polε in
BER also makes them reasonable candidates (32,33). Polφ, encoded by the POL5 gene, is required for rRNA synthesis (34). Alternative approaches will be needed to gain further insight into which of these polymerases might act in yeast NHEJ.

**Pol4 is dispensable when only one strand requires gap filling.** To gain insight into the order of events in NHEJ, we next created DSBs identical to the gap-containing 3’ overhang used in Figure 2A, except with a gap on only one strand. In this configuration, Pol4 was largely dispensable for joining, but Dnl4 was not (Figure 3). Joint sequences showed that the duplex was restored as expected in both wild-type and pol4 strains (Figure 3). This indicates that even in the absence of Pol4, the NHEJ machinery can ligate the one nicked strand, as has been observed in vitro (16). It is possible that the now stable primer could be extended by another polymerase to fill the gap during NHEJ, but it seems more likely that disengagement of the NHEJ proteins results in a single-strand 1-nt gap. This would not affect the outcome because the plasmid could be replicated by copying of the intact strand or simply be repaired by BER since the critical need for reassociation of DSB ends has been satisfied.

**Pol4 is not required for NHEJ via a recessed microhomology.** Data in Figure 1 indicated that Pol4 is not required for MMEJ joints that utilize recessed homologies on each side of the break. To explore this further, we designed a joint in which a 4-base 3’ overhang pairs with a recessed microhomology immediately adjacent to a blunt end. Like MMEJ, such repair correlates with a “last-ditch” repair attempt when overhang-to-overhang joining is not possible. Unlike MMEJ, however, this event showed strong dependence on both DNA ligase IV and Ku (Figure 4), indicating that NHEJ is primarily responsible for repair in such cases. Joining occurred at wild-type levels in pol4 yeast, and sequencing revealed that the joint occurred as designed, again indicating that Pol4 is dispensable for recessed microhomology joining, whether catalyzed by NHEJ or MMEJ (Figure 4).

Based on current data, we cannot determine whether other polymerases are recruited in association with recessed microhomology searching or simply that the search does not typically entail overcleavage and therefore does not require base resynthesis.

*Pol4 can fill gaps greater than 1-nt in length.* Pol X polymerases are able to efficiently fill gaps of various lengths *in vitro* as long as a 5’ terminus is provided (27,35). To test this ability during NHEJ, OMPs similar to those used in Figure 2A were generated in which the 4-base microhomology was kept constant but the gap on each side was extended to 2, 3, or 4 bases. Such DSB structures may be formed directly by DNA damaging agents, and are also thought to occur *in vivo* during the early stages of 5’ resection. Ends that formed 1- and 2-base gaps were joined at similar rates in wild-type yeast and showed >100-fold reductions in the pol4 and dnl4 mutant strains (Figure 5). Extending the gap to 3 and 4 bases reduced the joining efficiency substantially in the wild-type strain (Figure 5), consistent with the notion that resection inhibits NHEJ and commits DSB repair to recombination (36). The reduced repair rate made it difficult to reliably determine whether these events were Pol4-dependent. We nonetheless did observe more Ade+ colonies in wild-type yeast (93 for the 3-base gap and 41 for the 4-base gap) than in the pol4 strain (0 for the 3-base gap and 4 for the 4-base gap) for these DSBs. Thus, while there is an upper limit to the gap length that can be efficiently repaired by NHEJ, the few successful NHEJ events at longer gaps in wild-type yeast likely utilize Pol4.

**Pol4 is only required for gap filling in Dnl4-dependent joints.** Previous work in our laboratory showed that DSBs with overhangs of fewer than 6 bases strongly depend on NHEJ for repair, whereas DSBs with longer overhangs can be rejoined very efficiently by mechanisms other than NHEJ (21). We therefore next created a panel of DSBs containing 3’ overhangs with 4, 6, 8, and 10 bases of homology and either ligatable ends or 1-base gaps. These substrates represent the natural spectrum of DSBs expected to arise from closely spaced single-strand breaks. Gaps led to a substantial decrease in rejoining efficiency compared to the corresponding compatible overhangs at all overhang lengths tested, showing that precise rejoining is generally more efficient than imprecise rejoining (Figure 6). Gap-containing overhangs with 6 bases of homology were less Pol4-dependent than those with 4 bases,
showing a reduction of only 3.1-fold in the pol4 strain (Figure 6). This directly paralleled a reduced dependence on Dnl4 at 6 bases (Figure 6). DSBs with 8 and 10 bases of homology and 1-nt gaps were repaired at about 10-fold greater efficiency than DSBs with shorter overhangs, consistent with previous data, and were not impaired in the absence of Pol4 or Dnl4 (Figure 6) (21). Thus, Pol4 is only required for gap filling during Dnl4-dependent NHEJ. These data strengthen our model that NHEJ is specifically suited to rejoining DSBs with unstable termini (21), as they show that only NHEJ employs a specialized polymerase, Pol4, that can act on such termini.

Pol4 is required at DSBs containing 5' hydroxyls only when gaps are also present. DSBs caused by DNA damaging agents frequently contain 5' hydroxyls that must be repaired before ligation can occur (6,7). Pol4 can efficiently fill gaps with 5' hydroxyl termini in vitro, even though Pol β and Pol λ require a 5' phosphate for full activity (27,37). We therefore asked whether Pol4 is required for NHEJ of a DSB containing 3' overhangs, a 1-nt gap, and 5' hydroxyls. This joint was formed less efficiently than the corresponding 5' phosphate joint, consistent with previous results (21), but remained strongly Pol4-dependent (Figure 7, third and fourth joints). Thus, Pol4 is indeed utilized in vivo when the NHEJ machinery encounters gaps with 5' hydroxyl termini. But how is the 5' hydroxyl resolved? The terminal base containing the 5' hydroxyl could be removed by a nuclease either before or after its resynthesis by a polymerase. Initial removal of the base would result in a gapped joint demonstrated above to require Pol4. Moreover, Pol4 interacts with the 5' nuclease Rad27 in a manner consistent with their concerted repair of damaged 5' termini (38). Therefore, we next asked whether NHEJ of DSBs with 5' hydroxyl termini but no gaps requires Pol4. Surprisingly, wild-type levels of repair were observed in the absence of Pol4 regardless of whether the termini contained 5' hydroxyls or 5' phosphates (Figure 7, first and second joints). The simplest explanation for these data is that the 5' hydroxyl is converted to a phosphate by a kinase. Indeed, mammalian polynucleotide kinase / 3' phosphatase is thought to act during NHEJ (39,40). Its yeast homolog, Tpp1, contains only the 3' phosphatase domain, however, and we correspondingly failed to detect a 5' kinase activity in yeast (22). It is thus unclear whether a cryptic kinase activity is present or the removed 5' terminal base is unexpectedly resynthesized by a polymerase other than Pol4.

The mammalian Pol X polymerases can differentially complement pol4 mutation. The above results establish many features of DSB joints that determine Pol4 dependence in a fashion consistent with its enzymatic properties. Because the mammalian Pol X polymerases are structurally and enzymatically similar to Pol4 (27,28,35), we next sought to determine whether human Polβ, Polλ, and/or Polµ could complement pol4 mutation in yeast NHEJ at these same joints. Each polymerase, including Pol4, was expressed from the ADH1 promoter on a plasmid with an amino-terminal NLS-Myc tag. There was surprisingly wide variation in expression levels, with Pol4 paradoxically showing the lowest level of expression and Polβ the highest (Figure 8A). This is important when considering the relative NHEJ function of these polymerases below.

NHEJ was measured using the above plasmid assays as well as a previously described chromosomal assay. Broadly, the results indicated that the mammalian Pol X polymerases can indeed act during yeast NHEJ, although generally at lower efficiency. Strikingly, each polymerase showed a distinct pattern of joint formation. In the plasmid assays, a joint containing 2 homologous bases and 1-nt gaps was completed by Polλ and Polµ, but not Polβ (Figure 8B, first joint). Only Polµ was able to catalyze formation of a joint with one homologous base, a 1-base gap and a 1-base flap, however (Figure 8B, second joint). Strains expressing Polβ or Polλ formed only the MMEJ event also detected by this plasmid. All of the mammalian polymerases, including Polβ, were able to complete a joint containing 4 complementary bases and 1-nt gaps, although Polµ was now paradoxically less efficient (Figure 8B, third joint). Adding 1-nt nonhomologous flaps to this joint produced a similar pattern (Figure 8B, fourth joint). The chromosomal “suicide deletion” assay selects for imprecise NHEJ in the +2 reading frame at an HO endonuclease-created DSB (18). Such joining occurs in 1-2% of all surviving cells and is strictly Pol4 dependent (9,18). When Pol4
is present, nearly all events correspond to a joint with a 2-base insertion due to mispairing of the 3’ overhangs, consistent with above results (Figure 8C). The mammalian Pol X polymerases each resulted in a slight but detectable increase in colony recovery compared to the pol4 strain, but these nearly all corresponded to other imprecise joints (Figure 8C). Most common was a loss of 1 base resulting from a different overhang mispairing.

These data establish that each mammalian Pol X polymerase is capable of participating in NHEJ when expressed in yeast. The significance of this finding is most clear for Polλ and Polµ. These polymerases have been directly implicated in NHEJ based on in vitro results (14,16), and were only modestly overexpressed in yeast (Figure 8A). Their action in NHEJ likely reflects in part their similar apparent reduced dependence on a stable primer-template pair (26,41). Pol4, Polµ and Polλ are not biochemically identical, however (27,28,35), which likely explains their differential ability to catalyze various NHEJ joints. Our data are not sufficient to fully characterize the variable joining patterns, but they nonetheless support the idea that the seemingly redundant mammalian NHEJ polymerases evolved to deal with different end structures (10). Pol4, Polλ and Polµ also each contain an amino-terminal BRCT domain which interacts with various components of the NHEJ core machinery (15,42,43). It is likely that this domain contributes to the ability of Polλ and Polµ to function in yeast NHEJ, with species divergence in protein-protein interactions accounting for their lesser overall activity. We are currently exploring these possibilities. It is much less certain whether Polβ is a bona fide NHEJ polymerase as it was less consistently able to complement pol4 mutation despite being expressed at a much higher level. We argue that this overexpression most likely helped Polβ overcome its lack of a BRCT domain. Moreover, Polβ has never been functionally linked to mammalian NHEJ.

In summary, we have found that Pol4 is uniquely required for yeast NHEJ when joining occurs via pairing of 3’ overhangs that necessitates gap filling on both strands. Microhomology length, gap length and the presence of terminal mismatches affect the overall efficiency but not the Pol4 dependence as long as joints are still formed by Ku- and DNA ligase IV-dependent NHEJ. Pol4 may act at 5’ overhangs, but this function can also be carried out by unidentified, and presumably replicative, polymerases. Pol4 was not required for any joint tested that used recessed microhomologies. This pattern strongly indicates that the ability of Pol4 to extend weak primer-template pairs, and perhaps its ability to access ends bound by Ku and DNA ligase IV, accounts for its unique function in NHEJ. These conclusions are supported and extended to mammalian Pol X polymerases by the observation that Polµ, Polλ, and perhaps Polβ, can catalyze formation of these same “challenging” NHEJ joints. Further work is required to correlate the differing NHEJ outcomes with the biochemical properties of these various polymerases.

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FOOTNOTES

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1The abbreviations used are: DSB – double strand break, NHEJ – nonhomologous end joining, MMEJ – microhomology-mediated end joining, BER – base excision repair, NLS – nuclear localization signal.

FIGURE LEGENDS

Fig. 1. Pol4 is required for gap filling during NHEJ of 3’ overhangs regardless of whether nonhomologous flaps are present. Plasmids cut with SfiI were transformed into yeast. The Ade+ outcome selected for NHEJ events predicted to occur in the indicated joint configurations (as well as other joints in the same relative reading frame). Repair frequencies for each genotype are expressed as the ratio of Ade+ colonies over Leu+ colonies resulting from uptake of a cotransformed supercoiled plasmid (upper panel). Each bar represents the mean ± standard deviation from at least three independent transformations performed with the same reagents. A sampling of independent joints were recovered, amplified, and sequenced with primers within ADE2 (lower panel). Bases annealed in the joint are shown in bold, vertical lines indicate the location of the overhangs, and dashes indicate gaps in the sequence. Light shading highlights bases that diverged from the expected plasmid sequence. Numbers indicate how many times a joint was recovered for a given genotype. Assessment of the efficiency of any particular joint must consider both the transformation efficiency and its frequency in the recovered sequences.

Fig. 2. Overhang polarity determines Pol4 dependence. A, OMPs with 3’ overhangs for which the Ade+ phenotype selects for the indicated combinations of flaps and gaps. Repair frequencies and recovered joints are shown as in Figure 1. B, Rejoining of similar DSBs with 5’ overhangs. C, The translesion polymerases are not required for rejoining 5’ overhang DSBs containing a gap. Note that different carrier DNA preparations were used in B and C, resulting in a slightly different range in the transformation ratio due to nonequivalent effects of carrier DNA on the uptake of linear and supercoiled DNA.

Fig. 3. DSBs containing one ligatable strand and one gapped strand are repaired in the absence of Pol4. DSBs with mixed nicks and gaps were generated using OMPs that selected for the indicated joints, with data shown as in Figure 1.

Fig. 4. NHEJ that utilizes a recessed microhomology does not require Pol4. DSBs with or without a recessed microhomology were generated using OMPs that selected for the indicated joints, with data shown as in Figure 1.

Fig. 5. Pol4 can fill gaps greater than 1 nucleotide during NHEJ. DSBs with 4-base microhomologies in 3’ overhangs and variable gaps on each strand were generated using OMPs that selected for the indicated joints, with data shown as in Figure 1.

Fig. 6. Pol4 is only required for gap filling in Dnl4-dependent joints. DSBs with variable length 3’ overhangs were generated using OMPs that selected for the indicated joints, with data shown as in Figure 1. Data are separated onto two graphs because DSBs with 8- and 10-base overhangs are repaired about 10-fold more efficiently than DSBs with shorter overhangs. The Ade+/Leu+ ratio can exceed one for such plasmids because 10-fold more linear plasmid than circular plasmid is transformed, and yeast take up linear and supercoiled plasmid DNA with different efficiencies.
Fig. 7. The presence of terminal 5’ hydroxyls does not affect the requirement for Pol4. DSBs with variable 5’ phosphorylation status were generated using OMPs that selected for the indicated joints, with data shown as in Figure 1.

Fig. 8. Complementation of pol4 mutation by mammalian Pol X polymerases. A, Expression was verified by Western blot of whole cell lysates using the 9E10 Myc antibody (Santa Cruz Biotechnology). The blot was substantially overexposed so that the low level of Pol4 could be visualized. Note the much higher expression of Polβ. B, DSB rejoining was assessed by plasmid recircularization. The first two DSBs were made by SfiI digestion as in Figure 1, and the second two were generated using OMPs as in Figure 2. C, The HO(+2) suicide deletion assay was used to quantify the percentage of cells that undergo imprecise NHEJ of a chromosomal DSB. Repair is expressed as the fraction of all NHEJ events in the +2 reading frame (top panel). Recovered joints are shown as in Figure 1.
Figure 1
Figure 2

A 3’ Overhangs

- Wild-Type
- pol4
- dnl4

Ade+ Colony Sequences

Target Joint: CTTACCAATTGTAGAGCTATCACAAGGACA

Wild-Type 5x: CTTACCAATTGTAGAGCTATCACAAGGACA

pol4 5x: CTTACCAATTGTAGAGCTATCACAAGGACA

B 5’ Overhangs

- Wild-Type
- pol4
- dnl4

Ade+ Colony Sequences

Target Joint: CTTACCAATTGTAGAGCTATCACAAGGACA

Wild-Type 5x: CTTACCAATTGTAGAGCTATCACAAGGACA

pol4 5x: CTTACCAATTGTAGAGCTATCACAAGGACA
Figure 3

Ade+ Colony Sequences

Target Joint

Wild-Type

pol4

5x

4x

- CTTACC AATT GTAGA GACT ATCCACAAGGACA
- CTTACC AATT GTAGA GACT ATCCACAAGGACA
- CTTACC AACT GTGTA GACT ATCCACAAGGACA
Figure 4

Bar graph showing the Ade+/Leu+ colony counts for different strains:
- Wild-Type
- pol4
- yku70
- dnl4

Ade+ Colony Sequences:

Target Joint: CTTACCCAAATTGTAGAGACTATCCACAAGGACA

Wild-Type: 4x CTTACCCAAATTGTAGAGACTATCCACAAGGACA

pol4: 5x CTTACCCAAATTGTAGAGACTATCCACAAGGACA
Figure 6
Figure 7

Ade+ Colony Sequences

Target Joint: CTTACCCAATTTGTAGAGACTATCCACAAGGACA

Wild-Type 5x CTTACCCAATTTGTAGAGACTATCCACAAGGACA

pol4 5x CTTACCCAATTTGTAGAGACTATCCACAAGGACA
Figure 8
Supplemental Figure 1. A. Schematic of the oligonucleotide-modified plasmid method. B. Structure and ligation efficiency of oligonucleotide-modified plasmids. Primer extension was used to measure the ligation efficiency of each oligonucleotide-modified plasmid. Primer annealing sites are indicated as arrows in A.
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