Spring 2019

Effects of pol zeta, pol eta, and Polymerase delta Mutants on the Genetic Instability of Inverted Repeat InsH during Break-Induced Replication

Tyler Jackson

EFFECTS OF POL ZETA, POL ETA, AND POLYMERASE DELTA MUTANTS ON THE GENETIC INSTABILITY OF INVERTED REPEAT INSH DURING BREAK-INDUCED REPLICATION

by

Tyler Jackson

A thesis submitted in partial fulfillment of the requirements
for graduation with Honors in the Biology

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Anna Malkova
Thesis Mentor

Spring 2019

All requirements for graduation with Honors in the
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Lori Adams
Biology Honors Advisor

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Abstract

Break-induced replication (BIR) is a type of DNA repair pathway that specializes in the repair of one ended double-stranded breaks (DSBs). The pathway is necessary to preserve genetic information in the presence of DNA damage. However it is not currently known how BIR proceeds through inverted repeats (IRs) that are capable of forming secondary structures. Not only that, the specific subset of proteins required to assist BIR through IRs is also unknown. *Saccharomyces cerevisiae* is a suitable organism for the investigation of BIR because it allows for easy investigation into the role of highly conserved proteins. *S. cerevisiae* mutant experimental strains were generated using transformation techniques and then coupled with a chromosome III disome system containing a *LYS2*-InsH construct at the 16 kb position. BIR does not appear to require translesion polymerases to assist the bypass of IRs. Polymerase δ mutants investigated did not appear to significantly affect the progression of BIR through IRs raising additional questions as to the mechanism through these unstable elements. These results help clarify the mechanism by which BIR proceeds through IRs and eliminates the possibility that translesion polymerases, often recruited to bypass unstable DNA elements, are involved. Polymerase δ is supported by numerous studies to be the driver of replication during BIR synthesis and this study elucidates how a few of the mutants of polymerase δ did not affect the progression through IRs but raises questions for other polymerase δ mutants that could affect the progression of BIR through IRs.

ACKNOWLEDGEMENTS

A very special thanks to Dr. Anna Malkova who's help and guidance through this honors thesis project was instrumental to its subsequent success. Graduate student in Dr. Malkova's lab, Beth Osia, designed and helped me design several of the figures an tables shown in the thesis as well as assisting with the experiments. She has been a mentor to me throughout the majority of my years as an undergraduate at the University of Iowa and continues to be one.

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Introduction

Double-stranded DNA breaks (DSBs) are dangerous and potentially lethal events that can result from cellular sources such as DNA replication, mutagens, or problems with chromosomal segregation. Repair of these DNA breaks is crucial for the genomic stability and integrity of DNA within a cell. To mitigate the damage presented by DSBs, several DNA repair pathways are utilized depending on the circumstances of the DNA break. During the G1 phase of the cell cycle in *Saccharomyces cerevisiae,* non-homologous end joining (NHEJ) is the primary mechanism of repair. NHEJ repairs the breaks of DNA by resealing the DSB made by the damage through fusion with chromosomal ends (Rodgers and McVey, 2016). Consequently, this process is very error-prone because it does not take advantage of a homologous template for repair and may involve loss of genetic information (Rodgers and McVey, 2016). In contrast to G1 phase repair, DSBs occurring in the G2 phase of the cell cycle utilize homologous recombination (HR) pathways for repair the DNA breaks. Sub-pathways of HR include gene conversion (GC), double Holliday junction (dHJ), synthesis-dependent strand annealing (SDSA), single-strand annealing (SSA), and break-induced replication (BIR).

Each HR pathway is similar in its mechanism of repair starting by the resection of one strand to generate a 3' ssDNA overhang for invasion into a homologous template of DNA. This event is mediated by the Rad51 protein which forms a filament on the invading 3' ssDNA to protect it from degradation and assist in its search for homology (Janis and Rothstein, 2013). After invasion into the template, DNA synthesis proceeds in the 5'-3' direction recreating the double-stranded DNA formerly present before the DSB. BIR is specifically activated during instances where only one end of the DSB can find homology (Sakofsky et al., 2012). Possible scenarios for this type of repair occur during erosion of telomeres, when a replication fork

encounters nicked DNA that is converted into a DSB, or when one of two broken DSB ends fails to find homology (Malkova and Sakofsky, 2017). Two broken DSB ends may also find homology independent of each other therefore invading and synthesizing at ectopic locations (Smith et al., 2007). This ectopic recombination event often occurs with repetitive sequences in the site of invasion and can lead to chromosomal rearrangements (Smith et al., 2007).

BIR, like other homologous recombination pathways, starts with 5'-3' resection of a one-ended DSB and the ssDNA is coated with a Rad51 protein filament. The 3' end of the donor strand invades the recipient strand to initiate synthesis of nascent DNA proceeding via a migrating bubble. Polymerase δ is the main replicative polymerase likely catalyzing synthesis of both tbe leading and lagging strands during BIR. Although long synthesis of BIR does look to require pol ε, the exact role of this polymerase still remains under investigation (Malkova and Sakofsky, 2017). BIR is a topic of interest due to the numerous cancers and diseases it has been implicated in. Aberrant DNA repair by BIR that increases the prevalence of complex genomic rearrangements has been implicated in several different cancers (Kramara et al., 2018). BIR has also been implicated as a cause for trinucleotide repeat expansion leading to Huntington's disease as well as fragile X-syndrome (Mirkin, 2007). Thus, it is critical to have a deeper understanding of BIR so that diseases like the aforementioned ones could be perhaps treated by drug therapies.

There are several methods to studying BIR in an experimental setting using *Saccharomyces cerevisiae*. One method is to introduce a linear DNA fragment into the yeast genome where it will prompt strand invasion of the homologous template of DNA into the genomic DNA via BIR (Morrow et al., 1997). BIR will then initiate synthesis for hundreds of kilobases along the chromosome. The second method, which is used in this study, is to initiate

BIR using the HO site-specific endonuclease cut site in the *MAT-a* loci (Malkova et al., 1996). When yeast cells are exposed to galactose, the HO site-specific endonuclease, which is under the control of a Gal4 promoter, is recruited and creates a DSB at the cut site region on the newly introduced chromosome III homolog to the endogenous copy (Deem et al., 2008). After the cut has been made, BIR initiates synthesis of the broken donor strand into a homologous template in the yeast genome. This disomic system has been instrumental in studying the peculiarities of BIR. Another way that BIR can be studied but not experimentally introduced is at the telomeres. When telomeres erode to a critical point, ALT (alternative lengthening of telomeres) is activated and is driven by BIR to replenish the lost genetic material at the telomere (Lydeard et al., 2007).

A sequence that often promotes the onset of genomic instability is an inverted repeat (IR). These sequences have an increased tendency to form secondary structures like hairpins due to the palindromic sequences. IRs are a type of DNA motif that have been shown to create an elevated level of genomic instability associated with S-phase replication (Gordenin et al., 1993). Because BIR involves synthesis by pol δ of a homologous template to repair the DNA, there may be some parallels between S-phase replication and BIR as to how IRs introduce genetic instability. It is still unknown which particular aspects of BIR are affected by the presence of IRs on the track of synthesis. The question after these studies then was which proteins were involved with the instability of IRs.

Unpublished data by the Malkova lab reveals the unstable nature of IRs during BIR progression (Figure 1). At all positions, using the LYS+ reversion rate as a way to detect mutagenicity during BIR progression through IRs, there was a significant increase in rates of reversions during BIR (comparisons between 0 and 7 hours). It is likely that the genetic environment and the mechanism of BIR at each position have an effect but it's clearly ubiquitous

in its effect. To observe see how these deletions of sequences looked on a base by base level, unpublished data displaying sequencing results of the LYS+ outcomes is also shown (Figure 2). A stunning finding by this analysis is the fact that there is the presence of leading strand deletions during BIR but not in S-phase replication (Type I vs. Type II deletions). These results give further reason to study the mechanism of BIR progression through IRs to understand why such instability is generated.

Moreover, certain polymerases were identified as affecting the progression of S-phase replication through these hairpin structures. Experiments with the temperature sensitive polymerase δ allele indicated elevated levels of deletions and recombination events when encountering an IR capable of hairpin formation, suggesting a role of the catalytic subunit of polymerase δ for progression through IRs (Tran et al., 1995). Polymerase δ mutant *pol3-Y708A*, which causes a mutator phenotype in pol δ, also displayed an increased rate of deletion and recombination when encountering GAA microsatellites and even more so when coupled with the polymerase ζ mutation (Shah et al., 2012). A common IR used in studies, InsH, has been especially conducive in studying which proteins assist the replication fork during S-phase replication with progressing through IRs (Lobachev et al., 1998). The InsH sequence is comprised of two 69 nt. IRs containing a 9 nt spacer between each repeat that is all flanked by two 9 nt. direct repeats (DRs) (Gordenin et al., 1993).

This study used the InsH sequence to study genomic instability of IRs at a locus on chromosome III of *Saccharomyces cerevisiae* and what effects polymerase δ, ζ, and η mutants had on progression through IRs via BIR. The InsH sequence was inserted into the *LYS2* gene creating a LYS- phenotype and if removed would create a LYS+ phenotype offering a convenient reporter system. If translesion polymerases are truly important for progression

of BIR through IRs, one would expect to see some kind of effect in the rates of InsH deletions (failure of BIR to replicate through this region). A contrary effect would lead to the conclusion that these translesion polymerases would not be important. Likewise if polymerase δ mutants display altered LYS+ reversion rates, one would conclude that the polymerase δ enzyme carries some sort of significant function for helping BIR progress through the IRs. Sequencing can reveal whether or not the effect is also in the spectrum of deletions were altered in any way. Alterations in the spectrum as compared to WT would perhaps indicate a difference in the mechanism by which BIR progresses through IRs if there is a mutation. The data in this study indicated no significant change in the amount of InsH deletions in all translesion polymerase mutants tested as well as all polymerase δ mutants tested. Sequencing of the pol δ exonuclease deficient mutation *pol3-01* displayed no change in the spectrum of deletions either. These negative results indicate a lack of any effect on the InsH deletion rates during the progression of BIR through InsH and thus little to no role in assisting BIR over these sequences.

LYS+ Reversion Rates at Different Positions away from DSB

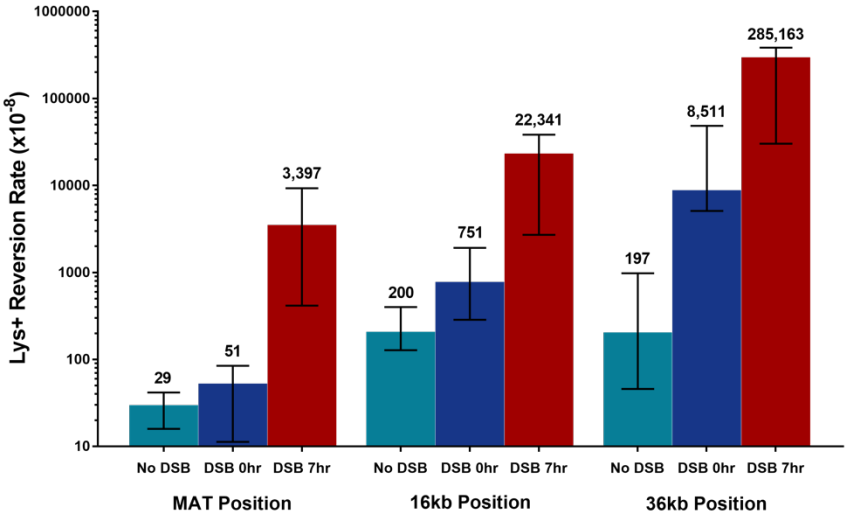

Figure 1 LYS+ reversion rates from unpublished data. The design of these experiments as well as the reporter system used are as described in the Materials and Methods section. The MAT, 16, and 36 kb positions were tested and the LYS+ reversion rates determined from the frequencies using a modified version of the Drake equation.

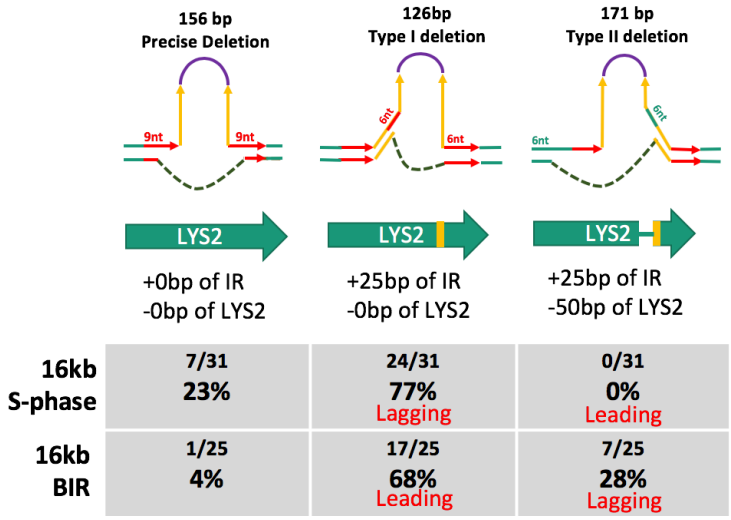

Figure 2 Deletion types from LYS+ reversion outcomes based on unpublished data. The classification of the deletion types as well as the polarities of the deletion types are as described in the Materials and Methods section. The numbers above the percentages are based on the number of LYS+ reversion outcomes sequenced.

Materials and Methods

Generation of Yeast Strains

The parent *Saccharomyces cerevisiae* strain used for the purposes of this experiment was AM 4466, which contained the LYS2-InsH sequence 16 kb from the HO endonuclease cut site (Figure 1). This strain was generated by transformation of a plasmid that contained the InsH sequence as well as a full sequence of LYS2 homology flanking each side of the InsH region and integrated into the *S. cerevisiae* AM 1003 strain. This was the strain used for further DNA transformation procedures. To construct the translesion polymerase mutants, DNA from strains AM 1702 (*RAD30*::KAN) and AM 4127 (*REV3*::BSD) were extracted using the glass bead DNA preparation protocol and then the DNA was PCR amplified using the appropriate primers flanking the antibiotic markers (these primers are the same as in the confirmation of strains Materials and Methods section). The DNA from both of these strains were used for two-step transformation into the AM 4466 strain to generate the following mutants: AM 4722 (4466>*RAD30*::KAN), AM 4774 (4466>*REV3*::BSD), and a double deletion translesion polymerase mutant AM 4848 (contains both of the gene replacements described for the single mutants).

The *pol3-Y708A* mutant was created by digesting the plasmids of *Escherichia coli* strains from the strain collection with HpaI and integrated into AM 4466 via "pop-in." The *pol3-Y708A* was then "popped-out." The plasmid DNA preparation was purified using the QIAGEN© Plasmid Miniprep Kit (Cat No. 27104). The purified plasmid DNA for both of the mutations was then transformed into the AM 4466 strain using the one-step OSB transformation protocol (Chen et al., 1992). This protocol generated the strains AM 4787 and AM 4789, which contain the *pol3-Y708A* mutation.

CRISPR/Cas9 Gene Editing in Saccharomyces cerevisiae

The *pol3-01* strain was created by CRISPR/Cas9 gene editing as described in the protocol by Anand et al. (2017). The *pol3-01* strain created by this method was denoted AM 4801.

Confirmation of Strains

The AM 4722 strain (4466>*RAD30*::KAN), AM 4718/AM 4774 (4466>*REV3*::BSD) and double deletion mutant strain AM 4898 strains were checked by genotyping on several types of selective medium. The medium used for genotyping were adenine, lysine, leucine, histidine, uracil dropout and then nourseothricin and hygromycin antibiotic media. The expected genotypes should have been ADE+, HIS+, LYS-, LEU+, URA+, NAT+, and HYG+. Positive and negative PCR was then carried out to confirm the validity of the gene replacement. The primers used were designed to anneal at the left and right junctions of the newly transformed marker or of the gene that used to be present. A positive left side confirmation using the primers OL 1196 (5' GTTCCATTCCACTCAAATTTGGG 3') and OL 3850 (5' AGCTGGCAACCTGACTTGTATC 3') and a positive right side confirmation using primers OL1195 (5' CTTAGAGGATACGAAGATTCCTCA 3') and OL 3851 (5' TGTTGATTGTAGCCGTTGCTC 3') indicated the presence of the correct replacement of *REV3* with the BSD marker used to produce AM 4718 and AM 4774. A negative left side confirmation using primers OL 1196 and OL 1203 (5' GCAGGCTTTCACCGTGCGATGGG 3') and a negative right side confirmation using primers OL 1195 and OL 1197 (5' CGTGTGCAGGACGTGCAGTTATCGT 3') were used

to check for the absence of the *REV3* gene in the AM 4718 and AM 4774 strains. The KAN marker was checked by positive right side confirmation using primers OL 1902 (5' CCACTTATTGTAGTCTTCTAGCGC 3') and OL 27 (5' CAGCGAGGAGCCGTAATTTT 3') and by positive left confirmation using primers OL 1903 (5' CACTTTGATCAAAAGCGAAGTCTC 3') and OL 26 (5' CCTCGACATCATCTGCCC 3') indicating the correct insertion of the KAN marker in the AM 4722 strain. A negative right side confirmation using primers OL 1902 and OL 1904 (5' CCCTCCTATATGGTTCCAGTGATAC 3') and a negative left side confirmation using primers OL 1903 and OL 1905 (5' GTTTGGCAATCAGGTCCATACTTTC 3') were also used to check for the absence of the *RAD30* gene in the AM 4722 strain.

The *pol3-Y708A* mutation containing strain was checked several different ways. First the three strains AM 4787, AM 4788, and AM 4789 were checked by genotyping onto adenine, lysine, uracil, and leucine dropout. The expected genotypes for these platings should be ADE+, LYS-, URA-, and LEU+. All strains gave these expected phenotypes. The next approach for checking the validity of the transformants was by canavanine (CAN) plating. Pavlov et al. (2001) found that strains containing the *pol3-Y708A* mutation displayed an elevated mutation rate when plated onto CAN media than wild-type *S. cerevisiae* strains. To check for this mutation rate, all three strains were replica-plated onto the same CAN plate as AM 1003 (wild-type serving as the negative control) and an existing *pol3-Y708A* mutant in the collection AM 1241 (serving as the positive control). The results of these platings were not as clear so a third method for checking was employed. The third method for checking was by galactose plating of the strains and observing half crossover rates as the ability to form HIS+ and ADE+ colonies when replica-plated off of GAL media. Vasan et al. (2014) recorded an 18% half-crossover rate associated

with this mutation. To check for this rate, the three strains were replica-plated twice onto galactose media and then replica-plated onto ADE, HIS, and LEU dropout medium to observe the repair outcomes. The results gave many chromosome losses as opposed to BIR but BIR was still present. Lastly, the DNA fragments from each strain were PCR amplified in the region containing the mutation and sent for sequencing. The results of sequencing should have detected a single base change at amino acid position 708 on the *pol3* gene responsible for converting the amino acid tyrosine to alanine.

BIR Induction Using DSBs in Saccharomyces cerevisiae

BIR can be induced by exposing *Sacchromyces cerevisiae* cells to galactose causing a break at a site called GAL-HO (Figure 1). The chromosome III disome system was used where one copy of the chromosome (donor) contained the LYS2-InsH construct 16 kb away from the BIR invasion site as previously described (Figure 1). The other copy of chromosome III (recipient) contains the *MAT*-a locus that contains a galactose inducible HO-endonuclease site (Figure 1). When cells are exposed to galactose, a break occurs at this site and BIR is initiated on chromosome III.

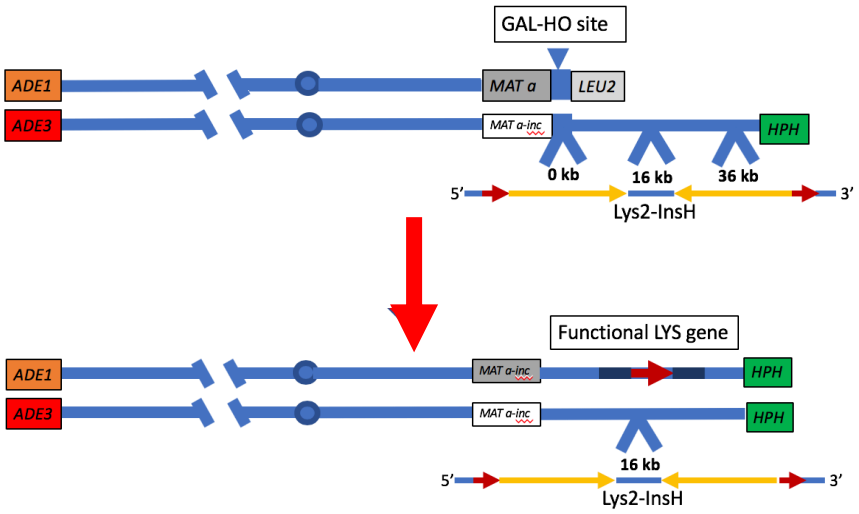

Figure 3 The gal inducible HO endonuclease system is shown in the following depiction. An endonuclease cuts one homolog of chromosome III and then this induces BIR which copies the sequence on the other homolog for repair.

Lysine Dropout and YEPD Frequency Experiments

All aforementioned strains were tested for their ability to revert to a LYS+ phenotype, which used as a measure for genetic instability due to InsH. This was achieved using the protocol depicted in Figure 4. The mutant strain of interest and the AM 4466 strain were grown on LEU dropout agar media for three days for growth of single colonies. The single colonies were then transferred to approximately 2-5 mL of liquid LEU dropout culture overnight. The next day, 1 mL these same cultures were transferred to 10 mL of lactate culture to be grown for 16-18 hours. Once these cultures have grown for this amount of time, frequency plating experiments using LYS dropout agar medium begin. 1.0 mL of culture was transferred over 5 LYS dropout plates and 0.3 mL of culture, serially diluted 1:10000, was plated over 3 YEPD plates. These were denoted as the 0 hour plates. The purpose of the 0 hr plating was to normalize the data for any pre-existing deletion of InsH events. 1.1 mL of 20% galactose was then added to the lactate cultures to induce the HO endonuclease to create a DSB for BIR to repair. The lactate cultures were extracted again at 7 hours for more frequency experiment plating. This time however 0.1 mL of culture was removed and plated over 5 LYS dropout plates. 0.3 mL of culture, serially diluted as for 0 hr was plated over 3 YEPD plates. Both the 0 hour and the 7 hour plates were grown for 7 days and then counted for recording. There were six experiments produced for each mutant strain and corresponding AM 4466 strain.

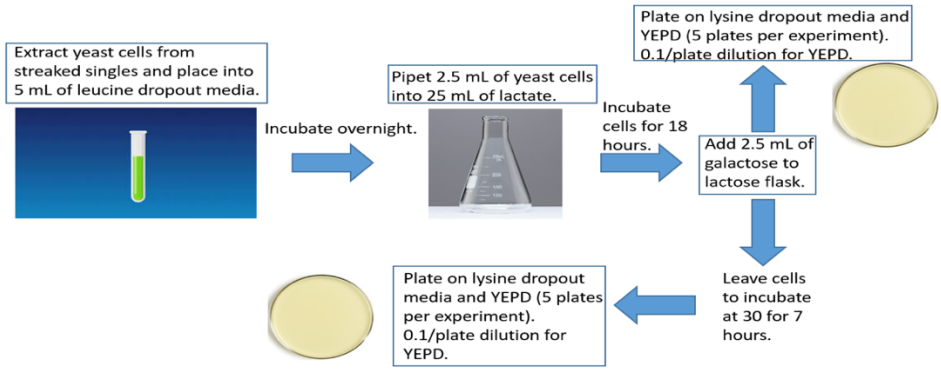

Figure 4 Depiction of the frequency experiment protocol. The strains were grown first in liquid leucine dropout, transferred to lactate after one day, then plated onto YEPD and Lys dropout agar media.

No Cut Plating Experiments

To test the spontaneous mutation rate of the LYS+ reversion, no cut strains were tested as described in the plating experiments above Leucine selects for outcomes strictly related to BIR and no other event that may have produced a LYS+ reversion genotype. Once the colonies on the LEU dropout plates were grown, 2 or 3 of the colonies on each plate were isolated and streaked for singles on more LEU dropout media to grow for about 3 days. These were then used for the lysine dropout frequency experiments described above.

Quantification of InsH Deletions

InsH deletions, characterized by the ability to grow on LYS dropout medium due to the failure to replicate through the InsH region (Figure 5), were quantified by counting the number of colonies that grew on the 0 hour LYS plates and the 7 hour LYS plates. These counts were converted to LYS+ reversion rates by applying a LYS+ reversion frequency equation based off of the methods described in the Materials and Methods of Deem et al. (2011). The

mutagenesis rates were determined by using a modified version of the Drake equation $u=f_7-f_0$. The 0 hour DSB's were calculated using the same modified formula but used different frequencies to determine the LYS+ reversion rate. This was calculated by taking the difference between the 0 hour and no DSB frequencies.

Qualification of InsH Excisions

The InsH excisions observed using the LYS dropout frequency experiment protocol described above. Colonies that grew on the 7 hour platings from each experiment done had the DNA purified and then used primers that annealed to the left OL 1104 (5' AAATGTCACTGCAAATTATGCGGAAGAC 3') and right junctions OL 1181 (5' CCATCCACTTCTCATCTGAAAGACC 3') with *LYS2* gene homology flanking the InsH sequence or what was left of the InsH sequence after excision. Percentages for the different types of excision types were determined by amplifying a region of *LYS2* from each side that included the "deleted" InsH sequence and then using a sequencing primer to determine the excision type. The three types of excision types were precise, type I imprecise, and type II imprecise (Figure 5).

Statistics Test for Significance

To determine whether the InsH excision frequencies were significantly different from the WT strain containing the LYS-InsH construct at 16 kb position (AM 4466), the Mann-Whitney nonparametric statistics test was used. The two groups for comparison were the mutant strain containing the LYS2-InsH construct and the WT strain AM 4466. P-values were calculated corresponding to each of the comparisons and anything $p<0.05$ was deemed significant. The Mann-Whitney statistics test was also applied to determining the significance of differences in excision types for the strains sequenced.

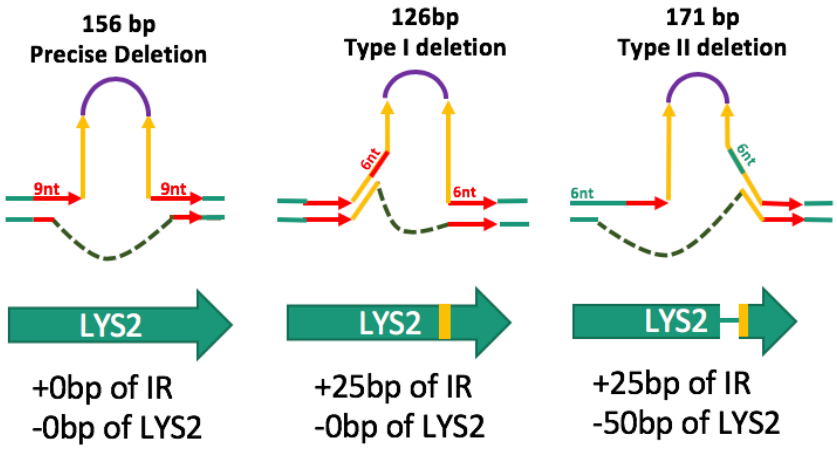

Figure 5 Three main categories of deletion types that can occur. The event is shown in the depictions and the change in base pair amount due to the deletion event is shown below the deletion event depictions.

Results:

Translesion Polymerases are not Important for Effective Progression through IRs by BIR

In order to study whether or not translesion polymerases are implicated in BIR progression through IR's in *S. cerevisiae,* strains AM 4722, AM 4772, and AM 4848 were investigated in their abilities to produce differences in LYS+ reversion rates. These strains were grown in LEU dropout liquid culture, transferred to lactate culture, and plated onto media at various time points. The frequencies were quantified on the basis of LYS+ outcomes on Lys dropout selective media. The frequencies of excisions for S-phase replication and BIR events were used to calculate the LYS+ reversion rates using a modified version of the Drake equation (Materials and Methods). The Mann-Whitney nonparametric statistics test was performed by comparing each translesion mutant strain's LYS+ reversion rates to the LYS+ reversion rates of the WT strain in this experiment (Figure 6). When this analysis was carried out, there was no significant difference in the LYS+ reversion rates between translesion mutants and the WT strain giving a p-value > 0.05 for each comparison (Figure 6). These results strongly suggest that translesion polymerases are not significantly assisting BIR with progression through IRs. Sequencing of the LYS+ reversion outcomes was not performed because there was a lack of any significant change in deletion frequency from the experiment so it would not be expected that these translesion polymerases would change the spectrum of the deletion types. The overall insignificance in LYS+ reversion rates are an interesting result given polymerase ζ is important for bypass through DNA lesions and may be expected to assist BIR in progression through IRs. Polymerase ζ has also implicated in template switching during microhomology-mediated break-induced replication (MMBIR), a form of BIR (Sakofsky et al., 2015). This result here however

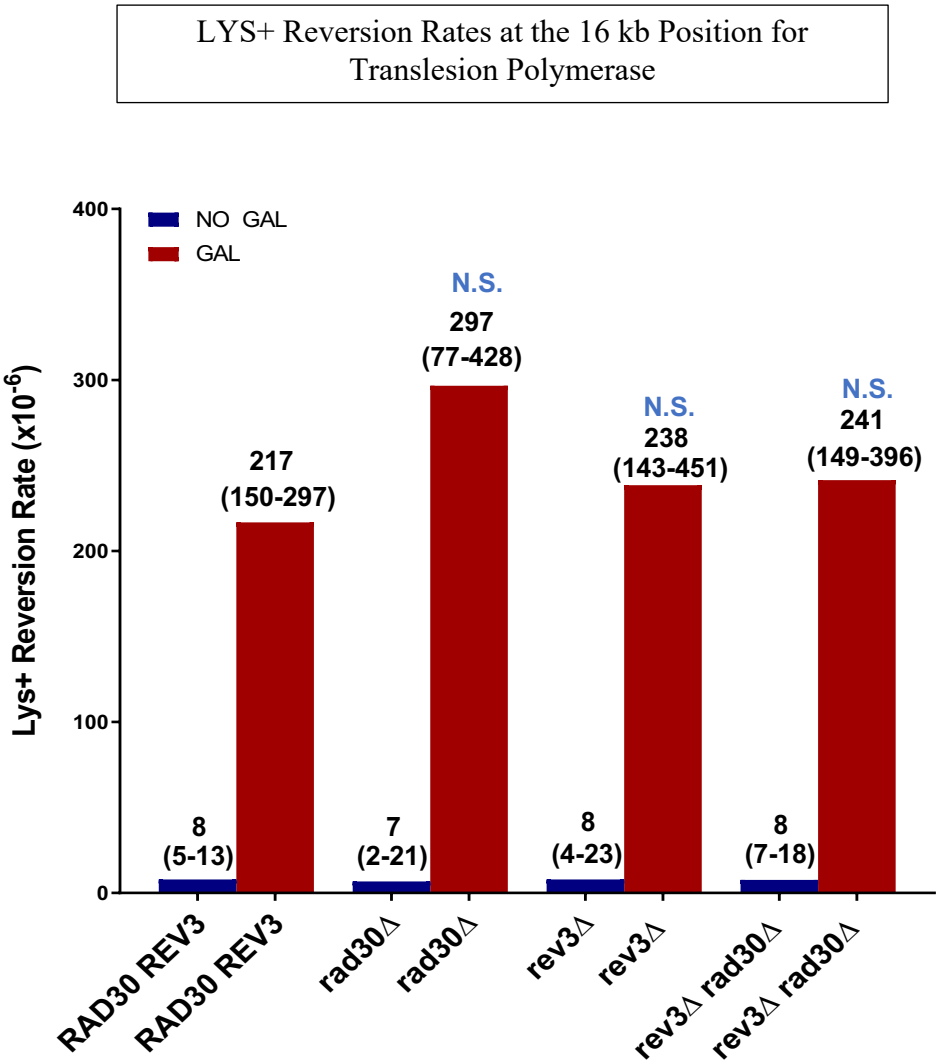

Figure 6 No significant changes observed in DSB repair between DNA pol translesion mutants and WT strains of yeast. The 0 hour bars (blue) represent the platings done at 0 hour and the 7 hour bars (red) represent the platings done at 7 hour described in the Materials and Methods section. The horizontal axis displays the mutant strains tested while the vertical axis displays the Lys+ reversion rate. There were a total of 6 experiments performed for each strain. Significance values were assigned accoridng to the Mann Whitney nonparametric statistical test was performed comparing the WT strain with each of the mutant strains. N.S. denotes no significance according to the Mann-Whitney test.

contradicts expectations and suggests that perhaps there is no template switching during the progression of BIR through IRs.

pol3-Y708A and pol3-01 do not Show an Elevated Level of InsH Excision during BIR

Likewise with the translesion polymerase mutants, polymerase δ mutants *pol3-01, pol3-t,* and *pol3-Y708A* were grown in LEU cultures, transferred to lactate cultures, and then plated as described in the previous results. The Mann-Whitney nonparametric statistic test was applied to determine whether or not the LYS+ reversion rates were the same or different between wild-type and mutant strains. The Mann-Whitney test detected no such significance giving $p<0.05$ with the comparison of the *pol3-01* strain LYS+ reversion rates and the WT LYS+ reversion rates. The *pol3-01* mutant has a defective exonuclease activity in polymerase δ and it looks as if this component is not highly involved in assisting BIR through IRs. This is contrary to what was expected, since this mutant is involved with eliminating template jumps in BIR (Anand et al., 2013). It has also been found to increase the ability of polymerase δ to undergo displacement synthesis and perhaps there is no way to increase it any more for progressing through IRs (Jin et al., 2003). On the other hand, the *pol3-Y708A* mutation did significantly decrease the deletion frequencies of InsH (Figure 7). The *pol3-Y708A* mutation is located in the nucleotide binding pocket of polymerase δ in the active site and decreases the processivity of polymerase δ (Pavlov et al., 2001). There was a $p<0.001$ significance detected by the Mann-Whitney test showing a significant decrease in the number of LYS+ reversion outcomes. However much of this decrease may be explained by the defective nature of this mutation. A majority of the events comprise chromosome loss and BIR actually makes up a much smaller portion of the repair outcomes, which had a large effect on the actual number of BIR outcomes produced.

LYS+ Reversion Rates at the 16 kb Position for Polymerase δ Mutants

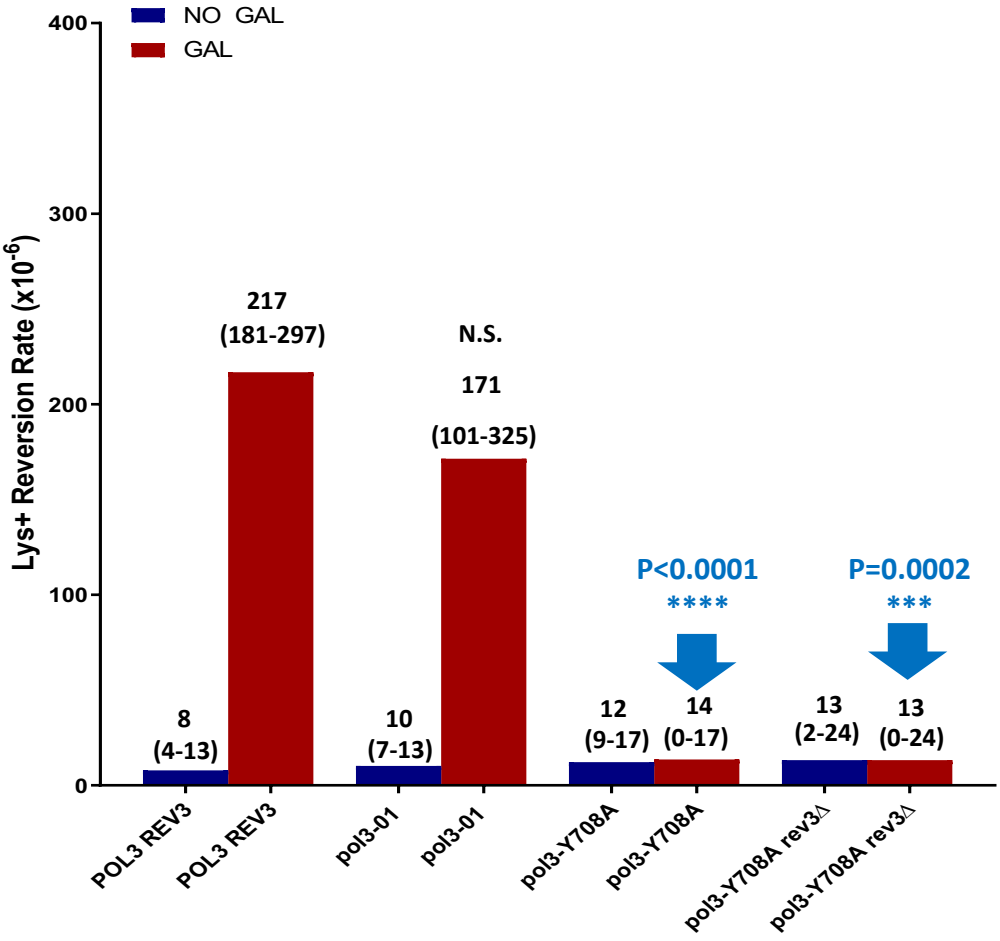

Figure 7 The 0 hour bars (blue) represent the 0 hour plating experiments and the 7 hour bars (red) represent the 7 hour plating experiments described in the Materials and Methods section. The identity of the strains is shown on the horizontal axis and the Lys+ reversion rate is shown on the vertical axis. There were a total of 6 experiments conducted for each mutant strain displayed on the figure. Significance values were assigned according to the Mann-Whitney nonparametric statistics test comparing WT with each mutant strain. N.S. denotes no significance. The Mann-Whitney test did assign significant p-values for the *pol3-Y708A* and the *pol3-Y708A rev3::BSD* mutant strains but these values are not shown (see Results for rationale).

Sequencing of Results of LYS+ Reversions in WT and pol3-01 Backgrounds

To confirm whether or not the *pol3-01* mutant strain had any effect on the spectrum of InsH deletion types, sequencing of the detectable LYS+ reversion outcomes was performed. There were 25 total WT LYS+ reversion outcomes sequenced and 51 LYS+ reversion outcomes in the *pol3-01* background. For the WT strain, 1/25 showed a precise deletion type, 17/25 showed a type I deletion, and 7/25 showed a types II deletion (Table 1). For the *pol3-01* strain, 1/51 showed a precise deletion type, 33/51 showed a type I deletion, and 17/51 showed a type II deletion. The results showed no significant difference in the spectrum of outcomes obtained in the *pol3-01* strain as compared to the WT strain using the Mann-Whitney Statistic test (Table 1). Even though *pol3-01* has been implicated as increasing the ability for polymerase δ to undergo displacement synthesis, it does not seem to have much of an effect upon the LYS+ reversion rates or types of excisions generated. Likely, the exonuclease activity of polymerase δ has little to no role in the processivity of polymerase δ through IRs. There was no BIR deficiency in the *pol3-01* strain suggesting these results can be interpreted as such.

Strain	Precise Deletion	Type I Deletion	Type II Deletion
POL3	4%	68%	28%
pol3-01	2%	65%	33%

Table 1 Percentages of deletion types for both the WT polymerase δ and mutant *pol3-01* strains. The Mann-Whitney nonparametric statistic test was run between the WT and mutant strains for all three deletion types and found none to be significant.

Discussion:

Experiments conducted in this study attempted to determine a mechanism of BIR in the presence of IRs such as InsH. Polymerase mutant strains were isolated and each mutant strain was analyzed in its ability to produce InsH excisions during BIR. The mutant LYS+ reversion rates were compared to the WT LYS+ reversion rates using the Mann-Whitney nonparametric statistics test. There was no significant difference in LYS+ revertants when the WT and translesion polymerase mutant *LYS2*-InsH strains were compared to one another (Figure 6). This suggests that translesion polymerases are likely not recruited to the site of BIR synthesis through IR's. Translesion polymerases are generally used to bypass lesions and have been implicated in S-phase replication as a solution to passing through IRs (Northam et al., 2014). MMBIR requires polymerase ζ for template switching as determined by Sakofsky et. al and seeing that it is not required using the LYS+ reversion system, there is likely no template switching involved with BIR progression through IRs via translesion polymerases (2015). Polymerase η is not required for template switching but it may have an opportunity to replace polymerase ζ upon deletion. The results show otherwise though both in the double translesion polymerase deletion strain, leading to the conclusion that translesion polymerases are not a significant protein needed for the progression of BIR through IR's, nor do they mediate IR deletions by BIR (Figure 6).

This poses the question of which other polymerases would be required to bypass IRs during BIR, and the next likely candidate would be the main replicative polymerase in BIR synthesis polymerase δ. The *pol3-01* mutant was designed and tested to determine whether exonuclease activity was needed to progress through the IR. This also tested how much polymerase δ's displacement synthesis method could be improved for progression through IRs

since researchers have found that this mutant improves the displacement synthesis function of polymerase δ (Jin et al., 2003). However, no significance in the rate of LYS+ reversion, or InsH excision rate, was found (Figure 7). This implied that exonuclease activity likely didn't have a role in the progression of polymerase δ through IR's. To ascertain whether perhaps the strand displacement aspect of *pol3-01* was important, sequencing of the LYS+ reversion cells was carried out. This also displayed no difference to the WT polymerase δ thus supporting the insignificance of exonuclease activity in this situation. Since the IRs present a similar situation to the processing of primers during S-phase replication, it's likely that displacement synthesis would play a role in the progression through IRs. Another mutation analyzed was the *pol3-Y708A* mutation and this mutation did show a significant difference, but it was due to a decrease rather than an increase in LYS+ reversion rates. This mutation is located in the active site of polymerase δ where the nucleotide binding pocket is and reduces the processivity this polymerase. The effect looked to be significant from the number of LYS+ reversions obtained in BIR, but upon further observation of repair outcomes found that more than 80% of the repair outcomes were half-crossovers, chromosome losses, or gene conversion, rather than BIR (data not shown)m and this mutant phenotype matches what was observed in Vasan et al. (2014). The apparent decrease is therefore likely to be a problem of this mutant polymerase δ reaching the reporter system because of massively reduced BIR efficiency rather than an actual decrease in the rate of LYS+ reversions caused by the mutant polymerase δ.

There are a few notable concerns with the current study that could affect observed significance in LYS+ reversion rates. InsH excisions do not always have to produce a detectable LYS+ phenotype and may even produce a very weak LYS+ phenotype (too difficult to visually detect).

Despite several negative results, this study addressed several questions about the progression of BIR through IRs and suggests potential candidates in future studies. First, there seems to be no relationship between translesion polymerases and polymerase δ exonuclease (tested by *pol3-01*) activity with progression of BIR through IR's according to detecting of LYS+ reversion rates. This means that these are likely not proteins/subunits involved with BIR's progression through IRs. Second, it is still uncertain what the effects of reduced processivity of polymerase δ are given the *pol3-Y708A* BIR deficiency. Polymerase δ is likely the polymerase that assists BIR through IR's and this makes sense because this is the main replicative polymerase during BIR on both leading and lagging strands (Smith et al., 2009). Several studies on S-phase replication have implicated the temperature sensitive polymerase δ mutant *pol3-t* as increasing deletion and recombination rates with different types of IRs including InsH (Gordenin et al., 1993) (Tran et al., 1995) (Lobachev et al., 1998). Unpublished work has actually found that the LYS+ reversion rates increased significantly in strains of *pol3-t* background in comparison to the WT suggesting this polymerase is involved in the progression of BIR through IRs. Lastly, the excision spectrums produced by the mutants studied provide valuable insight into how easily BIR machinery can enter the hairpin structure. The *pol3-01* mutation did not produce a significant difference in excision types but the temperature sensitive polymerase δ mutant *pol3-t* did in unpublished work. Perhaps this mutation slows down polymerase δ to a rate where the hairpins can form fully and make it harder for polymerase δ to traverse the IR's producing a high number of precise excisions.

Future directions include temperature experiments with the WT strain to gain insight into the mechanics of hairpin formation. Lower temperatures may stabilize the hairpin thus making it more difficult for polymerase δ to enter and theoretically producing more precise excisions. The lower temperature may also affect pol δ's ability to use displacement synthesis through the hairpin thus creating a difference in the InsH deletions observed. Other proteins would be interesting to investigate as well like helicases. The *pif1* helicase is a candidate for study since this is important for unwinding the migrating bubble ahead of polymerase δ during BIR synthesis (Wilson et al., 2013). *Pif1* is important for the efficient DNA synthesis by polymerase δ and if not present will cause DNA synthesis to decrease (Wilson et al., 2013). One would expect for the frequency of excision to perhaps decrease due to the decrease in DNA synthesis causing a similar problem that *pol3-Y708A* had in not reaching the reporter gene. It could also be likely that precise excisions would increase as a result of the haiprin having more time to form before polymerase δ could reach the position especially on the leading strand. Studying BIR mechanics at other positions like the *MAT* locus and 36 kb away from the DSB would be another potential direction for observing the effects of IRs on BIR (Figure 1). Different positions during BIR have yielded different results in terms of proteins implicated, mechanism of BIR synthesis, and the mutagenicity of BIR using a LYS+ reversion reporter as described in this study (Saini et al., 2013). More investigation into this difference could yield interesting insight into the mechanism of InsH excision during BIR at farther or closer distances to the DSB and whether these three positions differ from one another.

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