The Role of DNA Polymerase 4 in Homologous Recombination

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Abstract

DNA double-strand breaks (DSBs), caused by either exogenous or endogenous factors, will lead to cell death unless repaired by DNA repair pathways that maintain genome stability. DNA polymerase 4 (Pol4) has been implicated in a pathway of DSB repair called microhomology-mediated end-joining (MMEJ), which uses short segments of microhomologies (5-25 bps) to repair DSBs. To determine the role of Pol4 in DNA repair, pol4 mutants (catalytic point mutants and deletion mutants) were tested in novel MMEJ interchromosomal (inter) and intrachromosomal (intra) recombination assays containing microhomologies using the model organism, Saccharomyces cerevisiae. The pol4 catalytic and deletion mutant showed statistically significant decreases (ranging from 2- to 37-fold) in recombination. The pol4 mutants were also tested for sister chromatid exchange and direct repeat recombination. The results showed the pol4 catalytic point mutant had a 2-fold decrease in direct repeat recombination.

Introduction

DNA can be damaged by endogenous factors (e.g., reactive oxygen species) and environmental factors (e.g., UV radiation). The preservation of the genome is essential to life and the genetic information is maintained by various DNA repair and checkpoint proteins. When DNA damage occurs, it must be properly repaired through the various cellular maintenance mechanisms (e.g., nucleotide- or base excision repair, mismatch repair, non-homologous end-joining [NHEJ], and homologous recombination [HR]) (Hoeijmakers 2001; Friedberg et al. 2005) otherwise it may lead to senescence, mitotic catastrophe, apoptosis, or uncontrolled cellular growth (cancer). HR functions in the template-dependent, high-fidelity repair of DNA single stranded gaps, DNA double-strand breaks (DSBs), stalled/broken replication forks and inter-strand crosslinks.

Repair of DSBs is vital because unrepaired DSBs are lethal in all organisms (Bennett et al. 1993). Figure 1 shows the two pathways available to repair a DSB: NHEJ and HR. Which of the two pathways is used depends on the species, cell cycle phase, tissue type, and lesion type. In NHEJ, Ku80 binds to the exposed ends of the DNA and brings them together, thereby facilitating DNA polymerase mediated gap filling and strand ligation by Dnl4. This form of repair is prone to error because it uses less than 4 base pairs (bps) of homology and does not require a template to repair the break. In HR, when a DSB is created, the MRX complex (Mre11, Rad50, Xrs2), together with Sgs1, Dna2, Exo1 and Sae2, resects the DNA end, 5’ to 3’ resuming in a 3’ single –stranded DNA tail. HR uses homology of 30 bps or more to perform strand invasion and repair, by using either the sister chromatid or a homolog as a template to repair the DSB. Because HR uses a template to repair the break, it has a higher fidelity of repair than NHEJ. As shown in the model, there are several different DNA polymerases which function to restore DNA stability.
Pol4 is one of seven nuclear polymerases in budding yeast, but its role in DNA metabolism is not well understood. Pol4 is a conserved eukaryotic polymerase and its human homolog is DNA polymerase \( \lambda \) (Pol\( \lambda \)) (Shimizu et al. 1993; Garcia-Diaz et al. 2002). Because Pol4 and Pol\( \lambda \) are homologs, \textit{S. cerevisiae} can serve as a model to reveal the function of this polymerase in the mammalian system. The existing research implicates Pol4 in having a role in DSB repair, through a pathway similar to HR called microhomology-mediated end-joining (MMEJ). MMEJ is a newly characterized DNA repair pathway that uses small stretches of DNA homology (5-25 bps) to repair DSBs (Decottignies 2007; McVey and Lee 2008). Pol4 functions in MMEJ by gap-filling and suppression of mismatched nucleotides following homology pairing during the repair of a DSB (Lee and Lee 2007).

**DNA Double-Strand Break (DSB) Repair**

![Figure 1. This illustration shows the different pathways to repair a double-strand break.](image)

Another form of genomic instability is spontaneous homologous recombination between repeats, which causes genetic material to be lost. Eukaryotes contain many repetitive elements that, when recombination occurs between them, can lead to genome rearrangements and disease (Strout et al. 1998; Nakaya et al. 2004). About 43% of the human genome is occupied by four major classes of interspersed repetitive elements: short interspersed elements (SINEs), long interspersed elements (LINEs), elements with long terminal repeats (LTR elements), and DNA transposons (Li et al. 2001). The most frequent repetitive elements in the human genome are the LINEs and the \textit{Alu} elements. Recombination between \textit{Alu} elements results in genomic deletions associated with many human genetic disorders (Sen et al. 2006). The inability to preserve the genome is one of the major reasons for many cancers, such as Acute Myeloid Leukemia, as well as other human diseases (Schichman et al. 1994).

In this study Pol4 was shown to have a role in induced DSB repair. Although past research has speculated on Pol4’s possible function in MMEJ, there has not
been complete characterization of MMEJ as a pathway or Pol4 in MMEJ. This analysis characterizes the frequencies of repair in interchromosomal and intrachromosomal MMEJ following the creation of a DSB. In addition, Pol4 was also examined for a role in sister chromatid exchange (SCE) and direct repeat recombination (DRR). The results of this study indicate that Pol4 has a potential role in DRR.

**Methods and Procedures**

**Strain construction.**

Standard techniques for yeast growth, genetic manipulation and plasmid construction were used throughout this study (Sherman *et al.* 1986; Maniatis *et al.* 1989). All yeast strains used in this study were isogenic with W303-1A (Thomas and Rothstein 1989) but were wild type at the *RAD5* locus. The *pol4::KAN-MX* allele was created by single-step gene disruption of the *POL4* locus using a *pol4::KAN-MX* PCR construct. The PCR construct was derived from pWDH516. A 1.7 kb fragment containing the *KAN-MX* cassette, flanked on the 5’ end by the first 50 bp of the *POL4* coding sequence, and on 3’ end by the last 50 bps of the *POL4* coding sequence, was amplified using primer-1 (5’-ATG TCT CTA AAG GGT AAA TTT TTC GCC TTT TTA CCT AAT CCT AAC TAC TCC AGC TGA AGC TTC GTA CGC-3’) and primer-2 (5’-TTA TGC AGT TTT TTT TTC CCA TTC GAT ATT TCT ATG TTC GGG TTC AGC GTG CAT AGG CCA CTA GTG GAT CTG-3’). This PCR fragment was transplaced into the *POL4* locus, selecting for resistance to G418. Integration into the *POL4* locus was verified by genomic PCR (D. Meyer, personal communication).

The *pol4-D367E* catalytic point mutant was generated using site-directed mutagenesis (Zheng *et al.* 2004) with primer-3 (5’-CAA GTG TGG TGA AAT CGA TCT TTT ATT TTT CAA GCC GTT G-3’) and primer-4 (5’-AAA TAA AAG ATC GAT TTC ACC ACA CTT GGA ATA GCC CCT A-3’). A DNA fragment containing *pol4-D367E* was digested with *PstI* and *BamHI* then cloned into YIp365R, which possesses a *URA3* selectable marker, to create WDH975. Following transformation and selection for uracil prototrophy, WDH975 was linearized by digestion with *NsiI* to target its transplacement into the *POL4* locus. Uracil prototrophs were plated to 5-FOA media to select for plasmid loss and the resulting 5-FOA' colonies assessed by PCR analysis to confirm integration of the *pol4-D367E* construct into the *POL4* locus (D. Meyer, personal communication).

The *his3-Δ3’HOcs* interchromosomal MMEJ substrates were created by PCR using WDH954 as a template to make three constructs that share varying amounts of homology with the *his3-Δ5’HOcs* substrate. The first construct shares 14 bps of homology (TTA AAG AGG CCC TA; Inter14); the second construct shares 14 bps of homology followed by a 2 bp mismatch and then 4 bps of homology (TTA AAG AGG CCC TGG CC; Inter14-2-4); and the third construct has 14 bps of homology followed by a 2 bp mismatch and then 9 bps of homology (TTA AAG AGG CCC TAG GGG CCG TGC G; Inter14-2-9). The PCR constructs were each digested with *BamHI* and cloned individually into YIp365R, which possesses a *URA3* selectable marker, which had been digested with *BamHI* to create WDH1006 (Inter14), WDH1007 (Inter14-2-4) and WDH1015 (Inter14-2-9). The resulting plasmids were linearized by digestion with *MscI* to target their transplacement into the *HIS3* locus following transformation and selection for uracil prototrophy. Uracil prototrophs were plated to 5-FOA media to select for plasmid loss and the resulting 5-FOA' colonies.
assessed for histidine auxotrophy by replica plating to synthetic medium-lacking histidine. 5-FOA’ resistant and histidine auxotrophic colonies were subjected to PCR analysis to confirm integration of the his3-Δ3’-HOcs MMEJ constructs into the HIS3 locus (D. Meyer, personal communication). Construction of the other MMEJ and translocation assay components (leu2::HOcs-his3-Δ5’(300), and trp1::GAL1-HO-KAN-MX) have been discussed previously (Pannunzio et al. 2008).

The intrachromosomal MMEJ assay was created using the cloning free genomic integration method (Erdeniz et al. 1997), using the his3-Δ5’, Kluyveromyces lactis URA3 gene and the newly constructed interchromosomal MMEJ constructs as substrates. The PCR generated substrates were targeted to the HIS3 locus following transformation and selection for uracil prototrophy and histidine auxotrophy. The resulting colonies were verified by PCR and sequencing to confirm integration.

All interchromosomal MMEJ and translocation strains possess the his3-Δ5’ substrate at the LEU2 locus on one copy of chromosome III. The intrachromosomal MMEJ strains possess the his3-Δ5’ substrate at the HIS3 locus just downstream of the his3-Δ3’ substrate. The his3-Δ3’ substrate is located at the HIS3 locus on one copy of chromosome XV (XV/III). The his3-Δ5’ and his3-Δ3’ substrates share 14 bps (Inter 14), 14+4 bps (Inter14-2-4), 14+9 bps (Inter14-2-9) or 311 bps (translocation) of HIS3 coding sequence. These strains also possess one copy of the his3-Δ200 allele at the HIS3 locus that is unable to form an intact HIS3 gene by recombination with either substrate.

The sister-chromatid and direct repeat recombination strain was a generous gift from Dr. Lorraine Symington (Fung et al. 2009).

The strains for the URA3 mutation assay were created by crossing wild type strains with a URA3 gene to the pol4 catalytic and deletion mutant. PCR and selectable markers were used to identify mutants in assay strains.

**HO-stimulated MMEJ DSB frequencies.**

Two ml cultures of YP-Raffinose were inoculated with single colonies of appropriate genotypes, and incubated for 24 hours at 30°C. Galactose is added to the cells to a final concentration of 2% to induce expression of the HO endonuclease. After four hours of expression, the cells were plated to medium-lacking histidine to select for recombinants, and dilutions plated to YPD to determine viability. The frequency was determined by dividing the number of histidine prototrophic colonies by the total number of viable cells plated. Median frequencies from at least 25 independent cultures per growth period and for each genotype were reported and the 95% confidence intervals calculated using a table (Knight 2006). Differences between frequencies for which the 95% confidence intervals did not overlap were considered statistically significant.

**Spontaneous SCE/DRR rates.**

One ml cultures of sterilized dH20 were inoculated with single colonies of cells from the appropriate genotypes. Cells were diluted and plated to medium-lacking adenine to select for SCE recombinants and plated to YPD medium to determine viability. The colonies that grew on medium-lacking adenine are then replica plated onto medium-lacking uracil. Colonies that grew on the medium-lacking adenine and not the medium-lacking uracil are DRR recombinants, while
colonies that grew on both medium-lacking adenine and uracil are SCE recombinants. Rates were determined from at least 50-60 independent cultures by the method of the median (Lea and Coulson 1949). The 95% confidence intervals were determined using a table (Knight 2006). Differences between rates for which the 95% confidence intervals did not overlap were considered statistically significant.

**URA3 Mutation rates.**
Two ml cultures of YPD were inoculated with colonies of the appropriate genotypes and allowed to grow 24 hours in 30°C. Cells were diluted and plated on YPD and 5-FOA medium. Colonies that grew on the 5-FOA medium-contained mutated URA3 genes. The rates were determined by the method of the median (Lea and Coulson 1949). The 95% confidence intervals were determined using a table (Knight 2006). Differences between rates for which the 95% confidence intervals did not overlap were considered statistically significant.

**Figure 2:** Mutants were tested in the sister chromatid exchange assay. There are two possible recombinants: Ade+ Ura+ (unequal sister chromatid gene conversion) and Ade+ Ura- (deletion formation).
Figure 3: This figure shows the two different MMEJ genetic assays: interchromosomal (left) and intrachromosomal (right).

Results

**Interchromosomal and Intrachromosomal MMEJ assays.**

The pol4 catalytic (pol4-D367E) and deletion mutant (pol4::KANMX) were analyzed in the interchromosomal (inter) and intrachromosomal (intra) MMEJ assays (Figure 3) containing 14 bps of perfect homology, 14 bps of perfect homology with a 2 bp mismatch followed by 4 bps of perfect homology (14-2-4), or 14 bps of perfect homology with a 2 bp mismatch followed by 9 bps of perfect homology (14-2-9). The pol4 catalytic mutant showed a statistically significant decrease of 2-fold in the 14 bp inter (Figure 4) and a 2.5-fold for the 14 bp intra (Figure 5) MMEJ assays. The pol4 catalytic mutant also showed a 4.5-fold decrease in the 14-2-4 bps inter (Figure 4) and a 5-fold decrease in the 14-2-4 intra (Figure 5) MMEJ assays. The pol4 deletion mutant showed a decrease in recombination of 37-fold in the 14 bps inter (Figure 4) and a 4-fold decrease for the 14 bps intra (Figure 5) MMEJ assays. For the 14-2-4 bps MMEJ assays the deletion mutant showed a 2-fold decrease in the inter (Figure 4) and a 9-fold decrease for the intra (Figure 5) MMEJ assays. The pol4 mutants did not show any difference from wild type in either the inter (Figure 4) or intra (Figure 5) 14-2-9 bps MMEJ assays. The above results suggest Pol4 is involved in repairing DSBs, which contain
microhomologies shorter than the homology provided by the 14-2-9 bp MMEJ substrate.

To further examine the role of Pol4 in repairing DSBs containing longer stretches of homology on either side of the break, the pol4 catalytic mutant was tested in an interchromosomal translocation assay that has 311 bps of homology between the translocation substrates (Pannunzio et al. 2008). The translocation assay mechanism of repair is the same as the interchromosomal MMEJ assay, except for the amount of homology shared by the truncated his3 alleles. The pol4 catalytic mutant was tested in the translocation assay and it showed no difference from wild type (Figure 6). This result further supports the results mentioned above, which suggest that the function of Pol4 is in promoting DSB recombination using microhomologies.

SCE/DRR assay.

The mechanism of repair in spontaneous homologous recombination is different than induced DSB repair in that the initiation event need not involve a DSB. Therefore, the analysis of Pol4 in spontaneous homologous recombination may provide further insight into how Pol4 functions to support genome stability through different DNA repair mechanisms. Therefore, pol4 catalytic and deletion mutants were tested using a spontaneous recombination system developed in the Symington laboratory (Fung et al. 2009), which measures SCE and DRR (Figure 2). The SCE rate of the pol4 catalytic and deletion mutants showed no difference from wild type (Figure 7). However, the pol4 catalytic mutant had a 2-fold decrease in the DRR rate (Figure 7), while the deletion mutant did not show the same effect as the catalytic mutant in DRR (Figure 7). These results indicate a novel role for Pol4 in promoting DRR during spontaneous recombination.

In addition to the SCE and DRR assays, Pol4 was also tested in the URA3 mutation assay to determine whether it had any role in spontaneous mutagenesis. The URA3 mutation assay uses a toxic intermediate of uracil, 5-fluorouracil, to test for the mutagenesis of the wild type URA gene. The pol4 catalytic and deletion mutants were tested in the URA3 mutation assay because of the possibility that the DRR (ADE+ ura-) recombinants had begun as SCE recombinants but had acquired a mutation in URA3. The pol4 mutants did now show statistically significant differences compared with wild type in the URA3 mutation assay (Figure 8). This confirms that the direct repeat recombinants from the SCE/DRR assay are due to DRR and not SCE events, which acquired a subsequent mutation in URA3.
Figure 4: Interchromosomal MMEJ Assay.

Figure 5: Intrachromosomal MMEJ Assay.
Figure 6: Translocation Assay.

Figure 7: SCE/ DRR Assay.
Discussion

**The role of DNA polymerase 4 in MMEJ.**

The analysis of Pol4 indicates it has a role in MMEJ with complete microhomologies (14 bps) and microhomologies with mismatched nucleotides (14-2-4 bps). In inter and intra MMEJ assays, the pol4 mutants had statistically significant differences compared with wild type using 14 and 14-2-4 bp substrates but not 14-2-9 bp substrate. To further support that Pol4 has a role specifically in MMEJ, Pol4 was tested in an interchromosomal translocation assay that uses 311 bps of homology between truncated his3 alleles to repair an induced DSB. The identical translocation frequencies observed between pol4 catalytic mutants and wild type suggests that Pol4’s role in DSB repair is limited to using less than 23 bps of homology.

That the 14-2-4 bp MMEJ substrate contained 2 bps mismatch showed a more severe decrease in recombination compared with the 14 bps complete homology MMEJ substrate, suggesting that Pol4 has a role in mismatch suppression following the repair of a DSB. Adding just 2 bps of mismatches to the microhomologies in the 14-2-4 bp MMEJ substrate results in a decrease of the pol4 catalytic mutant of 4.5-fold for the inter and 5-fold for the intra MMEJ assay. The deletion mutant showed a 2-fold decrease in the inter and a 9-fold decrease for the intra 14-2-4 bp MMEJ assay. In addition, the deletion mutant has a more severe phenotype than the catalytic point mutant. This implies that the function of Pol4 may extend beyond gap filling in DSBs. The complete deletion of Pol4 seems to inhibit the ability of a cell to repair a DSBs using microhomologies, which suggests that the presence of Pol4 is essential in repairing the break and that there is no other protein or polymerase that fully substitutes for Pol4’s function.
The role of DNA polymerase 4 in SCE/DRR.
The analysis of Pol4 in the Symington SCE/DRR assay indicates that Pol4 does not have a role in SCE but has a role in spontaneous DRR. Figure 7 shows that both the catalytic mutant and the deletion mutant have the same rates of SCE as wild type. The only statistically significant difference was a 2-fold decrease in the DRR for the pol4 catalytic mutant. This result implies that Pol4 may have a function in deletion formation using homology between substrates. The substrates in the assay share all but 2 bps of homology in the ade2 gene on either side of the URA3 gene. The mechanism of DRR is unique from the MMEJ assay tested above since it does not require a DSB to form an ADE2 recombinant. Furthermore, recombination in the DRR assay uses a greater amount of homology compared with the MMEJ assay. This result suggests that Pol4 may have a function in general HR and is not specialized for dealing with the repair of DSBs, which contain microhomologies.

Pol4 has not been implicated in the scientific literature to have an effect on DRR. To further confirm the result of the SCE/DRR assay, the pol4 mutants were tested in the URA3 mutation assay. The URA3 mutation assay was performed as a control to insure that the pol4 catalytic mutant had a true decrease in forming direct repeat recombinants and is not just forming SCE recombinants which had acquired a mutation in the URA3 gene. The mutation assay showed that Pol4 and wild type had no difference in mutation rates, thus confirming the SCE/DRR assay result. Both the SCE/DRR assay and the URA3 mutation assay results suggest that Pol4 has a role in HR. Pol4 may have a role assisting in the formation of deletions using homologous direct repeats.

Speculative Model of Pol4’s function.
Previous research has characterized Pol4 to have gap-filling abilities (with preferences for small gaps with a 5’-phosphate group) and deoxyribose phosphate (dRP) lyase activity, but Pol4 lacks a 3’ to 5’ exonuclease activity (Gonzalez-Barrera et al. 2005). Based on the collected data, Pol4 does play a role for both the 14 bps complete microhomology substrates and the 14-2-4 bps mismatch MMEJ substrates. The data suggest that Pol4 does participate in gap-filling in MMEJ and in stabilizing mismatched nucleotides in DSB repair. Figure 9 shows a hypothesized model for the role of Pol4 in MMEJ with and without mismatched nucleotides. Following the creation of a DSB, the MRX complex helps to promote 5’ -> 3’ resection that results in a 3’ single-stranded tail containing the microhomology and binding of the single-stranded binding protein, RPA. For the complete homology Pol4 has a role in extending DNA heteroduplex formed following microhomology pairing. For microhomologies containing a mismatch, the backbone of the DNA structure will become disfigured from the normal double helix of B-DNA. Mismatch repair (MMR) proteins will relieve the stress of the mismatched nucleotides by nicking the DNA backbone to produce a 5’ dRP overhang. Under these circumstances, Pol4 is able to remove the 5’ dRP overhang with its dRP lyase activity, thus allowing DSB repair to continue.
Figure 9: This figure shows two possible roles of Pol4 in MMEJ. Pol4 may function in gap filling or in DNA extension of terminal mismatch substrates.
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