Published in final edited form as:

DNA Repair (Amst). 2013 December ; 12(12): . doi:10.1016/j.dnarep.2013.10.001.

Roles of exonucleases and translesion synthesis DNA polymerases during mitotic gap repair in yeast

Xiaoge Guo^a and Sue Jinks-Robertson^{b,*}

^aGraduate Program in Molecular Cancer Biology, Duke University, Durham, NC 27710

^bDepartment of Molecular Genetics and Microbiology, Duke University, Durham, NC 27710

Abstract

Transformation-based gap-repair assays have long been used to model the repair of mitotic double-strand breaks (DSBs) by homologous recombination in yeast. In the current study, we examine genetic requirements of two key processes involved in DSB repair: (1) the processive 5'-end resection that is required to efficiently engage a repair template and (2) the filling of resected ends by DNA polymerases. The specific gap-repair assay used allows repair events resolved as crossover versus noncrossover products to be distinguished, as well as the extent of heteroduplex DNA formed during recombination to be measured. To examine end resection, the efficiency and outcome of gap repair were monitored in the absence of the Exo1 exonuclease and the Sgs1 helicase. We found that either Exo1 or Sgs1 presence is sufficient to inhibit gap-repair efficiency over 10-fold, consistent with resection-mediated destruction of the introduced plasmid. In terms of DNA polymerase requirements for gap repair, we focused specifically on potential roles of the Pol ζ and Pol η translesion synthesis DNA polymerases. We found that both Pol ζ and Pol η are necessary for efficient gap repair and that each functions independently of the other. These polymerases may be either in the initiation of DNA synthesis from the an invading end, or in a gap-filling process that is required to complete recombination.

Keywords

Sgs1; Exo1; Pol ζ ; Pol η ; recombination

1. Introduction

Mitotic double-strand breaks (DSBs) are potentially toxic lesions that arise spontaneously or are induced by exogenous agents such as ionizing radiation. Cells have evolved two highly conserved mechanisms to restore broken chromosomes: homologous recombination (HR) and nonhomologous end-joining [1]. Though both pathways efficiently seal DSBs, HR is generally considered to be of higher fidelity because it uses an intact, homologous sequence as a template for the repair reaction. In the current study, we use a plasmid-based, gap-repair assay to model the mitotic repair of a defined DSB via the HR pathway in *Saccharomyces cerevisiae*.

© 2013 Elsevier B.V. All rights reserved.

Corresponding author: Sue Jinks-Robertson, Department of Molecular Genetics and Microbiology, 213 Research Dr., DUMC 3020, Duke University Medical Center, Durham, NC 27710, Phone 919-681-7273, sue.robertson@duke.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

As illustrated in Fig. 1, the HR repair program in yeast is initiated by degradation of the 5' ends of the broken molecule (for a general review of recombination, see [2]). Resection is a two-step process in which the Mre11-Rad50-Xrs2 (MRX) complex together with Sae2 initiates 5'-end removal, generating single-strand tails up to ~700 nucleotides [3, 4]. Two redundant pathways that involve either Exo1 or Sgs1-Dna2 then processively resect the 5' ends to generate long, 3' tails that become coated with the Rad51 protein to initiate a homology search. Once sequence identity is located, the nucleoprotein filament invades the homologous region, pairing with the complementary strand to form heteroduplex DNA (hetDNA) and displacing the non-complementary strand to yield a displacement loop (D-loop) intermediate. Following strand invasion, DNA synthesis-driven extension of the D-loop generates a segment of the displaced strand that is complementary to the 3' tail on the other side of the DSB. In the classical DSB repair (DSBR) model of recombination, engagement of the second end of the break by the D-loop (second-end capture) results in a double Holliday junction (HJ)-containing intermediate that can either be dissolved by the joint action of a helicase and topoisomerase, or cleaved by a structure-specific endonuclease (reviewed by [5]). As an alternative to second-end capture, the D-loop can be dismantled by helicases. The displaced, newly-extended 3' end can then anneal to the complementary tail on the other side of break, a mechanism referred to as synthesis-dependent strand annealing (SDSA). Whereas DSBR can generate either crossover or non-crossover (CO and NCO, respectively) events, SDSA yields only NCO products.

Both the DSBR and SDSA models predict at least two rounds of DNA synthesis: one to extend the invading end and a second to extend the captured end. DNA synthesis may additionally be required to fill remaining gaps if 5'-end resection exceeds 3'-end extension. Determining which DNA polymerases are important during HR has been complicated by the essential nature of some polymerases and by potential functional redundancy (e.g., see [6]). The inherent high fidelity of the Pol delta (δ) and/or Pol epsilon (ϵ) replicative DNA polymerases makes these prime candidates, however, and non-null alleles of Pol δ have implicated this polymerase in both spontaneous and DSB-induced recombination [7]. In addition to replicative DNA polymerases, yeast has two low-fidelity translesion synthesis (TLS) DNA polymerases that are specialized to replicate damaged DNA templates: Pol zeta (ζ) and Pol eta (η). A third TLS polymerase, Rev1, has both structural and enzymatic activities, and functions only in concert with Pol ζ (reviewed in [8]). Yeast studies have failed to reveal a requirement for either Pol ζ or Pol η in mitotic DSB repair [9, 10], but Pol ζ localizes to HO-induced DSBs [11] and is important in at least some types of mutagenesis associated with mitotic DSB repair [10]. During meiosis, a lack of an effect on spore viability similarly suggests that yeast TLS polymerases are not essential for DSB repair, but their absence does affect gene conversion [12].

In contrast the lack of a requirement for yeast TLS polymerases during HR, the activity of each is important in higher eukaryotes. Human Pol η , for example, was identified biochemically by its ability to robustly extend protein-free D-loops [13]. As predicted for TLS polymerases in general, however, recent work has shown that Pol η is much less processive than Pol δ in extending a Rad51-generated D-loop [14, 15]. In chicken DT40 cells, Pol η is required both for HR-mediated somatic hypermutation of immunoglobulin genes and for DSB-induced recombination [16]. Pol ζ has additionally been implicated in gene targeting in DT40 cells [17]. In *Drosophila*, both Pol ζ and Pol η are important during gap-repair and appear to compete with Pol δ [18]. Finally, human cells depleted for Pol ζ exhibit a deficiency in DSB repair [19].

In prior studies, we focused on the position of hetDNA in NCO products generated by gap repair and used this information to infer recombination mechanism and the roles of individual helicases during DSBR and/or SDSA [20, 21]. As illustrated in Fig. 1, an NCO

event produced by the SDSA pathway is expected to have a tract of hetDNA only in the repaired, recipient allele and only on one side of the gap. By contrast, an NCO that goes through an HJ-containing intermediate is expected to have a tract of hetDNA on each side of the gap. The presence of both tracts in the repaired allele is consistent with helicase-driven dissolution of HJs, while the presence of one tract in the recipient and one in the donor allele is indicative of HJ cleavage. In the current study, we focus on the genetic control of processes shared by the DSB and SDSA pathways: (1) the end resection that precedes formation of an invasion-competent nucleoprotein filament and (2) the DNA synthesis required to extend the invading end and/or fill gaps created by strand annealing. We find that presence of Exo1 or Sgs1 severely limits gap-repair efficiency, suggesting that resection by either can destroy the introduced substrate. With respect to HR-related DNA synthesis, loss of either Pol η or Pol ζ reduces gap-repair efficiency and the effect of losing both is additive. Their presence does not affect the CO-NCO outcome or the structures of NCO products, however, indicating a general role for each during mitotic DSB repair.

2. Materials and Methods

2.1. Strains and plasmids

All strains were isogenic to HK2210 or HK2211, W303 (*MATa leu2-3,112 his3 Δ ura3-1 trp1 RAD5*) derivatives obtained from Hannah Klein that contain a chromosomal *his3* allele that is identical (*can1 Δ ::his3 Δ 3'0*) or diverged (*can1 Δ ::his3 Δ 3'18*), respectively, in relation to the gapped *his3* allele used in transformation assays. SJR3409 and SJR3410 were constructed by deleting *MLH1* (*mlh1 Δ ::kan*) from HK2210 and HK2211, respectively, and TLS polymerase-deficient derivatives were constructed using PCR-generated deletion cassettes. A mismatch repair- and resection-defective strain was isolated by crossing strains SJR3688 (HK2210 *mlh1 Δ ::kan exo1 Δ ::loxP-TRP1-loxP*) and SJR3720 (HK2210 *MATa sgs1 Δ ::loxP-hyg-loxP*).

The *HIS3-URA3-ARS* plasmid pSR1062 [20] was used to measure gap-repair efficiency and to determine numbers of NCO and CO products. pSR1059 [20] is a *CEN*-containing plasmid with the same *HIS3* allele as pSR1062 and was used to isolate NCO events for sequence analysis. The *CEN*- and *LEU2*-containing plasmid pRS315 [22] was used to control for transformation efficiency in experiments with pSR1062. Plasmid DNA was isolated from a *dam⁻ dcm⁻ Escherichia coli* strain obtained from New England Biolabs [*ara-14 leuB6 fhuA31 lacY1 tsx78 glnV44 galK2 galT22 mcrA dcm-6 hisG4 rfbD1 R(zgb210::Tn10) Tet^S endA1 rspL136 (Str^R) dam13:: Tn9 (Cam^R) xylA-5 mtl-1 thi-1 mcrB1 hsdR2*].

2.2. Media and growth conditions

Cells were grown nonselectively in YPD (1% Bacto-yeast extract, 2% Bacto peptone, 2% dextrose; 1.5% agar for plates) supplemented with 500 μ g/ml adenine hemisulfate. His⁺ or Leu⁺ colonies were selected on synthetic complete (SC) medium deficient in histidine (SC-his) or leucine (SC-leu), respectively. Ura⁻ colonies were identified on SC medium containing 0.1% 5-fluoroorotic acid (5-FOA). All growth was at 30°C.

2.3. Gap-repair experiments

Cells growing exponentially in YPD were transformed with a 10:1 weight ratio of *Bss*HII-digested pSR1062 and uncut pRS315. As indicated in Results, the digested plasmid was either directly mixed with pRS315 or was purified from an agarose gel prior to mixing. Except where noted in the text, assays were done using gel-purified plasmid. His⁺ or Leu⁺ transformants were independently selected on SC-his or SC-leu plates, respectively, and gap-repair efficiencies were measured as the ratio of His⁺ to Leu⁺ colonies. To determine proportions of NCO and CO events, the stability of the *URA3* marker in individual

transformants was assessed. His⁺ colonies were grown in SC-his medium for three days, diluted 1:100 in YPD and grown overnight before spotting onto 5-FOA plates. NCOs grew confluent on 5-FOA while COs produced few, if any, colonies.

For DNA sequence analysis of NCO products, strains were transformed with *Bss*HII-digested pSR1059. His⁺ colonies were directly inoculated, without prior colony purification, into SC-his in 96-well microtiter plates. DNA was extracted after three days of growth and PCR-amplified products were sequenced by Duke University DNA Analysis Facility.

2.4 Statistical analysis

A Student's t-test was used to determine the statistical significance of differences in mean values of His⁺:Leu⁺ transformants between strains. A two-tailed Fisher Exact Test was used to compare distributions of NCOs versus COs events as well as the distributions of heteroduplex DNA-containing events among NCOs. $p < 0.05$ was considered significant.

3. Results

3.1. The gap-repair assay

The roles of TLS DNA polymerases and exonucleases in DSB-initiated mitotic recombination were examined using the transformation-based, gap-repair assay illustrated in Fig. 2. The introduced plasmid shares ~800 bp of homology with a 98%- or 100%-identical template located at the *CAN1* locus on chromosome V. The recipient, plasmid allele is a full-length *HIS3* gene containing a centrally located 8-bp gap created by restriction digest, while the chromosomal *his3* donor allele is missing the C-terminal 11 amino acids of the protein [20, 23]. The efficiency of gap repair was determined by measuring the number of His⁺ transformants relative to the number of Leu⁺ transformants obtained with an uncut, *LEU2*-marked plasmid.

In addition to assessing gap-repair efficiency, CO and NCO events were distinguished by monitoring the mitotic stability of a *URA3* marker present on the repaired plasmid. CO events integrate the plasmid into chromosome V and have a stable Ura⁺ phenotype, while NCO events have an unstable Ura⁺ phenotype and give rise to a high frequency of Ura⁻ colonies. Finally, the 19 silent, single-nucleotide polymorphisms (SNPs) engineered to create the 98%-identical donor allele were used to track the extent of strand transfer during individual gap-repair events [20]. In the absence of mismatch repair (MMR), mismatches present in hetDNA intermediates are expected to persist and segregate at the next round of DNA replication. The resulting His⁺ colony is thus "sectored" with respect to the SNPs covered by hetDNA, which can be detected as dual peaks on sequence chromatograms of DNA isolated from an unpurified colony. To monitor hetDNA, all gap-repair experiments were performed in the absence of MMR (*mlh1Δ* background), and we focus here specifically on the structures of NCO intermediates. The parental strain with only *MLH1* deleted is designated as wild type (WT) in the sections that follow. Because our previous analyses demonstrated that Dam-methylated DNA can trigger MMR-independent mismatch correction [24], all plasmid DNA was isolated from a *dam*⁻ (and *dcm*⁻) *E. coli* strain.

3.2. Sgs1 and Exo1 reduce gap-repair efficiency

Resection mediated by Sgs1-Dna2 and/or Exo1 on each side of chromosomal DSB has been estimated to be 2000–4000 nucleotides when a repair template is available, but can be much more extensive if repair kinetics are slow [25]. The plasmid used here is 7 kb in size, but the length of homology to the chromosomal repair template is only ~800 bp. Even though the amount of resection can exceed the homology present in a repair template [26], it seemed possible that processive resection by Sgs1 and/or Exo1 might be sufficient to destroy the

entire plasmid before repair occurs. We previously examined the role of Sgs1 during gap repair and found that it promotes repair, specifically facilitating formation of NCO products [21]. Here, we examined how loss of Exo1 alone, or the combined loss of Exo1 and Sgs1, affects gap repair between 98%-identical substrates.

In an *exo1Δ* background, there was no change in either the mean His⁺:Leu⁺ ratio ($p=0.85$) or in the distribution of CO and NCO products ($p=0.33$) relative to WT (Table 1). In the *sgs1Δ exo1Δ* double mutant, however, there was a 15-fold increase in the overall transformation efficiency ($p<0.0001$) and an elevated proportion of CO events among repaired plasmids. COs increased from 13% in WT to 39% in the double mutant ($p<0.00001$), an increase greater than previously observed in an *sgs1Δ* single mutant (from 9% to 15%; [21]).

3.3. hetDNA profiles are not altered in the absence of Sgs1 and Exo1

NCO products from the WT and *sgs1Δ exo1Δ* backgrounds were sequenced and two hetDNA parameters were compared: the distribution of bidirectional versus unidirectional hetDNA tracts and the length of unidirectional hetDNA tracts. As in prior analyses three classes of hetDNA-containing events were observed in WT (see Fig. S1). Class I events predominated (106/136 hetDNA-containing products) and had a continuous tract of hetDNA that extended from the gap, as is expected in an MMR-defective background. Class II events (12/136) had regions of restoration and/or gene conversion interspersed with hetDNA, indicative of residual MMR-independent removal of mismatches. Finally, Class III events (18/136) had a tract of gene conversion immediately adjacent to the gap, followed by a continuous tract of hetDNA; this pattern could reflect either gap expansion or mismatch removal via an MMR-independent repair pathway. Because of the ambiguities associated with Classes II and III, only Class I events were used to compare hetDNA patterns.

As previously reported [20, 21], hetDNA was detected only in the plasmid allele, suggesting that HJ cleavage is strongly biased to generate CO products and makes a negligible contribution to NCOs in this system. In the WT background, 90% of NCOs had hetDNA on only one side of the gap, consistent with SDSA (Table 1). Though there was a 2-fold decrease in the proportion of bidirectional hetDNA tracts in the *sgs1Δ exo1Δ* background (from 11/106 in WT to 2/41), this decrease was not significant ($p=0.35$). To examine whether lengths of hetDNA tracts were altered in the absence of Sgs1 and Exo1, the proportion of unidirectional tracts that extended from the gap to each flanking SNP was plotted (Fig. 3). There was no detectable difference between the lengths of hetDNA tracts in the WT and *sgs1Δ exo1Δ* backgrounds.

3.4. Pol ζ and Pol η independently promote gap repair

The roles of Pol ζ and Pol η TLS polymerases during gap repair initially were examined using the 98%-identical substrates. Either *REV3*, which encodes the catalytic subunit of Pol ζ, or *RAD30*, which encodes Pol η, was deleted. To facilitate comparisons of gap-repair efficiencies in different strain backgrounds, the average ratio of His⁺ to Leu⁺ colonies in WT was set to 1.00. As shown in Fig. 4, there was a significant, ~40% decrease in gap-repair efficiency in the absence of either Pol ζ or Pol η ($p<0.0001$). To assess whether Pol ζ and Pol η function together or separately during recombination, we examined gap repair in a *rad30Δ rev3Δ* double-mutant background. Gap-repair efficiency was decreased ~70% in the double mutant, a decrease significantly greater than that in either single mutant ($p<0.0001$). The effect in the double mutant was close to additive, indicating that Pol ζ and Pol η independently promote gap repair in this system. Although the gap-repair efficiencies were reduced in the single and double mutants, the distributions of CO and NCO events were indistinguishable from that in WT (Table 2, $p>0.05$). Finally, the gap-repair efficiency in a *rev1Δ* or *rev1Δ rev3Δ* background was the same as in the *rev3Δ* background, and that in a

rev1Δ rad30Δ rev3Δ triple mutant was equivalent to that in the *rad30Δ rev3Δ* double mutant (data not shown). Thus, as during lesion bypass, Rev1 appears to function with Pol ζ during gap repair.

Gel-purified, linear DNA fragments are typically used in gap-repair assays (e.g., [27, 28] and our own studies), and we recently found that substantial UV damage is introduced during purification. Thus, in the absence of nucleotide excision repair (a *rad14Δ* background), the transformation efficiency of gel-purified plasmid was reduced 4-fold; in the absence of gel purification, loss of Rad14 had no effect on gap-repair efficiency [24]. Because both Pol ζ and Pol η are normally involved in bypassing UV-induced DNA damage (reviewed by [8]), their apparent roles during gap repair could reflect the use of a gel-purified fragment in transformation experiments. We thus repeated the gap-repair assay using digested plasmid that had not been purified. A similar 4-fold decrease in repair efficiency was observed in the *rad30Δ rev3Δ* double mutant when unpurified DNA was used for transformation (Fig. 4). We conclude that the positive roles played by Pol ζ and Pol η during gap repair do not reflect a requirement for bypassing UV-induced DNA damage.

3.5. Roles of Pol ζ and Pol η during gap repair are not specific to diverged substrates

A defining property of Pol ζ is its ability to extend an unpaired primer-template terminus (reviewed by [8]), suggesting that its role in recombination, and perhaps that of Pol η, might be specific for interactions that involve non-identical substrates. In the gap-repair system used here, the first SNP on each side of the gap is only 20 bp away. It is possible that the resulting mismatch destabilizes and/or reduces extension of an invading 3' end by the Pol δ or Pol ε replicative polymerase, and hence could account for the TLS polymerase requirement. To test this, we examined gap-repair efficiency in WT and *rad30Δ rev3Δ* strains containing the 100%-identical chromosomal repair template. There was no significant difference in gap-repair efficiency in a WT background containing the 98%- versus 100%-identical chromosomal repair template (Fig. 4; $p=0.06$) consistent with prior gap-repair results obtained using a different gapped plasmid [23]. Importantly, there was an ~4-fold reduction in repair efficiency in the *rad30Δ rev3Δ* double mutant relative to WT when the 100%-identical allele was used as the repair template. We conclude that the independent roles of Pol ζ and Pol η in the gap-repair assay reflect a general requirement for each during HR-associated DNA synthesis.

3.6. Pol ζ and Pol η do not affect hetDNA profiles in NCO products

Given that both Pol ζ and Pol η promote gap repair but are assumed to only do limited DNA synthesis, it seemed likely that the extent and/or position of hetDNA in NCO products might be altered in their absence. In particular, their loss might be expected to result in shorter hetDNA tract lengths. Among the unidirectional hetDNA tracts, however, the proportion of tracts that extended from the gap to each flanking SNP was unaffected by loss of Rev3 and Rad30 (Fig. 3). In addition, neither the proportion of NCO events with unidirectional hetDNA nor the distribution of upstream versus downstream tracts was altered in the *rad30Δ rev3Δ* background (Table 1; $p=1$ and $p=0.16$, respectively).

4. Discussion

In the current study, a transformation-based gap-repair assay was used to focus on two key processes that are required for DSB repair: (1) the resection of the broken ends by Exo1 and Sgs1-Dna2 to generate 3' tails and (2) the restoration of resected sequence by DNA polymerases. In particular, we examined whether the Pol ζ or Pol η TLS DNA polymerase is required during or influences the gap-repair process. The overall efficiency of gap repair was quantitated via His⁺ prototroph production and individual repair events were designated

as a CO or NCO based on stability of a plasmid-encoded marker. Importantly, the use of a diverged, chromosomal repair template allowed the molecular structures of NCO intermediates to be inferred by sequencing recombination products.

As reported previously [20, 21], ~90% of hetDNA tracts associated with NCO events were confined to the broken plasmid allele and were only on one side of the gap, consistent with SDSA as the predominant molecular mechanism (see Fig. 1). Unidirectional hetDNA tracts thus are assumed to reflect pairing between the extended, invading 3' end and the recessed 5' end on the other side of the DSB. As illustrated in Fig. 5, there are three possible scenarios for the relationship between end resection and DNA synthesis during SDSA. First, it is possible that the amount of DNA synthesis precisely matches the extent of 5'-end resection. Though it is difficult to imagine how this might occur during SDSA, it has been suggested that DNA synthesis-driven extension of a captured D-loop could extend to and be limited by a homology border during DSBR [26]. A second possibility is that DNA synthesis primed from the invading 3' end is less than the extent of resection on the other side of the DSB. The resulting gap created by annealing of the ends would then be filled by a standard gap-filling reaction. In this case, hetDNA would directly reflect DNA synthesis rather than end resection. A third possibility is that extension of the invading end exceeds resection on the other side of the break so that processing of the resulting over-replicated end (e.g., by Rad1-Rad10 [29] or Mus81-Mms4 [30]) would have to precede ligation. In this case, the detected hetDNA would reflect the extent of end resection rather than of DNA synthesis.

Loss of Exo1- and Sgs1-dependent resection pathways reduces the repair of chromosomal DSBs generated by the HO endonuclease [3, 4] as well as DSBs introduced during replication of a nicked plasmid [31]. By contrast, the repair of DSBs introduced into a replicating plasmid is stimulated upon loss of Exo1 and Sgs1 [32]. The “ends-out” and “ends-in” integration of linear fragments introduced by transformed similarly is increased in an *sgs1Δ exo1Δ* background [25, 33]. In the absence of both Sgs1 and Exo1, but not in the absence of either alone, we observed a 15-fold increase in the efficiency of gap repair (Table 1). The lack of an increase in repair efficiency in either single mutant is consistent with the functionally redundant roles of these proteins observed during the resection of a chromosomal DSB [3, 4]. As previously proposed, we assume that long-range resection by either Exo1 or Sgs1-Dna2 destroys fragments transformed into yeast and infer that the His⁺ plasmids isolated correspond to a minority that escape such resection. The lack of a difference in hetDNA profiles in the presence versus absence of Exo1 and Sgs1 is consistent with this interpretation. Any 5'-end resection that does occur is presumably catalyzed by MRX and Sae2, which together process chromosomal DSBs in approximately 100 nt steps [4]. It should be noted that we identified hetDNA tracts that included only the gap-proximal SNP and hence were much shorter than expected 100 bp minimum. In addition, there were no obvious steps in terms of hetDNA length among SDSA events. We thus suggest that the observed hetDNA profiles reflect primarily random termination of DNA synthesis rather than the extent of end resection.

Studies of spontaneous and DSB-induced mitotic recombination in yeast have failed to reveal a requirement for either Pol ζ or Pol η [9, 10], although each TLS polymerase is important during HR in metazoans [16–19]. Use of a gap-repair assay in the current study has clearly demonstrates a requirement for both Pol ζ and Pol η during at least some types of homology-directed repair in yeast. The trivial explanation that these requirements reflect a need for the TLS polymerases to bypass UV damage introduced into the plasmid during gel purification was ruled out, but we cannot discount the presence of other types of plasmid damage (e.g., damage accumulated during transit from the cytoplasm to the nucleus). Because epistasis analysis indicates that Pol ζ and Pol η act independently during gap repair, however, one would have to assume that most plasmids contain a lesion that is a cognate

substrate for one or the other, but not both, TLS polymerases. Given the unique ability of Pol ζ to extend mispaired termini, we also considered the possibility that its role might reflect the use of a diverged repair template. That the effect is more general, however, is indicated by an equivalent requirement for Pol ζ as well as Pol η when an identical repair template was present. We thus favor a model in which these TLS polymerases act at distinct steps during the gap-repair process. Because neither affects the CO-NCO distribution, the relevant DNA synthesis step is likely common to both the DSBR and SDSA pathways.

Given the distributive nature of TLS polymerases *in vitro* and their failure to affect hetDNA tract lengths, it seems unlikely that either Pol ζ or Pol η is required for the bulk of HR-associated DNA synthesis. Instead, TLS polymerases might be involved in initiating extension of an invading 3', in initiating DNA synthesis after second-end engagement, or in gap filling if resection exceeds DNA synthesis. With regard to a possible gap-filling role, it is well known that single-strand DNA is more chemically reactive than duplex DNA [34]. If repair kinetics are slow in a plasmid relative to a chromosomal context, damage accumulation in the exposed single-strand tails would be enhanced in plasmid-based systems and could explain a requirement for TLS polymerases. This would be analogous to the accumulation of damage in extensive regions of single-strand chromosomal DNA generated by resection [35]. A final possibility incorporates the observation that the presence of Sgs1 or Exo1 strongly inhibits gap repair in the system used here. If resection is indeed limited (see above), then processive DNA synthesis by Pol δ or Pol ϵ could lead to over-replication of an invading end relative to the complementary, resected tail to which it must anneal, which would then necessitate additional processing steps [26]. Limited resection might, therefore, favor an involvement of the much less processive TLS polymerases. This specific idea can be tested in a chromosomal context, where a requirement for TLS polymerases would be expected to become evident only in an *sgs1 Δ exo1 Δ* background.

The results reported here establish a requirement for the yeast TLS polymerases during mitotic DSB repair in some contexts, bringing yeast in line with the requirements reported in metazoans [16–19]. Though the involvement of Pol ζ and Pol η in yeast mitotic DSB repair could be specific to a plasmid-based assay used here, the recent report of a role for each during meiotic gene conversion [12] suggests a more general requirement. Whether such a requirement is detected, however, may depend on the sensitivity of the specific assay being used as well as a variable degree of TLS involvement. In a chromosomal context, for example, each TLS polymerase might be required for only 10% of DSB repair, a level that would be too low to be detected. With regard to end-resection roles of Exo1 and/or Sgs2, the large increase in gap-repair efficiency observed in their absence suggests that the recovered plasmids likely were processed only by MRX-Sae2. Given the limited resection that presumably occurs in the gap-repair assay, the more general question of how hetDNA length relates to end resection will need to be addressed in a chromosomal context where Sgs1 and Exo1 promote DSB repair. If the primary function of Sgs1- or Exo1-mediated resection is to produce an invasion-competent 3' end and/or to allow second-end engagement, then hetDNA length would be expected to approximate the extent of end resection. If the primary function of processive resection is to trigger a checkpoint and provide time to complete HR [4, 36], however, then there is no *a priori* reason why hetDNA tracts should mirror end resection. Combining a sequencing-based approach analogous to that used here with the tools developed to physically monitor 5'-end resection from chromosomal DSB (e.g., see [3, 4]) will provide a more detailed understanding of molecular mechanisms of SDSA and of DSBR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Tom Petes and members of the SJR lab for helpful discussions and for comments on the manuscript. This work was supported by grant GM38464 from the NIH.

References

1. Symington LS, Gautier J. Double-strand break end resection and repair pathway choice. Annual review of genetics. 2011; 45:247–271.
2. Krogh BO, Symington LS. Recombination proteins in yeast. Annu Rev Genet. 2004; 38:233–271. [PubMed: 15568977]
3. Mimitou EP, Symington LS. Sae2, Exo1 and Sgs1 collaborate in DNA double-strand break processing. Nature. 2008; 455:770–774. [PubMed: 18806779]
4. Zhu Z, Chung WH, Shim EY, Lee SE, Ira G. Sgs1 helicase and two nucleases Dna2 and Exo1 resect DNA double-strand break ends. Cell. 2008; 134:981–994. [PubMed: 18805091]
5. Mimitou EP, Symington LS. Nucleases and helicases take center stage in homologous recombination. Trends Biochem Sci. 2009; 34:264–272. [PubMed: 19375328]
6. Wang X, Ira G, Tercero JA, Holmes AM, Diffley JF, Haber JE. Role of DNA replication proteins in double-strand break-induced recombination in *Saccharomyces cerevisiae*. Molecular Cell Biol. 2004; 24:6891–6899.
7. Maloisel L, Fabre F, Gangloff S. DNA polymerase δ is preferentially recruited during homologous recombination to promote heteroduplex DNA extension. Mol Cell Biol. 2008; 28:1373–1382. [PubMed: 18086882]
8. Waters LS, Minesinger BK, Wiltrot ME, D'Souza S, Woodruff RV, Walker GC. Eukaryotic translesion polymerases and their roles and regulation in DNA damage tolerance. Microbiol Mol Biol Rev. 2009; 73:134–154. [PubMed: 19258535]
9. Hicks WM, Kim M, Haber JE. Increased mutagenesis and unique mutation signature associated with mitotic gene conversion. Science. 2010; 329:82–85. [PubMed: 20595613]
10. Rattray AJ, Shafer BK, McGill CB, Strathern JN. The roles of *REV3* and *RAD57* in double-strand-break-repair-induced mutagenesis of *Saccharomyces cerevisiae*. Genetics. 2002; 162:1063–1077. [PubMed: 12454056]
11. Hirano Y, Sugimoto K. ATR homolog Mec1 controls association of DNA polymerase ζ -Rev1 complex with regions near a double-strand break. Curr Biol. 2006; 16:586–590. [PubMed: 16546083]
12. Arbel-Eden A, Joseph-Strauss D, Masika H, Printzentel O, Rachi E, Simchen G. Trans-lesion DNA polymerases may be involved in yeast meiosis, G3 (Bethesda). 2013 epub ahead of print.
13. McIlwraith MJ, Vaisman A, Liu Y, Fanning E, Woodgate R, West SC. Human DNA polymerase η promotes DNA synthesis from strand invasion intermediates of homologous recombination. Mol Cell. 2005; 20:783–792. [PubMed: 16337601]
14. Sneed JL, Grossi SM, Tappin I, Hurwitz J, Heyer WD. Reconstitution of recombination-associated DNA synthesis with human proteins. Nucleic Acids Res. 2013; 41:4913–4925. [PubMed: 23535143]
15. Sebesta M, Burkovics P, Juhasz S, Zhang S, Szabo JE, Lee MY, Haracska L, Krejci L. Role of PCNA and TLS polymerases in D-loop extension during homologous recombination in humans. DNA Repair (Amst). 2013; 12:691–698. [PubMed: 23731732]
16. Kawamoto T, Araki K, Sonoda E, Yamashita YM, Harada K, Kikuchi K, Masutani C, Hanaoka F, Nozaki K, Hashimoto N, Takeda S. Dual roles for DNA polymerase η in homologous DNA recombination and translesion DNA synthesis. Mol Cell. 2005; 20:793–799. [PubMed: 16337602]
17. Sonoda E, Okada T, Zhao GY, Tateishi S, Araki K, Yamaizumi M, Yagi T, Verkaik NS, van Gent DC, Takata M, Takeda S. Multiple roles of Rev3, the catalytic subunit of pol ζ in maintaining genome stability in vertebrates. EMBO J. 2003; 22:3188–3197. [PubMed: 12805232]
18. Kane DP, Shusterman M, Rong Y, McVey M. Competition between replicative and translesion polymerases during homologous recombination repair in *Drosophila*. PLoS Genet. 2012; 8:e1002659. [PubMed: 22532806]

19. Sharma S, Hicks JK, Chute CL, Brennan JR, Ahn JY, Glover TW, Canman CE. REV1 and polymerase ζ facilitate homologous recombination repair. *Nucleic Acids Res.* 2012; 40:682–691. [PubMed: 21926160]
20. Mitchel K, Zhang H, Welz-Voegele C, Jinks-Robertson S. Molecular structures of crossover and noncrossover intermediates during gap repair in yeast: implications for recombination. *Mol Cell.* 2010; 38:211–222. [PubMed: 20417600]
21. Mitchel K, Lehner K, Jinks-Robertson S. Heteroduplex DNA position defines the roles of the Sgs1, Srs2, and Mph1 helicases in promoting distinct recombination outcomes. *PLoS Genet.* 2013; 9:e1003340. [PubMed: 23516370]
22. Sikorski RS, Hieter P. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics.* 1989; 122:19–27. [PubMed: 2659436]
23. Welz-Voegele C, Jinks-Robertson S. Sequence divergence impedes crossover more than noncrossover events during mitotic gap repair in yeast. *Genetics.* 2008; 179:1251–1262. [PubMed: 18562664]
24. Guo X, Jinks-Robertson S. *N*-6-methyladenine triggers repair of mismatches in yeast gap-repair intermediates. *DNA Repair in revision.*
25. Chung WH, Zhu Z, Papusha A, Malkova A, Ira G. Defective resection at DNA double-strand breaks leads to *de novo* telomere formation and enhances gene targeting. *PLoS Genet.* 2010; 6:e1000948. [PubMed: 20485519]
26. Mazón G, Lam AF, Ho CK, Kupiec M, Symington LS. The Rad1-Rad10 nuclease promotes chromosome translocations between dispersed repeats. *Nat Struct Mol Biol.* 2012; 19:964–971. [PubMed: 22885325]
27. Symington LS, Kang LE, Moreau S. Alteration of gene conversion tract length and associated crossing over during plasmid gap repair in nuclease-deficient strains of *Saccharomyces cerevisiae*. *Nucleic Acids Res.* 2000; 28:4649–4656. [PubMed: 11095674]
28. Haghnazari E, Heyer WD. The DNA damage checkpoint pathways exert multiple controls on the efficiency and outcome of the repair of a double-stranded DNA gap. *Nucleic acids research.* 2004; 32:4257–4268. [PubMed: 15304563]
29. Lyndaker AM, Alani E. A tale of tails: insights into the coordination of 3' end processing during homologous recombination. *Bioessays.* 2009; 31:315–321. [PubMed: 19260026]
30. Hollingsworth NM, Brill SJ. The Mus81 solution to resolution: generating meiotic crossovers without Holliday junctions. *Genes Dev.* 2004; 18:117–125. [PubMed: 14752007]
31. Munoz-Galvan S, Lopez-Saavedra A, Jackson SP, Huertas P, Cortes-Ledesma F, Aguilera A. Competing roles of DNA end resection and non-homologous end joining functions in the repair of replication-born double-strand breaks by sister-chromatid recombination. *Nucleic Acids Res.* 2013; 41:1669–1683. [PubMed: 23254329]
32. Marrero VA, Symington LS. Extensive DNA end processing by Exo1 and Sgs1 inhibits break-induced replication. *PLoS Genet.* 2010; 6:e1001007. [PubMed: 20628570]
33. Stafa A, Svetec IK, Zgaga Z. Inactivation of the *SGS1* and *EXO1* genes synergistically stimulates plasmid integration in yeast. *Food Technol Biotechnol.* 2005; 43:103–108.
34. Lindahl T. Instability and decay of the primary structure of DNA. *Nature.* 1993; 362:709–715. [PubMed: 8469282]
35. Yang Y, Sterling J, Storici F, Resnick MA, Gordenin DA. Hypermutability of damaged single-strand DNA formed at double-strand breaks and uncapped telomeres in yeast *Saccharomyces cerevisiae*. *PLoS Genet.* 2008; 4:e1000264. [PubMed: 19023402]
36. Tan FJ, Hoang ML, Koshland D. DNA resection at chromosome breaks promotes genome stability by constraining non-allelic homologous recombination. *PLoS Genet.* 2012; 8:e1002633. [PubMed: 22479212]

Model

Figure 1.

Gap-repair mechanisms. The broken DNA molecule and the intact repair template are represented by solid orange and black lines, respectively; arrowheads indicate 3' ends. New DNA synthesis is indicated by dotted lines that are colored to match the template. Boxes indicate hetDNA. See text for additional details.

Gap-repair assay

Figure 2.

Gap-repair assay. The gapped *his3* allele and *URA3* marker contained on the plasmid are depicted by open and hatched boxes, respectively. The black box corresponds to the chromosomal *his3Δ3'* allele used as a repair template. Gray boxes reflect hetDNA in *His*⁺ transformants.

hetDNA length in unidirectional NCO events

Figure 3.

hetDNA length in unidirectional NCO events. The percentage of events that include a given SNP is plotted. The vertical line indicates the position of the gap in the plasmid; distances upstream and downstream of the gap (negative and positive numbers, respectively) are given in nucleotides. The position of points along the horizontal axis correspond to SNP positions.

A. Gap-repair efficiency

Figure 4.

Gap-repair efficiencies in TLS-deficient strains. Each dot corresponds to the His⁺:Leu⁺ value obtained from transformation of an independent culture and the mean value is given. For strains that were transformed with an identical plasmid mix (indicated by horizontal bars under the genotype), each individual His⁺:Leu⁺ ratio was normalized to the mean value obtained in WT.

Resection vs Synthesis model

Figure 5.

Relationship between 3'-end extension and resection during SDSA. The hetDNA (gray boxes) present in NCOs generated by SDSA reflects annealing of the extended, invading end to the resected end on the other side of the DSB. See text for additional details.

Table 1

Gap repair in *exoI*Δ single and *exoI*Δ *sgsI*Δ double mutants

Strain genotype	Gap-repair efficiency (relative to WT)	% CO (proportion)	% NCO (proportion)	Position of hetDNA in NCOs	
				% unidirectional (proportion)	% bidirectional (proportion)
WT	1.00	13% (24/191)	87% (167/191)	90% (96/106)	10% (11/106)
<i>exoI</i> Δ	0.99	8% (8/96)	92% (88/96)	ND	ND
<i>exoI</i> Δ <i>sgsI</i> Δ	14.7	39% (73/186)	61% (113/186)	95% (39/41)	5% (2/41)

ND, not determined.

Table 2

Gap repair in TLS polymerase-defective strains

Strain genotype	Gap-repair efficiency (relative to WT)	% CO (proportion)	% NCO (proportion)	Position of hetDNA in NCOs	
				% unidirectional (proportion)	% bidirectional (proportion)
WT	1.00	13% (24/191)	87% (167/191)	90% (96/106)	10% (11/106)
<i>rad30Δ</i>	0.58	16% (15/96)	84% (81/96)	ND	ND
<i>rev3Δ</i>	0.62	17% (16/95)	83% (79/95)	ND	ND
<i>rad30Δ rev3Δ</i>	0.28	17% (31/181)	83% (150/181)	89% (62/70)	11% (8/70)

ND, not determined.